

Understanding Immune Responses to Severe Influenza Disease

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ABSTRACT

Respiratory viral infections caused by Influenza A viruses (IAVs) are responsible for annual seasonal epidemics that cause mild, severe or fatal pulmonary disease and estimated to result in 243,000-640,000 deaths globally every year. Severe infections can result in lung tissue damage, acute respiratory distress syndrome (ARDS) as well as secondary bacterial pneumonia, all of which are detrimental to the host and can potentially lead to a fatal disease outcome. Many of those at high-risk of developing severe disease include children, the elderly and immunocompromised individuals. Although current influenza vaccines are the best way to combat the seasonal influenza epidemics, the vaccines need to be updated annually to match strains predicted to circulate in the upcoming influenza season. Frequent mutations in the virus can lead to strain mismatches, which can affect the protective efficacy of these vaccines and result in significant increases in infection rates, putting those who are most vulnerable at risk for developing severe disease. The aim of this PhD thesis was to investigate host factors and mechanisms associated with severe influenza disease to provide insights into identification and prediction of severe disease outcomes.

Co-expression of surface CD38 and MHC-II on CD8⁺ T cells is recognized as a classical hallmark of activation in many acute and chronic viral infection settings. However, high and prolonged CD38⁺MHC-II⁺ expression can be also associated with severe and fatal disease outcomes. Our previous studies have found that the persistence of CD38⁺MHC-II⁺ CD8⁺ T cells is associated with impaired IFN- γ production in H7N9-infected patients with fatal outcomes. In Chapter III, we sought to investigate the mechanisms underpinning the expression of CD38⁺MHC-II⁺ markers using wild type and transgenic mouse models of influenza infection. We provided evidence that CD38⁺MHC-II⁺ CD8⁺ T cells are recruited early to site of infection during severe influenza A virus infection and can be activated in an antigen-driven as well as bystander manner. Our adoptive transfer experiments also demonstrated that MHC-II molecules were not expressed intrinsically by CD38⁺CD8⁺ T cells, but acquired from antigen presenting cells by trogocytosis, and the CD38⁺MHC-II⁺ phenotype was crucial for optimizing T cell recall ability.

In Chapter IV, we investigated disease signatures during the early phases of infection to identify features associated with clinical outcomes. While comparable viral titres were observed

between H7N9 patients who survived or succumbed to infection, there was a trend of higher level of inflammation responses in those who died. Our transcriptome analyses of blood samples from fatal and recovered H7N9 patients found that the gene *Olah*, encoding for a fatty acid hydrolase, was expressed more than 80-fold higher in fatal H7N9 patients compared to those that recovered at early days after disease onset. We established an *Olah*-overexpressing A549 cell line, which allowed us to develop a qPCR platform to screen *Olah* expression in human tissues and cells. Our analyses of *Olah* distribution across different anatomical sites found *Olah* expression in human lung, spleen and PBMCs, with the most prominent expression detected in CD14⁺ cells within the human lungs.

Subsequently in Chapter V, we generated and established OLAH knockout (OLAH KO) mice to further investigate the role of *Olah* during severe influenza A virus infection. Compared to wild-type mice, OLAH KO mice displayed milder disease symptoms, less body weight loss and were protected from severe and fatal disease outcomes following IAV infection. In addition, OLAH KO lungs exhibited lower viral titres, less tissue damage and had significantly reduced levels of inflammatory cytokines and infiltrating innate immune cells, indicating the importance of *Olah* in controlling early excessive host responses and disease severity. These effects were associated with reduced lipid droplets in virus-infected epithelial cells lining the bronchioles, thus defining a role for OLAH in driving lipid metabolism during IAV disease.

Overall, the findings from this PhD thesis highlight the importance of CD38⁺MHC-II⁺ CD8⁺ T cells during severe influenza A virus infection and identified the mechanisms underlying severe IAV disease. We also identified *Olah* as a potential biomarker of severe disease and demonstrated its role in modulating early host immune responses via the formation of lipid droplets and defined its impact on disease outcomes. Overall, data generated in this PhD thesis advance our understanding of factors underlying fatal clinical outcomes and provide insights into the development of novel treatment strategies and biomarkers against severe influenza disease.

DECLARATION

With this I certify that,

- This study contains my own original work towards the PhD.
- The work in this study was conducted by myself with collaborations such as materials used highlighted and acknowledged.
- The thesis is less than 100,000 words, excluding tables, references, and appendices.

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“Take pains, be perfect.”

(quote from A Midsummer Night's Dream, Shakespeare)

RESEARCH OUTPUT

Publications

- Louise C Rowntree, Brendon Chua, Suellen Nicholson ... **Xiaoxiao JIA**... Katherine Kedzierska. Robust correlations across six SARS-CoV-2 serology assays detecting distinct antibody features. *Clinical & Translational Immunology*. (2021, 10(3))
- Marios Koutsakos^{1*}, Louise C Rowntree^{1*}, Luca Hensen^{1*}, Brendon Chua^{1*}, Carolien van de Sandt^{1*}... **Xiaoxiao JIA** ... Thi H. O. Nguyen[#], Katherine Kedzierska[#] and Allen C. Cheng[#]. Integrated immune dynamics define correlates of COVID-19 severity and antibody responses. *Cell Reports Medicine*. (2021, 2:100208)
- Irani Thevarajan, Thi H. O. Nguyen, Marios Koutsakos ... **Xiaoxiao JIA**... Katherine Kedzierska. Breadth of concomitant immune responses prior to patient recovery: a case report of non-severe COVID-19. *Nature Medicine*. (2020, 26: 453-455).
- **Xiaoxiao JIA**, Brendon Chua, liyen Loh, Marios Koutsakos, Lukasz Kedzierski, Moshe Olshansky, William R Heath, Jianqing Xu[#], Zhongfang Wang[#], Katherine Kedzierska[#]. High expression of CD38 and MHCII on CD8⁺ T cells during severe influenza disease reflects bystander activation and trogocytosis. Under review at *PLoS Pathogens*.
- Thi H.O. Nguyen, Marios Koutsakos, Carolien E. van de Sandt ... **Xiaoxiao JIA** ... Paul G. Thomas, Allen C. Cheng[#] and Katherine Kedzierska[#]. In-concert immune dynamics during natural influenza virus infection and recovery in acute hospitalized patients. Under review at *Science Immunology*.
- Georgia Deliyannis^{1*}, Chinn Yi Wong^{1*}, Hayley A. McQuilten^{1*} ... **Xiaoxiao JIA** ... David C. Jackson[#], Brendon Y. Chua[#]. TLR-2-mediated activation of innate responses in the upper airways confers antiviral protection of the lungs. *JCI Insight*. (2021, 6(5):e140267).
- Jennifer Habel, Carolien van de Sandt, Thi H. O. Nguyen ... **Xiaoxiao JIA** ... Louise C Rowntree^{*} and Katherine Kedzierska^{*}. Suboptimal SARS-CoV-2-specific CD8⁺ T cell response associated with the prominent HLA-A*02:01 phenotype. *PNAS*. (2020, 117(39):24384-24391)
- Maria Auladell^{1*}, Xiaoxiao JIA^{1*}, Luca Hensen^{1*}, Annette Fox, Thi H.O. Nguyen, Peter C. Doherty and Katherine Kedzierska. Recalling the future: immunological memory towards unpredictable influenza viruses. Invited review for *Frontiers in Immunology*, *Frontiers in Immunology*. (2019, 10:1400) *Equal author contributions.
- van Wilgenberg Bonnie, Loh Liyen, Chen Zhenjun... **Xiaoxiao JIA** ...Kedzierska Katherine, Hinks Timothy, et al. MAIT cells contribute to protection against lethal influenza infection in vivo. *Nature Communication*. (2018, 9(4076))

Awards

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Presentations

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Mechanisms underlying Expression of CD38 and MHCII on CD8+ T Cells following Severe Influenza Virus Infection

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Oral presentation

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Expression of CD38 and MHCII on CD8+ T Cells following Severe Influenza Virus Infection

2018 47th Annual Scientific Meeting of The Australasian Society for Immunology

Oral presentation

Expression of CD38 and MHCII on CD8+ T Cells following Severe Influenza Virus Infection

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Poster presentation

CD38+MHCII+CD8+ T Cells Expression upon Influenza Virus Infection

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2018 Doherty Institute Immunology Theme

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Understanding Immune Responses to Severe Influenza Disease

2017 46th Annual Scientific Meeting of The Australasian Society for Immunology

Poster presentation

Regulation of CD38 and HLA-DR Expression During Severe and Mild Influenza Virus Infection

Jia X, Loh L, Wang Z[†], Kedzierska K[†]

2017 NH&MRC Influenza Program Retreat

Oral presentation

Regulation of HLA-DR and CD38 Expression in Mild and Severe Influenza Virus Infection

2017 VIIN Young Investigator Symposium

Poster presentation

CD38⁺MHCII⁺CD8⁺ T Cells Expression upon Influenza Virus Infection

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2017 10th Biennial Scientific Conference

Oral presentation

Regulation of HLA-DR and CD38 Expression in Mild and Severe Influenza Virus Infection

ABBREVIATIONS

A	ACCs	Acetyl-CoA carboxylases
	ACs	acyl-CoA synthetases
	ADCC	antibody-dependent cellular cytotoxicity
	ADPRC1	ADP ribose hydrolase 1
	AMPK	5'-AMP-activated kinase
	APCs	professional antigen presenting cells
	ARDS	acute respiratory distress syndrome
	ATC	Ammonium Tris Chloride
B	ATP	adenosine triphosphate
	B2M	β_2 microglobulin
	BAL	bronchoalveolar lavage
	BCR	B cell receptor
C	bp	base pair
	CBA	Cytometric Bead Array
	CLIP	class II-associated invariant chain peptide
	CLPs	common lymphoid progenitors
	CMV	human Cytomegalovirus
	COL27A1	collagen type XXVII alpha 1 chain
	COVID	coronavirus disease
	cRPMI	complete Roswell Park Memorial Institute media 1640
	CRISPR	clusters of regularly interspaced short palindromic repeats
	cTECs	cortical thymic epithelial cells
D	CTLs	cytotoxic T lymphocytes
	DC	dendritic cell
	DMEM	Dulbecco's Modified Essential Media
	DN	double negative
	DP	double positive
	DRiPs	defective ribosomal products
E	ER	endoplasmic reticulum
	EBV	Epstein-Barr virus
	EID ₅₀	Median embryo infectious dose
	EVD	Ebola virus disease
F	FACS	fluorescence activated cell sorter
	FAS	fatty acid synthesis
	FAO	fatty acid oxidation
	FFAR4	free fatty acid receptor 4
	FITC	fluorescein isothiocyanate
G	GFP	green fluorescent protein

	GLUT1	glucose transporter 1
H	HA	hemagglutinin
	HAV	hepatitis A virus
	HBV	hepatitis B virus
	HCV	hepatitis C virus
	HIF1 α	hypoxia-inducible factor
	HI-FCS	heat inactivated fetal calf serum
	HIV	human immunodeficiency virus
	HLA	human leucocyte antigen
	HSV-I	herpes simplex virus 1
I	IAV	influenza A virus
	IBV	influenza B virus
	ICV	influenza C virus
	IFNs	interferons
	IFTM3	IFN-induced transmembrane protein-3
	Ig	immunoglobulin
	IL	interleukin
	i.n.	intranasal
	IRF4	interferon regulatory factor 4
	i.v.	intravenous
K	KO	knockout
	KIR	killer cell immunoglobulin-like receptor
L	LCMV	lymphocytic choriomeningitis virus
	LD	lipid droplet
	LN	lymph node
M	M1	matrix 1 protein
	M2	matrix 2 protein
	MDCK cell	Madin-Darby Canine Kidney cell
	mDCs	myeloid-derived dendritic cells
	MHC-I	major histocompatibility complex I
	MHC-II	major histocompatibility complex II
	MIIC	MHC-II compartment
	MIP	macrophage inflammatory protein
	MLNs	mediastinal lymph nodes
	MPECs	memory precursor effector cells
	mTOR	mammalian target for rapamycin
	mTORC1	mTOR complex 1
	MYO1B	myosin-IB
N	NA	neuraminidase
	NALT	nasal-associated lymphoid tissue
	NK	natural killer
	NOD	Nucleotide-binding oligomerization domain
	NP	nucleoprotein

O	OLAH	oleoyl-(acyl-carrier-protein) hydrolase
	OCT	optimum cutting temperature
	OT-I cell	MHC class I-restricted, ovalbumin-specific, CD8 ⁺ T cell
	OVA	Ovalbumin
P	OXPHOS	oxidative phosphorylation
	PA	polymerase acidic protein
	PB1	polymerase basic 1
	PBMC	peripheral blood mononuclear cells
	PBS	phosphate-buffered saline
	PD-1	programmed death-1
	PDK4	pyruvate dehydrogenase kinase 4
	PE	phycoerythrin
	PerCP-Cy5.5	Peridinin Chlorophyll Protein Complex Cyanin5.5
	PFA	paraformaldehyde
	PFU	plaque forming units
	p.i.	post infection
	PI3K	phosphoinositide 3-kinase
	pMHC	peptide-MHC
Q	qPCR	quantitative polymerase chain reaction
R	RIG-I	Retinoic acid-inducible gene-I
	RNA	ribonucleic acid
S	SA	sialic acids
	SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
	SD	standard deviation
	SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
	SF	serum free
	sgRNA	single guide RNA
	SNPs	single nucleotide polymorphisms
	SLECs	short-lived effector cells
	SRC	spare respiratory capacity
T	TAP	transporter associated with antigen processing protein
	T _{CM}	central memory T cell
	TCR	T cell receptors
	T _{EM}	effector memory T cell
	Th	T helper
	TI	T cell-independent
	TKO	triple knockout
	TLR	Toll-like receptor
	TNF	tumor necrosis factor
	TRAF	tumour-necrosis receptor-associated factor
	T _{RM}	tissue-resident memory CD8 ⁺ T cell

	TSC2	tuberous sclerosis complex 2
V	vPNPs	ribonucleoprotein
W	WT	wild type

CHAPTER I: INTRODUCTION

1.1 Influenza

1.1.1 Seasonal and pandemic influenza

Respiratory viral infections caused by Influenza A virus (IAV) strains are responsible for annual seasonal epidemics that cause mild, severe or fatal pulmonary disease and estimated to result in 243,000-640,000 deaths globally every year¹. In 2017 alone, ~9.5 million people were hospitalized with lower respiratory tract influenza infections for a total of 81.5 million hospital days². Infection can typically cause mild symptoms such as high fever, coughing, muscle pains, and headaches which tend to resolve within days to weeks. However, severe infection can result in lung tissue damage, acute respiratory distress syndrome (ARDS) as well as secondary bacterial pneumonia, all of which is detrimental to the host and can result in a fatal disease outcome. Many of those at high-risk of developing severe disease include children, the elderly and immunocompromised individuals.

The most effective way to protect against influenza is by vaccination using inactivated virus formulations which induce neutralizing antibody responses towards the viral surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins³. Given the frequent mutations that occur in these antigens, vaccines only provide strain-specific protection and offer little cross-protection against strains not covered in the formulations. Thus, seasonal influenza vaccines need to be updated annually to match strains that are predicted to circulate in the upcoming influenza season. According to the WHO, it is recommended to be vaccinated in September in the Northern Hemisphere and February in the Southern Hemisphere, well before the advent of influenza seasons in these regions. However, reports of low vaccine efficacies, especially towards H3N2 strains have recently raised concerns. For example, in Australia, compared to previous years, the estimated 2017 vaccine efficacy against H3N2 strains was only 10% (Australian Influenza Surveillance Report 2017, The Department of Health, Australian Government), whilst in the US, this vaccine effectiveness was only 13% in the 2014-2015 influenza season⁴. Although, mismatch of vaccine strains with those in circulation is a major reason for such low vaccine effectiveness, even when matched, the overall estimated efficacy of inactivated influenza vaccines is only 40% to 60%. Additionally, there are other factors such as age, underlying medical conditions, childhood imprinting and vaccination status of recipients

which are contributing factors⁴⁵. Furthermore, since the majority of influenza strains for these vaccines are generated in chicken eggs, significant egg-adapted changes in the viral HA can occur, and as a consequence, can also lead to low effectiveness of these vaccines⁶.

Conversely to seasonal strains, influenza pandemics can be caused by the emergence of novel strains towards which the population has minimal immunity against and can result in very high morbidity and mortality rates⁷. Major IAV pandemics that have occurred in the past century include the 1918 Spanish flu (H1N1)⁸⁹, which caused 50 to 100 million deaths and was the most lethal pandemic across the globe, followed by Asian influenza (H2N2)^{10,11} in 1957 causing 2 million deaths and Hong Kong influenza (H3N2)¹² in 1968 causing 1 million deaths. Furthermore, in April 2009, a swine-derived H1N1-pandemic emerged, spread rapidly and caused more than 284,000 deaths worldwide¹³. In 2013, an outbreak of a novel H7N9 strain of avian-origin H7N9 occurred in China and resulted in a 40% mortality rate in infected individuals¹⁴. Five years onwards, H7N9 virus continues to circulate in poultry and China has since experienced 5 H7N9 epidemic waves.

1.1.2 Types of influenza virus

Influenza viruses are divided into four subtypes: A¹⁵, B^{16,17}, C¹⁸ and D¹⁹ according to their host range and pathogenicity (**Table 1.1**). While influenza A virus (IAV), influenza B virus (IBV) and influenza C virus (ICV) are all transmissible among humans, ICV usually causes mild disease and has not been reported to cause global epidemics. IAV on the other hand, which belongs to the Orthomyxoviridae family, causes the highest mortality and morbidity rates compared to influenza B and C viruses. Apart from infecting humans, IAVs are also recognised as zoonotic pathogens. Strains such as H5N1 and H9N2 are able to infect a wide range of avian and mammalian animal hosts (eg. pigs, chickens), which can transmit to humans after genomic re-assortment. To date, IAVs are the only type that cause global epidemics and pandemics. Generally, annual seasonal influenza cases peak during colder winter months in both Northern and Southern Hemispheres, as these temperatures and environments tend to provide optimal conditions for transmission to occur via inhalation of aerosols from infected individuals or through contact with contaminated objects (e.g. doorknobs).

Table 1.1 The types of influenza virus

Influenza type	Host range	Pathogenicity	Subtype/Lineages
A	Human, Avian and Mammalian animals	Moderate to severe illness, causes global epidemics and pandemics	H1-H18, N1-N11
B	Human	Mild to severe, causes seasonal epidemics	Victoria, Yamagata
C	Human, Pig	Rare in human, caused local epidemics	N/A
D	Cattle	N/A	N/A

1.1.3 The structure of influenza A virus

IAVs are single-stranded negative-sense RNA viruses with a genome consisting of 8 segments encoding for multiple proteins, including nucleoprotein (NP), hemagglutinin (HA), neuraminidase (NA), matrix protein (M1), ion channel protein (M2), three polymerase proteins (PB1, PB2, PA (polymerase acidic protein), NS2(NEP)) and non-structural proteins NS1, PA-X and PB1-F2²⁰ (**Fig 1**)). The major proteins involved in viral host cell entry and the release of infectious virion progeny from infected cells are the HA and NA, respectively. HA is a glycosylated type-I integral membrane protein, involved in recognising sialic acids (SA, N-acetyl neuraminic acid) in host cells^{21,22}. IAVs that are adapted to infect birds, have HAs that can bind SA linked to galactose in a α 2,3-linkage, whereas the HAs of IAVs adapted to infect humans preferentially bind to α 2,6-linked SAs²³. Therefore, HA plays an important role in host range and specificity²⁴. Conversely, NA is a type-II integral membrane glycoprotein with sialidase (neuraminidase) enzyme activity and its function is to cleave host cells SA for virus release after viral replication²⁵.

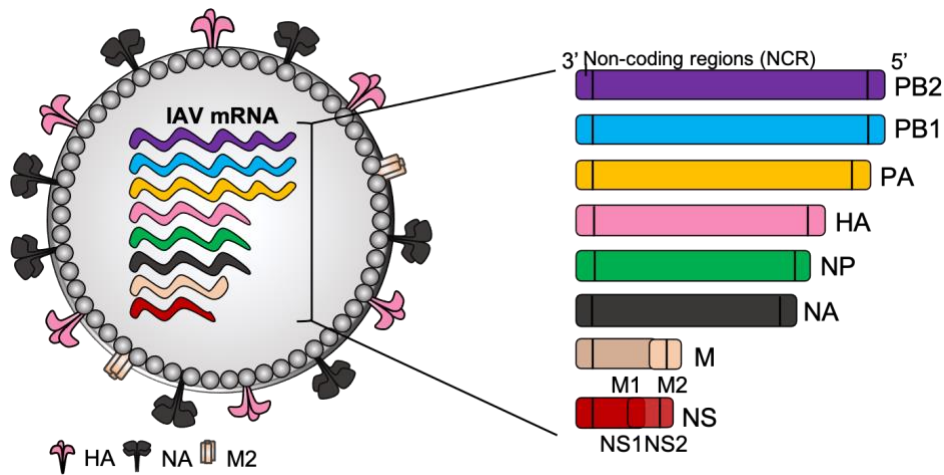


Fig 1. The structure of influenza A virus

The figure illustrates the structure of influenza A virus (IAV). The viral genome is contained within a lipid bilayer, with viral protein Hemagglutinin (HA), neuraminidase (NA) and ion channel matrix 2 (M2) on the surface. HA and NA are the major proteins involved in viral host cell entry and the release of infectious virion progeny from infected cells, respectively. The IAV genome is comprised of 8 negative-sense single-stranded RNA segments including genes that encode for three polymerase proteins (PB1, PB2, PA (polymerase acidic protein), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein (M1) - ion channel protein (M2), and the non-structural proteins NS1 and NS2.

IAVs have been classified into different strains according to HA and NA subtypes expressed on the virion surface. Both viral proteins are the major antigenic targets of the host immune antibody response. Additionally, NA is the target of the antiviral drugs oseltamivir and zanamivir^{26,27}. Novel IAV strains that emerge throughout the influenza season arise as a result of mutations via amino acid substitutions on the HA and NA glycoproteins in a process known as “antigenic drift”^{15,28,29}. Mutations arise as a result of error-prone viral replication, which is caused by inefficient or lack of proofreading capacity of the viral RNA-dependent RNA polymerase³⁰. Additionally, novel IAVs that contain more dramatic changes in their surface glycoproteins can arise via “antigenic shift”³¹. This can occur when two different IAV strains infect the same host cell, enabling the exchange of RNA segments with each other in a process known as gene reassortment, generating a novel strain that have pandemic potential such as the H1N1 swine influenza, H5N1 avian influenza as well as chicken-derived H7N9 avian influenza. These two mechanisms are extremely important for adaption of IAVs to different environments and hosts. To date, there are 18 subtypes of HA and 11 subtypes of NA³² with H1N1 and H3N2³³ being the most common human-adapted strains at present.

1.1.4 Infectious cycle of influenza A virus

Attachment of the HA to α -2,3- or α -2,6-linked SAs on the host cell surface triggers fusion with the plasma membrane, endocytosis and trafficking into endosomes³⁴. Acidic pH changes in the endosome lead to HA conformation changes leading to endosomal membrane fusion and allowing the virus genome ribonucleoprotein (vRNPs), a complex consisting of each genomic segment bound to viral NP and PA, to be released into the cytoplasm. In addition, activation of the M2 ion channel allows acidification of the virion and dissociation of the viral genome from M1 protein, altogether resulting in its transport into the host cell nucleus³⁴. Once there, vRNAs are transcribed and replicated by the viral PA to form new vRNP-M1 complexes, as well as translated in either cytosolic ribosomes (NP, PA, PB1, PB2, M1, NS1, NS2) or endoplasmic reticulum (ER)-associated ribosomes (HA, NA, M2)³⁵. Translated HA, NA and M2 together with newly formed vRNP-M1 complexes, are then transported to cell surface to be packaged into progeny viral particles. The liberation of these particles from the plasma membrane is the last step whereby M2 promotes specific membrane curvature of budding virions necessary for scission and NA cleaves the binding between HA and SAs on host cell surface to facilitate release³⁵.

1.2 Innate immunity to influenza A virus

The innate immune system is the first line of host defence against pathogens and is activated rapidly after IAV infection³⁶. It is comprised of physical barriers, mediators and effector immune cells. Infection of respiratory epithelial cells leads to the activation of at least three signalling pathways including the Retinoic acid-inducible gene-I (RIG-I)-like receptor³⁷, Toll-like receptor (TLR)³⁸ and Nucleotide-binding oligomerization domain (NOD)-like receptor pathways^{39,40}. During infection, TLR3⁴¹ and TLR7⁴², together with RIG-I⁴³ and NOD2⁴⁴ recognise virus at different stages of replication and activate downstream pathways such as MyD88-NK κ B and STAT1⁴⁵. As a consequence, these pathways trigger anti-viral signalling cascades leading to the production of interferons (IFNs), cytokines and chemokines⁴⁶. Type I (IFN- α and IFN- β), type II (IFN- γ) and type III (IFN- λ) IFNs in particular are important in fighting against viral infection^{47,48}. Studies have also shown NLRP3, which belongs to NOD-like receptor family, drives important inflammatory responses by producing IL-1 β and IL-18 by activating caspase-1⁴⁰. Expression of these molecules amongst others such as CXCR2⁴⁹,

CCR2, CCR5⁵⁰, CCR7⁵¹ attracts other innate cells, including neutrophils, macrophages, natural killer (NK) cells and dendritic cells (DCs).

IAV-infected epithelial cells can also secrete CCL2⁵², which can activate macrophages and which in turn can produce pro-inflammatory cytokines (e.g. IL-6 and TNF)⁵³ to promote further inflammation and cellular recruitment⁵⁴. In addition, alveolar macrophages can also be infected and in the case of seasonal IAV strains, this results in an abortive infection⁵⁵. This, together with their ability to phagocytose apoptotic-virus infected cells⁵⁶ helps limit virus spread⁵⁷.

Natural Killer (NK) cells also play an important anti-viral role by killing influenza-infected cells upon recognition of the Fc portion of bound antibodies via their surface receptor CD16. This mechanism is known as antibody-dependent cellular cytotoxicity (ADCC)⁵⁸. During IAV infection, NK cells are recruited to the site of infection within hours after the initiation of inflammatory responses to perform their killing function as well as produce IFN- γ ^{59,60}.

A key cell population linking innate and adaptive immunity are dendritic cells (DCs)⁶¹. These professional antigen presenting cells (APCs) can phagocytose infected cells⁶² and also have the ability to efficiently capture, process and present viral antigens to activate naïve T cells. Peptides presented on major histocompatibility complex II (MHC-II) molecules are recognized by antigen-specific CD4⁺ T cells, which are activated to help promote the development of CD8⁺ T cell responses⁶³. In addition, peptide antigens produced from viral proteins can be cross-presented via major histocompatibility complex I (MHC-I) to directly activate CD8⁺ T cells. Presentation of peptide-MHC-I molecules on surface of infected cells represents also a way to mark virally-infected cells for killing by activated CD8⁺ cytotoxic T lymphocytes (CTLs) to clear the virus⁶⁴. This will be discussed in more detail in the next section on “Antigen Presentation”. Interestingly, despite the important role of DCs in T cell priming, a subset of conventional DCs that express CD11b or XCR1 in nasal-associated lymphoid tissue (NALT) can suppress T cell expansion during steady state conditions. This process can be reversed by inflammation induced recruitment of monocyte-derived DCs to site of viral infection⁶⁵.

1.3 Adaptive immunity: Antigen presentation and recognition

1.3.1 Structure of MHC molecules

Genes encoding for major histocompatibility complex molecules first appeared in cartilaginous fish⁶⁶ around 500 million years ago⁶⁷. Classical MHC molecules, defined by MHC-I and MHC-II, are transmembrane molecules that contain polypeptide α and β chains and are responsible for presenting processed antigen to CD8⁺ and CD4⁺ T cells respectively.

The MHC-I complex is composed of 3 extracellular α domains; $\alpha 1$, $\alpha 2$, $\alpha 3$, and a non-polymorphic β_2 microglobulin (B2M) domain. The $\alpha 1$ and $\alpha 2$ domains form a peptide-binding groove that present processed antigenic peptides as well as facilitates interaction with T cells via their T cell receptors (TCRs). The $\alpha 3$ domain on the other hand plays a role in stabilizing the complex on the cell surface and also interacts with the T cell co-receptor CD8 during antigen presentation⁶⁸. B2M lies under the $\alpha 1$ domain and although it does not contain a transmembrane domain, it is important for stabilizing the tertiary structure of MHC-I molecule complexes for antigen presentation⁶⁹. MHC-I complexes form a closed binding groove for peptides and preferentially binds peptides that are most commonly 8-10 and at most 14 amino acids in length⁷⁰.

MHC-II complexes are heterodimers that contain two α domains; $\alpha 1$, $\alpha 2$, and two β domains; $\beta 1$, $\beta 2$. The $\alpha 2$ and $\beta 2$ domains are transmembrane proteins that anchor into the cell surface, with the $\beta 2$ domain associating with the T cell co-receptor CD4 during antigen presentation. Above these domains lie the $\alpha 1$ and $\beta 1$ chains, together forming the peptide-binding groove. Given the open “overhanging” region at C terminal of the MHC-II peptide binding groove, MHC-II molecules can bind longer peptides than MHC-I^{70,71}.

Both MHC-I and MHC-II molecules are high polymorphic. To date, there are three subclasses of classical MHC-I molecules in humans (HLA-A, HLA-B, HLA-C) and mice (H2-K, H2-D, H2-L). Similarly, classical MHC-II molecules can be subdivided into HLA-DR, HLA-DQ and HLA-DP in humans and H-2A(I-A) and H-2E(I-E) in mice⁷² (**Fig 2**).

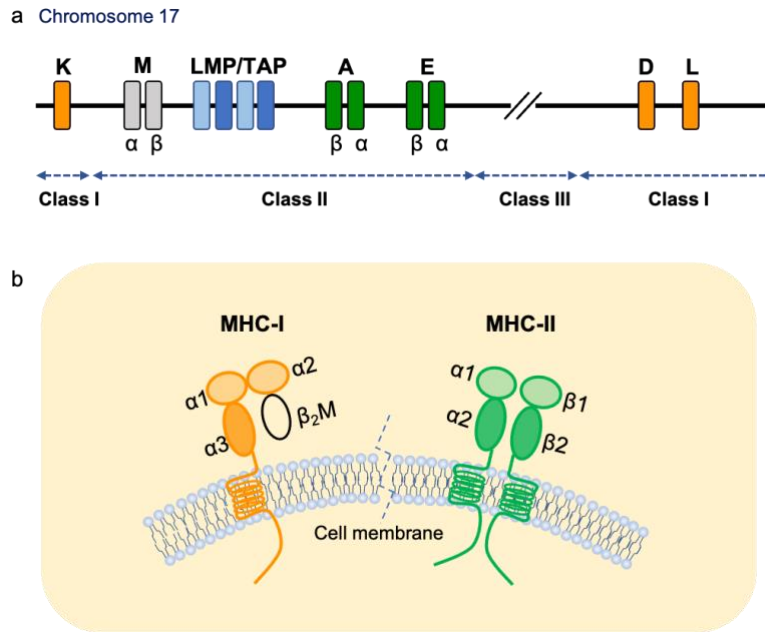


Fig 2. The structure of murine MHC molecules

(a) Schematic representation of the gene locations of different haplotypes of murine MHC class I (K, D, L), II (A, E) and III molecules and their components on chromosome 17. (b) The structure of the MHC-I and MHC-II molecule. The MHC-I complex is composed of 3 extracellular α domains: $\alpha1$, $\alpha2$, $\alpha3$, and a non-polymorphic β_2 microglobulin (β_2M) domain. The $\alpha3$ domain plays a role in stabilizing the complex on the cell surface. β_2M lies under the $\alpha1$ domain and does not contain a transmembrane domain. MHC-II complexes are heterodimers that contain two α domains; $\alpha1$, $\alpha2$, and two β domains: $\beta1$, $\beta2$. The $\alpha2$ and $\beta2$ domains are transmembrane proteins that anchor into the cell surface.

1.3.2 Expression of MHC I and MHC II molecules

MHC I molecules

MHC-I molecules are ubiquitously expressed on all nucleated cells and typically can present endogenously processed intracellular-derived antigens. Empty MHC-I molecules devoid of peptide are assembled in the endoplasmic reticulum (ER). Concurrently, intracellular antigens are proteasomally degraded in the cytosol and processed into peptides. A heterodimer protein called transporter associated with antigen processing protein (TAP) then mediates trafficking of processed peptides from the cytosol to the ER to bind to the MHC-I peptide binding groove with the help of Tapasin and ERp57^{73,74}. Once MHC-I-peptide complex are successfully formed, they are transported to the Golgi and then to the plasma membrane surface for presentation to T cells.

While most intracellular antigens are cleaved and processed by the conventional proteasome, a specific type of immunoproteasome, which consists of the subunits LMP2 ($\beta1i$), MECL-1($\beta2i$)

and LMP7 ($\beta 5i$), is utilized when cells are stimulated by IFN- γ or TNF- α ⁷⁵. This changes the cleavage pattern of antigens for optimal generation of peptides to be loaded onto MHC-I molecules^{76–78}. Mice deficient in these three immunoproteasome subunits (triple knockout mice; TKO) express reduced amounts of MHC-I on the cell surface of myeloid and lymphoid cells and exhibit impaired antigen presentation during influenza virus and LCMV infection⁷⁹. Moreover, MHC-I peptide repertoire also differs significantly in TKO mice compare to WT controls. Another set of β subunits ($\beta 1i$, $\beta 2i$, $\beta 5t$) is specifically utilized in the thymoproteasome of cortical thymic epithelial cells and plays an essential role in the development of CD8⁺ T cells during positive and negative thymic selection⁸⁰.

Peptide antigens can also be derived from “defective ribosomal products” (DRiPs)⁸¹. The discovery of this process was based on observations that viral-derived peptides could be generated rapidly in cells^{82–84} (often within one hour) after infection and before whole viral proteins could be detected⁸⁵. These DRiPs comprise mainly of peptides generated in error during the translation of genetic information such as excessive production of multiprotein complexes⁸⁶, mistranscription⁸⁷ and mistranslation⁸⁸. Studies show DRiPs can be generated by translational modifications occurring in the expression of IAV nucleoprotein (NP) on an Alphavirus vector and comprise of more than 50% of expressed antigens on cells⁸⁹. Moreover, DRiPs can be major source of IAV neuraminidase (NA)-derived peptides⁹⁰. Thus, DRiPs are crucial for viral immunosurveillance given the fast generation of antigenic peptides so that rapid detection by CD8⁺ T cells^{83,84} can occur.

Over time, some viruses have evolved ways to interfere with the antigen loading process^{91–93} by either inhibiting the peptide transporter TAP⁹⁴, induce degradation of MHC-I molecules in the ER or at the plasma membrane, prevent the transport of MHC-I molecules to the cell surface from ER⁹⁵, or even inhibit MHC-I gene transcription⁹⁶. For example, HIV expresses protein called Nef capable of retaining newly synthesized MHC-I molecules in the cell and target them to lysosome for degradation⁹⁷. Studies have shown that both influenza A and influenza B viruses can manipulate MHC-I expression on infected cells via distinct mechanisms⁹⁸, thus leading to reduced CD8⁺ T cell responses⁹³. There are, however, ways that the immune system can overcome some of these impairments to resolve infection. For example, NK cells can recognize non-MHC-I presenting or down-regulated MHC-I expressing cells as potentially infected cells affected by these viral escape mechanisms⁹⁹.

MHC-II molecules

MHC-II molecules are mainly expressed on antigen presentation cells (APCs), including B cells, DCs, monocytes and macrophages. A transcription factor called CIITA is essential for MHC-II expression in these cells. Studies show that MHC-II expression and CIITA upregulation on many cell types such as epithelial cells and tumor cells can help boost T cell immune responses^{100–102}. Interestingly, high levels of MHC-II expression on CD8⁺ T cells can be detected in patients who recovered or succumbed to disease¹⁰¹ during acute influenza virus H7N9 infection and Ebola¹⁰³.

Similar to MHC-I, MHC-II molecules are assembled in ER. An assembled MHC-II molecule is associated with an invariant chain which occupies the peptide binding groove to prevent premature binding of antigen before entering the endosomal pathway to encounter extracellular-derived foreign antigens or degraded self-proteins^{104,105} (**Fig 3**). Once MHC-II molecules are shuttled into the late endosomal MHC-II compartment (MIIC)¹⁰⁶, the bound invariant chain is degraded by proteases leaving a fragment in the peptide binding groove called CLIP, which can be displaced in the presence of higher affinity peptides that can bind to the MHC-II molecules¹⁰⁷. In humans, this process is mediated by an MHC-II like molecule called HLA-DM in the MIIC and promotes the release of CLIP from the peptide binding groove. A completely formed MHC-II-peptide complex will then be transported to plasma membrane and expressed on the cell surface^{108,109} ready for presentation to CD4⁺ T cells. MHC-II-peptide complexes are also continuously recycled from the surface and internalized in a process driven by E3 ubiquitin ligase (MARCH1)-induced ubiquitylation for degradation¹¹⁰. MHC-II molecules can also be recycled from the surface to be loaded with new peptide in early endosomes¹¹¹. Mature DCs, however, prevent internalization of peptide-loaded MHC-II by reducing the expression of MARCH1¹¹².

Given the important role MHC molecules play in pathogen infection¹¹³, organ transplantation¹¹⁴, auto-immunity¹¹⁵, and cancer therapies¹¹⁶, deficiency of either or both MHC-I and MHC-II will lead to impaired antigen presentation. For example, cells in mice lacking MHC-I molecule expression are more susceptible to killing by NK cells¹¹⁷ and failure of allogeneic bone marrow rejection in these mice also suggests MHC-I plays a role in tolerance¹¹⁷. Not surprisingly, deficiency of MHC-I molecules leads to a lack of CD8⁺ T cells⁶⁹ and impaired CD4⁺ T cell generation and function¹¹⁸.

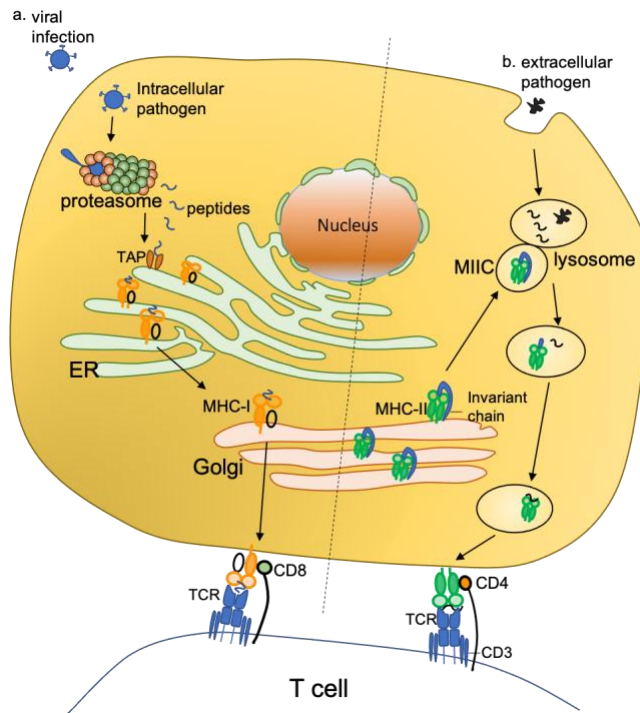


Fig 3. Antigen presentation by MHC-I and MHC-II molecules

Antigen presentation by MHC-I and MHC-II molecules resulting from pathogen infection are shown. (a) During viral infection, antigens are cleaved and processed by the conventional proteasome into peptides. The processed peptides are then loaded onto MHC-I molecules via TAP at ER. The MHC-I-peptide complexes are then transported through the Golgi and to the cell membrane to be presented to CD8⁺ T cells. (b) Extracellular pathogens taken up by the cell by endocytosis end up in the lysosome. Newly synthesized MHC-II molecules containing an invariant chain (to prevent pre-mature binding of antigen) are shuttled into the late endosomal MHC-II compartment (MIIC). The bound invariant chain is degraded and high affinity peptides then bind to the MHC-II molecules. The MHC-II-peptide complexes are then transported to the cell membrane for presentation to CD4⁺ T cells.

Studies further demonstrate that viruses can take advantage of host MHC molecules for their replication and transmission. Researchers found that viruses utilize “licensed” NK cells (in which the NK cell inhibitory Ly49 receptors interacted with MHC-I molecules during maturation) to escape the immune surveillance after infecting mice¹¹³. Newly-defined bat influenza A viruses can replicate in wildtype mice but not MHC-II KO mice and transcriptomic profiling accompanied by the use of CRISPR-Cas9 screening, suggesting that MHC-II molecules, more specifically HLA-DR, could be a potential receptor for these viruses¹¹⁹. Other than viruses, many studies demonstrate that although MHC-II expressing tumor cells are beneficial for induction of host immune responses, there are also reports this type of cell can accelerate disease progression^{120,121}.

Several conditions and factors can alter the expression of MHC molecules, such as the course of pathogenic infection, auto-immune disease as well as different stages of an immune response.

Notably, pro-inflammatory cytokines, including TNF- α and IFN- γ can affect MHC-I expression by modulating the immunopeptidome landscape of human lung cancer cells¹²². A study on human breast cancer shows that IFN- γ can alter the MHC peptidomes, and MHC-I molecule expression may be affected by the availability of empty MHC molecules¹²³.

1.3.3 Antigen presentation

Given that all the nucleated cells can express surface MHC-I molecules, theoretically all of these cells are capable of presenting processed antigens. For example, influenza virus infected epithelial cells present viral peptide via MHC-I for recognition by immune cells, including NK cells and CD8⁺ T cells.

Inhibitory and active receptors on NK cells can recognize pMHC-I to determine whether or not to initiate killing¹²⁴. In humans, such inhibitory receptors include those belonging to killer cell immunoglobulin-like receptor (KIR) and C-type lectin-like receptor (CD94:NKG2A) families. KIR has higher diversity for recognition compared to NKG2A, which only recognizes human HLA-E, and can discern specific MHC-I alleles (eg. HLA-A, HLA-B, HLA-C)¹²⁵. NK cells also use inhibitory receptors to interact with MHC-I molecules loaded with either “self” or “foreign” antigens to distinguish between healthy and infected cells and avoid mis-killing.

CD8⁺ T cells kill infected cells by recognizing viral peptides presented with MHC-I complexes but not non-infected cells. However, studies showed that neighboring non-infected cells adjacent to infected cells can still present viral antigens to activate CD8⁺ T cells. In this setting, viral-derived peptides can be directly transferred from one cell to another through a gap-junction-mediated pathway mediated by cytosolic peptidase activity^{126,127}. For example, HLA-A2 expressing cells can acquire the HLA-A2-restricted influenza virus Matrix protein (M1)-derived peptide epitope M1₅₇₋₆₅ from donor peptide-expressing cells in this manner to subsequently activate M1-specific T cells¹²⁶.

Professional APCs also exhibit the ability to cross-present exogenously-derived antigens to activate naïve CD8⁺ T cells without getting infected themselves¹²⁸ with dendritic cells (DCs) being the most proficient at this¹²⁹. There are multiple ways for uninfected DCs to gain processed antigens from infected cells such as by recognizing and engulfing dead or apoptotic

infected cells through phagocytosis¹³⁰ and by “cross-dressing” (**Fig 4**), wherein pMHC-I complexes are acquired directly from infected cells and presented without the need for any further processing^{130,131}. It has been postulated that this can occur by direct cell-cell membrane interaction or the capture of exosomes containing pMHC-I complexes, with the former being the more likely scenario *in vivo*¹³⁰.

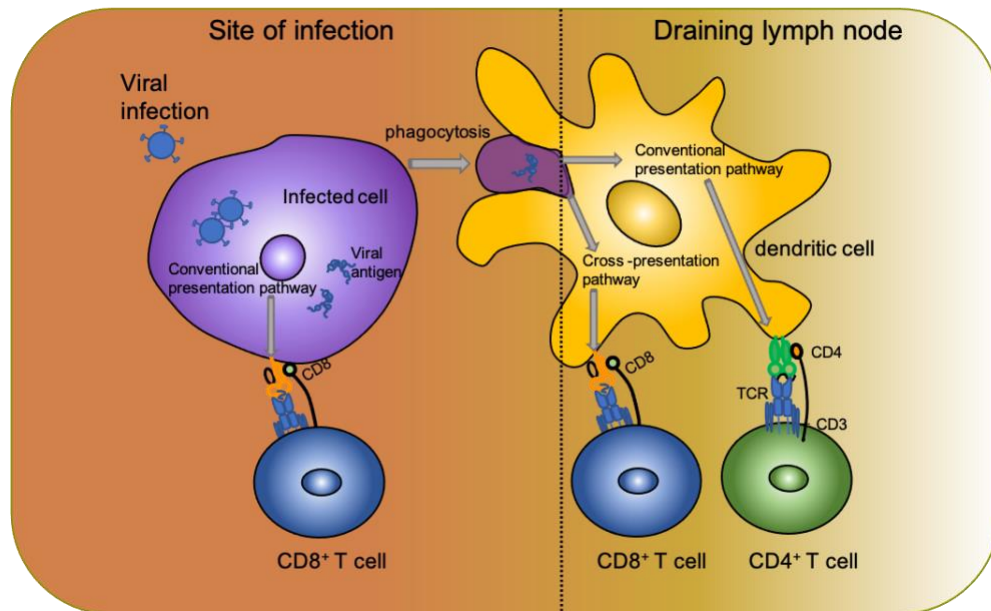


Fig 4. Antigen cross-presentation by dendritic cells

During virus infection, in addition from infected host cells presenting pMHC-I to CD8⁺ T cell or APCs such as DCs processing antigens acquired from infected cells then present pMHC-II to CD4⁺ T cells, DCs at site of infection are able to recognize and engulf dead or apoptotic infected cells through phagocytosis. pMHC-I complexes acquired from infected cells can then be presented without the need for any further processing to T cells.^{130,131}

Various subsets of DCs exist in both humans and mice, with inflammatory DCs, CD8 α ⁺ lymphoid organ-resident DCs and CD103⁺DCs playing major roles in cross-presentation¹²⁸ during viral infection. For example, during herpes simplex virus 1(HSV-I) infection, dermal DCs capture antigen in the periphery and migrate to the draining lymph nodes where antigen is transferred to CD8 α ⁺ DCs for cross-presentation^{132,133}. In addition, other studies show that CD103⁺DCs and are the main migratory DC subtypes which are capable of cross-presenting viral- and self-peptides¹³⁴ whilst during influenza virus infection, inflammatory DCs are the dominant cross-presenting subset¹³⁵. In general, although myeloid-derived DCs (mDCs) are susceptible to viral infection, they are ~300-fold less efficient at presenting antigens via MHC-I compared to uninfected cross-presenting mDCs¹³⁶.

Recent research also shows that immune system has the ability to distinguish whether viral peptides are directly-presented (i.e. endogenously derived antigens presented by MHC-I molecules) or cross-presented in order to optimize T cell activation. During IAV infection in mice, optimal NP₃₆₆₋₃₄₇ presentation is driven by direct-presentation whereas cross-presentation is required for PA₂₂₄₋₂₃₃ presentation¹³⁷. Furthermore, CD8⁺ T cells can acquire MHC-I directly from APCs via trogocytosis, a process involving the transfer of plasma membrane portions during cell-cell interactions¹³⁸, as was first observed in experiments involving the adoptive transfer of mis-matched MHC molecules on recipient T cells¹³⁹. *In vitro* experiments showed that T cells can gain and internalize pMHC complexes within minutes when co-incubated with APCs, increasing their sensitivity to fratricide by neighboring T cells¹⁴⁰. Moreover, this process also leads to the acquiring of costimulatory molecules such as B7 ligands, which are crucial for T cell signaling and activation¹⁴¹.

As described previously, MHC-II molecules present longer peptide lengths than MHC I because of the open structure of its peptide-binding groove, and present antigens from extracellular-derived sources or degraded self-proteins in endosomes. However, MHC-II molecules can also present endogenously-derived antigens as has been reported during influenza virus challenge, where antigen-specific CD4⁺ T cells responses can be driven by infected APCs through MHC-II presentation of viral antigens following processing and degradation by the proteasome¹⁴². CD4⁺ T cells can also acquire pMHC-II complexes through trogocytosis from APCs to boost antigen-specific CD4⁺ T cells responses¹⁴³. Furthermore, pMHC-II acquired by CD4⁺ T cells from DCs has been shown to serve as a mechanism by which excessive clonal expansion can be regulated¹⁴⁴. “Snatching” of MHC-II is not limited to T cells and can occur during interactions with other cell types. For example, NK cells can acquire MHC-II and costimulatory molecules from DCs resulting in reduced antigen presentation to CD4⁺ T cells ultimately leading to decreased T cell responses¹⁴⁵. Furthermore, basophils can acquire MHC-II from dendritic cells to promote T cell differentiating to Th2 cells¹⁴⁶. Notably, an *in vitro* study showed that murine CD8⁺ T cells that have acquired MHC-II molecules from dendritic cells can stimulate CD4⁺ T cells in culture¹⁴⁷. How antigen presentation through MHC molecules play critical roles in T cell development will be discussed in the next section.

1.4 Adaptive immunity to virus infection

Although slower to develop than innate immune responses, the adaptive response arises several days after the initial infection¹⁴⁸ and involves a broad range of specialised highly polymorphic receptors to specifically recognize virus antigens and kill infected cells. Additionally, adaptive responses can generate immunological memory, which can be rapidly and efficiently recalled upon secondary encounter with the same pathogen.

1.4.1 T and B cells

T and B cells are the two main cell types in the adaptive immune system that drive cellular and humoral immunity, respectively. Arising from bone-marrow hematopoietic stem cells, common lymphoid progenitors are transported to thymus for development into T cells, whilst those destined to become B cells are retained and develop in the bone marrow (or fetal liver).

B cell progenitors undergo a series of developmental stages to become mature naïve B cells, during which rearrangement of the immunoglobulin (Ig) loci on heavy and light chains as well as V-D-J segments occurs and is regulated to generate a diverse B cell receptor (BCR) repertoire^{149,150} that can recognize and bind to a wide range of epitopes. Immature B cells migrate to secondary lymphoid organs, including the lymph nodes and spleen where they complete their development in the germinal centers. Mature B cells are able to mount antigen-specific responses in either APC/T cell dependent- or independent-manner to further become short-lived or long-lived plasma cells, and drive humoral immunity by secreting different isotypes of Ig. T-independent antigens also called TI antigens, leads to elicit B cell expansion and activation without help from T cells. For example, the polysaccharide on extracellular bacteria contains a repetitive molecular pattern that can bind to Ig receptor clusters on B cell surface, thus together with help from several other signals (eg. engagement of TLRs) can lead to B cell proliferate without help from T cells^{148,151}. The majority of antibody responses elicited, however, requires help from T cells. B cells firstly require cross-linking of their Ig receptors upon binding of antigens presented by APCs (eg. follicular DCs), which will subsequently trigger their activation. Following this, B cells can present these antigens via MHC-II molecules to T cells. When MHC-II restricted CD4⁺ T cells recognize the specific antigen presented by B cells, they help promote B cell activation and their differentiation into short-lived plasma cells that secrete low-affinity antibodies or into long-lived memory B cells that are capable of secreting high affinity antibodies during secondary infection¹⁵¹.

IgM is the first antibody isotype produced shortly after viral infection and although has low binding affinity, is capable of neutralizing virus as well as activating the complement system¹⁵². IgG and IgA isotypes arise around 2 weeks (or longer) after the onset of infection. In contrast to IgM, which typically decrease in levels over time, these isotypes are longer lived and can bind antigens with greater affinity. IgA confers protection at the mucosal surfaces such as within the respiratory tract¹⁵³ and whilst IgG is involved in long-term systemic protection¹⁵⁴. Memory B cells are generated along with recovery from primary immune responses to viral challenge and will elicit rapid response to secondary challenge to the same pathogen by producing profound and high-affinity Ig especially IgG¹⁴⁸.

During influenza virus infection, B cells form a critical arm of the humoral response by producing IAV-specific antibodies, especially those directed against surface glycoproteins, HA and NA^{155,156}. HA-specific antibodies can block interaction between the virus HA and host sialic acid receptors^{157,158} and prevent viral entry whilst NA-specific antibodies prevent the release of newly formed virions from the host cell membrane by inhibiting the cleavage function of this glycoprotein. Non-neutralizing antibodies against other internal viral proteins such as NP can contribute to virus elimination. Researchers have observed high levels of NP-specific antibodies after immunisation of mice with soluble recombinant NP. In the absence of NP-specific T cells responses, these immunized mice showed significantly lower viral titre and better survival rate¹⁵⁹. Other non-neutralizing antibodies, such as against M2e can provide protection in ADCC-dependent manner¹⁶⁰.

Antibodies driven by natural influenza virus infection can last for more than 50 years and can even provide some degree of protection against encountered strains which share similar epitopes from a previously encountered strain. Known as “immunological imprinting”¹⁶¹, a classical example is typified by individuals who were infected with the 1918 A/H1N1 pandemic strain. These individuals retained HA-specific antibodies for up to 90 years¹⁶² and were better protected during the 2009 A/H1N1 pandemic¹⁶³. Thus, encounter with influenza virus in childhood can contribute to an extent result in lifelong protective antibody responses to antigenically similar strains^{164,165}. Furthermore, H3N2 subtypes have been one of the dominant circulating strains for more than 50 years (since 1968) and serological studies show individuals born after 1968 are likely to be imprinted with immunity towards these strains but those born before are not¹⁶⁶. Immune responses against a specific clade of a virus strain

encountered early in life can, however, also have a detrimental effect later on future similar strains that have drifted. Middle-aged individuals who were born around 1968, although exposed to H3N2 strains in childhood, mainly produce non-neutralizing antibody responses upon encounter with more recent circulating 3c2.A H3N2 strains and thus are still susceptible¹⁶⁶.

Compared to antibody responses, CD8⁺ T cells can cross-protect against different subtypes of influenza virus, including influenza A, B and C¹⁶⁷. This is because CD8⁺ T cells can recognize highly conserved epitopes presented by infected cells, which are generated not only from viral surface proteins but also internal proteins such as NP, M1, PA and PB1 commonly shared by different strains¹⁶⁸. Thus, in addition to significant and ongoing efforts to improve antibody-based vaccines to offer better protection against influenza, much work is also now focused on harnessing the role of cross-protective CD8⁺ T-cell immunity for developing universal influenza virus vaccines that can protect against influenza irrespective of subtype. The importance of T cell responses will be discussed in following sections (1.4.3-1.4.5).

1.4.2 T cell development

T cells are generated from common lymphoid progenitors (CLPs) that migrate from the bone marrow to thymus via the blood and is driven by the expression of chemokine receptors such as CCR7 and CCR9. Studies show that deficiency of CCR7 in mice greatly impacts CLPs settlement in the thymus, while CCR7 can sustain CLPs migration to thymus in the absence of CCR9¹⁶⁹. T cells in the thymus begin their development as double-negative (DN) cells, wherein they do not express CD4 and CD8 on the cell surface nor do they express CD3. Where CD8 and CD4 are co-receptors that help signaling between TCR-pMHC-I and TCR-pMHC-II complexes during antigen presentation, respectively, CD3 is a heterotetrametric complex composed of 4 components including two CD3 ϵ chains, CD3 γ as well as CD3 δ chains which are essential for stabilization of TCRs¹⁷⁰.

During this DN stage, there are also different sub-stages (**Fig 5**), DN1 to DN4, which can be distinguished by surface expressing markers including CD44 (an adhesion protein, lymphocytes homing and activation maker), CD25 (interleukin-2 receptor or IL-2RA, cell proliferation marker) and c-kit (cytokine receptor) in mice. DN1 cells are c-kit⁺CD44⁺CD25⁻,

where they are adept at binding to extracellular matrix components produced by fibroblasts such as collagen or cell surface ligands expressed by cortical thymic epithelial cells (cTECs) such as Notch 1¹⁷¹, all of which are essential for survival in the cortex of the thymus. When thymocytes move from the DN1 to the DN2 stage, CD25 expression is increased and these cells proliferate as well as gradually migrating to the subcapsular regions of the thymus towards the outer cortex. This is also accompanied by the expression of CD3 chains. As cells progress to DN3, their expression of CD25 is maintained but c-kit and CD44 is downregulated, whilst proliferation ceases. During this critical stage, thymocytes become $\alpha\beta$ T cells in which functional pre-TCR complexes are generated. Cells further undergo β -selection during which cells with functional TCR β will be able to survive and actively proliferate as $\alpha\beta$ T cells. These cells are also called pre-T cells during which only about 10% establish productive TCR β rearrangement and survive to undergo further development¹⁷². Procession to DN4 is characterized by decreased expression of CD25, while low level of CD4 and CD8 can be detected on the cell surface.

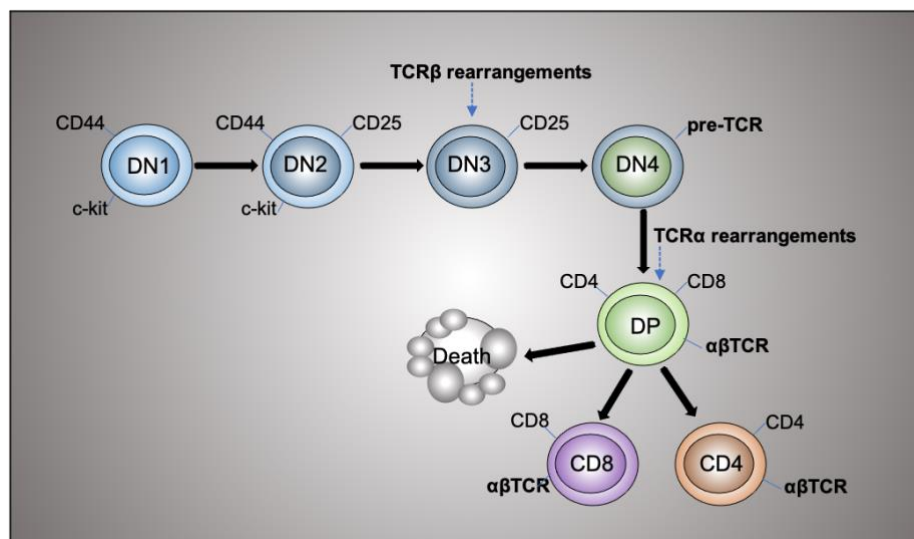


Fig 5. T cell development

Derived from common lymphoid progenitors (LPs) that migrate from the bone marrow to thymus, T cells experience several stages of development to become mature naïve T cells. At the first DN1 stage, cells are c-kit⁺CD44⁺CD25⁻ and are they are adept at binding to extracellular matrix components that are essential for survival. CD25 expression is increased when move from the DN1 to DN2 stage to promote proliferation. While expression of CD25 is maintained through to the DN3 stage, c-kit and CD44 is downregulated combined with decreased proliferation. During this stage, thymocytes become $\alpha\beta$ T cells in which functional pre-TCR complexes are generated at the cell surface with rearrangement of TCR β V(D)J segments occurring. The DN4 stage is characterized by expression of pre-TCR complexes and decreased expression of CD25. After passing through all 4 DN stages, CD4 and CD8 expression levels are significantly upregulated on cell surface where they then enter the “double-positive (DP) stage”. DP cells that survive positive and then negative selection finally become mature naïve T cells.

After passing through all 4 DN stages, CD4 and CD8 expression levels are significantly upregulated on cell surface where they then enter the “double-positive (DP) stage”. Herein, cells experience positive selection where they are exposed to self p-MHC complexes presented by cortical epithelial cells. DP thymocytes that are incapable of interacting with p-MHC complexes with a moderate affinity will undergo apoptosis, as only T cells that are able to recognize and bind to both antigen and host MHC molecules can mount an efficient immune response. This is then followed by negative selection which occurs in the medulla of the thymus where cells that bind to self p-MHC complexes with a strong affinity are eliminated. This process removes self-reactive T cells which could otherwise lead to the development of severe autoimmune diseases. Furthermore, DP thymocytes will be selected based on binding to either MHC-I or MHC-II to instruct their development into either CD8 or CD4 single positive T cells, respectively. T cells that complete these development stages become mature naïve CD4⁺ or CD8⁺ T cells that migrate out from thymus and ready for further activation as a result of pathogen invasion.

1.4.3 CD8⁺ T cell activation

During primary influenza virus infection, naïve CD8⁺ T cells require signals from DCs in the draining lymph node to become activated and differentiated into effector cells. There are three essential signals that trigger T cell activation: engagement of TCR and pMHC-I complexes, binding of costimulatory receptors and cytokine stimulation. When infected by influenza A virus, several type of DCs, including migratory CD103⁺ DCs and resident CD8 α ⁺ DCs can be recruited to the draining lymph node (LN) from site of infection (lung) driven by CCR7, which was showed to be more crucial for priming influenza-specific CD8⁺ T cells than CD4⁺ T cells¹⁷³. In addition, CD103⁺ DCs were also demonstrated to drive antigen presentation by migrating to mediastinal lymph nodes (MLNs) to activate naïve T cells^{174,175}. *Ex vivo* experiments show that migratory CD103⁺ DCs in primary influenza virus infected mice dominant over resident CD8 α ⁺ DCs for boosting influenza-specific CD8⁺ T cell proliferation¹⁷⁶. Naïve CD8⁺ T cells are activated upon recognizing pMHC-I complexes on antigen presenting DCs in concert with receiving costimulatory signals. These signals are provided by a heterogenous group of cell surface molecules including CD80 and CD86 that bind to CD28 on naïve T cells, which not only maintain T cell survival but also boost T cell activation by triggering subsequent signaling pathways and lead to production of cytokines. Signals can also be provided by binding of

several key molecules such as CD137 (4-1BB), CD134 (OX40) and inducible T cell co-stimulator (ICOS, CD278) on APCs with their respective ligands 41BBL, OX40L and ICOSL. Costimulatory signals are essential for naïve T cell activation and without them, can lead to T cell anergy.

The secretion of IL-12 and type I IFNs by DCs serves as a third requirement for the activation of CD8⁺ T cells. In the case of the IAV strains A/X31 (H3N2; X31) or A/Puerto Rico/8/1934 (H1N1; PR8), infection of C57BL/6 mice leads to the induction of virus-specific CD8⁺ T cells that recognize D^bNP₃₆₆₋₃₇₄ and D^bPA₂₂₄₋₂₃₃ as the most dominant primary responses. Activated CD8⁺ T cells can differentiate into cytotoxic T lymphocytes (CTLs) which are able to kill infected cells through the secretion of perforin and granzymes into the area of contact or immunological synapse¹⁷⁷. Another effector function of CTLs is to secrete pro-inflammatory and anti-viral cytokines, including TNF and IFN- γ to render the local environment non-permissive for viral replication¹⁷⁸. These cytokines work by inhibiting various steps of viral invasion process including viral entry, replication, release and transmission¹⁷⁹. For example, IFN- γ inhibits HCV entry by downregulating the expression of Claudin-1 on host cells, an essential viral receptor¹⁷⁹, and is also able to control infection by enhancing the lysis of IAV infected cells¹⁸⁰. In addition, CTLs produce chemokines including CCL3, CCL4 and CCL5 to recruit other immune cells to site of infection¹⁸¹. The cytotoxicity of CTLs is highly dependent on the type of APCs inducing their activation as well as tissue location. For example, draining LN-activated CTLs produce high levels of IFN- γ to aid in killing, whereas lung activated CTLs express lower levels of IFN- γ but high levels of the regulatory cytokine IL-10 to protect tissue from severe damage caused by excessive inflammation¹⁸². Furthermore, IL-15 produced by local DCs is vital for expansion and survival of effector CD8⁺ T cells¹⁸³.

Activated CD8⁺ T cells, including CTLs, can be phenotypically defined by the expression of several cell surface markers and transcription factors^{184,185}. For example, interferon regulatory factor 4 (IRF4), which is a member of the IRF transcription factor family, has been shown crucial for CD8⁺ T cell proliferation after activation as well as effector CD8⁺ T cell differentiation by modulating expression of other two transcription factors that also contribute to CD8⁺ T cell differentiation: Blimp1 and Tbet¹⁸⁶. The importance of IRF4 was demonstrated whereupon its depletion greatly impairs effector CD8⁺ T cell function and viral clearance¹⁸⁶. Of note, high surface expression of CD38 and HLA-DR can correlate with

activation in several acute and chronic viral infections in human, including HIV¹⁸⁷, HCV¹⁸⁸, EVD (Ebola virus disease)¹⁰³. CD38, also known as cyclic ADP ribose hydrolase 1 (ADPRC1), is a type II glycoprotein expressed on the surface of different immune cells, including T cells, B cells and NK cells. It is involved in cell adhesion, signal transduction during cell activation and calcium signalling after activation of ion channels in the cell¹⁸⁹. On the top of these, CD38 are indicated to be able to interact with cell membrane proteins such as TCR/CD3 complexes and CD19/CD81 complexes on B cells, therefore contributes to immune cell activations⁴³. Although HLA-DR is typically more associated with the presentation of antigens by APCs, it is upregulated on CD8⁺ T cells during the early phase of an infection, then gradually downregulated during the recovery phase¹⁹⁰. However, the persistence of CD38⁺HLA-DR⁺ CD8⁺ T cells is often associated with detrimental outcomes. It has been found that fatal EVD correlates with a higher proportion of CD38⁺HLA-DR⁺ CD8⁺ T cells with limited functional capacity¹⁰³. Furthermore, during HIV-1 infection in human, HLA-DR⁺ but not CD38⁺HLA-DR⁺ CD8⁺ T cells in patients are more effective in secreting IFN- γ ¹⁹¹. Recent findings in our laboratory found that fatal H7N9 infection is associated with the presence of a large population of CD38⁺HLA-DR⁺ CD8⁺ T cells that are unable to secrete IFN- γ ¹⁹². Furthermore, these T cells also express the marker PD-1 reflecting an exhausted phenotype and thus indicating that expression of CD38 and HLA-DR serves as a measure of influenza disease severity.

1.4.4 Memory CD8⁺ T cells establishment

Following disease resolution, most effector CD8⁺ T cells will undergo apoptosis, while a small population remains to form a memory pool that can recalled effectively upon re-infection. Effector CD8⁺ T cells can be divided into either memory precursor effector cells (MPECs) or short-lived effector cells (SLECs), wherein MPECs are destined to become long-lived memory CD8⁺ T cells while SLECs will not (**Fig 6**). Following recovery from influenza in mice, memory D^bNP₃₆₆₋₃₇₄ and D^bPA₂₂₄₋₂₃₃ specific CD8⁺ T cells can persist for at least 570 days after infection¹⁹³. Notably, optimal generation of memory CD8⁺ T cells can be established in draining lymph node as early as 3.5 days after influenza infection¹⁹⁴.

Memory CD8⁺ T cells are further divided into different subsets based on their expression of CD62L, from which CD62L^{hi} cells are classed as “central memory” cells (T_{CM}) and CD62L^{lo} cells as “effector memory” cells (T_{EM}). T_{CM} cells maintain high proliferative capacity but

exhibit low levels of cytotoxicity, preferring to circulate through secondary lymphoid organs and entering lymph nodes by moving through high endothelial venules in a CCR7-dependent manner. In contrast, T_{EM} cells are mainly located in the red pulp of the spleen as well as in non-lymphoid tissues¹⁹⁵ and do not depend on CCR7 for trafficking¹⁹⁶. These cells have a lower potential for proliferation but exhibit high levels of cytotoxicity.

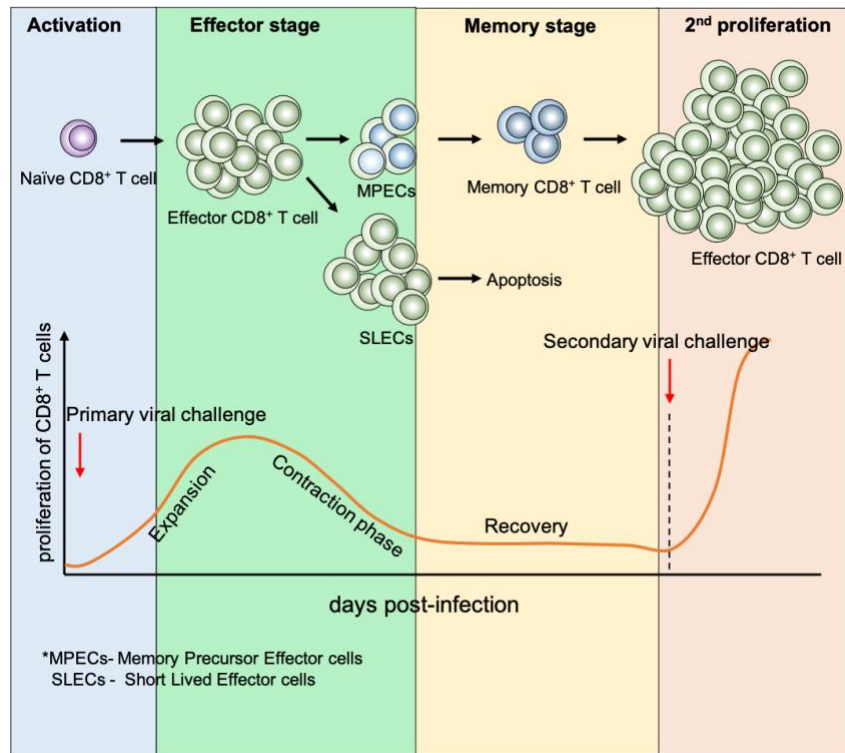


Fig 6. Dynamics of CD8⁺ T cell responses during virus infection

Following virus infection for the first time, naïve CD8⁺ T cells are activated and become effector cells and proliferate quickly. Effector CD8⁺ T cells are grouped into either short-lived effector cells (SLECs) that will eventually undergo apoptosis, or memory precursor effector cells (MPECs) which are able to survive after resolution of disease and become memory CD8⁺ T cells. Upon encounter with the same pathogen again, these memory CD8⁺ T cells can proliferate in a shorter time to perform their effector functions.

However, the generation of memory CD8⁺ T cells is not wholly dependent on CD62L expression but rather their tissue location¹⁹⁴. In contrast, downregulation of KLRG1 on KLRG1⁺ effector CD8⁺ T cells during the contraction phase is crucial for developing into long-lived memory CD8⁺ T cells¹⁹⁷. It also was shown that CD4⁺ T cells are important for CD8⁺ T cell memory formation. In CD4-deficient mice, CD8⁺ T cell priming and function induced by infection is not impacted but results in less functional memory CD8⁺ T cells compared to that in WT mice¹⁹⁸.

Another important type of memory CD8⁺ T cell are tissue-resident memory CD8⁺ T cells (T_{RM}). T_{RM} cells do not circulate through blood but instead persist at the location where infection occurred, which may include a variety range of tissue and organs. Their phenotype is mainly defined by the expression of CD69 and CD103^{199,200}. Although longevity is a characteristic of these cells, T_{RM} cell populations are replenished from T_{EM} pool²⁰¹. During influenza virus infection, T_{RM} cells located in both upper respiratory and lung are crucial for protecting the host from secondary infection. In the nasal epithelia, T_{RM} cells are able to prevent viral progression from upper respiratory into lung thus preventing the development of severe secondary influenza virus infection²⁰². Interestingly, due to constant exposure to pathogens in the respiratory tract, T_{RM} cells can themselves be susceptible to viral infection²⁰³. Studies have found that lung T_{RM} cells can also selectively express IFITM3, a key anti-viral mediator, in order to resist infection²⁰³. The establishment of influenza-specific CD8⁺ T_{RM} cells is not only dependent on the presence of cytokines and expression of transcription factors such as IL-15, TGF-β and T-bet^{204,205}, but can also be modulated by local environmental cues during the effector stage of the immune response. Alveolar macrophages can be a negative regulator of T_{RM} cells, as demonstrated by the increase in number of influenza-specific lung T_{RM} but not T_{EM} and T_{CM} cells following depletion of these macrophages in a CD169-DTR mouse model during the early stages of infection²⁰⁶. CD4⁺ T cells producing IFN-γ can be crucial for creating optimal conditions to generate influenza-specific T_{RM} cells²⁰⁷. Furthermore, inducing expression of 4-1BB on CD8⁺ T cells greatly boosts the generation of influenza-specific T_{RM} cells that can persist for more than 200 days, whereas of 4-1BB deficiency leads to failure of T_{RM} cells to form²⁰⁸. Notably, the proliferation of influenza-specific T_{RM} cells depends on local but not systemic administration of 4-1BBL²⁰⁸.

1.4.5 Recalling ability of memory CD8⁺ T cells

Recalling influenza-specific memory CD8⁺ T cell responses upon secondary exposure to a virus is generally more rapid than when the same virus is encountered for the first time (usually 2 days quicker than a primary infection in mice)¹⁹³. Several transcription factors can contribute to recall ability of memory CD8⁺ T cells. For example, Blimp-1 is essential for the differentiation of naïve CD8⁺ T cells into functional effector cells and although Blimp-1 deficiency does not impact memory formation, it can result in impaired recall responses²⁰⁹. In

addition, IRF4 has been proven to be vital for T_{RM} cells formation as well as recall ability and proliferation of IFN- γ ⁺ CD8⁺ T cells during heterosubtypic IAV infection²¹⁰.

The cytokine producing profiles of T_{CM} and T_{EM} cells can also differ with T_{EM} cells mainly producing Th1-type cytokines including IL-12 and IFN- γ while T_{CM} cells produce Th2-type cytokines such as IL-4 and IL-10 during antigenic stimulation²¹¹. Studies also found that despite their robust proliferative capacities and circulation throughout the body during secondary viral challenge, T_{CM} and T_{EM} cells do not appear to contribute to reducing viral infection as much as T_{RM} cells¹⁹⁹. While similar frequencies of D^bNP₃₆₆₋₃₇₄ and D^bPA₂₂₄₋₂₃₃ specific CD8⁺ T cells are found following primary infection, D^bNP₃₆₆₋₃₇₄ CD8⁺ T cells proliferate more rapidly upon recall and dominate secondary T cell responses^{193,212}. This may be due to several reasons: the persistence of antigen at the site of infection given NP expression can be sustained in the lung for weeks after recovery from primary viral infection²¹³, as well as antigen load and signals received from interacting with a wide spectrum of cell subsets²¹⁴. Studies show that D^bNP₃₆₆₋₃₇₄ CD8⁺ T cells are predominantly in contact with CD103⁺CD11b^{hi} DCs that express CD40 and CD70 during the late stages of primary infection. This allows memory CD8⁺ T cells to retain CD25 expression and thus responsiveness to IL-2 leading to profound proliferative capacities and cytotoxicity during secondary viral challenge. In contrast, D^bPA₂₂₄₋₂₃₃-specific CD8⁺ T cells do not interact with these specific DCs during primary infection, leading to reduced proliferation potential and cytotoxicity upon recall²¹⁵.

D^bNP₃₆₆₋₃₇₄- and D^bPA₂₂₄₋₂₃₃-specific T_{RM} cells are also generated differently depending on the manner of their TCR-p-MHC-I interaction, which can lead to distinct transcriptomic signatures between these populations^{216,217}. For example, lung D^bNP₃₆₆₋₃₇₄ T_{RM} cells persistently stimulated via their TCR leads to the formation of two distinct phenotypes reminiscent of conventional or exhausted-like memory CD8⁺ T cells, the latter of which is associated with the upregulation of exhaustion-related genes *Pdcd1*, *Tigit*, *Havcr2* and *Lag3*²¹⁷. Interestingly, compared to D^bPA₂₂₄₋₂₃₃ T_{RM} cells and splenic memory T cells, these exhausted-like D^bNP₃₆₆₋₃₇₄ T_{RM} cells still appear to play an important role not only offering efficient protective capabilities during acute secondary viral infection but also regulating immune responses to avoid excessive tissue damage²¹⁷.

1.5 Metabolism and T cell immunity

1.5.1 T cell metabolism during effector immune responses

T cell activation, differentiation as well as their functions are highly regulated by their metabolic state during different stages of the immune response. This can greatly affect epigenetic changes by DNA methylation and/or histone modification²¹⁸ and impact their fate²¹⁹. During viral infection or tumour invasion, the metabolic signature in T cells alters as cells differentiate from resting naïve to activated effector cells. Naïve T cells tend to have relatively low energy requirements and maintain low rates of glycolysis and nutrient uptake, preferentially using oxidative phosphorylation (OXPHOS) or fatty acid oxidation (FAO) to produce adenosine triphosphate (ATP) which carry and offers energy to cellular processes (**Fig 7**). Their catabolism changes, however, when they transit to become effector T cells^{220,221}.

Following T cell activation, especially when T cells migrate from lymphoid organs to the site of infection, microenvironment changes which include nutrient availability and oxygen levels can lead to dramatic alterations of cellular metabolic pathways due to an increase in energy requirements needed for trafficking, proliferation and performing effector functions. To meet these requirements, increased uptake of both glucose and amino acids are necessary to meet high energy demands for *de novo* fatty acid, amino acid and protein synthesis^{222,223}. Consequently, T cells switch to anaerobic glycolysis which although is a less efficient way to generate ATP than aerobic glycolysis, it is rapid and more sustainable. Generally, when T cells activated by TCR-pMHC interaction, downstream CD28-dependent signalling pathways are triggered, during which phosphoinositide 3-kinase (PI3K) activation enhances glucose transporter 1 (GLUT1 or SLC2A1) expression on cell surface to increase glucose transport and uptake^{224,225}. PI3K also triggers activation of AKT and mTOR complex 1 (mTORC1), which is one of the central regulators for metabolic reprogramming during the effector stage to boost glycolysis and is crucial for efficient T cell activation as well as subsequent metabolic reprogramming²²⁶. Apart from enhanced uptake of glucose, activation of T cells also leads to greater uptake of amino acids under control of the metabolic regulator Myc²²⁷ for *de novo* biosynthesis of cellular products. For example, glutamine and leucine are essential for mTORC1 activation²²⁸ with the former also essential for fatty acid synthesis via glutaminolysis²²³.

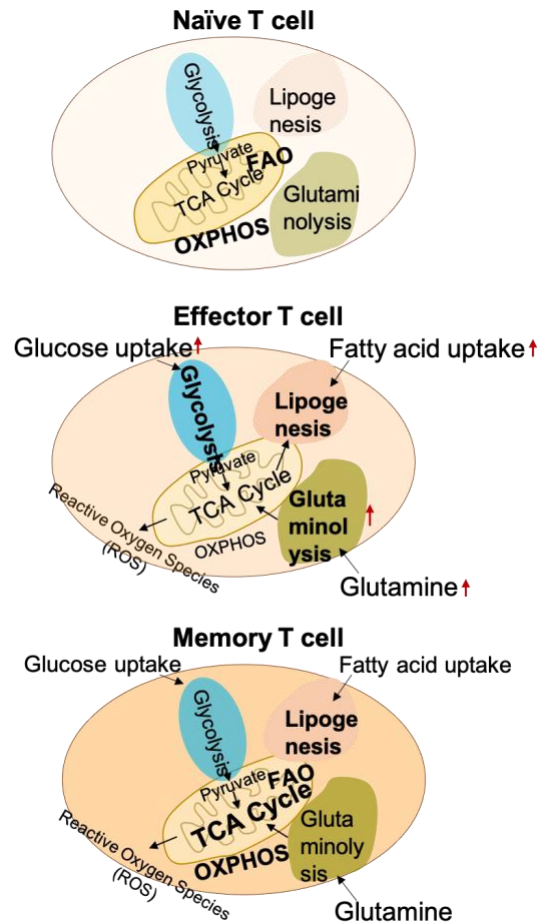


Fig 7. Dynamics of T cell metabolism

Naïve T cells tend to have relatively low energy requirements and maintain low rates of glycolysis and nutrient uptake, preferentially using oxidative phosphorylation (OXPHOS) or fatty acid oxidation (FAO) to produce adenosine triphosphate (ATP) as an energy source to drive cellular processes. When naïve T cells are activated to become effector cells, due to an increase in energy requirements needed for trafficking, proliferation and performing effector functions, they switch to anaerobic glycolysis where glucose, glutamine and fatty acid uptake are increased to meet high demand for energy and cellular processes. Following contraction of the immune response and as effector T cells progress to become long-term memory cells, their metabolic pathways are reprogrammed where mitochondrial OXPHOS and FAO become the predominant source of energy and glycolytic flux is blocked to support the generation of memory populations, together with decreased uptake of nutrients from external environment.

Metabolic reprogramming not only occurs during T cell activation but also throughout their differentiation into various subtypes wherein their usage of glycolysis and amino acids as well as degree of biosynthesis can be distinct²²³. In the case of CD4⁺ T cells, deficiency of mTOR leads to impaired effector CD4⁺ T cell generation and instead promotes the development of regulatory T (Treg) cells²²⁹. Both mTORC1 and mTORC2 can also show essential for Th1/Th17 and Th2 generation, respectively, due to the engagement of different pathways utilised for glycolysis. Selective depletion of mTOR1 activator leads to failure of cells to differentiate into Th1 and Th17 types but does not affect the development of Th2 cells

which exhibit high glycolytic activity²³⁰. Inversely, deficiency of mTORC2 suppresses Th2 differentiation but does not impact their development into Th1 or Th17 cells²³¹. In the case of CD8⁺ T cells, mTOR also plays a central role in modulating CTL function and IFN- γ production^{232,233}. CTLs function can depend on glucose uptake, which can be stimulated by mTORC1 via modulating hypoxia-inducible factor (HIF1 α)²³⁴, including production of IFN- γ , GM-CSF as well as Granzyme B protein which are crucial for CTLs cytotoxicity and proliferation²³⁵.

Apart from mTOR induced metabolic reprogramming, studies have shown that autophagy plays an important role in directing glucose metabolism. Disrupting the autophagy pathway in cells can enhance glucose metabolism boosting the effector function of tumor antigen-specific CD8⁺ T cells²³⁶. Concomitant with enhanced glycolysis as well as nutrient uptake, T cell activation also leads to increased OXPHOS and mitochondrial biogenesis including mitochondrial fusion²³⁷ to meet the energy requirements²²³. Recent studies showed the importance of OXPHOS on the self-renewing capacities of T cells with impaired OXPHOS leading to failure of T cells to self-renew and contributing to T cell exhaustion²³⁸. Furthermore, without CD4⁺ T cell help in regulating metabolic channels during the primary effector phase, influenza-specific memory CD8⁺ T cells display defective OXPHOS and an exhaustion phenotype at the transcriptional level which can limit their recall ability during viral re-challenge²³⁹.

1.5.2 Metabolic reprogramming in memory T cells and recall responses

During the contraction of the T cell response, the metabolic pathways utilised by effector T cells destined to become long-term memory T cells are reprogrammed as mitochondrial OXPHOS and FAO become the predominant source of energy and glycolytic flux is blocked to support the generation of memory T cells²⁴⁰. Another important change is the suppression of mTOR activation by 5'-AMP-activated kinase (AMPK), a kinase involved in metabolic sensing, which inhibits mTOR activity by activating tuberous sclerosis complex 2 (TSC2) to subsequently block glycolytic flux^{241,242}. Different subtypes of memory T cells also differ in their metabolic profile for energy generation. As T_{CM} cells maintain higher potential for proliferation compared to T_{EM} cell, their mitochondrial respiratory capacity is increased²⁴³. Conversely, given that increased glycolysis favours the generation of subsequent T_{EM} cells²⁴³,

they are more resistant to a hypoxic environment compared to T_{CM} cells²⁴⁴. In the case of T_{RM} cells, their persistence and survival is largely dependent on the uptake of exogenous free fatty acids (FFA) as well as using mitochondrial FFA β -oxidation. This is in line with their use of fatty-acid-binding proteins FABP4 and FABP5 for maintaining longevity and function^{245,246}. Interestingly, in comparison to naive or effector T cells, increased mitochondrial mass in memory T cells have been observed resulting in better mitochondrial spare respiratory capacity (SRC) which helps them to generate more energy to overcome environmental stresses or low nutrient supply²⁴⁷. Previous studies also found that IL-15 can promote mitochondrial biogenesis and induce this catabolic switch²⁴⁸. Overall, these metabolic changes help memory T cells to rapidly react to secondary pathogen challenge²⁴⁹.

Fatty acid synthesis is also an important process for T cell differentiation and formation of memory. Acetyl-CoA carboxylases (ACCs) are enzymes crucial for regulating cellular fatty acid metabolism. In the absence of ACC1 (or ACACA), an isoform in humans, formation of T helper 17 (Th17) cells can be suppressed by blocking fatty acid synthesis and can lead to the development of Treg cells²⁵⁰. Moreover, tumour-necrosis receptor-associated factor-6 (TRAF6) is an adapter protein that regulates memory CD8⁺ T cell development by modulating fatty acid metabolism and mice lacking TRAF6 experience strong effector CD8⁺ T cell responses but lack the ability to generate memory T cells²⁵¹.

While the mechanisms and processes involved in the transition of naïve CD8⁺ T cells into effector and memory populations during viral infection have been well studied, there is still much to learn about how cellular metabolism regulates and affects these outcomes. The findings from the studies highlighted here clearly underscore the importance that different metabolic pathways play in the induction and maintenance of T cell immunity. By interfering with autophagy pathways involved in CD8⁺ T cell metabolism, researchers found these results in a large skew towards CD8⁺ T cells with an effector-like phenotype and increased cytokines production, resulting in controlling and slowing tumor growth²³⁶. Moreover, metabolic conditions such as obesity are recognised as a contributing factor in the impairment of T cell immunity during influenza infection^{252,253} and cancer models²⁵⁴. Of relevance to the topic of this thesis, during severe influenza virus infection, T cell effector function is greatly influenced by modulating insulin receptor which is essential for glucose uptake²⁵⁵. Researchers found lack of T cell-specific insulin receptor greatly impairs antigen-specific immune responses in severe influenza disease model²⁵⁵. These findings and more highlight the relationship between T cell

metabolism and immunity and further studies into their underlying mechanisms and links in the context of disease are crucial for providing ways of how we can improve protection against tumors and infections.

1.6 Rationale and Objectives

Understanding host immune responses to against viral infections is key to developing new strategies that can protect against pathogen invasion. T cell immune responses as well as cellular metabolism are crucial factors that can alter the disease outcomes in influenza-infected patients. This PhD thesis sought to investigate the mechanism(s) how an activated subset of T cells and a host metabolism-associated gene in impacting immune responses during severe influenza disease.

The specific aims and hypothesis of this PhD study were:

1. To understand the role of CD38⁺MHC-II⁺ CD8⁺ T cells during severe influenza A virus infection. We hypothesised that different activation patterns of CD38⁺MHC-II⁺ CD8⁺ T cells impact the function of CD8⁺ T cells and contribute to disease severity.
2. To define early bio-markers of disease severity in fatal H7N9 infection. We hypothesised that key gene features will be associated with fatal outcome of H7N9 disease.
3. To determine the impact of OLAH expression in regulating host immune responses in severe influenza A virus infection. We hypothesised that the host metabolism related gene, OLAH, can greatly impact immune responses towards severe influenza disease and can be a potential early stage bio-marker to benefit future clinical treatment.

CHAPTER II: MATERIALS AND METHODS

2.1 Materials

2.1.1 Mice

All mice strains used for experiments described in this thesis were bred and maintained at the Animal Facility in the Department of Microbiology and Immunology at the University of Melbourne (Parkville, VIC, Australia). Mice aged at least 6 weeks old (**Table 2.1**) were used in all experiments. C57BL/6 mice were used throughout this thesis. OT-I/Ly5.1 and MHC-II^{-/-} mice were used in chapter III, while OLAH^{-/-} mice were used in chapter V.

Table 2.1 Mouse strains

Mouse strain	MHC-I haplotype	Description	Chapter
C57BL/6 (B6)	H2-D ^b , H2-K ^b	Expresses the congenic marker Ly5.2	III, IV, V
OT-I/Ly5.1	H2-D ^b , H2-K ^b	B6 background, expresses a T cell receptor that recognizes the OVA ₂₅₇₋₂₆₄ peptide (SIINFEKL) presented on H2-K ^b ; expresses the congenic marker Ly5.1	III
MHC-II ^{-/-}	H2-D ^b , H2-K ^b	B6 background; MHC-II I-A ^b gene is deleted	III
OLA ^H ^{-/-} (OLA ^H KO)	H2-D ^b , H2-K ^b	B6 background, Oleoyl-ACP Hydrolyse (OLA ^H) gene is deleted	V

2.1.2 Influenza A virus strains

Influenza A virus strains used for experiments are listed and described in **Table 2.2**. All influenza A virus strain stocks were grown in the allantoic cavity of 10-day old embryonated chicken eggs for 72 hours at 37°C and viral titres were determined by plaque assays (**section 2.2.6**).

Table 2.2 Influenza A virus strains used in this thesis

Virus strain*	Subtype	Additional information	Chapter
A/Hong Kong/1/1968	H3N2	Abbreviation X31	III, V
A/PR/8/1934 (PR8)	H1N1	Abbreviation PR8	V
X31-OVA	H3N2	recombinant X31 virus containing the H2k ^b restricted OVA ₂₅₇ epitope	III
PR8-OVA	H1N1	recombinant PR8 virus containing the H2k ^b restricted OVA ₂₅₇ epitope	III

*All strains were originally obtained from the Department of Immunology, St Jude Children's Research Hospital, Memphis, TN, USA.

2.1.3 CD8⁺ T cell peptides

Peptides used for experiments are listed and described in **Table 2.3**. Peptides were purchased from GL Biochem (Shanghai, China; at least 80% purity) and resuspended at a concentration of 1mM in HBSS and stored at -80°C.

Table 2.3 Peptides used in this thesis

Peptide	Abbreviation	Sequence	MHC-I restriction	Chapter
Nuclearprotein ₃₆₆₋₃₇₄	NP ₃₆₆₋₃₇₄	ASNENMETM	H2-D ^b	III, V
Polymerase acidic protein ₂₂₄₋₂₃₃	PA ₂₂₄₋₂₃₃	SSLENFRAYV	H2-D ^b	III, V

2.1.4 Buffers and Media

MDCK cell media

Dulbecco's Modified Essential Media (DMEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 ug/ml streptomycin and 10% Heat-inactivated (incubated at 56°C for 30 minutes) fetal calf serum (HI-FCS) (all Gibco, Invitrogen Cell Culture, Carlsbad, CA, USA).

SF-MDCK media

DMEM supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 10 U/ml penicillin, 100µg/ml streptomycin (all Gibco).

Complete RPMI (cRPMI)

RPMI-1640 media supplemented with Enriched supplement (2mM L-glutamine, 1mM MEM sodium pyruvate, 100µM (1x) MEM non-essential amino acids, 5mM HEPES buffer solution, 55µM 2-mercaptoethanol), 100 U/ml penicillin, 100 µg/ml streptomycin and 10% HI-FCS (all Gibco).

2× L15 media

27.4 g of Leibovitz L-15 medium powder (Gibco), 0.56 mg/ml of NaHCO₃ (in Hanks Buffered Salt Solution (HBSS; Media Preparation Unit, Department of Microbiology and Immunology, the University of Melbourne), 800 µM Hepes buffer (pH 6.8), 200 U/ml penicillin (Gibco), 200 µg/ml streptomycin (Gibco), were mixed and dissolved in 1L Baxter water (Baxter Healthcare, Old Toongabbie, NSW, Australia).

RPMI-anti

RPMI media (Media Preparation Unit, Department of Microbiology and Immunology, the University of Melbourne) supplemented with 100 U/ml penicillin (Gibco) and 100 mg/ml streptomycin (Gibco).

MACS buffer

PBS (Media Preparation Unit, Department of Microbiology and Immunology, the University of Melbourne), supplemented with 1% v/v Bovine Serum Albumin (BSA; Gibco) and 0.02% v/v Sodium Azide (Sigma Aldrich).

1% Paraformaldehyde (PFA)

37% PFA (Sigma) diluted in MACS buffer (MDU).

Mouse FcR block

2.4G2 hybridoma supernatant (containing anti-CD16/CD32) with 1% (v/v) mouse and 1% (v/v) rat serum.

Sort buffer

PBS (Media Preparation Unit, Department of Microbiology and Immunology, the University of Melbourne) supplemented with 1% FCS.

Ammonium Tris Chloride (ATC) buffer

0.14M NH₄Cl and 0.017M Tris dissolved in 500ml distilled H₂O.

Lung digestion buffer

2mg/ml collagenase III powder (Worthington Biochemical Corporation) and 100µg/ml DNase-I were dissolved in RPMI-anti and pre-warmed to 37°C before use.

NP-40 buffer

30 ml of 5 M NaCl, 100 ml of 10% NP-40, 50 ml of 1 M Tris (pH 8.0) dissolved in 820 ml of H₂O. Store at 4°C.

10% SDS PAGE gel (amounts to make up 2 gels)

Lower gel	Upper gel
dH ₂ O 4ml	dH ₂ O 1.58ml
Lower gel buffer 2ml	Upper gel buffer 0.63ml
40% acrylamide 2ml	40% acrylamide 0.25ml
10% APS 40.5ul	10% APS 100ul
TEMED 6ul	TEMED 6ul

Tris/glycine transfer buffer (make in advance and store at 4 degrees):

25mM Tris, 192mM glycine, 10% methanol, 0.1% SDS dissolved in MiliQ to 1L.

Blocking reagent

50ml 10xPBS, 2.5g BSA (5%), 0.5ml Tween-20 (0.1%) dissolved in 440ml MiliQ.

PBST (wash buffer):

100ml 10xPBS, 0.5ml Tween-20 (0.05%) dissolved in MiliQ to 1L.

2.1.5 Antibodies

Anti-mouse monoclonal antibodies used in this thesis for flow cytometry are listed in **Table 2.4**.

Table 2.4 Antibodies for flow cytometry used in this thesis

Antibody	Conjugated fluorochrome	company	clone
CD3	AF700	Invitrogen	eBio500A2
	Percp-Cy5.5	BD Biosciences	145-2C11
CD8	AF700	Biolegend	53-6.7
	PE	Biolegend	53-6.7

	Percp-Cy5.5	BD Biosciences	53-6.7
CD4	APC-Cy7	BD Biosciences	GK1.5
CD38	PE-Cy7	Biolegend	90
	BV711	BD Biosciences	90
I-Ab	Pacific Blue	Biolegend	AF6-120.1
CD44	FITC	BD Biosciences	IM7
	AF700	BD Biosciences	IM7
CD62L	BV570	Biolegend	MEL-14
CD45.1	APC-Cy7	Biolegend	A20
	PE	BD Biosciences	A20
CD45.2	BV711	BD Biosciences	104
TCRva2	PE-Cy7	BD Biosciences	B20.1
CD11b	FITC	Invitrogen	M170
	BV605	BD Biosciences	M170
CD11c	FITC	eBiosciences	N418
	PE	BD Biosciences	HL3
B220	FITC	Biolegend	RA3-6B2
CD19	FITC	Biolegend	6D5
NK1.1	FITC	Biolegend	PK136
	PE	BD Biosciences	PK136
F4/80	FITC	Biolegend	CI:A3-1
Ly6G	PE-Cy7	BD Biosciences	1A8
CD64	AF647	BD Biosciences	X54-5/7.1
	BV421	Biolegend	139309
SigLecF	PECF594	BD Biosciences	E50-2440
Ly6C	AF700	BD Biosciences	AL-21
EpCam	APC-Cy7	Biolegend	G8.8
CD31	BV421	Biolegend	390
CD45	PerCP	BD Biosciences	30-F11
CD36	APC	BD Biosciences	562744
TCRgd	BV421	BD Biosciences	GL3
TCRβ	BV711	BD Biosciences	H57-597
CD27	Percp-Cy5.5	Biolegend	LG.3A10
IFN-γ	FITC	Biolegend	XMG1.2
TNF-α	APC	BD Pharmingen	554420
IL-2	PE	BD Pharmingen	554428
Flu A- NP	FITC	GeneTex	GTX36902

2.1.6 Peptide-MHC class I (pMHC-I) tetramers

pMHC-I tetramers used in this thesis are listed and described in **Table 2.5**. All biotinylated pMHC-I monomers were made by Dr. Jie Lin (Department of Microbiology and Immunology, the University of Melbourne). For tetramerization, fluorochrome were added in 6 separate aliquots to monomers (1mg/ml) and held at room temperature in the dark for 10 min incubation following each aliquot. Streptavidin (SA) conjugated fluorophores (SA-PE or SA-APC, 2mg/ml, Invitrogen) was used to achieve a final monomer/SA ratio of 4:1. mCD1d- α -GalCer tetramer was provided by Professor Dale Godfrey (Department of Microbiology and Immunology, the University of Melbourne).

Table 2.5 pMHC-I Tetramers used in this thesis

Tetramer	Peptide/Glycolipid	Chapter
H2-D ^b NP ₃₆₆	NP ₃₆₆₋₃₇₄	III,V
H2-D ^b PA ₂₂₄	PA ₂₂₄₋₂₃₃	III,V
mCD1d- α -GalCer	α -GalCer	V

2.1.7 Plasmids used for retroviral transduction

pMIG-II-OLAH plasmid was generated by Prof. Zhongfang Wang from Guangzhou Medical University, China. pPAM-E (shuttle vector) and pVSVG (encoding viral glycoprotein envelope) plasmids for retroviral transduction were provided by Dr. Zhenjun Chen from Department of Microbiology and Immunology, University of Melbourne.

2.2 Methods

2.2.1 Infection of mice with influenza virus

Mice were anaesthetized by isoflurane (Veterinary Companies of Australia, Kings Park, NSW, Australia) inhalation using an anaesthetic machine (Advanced Anaesthesia Specialists, Sydney, NSW, Australia) and infected intranasally with influenza virus in 30 μ l of PBS. Mice were infected with 10² (mild), 10⁴ (sub-lethal) and 10⁵ (severe)PFU of X31 or X31-OVA virus and

re-challenged with 10^4 PFU of PR8 or PR8-OVA virus according to the experiment design. Primary and secondary infections were performed 4-6 weeks apart.

2.2.2 Adoptive transfer of cells

Cells were resuspended in PBS to achieve a desired concentration in 200 μ l per mouse and intravenously administered via the tail vein using an insulin syringe (BD) equipped with a 29G needle. Mice were rested for 24 hrs following transfers before experiments were conducted.

2.2.3 Bone-marrow chimera adoptive transfers

CD45.1 or CD45.2-expressing C57BL/6 wildtype and CD45.2-expressing OLAH KO mice were lethally irradiated with 2 doses of 550 rad 3 hours apart and reconstituted with 5×10^6 T cell-depleted bone marrow cells from CD45.1- or CD45.2-expressing wildtype or CD45.2-expressing OLAH KO mice. Donor and recipient cells were discriminated by isoform expression *in vivo*. Mice were depleted of radioresistant T cells on the following day by intraperitoneal injection with anti-Thy1 antibody (clone T24). Animals were maintained on antibiotics (neomycin and polymyxin B, Sigma-Aldrich) contained in drinking water for 6 weeks. Chimeric mice were allowed to reconstitute for at least 8 weeks by which time they reached an average reconstitution of >90%.

2.2.4 Tissue sampling and cell counting

To assess immune responses during influenza A virus infection, bronchoalveolar lavage (BAL), nasal turbinate, lung, mediastinal lymph nodes (MLN) and spleens were collected. For the collection of BAL, a gavage catheter was inserted into an incision made in the trachea of a mouse. A 1ml syringe (Terumo Corp.) containing PBS was attached to this catheter and used to instill the bronchioles. The instilled fluid was gently retracted, and the action was repeated 3 times. The collected fluid was then centrifuged for 6 min at 500g at 4°C to pellet cells. Following discard of the supernatant, the cell pellet was resuspended with cRPMI.

For the collection of nasal turbinate, the nasal cavity (including the upper palette) was dissected from the skull, spliced into 2 halves with scalpel blade and transferred into 5ml bijoux tubes. 2ml of digestion buffer was added and incubated for 30min at 37°C before topping up

with 2ml of cRPMI. The digested tissue was passed through a metal sieve and meshed with a 10ml syringe plunger in a petri dish to obtain a single cell suspension. This was collected into a 10ml centrifuge tube (Sarstedt, Germany) and centrifuged at 6 min at 500g. Supernatant was aspirated and cells were resuspended in 3ml of pre-warmed ATC buffer and incubated at 37°C for 5 min. Samples were centrifuged again and cells were resuspended in cRPMI. For the collection of lungs, a perfusion was performed with 3ml PBS using 3ml syringe (Terumo Corp.) and 21-G needle (TERUMO) by injection into the right ventricle. Whole lung tissue was then collected into plastic bijoux tubes and finely minced before adding 2ml of digestion buffer and incubated for 30min at 37°C. The sample was then topped up with 2ml of cRPMI and meshed through a 70µm sieve strainer (Greiner) attached to a 50ml centrifuge tube (Falcon) using a 5ml syringe plunger. Cells were then centrifuged for 6 min at 500g and the pellet resuspended in 3ml of pre-warmed ATC buffer and incubated at 37°C for 5min. Samples were centrifuged again and cells were resuspended in cRPMI. For the collection of MLN and spleens, each tissue was collected into a plastic bijoux tubes containing 3ml PBS and then meshed through 70µm sieve strainer (Greiner), as described above for the lung tissue and in the case of the spleens, also treated with pre-warmed ATC.

For the analysis of viral titres by plaque assay and cytokines levels by Cytokine Bead Array (CBA), BAL and lungs were collected as described above with minor modifications. Lungs were not perfused prior to harvesting and were instead collected in 13ml round bottom tubes (Sarstedt, Nümbrecht, Germany) filled with 2ml of RPMI-anti medium. Samples were then disrupted using a homogenizer (Kinematica, Lucerne, Switzerland) and centrifuged at 6 min at 500g at 4°C. Clarified supernatants from centrifuged homogenized lungs as well as BAL were collected and stored at -80°C. All cell suspensions were counted by trypan blue exclusion using a hemocytometer or diluted at 1:100 in isotonic solution IsoFlow (Beckman Coulter Life Sciences, Australia) and counted by size exclusion using a Coulter-counter machine (Beckman Coulter Life Sciences, Australia).

To prepare samples for histopathology, lungs were collected as described above with minor modifications. Lungs were not perfused prior to harvesting and were instead collected in a 5ml tube containing 10% neutral formalin buffer at room temperature. Collected samples were then sent to the Department of Anatomy & Neuroscience, University of Melbourne for analysis.

To prepare samples for immunofluorescence staining, mice lungs were slowly inflated and embedded with optimum cutting temperature (OCT) fluid and stored at -80°C. Frozen lung sections were cut using a Leica CM 3050 S cryostat in 14 µm sections, mounted on microscope slides and stored at -80°C until analysis.

2.2.5 Intracellular protein detection by immunofluorescence microscopy

Thawed mouse lung sections were fixed with 4% paraformaldehyde (Sigma) for 15 min at room temperature and then washed with PBS. Samples were then permeabilised with 0.1% Triton X-100 (Sigma T9284-500ml) for 10 min at room temperature then washed with PBS and followed by blocking with 1% BSA for 30 mins. After washed with PBS, blocked sections were stained with primary α Influenza A virus nucleoprotein (NP) (Kindly provided by Prof, Weisan Chen, La Trobe University) for 1 hour, washed and stained with secondary antibody Alexa Fluor 555 (Life technologies) for 1h. Lipid droplets (LDs) were stained by BODIPY 493/505 (Life technologies) for 1h at room temperature and nuclei were stained with DAPI (Sigma) for 5 min. Sections were mounted using Vectashield Antifade Mounting Medium (Vector Laboratories) prior to imaging on a ZEISS 800 confocal microscope. Images were processed using Zeiss ZEN blue software and ImageJ analysis software. LD numbers were analysed from acquired z-stack maximum Intensity projection images (Zeiss ZEN blue addition software) in ImageJ using the particle analysis tool and normalised to average LD per cell. For each mouse lung sample, at least 9 fields of view from different locations across the coverslip were imaged at 63X magnification.

2.2.6 Madin-Darby Canine Kidney (MDCK) cell plaque assays

Supernatant from collected BAL samples or lung homogenates were used to determine viral titres by plaque assays on Madin-Darby Canine Kidney (MDCK) cells. MDCK cells were thawed from Liquid Nitrogen and passaged for 2-3 times prior to use in these assays. Cells were seeded in 6-well culture plates (Techno Plastic Products, VIC, Australia) at 1.2×10^6 cells in 3ml of MDCK media per well and incubated at 37°C (5% CO₂) incubator for 24 hours to allow formation of a confluent monolayer. On the day of the assay, samples were thawed and serially diluted 10-fold in SF-MDCK media. MDCK cells in 6-well culture plates were washed with PBS or RPMI-anti before the addition of 120-150µl of each diluted sample (in duplicate

wells) and incubated for 45min at 37°C (5% CO₂). 2×L15 media was pre-warmed in a 56°C water bath and diluted 1:2 with water (Baxter, country) containing 1.8% agarose (Sigma-Aldrich) supplemented with 2ug/ml Trypsin Worthington (Worthington Biochemical Corporation) right before overlaying onto MDCK cells at 3ml per well. Plates were incubated at 37°C (5% CO₂) for 72 hours and plaques manually counted.

2.2.7 Cytometric Bead Array (CBA)

Supernatant from collected BAL samples or lung homogenates were used to measure the concentration of cytokines using a BD CBA flex set (BD Bioscience) as per the manufacturer's instructions. Samples were analysed using a Becton Dickinson FACS Canto II flow cytometer and data analysed using FCAP Array software (Soft Flow Inc., Pecs, Hungary).

2.2.8 Tetramer and antibody staining

Cells collected from the BAL, nasal turbinate, lung, MLN and spleens were seeded onto U-bottom 96-well plates (Sigma-Aldrich), washed twice with PBS and stained with Live/Dead viability dye (Thermo Fisher) at room temperature in the dark for 10 mins. Cells were then washed with MACS buffer and stained with tetramer in MACS buffer for 1 hour at room temperature in the dark and washed with MACS buffer. Antibody cocktails containing fluorochrome-conjugated antibodies supplemented with mouse FcR block were then used to subsequently stain cells for 30 mins on ice in dark. After washing, cells were fixed with 1% paraformaldehyde before analysis by flow cytometry. Samples were analysed on an LSR Fortessa (BD Biosciences). All flow cytometric data were analysed on FlowJo software version X (FlowJo LLC, Becton Dickinson).

2.2.9 Peptide stimulation and intracellular antibody and cytokine staining

Harvested BAL, nasal turbinate, lung, MLN and spleen cells were seeded onto U-bottom 96-well culture plates (Sigma-Aldrich) and resuspended with cRPMI containing 1µM peptide, 1 µg/ml Golgi-Plug (BD Biosciences) and 10 U/ml recombinant human IL-2 (Roche Diagnostics, Basel, Switzerland) and incubated at 37°C (5% CO₂) incubator for 5 hours. Cells from each

sample were also treated under the same conditions but in the absence of peptide as stimulation controls.

For intracellular antibody and cytokine staining, cells were washed twice with PBS and stained with Live/Dead viability dye for 10 min at room temperature in the dark. Samples were then washed with MACS buffer and stained with surface antibody cocktail at 4°C on ice in the dark. Following 2 washes of MACS buffer, cells were fixed and permeabilized by using BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences) for 20 minutes on ice and then washed with Perm Wash buffer (BD Biosciences). Fluorochrome-conjugated antibodies for intracellular markers or cytokines were made up in Perm Wash Buffer and incubated with cells for 30 minutes on ice, then washed and resuspended with MACS buffer. Stained samples were acquired on an LSR Fortessa (BD Biosciences) flow cytometer. For influenza A virus nucleoprotein (Flu-NP) staining, cells were stained as described above with minor modifications. Cells were fixed and permeabilized by using eBioscience FOXP3 staining kit (Thermo Fisher) in same conditions and then washed with Perm Wash buffer (Thermo Fisher). Fluorochrome-conjugated α Flu-NP antibody was made up in Perm Wash Buffer and incubated with cells for 30 minutes on ice, then washed and resuspended with MACS buffer. Data from control samples, incubated in the absence of peptide, were used for subtracting background staining.

2.2.10 Cell sorting

Cells were stained with Live/Dead viability dye and fluorochrome-conjugated antibodies for surface markers, washed and resuspended with MACS buffer followed by filtration through 70 μ m or 40 μ m sieve strainers. Cells were resuspended in sorting buffer and sorted on a FACS Aria III (BD Biosciences) flow cytometer. Sorted cells were collected in filtered HI-FCS and washed with PBS prior for use subsequent experiments. Purity checks were routinely performed upon completion of each sort.

2.2.11 Microarray

Peripheral blood (2 ml) was collected from each patient, preserved in PAXgene Blood RNA

Tubes (QIAGEN) and stored at -80 °C until use. Total RNA was extracted by using PAXgene Blood RNA Kit (QIAGEN). Microarray-based transcriptome assay was performed by the Gene Tech (Shanghai) company using Affymetrix GeneChip Human Gene 2.0 ST Array. All analyses used the R statistical programming environment (v3.03). Raw CEL intensity files underwent 'Perfect Match' probe set-level normalization including background correction using robust multiarray averaging (RMA), quantile normalization and summarization of gene expression using the median published algorithm²⁵⁶. Probe set intensity distributions between arrays (before and after normalization) and plots of the log intensity ratio versus the average log intensity (MA plots) were assessed to determine whether any arrays showed excessive technical variability with all arrays passing inspection. Unexpressed probe sets, which did not have significantly more fluorescence than background probes, were identified as those with a PSDABG P value > 0.05 and were removed from subsequent analyses. The LIMMA package was utilized to identify differentially expressed probe sets²⁵⁷. The death and recovery sets were compared using an empirical Bayes procedure to moderate standard error, and a t-test was used to test for difference in expression levels. Corresponding P values were adjusted for multiple testing using the Benjamini & Hochberg approach. Differentially expressed genes were defined by adjusted P value < 0.05 and an absolute log₂ fold-change of ≥ 2.0 .

2.2.12 Generation of *Olah*-overexpressing A549 cell line

1×10^6 293T cells were cultured in 10 ml of cDMEM in a 10 cm tissue culture plate and incubated at 37°C and 5% CO₂ overnight. The next day, 470 μ l of OptiMEM (Gibco) media was gently mixed with 30 μ l Eugene 6 reagent and incubated at room temperature for 5 min. This solution was added dropwise into a tube containing 4 μ g pMIG-II, 4 μ g pPAM-E and 2 μ g pVSVG plasmids and incubated for 15min at room temperature before adding dropwise into 293T cell cultures. Following incubation for 24hrs, cell culture media was then aspirated and replaced with 10ml of fresh cDMEM media. Meanwhile, 1×10^5 A549 cells in 10 ml cDMEM was plated in a 10 cm tissue culture plate and 12hrs later, media was aspirated and replaced with pre-filtered media from 293T cells. 10 μ l of polybrene was then added into A549 cell cultures, and 10 ml fresh cDMEM was added into the 293T cell cultures. This media transfer procedure was repeated every 12hrs for 7 days. The last transferred media from A549 cells were then aspirated and replaced with 10 ml of fresh cDMEM and passaged until a confluent monolayer.

2.2.13 Western blot

Samples containing equal amounts of protein were heated at 90°C on heat block and cooled on ice for 5 mins prior to loading. 24µl of sample was mixed with 6µl 5X loading buffer and loaded onto a 10% SDS-PAGE gel. The gel was run at 125V for 60 mins, and separated protein then transferred to a nitrocellulose (PVDF) membrane. For this, the PVDF membrane was soaked for 15sec in methanol, 3mins in MiliQ water and 5 mins in transfer buffer before use. The gel was then placed on the PVDF membrane (Immobilon) and covered by 3 blotting papers and 1 sponge at the top and bottom. The membrane transfer was run at 100V for 60 mins at 4°C and then incubated in blocking reagent at room temperature for 1 hour followed by wash buffer containing primary antibody overnight at 4°C. Following three washes with PBST (10 min per wash), the membrane was incubated with wash buffer contained secondary detecting antibody for 2 hrs at room temperature in the dark. The stained membrane was then been detected using Pharos FX Plus Imager (BIO-RAD).

2.2.14 Quantitative Polymerase Chain Reaction (qPCR)

RNA was extracted from tissue or cell samples using RNeasy Mini Kit (QIAGEN) and quantified by NanoDrop (Thermo Fisher). QIAGEN OneStep RT-PCR Kit (QIAGEN), together with probes for OLAH (human: Hs00217864_m1, mouse: Mm00521437_m1, Thermo Fisher) and actin (human: Hs01060665_g1, mouse: Mm00607939_s1, Thermo Fisher) were used for extracting RNA samples and performing reverse transcription to determine gene expression levels. qPCR was performed and analysed on QuantStudio 6 and 7 Pro Real-Time PCR Systems (Applied Biosystems) in the Department of Microbiology and Immunology, University of Melbourne.

2.2.15 Statistical analysis

All statistical analysis was performed in Prism 7 or 8 (GraphPad, CA, USA) unless specified. Unpaired t-tests was performed to compare bodyweight between mice groups. Mann-Whitney tests was used for comparison variable between two mice groups that were not normally distributed, and 2-way ANOVA was used according to each experimental design to compare

independent variables in different groups of mice. Significance was defined as * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

CHAPTER III: HIGH EXPRESSION OF CD38 AND MHC CLASS II ON CD8⁺ T CELLS DURING SEVERE INFLUENZA DISEASE REFLECTS BYSTANDER ACTIVATION AND TROGOCYTOSIS

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3.3 Abstract

Although co-expression of CD38 and HLA-DR on T-cells reflects activation during viral infections, high and prolonged CD38⁺HLA-DR⁺ expression is associated with severe disease.

As the expression of CD38⁺HLA-DR⁺ is poorly understood, we used mouse models of influenza A/H7N9, A/H3N2 and A/H1N1 infection to investigate mechanisms underpinning CD38⁺MHC-II⁺ phenotype on CD8⁺ T-cells. Beyond observation of CD38⁺MHC-II⁺PD-1⁺ activation profiles in mice, the analysis of influenza-specific immunodominant D^bNP₃₆₆⁺CD8⁺ T-cell responses showed that CD38⁺MHC-II⁺ co-expression was detected on both virus-specific and bystander CD8⁺ T-cells, with increased numbers of both CD38⁺MHC-II⁺CD8⁺ T-cell populations observed in lungs during severe infection. To understand mechanisms underlying CD38/MHC-II co-expression, we used adoptively-transferred transgenic OT-I CD8⁺ T-cells and A/H1N1-SIINKEKL infection. OT-I cells adoptively-transferred into MHC-II^{-/-} mice had no MHC-II after infection, suggesting that MHC-II was acquired via trogocytosis. Detection of CD19 on CD38⁺MHC II⁺ OT-I cells supports that MHC-II was acquired by trogocytosis sourced from B-cells. Co-expression of CD38⁺MHC-II⁺ on CD8⁺ T-cells was needed for optimal recall following secondary infection. Overall, our study demonstrates that both virus-specific and bystander CD38⁺MHC-II⁺ CD8⁺ T-cells are recruited to the site of infection during severe disease, and that MHC-II expression occurs via trogocytosis from antigen-presenting cells. Our findings highlight the importance of the CD38⁺MHC II⁺ phenotype for CD8⁺ T-cell memory establishment and recall.

3.4 Summary

T cell activation markers CD38 and MHC-II were found highly and continuously expressed on CD8⁺ T-cells in patients succumbed to severe H7N9 virus infection. However, the mechanisms underlying CD38⁺MHC-II⁺ co-expression on CD8⁺ T cells during viral infections remains unclear. This study reveals activation manners of this subset of T cells via both antigen-specific and bystander stimulation, demonstrating early recruitment of CD38⁺MHC-II⁺ CD8⁺ T cells at the site of infection during severe influenza A virus challenge. Importantly, we also showed trogocytosis of MHC-II on murine CD8⁺ T cells from other antigen-presenting cells rather than intrinsically expressing, at least partially from B cells during infection, and such snatching process is crucial to enhance recall ability during severe influenza virus challenge. Our research work helps to better understand the mechanism underlying the regulation of CD38⁺MHC-II⁺ CD8⁺ T cells during severe influenza virus infection, and expression pattern of this subsets could potentially become an early biomarker to predict the disease outcomes.

3.5 Introduction

Influenza virus infections lead to annual seasonal epidemics that result in 243,000-640,000 deaths globally every year¹. In 2017 alone, ~9.5 million people were hospitalized with lower respiratory tract influenza infections for a total of 81.5 million hospital days². Furthermore, disease severity and mortality rapidly escalate when a new influenza A virus (IAV) emerges, for which a matching vaccine is unavailable, as exemplified by the catastrophic 1918-1919 H1N1 pandemic and the emergence of avian-derived H5N1 and H7N9 strains in 2003³ and 2013⁴, respectively. In the absence of neutralizing antibodies, the recall of pre-existing broadly cross-reactive memory CD8⁺ T cells that recognize conserved peptide epitopes derived from internal viral proteins presented by infected cells, can promote recovery against novel influenza A viruses (IAVs) and even different influenza types^{5,6,7}. Activated CD8⁺ T cells can kill virus-infected cells via perforin- and granzyme-mediated lysis but also produce cytokines including IFN- γ and TNF- α , which render the local environment non-permissive for virus replication and ultimately provide some degree of heterosubtypic protection in the antibody-naïve individuals.

The importance of broadly cross-reactive CD8⁺ T cell responses in mitigating influenza disease severity has been shown during outbreaks caused by the emergence of novel subtypes, including the 2003 H5N1⁸, 2009 pandemic H1N1⁷ and 2013 H7N9 strains^{9,10}. Our analysis of immune responses in H7N9-infected hospitalized patients in China demonstrated that early proliferation of H7N9-specific CD8⁺ T cells contribute to disease resolution and survival, as reflected by mild to moderate clinical scores and early time to discharge from hospital⁹. In particular, we showed that early, transient co-expression of CD38⁺ and HLA-DR⁺ (MHC-II⁺) on CD8⁺ T cells during the early stages of H7N9 infection was strongly associated with patient survival¹⁰.

Co-expression of CD38, a cyclic ADP ribose hydrolase and cell surface glycoprotein involved in signal transduction, adhesion and calcium signaling¹¹, and MHC-II on CD8⁺ T cells is recognized as a classical hallmark of activation during viral infections. CD38⁺MHC-II⁺CD8⁺ T cells can be detected transiently at high levels in patients infected with viruses such as Ebola¹², HCV¹³, HIV^{14,15}, Dengue¹⁶, pandemic H1N1 IAV¹⁷, and more recently SARS-CoV-2^{18,19} during the acute phase of infection. CD38⁺MHCII⁺CD8⁺ T cells are known for their proliferative capacity, exhibit effector functions such as cytotoxicity and cytokine production^{20,21,22} and typically decrease in frequency following the resolution of infection^{21,22,18}.

However, in converse prolonged expression and high frequency of CD38⁺MHCII⁺CD8⁺ T cells can be associated with a loss of effector function, as shown during chronic viral infections^{23,24}. Moreover, H7N9-infected patients with fatal disease outcomes displayed high and prolonged expression of CD38⁺MHC-II⁺ on CD8⁺ T cells, which had minimal capacity to produce IFN- γ , thus linking the prominence of CD38⁺MHC-II⁺ phenotype to fatal outcomes in severe IAV-mediated pneumonia¹⁰.

Despite the important role of CD38⁺MHCII⁺CD8⁺ T cells in viral control, the underlying mechanisms driving their activation and regulation during viral infections are still poorly understood. Here, using our established H7N9 and H3N2 influenza wild-type and transgenic mouse models, we determined how disease severity affected the activation of IAV-specific CD38⁺MHC-II⁺CD8⁺ T cell responses *in vivo* and the antigenic determinants that drive their activation and expansion. Our study also revealed how this population acquires MHC-II expression from antigen-presenting cells by trogocytosis and revealed superior memory persistence of CD38⁺MHC-II⁺CD8⁺ T cells.

3.6 Materials and Methods

Ethics statement. Animal experiments were conducted according to the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes Guidelines for the housing and care of laboratory animals. Ethics was approved by the University of Melbourne Animal Ethics Experimentation Committee (1714108). Animal experiments involving H7N9 infection were approved by the Shanghai Public Health Clinical Center in China (2014-E032-01).

Mice and viral infections. For primary infections, 6-8 week old male or female C57BL/6 mice were lightly anaesthetised with isofluorane and infected by intranasal (i.n.) instillation (30 μ l) with 10² and 10⁵ PFU of A/HK/x31 (X31; H3N2). To examine secondary CD8⁺ T cell responses in mice following H7N9 infection, mice were first primed i.n. with 10⁴ EID₅₀ of A/Chicken/Shanghai/F/98 (H9N2) virus, followed 60 days later with 10⁶ pfu of A/Shanghai/4664T/2013 (H7N9) virus.

To investigate mechanisms underlying MHC-II expression on murine CD8⁺ T cells, 10⁴ naïve OT-I cells were adoptively transferred into C57BL/6 mice or MHC-II^{-/-} mice 24 hours prior to

infection with X31-OVA. After 8 days, CD45.1⁺CD8⁺V α 2⁺ OT-I cells in the BAL, mediastinal lymph node and spleen were assessed. The experiment was repeated twice with 3-5 mice per group for each repeat.

To examine the recall ability of CD38⁺MHC-II⁺CD8⁺ T cells, 10⁴ naïve OT-I cells were adoptively transferred into C57BL/6 mice 24 hours prior to infection with X31-OVA. After 8 days, CD45.1⁺CD8⁺V α 2⁺ OT-I cells in the lymph node and spleen were four-way sorted based on their expression of CD38 and MHC-II. Each sorted population was adoptively transferred into separate naïve C57BL/6 recipient mice (5x10³ cells per mouse) and 30 days later infected with X31-OVA. The experiment was repeated twice with 3-5 mice per group for each repeat.

Tissue sampling and cell preparation. Lungs, spleens, mediastinal lymph nodes (MLN) and bronchoalveolar lavage (BAL) were collected from mice at various time points after infection. Lungs were either homogenized and centrifuged to obtain clarified supernatants to assay for viral titres or enzymatically digested in collagenase III (Worthington Biochemical Corporation, USA; 1mg/ml) and DNase I (Sigma-Aldrich, Germany; 0.5mg/ml) before passing through cell sieves to obtain single cell suspensions for analysis. Where necessary, cell suspensions from tissues were incubated with 0.15M NH₄Cl and 17 mM Tris- HCl at pH 7.2 for 5 mins at 37°C to lyse red blood cells.

Measurement of lung viral load. Titres of infectious virus were determined by plaque assays on confluent Madin Darby canine kidney (MDCK) cell monolayers cultured in six-well plates. Cells were infected with lung homogenate supernatants at varying dilutions for 45 min at 37°C before the addition of Leibovitz L15 or MEM medium containing 0.9% agarose overlay containing Trypsin (Worthington Biochemical, NJ, USA), as previously described²⁵. Plates were incubated at 37°C 5% CO₂ for 3 days and virus-mediated cell lysis then counted as plaques on the cell layer and expressed as plaque forming units (PFU).

Analysis of cytokine levels. Cytokines present in lung homogenate supernatants were measured using a BD CBA flex set (BD Bioscience) as per the manufacturer's instructions²⁵. Samples were analysed using a Becton Dickinson FACS Canto II flow cytometer. Data were analysed using FCAP Array software (Soft Flow Inc., Pecs, Hungary).

Tetramer and antibody staining. MHC-I tetramer targeting the immunodominant epitope of the influenza nucleoprotein (D^bNP₃₆₆₋₃₇₄ – ASNENMETM, D^bPA₂₂₄₋₂₃₃ - SSLENFRAYV) was produced in-house and conjugated to streptavidin-APC/PE (Life Technologies Australia Pty Ltd) at a 1:250 dilution at room temperature for 1h. Cells were stained with combinations of fluorochrome-conjugated antibodies. PerCP-Cy5.5-CD3(#551163), PE-CD8(#561095), BV421-I-Ab(#562928), APC-CD44(#553133), FITC-CD44(#553133), BV711-CD38(#740697), PerCP-Cy5.5-CD8(#551162), APC/eF780-CD62L(#47-0621-82), APC-CD11c(#17011481), PE-Cy7-TCR-va2(#560624) from BD Biosciences, USA etc. PE-Cy7-CD38(#102718), BV785-PD-1(#329908), APC-Cy7-CD45.1(#110716), FITC-I-Ab(#116406), Percific Blue-I-Ab(#116422), FITC-CD19(#115506) from Biolegend, USA. AF700-CD3(#56003382) was purchased from Invitrogen, USA.

Live/Dead-aqua 525 were purchased from Invitrogen. Briefly, cell suspensions were stained with Live/Dead Aqua viability dye at room temperature for 10 mins followed by staining with tetramer for 15 mins and cell surface marker antibodies for 30 mins. Cells were fixed with 1% paraformaldehyde before analysis by flow cytometry. All antibody and tetramer staining were performed at 4°C and in the dark. Samples were subsequently acquired on a Becton Dickinson LSR Fortessa or Aria III flow cytometer and data analyzed by Flowjo Software (Tree Star Inc, USA).

Statistical analyses. The comparison between absolute numbers of cells were analysed using two-way ANOVA analysis. Body weight between mice groups were compared by multi-T test. Absolute numbers of OT-I cells from different mice groups were compared using ordinary one-way ANOVA analysis.

3.7 Results

3.7.1 H7N9 infection in mice induces CD38⁺MHC-II⁺PD-1⁺ activation profiles

Our previous data revealed that patients hospitalized with severe H7N9 influenza had high and prolonged expression of CD38, MHC-II and PD-1 on CD8⁺ T cells¹⁰. To understand the mechanisms underlying such hyper-activated CD38, MHC-II and PD-1 phenotype, we first investigated the recruitment kinetics and specificity of CD38⁺MHC-II⁺ and CD38⁺PD-1⁺ CD8⁺ T cells in a mouse model of H7N9-infection in C57BL/6 (B6) mice. To minimize H7N9-

induced mortality and mimic populations that have a previous influenza A virus infection history, B6 mice were first primed intranasally (i.n.) with 10^4 EID₅₀ of the avirulent strain A/Chicken/Shanghai/F/98 (H9N2) 60 days prior to i.n. challenge with 10^6 pfu of A/Shanghai/4664T/2013 (H7N9) virus (**Fig 1a**). Overall, ~20% of CD8⁺ T cells in the spleen or mediastinal lymph node (MLN) were CD38⁺MHC-II⁺ (20.2% for both spleen and MLN) (**Fig 1ci,ii, Fig S1**) or CD38⁺PD-1⁺ (range 17.8-20.6%) (**Fig 1cii,iii**) on d10 after H7N9 exposure. Analysis of CD8⁺ T-cell responses directed at the immunodominant D^bNP₃₆₆ epitope showed 3-5-fold enrichment of D^bNP₃₆₆⁺CD8⁺ T cells within the CD38⁺MHC-II⁺ or CD38⁺PD-1⁺ sets from the spleen and MLN (**Fig 1b, Fig S1**). Alternatively, when gated on the D^bNP₃₆₆⁺CD8⁺ set, the CD38⁺MHC-II⁺ (59.5% and 81.1% for spleen and MLN) and CD38⁺PD-1⁺ (90.2% and 93.4%) phenotypes were prominent (**Fig 1c**), with the CD38 and MHC-II levels (measured by frequency) being higher for MLN than spleen ($p < 0.05$) (**Fig 1c**). Such high expression of CD38⁺MHC-II⁺ or CD38⁺PD-1⁺ on tetramer-specific CD8⁺ T cells after H7N9 infection in mice supports the observations from H7N9 patients. As expected, the majority of CD38⁺MHC-II⁺ or D^bNP₃₆₆⁺CD8⁺ T cells in MLN and spleen were CD44^{hi} (antigen-experienced), though expression of CD62L, a molecule that mediates trafficking into lymph nodes via high endothelial venules, differed for these two sites (**Fig 1d**).

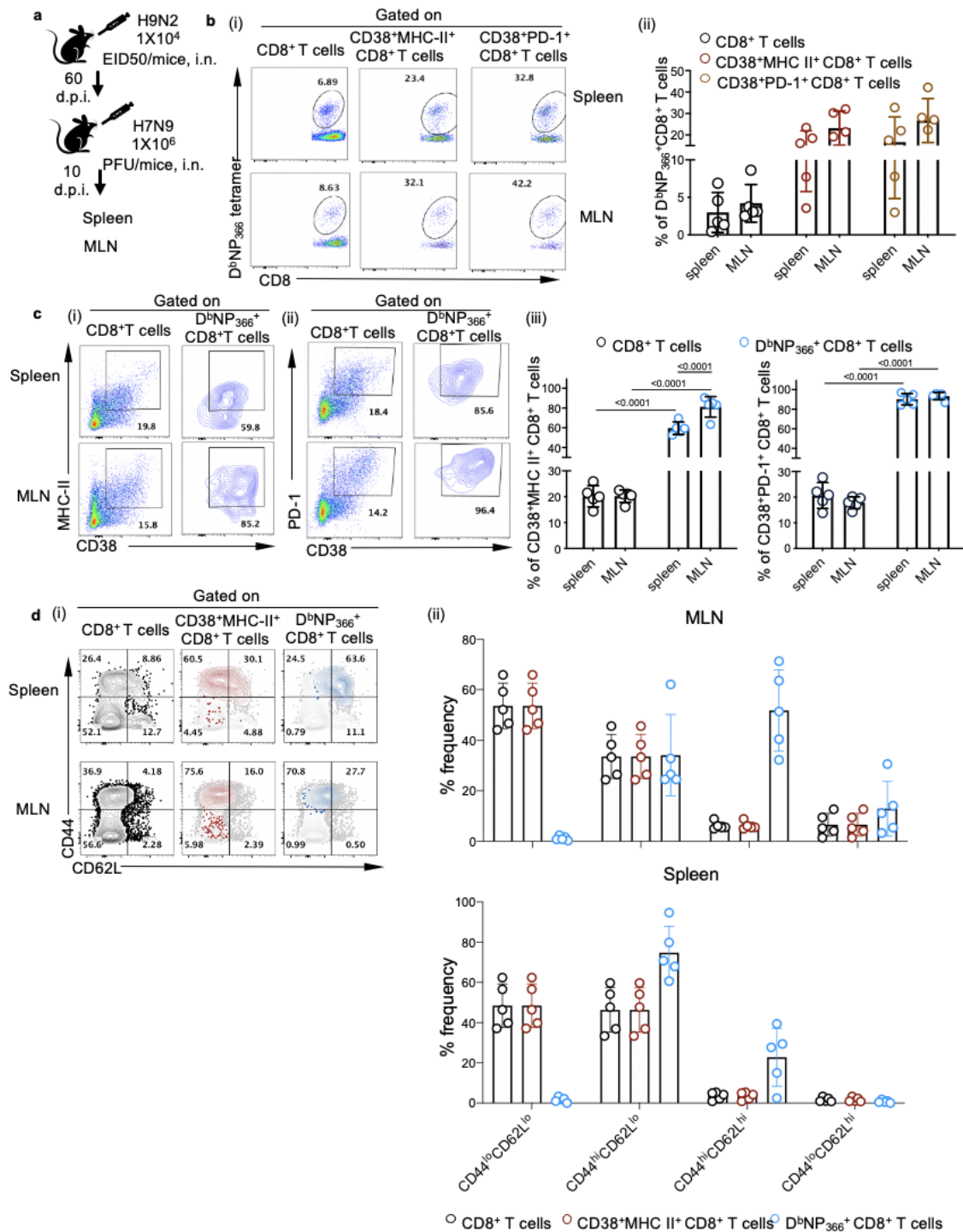


Fig 1. H7N9 infection in mice induces $CD38^+MHC-II^+PD-1^+$ activation profiles

(a) C57BL/6 Mice (n=5) were infected i.n. with H9N2 (A/Chicken/Shanghai/F/98) (10^4 EID50) virus and challenged i.n. at 60 d.p.i. with H7N9 (A/Shanghai/4664T/2013) (10^6 PFU) virus. Spleens and MLNs were harvested 10 days after H7N9 challenge and phenotype of $CD8^+$ T cells analysed. (b)(i) Representative dot plots and (ii) frequencies of $D^bNP_{366}^+$ $CD8^+$ T cells within $CD8^+$, $CD38^+MHC-II^+$ $CD8^+$ and $CD38^+PD-1^+$ $CD8^+$ gated populations are shown. (c)(i) Depicted are representative dot plots and (ii) frequencies of $CD38^+MHC-II^+$ or $CD38^+PD-1^+$ within $CD8^+$ and $D^bNP_{366}^+$ $CD8^+$ T cell populations. (d) Memory profiles were also analysed based on co-expression of CD62L and CD44. Statistics were performed using Mann-Whitney test.

As CD8⁺ T cells from H7N9-infected hospitalized patients displayed high PD-1 levels, we compared PD-1 expression on H7N9-specific D^bNP₃₆₆⁺CD8⁺ T cells to that on CD38⁺MHC-II⁺ or CD38⁺MHC-II⁻ CD8⁺ T cells. D^bNP₃₆₆⁺CD8⁺ T cells in both the MLN and spleen displayed the highest level of PD-1 (**Fig 2**). As expected, PD-1 levels on CD8⁺ T cells negative for dual expression of CD38⁺MHC-II⁺ were minimal. Thus, high CD38⁺MHC-II⁺PD-1⁺ expression on D^bNP₃₆₆⁺CD8⁺ and CD38⁺MHC-II⁺ CD8⁺ T cells confirmed the correlation between high CD38⁺MHC-II⁺PD-1⁺ CD8⁺ T-cell prevalence and severe disease.

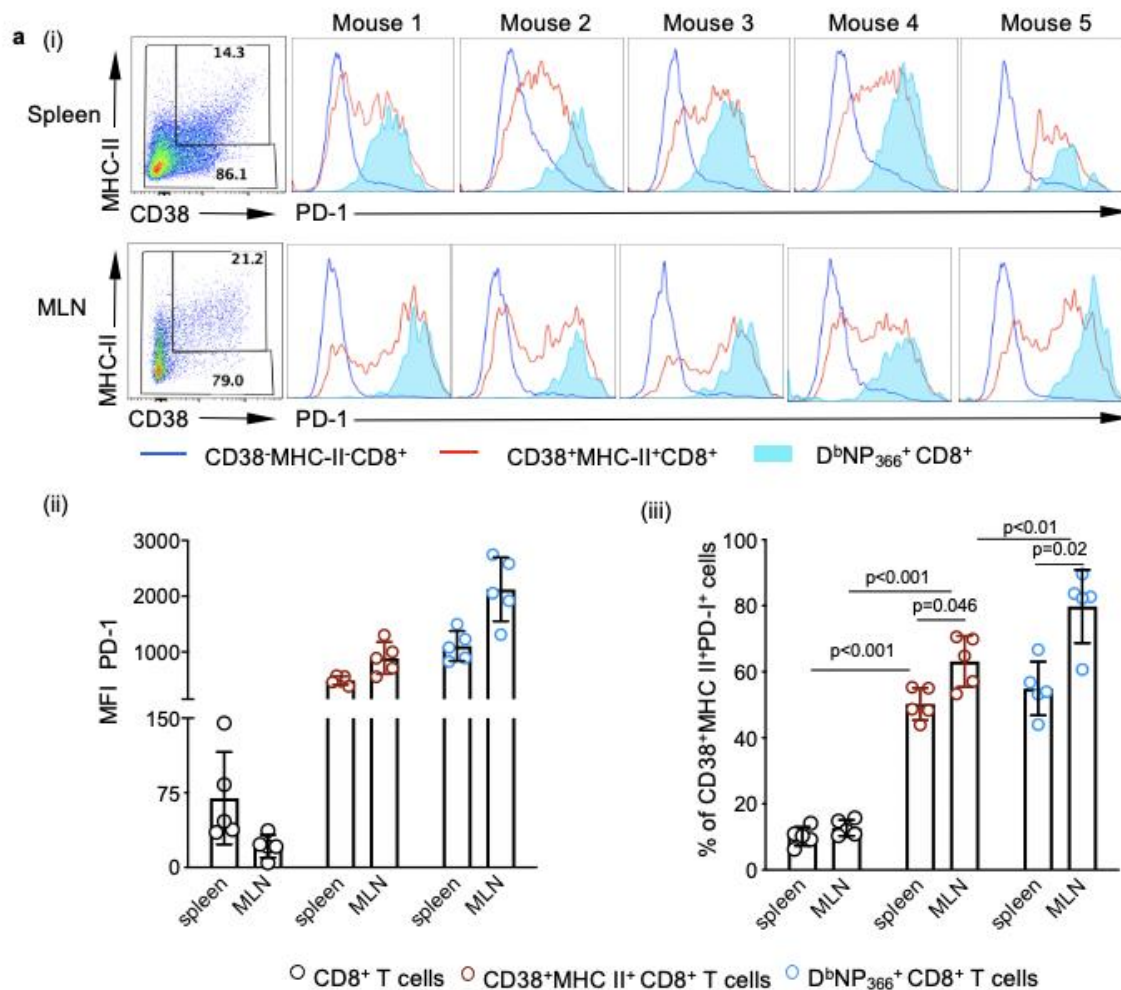


Figure 2. Jia X et al

Fig 2. CD38⁺MHC-II⁺CD8⁺ T cells express higher levels of PD-1

Spleens and MLNs of C57BL/6 mice (n=5) infected i.n. with H9N2 (10⁴ EID₅₀) and again 60 days later with H7N9 (10⁶ PFU) were obtained 10 days following the last challenge. (a) The expression of PD-1 on D^bNP₃₆₆⁺CD8⁺, CD38⁺MHC-II⁺CD8⁺ T cells and CD38⁺MHC-II⁻CD8⁺ T cell populations from each animal is depicted as (i) histograms, (ii) mean fluorescence intensity (MFI) and (iii) frequency. Statistics were performed using Mann-Whitney test.

3.7.2 Recruitment of CD38⁺MHC-II⁺ and CD38⁺PD-1⁺CD8⁺ T cells to the site of infection

To determine how virus load and disease severity affect the recruitment kinetics and phenotype of CD38⁺MHC-II⁺ and CD38⁺PD-1⁺ CD8⁺ T cells, we infected B6 mice intranasally with a low (10² pfu) or high (10⁵ pfu) dose of A/HK/x31 (H3N2; X31) virus (**Fig 3a**). While comparable virus replication kinetics were observed at d3, d5, d7 and d10 after infection (**Fig 3b**), the high dose caused more severe disease, as evidenced by significantly greater body weight loss between d3 and d7 ($p<0.001$) (**Fig 3c**), and increased lethality ($p=0.003$) (**Fig 3d**) as well as excessive inflammation in the lung (**Fig 3e**). The 10⁵ pfu X31 virus challenge was associated with more rapid acquisition of a PD-1^{hi} phenotype ($p<0.05$ on d7) on D^bNP₃₆₆⁺CD8⁺ T cells (**Fig 3f**), relecting the pattern found previously for H7N9-infected mice and patients who succumbed to H7N9 disease.

Although cell numbers for all CD38⁺MHC-II⁺ and CD38⁺PD-1⁺ CD8⁺ T cells in the regional MLN were comparable from d5 and beyond (**Fig 3hi**), only a small population of cells (11.8%) were D^bNP₃₆₆⁺ (**Fig 3hii**) within CD38⁺MHC-II⁺ CD8⁺ T cells (peak d10) and (17.3%) within CD38⁺PD-1⁺CD8⁺ T cells (peak d16), indicating that the majority of CD38⁺MHC-II⁺ and CD38⁺PD-1⁺ CD8⁺ T cells recruited during severe influenza disease were tetramer-negative. Furthermore, CD38⁺MHC-II⁺ and CD38⁺PD-1⁺ CD8⁺ T cells were increased and peaked earlier (d7) at the site of infection (BAL) following the high dose challenge (**Fig 3hi left, Fig S2**), while the IAV-specific CD8⁺ T cell responses peaked 3 days later (**Fig 3hii left, Fig S2**) and at comparable numbers for both (10⁵ and 10² pfu) groups. Together, these data suggest that D^bNP₃₆₆ tetramer-negative CD38⁺MHC-II⁺CD8⁺ T cells, many of which numerically will not be virus-specific, extravasate into the lung prior to recruitment of the majority of antigen-specific cells. Whether this is a consequence or a cause of more severe inflammation and pathology is currently unclear.

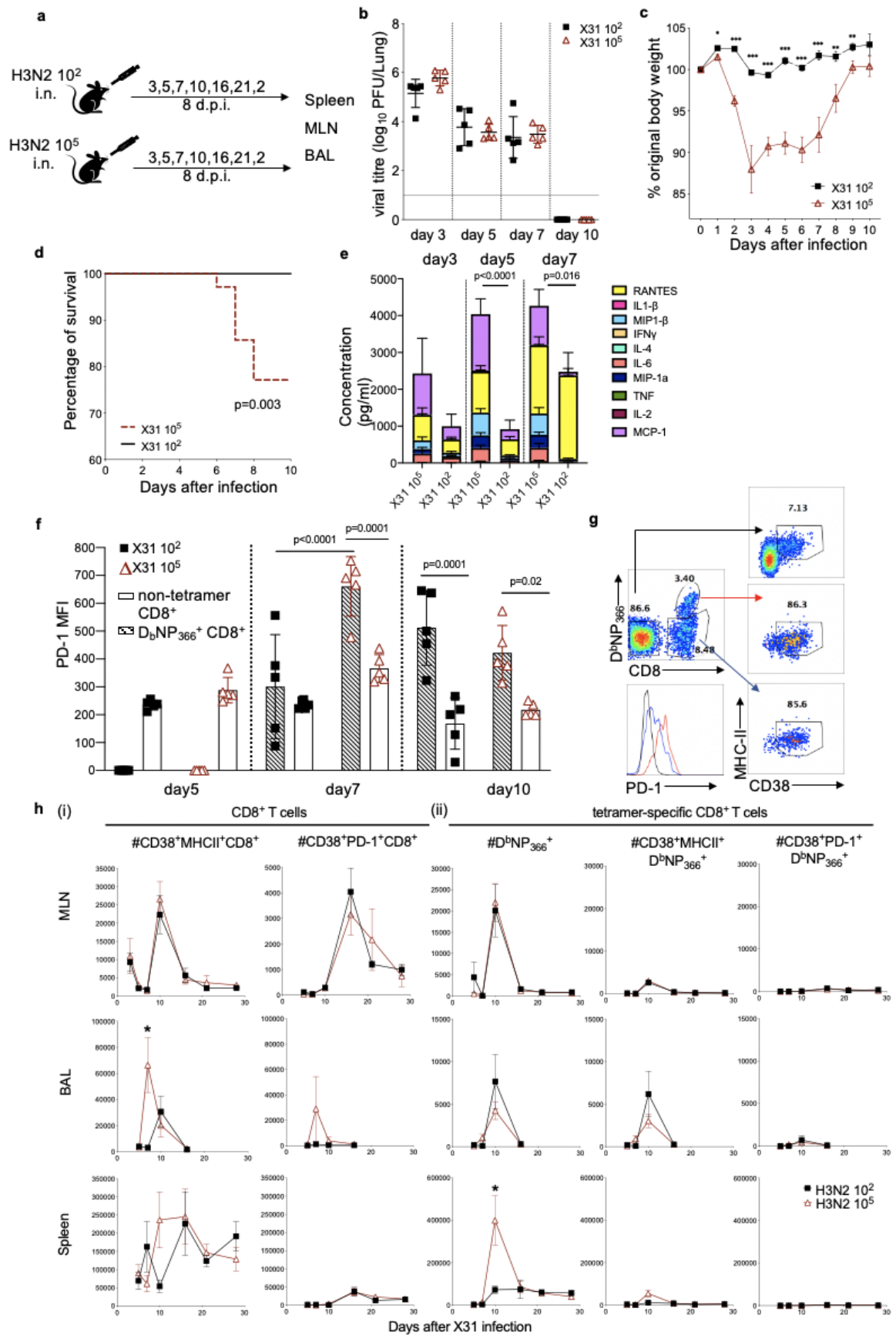


Figure 3. Jia X *et al*

Fig 3. H3N2 viral dose affects kinetics of CD38⁺MHC-II⁺ and CD38⁺MHC-II⁺PD-1⁺ at the site of infection. (a) C57BL/6 mice were infected i.n. with X31 at either a high (10⁵ PFU) or low (10² PFU) viral dose (n=5/group). (b) At various days post-infection (p.i.), lung viral titres were determined in MDCK plaque assays. Each symbols denotes an individual mice and the mean (±SD) is shown for each group. Mice were also monitored daily after infection for (c) weight loss and (d) survival (mice were humanely culled when a weight loss of >20% of their pre-experimental body weight was observed over a 2 day period and accompanied by clinical signs of disease, or when a weight loss of >25% of their pre-experimental body weight occurred). (e) Lung homogenates were assayed by cytometric bead array to determine the cumulative amount of total cytokines present and the amounts of each cytokine. (f) MFI of PD-1 on D^bNP₃₆₆⁺CD8⁺ T cells (dashed bars) or non-tetramer CD8⁺ T cells (open bars). (g) Representative histograms of PD-1 expression on D^bNP₃₆₆⁺ (red line) or D^bNP₃₆₆⁻specific (blue line) CD38⁺MHC-II⁺CD8⁺ T cells or remaining D^bNP₃₆₆⁻CD8⁺ T cells (black line). (h) Numbers of (i) non-tetramer- and (ii) D^bNP₃₆₆-tetramer specific CD38⁺MHC-II⁺ and CD38⁺MHC-II⁺PD-1⁺ CD8⁺ T cells in MLN, BAL and spleen at various time-points after infection. *p<0.05, **p<0.01 using Mann–Whitney test.

3.7.3 CD38⁺CD8⁺ T cells utilise trogocytosis to acquire expression of MHC-II

As expression of the *H2-Ab* (*MHC-II*) gene at the transcriptional level depends on the transcription factor *CIITA*²⁶²⁷, and murine CD8⁺ T cells lack intrinsically expressed *CIITA*²⁸, we further investigated how murine CD8⁺ T cells acquire expression of the MHC-II. We utilised a transgenic OT-I mouse model in the influenza setting. OT-I CD8⁺ T cells contain a TCRαβ specific for the K^bOVA₂₅₇₋₂₆₄ (OVA; SIINFEKL) epitope, which proliferate after infection with influenza-OVA virus (with SIINFEKL engineered into the neuraminidase stalk).

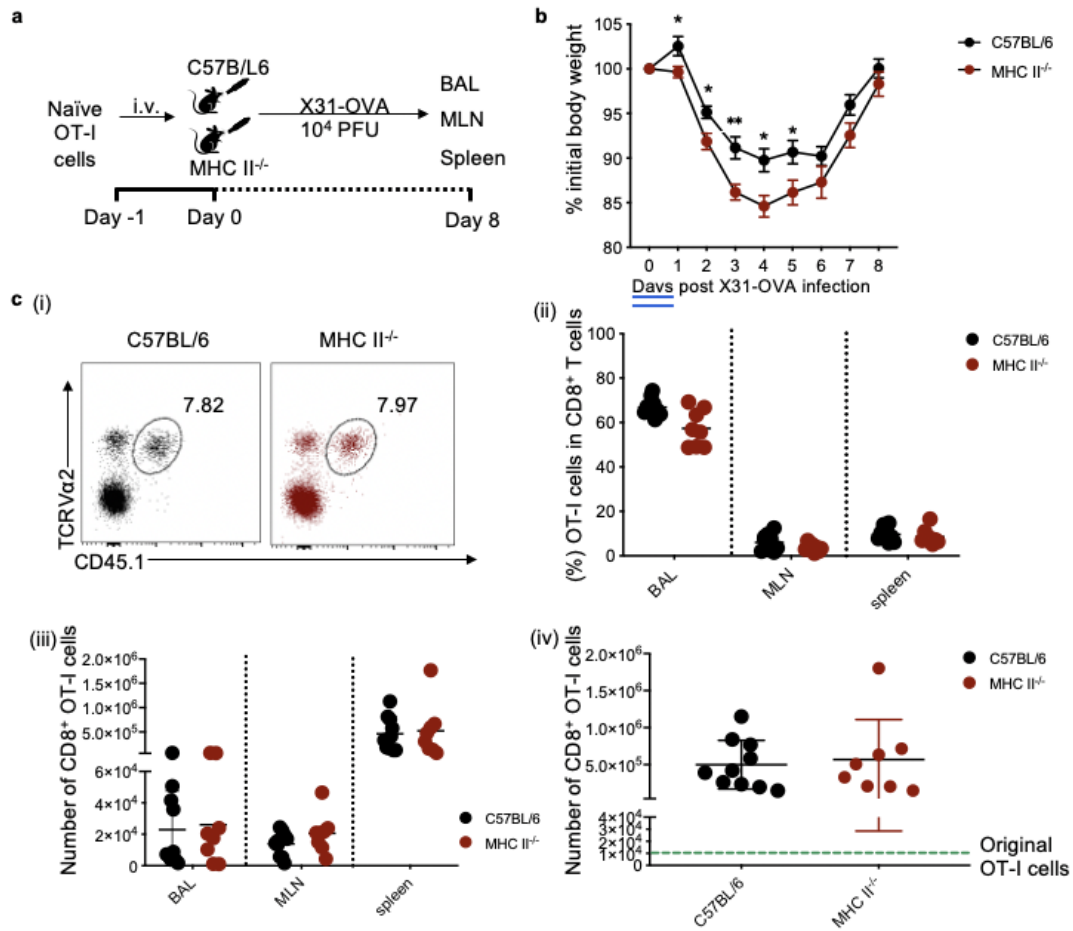


Figure 4. Jia X et al

Fig 4. Similar OT-I T cell proliferation in C57BL/6 and MHC-II^{-/-} mice after IAV infection

(a) C57BL/6 and MHC-II^{-/-} mice (n=5/group) received 10⁴ OT-I T cells via the i.v. route 24 hrs prior to infection. Mice were subsequently infected with 10⁴ PFU of X31-OVA (H3N2) and BAL, MLN and spleen were harvested at 8 d.p.i.. **(b)** Mice were monitored daily during the course of infection for weight loss before **(c)** OT-I T cell populations from each organ was enumerated. (i) Depicted are representative dot plots of CD45⁺Vα2⁺ OT-I T cells within the CD8⁺ T cell pool in the MLN. Shown are also (ii) percentages, (iii) numbers of OT-I T cells in each different organs and (iv) as a cumulative total. *p<0.05, **p<0.01.

We adoptively transferred naïve OT-I CD8⁺ T cells into MHC II^{-/-} or C57BL/6 wild-type mice prior to X31-OVA infection (**Fig 4a**). We observed greater body weight loss (5%) in MHC II^{-/-} mice compared to wild-type mice particularly at 3-5 days post-infection (**Fig 4b**), indicating that lack of MHC-II expression leads to perturbed immune responses and increased disease severity. When we analysed the expansion of OT-I cells (**Fig 4ci**) by frequency (**Fig 4cii**) or number (**Fig 4ciii**) of the total CD8⁺ T cell pool, we observed similar numbers in BAL, MLNs and spleens of both WT and MHC II^{-/-} mice. As there were also no differences in the combined total number of OT-I cells from these organs of each mouse (**Fig 4civ**), this indicates that OT-I cells proliferate to the same extent in both WT and MHC^{-/-} mice.

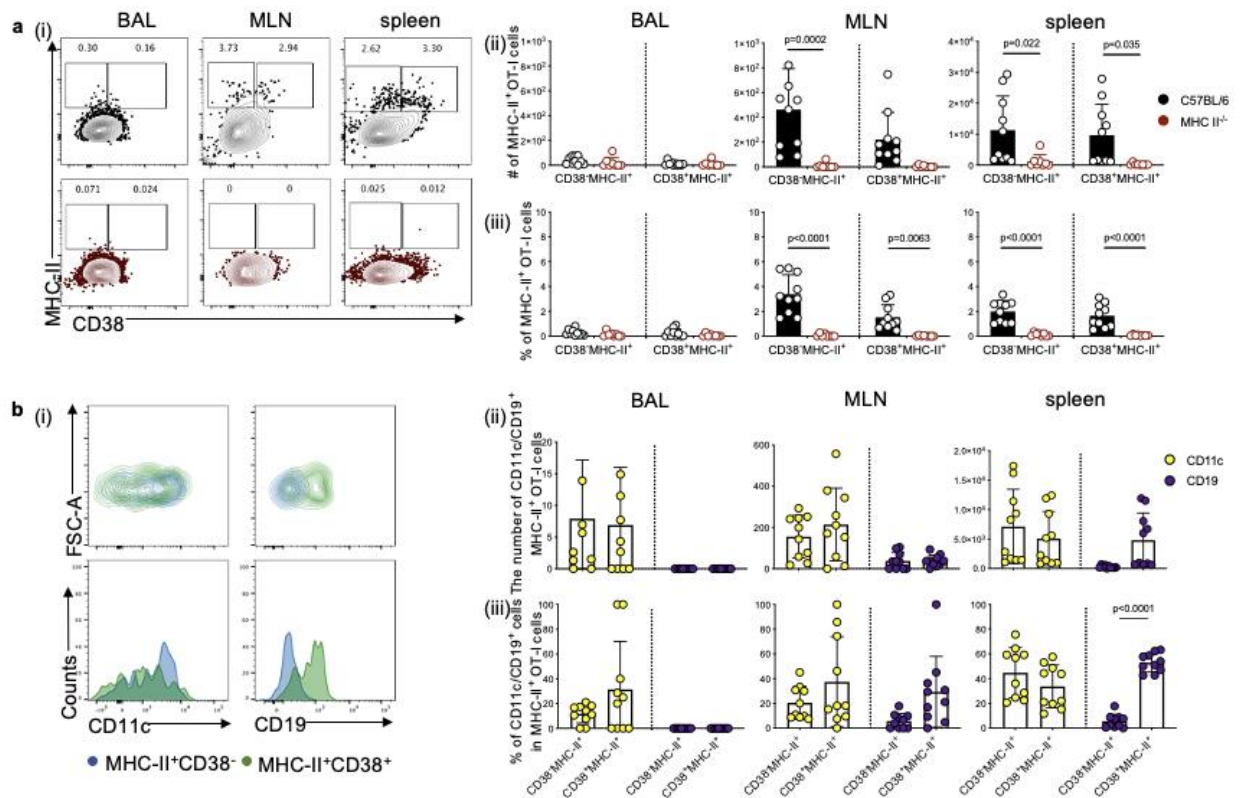


Figure 5. Jia X *et al*

Fig 5. CD38⁺ OT-I T cells do not intrinsically express MHC-II and acquire it via trogocytosis

(a) C57BL/6 and MHC-II^{-/-} mice (n=5/group) received 10⁴ OT-I T cells via the i.v. route 24 hrs prior to infection with 10⁴ PFU of X31-OVA. **(i)** Representative dot plots of CD38 and MHC-II expression on OT-I T cells in MLN, BAL and spleens 8 day p.i. **(ii)** Bar graphs show the (ii) number and (iii) frequency of CD38⁺MHC-II⁺ and CD38⁺MHC-II⁻ cells within the MHC-II⁺ OT-I T cell pool in each organ. **(b)** **(i)** Representative dot plots and histograms of CD11c and CD19 expression on CD38⁺MHC-II⁺ (green) and CD38⁺MHC-II⁻ (orange) OT-I T cells in C57BL/6 mice as well as (ii) their numbers and (iii) frequencies.

Analysis of MHC-II expression on OT-I cells in BAL, MLNs and spleens of MHC-II^{-/-} or B6 mice showed that MHC-II phenotype was only limited to OT-I cells transferred into B6 mice and absent in those transferred into MHC-II^{-/-} mice (**Fig 5a(i-iii)**). This observation extended to CD38⁺ OT-I cells, thus indicating that murine CD8⁺ T cells do not intrinsically express MHC-II but acquire it from other cells during influenza virus infection.

To determine how transferred OT-I cells could have acquired MHC-II, we stained OT-I cells in influenza-infected mice for markers associated with antigen presentation, including CD19 to represent B cells and CD11c as a surrogate DC and macrophage marker²⁹. In BAL, MLN and spleen, CD11c⁺ OT-I cells were consistently detected on both CD38⁺MHC-II⁺ and CD38⁺MHC-II⁻ populations in the BAL (mean 11.7% and 31.4%) as well as the MLN (mean

20.5% vs 37.5%) and spleen (average ~ 44.8% vs 33.7%) (**Fig 5bi,ii and iii**). In contrast, CD19 was associated mostly with CD38⁺MHC-II⁺ OT-I cells (mean 5.7% vs 29.4% in MLN and 5.6% vs 53.0% in spleen), while was rarely detected on CD38⁻MHC-II⁺ and no CD19 expression was observed on OT-I cells in BAL, suggesting that interaction of CD38⁺MHC-II⁺CD8⁺ T cells with CD19⁺ B cells via trogocytosis provides the source of MHC-II.

To further verify whether CD19, CD11c and MHC-II expression was intrinsic to OT-I cells, we measured CD19, CD11c and MHC-II (H2-Ab) mRNA levels in naïve, effector (8 d.p.i) and memory (30 d.p.i) CD8⁺ T cell populations following X31-OVA infection. Although gene expression of CD11c was not initially detected in naïve populations (**Fig 6a**; mean=14.67), a 2.4 x10²-fold increase was observed in effector (**Fig 6b**; mean=3494) and 1.4 x10²-fold increase in memory (**Fig 6c**; mean=1985.5) CD8⁺ T cell populations, demonstrating that CD11c is intrinsic to CD8⁺ T cells and upregulated as they differentiate. In contrast, this was not the case for CD19 and MHC-II (H2-Ab), with undetectable levels of gene expression observed in naïve, effector and memory CD8⁺ T cell populations, similar to levels observed for CD4 gene expression. Overall, our data support the notion that the surface expression of MHC-II on CD8⁺ T cells is potentially acquired from B cells and dendritic cells/macrophages during influenza virus challenge.

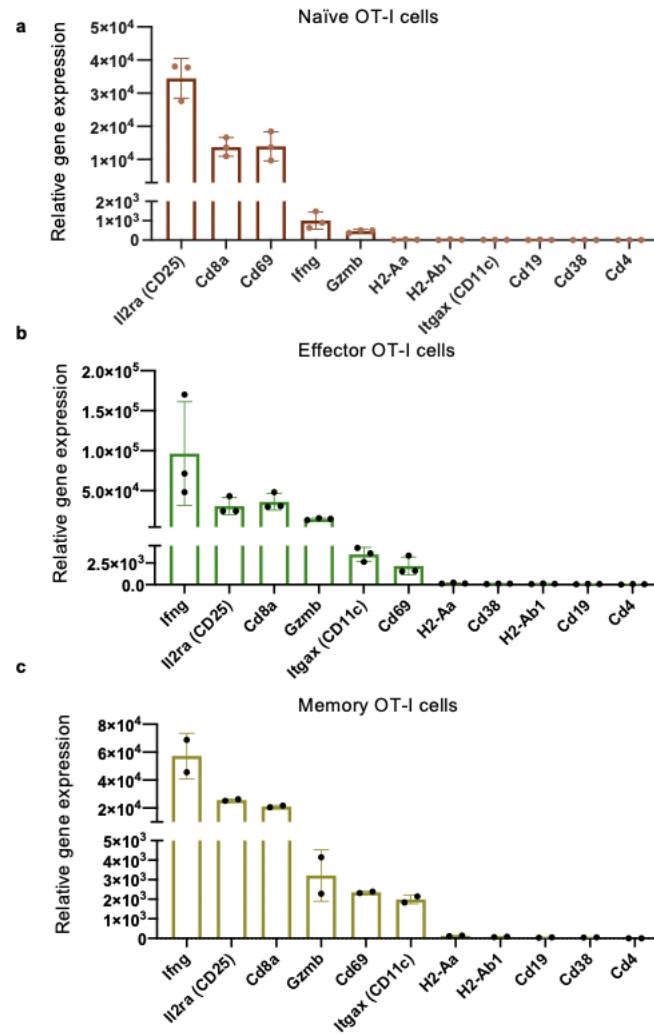


Figure 6. Jia X *et al*

Fig 6. Gene expression in CD8⁺ T cells at naïve, effector and memory stage following influenza virus infection. Naïve OT-I cells were adoptively transferred into C57BL/6 mice and recipient mice were subsequently infected with 10⁴ PFU of A/HKx31-OVA. Immune organs were harvested at **(a)** 0 (naïve OT-I cells), **(b)** 10 days (effector OT-I cells) or **(c)** 60 days (memory OT-I cells) post-infection, to analyse gene expression in CD8⁺ T cells by RNAseq. The original data were derived from Brendan E. Russ *et al*³⁰ and re-analysed for this study.

3.7.4 Superior recall capacity of CD38⁺MHC-II⁺ CD8⁺ T cells

To investigate the functional significance of CD38⁺MHC II⁺CD8⁺ T cells during IAV infection, we analysed the recall capacity of memory OT-I cells established from 4 subtypes of d8 effectors depicted by CD38 and MHC-II expression (CD38⁻MHC-II⁻, CD38⁺MHC-II⁻, CD38⁻MHC-II⁺, CD38⁺MHC-II⁺). OT-I cells adoptively transferred into B6 mice were expanded *in vivo* following infection with X31-OVA. On day 8 after infection, 4 populations

of OT-I cells were FACS-isolated (**Fig 7a**). Each (CD38⁻MHC-II⁻, CD38⁺MHC-II⁻, CD38⁻MHC-II⁺, CD38⁺MHC-II⁺) population was adoptively transferred into separate naive recipient B6 mice, and subsequently rested for 30 days to establish memory formation. All mice were then challenged with X31-OVA virus. Mice that received CD38⁻MHC-II⁻ OT-I cells exhibited significantly ($p<0.05$) more body weight loss than mice that received OT-I cells expressing only CD38 (CD38⁺MHC-II⁻), MHC-II (CD38⁻MHC-II⁺) or both these markers (CD38⁺MHC-II⁺) (**Fig 7b**), indicating an important contribution of these activation markers for protection against severe IAV disease. Importantly, the greatest recall numbers of memory OT-I cells were observed in both the BAL (**Fig 7ci,ii**) and spleen (**Fig 7ci,iii**) of mice that received CD38⁺MHC-II⁺ cells with OT-I numbers being ~3.1-fold and ~4.2-fold higher, respectively, when compared to other subsets.

Given that we observed the acquisition of MHC-II molecules by CD8⁺ T cells during primary infection (**Fig 5**), we investigated whether this process also occurred following secondary challenge. Irrespective of the original phenotype of OT-I cells transferred, MHC-II⁺ OT-I cells (mean range from 20.4-31.06% across four groups) were found in BAL (**Fig 7di,ii**). Furthermore, distinct populations of CD38⁺MHC-II⁺ OT-I cells were detected in the spleens of mice across all four groups (**Fig 7di,iii**), indicating that OT-I cells that did not previously exhibit this phenotype can differentiate into CD38⁺MHC-II⁺ expressing cells upon rechallenge. This, however, did not improve their recall ability and highlights the importance of establishing CD38⁺MHC-II⁺ phenotype prior to secondary exposure. Interestingly, while both CD19⁺ and CD11c⁺ OT-I cells could be found on CD38⁺MHC-II⁺ OT-I cells in the spleens of all animal groups (**Fig 7ei,iii**), only CD19⁻CD11c⁺ OT-I cells could be detected in BAL (**Fig 7ei,ii**). Overall, our findings highlight the importance of the CD38⁺MHC-II⁺ phenotype for CD8⁺ T-cell memory establishment and recall.

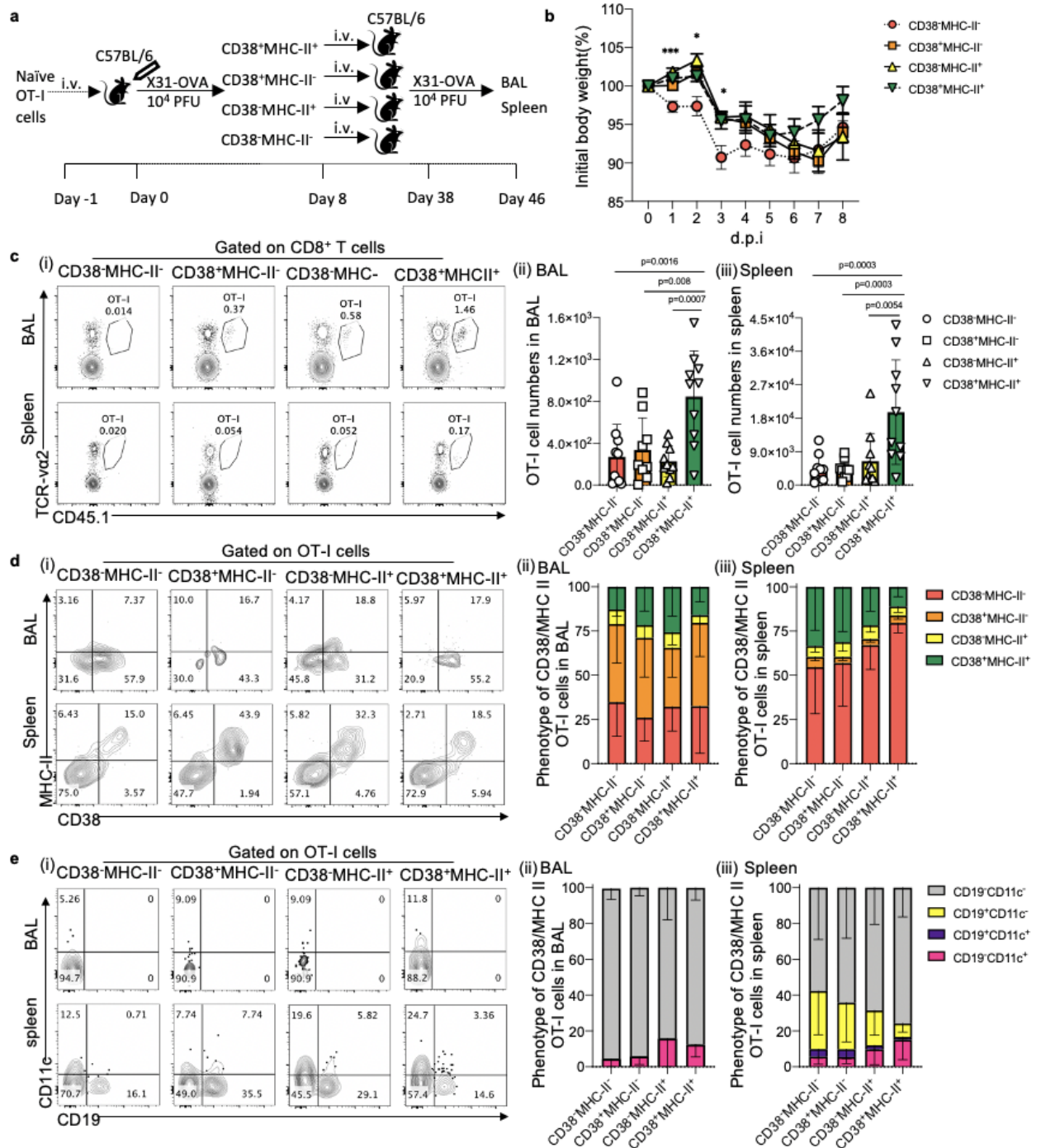


Figure 7. Jia X et al

Fig 7. Superior recall capacity of CD38⁺MHC-II⁺ CD8⁺ T cells

(a) Naïve OT-I cells (10⁴ per mouse) were transferred into C57BL/6 mice and the next day challenged with 10⁴ PFU X31-OVA virus. OT-I cells (from MLN and spleen) were sorted based on CD38 and MHC-II expression on day 8 p.i. Sorted OT-I cells were adoptively transferred into new recipient C57BL/6 mice (3x10⁵ cells per mice) and rested for 30 days. Mice were then challenged with 10⁴ PFU X31-OVA virus and sacrificed on day 8 p.i. (b) Mice body weight were monitored over the course of 8 days following secondary viral challenge. (c) (i) Representative FACS plots gated on CD8⁺ T cells across different recipient mice groups and showing the frequency of OT-I cells in BAL and spleen. Numbers of OT-I cells in (ii) BAL and (iii) spleen. (d) (i) Representative FACS plot for CD38 and MHC-II expression on OT-I cells from different recipient mice groups.

Percentage of phenotypes based on CD38 and MHC-II of OT-I cells in (ii) BAL and (iii) spleen. **(e)** (i) Representative FACS plot for CD19 and CD11c expression on OT-I cells from different recipient mice groups. Percentage of phenotypes based on CD19 and CD11c of OT-I cells in (ii) BAL and (iii) spleen.

3.8 Discussion

Co-expression of CD38⁺MHC-II⁺ on human CD8⁺ T cells is a hallmark of viral-specific T cell responses during many viral infections. In particular, early and rapid expansion of CD38⁺MHC-II⁺ CD8⁺ T cells can correlate with effective viremia control during acute HIV²⁰, Ebola¹², HCV³¹, Dengue¹⁶ infection. Our previous studies in H7N9-patients in China showed that the earlier recruitment of activated CD8⁺ T cells correlated with shorter duration of hospitalization, while late recruitment of CD8⁺ T cells leads to more diverse immune responses including upregulation of neutralizing antibodies, CD4⁺ T cells and NK cells⁹. While patients who recovered from H7N9 had early IFN- γ -secreting CD8⁺ T cells¹⁰, persistent and prolonged expression of CD38 and MHC-II on CD8⁺ T cells was associated with fatal outcomes¹⁰.

In this study, we demonstrate that IAV infection in a mouse model also induced the activation and expansion of CD38⁺MHC-II⁺ CD8⁺ T cells. Expression of CD38⁺ and MHC-II⁺ on CD8⁺ T cells recalled following H7N9 challenge correlated with high levels of PD-1 expression in line with our observations in H7N9-infected patients who survived¹⁰. CD38⁺MHC-II⁺PD-1⁺CD8⁺ T cells were also detected in the primary response following infection with the mouse adapted strain, X31. Of note, increased disease severity resulting from infection with a high dose of X31 was not only characterized by excessive cytokine production, reminiscent of early hypercytokinemia observed during fatal H7N9 infection in humans⁹, but also led to increased PD-1⁺ expression in CD38⁺MHC-II⁺CD8⁺ T cells. This is expected given that CD8⁺ T cell exhaustion, defined by PD-1^{high} expression, is a notable feature of disease conditions where excessive or prolonged inflammatory conditions are present, such as during acute and chronic infections caused by highly virulent IAV strains¹⁰, HIV^{32,33}, B type hepatitis (HBV)³⁴ as well as lymphocytic choriomeningitis virus (LCMV)³⁵.

Within the total primed CD38⁺MHC-II⁺ CD8⁺ T cell population in mice following influenza virus infection, a large proportion appeared to be tetramer-negative, which indicates recruitment of bystander CD8⁺ T cells at the site of infection. This was observed within populations of endogenous primary CD38⁺MHC-II⁺ CD8⁺ T cell populations (**Fig 3**) and

during the secondary recall of OT-I cells (**Fig 4**). This could be due to inflammation-induced expansion not only at the site of infection but also in peripheral immune organs as numerous studies show that cytokines³⁶ such as type I IFN³⁷, IL-15³⁸, IL-12/IL-18³⁹ are capable to recruit non-virus-specific CD8⁺ T cells during different infectious diseases. It is unclear however what impact these bystander CD38⁺MHC-II⁺ CD8⁺ T cells have on the overall immune response against IAV infection. Similar findings have been noted by researchers who found bystander CD8⁺ T cells activated during the early phases of HIV-1 infection with CD38⁺MHC-II⁺ phenotype and specificity for cytomegalovirus, Epstein–Barr virus and influenza antigens²⁰. Moreover, bystander CD8⁺ T cells and CD38⁺MHC-II⁺ CD8⁺ T cells can exhibit cytotoxic effects during acute Hepatitis A virus infection closely correlating with severe liver injury⁴⁰. Thus, while not much is known about the function of bystander CD38⁺MHC-II⁺ CD8⁺ T cells during influenza infection, it is possible that their presence may be related to the hypercytokinemia state at site of infection and contribute to lung damage leading to severe disease outcomes⁹.

In contrast to human CD8⁺ T cells, murine CD8⁺ T cells lack the transcription factor *CIITA*, a master regulator of *H2-Ab (MHC-II)* gene expression²⁶. However, MHC-II is highly expressed on antigen-presenting cells (APCs) and our results are the first to demonstrate *in vivo* that CD38⁺CD8⁺ T cells can “snatch” MHC-II, along with CD19 and possibly other cell surface markers from B cell populations. Trogocytosis of cell surface markers has been described for many immune cells in both mice and humans. For example, basophils can acquire MHC-II from dendritic cells to promote T cell differentiation to Th2 cells⁴¹. Similarly, T cells can acquire HLA-G from APCs changing their function as effector cells to regulatory cells⁴². Of relevance, CD38 expressed on B cells can establish lateral associations with various membrane proteins/complexes, including CD19 to help mediate intracellular signaling⁴³. The interaction of both these markers thus paints a plausible scenario where CD19 is ‘snatched’ from B cells via interaction with CD38 on CD8⁺ T cells. Recent evidence also indicates that the exchange of markers between CD8⁺ T cells and B cells populations is bi-directional. Strittmatter-Keller *et al* in particular have reported a population of human B cells expressing CD8 despite the absence of CD8 α or CD8 β message, suggesting that this marker is acquired via cell-to-cell interactions with T cells⁴⁴.

What is to be gained by CD38⁺CD8⁺ T cells acquiring MHC II expression? Our adoptive transfer results demonstrate that MHC-II on murine effector CD38⁺CD8⁺ T cells may play a role in their optimal survival and expansion following secondary challenge. This may be related to enhanced ability to migrate to site of infection or stimulate other immune subsets in order to boost immune responses towards viral challenge. In fact, *in vitro* studies have shown that murine CD8⁺ T cells that have acquired MHC-II molecules from dendritic cells are able to stimulate CD4⁺ T cells in culture⁴⁵. Given the importance of helper T cells in memory CD8⁺ T cell formation^{46,47} and in the recall of secondary responses^{48,49}, it can be assumed that CD8⁺ T cells expressing MHC-II molecules can also present antigen for recognition by CD4⁺ T cells to stimulate the secretion of cytokines that promote effector function and differentiation into memory populations. This could therefore constitute an additional mechanism to amplify T-helper responses⁵⁰ but may also contribute to excessive responses or even tolerance⁵¹, especially if immune-regulatory mechanisms are perturbed as reflected in dysfunctional innate immune responses observed during severe H7N9-infection⁹. Other implications can be gleaned from studies demonstrating snatching of peptide-MHC-I complexes (pMHC) by CD8⁺ T cells from APCs via cell-cell interactions^{52,53,54}. Here, trogocytosis can lead to fratricide of T cells or exhaustion due to prolonged engagement of TCRs with “snatched” MHC-II-antigen complexes especially in the presence of high densities of pMHC⁵². These scenarios highlight the impact of trogocytosis of MHC-II molecules, enhancing interaction between CD4⁺ and CD8⁺ T cells to boost memory CD8⁺ T cell recall responses but in the event of a severe viral infection, this process may impair CD8⁺ T cell responses as observed in fatal H7N9 patients. Therefore, this MHC-II trogocytosis by CD8⁺ T cells may be a double-edged sword during infection and balancing of the level on this specific immune subset may be one of the key factor for controlling disease outcomes.

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Author contributions: XJ, ZW, BC, LL, LK and MK performed and analysed the experiments. JX, ZW, JQ, WH and KK designed experiments. XJ, BC and KK wrote the manuscript. All authors revised the manuscript. KK led the study.

3.10 Competing Interests

Disclosures: The authors declare no competing interests exist.

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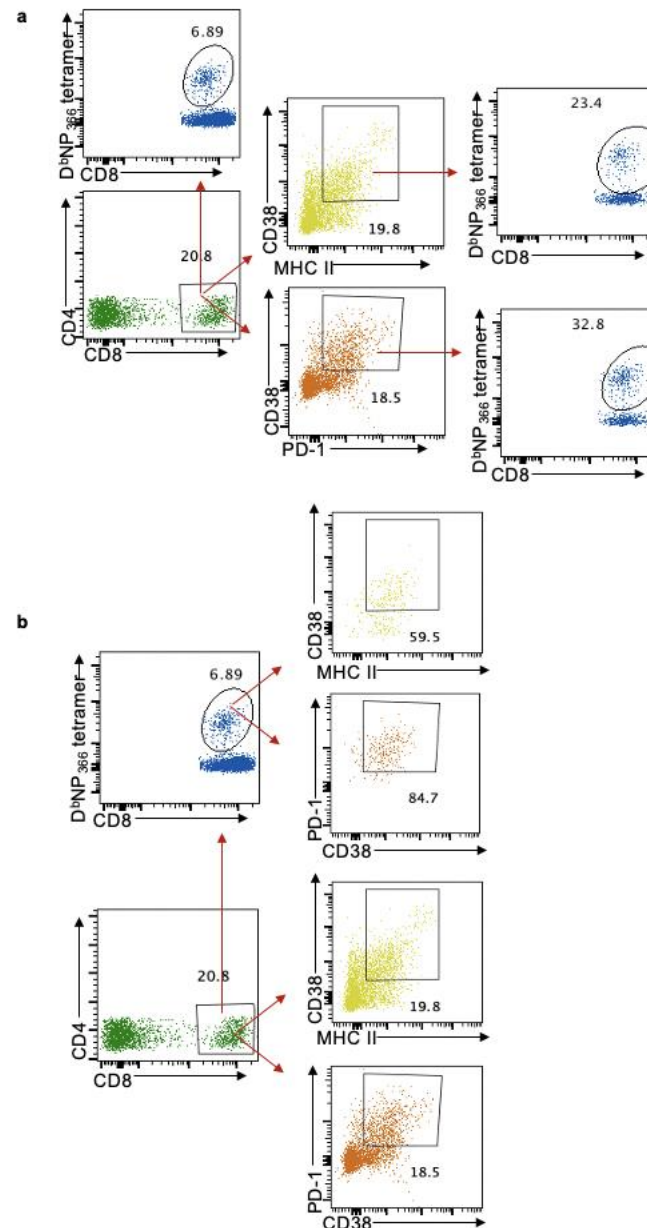
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3.12 Supplemental Files

Fig S1 shows gating strategies on different subsets of CD8⁺ T cells and phenotypes.

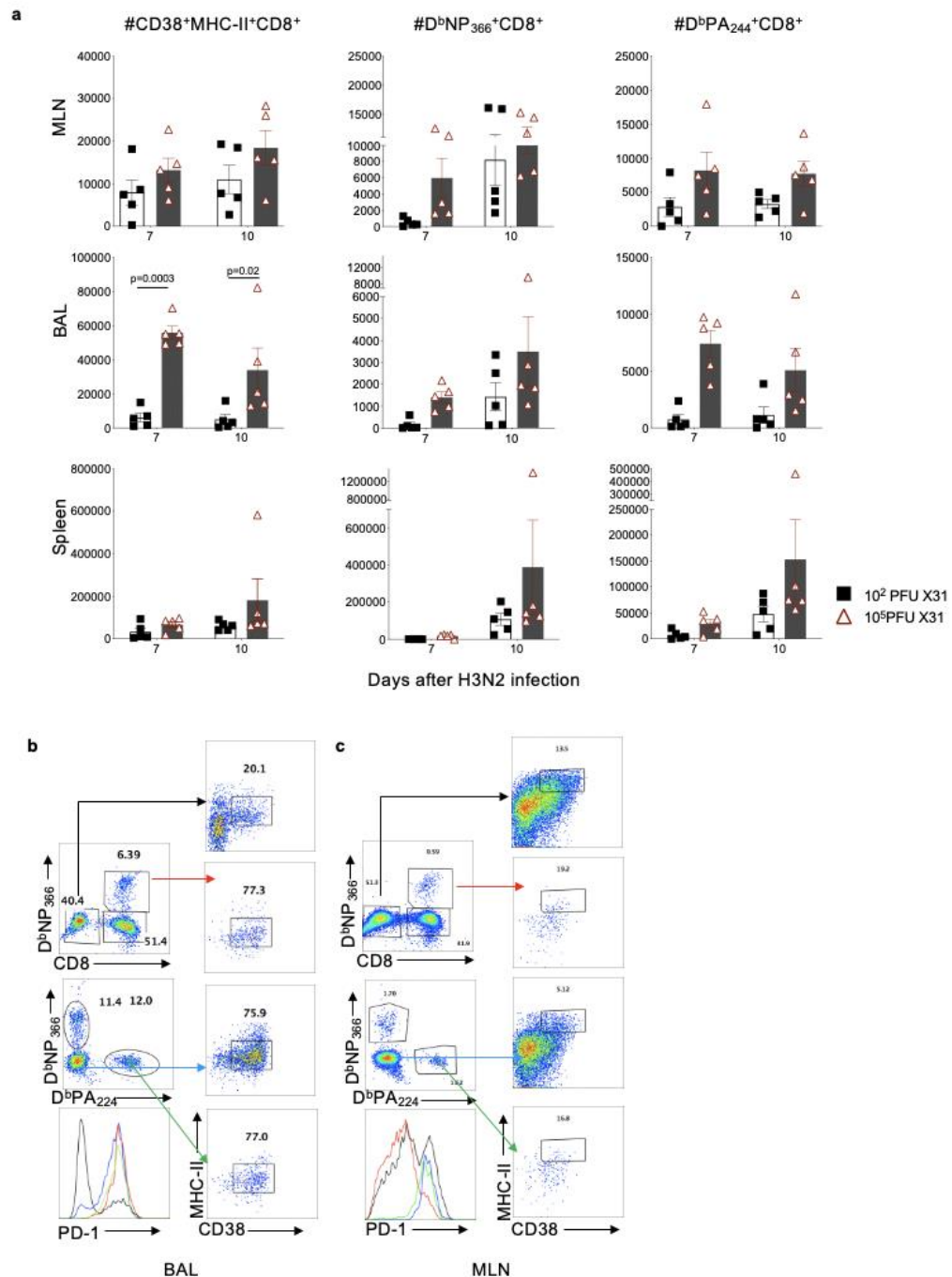


Supplementary Figure 1. Jia X *et al*

Fig S1. Gating strategy of D^bNP₃₆₆⁺CD8⁺ and CD38⁺MHC-II⁺CD8⁺ T cell populations

(a) Gating strategy to define D^bNP₃₆₆⁺CD8⁺ T cells from the pool of total CD8⁺ T cells, CD38⁺MHC-II⁺CD8⁺ T cells or CD38⁺PD-1⁺CD8⁺ T cells. **(b)** Gating strategy to define CD38⁺MHC-II⁺CD8⁺ and CD38⁺PD-1⁺CD8⁺ T cells from total CD8⁺ or D^bNP₃₆₆⁺CD8⁺ T cell populations.

Fig S2 shows (a) early recruitment of CD38⁺MHC-II⁺ CD8⁺ T cells and influenza-specific CD8⁺ T-cell to BAL after severe influenza virus challenge and (b) gating strategies on CD38, MHC-II and PD-1 phenotype on different subsets of CD8⁺ T cells.



Supplementary Figure 2. Jia X *et al*

Fig S2. Early recruitment of tetramer-negative CD38⁺MHC-II⁺CD8⁺ T cells to the site infection with a high viral dose of H3N2. B6 mice were infected with HKx31 (H3N2) at either a high (10⁵ pfu) or low (10² pfu) dose i.n.. At d7 and d10 after infection, BAL, spleen and MLN lymphocytes were stained with the D^bNP₃₆₆ and D^bPA₂₂₄ tetramers and anti-CD8 antibody, combined with a phenotyping panel for CD38, PD-1 and I-A^b. **(a)** Numbers of CD38⁺MHC-II⁺CD8⁺ T-cells, D^bNP₃₆₆⁺CD8⁺ and D^bPA₂₂₄⁺CD8⁺ T cells are shown in MLN, BAL and spleen at d7 and d10 after infection (n=5 mice). **(b, c)** Representative FACS plots are shown for CD38, MHC-II and PD-1 expression on D^bNP₃₆₆⁺CD8⁺, D^bPA₂₂₄⁺CD8⁺ T cells and tetramer-negative CD8⁺ T cells.

CHAPTER IV: TO CHARACTERISE DISEASE SIGNATURES DURING EARLY PHASES OF H7N9 VIRUS INFECTION *IN VITRO*

4.1 Introduction

When a novel avian A/H7N9 influenza virus emerged in China in 2013, previous work from our laboratory was the first to show that early CD8⁺ T cell responses drove recovery from severe influenza disease²⁵⁸. It also defined why patients succumbed to H7N9 infection by showing that fatal disease outcome was associated with over-activation of early innate immune responses and excessive inflammation²⁵⁹. Furthermore, the whole blood gene expression analysis found differential expression of a wide range of genes (> 2000) during late stages of disease when H7N9 patients who recovered versus those who succumbed to the disease were compared, particularly within the genes involved in TCR signaling pathways²⁵⁸.

In this study, we focused our analysis on the early stages of H7N9 disease between these two H7N9 patient groups, with the objective to uncover novel markers that could predict the outcome of influenza virus infection. Here, we report that a gene encoding for Oleoyl-(acyl-carrier-protein) hydrolase (OLAH), located on human chromosome 10, was significantly up-regulated at the transcription level in whole blood of patients who succumbed to infection compared to those that survived. OLAH is an enzyme that is involved in lipid biosynthesis where its function is to mediate the release of different lengths of free fatty acids to meet the energy needs of cells²⁶⁰.

A handful of previous clinical studies reported elevated OLAH gene expression in patients during early sepsis²⁶¹, and in a small sample size of IAV-infected (A/H1N1/pdm09) children experiencing neurological symptoms and pneumonia²⁶². Increased oleic acid, which is the major downstream product of OLAH was also detected in serum of patients with ARDS²⁶³, implicating OLAH's role in exacerbating inflammation and severe respiratory disease. However, these reports are observational and to date, the role of OLAH in mediating infectious disease outcomes and its involvement in modulating immune responses is unclear.

As discussed in Chapter I, *de novo* fatty acid metabolism can alter the activation and differentiation of CD8⁺ T cells due to its impact on energy support pathways²²². Moreover, it

has been found that lipid metabolism can greatly affect innate immune responses. For instance, differentiation of alveolar macrophages in lung is greatly dependent on the metabolic pathway within cells, as fatty acid synthesis (FAS) is required to generate pro-inflammatory macrophages named M1²⁶⁴, while enhanced fatty acid oxidation (FAO) can contribute to M2 macrophage polarization²⁶⁵, indicating that altering fatty acid metabolism can impact macrophage function.

In addition to having a direct impact on host immunity, pathogens are greatly dependent on host metabolism to benefit their own replication²⁶⁶. Of relevance to this study, infection by influenza virus can cause host cell metabolic disorders by increasing fatty acid synthesis²⁶⁷. The accumulation of metabolic mediators such as pyruvate dehydrogenase kinase 4 (PDK4), which is unfavorable for homeostasis regulated by glucose, together with dysregulation of ATP production particularly during the early stage of infection has been implicated in exacerbating the cytokine storm, suggesting that restoring long chain fatty acid oxidation particularly at the onset of disease may be associated with improved clinical outcomes in influenza-infected patients²⁶⁶.

Given the role of OLAH in lipid metabolism, it thus stands to reason that OLAH could constitute a key driver of severe outcomes caused by influenza virus infection. In this PhD Chapter, we extended our investigations of H7N9 transcriptome signatures to the early phases of infection in patients who recovered versus patients who succumbed to H7N9 disease. Our data provide evidence that OLAH gene expression is strongly linked to severe disease. In the current Chapter, we evaluated and developed reagents that enabled us to further investigate the distribution of OLAH across different immune tissues and cell types to gain further insight into OLAH's role in driving severe disease outcomes.

4.2 Results

In 2013, an avian-derived H7N9 subtype of influenza A virus emerged in China causing high hospitalization (>90%) and mortality (>30%) rates^{268,269}. During the first wave of the H7N9 outbreak, our laboratory in collaboration with Professor Jianqing Xu from Fudan University in Shanghai, collected nasal swabs and longitudinal blood samples from a range of H7N9 patients at different time points. In particular, we obtained samples from 8 individuals

within 17 days of hospital admission (average of 10 days), who then either recovered (n=4) or died from H7N9 infection (n=4) (**Fig 1a**). All patients in the recovered group were discharged from hospital within 23 days. Patients from this unique cohort were had a mean of 69 and 70 years of age in the recovered and severe/fatal groups, respectively. Two patients in the recovered group and 3 patients in severe/fatal group had underlying medical conditions including arrhythmia, diabetes, cholecystitis, hypertension, heart disease, chronic bronchitis and primary biliary cirrhosis (**Fig 1b**).

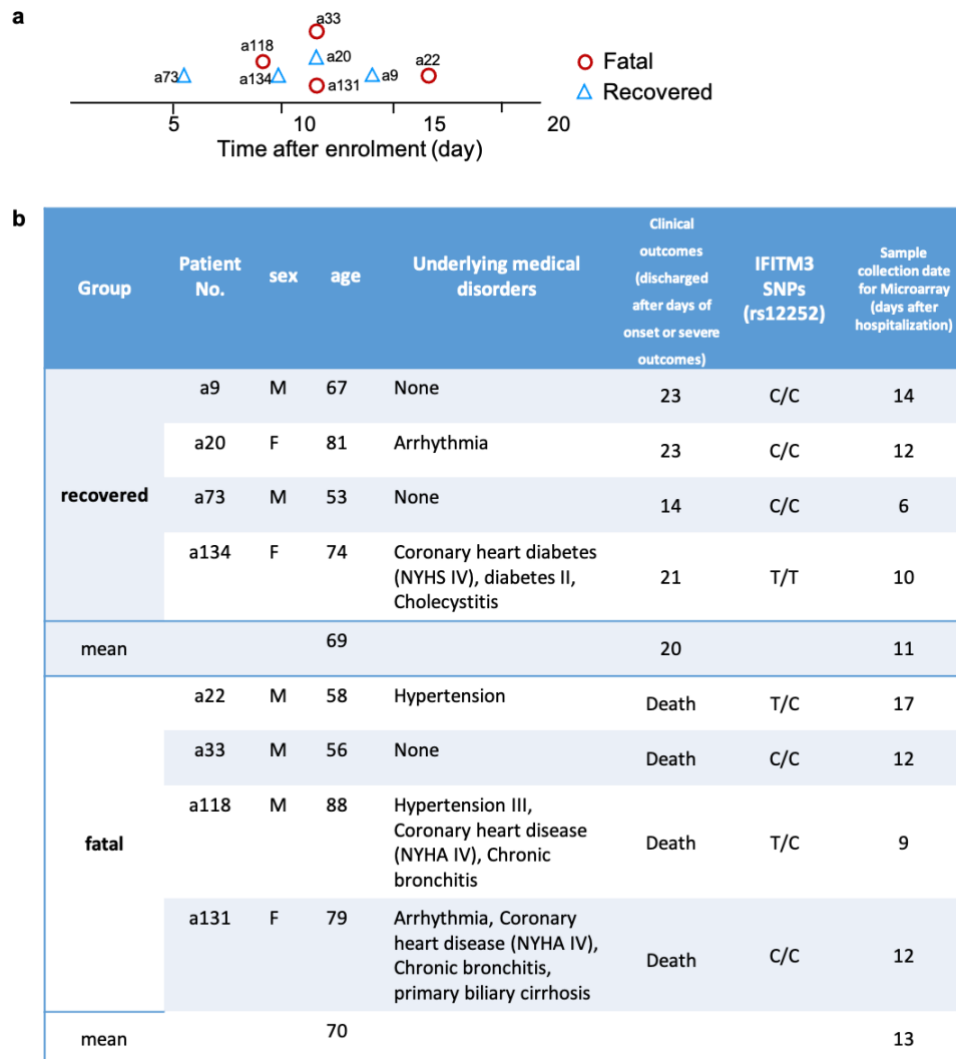


Fig1. Clinical information on H7N9 patients used in this Chapter

(a) Time points of whole blood sampling from hospitalized H7N9 patients who recovered or died from influenza disease. (b) Patient clinical information, including patient number, gender, age, underlying medical disorders, clinical outcomes, IFITM3 SNPs (rs12252) and day of sampling for Affymetrix analysis.

Our previous study showed that CD8⁺ T cells in patients who succumbed to H7N9 infection displayed an “exhausted” phenotype defined by high levels of the activation makers CD38 and HLA-DR on cell surface, and also with minimal secretion of IFN- γ ²⁵⁸. Severe

outcomes were also associated with delayed T cell expansion, clonally diverse responses¹⁹² as well as the presence of single nucleotide polymorphisms (SNPs) in the anti-viral mediator IFN-induced transmembrane protein-3 (IFITM3)²⁵⁹. In particular, the C/C phenotype in the Rs-12252 allele is associated with faster disease progression and a lower survival rate²⁵⁹. Analysis of Rs-12252 SNPs from DNA samples of patients from both groups found that all patients in the severe/fatal group expressed both the C/C or T/C phenotype (50% C/C and 50% T/C) (**Fig 1b**), indicating that these SNPs may play a role in mediating clinical outcomes. However, 75% of patients who recovered also expressed the C/C phenotype and yet still resolved disease, which further suggested that there are other host factors that mediate disease outcome.

4.2.1 Higher inflammation level in fatal H7N9 patients

Analysis of viral titres in nasal swabs of H7N9 patients found comparable levels between H7N9 patients belonging to the recovered and severe/fatal group (**Fig 2ai**). However, higher levels of cytokines were observed in the severe/fatal group (6-9 days after admission) (**Fig 2aii**), in particular IL-6, MIP-1 β and IL-8, culminating in clear differences in the total amount of cytokines detected.

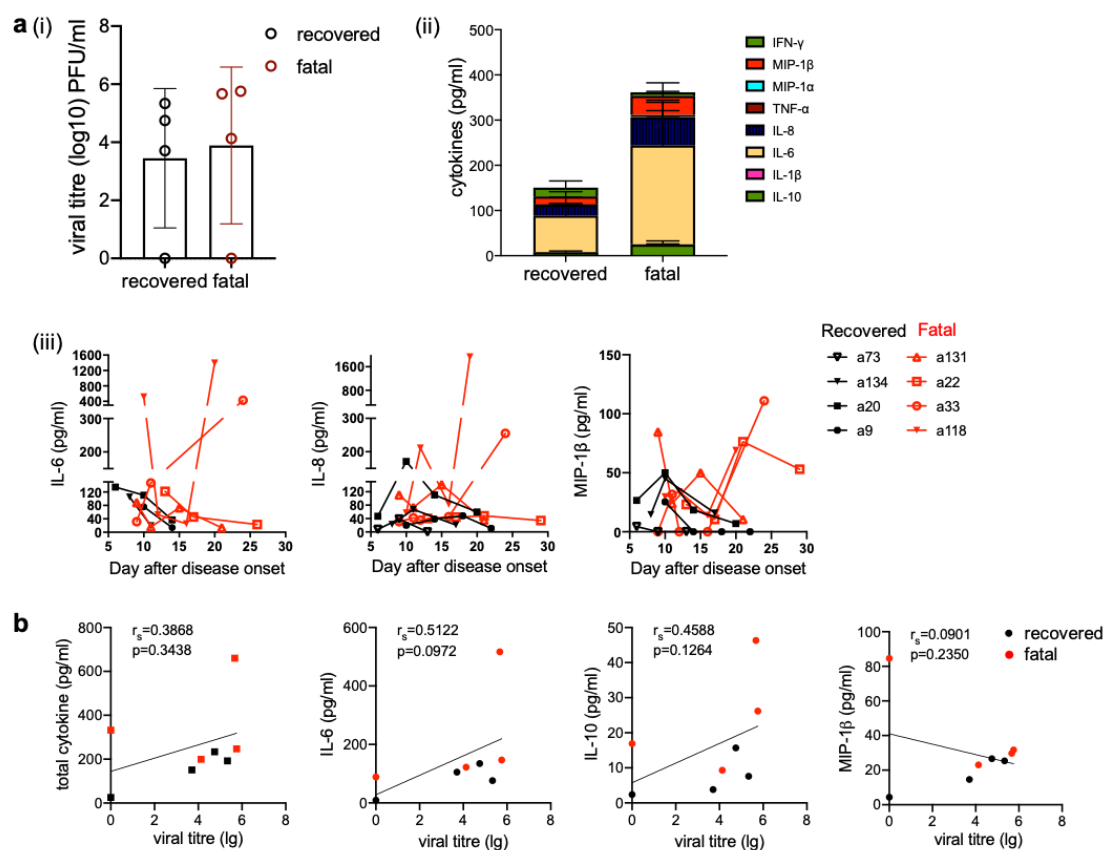


Fig2. Higher inflammation level in fatal H7N9 patients

(a)(i) Viral titres from patient nasal swabs and (ii) cytokines from patient plasma (average $\pm$ SD). (iii) Longitudinal analyses of individual cytokine levels in recovered and fatal patients. (b) Correlations of viral titre vs total cytokines, IL-6, IL-10 and MIP-1 β in recovered and fatal patient groups were calculated using Spearman Correlation and R^s represents R-squared value. Original data were obtained by Zhongfang Wang for a larger cohort of H7N9 patients and replotted for the purpose of this study. The thesis author has contributed to experimental design, acquiring samples, performing assays and data analyse.

Longitudinal analysis of IL-6, IL-8, and MIP-1 β levels in severe/fatal patients supported these results and showed that in some cases high amounts of these cytokines persisted throughout the course of the disease. In contrast, in the recovered group, there was an overall trend of lower levels of cytokines, which in many cases subsided upon discharge from hospital (**Fig 2a**).

Given that there were no differences in viral titres in both groups, we found that viral titres did not significantly correlate with total cytokine amounts, IL-6, IL-8, and MIP-1 β when data from all patients were combined (**Fig 2b**). Correlation analyses across multiple variables at the same time found strong positive correlations between some cytokines in all H7N9 patients, most notably between IL-6 and IL-10 (0.8) as well as IL-8 and MIP-1 β (0.8) (**Fig 3a**). Similar correlations between these cytokines were also found when we analysed results in fatal and recovered H7N9 patients groups separately (**Fig 3b,c**). In addition, we found significant correlation between IL-10 and IL-8 (0.86 in fatal and 0.8 in recovered), IL-10 and MIP-1 β (0.88 in fatal and 1 in recovered) in both patient groups, and IL-6 and IL-8 (1.0), IL-6 and MIP-1 β (0.8), IFN- γ and IL-10 (0.8), IFN- γ and MIP-1 β (0.8) in patients who recovered (**Fig 3c**), indicating a diverse cytokine profile associated with H7N9 disease. Importantly, in H7N9 recovered patients, there was a higher correlation between viral titres and total cytokine production (0.8), IL-10 (0.8), MIP-1 β (0.8) and IFN- γ (1) (**Fig 3b**), suggesting that recovery is reflected by a more controlled and focused cytokine response which was not as apparent in patients who succumbed to the infection (**Fig 3c**).

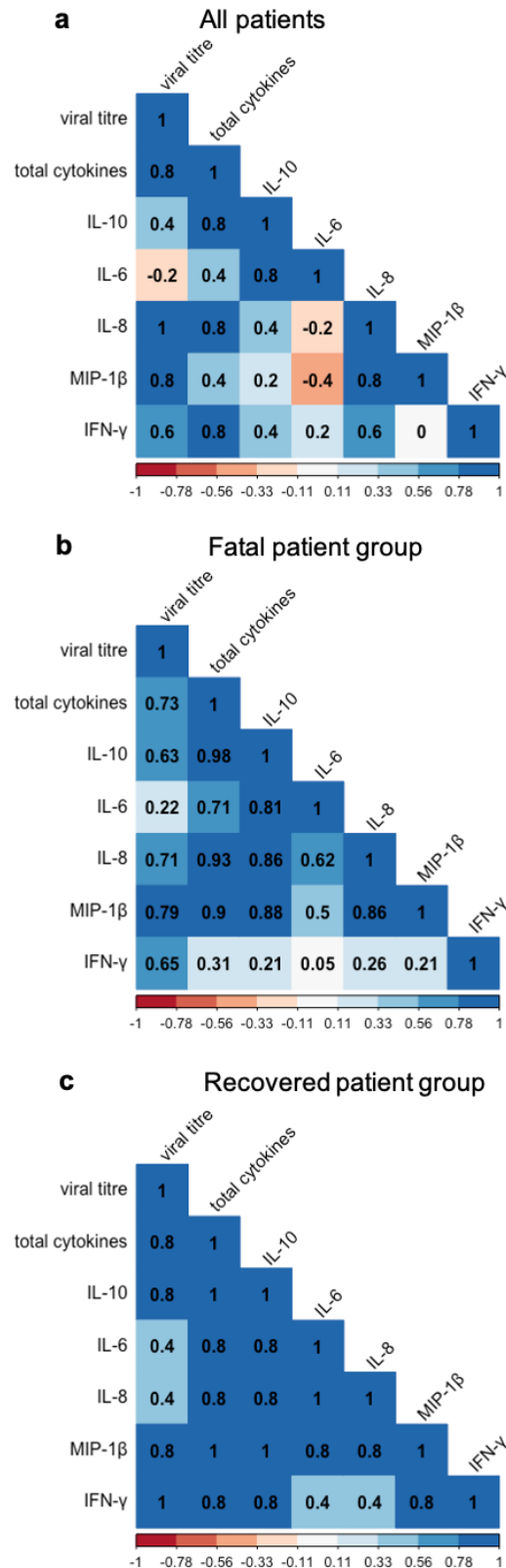


Fig.3 Correlations between cytokines and viral titre in H7N9 patients

Relationships between cytokine levels and viral titres in the (a) total patient, (b) fatal patient and (c) recovered patient groups were analysed by Spearman Correlation and R^s represents R-squared value. Original data were obtained by Zhongfang Wang for a large H7N9 cohort and replotted for the purpose of this study. The thesis author has contributed to experimental design, acquiring samples, performing assays and data analyse.

4.2.2 Higher OLAH expression in fatal H7N9 patients at early stage of disease

To investigate whether other potential host factors might contribute to different disease outcomes, we analysed transcriptomic gene expression levels by Micro-Array in whole blood samples from recovered and severe/fatal patients early after disease onset. In contrast to our previous Affymetrix Human Gene ST 2.0 arrays analysis of blood samples from H7N9 patients at the late stage of disease showing more than 2000 differentially expressed genes between both groups²⁵⁸, strikingly only three genes were differentially expressed during the early phases of disease (**Fig 4a,b**). Two of the genes encoded for collagen (*COL27A1*) and myosin (*MYO1B*), which were expressed at ~5-fold higher and ~10-fold lower in patients who succumbed infection.

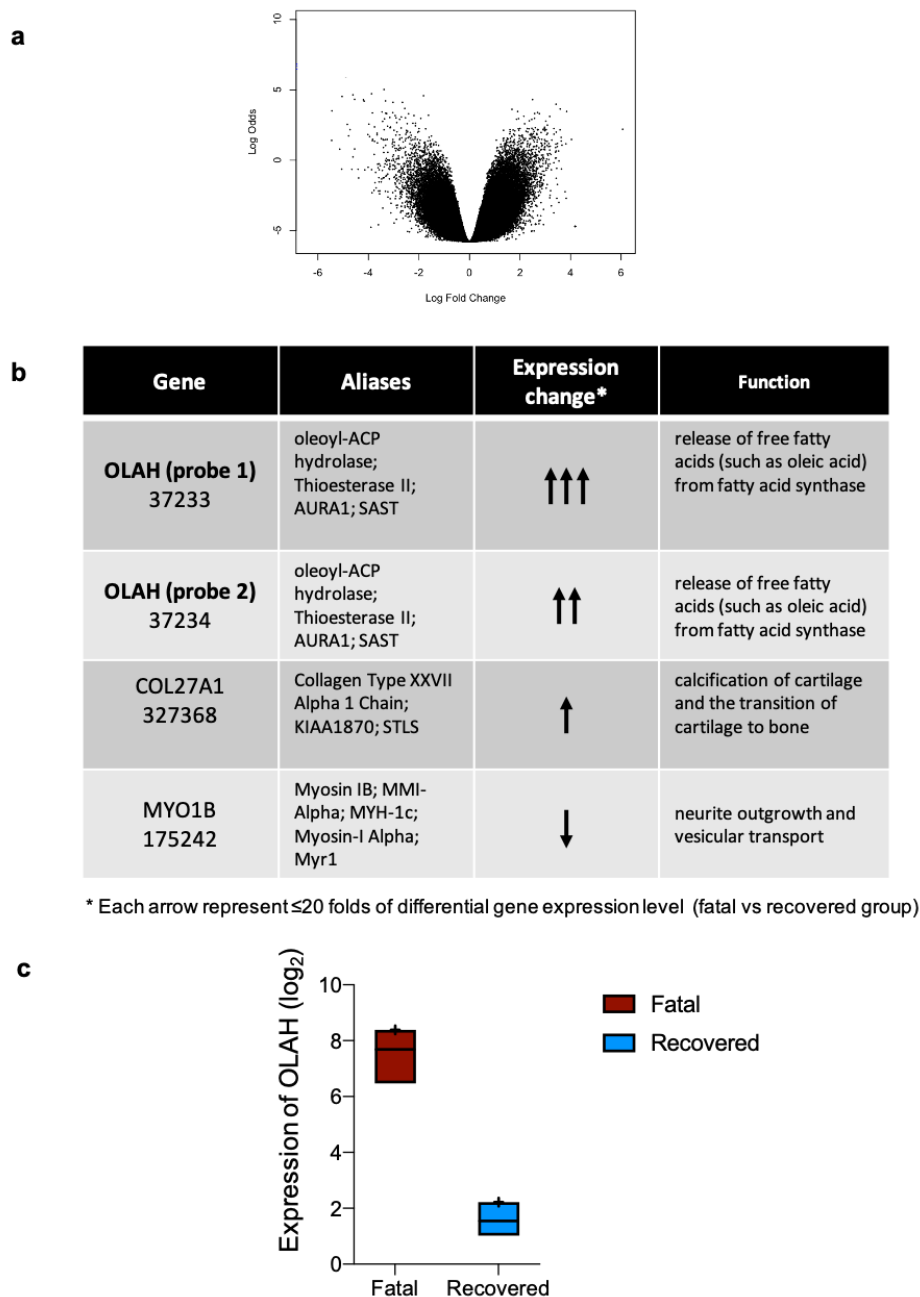


Fig4. Higher OLAH expression in fatal H7N9 patients

(a) Total RNA collected from peripheral blood of fatal and recovered patients during the early stage of disease onset and analysed on Affymetrix Human Gene ST 2.0 arrays. Volcano plot shows differential gene expression profiles between recovered and fatal H7N9 patient groups. (b) Three genes were differentially expressed between recovered and fatal patient groups at the early time-points after disease onset. (c) Transcriptional level of *Olah* expression between recovered and fatal patient groups during the early stages of disease onset.

However, an exciting finding was that two probes pointed to a gene encoding for OLAH (Fig 4b), found to be expressed at more than 80-fold higher in patients from the severe/fatal

groups than in those who recovered (**Fig 4c**), indicating a potential role of OLAH in mediating severe disease outcomes.

4.2.3 Trials on detecting OLAH in human/mouse cells using Western Blot

To further investigate the expression of OLAH across different anatomical compartments and cell types, we first purchased three commercially available antibodies for western blot detection of human and mouse OLAH. As the OLAH protein is not commercially available, protein lysates were prepared from the lung epithelial cell line, A549, human PBMCs and placenta-derived cells from a healthy human subject, in addition to testis-derived cells from uninfected C57BL/6 mice. However, despite several attempts, OLAH expression was not consistently detected by Western blotting using all three antibodies tested (Antibodies-online.com, AVIVA Systems Biology, MERCK) (**Fig 5**).

a

company	Catalog No.	name	clonality	Predicted reactivity
Antibodies - online.com	ABIN2786608	Oleoyl-ACP Hydrolase(OLA) (N-Term)	polyclonal	Horse: 83%, Human: 100%, Mouse: 77%, Pig: 79%, Rabbit: 83%
AVIVA Systems Biology	ARP56263_P050	OLA Antibody - N-terminal region (ARP56263_P050)	polyclonal	Horse: 83%; Human: 100%; Mouse: 77%; Pig: 79%; Rabbit: 83%
MERCK	HPA037948	Anti-OLA antibody produced in rabbit	polyclonal	human

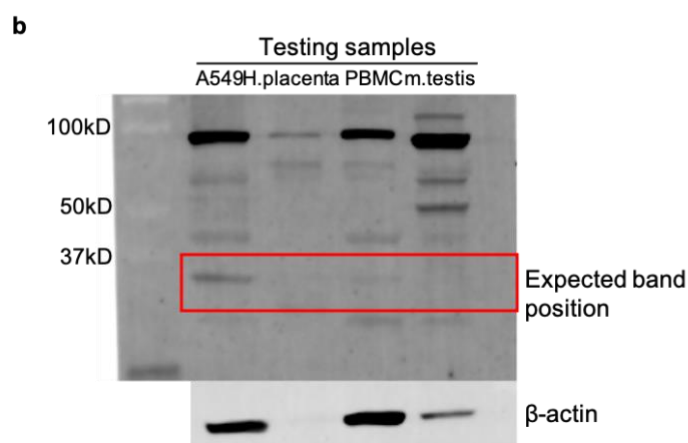


Fig 5. Detection of OLAH by commercially available antibodies and the western blot approach

(a) Information on selected commercial anti-OLAH antibodies used for evaluation in this PhD Chapter.
(b) Representative example of western blot on human A549 cells, human placenta cells, human PBMCs and mouse testis cells. Supernatants containing target protein from lysed cells were run on a 10% SDS-PAGE gel and transferred onto PVDF membrane. Purchased primary OLAH antibody (in rabbit host) with secondary anti-rabbit antibody conjugated with Alexa Fluor 488 (FITC) fluorescence were used to detect OLAH protein at the expected molecular weight. Fluorescence was detected using Pharos imager.

At best, one of the antibodies obtained from Antibodies-online.com, reported to recognize human and mouse OLAH, resulted in the detection of a faint band at ~30kD, corresponding to the expected molecular weight of OLAH (30kD) in A549 cells. An even less intense band was also detected in the human PBMC sample but this required large amounts of lysate as indicated by the strong β -actin levels (**Fig 5**). We could not detect similar bands in human placenta-derived cells nor using testis-derived mouse cells to confirm species cross-reactivity. However, these data were not convincing and additionally, we encountered difficulties in extracting sufficient amounts of protein from human and murine samples, as indicated by the relatively lower levels of β -actin present. Thus, given a lack of publications using these anti-OLAH antibodies, low intensity of staining and the requirement for large amounts of protein to be used, we were not convinced that the commercially available antibodies reliably detected OLAH, thus moved our efforts to qPCR for OLAH detection.

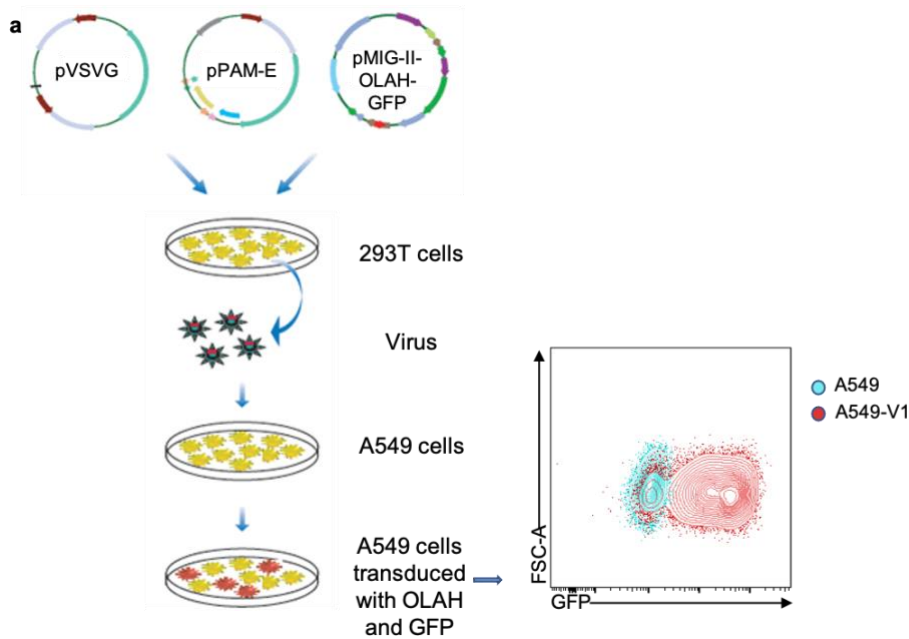


Fig 6. Generation of *Olah*-overexpressing A549 cell line

(a) Flow-chart for the generation of *Olah*-overexpressing A549 cell line using recombination of retro viruses. *Olah*-GFP inserted in pMIG-II plasmid (4μg), pVSVG (2μg) encoding viral glycoprotein envelope and pPAM-E (4μg) as a shuttle vector, together with OptiMEM and Fugene 6 reagent were added to 239T cell cultures for 24 hrs to generate *Olah*-GFP virus. The supernatant containing recombinant virus from transduced 239T cells were added to A549 cell cultures to generate a *Olah*-GFP transduced A549 cell line (A549-V1). Successfully transduced cells were identified and sorted by flow cytometry based on GFP expression.

4.2.4 Generation of *Olah*-overexpressing A549 cell line

We subsequently explored the use of qPCR as a method for us to analyse *Olah* expression (**Fig 7a**). To validate this method, we established an *Olah*-over-expressing human lung epithelial cell line A549 (A549-V1). To achieve this, we used recombinant retrovirus with an *Olah*-GFP inserted into a pMIG-II plasmid together with pVSVG plasmid encoding for viral glycoprotein envelope and pPAM-E as a shuttle vector, to generate virus that expresses *Olah*-GFP in 293T cells. The newly generated virus was then used to infect A549 cells in culture and OLAH expressing cells, based on the expression of GFP, were sorted by flow cytometry (**Fig 6**). RNA extracted from these A549 and A549-V1 cells was used to screen for OLAH gene expression using qPCR and our results showed that *Olah* expression was indeed higher in A549-V1 compare to A549 cells (**Fig 7b,c**).

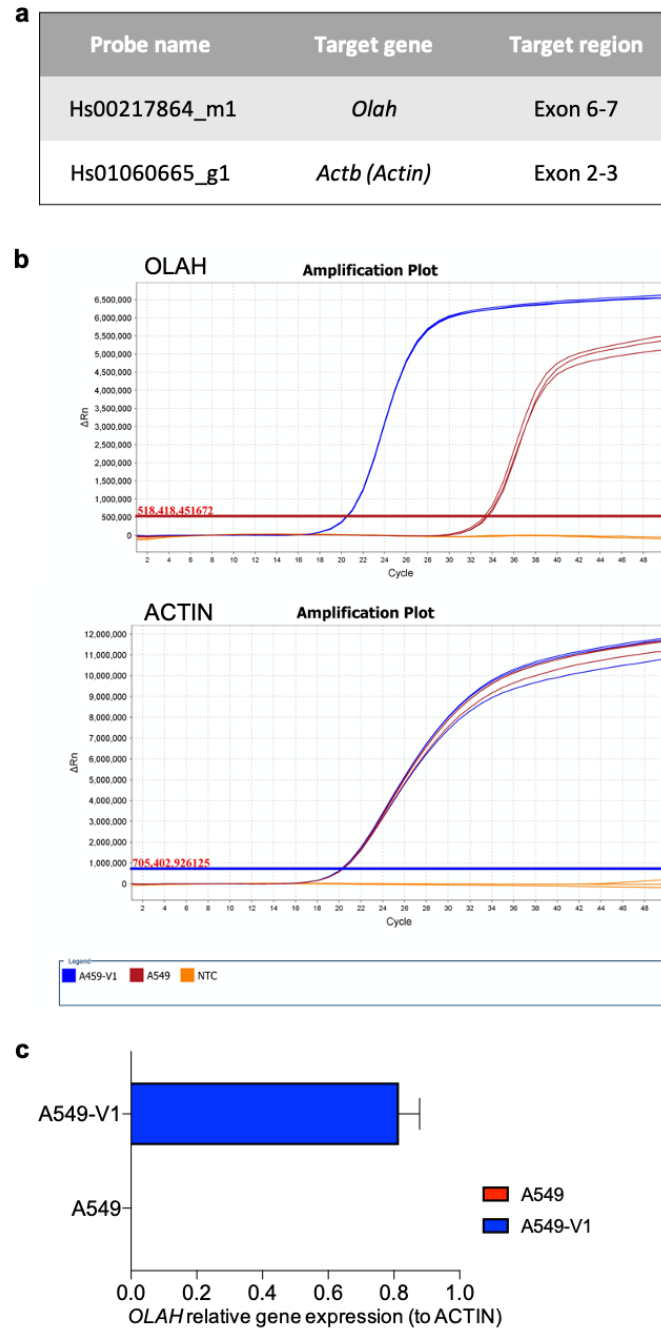


Fig 7. Identification of *Olah*-overexpressing A549 cell line via qPCR

(a) Probes for detecting *Olah* and *Actb* in qPCR. (b) RNA were extracted from single cell suspensions from A549 or *Olah*-overexpressing A549 cell lines (A549-V1). qPCR was performed to measure *OLAH* gene expression levels. Amplification plots of *OLAH* and house-keeping gene (*actin*) are shown in (b) and *OLAH* gene expression levels relative to *actin* levels in each cell population are shown in (c). Bar graphs and error bars depict the average levels ($\pm$ SD) from replicate samples (n=3).

4.2.5 Higher *Olah* expression in immune organs

Given little is known about OLAH, especially with regard to its expression levels in cells, we screened OLAH gene expression in cells from healthy human tissues, including PBMCs, spleen, thymus and lung from healthy adults as well as tonsils from healthy children. We performed qPCR on RNA extracted from these samples and found that OLAH is highly expressed in the lung, spleen, and PBMCs (**Fig 8a,b**), with lower expression in the tonsil and thymus.

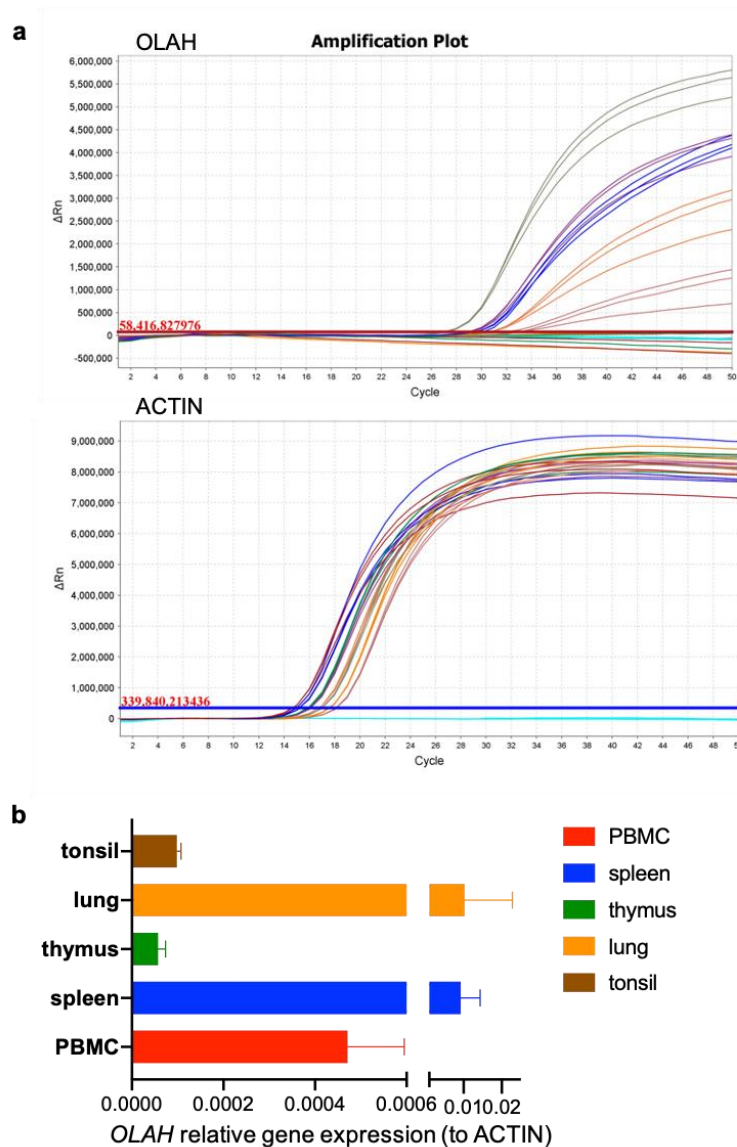


Fig 8. OLAH expression across different human cell/tissues

RNA samples were extracted from single cell suspensions from healthy human PBMCs, spleen, thymus, lung and tonsil and qPCR was performed to measure OLAH gene expression levels. Amplification plots of OLAH and house-keeping gene (*actin*) are shown in (a) and OLAH gene expression levels relative to actin levels in each cell population are shown in (b). Bar graphs and error bars depict the average levels ($\pm$ SD) from replicate samples (n=3).

4.2.6 Higher *Olah* distribution in human monocytes

Since lung is not only an important immune organ but also the primary site of influenza virus infection, we examined the distribution of OLAH expression across different immune cell populations in this organ. Our results showed that OLAH expression was found in cells that overlapped with CD14 expression. CD14 marker is predominantly expressed on cells of myelomonocyte lineage (**Fig 9**), including monocytes and macrophages and thus appears to suggest that OLAH-driven disease outcomes might be mediated by cells of the macrophage lineage.

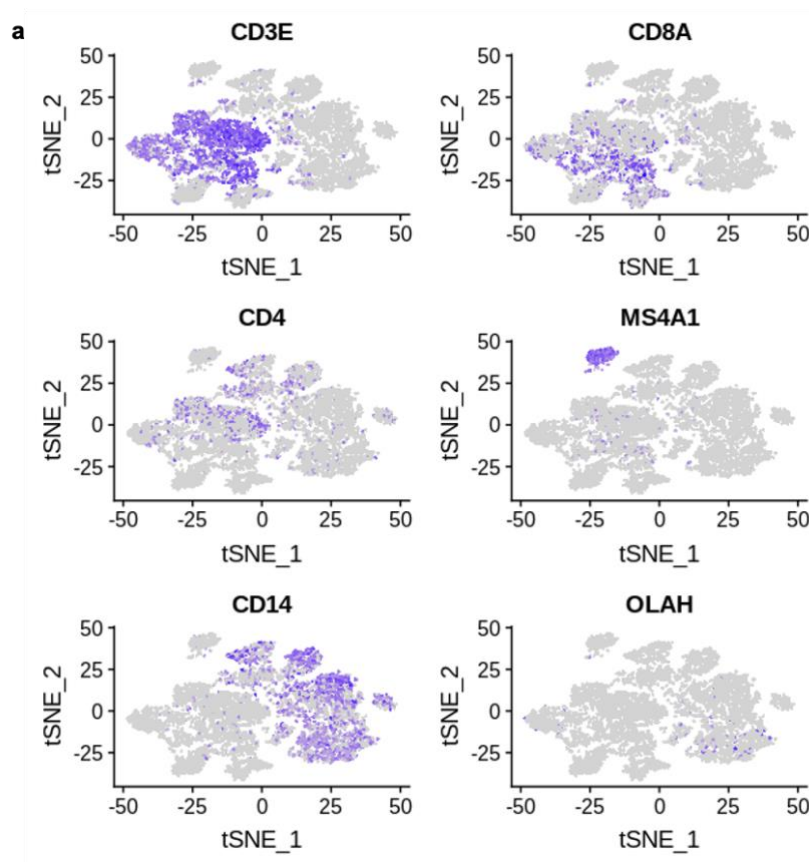


Fig 9. OLAH expression in human lung cells

(a) Single-cell suspension from healthy human lung tissue were processed and stained for different cell markers. Expression of immune cell markers were detected by flow cytometry. tSNE analysis on healthy human lung cells showing the distribution of OLAH expression on different cell types. Data were obtained in collaboration with Prof Paul G Thomas from St Jude Children's Research Hospital. The thesis author has contributed to experimental design and data analyse.

4.3 Discussion

H7N9 patients who fail to initiate early effective immune responses tend to also exhibit high levels of inflammatory responses subsequently resulting in pneumonia, tissue damage and

ultimately death²⁵⁸. These inflammatory responses are typified by high levels of IL-6, IL-10 and in particular MIP-1 β and IL-8, which are also detected at higher levels in patients with avian H5N1 severe infection²⁷⁰. Our previous study has shown that levels of these cytokines in the BAL of fatal patients, which represents the site of infection, are 10³-fold higher than in serum²⁵⁹, and indicative of severe cytokine storm. Access to this unique cohort of H7N9 patients allowed us to examine differential transcriptome features during the early phases of H7N9 disease. While we did not observe significantly increased viral titres in the fatal patient group, there was a clear trend showing that excessive inflammatory cytokines were detected, including IL-6, IL-8, IL-10 and MIP-1 β . This is in line with previous studies analysing cytokine levels in H5N1-infected patients^{271–273}. Of note, the number of individuals in our H7N9 patient cohort in this study is a limiting factor, and increasing the cohort number will undoubtedly result in more robust coefficients and results .

Interferon-inducible transmembrane (IFITM) protein family has been reported to be critical to limit viral replication during influenza virus infection^{274–276}, and the IFITM3 allele (SNP Rs-12252) C/C can be associated with impaired function in preventing influenza virus infection of cells²⁷⁷. Our previous analyses investigating the presence of IFITM3 Rs-12252 SNPs also found that frequencies of C/C and T/C alleles in H7N9 patients were significantly higher than in healthy individuals (81% vs 63%), with those having the C/C genotype likely to experience faster disease progression with lower survival rate²⁵⁹. In line with these findings, analysis presented in the current PhD Chapter showed that all patients in the severe/fatal group expressed either the T/C or C/C alleles, suggesting that they may be predisposed to severe clinical outcomes. However, the C/C allele was also observed in 3 out of 4 patients who recovered with the caveat that these are relatively small sample sizes and analysis in larger cohorts would reveal more significant differences. Nevertheless, these observations suggest that there are other host factors which may account for differences in the disease outcomes.

Our Micro-Array analysis revealed that in contrast to significant changes in gene signatures between the severe/fatal and recovered patient groups at the later stage of disease²⁵⁸, only three differentially expressed genes were detected during the early phase of infection. Of these, the gene for OLAH was profoundly expressed in patients who succumbed to the H7N9 infection, suggesting OLAH might be potentially associated with mediating different disease outcomes. While research on this gene is scarce, especially on its involvement in immune-

mediated functions, its role in fatty acid synthesis suggests that OLAH expression levels can impact lipid metabolism in host cells. In addition, OLAH may have downstream effects on ensuring immune responses and the regulation of physiological processes during infection. This hypothesis is supported by previous studies reporting its detection at high levels in during severe respiratory conditions^{261–263}. In line with this, enrichment of oleic acid, one of the downstream products of OLAH, can suppress the function of immune cells^{278–280}. Our A549 *Olah*-overexpressing cell line was confirmed by qPCR. In future studies, these newly generated cell lines can be tested by Western Blots. Given that high expression levels of OLAH can be found in human lung, spleen cells and followed by in PBMCs, it is possible that immune responses in these tissues might be affected. These effects may be associated with myeloid-derived cells such as monocytes and/or macrophages based on the co-expression of OLAH and the myelomonocytic marker CD14. The relatively low OLAH expression in tonsil may be due to different composition of lipids in this tissue compared to the spleen and lung, although further studies are needed to confirm this. It is well known that alterations to lipid metabolism can have an effect on the differentiation of macrophages into proinflammatory M1-type cells or immune-suppressive M2-type cells^{281,282}, impacting subsequent innate anti-viral responses. Moreover, OLAH expression may also contribute to the development of other diseases as excess oleic acid can impact adipogenesis in cells and thus play a role in driving obesity²⁸³, one of the high risk groups for severe influenza disease²⁸⁴.

Another possible effect OLAH may have is on the permissiveness of host epithelial cells or other immune cells such as macrophages to infection. HA-mediated membrane fusion is the first step for viral entry of influenza virus, and IFTIM proteins can prevent this via reducing host phospholipid bilayer cell membrane fluidity and inducing a positive spontaneous membrane curvature²⁸⁵. It has been found, however, that the presence of oleic acid within this bilayer can impart a negative spontaneous curvature and promote membrane fusion²⁸⁵. These effects can overall impact the environment within the site of infection through the dysregulation of cytokines affecting early recruitment of immune cells and even tissue integrity, thus contributing to different disease outcomes after infection.

Overall, the results presented in this PhD Chapter demonstrated that OLAH expression in H7N9 patients was significantly upregulated and might be associated with severe influenza disease outcomes. In evaluating and identifying reagents to analyse OLAH expression levels in tissues and cells provides tools for further studies determining the role of OLAH in mediating

disease severity. Furthermore, *in vitro* studies using our established A549 *Olah*-overexpressing cell line, and the use of *in vivo* OLAH-deficient mouse models can facilitate our understanding of the role of OLAH during influenza virus infection across different timepoints and the mechanisms that underpin these effects. This could ultimately lead to the development of novel therapies against severe influenza virus infection and could propose OLAH as a potential biomarker for predicting severe outcomes.

CHAPTER V: UNDERSTANDING THE ROLE OF OLAH IN REGULATING HOST IMMUNE RESPONSES UPON *IN VIVO* INFLUENZA A VIRUS INFECTION

5.1 Introduction

Chapter 4 of this thesis showed that high OLAH gene expression can be a strong predictive signature of fatal clinical outcomes in severe H7N9 patients. While elevated OLAH gene expression levels have been reported in clinical studies^{261,262}, OLAH's direct role in contributing to severe influenza or any other disease has not been reported to date.

IAV infection in C57BL/6 mice has been extensively characterised and serves as an accessible model for studying host-pathogen relationships, providing significant virological and immunological insights²⁸⁶. Low dose (10^2 - 10^4 pfu of H1N1 X31) IAV infection causes mild disease and up to 25% weight loss before recovery within 10 days, while high dose (10^5 pfu of H1N1 X31) IAV challenge results in severe disease and more than 25% body weight loss within 5 days, accompanied by extensive lung viral replication within 3 days²⁸⁷. In the latter case, this is accompanied by substantial infiltration and proliferation of innate immune cells, including NK cells, neutrophils, macrophages to the site of infection during the early stage of infection subsequently followed by the elicitation of adaptive immune responses, including the recruitment of T cells and B cells^{193,288,289}. While the presence of innate cells usually plays a protective role during IAV infection³⁶, elevated levels can however be detrimental to the host, particularly through the production of cytokines such as IFNs and TNF- α as well as mediators, such as reactive oxygen species which can cause significant lung tissue damage. Moreover, extensive infection of respiratory epithelial cells can also result in large amounts of cytokines being produced, exacerbating inflammation and contributing further to organ dysfunction and leakage²⁹⁰.

Severe disease caused by the IAV strain A/Puerto Rico/8/1934 (PR8; H1N1) has also been found to induce significant changes to the lipidomic profile in the lungs, in particular increased levels of linoleic acid²⁸⁷. Moreover, bronchoalveolar lavage fluid of IAV-infected mice has been shown to have significantly increased levels of long chain fatty acids, including palmitic acid, oleic acid, and linoleic acid²⁹¹. Amongst these, oleic acid and its derivative

linoleic acid²⁹² are highly relevant as OLAH catalyses the release of oleic acid from its acyl carrier protein following its synthesis.

Lipids not only serve as an essential source of energy but also play important structural, metabolic and physiological roles. Furthermore, lipids are involved in different aspects of viral infection and pathogenesis, from facilitating cell entry to providing fatty acids and energy for replication and assembly of viral particles^{293,294}. Lipids such as oleic acid are taken up and stored in cells within intracellular lipid droplets^{293–296}. These are multifunctional organelles which are involved in metabolism, signalling and anti-viral responses^{295,297–299}. Depending on the cell type, rapid and dramatic changes in lipid droplet size, number and distribution occur in response to changes in cellular conditions.

The role of lipid droplets in facilitating viral replication is now becoming more recognised. *In vitro* studies have shown that during flavivirus infection caused by Hepatitis C and Dengue virus, viral proteins are able to interact with lipid droplets to benefit viral assembly³⁰⁰. Co-localization of viral capsid proteins with lipid droplets in particular have been observed in infected host cells, indicating that these organelles are being ‘hijacked’ to produce new virions³⁰¹. Interestingly, the presence of high levels of oleic acid in the extracellular milieu results in increased lipid droplets in cells as a result of uptake. This is mediated by CD36, a glycoprotein expressed on a wide range of cell surfaces including both immune (eg. macrophage) and non-immune cells. CD36 is a fatty acid translocase belonging to the class B scavenger receptor family which facilitates the uptake of long-chain fatty acids as well as the low-density lipoproteins into cells. Although research on the function of CD36 in host cells in the context of infection is limited, it has been shown that HIV-1 can ‘hijack’ CD36⁺ compartments in macrophages to support replication³⁰². Moreover, oleic acid can accelerate cancer cell growth in a CD36-dependent manner³⁰³. Overall, these studies demonstrate a direct role for OLAH and its lipid products in promoting viral infection and hence point to its potential involvement in exacerbating viral disease.

In this Chapter, we investigated how expression of OLAH drives severe influenza disease. To examine this, we established and used OLAH knockout (OLAH-KO) mice which enabled us to use a mouse model of IAV infection to investigate how the presence or absence of OLAH affects viral disease outcome, the formation of lipid droplets in the lung and how this

relates to the induction of both anti-viral innate and adaptive immune responses during mild and severe IAV diseases.

5.2 Results

5.2.1 OLAH is expressed in immune and non-immune cells across different anatomical sites in naïve C57BL/6 mice

As there is paucity of data on the expression of OLAH at different anatomical sites in mice, we first investigated OLAH expression across different organs and tissues in naïve C57BL/6 (WT) mice. We harvested a wide range of immune and non-immune tissues, including lymph nodes (LNs), thymus, spleen, liver, lung, brain, testis and placenta, and performed qPCR to measure *Olah* mRNA expression levels. Interestingly, high levels of *Olah* mRNA expression were detected in the reproductive system, including placenta and most notably the testis (**Fig 1a**), as well as brain, lung and thymus. However, *Olah* mRNA expression was not detected in liver, spleen and LNs.

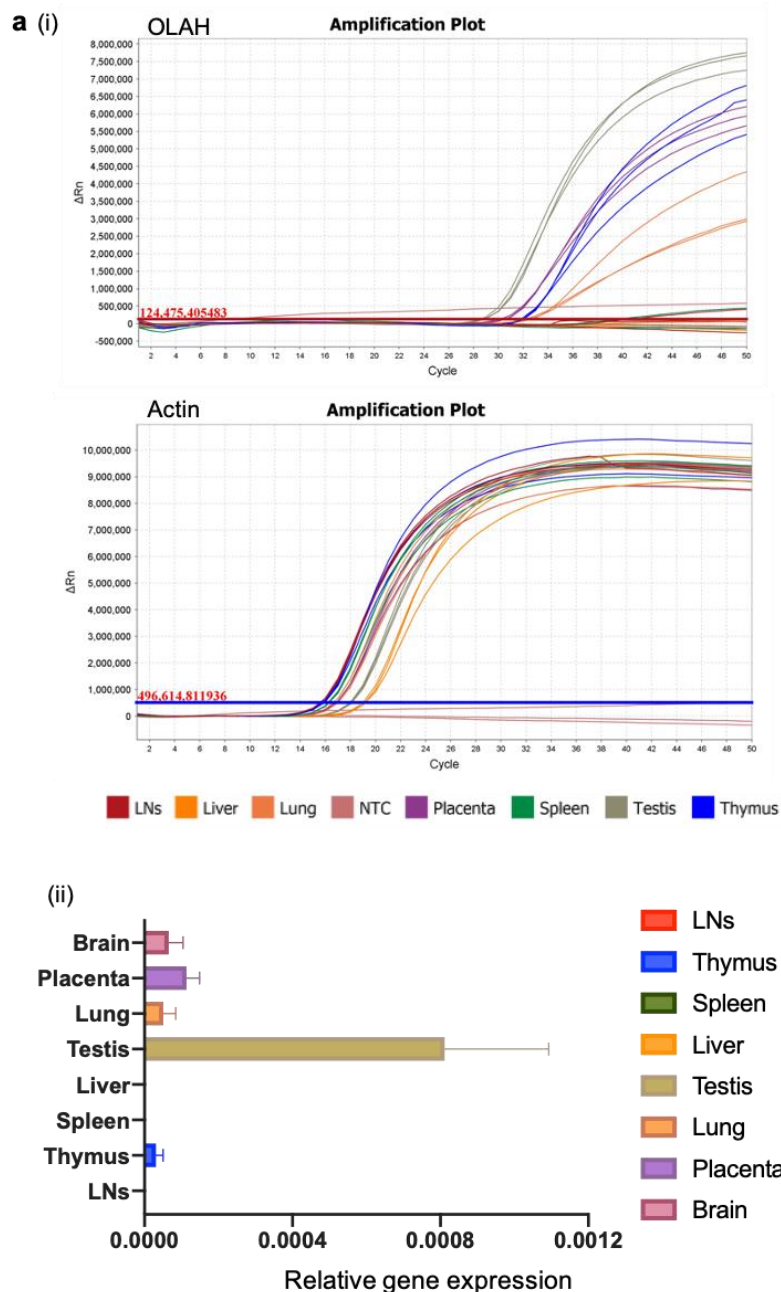


Fig 1. Higher *OLAHA* gene expression in reproductive organs and lungs of naïve mice

Tissue and organs, including LNs, thymus, spleen, liver, lungs, brains, testis (male) and placenta (female) were collected from naïve C57BL/6 mice. mRNA from these samples were extracted and qPCR was performed to measure *OLAHA* gene expression levels. Amplification plots of *OLAHA* and house-keeping gene (*actin*) products are shown in a(i) and *OLAHA* gene expression levels relative to actin levels in each tissue are shown in a(ii). Bar graphs and error bars depict the average levels ($\pm$ SD) from replicate samples (n=6 for all samples except n=3 for brain).

To further examine which cell types in these tissues expressed *OLAHA*, lung, thymus and testis were harvested, and RNA extracted from sorted immune CD45⁺ and/or non-immune CD45⁻ cells. While we could obtain CD45⁺ and CD45⁻ cells from lungs, sufficient numbers of CD45⁺ cells from testis and CD45⁻ cells from thymus could not be collected for this analysis.

As shown in **Fig 2**, the highest *Olah* mRNA expression was detected in CD45⁻ cells from testis (confirming our observations in Fig 1a) and lung. Furthermore, detectable levels of *Olah* mRNA were found in CD45⁺ cells from lung and thymus.

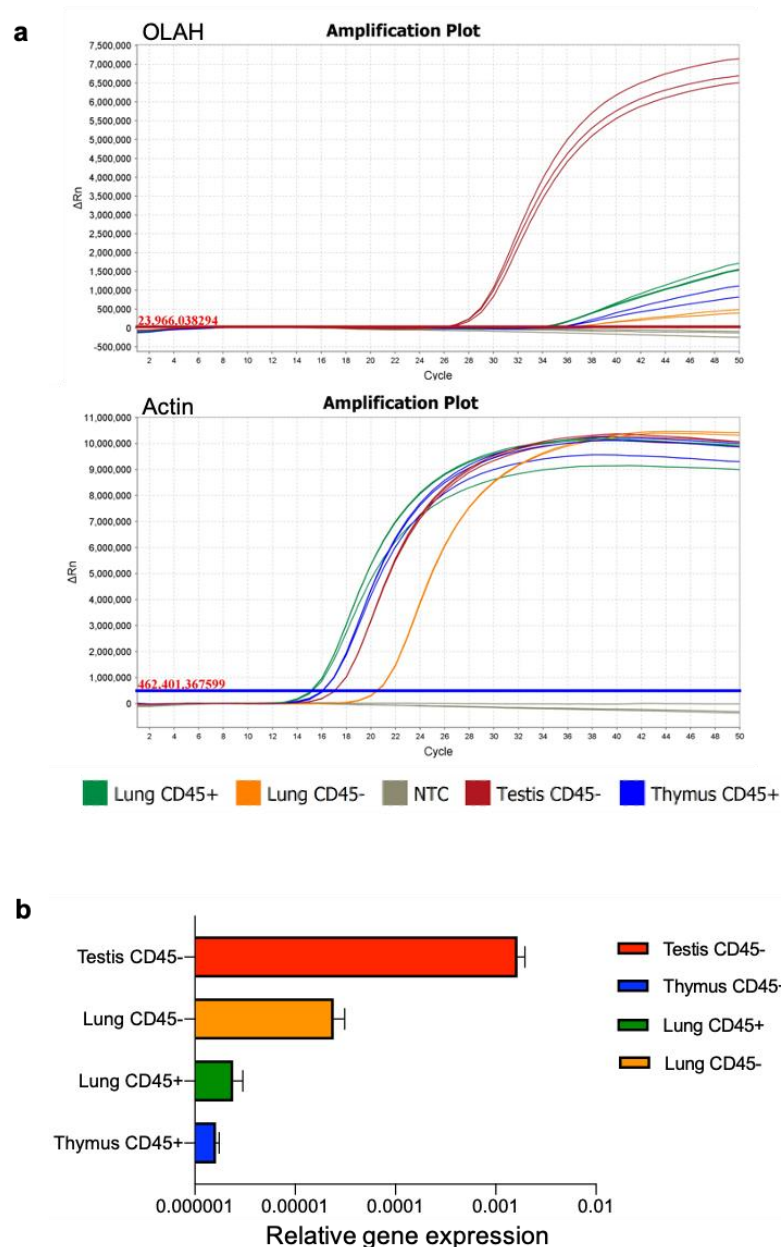


Fig 2. Higher *OLAH* gene expression on non-lymphocytes in naïve mice

Single cell suspensions were prepared from testis, thymus and lungs from naïve C57BL/6 mice and sorted to obtain CD45⁺ lymphocytes and CD45⁻ non-lymphocytes. mRNA was extracted from cells samples and qPCR was performed to measure *OLAH* gene expression levels. (a) Amplification plots of *OLAH* and house-keeping gene (*actin*) are shown, and (b) *OLAH* gene expression levels relative to actin levels in each cell population are shown. Bar graphs and error bars depict the average levels ($\pm$ SD) from replicate samples (n=3).

Altogether, our data indicate OLAH expression is mainly found in non-immune organs (especially the reproductive system), non-immune cells and to a lesser extent in lungs and cells of the immune system. The presence of *Olah* mRNA in these tissues and its role in fatty acid synthesis, suggests that OLAH might contribute to the modulation of immune responses induced by infection by altering the lipid metabolism in immune and non-immune cells at different anatomical locations.

5.2.2 Generation and establishment of OLAH KO mice

To understand the impact of *Olah* expression on host immune responses and disease severity during influenza virus infection, we first generated OLAH KO mice to compare the effects of infection to wild type C57BL/6 mice. In contrast to humans, where the *Olah* gene is located on chromosome 10, *Olah* is located on chromosome 2 in mice and contains 7 introns and 8 exons (**Fig 3a**). CRISPR/Cas9-targeted deletion was used to remove exon 1 and 2 of the *Olah* gene in C57BL/6 mice. Single guide RNAs (sgRNAs) were used to delete a region of the *Olah* gene comprising of 4467 base pairs (bps) (**Fig 3b**). This resulted in the establishment of 15 F0-generation mice.



Fig 3. Targeted deletion of the OLAH gene to generate heterozygous knockout mice

(a) Schematic representation of the OLAH gene on mouse chromosome 2 depicting the region within exon 1 and 2 targeted for deletion. (b) Sequence of the OLAH gene showing positions of exon 1 and 2. Also indicated are the 5' and 3' sgRNA sequences used to for CRISPR/Cas9-mediated deletion of this region. c(i) Primers were designed to detect the wildtype allele and resulting sequence following deletion of the target region. c(ii) DNA were extracted from F0 mice (n=15 lines) and screened for the presence of the WT allele and deleted region via PCR. The expected bands

To validate the genotype of these F0 mice, primers were designed to detect the presence of the gene sequence resulting from deletion of this region (e.g. containing the deletion; KO allele) or the intact wildtype allele (e.g. without the deletion; WT allele) (**Fig 3ci**) and used in a PCR on DNA obtained from ear notch samples. Our screening results show that all 15 mice expressed the WT allele PCR product (**Fig 3cii**), while 10 mice additionally expressed the KO allele product, indicating a heterozygous genotype. The absence of the KO allele product in the other 5 mice suggests that the *Olah* gene was not deleted and they remained WT homozygous for this allele.

DNA samples from all heterozygous mice were then sequenced to confirm the presence of the KO allele using the same forward and reverse primers used for PCR detection. The reads from each mouse aligned with the predicted sequence containing this deletion, confirming expression of the allele, although a proportion of these sequences contained truncations (**Fig 4a**). This is likely to be a result of Cas9-mediated excision of the DNA sequence during the early embryo stage followed by repairs to this break by insertion of InDel mutations (highlighted in yellow) between the 2 sgRNA cut sites.

Confirmed heterozygous mice were bred with WT mice to generate F1 heterozygotes, which were confirmed for KO allele expression via PCR (as in **Fig 3c**). This was followed by another round of breeding between F1 heterozygotes to generate offspring that were selected for homozygous expression of the KO allele to establish OLAH KO lines. qPCR on RNA samples from mice derived from these lines confirmed that in comparison to WT mice, *Olah* gene expression was absent at the transcriptomic level (**Fig 4b**).

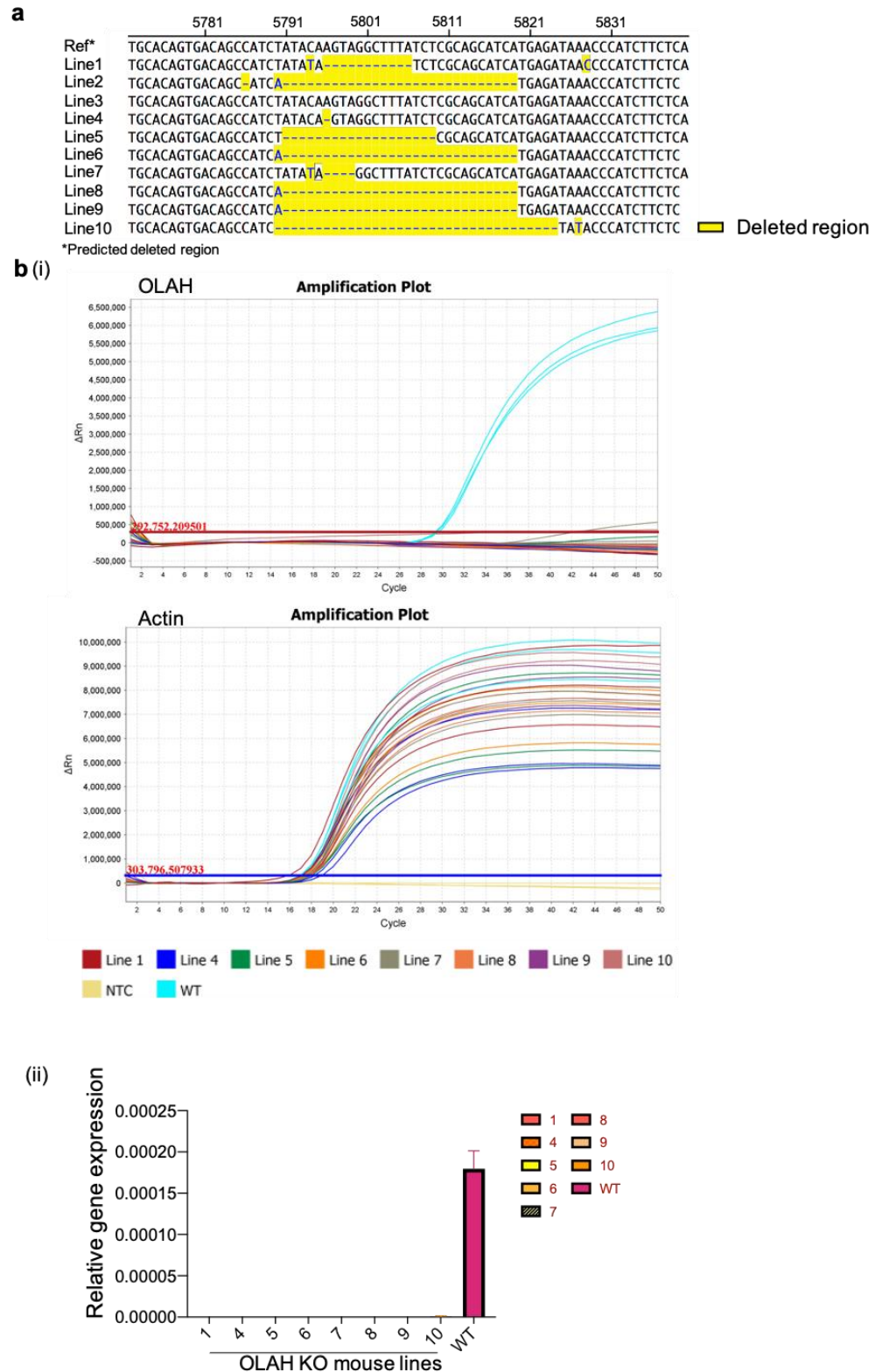


Fig 4. Validation of homozygous OLAH KO mouse lines

(a) Identified heterozygotes (n=10 lines) were genotyped to identify the sequence of deleted region (yellow) in comparison to the intact sequence (indicated as Ref). (b) Following further breeding of F0 mice to generate OLAH KO homozygotes, mRNA from testis were extracted and qPCR was performed to confirm the absence of the OLAH gene compared to WT mice. Amplification plots of OLAH and actin are shown in b(i) and OLAH gene expression levels relative to actin levels are shown in b(ii). Bar graphs and error bars depict the average levels ($\pm$ SD) from replicate samples (n=3).

5.2.3 Naïve OLAH KO and WT mice exhibit similar phenotypes and immune cell profiles

Having generated OLAH KO mice, we first asked whether there are any differences in phenotype of naïve OLAH KO and WT mice. Inspection of their outward appearance showed that both strains appeared visually similar (**Fig 5ai**). Moreover, there were no significant differences in the weights of similarly aged OLAH KO and WT mice (**Fig 5aii**). A wide range of tissue and organs from 7-8 weeks old mice from both strains were also harvested for histological analysis (**Fig 5b**). These include the adrenal glands, bladder, bone marrow, brain, cecum, colon, duodenum, epididymis, eyes, gall bladder, harderian glands, head, heart, hind leg (including the long bone, bone marrow, synovial joint and skeletal muscle), ileum, jejunum, kidney, liver, lungs, mesenteric lymph node, pancreas, penis, preputial gland, prostate glands, salivary glands and regional lymph nodes, seminal vesicles, skin, spinal cord, spleen, stomach, tail, testes, thymus, thyroid and trachea. Overall, there were no significant visual differences in tissues of the two strains aside from a permanent proteinaceous plug detected in the urinary tract of OLAH KO mice. This may indicate that the urinary bladder of these mice is affected by prominent urethral dilation and epithelial hyperplasia subsequently blocking their urinary flow, which also suggests early signs of urethral obstructive syndrome.

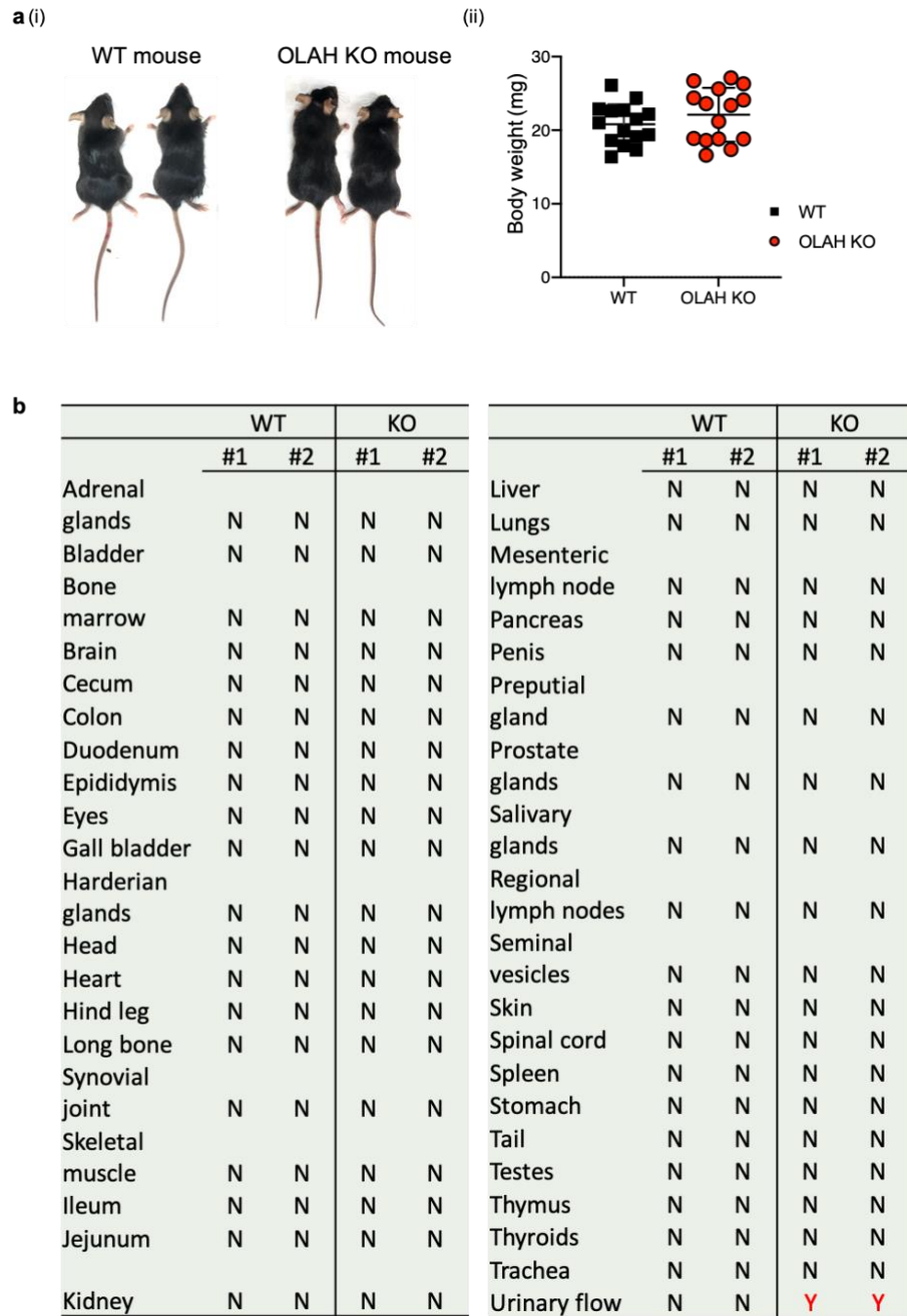


Fig 5. External and internal phenotype of naïve WT and OLAH KO mice

a(i) Outward appearance of OLAH KO and WT mice (7-8 weeks old) and a(ii) body weight (6-9 weeks old). (b) Histopathological examination of various organs and tissues of OLAH KO and WT mice (7-8 weeks old, n=2 per strain). Differences between strains are denoted by “Y” and no differences are indicated by “N”.

Subsequently, we analysed the phenotype and frequencies of immune cells in spleen, lung and thymus of both naïve OLAH KO and WT mice. Panels of immune cell markers were used to distinguish lymphocyte populations specifically B cells, T cells, CD4⁺ T cells, CD8⁺ T

cells, NK cells, iNKT cells, $\gamma\delta$ T cells, neutrophils, eosinophils, dendritic cells (DCs), alveolar macrophages and CD64⁺ pan macrophages (**Fig 6**).

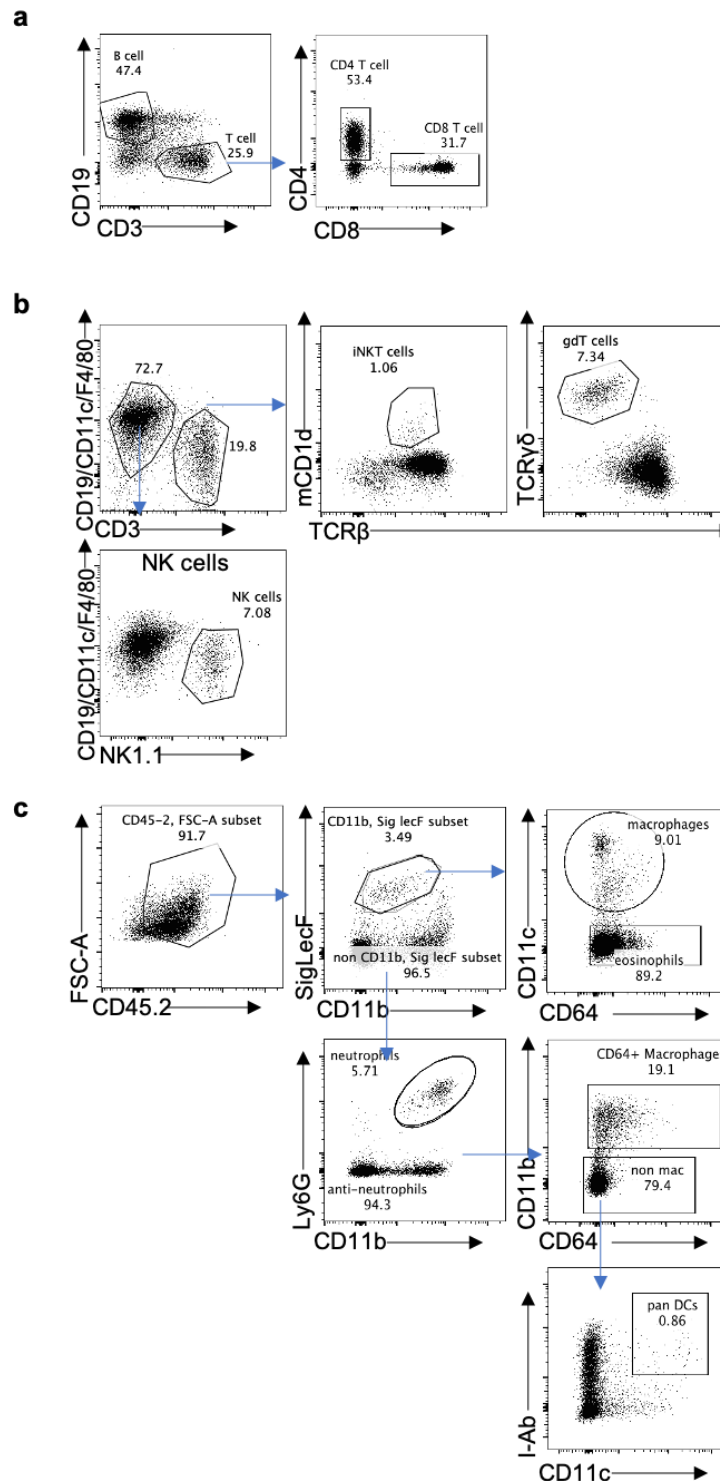


Fig 6. Gating strategies to distinguish immune cell populations in naïve mice

FACS gating strategies used to distinguish (a) B cells (CD19⁺), CD4⁺ T cells (CD3⁺CD4⁺) and CD8⁺ T cells (CD3⁺CD8⁺), (b) iNKT cells (CD3⁺NK1.1⁺), $\gamma\delta$ T cells (CD3⁺ $\gamma\delta$ ⁺) and NK cells (CD3⁺NK1.1⁺), and (c) alveolar macrophages (SigLecF⁺CD11b⁺CD64⁺CD11c⁺), neutrophils (CD11b⁺Ly6G⁺), eosinophils (SigLecF⁺CD11b⁺CD64⁺CD11c⁺), CD64⁺ pan macrophages and dendritic cells (CD11c⁺I-Ab⁺) in naïve WT and OLAH KO mice.

Our analysis revealed that both strains had comparable cell numbers across all populations in each organ, thus indicating that naïve OLAH KO mice exhibit similar immune cell profiles as their congenic WT counterparts (**Fig 7**).

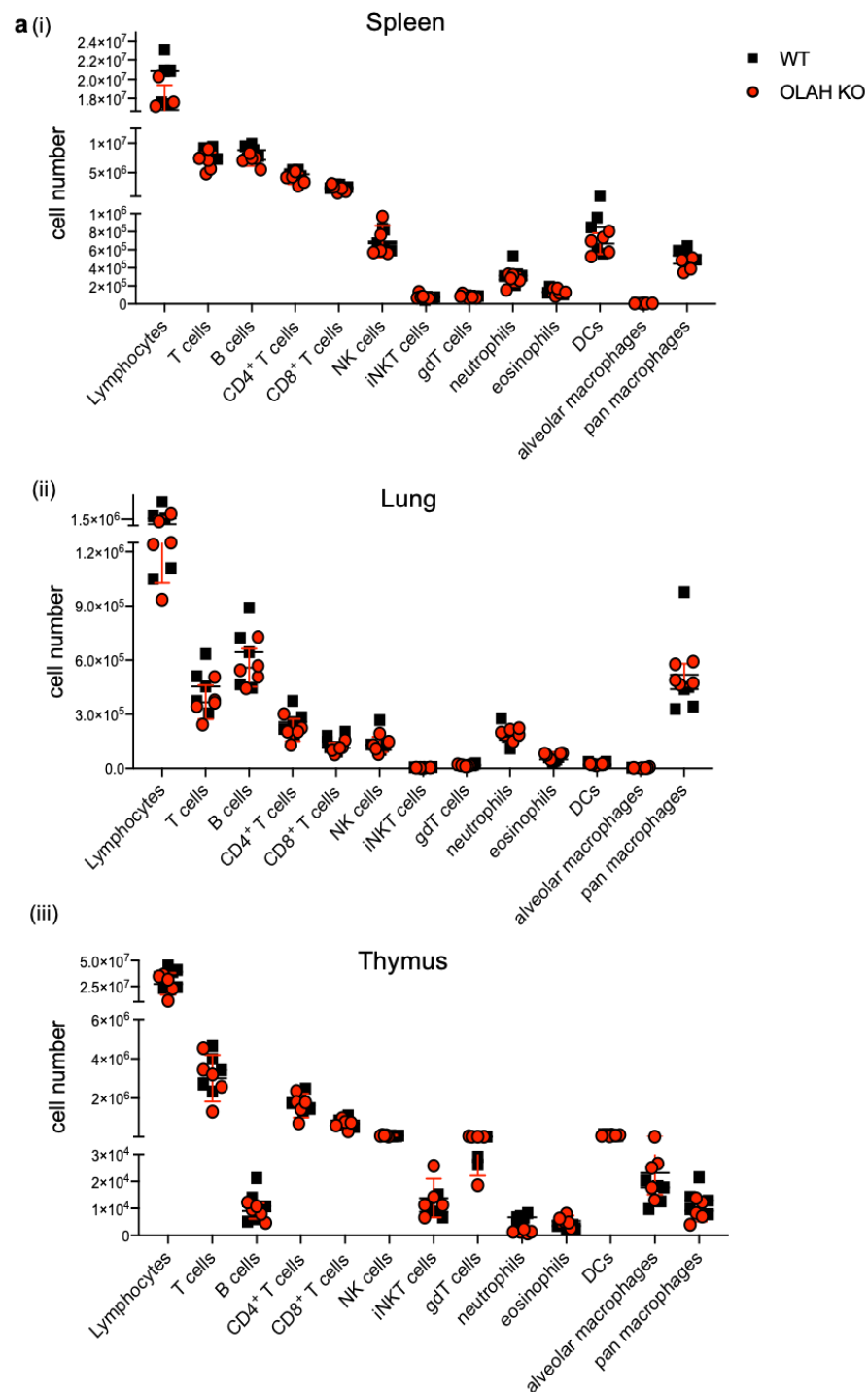


Fig 7. Comparable numbers of immune cell populations in naïve WT and OLAH KO mice
Cell numbers of different immune cell populations in a(i) spleen, a(ii) lung and a(iii) thymus of OLAH KO and WT mice (Error bars depict the average levels ($\pm$ SD) from replicate samples (n=5).

5.2.4 Reduced disease severity in OLAH KO mice following influenza virus infection

Since H7N9-infected patients who succumbed to infection displayed a high expression of OLAH, the next question we asked was whether the absence of OLAH results in a differential influenza disease outcome. We infected OLAH KO and WT mice with a mouse-adapted influenza A viral strain, X31 (H3N2), at 10^4 PFU or 10^5 PFU, causing mild or severe to fatal disease, respectively in WT mice. All OLAH KO and WT mice infected with 10^4 PFU survived over the course of 10 days after infection (**Fig 8ai**).

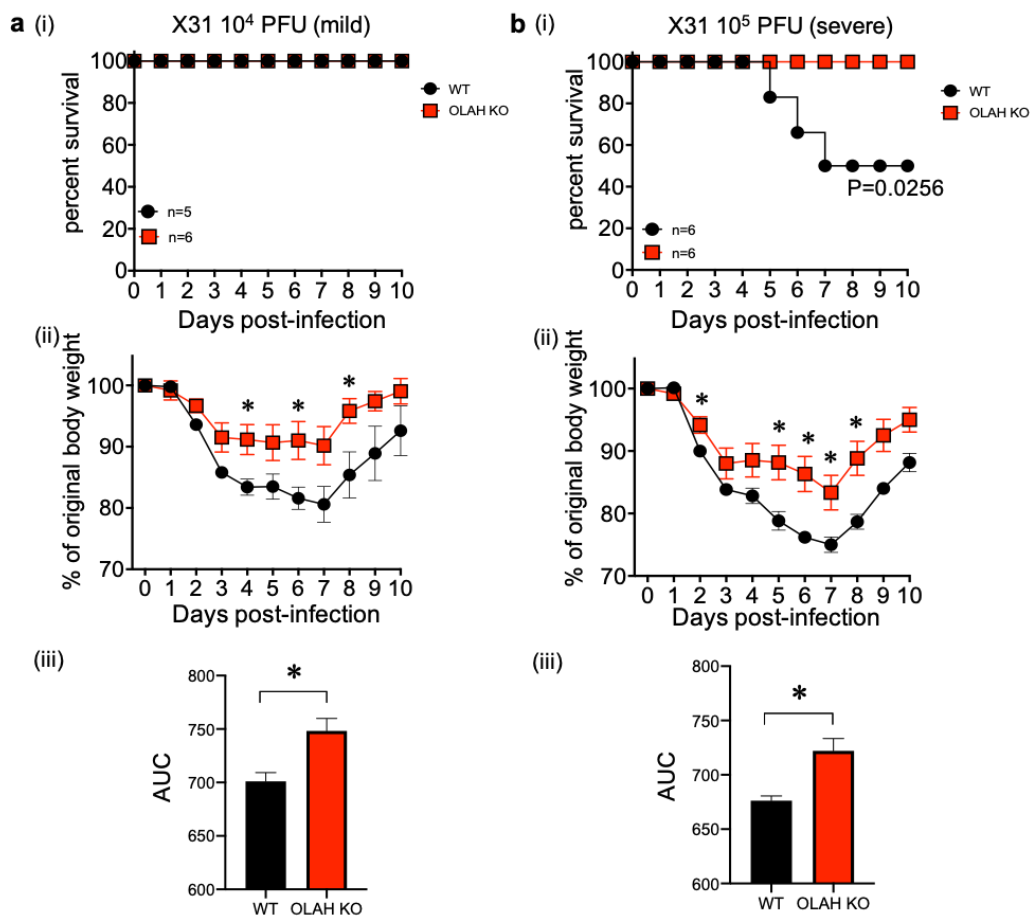


Fig 8. Reduced disease severity in OLAH KO mice following influenza virus infection

OLA KO and WT mice (6-9 weeks old; n=5 to 6 per group) were intranasally infected with X31 using (a) 10^4 PFU or (b) 10^5 PFU to establish mild or severe disease, respectively. Mice were monitored for body weight over 10 days and culled when >25% of their original weight was lost. a(i) and b(i) show the survival rate of both mild- and severely infected mice with their mean body weight profiles ($\pm$ SEM) depicted in a(ii) and b(ii). The total average ($\pm$ SEM) body weight in a(iii) mild- and b(iii) severely infected groups over the course of the experiment was determined by area under the curve analysis. (* p < 0.05).

However, the body weight loss was significantly more substantial in WT mice, compared to OLAH KO mice, particularly at 4-8 days after infection (~10% weight loss in OLAH KO vs 20% in WT) (**Fig 8aii**). This was also confirmed by an area under the curve

(AUC) analysis of weight loss profiles, which showed OLAH KO mice exhibited significantly higher weights than WT mice over the course of infection (**Fig 8a**). Differences in disease outcomes were even more striking during high dose IAV infection, with half of WT mice in the group succumbing to the infection and losing >25% of their weight. In contrast, all of the OLAH KO mice survived and none lost >20% of weight before recovering (**Fig 8b**). Again, AUC analyses of weights in both groups (**Fig 8c**) further confirmed that OLAH KO mice lost significantly less weight. These results thus recapitulate our observations in H7N9-infected patients who died and confirms that OLAH expression can be associated with disease severity.

5.2.5 Decreased viral-mediated lung damage in OLAH KO mice

The lung epithelium is the primary site of influenza virus replication and can result in tissue damage and exacerbate disease severity. Given the observed differences in disease outcomes following infection of OLAH KO and WT mice with 10^5 PFU of X31, we set out to ascertain the level of lung tissue damage in these mice. Histopathological analysis was performed on lung sections obtained at day 1,3,5 following infection. As expected, no lung immunopathology was observed in lungs of uninfected WT and OLAH KO mice (Day 0) (**Fig 9a,b**). However, once challenged with IAV X31, lung damage scores in WT mice rapidly increased, as evident by the appearance of widespread tissue abnormalities including peribronchiolitis, epithelial necrosis and lymphocytic infiltration. Damage was consistently observed in ~30% of each visualized section from WT mice on day 1 and 3 after infection and further increased by day 5, reaching up to 60% in some cases. In contrast, significantly lower scores were observed in the majority of sections from OLAH KO mice across all time points, although some abnormalities such as mild peribronchiolitis and haemorrhaging were observed. These results thus demonstrate that severe influenza disease driven by OLAH is associated with increased lung pathology and in line with our observations that OLAH KO mice are protected against severe disease outcomes.

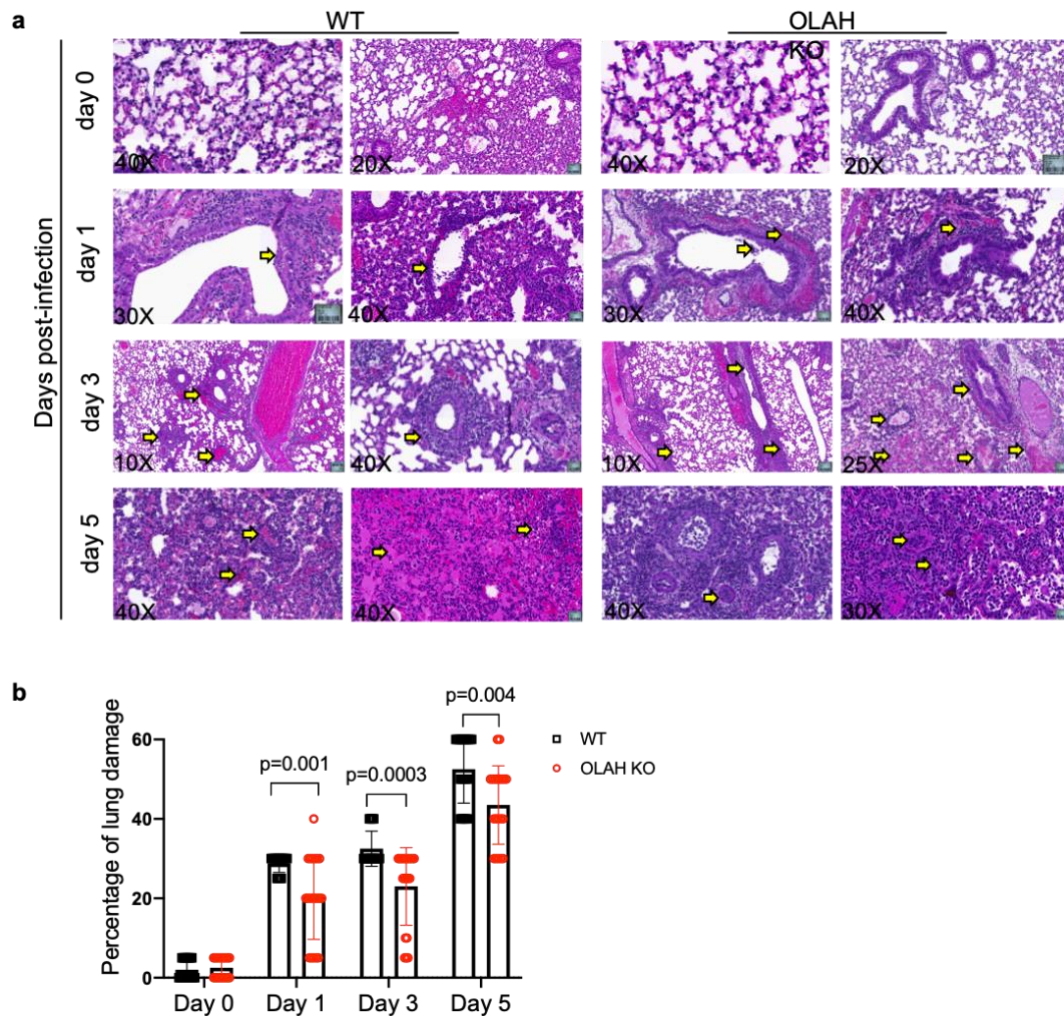


Fig 9. Less lung damage in OLAH KO mice during the early phases of high dose influenza virus infection

OLA KO and WT mice (6-9 weeks old; n=4 per group) were infected with 10^5 PFU of X31 and lungs harvested at 1,3,5 days after infection. Lungs were also harvested from naïve mice (Day 0). Lung sections were prepared and analysed for tissue integrity and abnormalities by histopathology. (a) Representative images of lung sections from WT and OLAH KO mice and (b) Independently assessed damage score for each sample (Average with $\pm$ SD). Yellow arrows in (a) indicate abnormal signatures within tissue section.

5.2.6 Decreased lipid droplet formation in OLAH KO mice

We further elucidated the mechanism(s) underlying OLAH-mediated influenza disease severity. Since OLAH is involved in lipid metabolism and given that viruses (especially flaviviruses) can hijack lipid droplets (LDs) in cells to benefit viral replication, we hypothesized that absence of OLAH leads to impaired LD formation to hinder IAV replication. Lung sections from OLAH KO and WT mice at d0, 12hrs and day1,2,3 after infection with 10^5 PFU of X31 were stained with BODIPY for LDs, influenza virus nuclear protein (NP) and the fatty acid receptor CD36. In lungs of uninfected OLAH KO mice, we detected higher numbers of LD per cell than WT mice (**Fig 10a,b**). This was unexpected given ablation of OLAH was

surmised to lower fatty acid synthesis but this finding might be due to compensative metabolic mechanisms such as acyl-CoA synthetases (ACSSs) that may be able to boost LD formation by activating major long-chain fatty acids synthesis³⁰⁴.

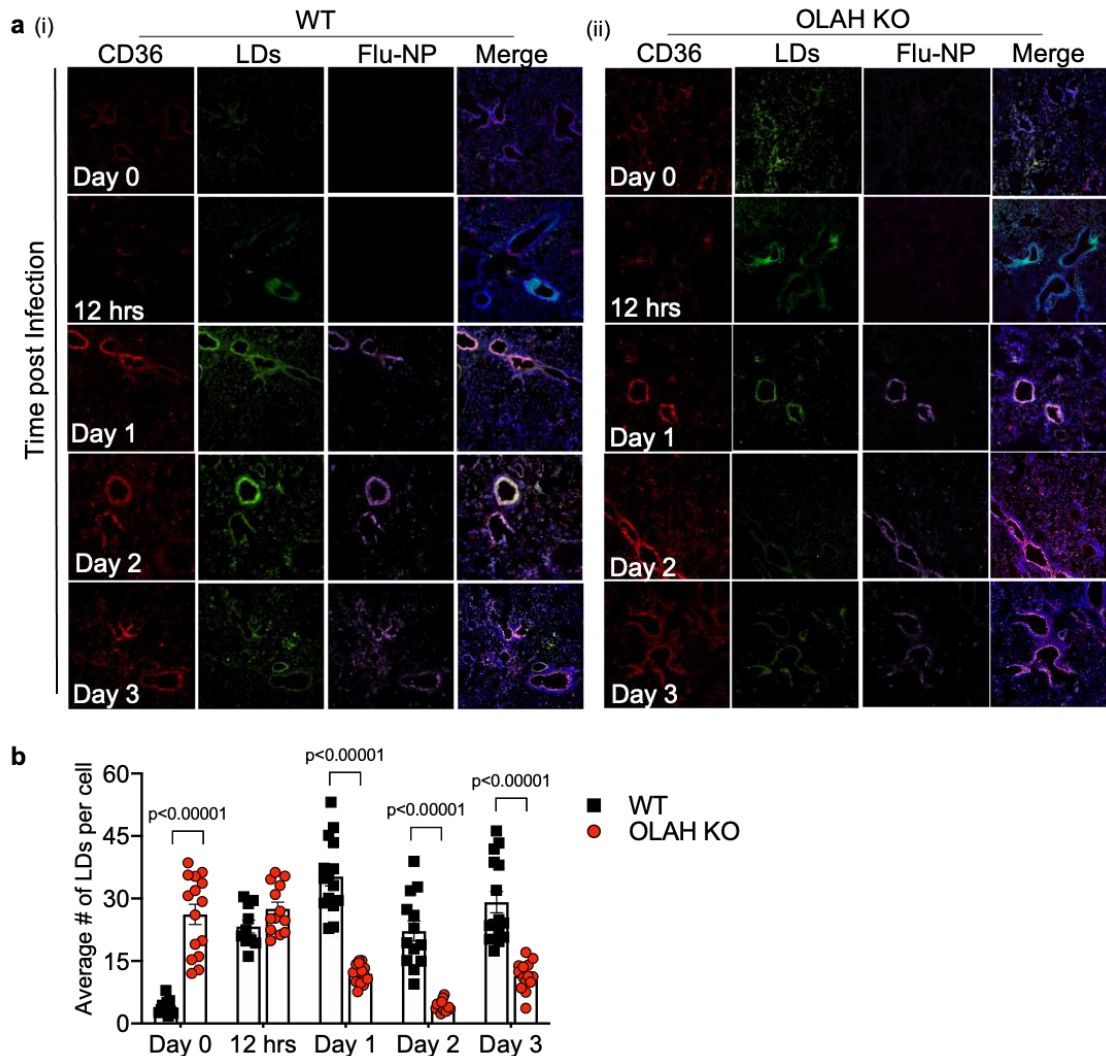


Fig 10. Less lipid droplets in lungs of OLAH KO mice during high dose influenza virus infection OLAH KO and WT mice (6-9 weeks old; n=5 per group at each timepoint) were infected with 10^5 PFU of X31 and lungs harvested at 12hrs, day 1, day 2 and day 3 after infection. Lungs were also harvested from naïve mice (Day 0). Lung sections were prepared and stained with PE-conjugated anti-CD36, AF555-conjugated anti-IAV-NP protein (Flu-NP) antibodies, BODIPY 395/505 and DAPI. Single and merged images for staining for each time point are shown in (ai) and (aii). (b) Lipid droplet numbers (in BODIPY⁺ cells) by were analysed from z-stack images acquired by confocal microscopy. The average number of lipid droplets (Average with $\pm$ SEM) in each lung was analysed in sections from at least 9 fields of view from different locations across the coverslip.

At 12 hours after infection, an increase in the number of LDs was detected in lung cells of WT mice (**Fig 10ai,b**), reaching similar levels to OLAH KO mice, which remained largely unchanged. However, while a decrease in LD numbers in OLAH KO mice was observed at 1

day after infection and continued to remain at low levels, the opposite occurred in WT mice with significantly increased LD numbers at 12hrs, day1,2,3 post infection compare to day0 (**Fig 10ai,b**). In addition, both CD36 and NP co-localized strongly with lung cells harboring LDs (**Fig 10ai**), especially in WT mice from 1 day onwards, indicating potential lipid uptake and viral infection taking place in these cells. These co-localizations in cells of OLAH-KO mice are less pronounced and correlate with the lower LD numbers (**Fig 10aii**). Overall, the above data indicate that reduced disease severity in OLAH KO mice might be linked to perturbed formation of LDs during IAV infection, and as a consequence leading to decreased viral replication.

5.2.7 Reduced lung viral titres and inflammation in OLAH KO mice

To investigate whether viral replication in OLAH KO mice is associated with reduced LD formation, we measured lung viral titres at day 1,2,3 after infection (**Fig 11ai,ii**). In comparison to lungs from WT mice, significantly lower viral titres were detected in the lungs of OLAH KO mice, most notably on day 1 post-infection (**Fig 11aii**), although there were no differences in their lung weights (**Fig 11aiii**). To determine whether there were any differences in inflammatory responses, we also measured cytokines in bronchoalveolar lavage (BAL) fluid and found that OLAH KO mice indeed had lower amounts of cytokines compared to WT mice on day 1 post-infection (**Fig 11aiv**), especially TNF- α by ~3.4-fold and MIP-1 β by ~3.4-fold. Excessive amounts of TNF- α in particular have been reported to cause tissue damage during virus infection³⁰⁵. In addition, we also observed lower total cell numbers in the BAL (on day 1 and 3) and lungs (on day 1) of OLAH KO mice than WT mice, while comparable numbers were found in nasal turbinate (**Fig 11b**), thus indicating markedly decreased inflammatory responses in OLAH KO mice especially during the early phases following high dose IAV infection. To determine correlations amongst these variables: viral titre, total cell number, total cytokine and TNF, we found that lung viral titres in both groups of mice positively correlated with total cell numbers, total cytokine production and TNF- α levels, indicating that the heightened inflammatory milieu in WT mice were associated with increased viral replication (**Fig.11c**).

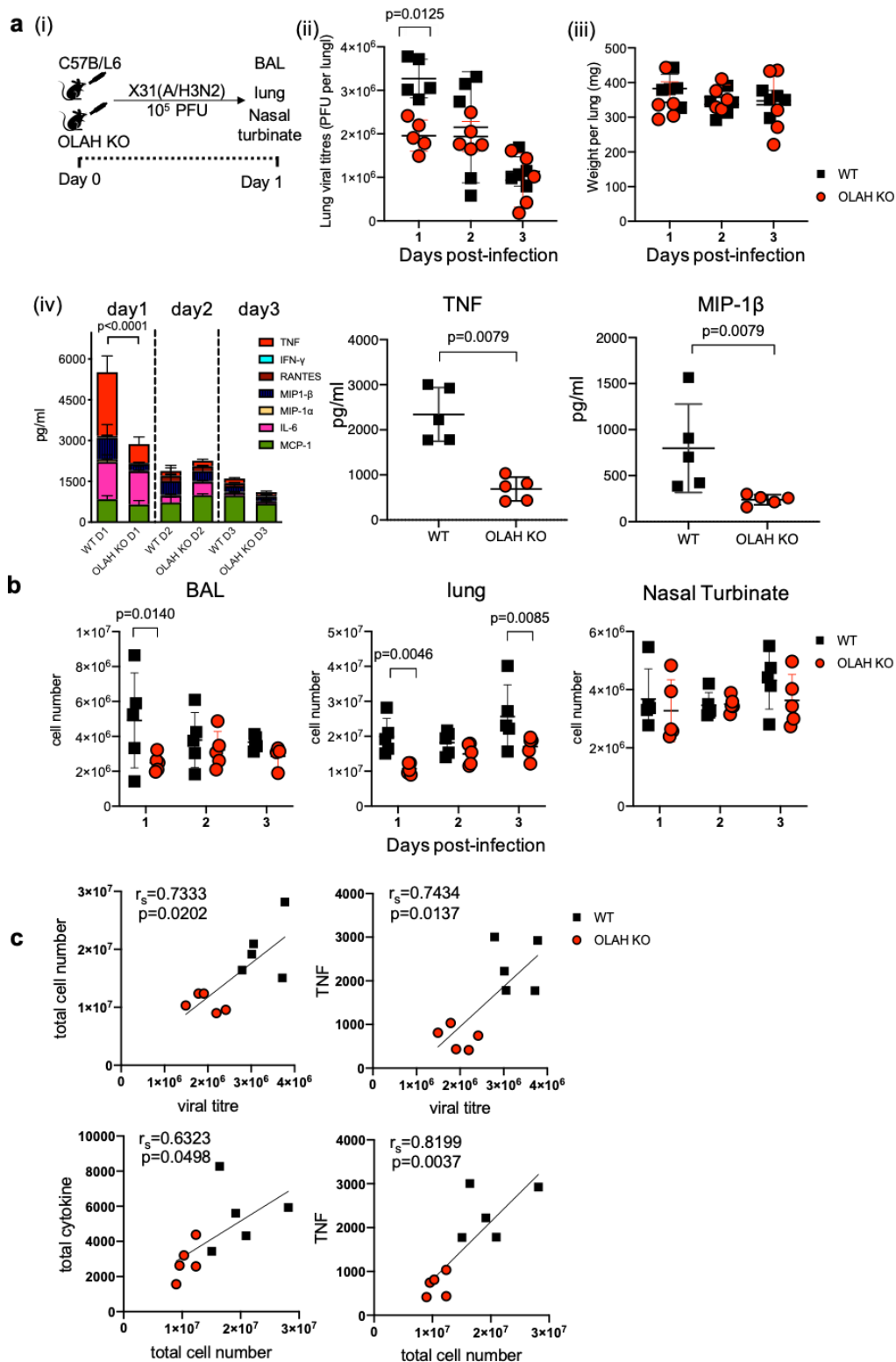


Fig 11. Reduced lung viral titres and inflammation in *OLA*H KO mice during influenza A virus infection at early stage of infection

a. (i) *OLA*H KO and WT mice (6-9 weeks old; $n=5$ per group at each timepoint) were challenged with A/H3N2 X31 virus at 10^5 PFU and BAL, lung and nasal turbinate were harvested at day1, day2 and day3 after infection. (ii) viral titre and (iii) weights of lungs were calculated in both mice groups (Average with $\pm$ SD). (iv) BAL supernatant samples were collected and performed with cytokine bead

array to identify cytokine production level in both mice groups after infection. (Average with $\pm$ SD). b. Total cell numbers across day1,2,3 in BAL, lung and nasal turbinate were calculated in both WT and OLAH KO mice groups. c. Correlations on viral titre vs total cell number, total cell number vs cytokines and total cell number vs TNF cytokine were calculated using Pearson Correlation and R^2 represents R-squared value.

5.2.8 Decreased innate immune responses in OLAH KO mice at early stages after IAV infection at the site of infection

Given that we observed differences in total cell numbers, we subsequently investigated magnitude of specific innate cell populations in these mice. To achieve this, we stained cells within nasal turbinate, BAL and lung with different markers for immune cells involved in innate immune responses, including neutrophils, $\gamma\delta$ T cells ($CD3^+\gamma\delta^+$), NK cells ($CD3^-NK1.1^+$), eosinophils ($SigLecF^+CD11b^+CD64^+CD11c^-$), macrophages ($SigLecF^+CD11b^+CD64^+CD11c^+$) and iNKT cells ($CD3^+NK1.1^+$). Lower cell numbers of immune cells, including neutrophils, $\gamma\delta$ T cells, NK cells, eosinophils, macrophages and iNKT cells were immediately apparent in the lungs of OLAH KO mice compared to WT on day 1 post-infection (**Fig 12a,b**).

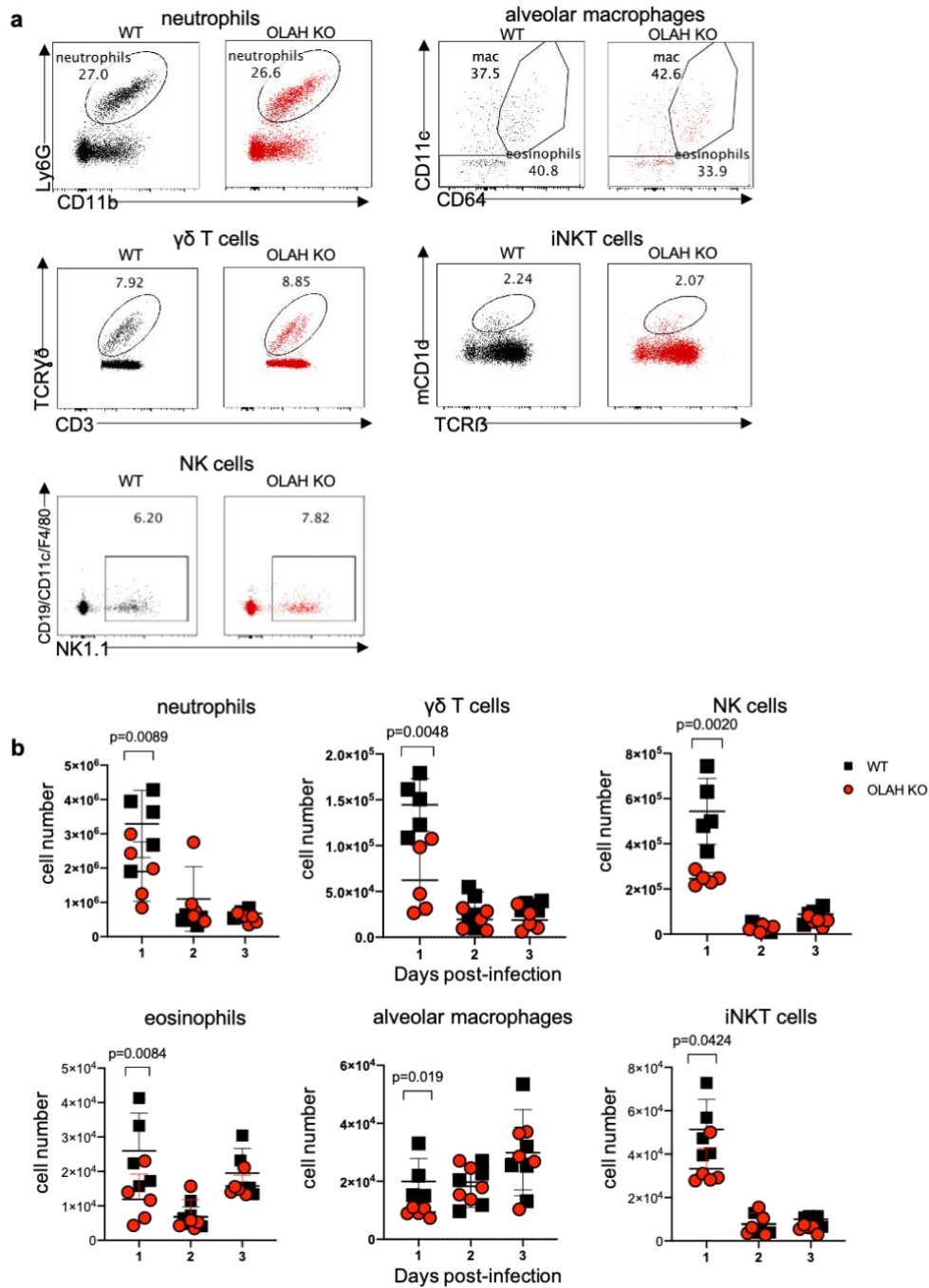


Fig 12. Decreased innate immune responses in *OLA*H KO mice at early stage of infection in the site of infection

OLA H KO and WT mice (6-9 weeks old; n=5 per group at each timepoint) were challenged with A/H3N2 X31 virus at 10^5 PFU and BAL, lung and nasal turbinate were harvested at day1, day2 and day3 after infection. a. Gating strategy to identify innate immune cells including neutrophils, alveolar macrophages, $\gamma\delta$ T cells, iNKT cells and NK cell in both mice groups. b. Cell number of different innate immune cells in lung from both OLA H KO and WT mice groups (Average with $\pm$ SD).

By day 2 and 3, cell numbers in both groups decreased and were at comparable magnitudes. No significant differences in cell numbers between WT and OLA H KO mice were observed in the nasal turbinate and BAL at these same time points, with the exception of

neutrophils which were found in lower numbers by ~1.5-fold in the BAL of OLAH KO mice on day 3, reflecting our observations in the lung (Fig 13). Taken together, with the increased lung viral titres and inflammation observed in WT mice on day 1 post-infection, our results indicate that OLAH plays a role at the very early stage of infection to enhance viral replication and excessive inflammation, leading to tissue damage and subsequently severe and fatal disease outcomes.

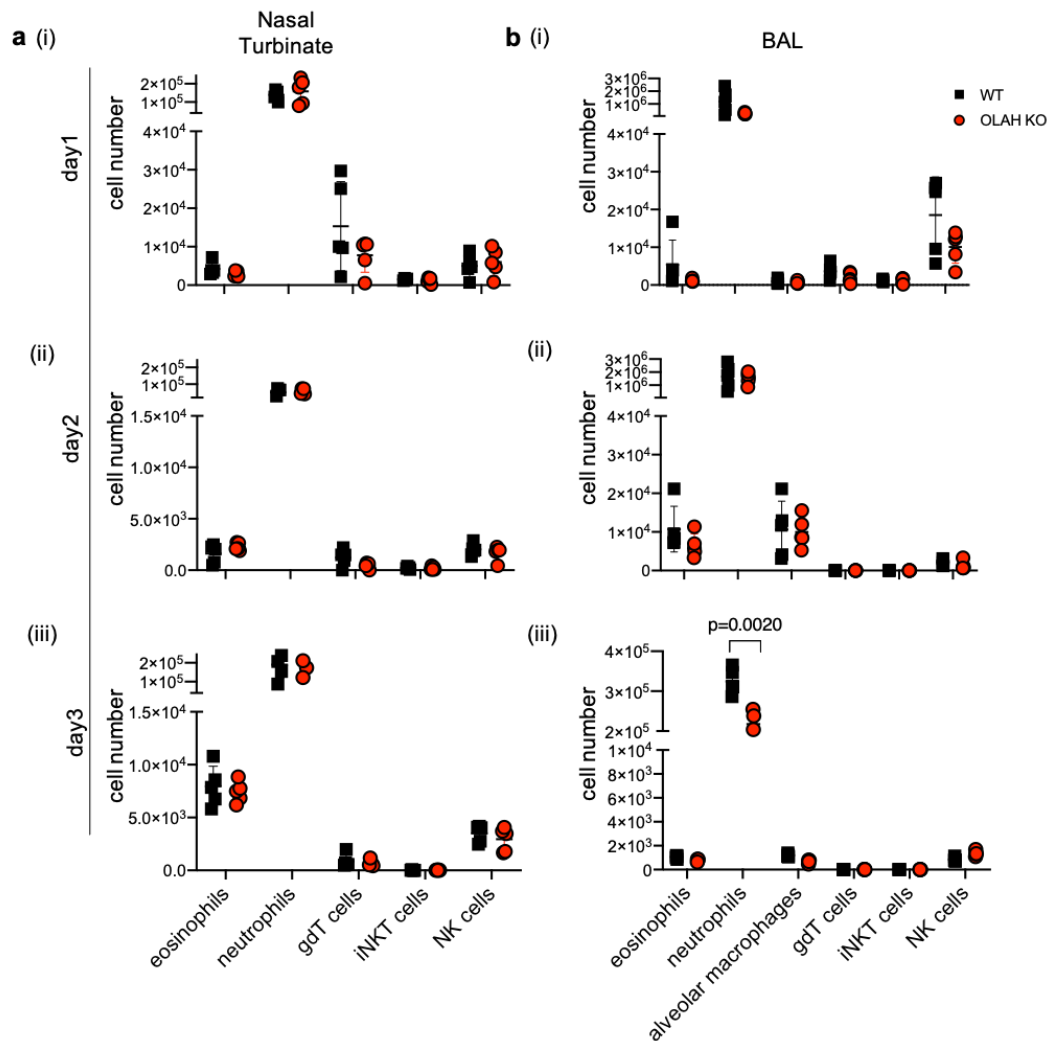


Fig 13. Comparable innate immune responses in OLAH KO mice at early stage of infection in nasal turbinate and BAL

OLA KO and WT mice (6-9 weeks old; n=5 per group at each timepoint) were challenged with A/H3N2 X31 virus at 10⁵ PFU and BAL, lung and nasal turbinate were harvested at day1, day2 and day3 after infection. Innate immune cells numbers including neutrophils, $\gamma\delta$ T cells, NK cells, eosinophils, alveolar macrophages and iNKT cells were calculated from both OLAH KO and WT mice groups in (a) nasal turbinate and (b) BAL across day1(ai,bi), day2 (aii, bii) and day3 (aiii, biii) (Average with $\pm$ SD).

5.2.9 Reduced T cell numbers and lower frequencies of effector IFN- γ ⁺CD8⁺ T cells in OLAH KO mice after low dose influenza infection

We next determined whether infection of OLAH KO and WT mice impacts generation and maintenance of virus-specific T cell responses. To address this and ensure that enough mice survive the course of infection until the experimental timepoint, we challenged mice with a lower dose of X31 (10^4 PFU). On day 8 post-infection, we collected mediastinal lymph nodes (MLN), BAL and spleens to analyze T cell immune responses. Our results showed no significant differences in the numbers of CD4⁺ T cells and CD8⁺ T cells, including antigen-specific D^bNP₃₆₆⁺CD8⁺ and D^bPA₂₂₄⁺CD8⁺ T cells in the BAL and MLN of both OLAH KO and WT mice (**Fig 14a,b**). There were, however, less overall numbers of CD4⁺ T cells and CD8⁺ T cells, particularly CD8⁺ T cells specific for D^bNP₃₆₆, in the spleens of OLAH KO mice. In examining the functional qualities of virus-specific CD8⁺ T cells, we detected lower frequencies of IFN- γ ⁺ CD8⁺ T cells within the D^bPA₂₂₄⁺ CD8⁺ T cell pool in the MLN and spleen of OLAH-KO mice (**Fig 14c**). These results again support the observation of decreased immune responses in OLAH KO mice. We however, did not observe different frequency of IFN- γ ⁺ CD8⁺ T cells in D^bNP₃₆₆⁺CD8⁺ T cell pool between 2 mice groups. This may be due to different dynamics of D^bPA₂₂₄- and D^bNP₃₆₆-specific CD8⁺ T cells after infection.

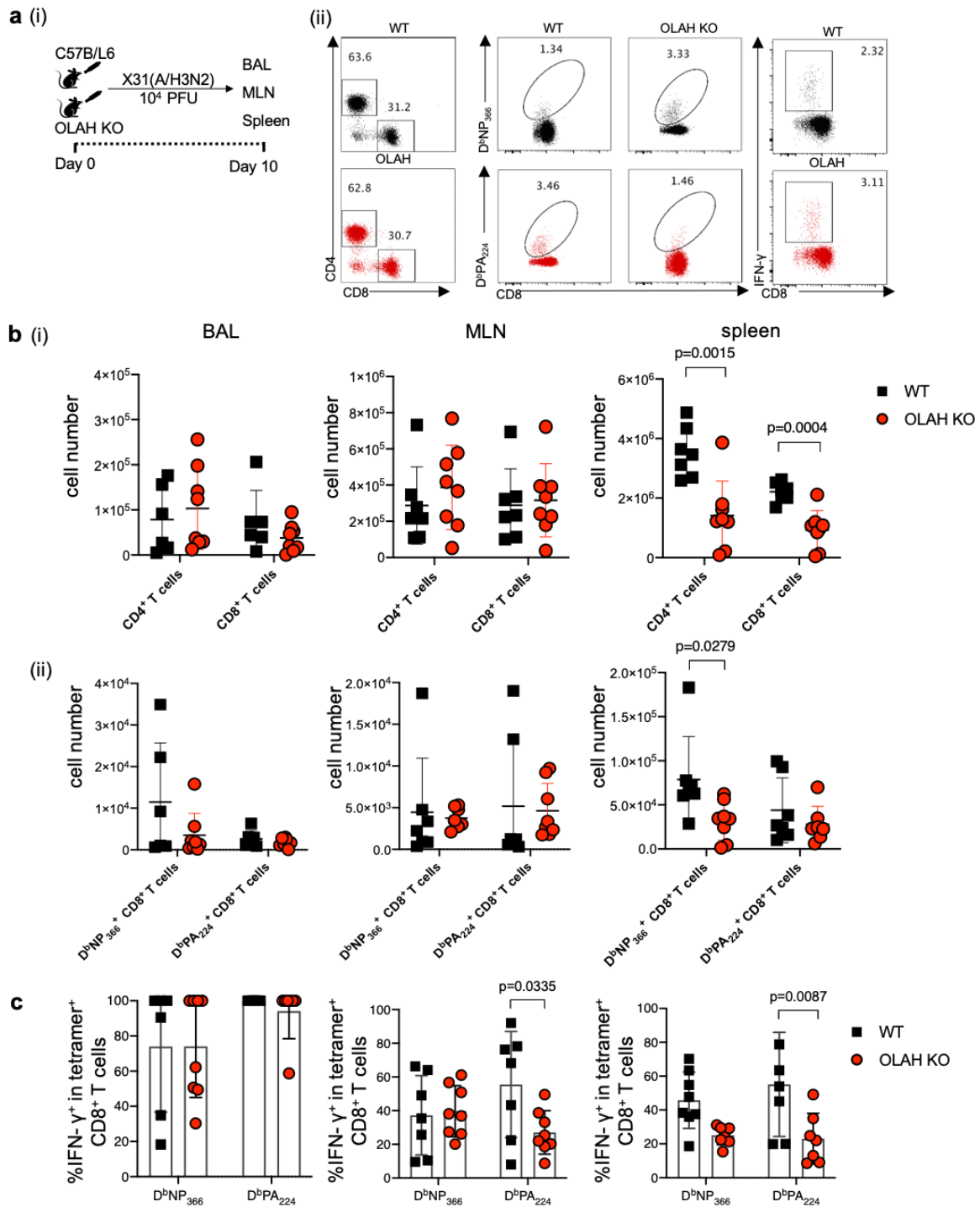


Fig 14. Reduced T cell numbers and Lower frequencies of IFN- γ ⁺CD8⁺ T cells in *OLAH* KO mice at effector stage after influenza A virus infection

a.(i) *OLAH* KO and WT mice (6-9 weeks old; n=8 mice per group) were challenged with A/H3N2 X31 virus at 10⁴ PFU and spleen were harvested at day10 after infection. (ii) Representative FACS plots on CD4⁺ T cells, CD8⁺ T cells, D^bNP₃₆₆⁺ CD8⁺ T cells, D^bPA₂₂₄⁺ CD8⁺ T cells and IFN- γ ⁺ CD8⁺ T cells in both mice groups. b(i) Cell number of CD4⁺ T cell and CD8⁺ T cell in BAL, MLN and spleen in *OLAH*

KO and WT mice. (ii) D^bNP₃₆₆⁺ CD8⁺ T cell and D^bPA₂₂₄⁺ CD8⁺ T cell number in BAL, MLN and spleen in OLAH KO and WT mice. c. Cell samples from BAL, MLN and spleen were stimulated with either D^bNP₃₆₆ or D^bPA₂₂₄ peptide (1μM) for 5h at 37°C. Frequency of IFN-γ⁺ CD8⁺ T cells within either D^bNP₃₆₆⁺ CD8⁺ T cells or D^bPA₂₂₄⁺ CD8⁺ T cells in BAL, MLN and spleen were shown. (Average with ±SD)

5.2.10 Similar memory T cell profiles between WT and OLAH KO mice after low dose influenza infection

To determine whether deletion of OLAH affect establishment and recall of memory T cells, spleens from OLAH KO and WT mice were collected on day 30 post-infection and virus-specific T cell responses analyzed. In both mouse groups, comparable CD4⁺ T cell, CD8⁺ T cell as well as virus-specific D^bNP₃₆₆⁺ and D^bPA₂₂₄⁺ CD8⁺ T cell numbers were detected (**Fig 15a**). We also found similar frequencies of D^bNP₃₆₆⁺ and D^bPA₂₂₄⁺ CD8⁺ T cells producing IFN-γ, TNF- α or TNF-α and IL-2, in both OLAH KO and WT mice groups (**Fig 15b**), thus demonstrating that despite lower T cell responses elicited in OLAH KO mice during the effector phase of the responses, they could generate similar memory profiles as WT mice.

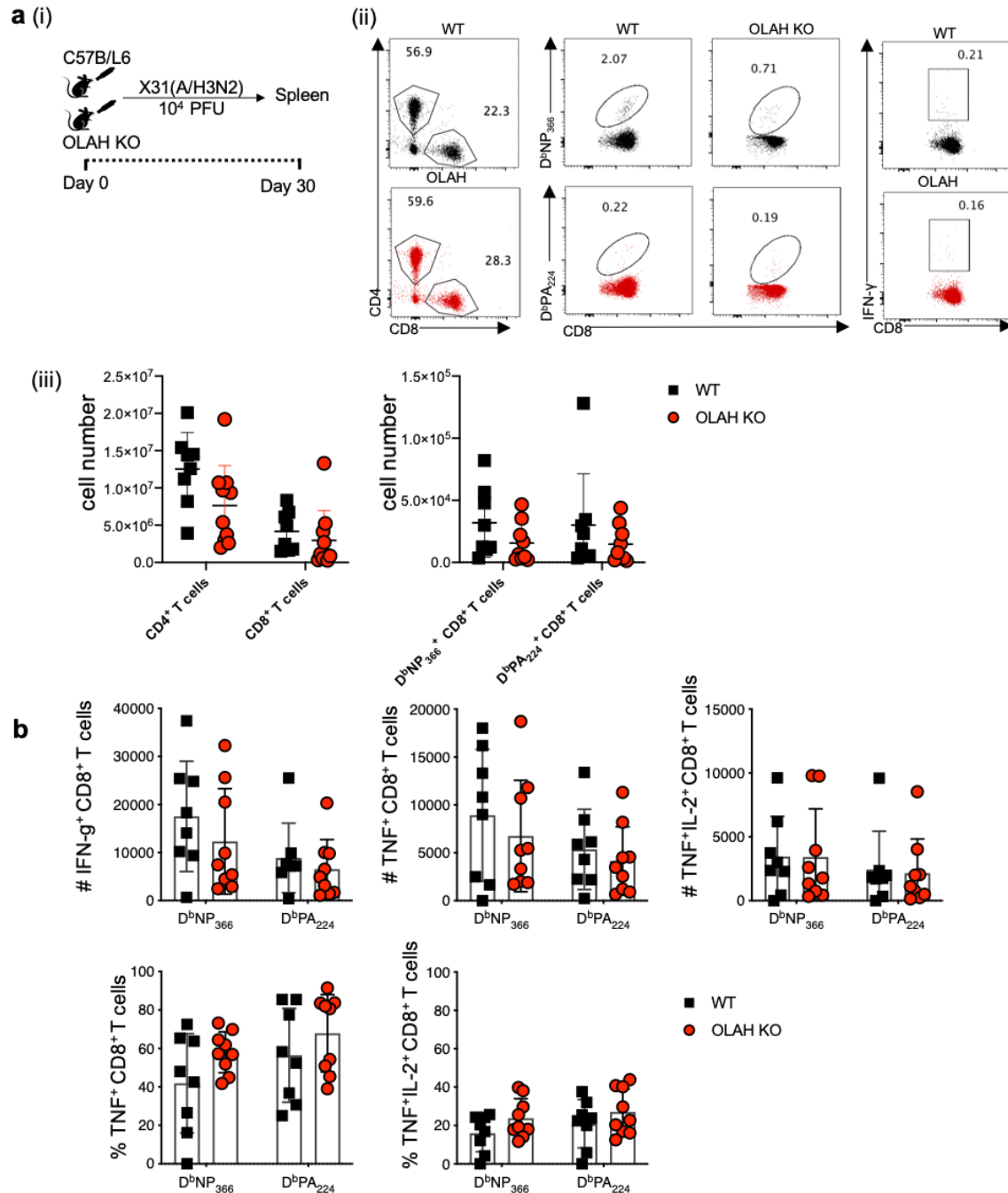


Fig 15. Similar memory profiles between WT and OLAH KO mice after influenza A virus infection

a.(i) OLAH KO and WT mice (6-9 weeks old; n=8 to 9 mice per group) were challenged with A/H3N2 X31 virus at 10^4 PFU and spleen was harvested at day30 after infection. (ii) Representative FACS plots on $CD4^+$ T cells, $CD8^+$ T cells, $D^bNP_{366}^+$ $CD8^+$ T cells, $D^bPA_{224}^+$ $CD8^+$ T cells and $IFN-\gamma^+$ $CD8^+$ T cells in both mice groups. (iii) Cell number of $CD4^+$ T cell, $CD8^+$ T cell, $D^bNP_{366}^+$ $CD8^+$ T cell and $D^bPA_{224}^+$ $CD8^+$ T cell in spleen from OLAH KO and WT mice groups. b. Cell samples from spleen were stimulated with either D^bNP_{366} or D^bPA_{224} peptide ($1\mu M$) for 5h at $37^\circ C$. Frequency of $IFN-\gamma^+$ $CD8^+$ T cells within $CD8^+$ T cells and $TNF^+/IL-2^+$ within $IFN-\gamma^+$ $CD8^+$ T cells in both mice groups were shown. (Average with $\pm SD$)

5.2.11 Reduced disease severity in OLAH KO mice following secondary influenza A virus infection

Having shown comparable memory CD8⁺ T cell responses in both WT and OLAH KO mice, we next asked whether there were differences in the recall capacity of these antigen-specific memory CD8⁺ T cell pools following secondary influenza virus infection. We first infected OLAH KO and WT mice with 10⁴ PFU of X31 and then challenged 30 days later with a heterologous strain, A/Puerto Rico8/8/34 (H1N1; PR8), which shares the same internal viral proteins. Although mice from both groups could survive secondary infection (**Fig16 ai**), OLAH KO mice displayed a trend of less body weight loss before their full recovery compared to WT (**Fig16 aii**), similar to what was observed during primary X31-infection.

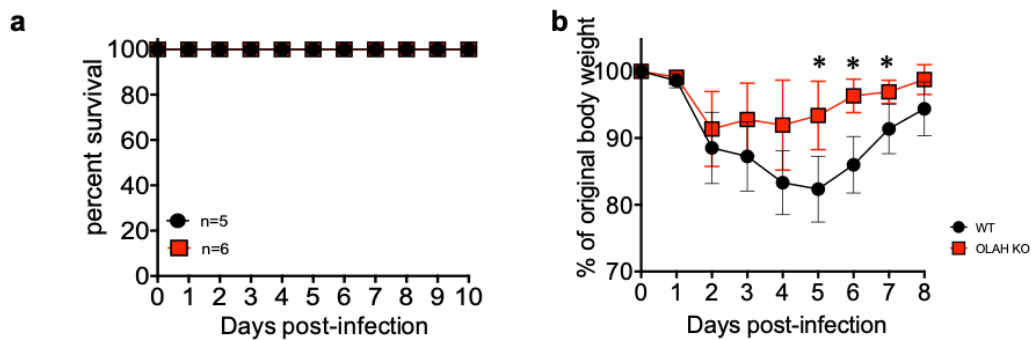


Fig 16. Less body weight loss in OLAH KO mice during secondary influenza A virus infection OLAH KO and WT mice (6-9 weeks old; n=5 to 6 per group) were intranasally infected with A/H3N2 X31 virus at 10⁴ PFU. Mice were rested for 30 days before given secondary challenge with A/H1N1 PR8 virus at 10⁴ PFU, and BAL, MLN and spleen were harvested at day 8 after infection. Mice were monitored for body weight over 10 days and culled when >25% of their original weight was lost. (a) show the survival rate of infected mice with their mean body weight profiles ($\pm$ SEM) depicted in (b). (* p < 0.05).

We then collected BAL, MLNs and spleens on day 8 post-infection with PR8 to analyze CD8⁺ T cell responses. Again, we found comparable numbers of CD4⁺ T cell and CD8⁺ T cells, as well as virus-specific D^bNP₃₆₆⁺CD8⁺ and D^bPA₂₂₄⁺ CD8⁺ T cells in all BAL and collected organs from mice of both groups (**Fig 17a,b**), in addition to similar frequencies of IFN- γ ⁺ and IFN- γ ⁺TNF- α ⁺ CD8⁺ T cells within each virus-specific CD8⁺ T cell pool (**Fig 17c**). These results demonstrate that OLAH does not affect generation of memory or secondary recall CD8⁺ T cell responses and the fact that OLAH-KO mice lose less weight during secondary infection further indicates that OLAH plays a role in mediating disease severity during early-stages of IAV infection.

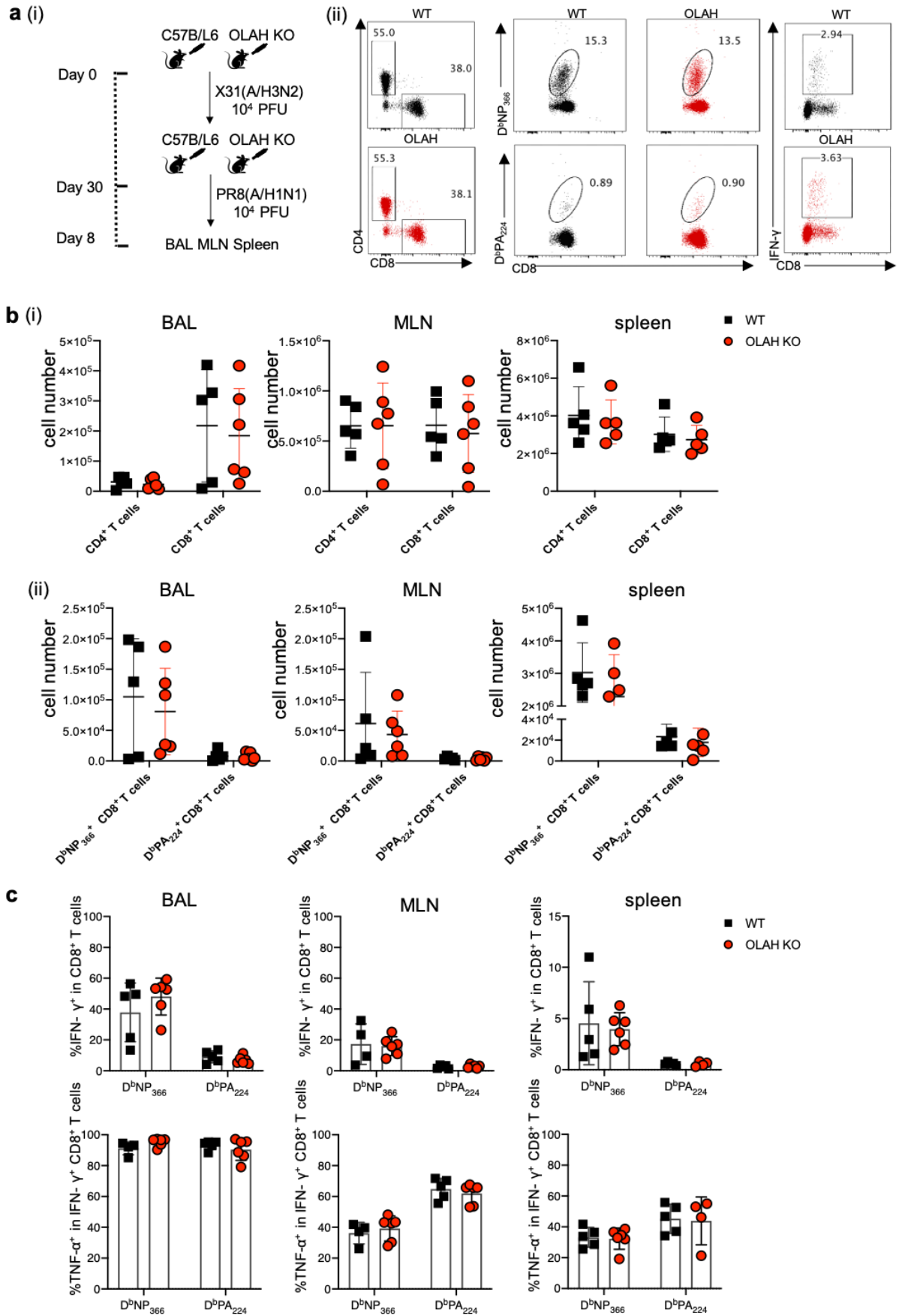
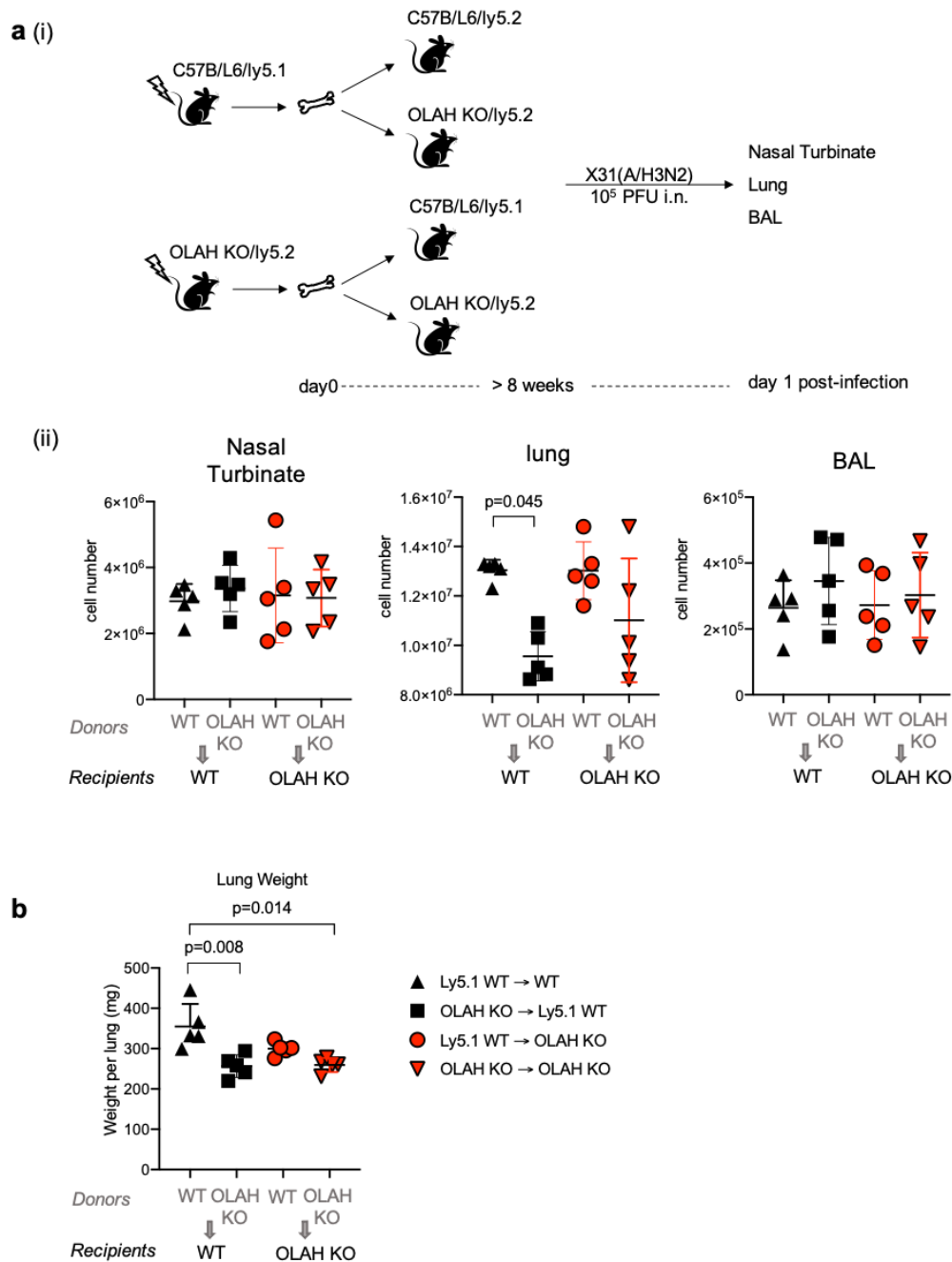


Fig 17. Comparable immune profiles between WT and OLAH KO mice after secondary viral challenge

a.(i) OLAH KO and WT mice (6-9 weeks old; n=5 to 6 per group) were intranasally infected with A/H3N2 X31 virus at 10^4 PFU. Mice were rested for 30 days before given secondary challenge with A/H1N1 PR8 virus at 10^4 PFU, and BAL, MLN and spleen were harvested at day 8 after infection. (ii) Representative FACS plots on CD4⁺ T cells, CD8⁺ T cells, D^bNP₃₆₆⁺ CD8⁺ T cells, D^bPA₂₂₄⁺ CD8⁺ T cells and IFN- γ ⁺ CD8⁺ T cells in both mice groups. b.(i) Cell number of CD4⁺ T cell and CD8⁺ T cell in BAL, MLN and spleen in OLAH KO and WT mice. (ii) Cell number of D^bNP₃₆₆⁺ CD8⁺ T cell and D^bPA₂₂₄⁺ CD8⁺ T cell in BAL, MLN and spleen in OLAH KO and WT mice. c. Cell samples from BAL, MLN and spleen were stimulated with either D^bNP₃₆₆ or D^bPA₂₂₄ peptide (1 μ M) for 5h at 37°C. Frequency of IFN- γ ⁺ CD8⁺ T cells within CD8⁺ T cells and TNF⁺ within IFN- γ ⁺ CD8⁺ T cells in BAL, MLN and spleen from both mice groups were shown. (Average with $\pm$ SD)

5.2.12 Altered immune responses in infected OLAH KO and WT bone marrow chimeras

Given that OLAH is expressed in both CD45⁻ non-immune- and CD45⁺ immune cells, we generated bone-marrow chimeras using OLAH KO and WT mice to assess the relative roles of these hemapoietic and non-hemapoietic cells during severe influenza virus disease. Adoptive transfer of bone marrow donor cells from WT mice into lethally irradiated OLAH KO recipient mice (WT $\rightarrow$ OLAH KO) provides a model in which only the CD45⁺ immune cells from the WT donor express OLAH (**Fig18 ai**). In the inverse (OLAH KO $\rightarrow$ WT), only CD45⁻ non-immune cells (e.g. lung epithelial cells) express OLAH. We also established chimeras where OLAH KO or WT donor bone marrow were transferred into the same irradiated recipient strains (OLAH KO $\rightarrow$ OLAH KO or WT $\rightarrow$ WT) as controls. After 8 weeks following transfer, we challenged mice with 10^5 PFU of X31 and analyzed cell infiltrates in respiratory tract samples on day 1 post-infection. We found that despite comparable total cell numbers in the nasal turbinate and BAL of all four chimeric mice groups, lung cell numbers in OLAH KO $\rightarrow$ WT mice were significantly lower than WT $\rightarrow$ WT mice ($p=0.045$) (**Fig18 aii**). Similar lower numbers were also observed in 3 out of 5 OLAH KO $\rightarrow$ OLAH KO mice and taken together suggests that absence of OLAH in immune cells results in less cell infiltration. Together with this, compared to WT $\rightarrow$ WT mice, reduced lung weights were evident in all other chimeric mice groups that had cells deficient in OLAH expression, including control OLAH KO $\rightarrow$ OLAH KO mice ($p=0.014$) (**Fig18 b**), altogether suggesting that absence of OLAH in either cell population, particularly immune cells (OLAH $\rightarrow$ WT; $p=0.008$), contributes to a lower inflammatory state.



Since influenza primarily targets epithelial cells and alveolar macrophages can also be abortively infected, we further analysed intracellular viral nucleoprotein (NP) expression in these cell populations to ascertain the contribution of OLAH to their infectivity. Compared to WT $\rightarrow$ WT, significantly lower frequencies of NP⁺ epithelial cells were detected in OLAH KO

→ WT mice only in the nasal turbinate ($p=0.0067$) (**Fig 19a,bi**), the point of entry for respiratory viruses, but not the lungs or BAL. It is interesting to note that less NP⁺ epithelial cells were also found in the nasal turbinate of WT → OLAH KO and OLAH KO → OLAH KO groups.

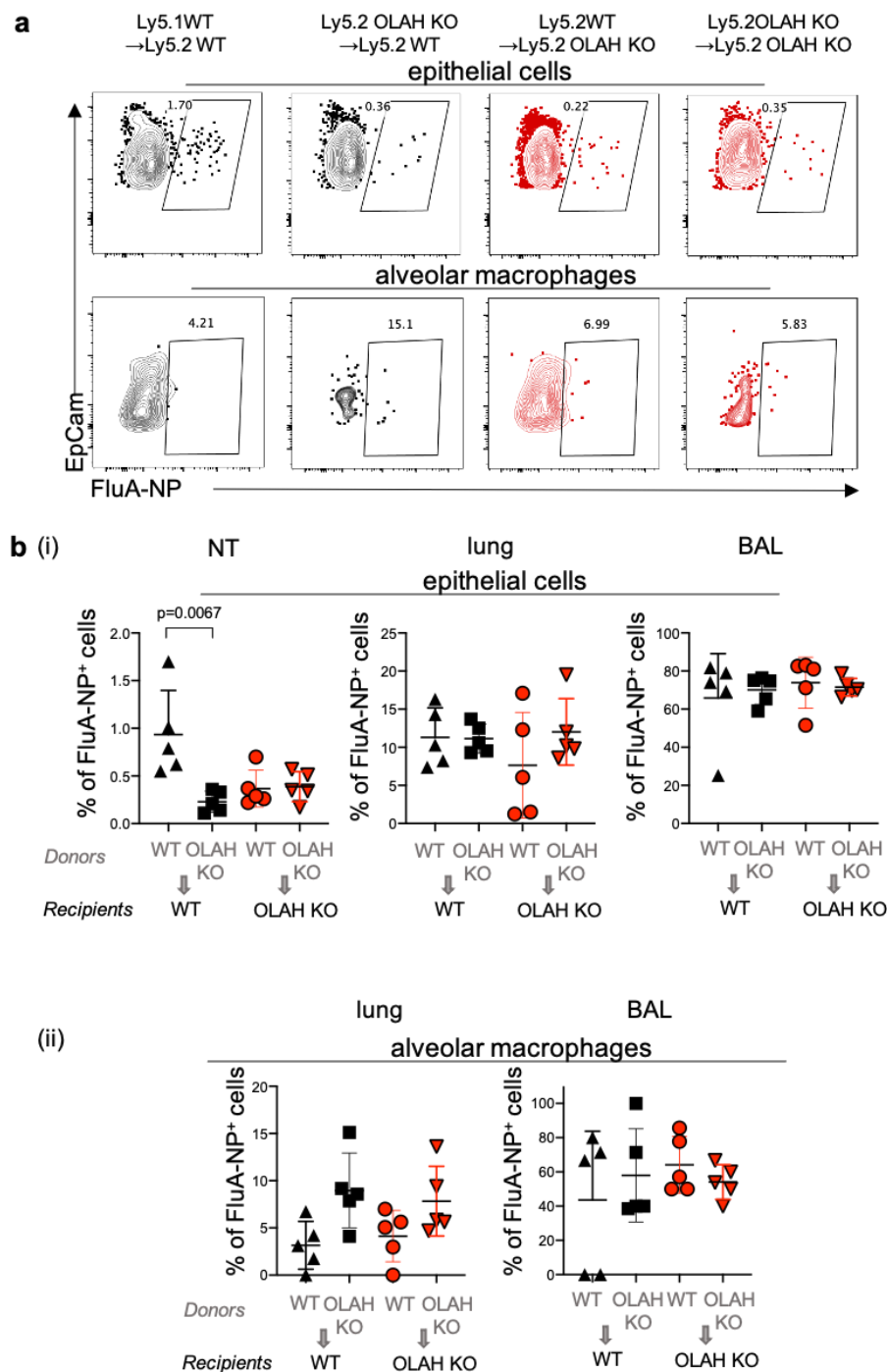


Fig 19. Reduced infection rate on epithelial cells in nasal turbinate of OLAH KO BM mice

Bone marrow chimeras were established by adoptive transfer of bone marrow cells from WT C57BL/6 or OLAH KO mice into irradiated recipient mice (n=5 per group). After 8 weeks, mice were challenged with X31 virus at 10^5 PFU, and nasal turbinate, lung and BAL were collected on day1 after infection. a. Representative FACS plots of FluA-NP staining on epithelial cells (nasal turbinate) and alveolar

macrophages (lung) from four mice groups. b. Frequency of FluA-NP⁺ cells in (i) epithelial cells and (ii) alveolar macrophages from four mice groups. Average with $\pm$ SD are shown.

In line with this, a similar trend was observed for frequencies of NP⁺ macrophages in the nasal turbinate, although not statistically significant (**Fig 19a,bii**). These results nonetheless reinforce the view that absence of OLAH in either of these CD45-epithelial cells or CD45⁺ macrophages cell populations may contribute to a better control of viral infection and thus disease outcome. Of note, in lungs of chimeras that had OLAH deficient immune cells (OLAH KO $\rightarrow$ WT and OLAH KO $\rightarrow$ OLAH KO), NP⁺ alveolar macrophage frequencies were elevated in several mice, as compared to those that had WT immune cells (WT $\rightarrow$ OLAH KO and WT $\rightarrow$ WT), which is consistent with their ability to limit infection by acting as a “sink” for virus³⁰⁶.

As we observed differences in amounts of lipid droplets in lungs of OLAH KO and WT mice before and after infection (**Fig 10**), we investigated whether this was associated with CD36 expression on epithelial cells and macrophages in the bone-marrow chimeric mice to gain an insight into which cells are involved in lipid uptake. In the nasal turbinate, compared to their WT $\rightarrow$ WT counterparts, we observed lower percentages of CD36⁺ epithelial cells in OLAH KO $\rightarrow$ WT mice ($p=0.0385$), and to some extent in WT $\rightarrow$ OLAH KO mice, but not OLAH KO $\rightarrow$ OLAH KO mice (**Fig 20a,bi**). This suggests that CD36 expression in epithelial cells is affected in situations where OLAH expression is varied amongst both immune and non-immune cells. However, this trend was only observed at the site of viral entry and no clear differences in each population in the lung and BAL were seen between any groups.

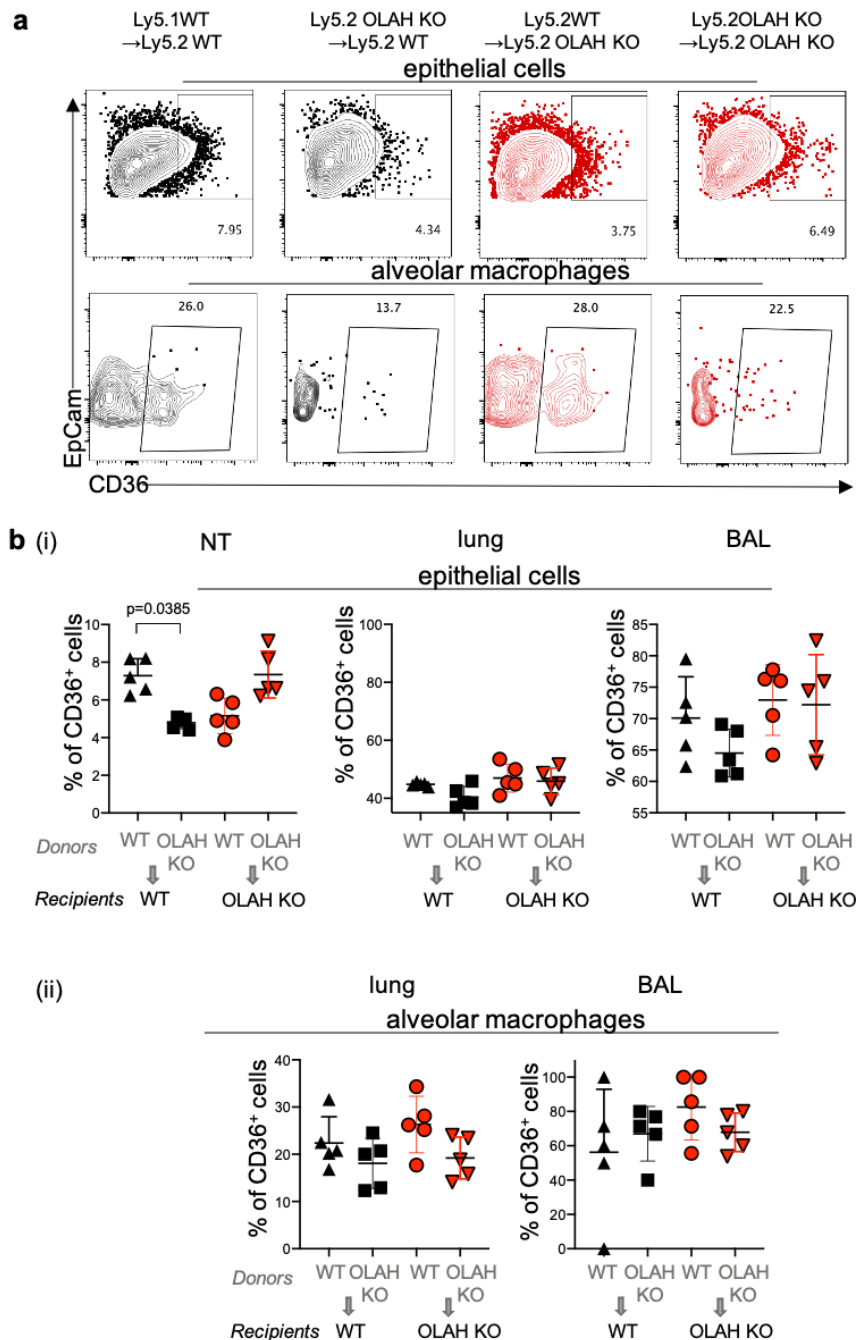


Fig 20. Reduced CD36 expression on epithelial cells in nasal turbinate of OLAH KO BM mice

Bone marrow chimeras were established by adoptive transfer of bone marrow cells from WT C57BL/6 or OLAH KO mice into irradiated recipient mice (n=5 per group). After 8 weeks, mice were challenged with X31 virus at 10^5 PFU, and nasal turbinate, lung and BAL were collected on day1 after infection. a. Representative FACS plots of CD36 staining on epithelial cells (nasal turbinate) and alveolar macrophages (lung) from four mice groups. b. Frequency of CD36⁺ cells in (i) epithelial cells and (ii) alveolar macrophages from four mice groups. Average with $\pm$ SD are shown.

In the case of macrophages, we detected increased CD36 expression in chimeric mice which received OLAH expressing immune cells (WT → WT and WT → OLAH KO) than those with OLAH deficient immune cells (OLAH KO → WT and OLAH KO → OLAH KO)

(Fig 20bii). Although these differences were not statistically significant, it is tempting to speculate that the increased CD36 expression on macrophages in the former mentioned groups have a greater capacity to facilitate lipid uptake and contribute to the increased lipid droplet formation. While further studies are needed to confirm these findings, overall our results allude to a complex interplay of mediators involved in driving OLAH-mediated disease severity and indicate a role for macrophages as well as other innate immune cells during the early stages of infection.

5.3 Discussion

Although OLAH is known to be involved in fatty acid biosynthesis and subsequently involved in lipid metabolism through its primary catalytic product oleic acid, little is known about its role in immune responses and driving influenza disease severity. To study the role of OLAH in anti-viral responses, we utilized a well characterized B6 mouse model of IAV infection. For the first time, we analysed OLAH gene expression in WT mice across different anatomical compartments and found that OLAH was expressed at higher levels in the testis and placenta compared to organs/tissues related to immune and respiratory function such as the thymus and lung. This might be as the reproductive tissues contain a greater abundance of adipose tissue, where fatty acid synthesis mainly takes place, which is also important for lipid homeostasis^{307,308}, especially in male fecundity^{309,310}. Undetectable OLAH expression in mouse spleen samples which is different from what we observed in human samples in Chapter IV might be explained by the fact that the immune system in an adult human spleen is experienced while our mice were naïve. In addition, the lipid usage and composition in the spleens of mice and humans may also contribute to this difference. Moreover, OLAH expression was also observed not only in CD45⁻ cells but also CD45⁺ lymphocytes, which could reflect its role in *de novo* fatty acid synthesis for cellular functions and processes. For instance, macrophages greatly depend on lipid metabolism to instruct their differentiation as fatty acid biosynthesis contributes to membrane remodeling and formation of inflammatory mediators in M1 macrophages, while activation of anti-inflammatory M2 macrophages are highly reliant on FAO³¹¹.

In successfully establishing OLAH KO mice, we found that compared to naive WT mice, OLAH deficiency did not affect their behaviour as well as outward and internal appearance or frequencies of immune cells, indicating that altered or impaired OLAH-mediated

fatty acid synthesis does not significantly impact their development and phenotype. Striking differences between these strains were, however, observed upon primary influenza A virus infection, wherein absence of OLAH not only led to milder disease, as evident by less body weight loss, but also complete protection from fatal disease outcomes, thus supporting our observations in H7N9-infected patients who died and verifying the importance of OLAH in influenza disease severity. These effects appear to be the consequence of reduced viral replication resulting in more controlled innate immune responses and inflammation, especially during the early stages of infection.

Hypercytokinemia is a typical hallmark of severe influenza disease^{271,312,313} and also present during ARDS³¹⁴ and other respiratory conditions such as chronic obstructive pulmonary disease³¹⁵ and asthma³¹⁶. This is often reflected by increased levels of cytokines due to excessive host responses, such as those that are especially proinflammatory, including IFNs, TNFs and IL-6³¹⁷. In line with this, we detected lower levels of TNF- α in the lungs of infected OLAH KO mice, thus implicating a causative role in the severe lung tissue damage observed in WT mice during disease progression and indicating that OLAH drives IAV-induced hypercytokinemia and immune dysfunction.

In investigating how OLAH was mediating these effects, our results indicating the involvement of lipid droplets in the lung where in the absence of OLAH, LDs formation during viral infection was perturbed particularly during the early stages of infection. Given the role of LDs in fatty acid synthesis, it was perhaps unexpected to see more lipid droplets initially in the lungs of naive OLAH KO mice than their WT counterparts. As mentioned earlier, this might be due to compensatory mechanisms at play to account for the absence of oleic acid and the lipid species within these droplets are likely to be different. Nonetheless, it is known that many viruses including those belonging to the Flaviviridae (HCV^{318,319}, Dengue virus³²⁰) and Reoviridae (rotavirus³²¹, orthoreovirus³²²) families can use host cell lipid droplets to enable their assembly and hence replication. For example, the capsid protein of dengue virus can trigger lipid droplet formation and using them as a platform for nucleocapsid formation during the encapsidation stage³⁰¹. While similar studies during influenza virus infection are limited, Episcopio *et al* demonstrated that influenza virus infection can stimulate formation of lipid droplets to accumulate near the cell nuclei, while inhibition of their formation can reduce virus replication³²³. These results together with ours show that limiting the usage of lipid droplets

by influenza virus, in our case by knocking out OLAH function, can significantly reduce viral replication. Furthermore, studies have shown that oleic acid, which is significantly increased in mice BAL during influenza virus infection²⁹¹, is a potent inducer of lipid droplet accumulation, a process that involves the long-chain fatty acid receptors FFAR4^{324,325} and CD36³²⁶, further providing evidence that OLAH-mediated lipid droplets contribute to viral replication and disease. Of note, the detailed composition of lipids in lipid droplet between WT and OLAH KO mice are not known, and future investigation to define specific lipids changes caused by *Olah* knocking-out may leading us to a clearer view of how does OLAH contribute to altering immune system.

A growing number of studies also indicate that lipid droplets not only regulate fatty acid homeostasis in a cell but are also involved in host immune responses. Their capacity to store cytokines, including IFNs²⁹⁶, TNF- α ^{327,328}, RANTES and IL-16³²⁹, indicate that they play a role in anti-viral responses and controlling inflammation. It is therefore possible that the lower levels of inflammation observed in OLAH KO mice, especially in TNF- α levels, may be directly related to reduced host cell lipid droplet numbers and size during infection. The role of lipid droplets also extends to cellular functions especially in macrophages^{298,299,330}, as a source of lipid precursors to support cell proliferation and induce inflammatory responses^{331,332}. Under excessive inflammatory conditions however, macrophages can differentiate from being involved in pathogen clearance to exhibiting immunosuppressive properties. In fact, proinflammatory responses of macrophages can be inhibited by esterification of fatty acids within lipid droplets³³³. In addition, oleic acid-induced lipid droplet formation leads to tumor-associated macrophages acquiring an immune suppressive phenotype by regulating mitochondrial respiration in the cell²⁹⁸. Given that elevated levels of macrophages were seen in lungs of infected chimeric mice that received OLAH KO immune cells, it is plausible that these cells represent effectors with anti-viral function since preventing OLAH-mediated lipid droplet formation can inhibit their differentiation into immune suppressive cells. This is also consistent with macrophages in chimeric mice that received WT immune cells and expressed higher levels of CD36, reflecting their potential for lipid uptake and thus lipid droplet formation. Further repeat of this experiment with larger sample sizes as well as new studies to examine macrophage cytokine production and mitochondrial respiration analyses in the context of oleic acid treatment will of course be needed to prove this theory.

OLAH deficiency also appears to affect the magnitude of primary effector anti-viral CD8⁺ T cell responses, which might be due to differences in the composition of their lipid content thus impacting the demand for *de novo* fatty acid synthesis in activated T cells. The lower magnitude of responses observed in OLAH-KO mice may also be due to lesser amounts of antigen present as a result of reduced viral load and possibly better control of innate immune responses during the early phases of infection. These differences, however, did not appear to affect the establishment of memory populations as OLAH KO mice generated similar memory profiles as WT mice in the spleen. It must be noted however that memory profiles at the site of infection (lungs) were not examined. T_{RM} cells in particular are crucial for eliciting effective secondary immune responses against influenza virus infection and studies have found D^bNP₃₆₆⁺ and D^bPA₂₂₄⁺ T_{RM} cells can be detected in the lung during the memory phase²¹⁷. It is therefore possible that given differences in primary effector D^bNP₃₆₆⁺CD8⁺ and D^bPA₂₂₄⁺CD8⁺ T cell responses, this may have translated into differences in T_{RM} cell profiles. While this may explain why OLAH KO mice lose less body weight than WT mice upon secondary infection, we did not observe any differences in the magnitude or quality of recall CD8⁺ T cell immune responses, although influenza-specific CD8⁺ T cell responses were only analysed at one time-point. Clearly there are other factors that could account for why OLAH KO mice are better protected in this situation. Given more time, examining lung viral titres, inflammatory and innate immune responses as well as CD8⁺ T cell expression of cytolytic mediators such as granzyme and perforin could shed light for the observed differences in secondary disease outcome. Another important caveat to consider is that these experiments were conducted in the context of a mild primary infection in accordance with our animal ethics requirements and that establishing a severe infection may elicit a different profile of primary immune responses and result in outcomes upon secondary challenge akin to those observed in severely infected H7N9 patients who exhibited ineffective CD8⁺ T cell responses.

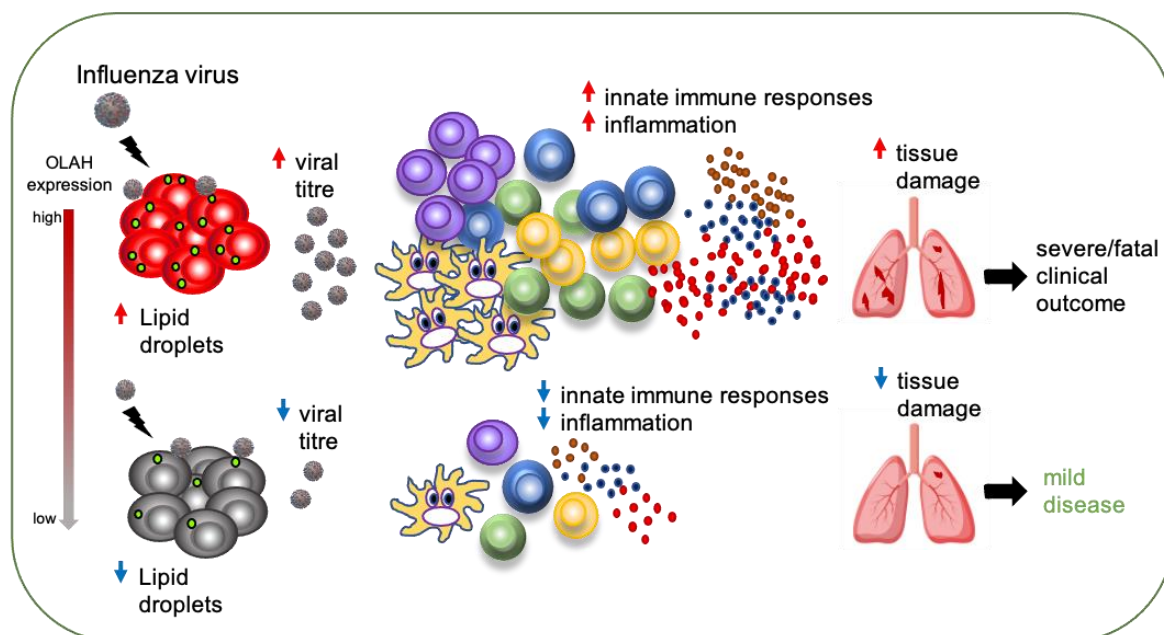


Fig 21. OLAH as a potential bio-marker for the early prediction of patients at the risk of fatal clinical outcomes

Schematic diagram showing the hypothetical model of OLAH functioning in host immune responses during severe influenza virus challenge.

In summary, our results in this Chapter describe a successful generation and establishment of an OLAH-deficient mouse model which allowed us to directly demonstrate that altered OLAH expression can impact influenza disease outcome. Our findings indicate that with OLAH expression present during high dose influenza virus infection, increased formation of lipid droplets in lungs can be observed, which correlates with higher viral titres compared to OLAH KO mice, thus triggering excessive inflammation and lung tissue damage (**Fig 21**). These effects are most pronounced during the early stages of infection and at a magnitude that can overwhelm the host and culminate in severe and fatal disease outcomes.

CHAPTER VI: CONCLUDING REMARKS

Despite intense research efforts, viral, host and immune factors determining IAV disease severity are still far from being completely understood. Published studies provided evidence that the elderly, pregnant women and individuals with underlying medical conditions are more susceptible to severe or even fatal disease outcomes^{284,334,335}. However, it is still unclear why influenza causes mild “asymptomatic” infection in some individuals while others, including previously healthy individuals with no prior risk factors, develop severe and fatal disease. Therefore, in this PhD thesis, we sought to understand host factors and mechanisms underlying severe influenza disease outcomes.

The upregulation of surface CD38 and MHC-II molecules on CD8⁺ T cells during H7N9 infection has been identified as a hallmark of either recovery or severe disease in humans where contraction of CD38⁺MHC-II⁺ CD8⁺ T cell response following their activation is associated with recovery but persistence leads to less favorable outcomes¹⁹². In Chapter III, we established and utilized a mouse model of influenza A virus infection to define activation, recruitment and survival of CD38⁺MHC-II⁺ CD8⁺ T cells *in vivo* as well as investigated the underlying mechanism. Similar to previous findings in H7N9 patients¹⁹², we observed early increased recruitment of CD38⁺MHC-II⁺ CD8⁺ T cells to the site of infection after severe influenza A virus infection, when compared with CD38⁺MHC-II⁺ CD8⁺ T cells during mild IAV disease. We found that activation of CD38⁺MHC-II⁺ CD8⁺ T cells occurs in an antigen and non-antigen driven manner, the latter indicating bystander activation. Similar findings have been seen in acute IAV-infected patients in whom bystander CD8⁺ T cells against Epstein-Barr virus (EBV) and human Cytomegalovirus (CMV) specific antigens were activated in IAV-infected patients PBMCs and display a CD38⁺MHC-II⁺ phenotype³³⁶. There is also evidence that CD8⁺ T cells activated in a non-antigen specific bystander manner can lead to immunopathology. This can be exemplified by studies showing that while herpesvirus-specific CD8⁺ T cells can be beneficial during heterologous viral challenge³³⁶, while bystander CD8⁺ T cells from human PBMCs activated via IL-15 stimulation (*in vitro*) are associated liver tissue injury in Hepatitis A virus (HAV) infected patients³³⁷.

This posits the question, how are bystander T cells activated if not in an antigen-driven manner? Studies have in fact shown that direct engagement of TLRs can directly activate memory CD8⁺ T cells without TCR engagement, in particular via TLR2 and TLR7³³⁸. While

influenza viral RNAs can be recognized by TLR3, TLR7 and TLR8³³⁹, the impact of this activation pathway on bystander CD8⁺ T cells remains unclear. Additionally, innate immune responses inducing inflammatory cytokines could be also, at least partially, responsible for the activation of bystander CD8⁺ T cells. Studies showed that type I IFN profoundly induced during viral infection can activate CD8⁺ T cells in the absence of TCR engagement³⁴⁰. Furthermore, cytokines such as IL-12, IL-15 and IL-18 can also activate CD8⁺ T cells in a non-virus-specific manner^{341,342}. Additionally, detection of CD19 on CD38⁺MHC-II⁺ OT-I cells further supported that MHC-II was acquired by trogocytosis, at least partially, sourced from B cells. Snatching of MHC molecules by both CD8⁺ T cells and CD4⁺ T cells from antigen presenting cells has been reported by previous studies in order to improve cell-mediated responses and promote memory formation^{143,147,343}. It is thus plausible that similar mechanisms are at play during influenza infection in humans³⁴⁴ but it remains to be seen whether these “snatched” MHC-II molecules on CD38⁺CD8⁺ T cells are empty or loaded with peptide antigens. One assumption is that CD38⁺CD8⁺ T cells acquire antigen-loaded MHC-II complexes that are then presented to CD4⁺ T cells promoting the formation of memory CD8⁺ T cells. A question remains whether the “snatched” empty MHC-II molecules are peptide-loaded, as this would have an impact on stimulation of CD4⁺ T cells. Collectively, our findings highlight the importance of the CD38⁺MHC-II⁺ phenotype for CD8⁺ T-cell memory establishment and recall, thus providing key insights into mechanisms underpinning CD38 and MHC-II co-expression on CD8⁺ T cells during viral infections, especially during severe influenza diseases. This CD38⁺MHC-II⁺ CD8⁺ T-cell immune subset may constitute a key signature of clinical outcome during the early stages of disease, and further investigations are therefore needed to determine whether CD38⁺MHC-II⁺ CD8⁺ T-cell presence and/or function can be modulated by medical treatment. Of note, our studies were performed in a mouse model, and whether trogocytosis occurs in humans during influenza diseases is not clear yet. Although human CD8⁺ T cells can express MHC-II molecule (HLA), it cannot exclude the possibility that extracellular MHC-II (empty or antigen-loaded) are acquired via cell-cell interactions.

In Chapter IV, with access to unique samples from a cohort of H7N9 patients who recovered or succumbed to severe influenza disease, we investigated disease signatures during the early phases of infection to identify key features associated with clinical outcomes. We observed that viral titres correlated with cytokine production in H7N9 patients who recovered, suggesting that recovery is associated with a controlled immune response. However, this was not the case for H7N9 patients who displayed dysregulated immune responses and had fatal

disease outcomes^{258,259}. While previous gene expression analysis in H7N9 patients at late time points after disease onset revealed profound differences in gene expression between patients who survived and died²⁵⁸, only 3 genes were differentially expressed during the early stage of disease (Chapter IV of this PhD thesis). Amongst those 3 differentially expressed genes, a gene encoding for *Olah* was 80-fold higher in H7N9 patients who died when compared to patients who survived. Our analyses of *Olah* gene expression at different anatomical sites showed high levels of *Olah* expression in the lung, spleen and PBMCs, supporting our hypothesis that *Olah* could be playing a role modulating host immune responses during severe influenza A virus infection. High *Olah* expression was particularly found in CD14⁺ cells, defining multipotent precursor cells of myelomonocytic lineage as well as their differentiated counterparts such as monocytes and macrophages, thus providing potential clues as to how OLAH could affect host immunity during the early phases of IAV infection. These findings provide the rationale for extending our analyses to future studies on OLAH expression in larger and stratified (e.g. age, gender) cohorts of severely-infected influenza patients and individuals with other respiratory diseases such as COVID-19. Using successfully established qPCR techniques in this PhD thesis, *Olah* distribution can be further analysed at different phases of influenza disease in large patient cohorts. Furthermore, given that respiratory epithelial cells are the primary targets for influenza virus infection, we cannot rule out the possibility that OLAH-mediated effects are present in non-immune cells within the respiratory tract. The establishment of the *Olah*-overexpressing human lung epithelial cell line A549-V1 in Chapter IV can thus serve as a useful and relevant platform to shed light on the susceptibility of the epithelium to viral infection and its effects.

While *in vitro* experiments and analyses are important for studying OLAH-mediated effects especially in clinical settings, *in vivo* experimentation is needed to investigate direct effects of OLAH on severe influenza disease as well as underlying mechanisms. Therefore, the aim of Chapter V was to generate and establish an OLAH KO mouse model to determine the role of *Olah* during severe influenza A virus infection. In doing so, we demonstrated that infection of OLAH KO mice with a high dose of virus, which would normally cause severe disease in WT mice, resulted in milder disease symptoms such as reduced body weight loss, greater survival rates and significantly diminished lung inflammation and damage. Of great importance was our finding on lower levels of cytokines associated with host tissue damage, particularly TNF- α ³⁰⁵, which can be produced by lung epithelial cells³⁴⁵, DCs and CD8⁺ T cells, and known to exacerbate influenza disease severity caused by highly pathogenic influenza

H5N1 infection in humans^{273,290} and mice²⁷². The role of TNF in driving inflammation-mediated disease severity is also supported by previous findings showing that TNF deficient mice exhibit lower levels of inflammation and better survival rates following influenza virus infection^{346,347}. MIP-1 β (CCL4) was also detected at significantly lower levels in OLAH KO mice, mainly produced by macrophages and associated with severe influenza disease outcomes^{259,348,349}. Although not examined, the importance of Type I and III IFNs should not be ignored, especially given their role in restricting viral replication during the early stages of infection^{350,351}. In particular, IFN- λ can be produced by epithelial cells³⁵⁰ to prevent influenza virus infection progressing from the upper respiratory tract into the lungs³⁵².

It should also be noted that while the focus of our studies have been based on using a high dose of virus to cause severe disease, inflammatory profiles during mild disease may present differently³⁴⁸. The metabolic profile in BAL, CD45⁺ cells and alveolar epithelial cells for example can differ in mice with severe and mild influenza A virus infection²⁸⁷, suggesting that OLAH's impact is conditional on the disease state. On another hand, it is also plausible that different inflammation levels also alter OLAH expression which subsequently leads to differences in ensuing immune responses. For example, TNF- α has been shown to induce *de novo* fatty acid synthesis and impact lipid metabolism³⁵³, which may make OLAH expression redundant. In this regard, this may explain why we did not observe major differences in ensuing T cell responses since the experiments to assess T cell immunity in OLAH KO mice were performed under mild disease conditions. Thus, the role of OLAH expression in the induction of adaptive immunity, including antibody responses during a severe disease state remains to be fully elucidated in the future studies. Furthermore, as we observed a correlation between high and prolonged expression of CD38 and MHC-II on CD8⁺ T cells with more severe disease outcome in H7N9 patients¹⁹², analyses of CD38⁺ and MHC-II⁺ phenotype on CD8⁺ T cells in WT and OLAH KO mice could reveal whether OLAH plays a role in modulating frequencies of this population.

Although anti-viral drugs such as adamantane and oseltamivir/zanamivir, which target viral proteins such as NA, can be effective in inhibiting virus release from infected host cells³⁵⁴, their use is often associated with the development of resistance^{355,356}. Given hypocytokinemia is often a feature of severe influenza infection, strategies to protect against disease have focused on anti-inflammation treatment at the early stage of disease³⁵⁷. For instance, anti-TNF

treatment in immunosuppressed patients with inflammatory bowel diseases, who are at a higher risk of influenza virus infection due to their dysregulated inflammation responses³⁵⁸, show increased antibody responses to influenza vaccination³⁵⁹, indicating that control of TNF-mediated effects can improve adaptive immunity. To date however, no clinical trials on anti-TNF therapy have been conducted on influenza-infected patients without underlying diseases due to concerns that it pre-disposes patients to higher risk of bacterial infections and other complications observed in previous anti-TNF clinical trials on sepsis patients³⁶⁰ and those with rheumatoid arthritis³⁶¹. Trials using glucocorticoids for treating severe influenza disease to boost anti-inflammatory activity have also shown suppression of cytokine production³⁶². However, while benefits from glucocorticoid treatment have been demonstrated³⁶³, there are opposing reports that using glucocorticoid as treatment results in comparable or higher mortality rates compared to control groups that did not receive glucocorticoid^{364–366}.

As influenza virus infection can impact cellular metabolic pathways, including interruption of glycolysis and fatty acid synthesis³⁶⁷ as well as increasing the production of reactive oxygen species (ROS)³⁶⁸, studies have been conducted to explore possible ways to target and tune specific metabolic pathways³⁶⁹ as a way to reduce disease severity. For example, it has been reported that suppression of Nox2 expression, which promotes production of ROS, can prevent lung tissue damage caused by influenza virus infection³⁷⁰. Since infection can cause significant metabolic changes in pathways involving c-Myc, glycolysis and glutaminolysis³⁶⁷, researchers found that the drug BEZ235, which acts as a regulator of PI3K/mTOR pathway, can control infection-mediated metabolic effects and in mice, thus lead to better survival rates and lower lung viral titres³⁶⁷.

Our findings that OLAH is involved in infection-driven lipid droplet formation postulates that infection requires *de novo* lipid synthesis for viral replication. LDs “hijacked” by virus can be not only the platform for viral assembly, but also an energy storage during virus replication^{300,371}. In addition to this, LDs can also be an organelle to store cytokines (as described in Chapter V), thus may be directly related to altered cytokine production during virus challenges²⁹⁶. Although most of the studies observed increased LDs formation in host cells induced by viral infection are in flavivirus genus, our study in Chapter V demonstrates this phenomenon also occurs during influenza A virus challenge, and viral replication can be impaired by limiting the formation of LDs as what we observed in OLAH KO mice, combined with a lower level of inflammation responses. However, given that inhibiting LD formation

contributes to impaired viral replication in influenza disease³²³, our view is that deficiency of OLAH plays an important role in controlling viral infection by limiting resources for replication and thus constitutes potential target for therapeutic intervention. On the other hand, it is also possible that OLAH is involved in the upsurge of diacyl glycerophosphocholine and diacyl glycerophosphoethanolamine which are associated with proinflammatory responses, tissue damage as well as disease severity³⁷². Therefore, targeting and suppressing the actions of OLAH, especially early in the disease phase constitutes a viable strategy which may positively impact host defenses by controlling excessive inflammation leading to better disease outcomes. Evidence from cancer studies in fact show that fatty acid uptake, most notably oleic acid, mediated by the fatty acid translocase CD36 contributes to growth of tumor cells^{303,373}. Furthermore, altered OLAH expression might be related to the expression of SREBP, a master regulator for transcription of genes related to fatty acid synthesis^{374,375}, which has been reported to promote production of oleic acid³⁷⁵, providing a link between SREBP expression and OLAH.

Having well-known host factors defining vulnerable populations during a virus outbreak and the ability to predict the severity of infection at early days after disease onset are critical measures necessary for alleviating disease and preventing deaths. In profiling and dissecting the mechanisms underpinning an important CD8⁺ T-cells population for memory establishment and recall as well as understanding how severe disease is driven by high OLAH expression in this PhD thesis has led to a greater understanding of what is lacking, impaired or overactive during severe and fatal influenza disease. Altogether, these findings have provided key insights into how we can control severe and fatal influenza disease and highlight potential bio-markers, which could assist to predict clinical outcomes and defined individuals at high risk of dying from severe influenza disease.

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APPENDIX

The following equal co-first and co-author publications beyond this thesis projects have been generated as part of work conducted during the duration of PhD study.



Recalling the Future: Immunological Memory Toward Unpredictable Influenza Viruses

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Persistent and durable immunological memory forms the basis of any successful vaccination protocol. Generation of pre-existing memory B cell and T cell pools is thus the key for maintaining protective immunity to seasonal, pandemic and avian influenza viruses. Long-lived antibody secreting cells (ASCs) are responsible for maintaining antibody levels in peripheral blood. Generated with CD4⁺ T help after naïve B cell precursors encounter their cognate antigen, the linked processes of differentiation (including Ig class switching) and proliferation also give rise to memory B cells, which then can change rapidly to ASC status after subsequent influenza encounters. Given that influenza viruses evolve rapidly as a consequence of antibody-driven mutational change (antigenic drift), the current influenza vaccines need to be reformulated frequently and annual vaccination is recommended. Without that process of regular renewal, they provide little protection against “drifted” (particularly H3N2) variants and are mainly ineffective when a novel pandemic (2009 A/H1N1 “swine” flu) strain suddenly emerges. Such limitation of antibody-mediated protection might be circumvented, at least in part, by adding a novel vaccine component that promotes cross-reactive CD8⁺ T cells specific for conserved viral peptides, presented by widely distributed HLA types. Such “memory” cytotoxic T lymphocytes (CTLs) can rapidly be recalled to CTL effector status. Here, we review how B cells and follicular T cells are elicited following influenza vaccination and how they survive into a long-term memory. We describe how CD8⁺ CTL memory is established following influenza virus infection, and how a robust CTL recall response can lead to more rapid virus elimination by destroying virus-infected cells, and recovery. Exploiting long-term, cross-reactive CTL against the continuously evolving and unpredictable influenza viruses provides a possible mechanism for preventing a disastrous pandemic comparable to the 1918-1919 H1N1 “Spanish flu,” which killed more than 50 million people worldwide.

Keywords: T cells, B cells, influenza, immunological memory, vaccine

INTRODUCTION

Successful vaccination relies on the induction of long-term immunological memory. Exposure to an infectious virus elicits acute effector responses that mediate acute pathogen control, along with the generation and maintenance of T cell and B cell memory capable of protecting against re-exposure. At sufficient levels, neutralizing antibody (Ab) can prevent re-infection while, especially if such protection is partial, the rapid recall of memory CD8⁺ cytotoxic T lymphocytes (CTLs) facilitates enhanced pathogen control. Seasonal influenza results from the emergence of an occasional, highly infectious variant selected as a consequence of Ab-driven mutational change in the viral envelope hemagglutinin (HA) and/or neuraminidase (NA) proteins. Pandemic influenza A viruses, on the other hand, arise from gene reassortment of two different influenza A virus (IAV) subtypes infecting the same cells. As a consequence, the influenza research and control community face the continuing challenge of producing new vaccines to control emerging threats.

Most of the existing products utilize inactivated virus, or isolated viral HA and NA proteins, that stimulate influenza strain-specific antibody immunity and B cell memory, but do not prime the much more cross-reactive CD8⁺ CTL compartment. The challenge is thus to add a T cell-targeted vaccine component that promotes CTL memory for the rapid recall of anti-viral CTL effectors to the respiratory tract for early virus control and/or induce cross-protective B cells. In this review, we focus on the nature of optimal memory B cell and T cell generation and ask how we might use this knowledge to overcome the limitations of seasonal influenza vaccines by developing feasible strategies for both inducing and maintaining long-term, cross-reactive immunological memory.

The Burden of Seasonal Influenza

Seasonal influenza virus is a global health problem. In the United States, influenza virus infections causes 9.2–35.6 million cases of illness, 140,000–710,000 hospitalizations and 12,000–56,000 deaths per year (1). Globally, it is estimated that every year 290,000–650,000 respiratory deaths are due to seasonal influenza (2). The World Health Organisation (WHO) recommends annual influenza vaccination for people at high risk of developing severe disease, and for those in contact with high-risk individuals. Vulnerable groups include the elderly (>65 years), young children (6–59 months), Indigenous populations, patients with chronic medical conditions, pregnant women, and health-care workers (3). National health authorities in the countries with an advanced public health system recommend annual vaccination for everyone 6 months of age and above, both to protect individuals and to limit the spread of the virus through the community (4, 5).

Influenza Virus Evolution Poses a Challenge for Long-Term Humoral Immunity and Vaccine Effectiveness

Influenza viruses attach to host cells via HA binding to cell surface sialic acids (6, 7). Protective antibodies (Abs) block virus attachment by binding to the antigenic sites (8–11) proximate to

the sialic acid receptor binding pocket on the HA head. Such Abs are the best correlate for influenza immunization and are measured using the hemagglutination inhibition (HAI) assay, which detects Abs blocking the capacity of the virus to agglutinate red blood cells by binding to sialic acids on their surface (12). The influenza virus RNA polymerase lacks proof-reading function, with the consequence that there is a constant emergence of mutants (affecting viral fitness and/or immune recognition) carrying substitutions that arise randomly across the genome. Antibody-mediated immune pressure drives the selection of viruses expressing variant HAs and NAs (13, 14) that, if their “fitness” is not unduly compromised, have the potential in nature to cause the process that has long been called antigenic drift (15, 16). Clearly, for a drifted strain to emerge as a clinical problem, its HA must be sufficiently changed to escape neutralization by pre-existing antibodies induced broadly in human populations by past infections and/or vaccinations. The reality that individuals who were once protected are now at risk from the new variant strain is the basis for frequently reformulating seasonal influenza vaccines (17). In contrast, through the process of antigenic shift, influenza viruses incorporate a completely new HA or NA (18), which adds a new virus into the epidemiological mix. When it comes to antibody-mediated selection, the A/H3N2 strains have consistently shown the greatest antigenic drift for the three types of influenza viruses that co-circulate globally and cause seasonal epidemics (A/H1N1, A/H3N2, and influenza B viruses) (16, 19). In general, more extensive epidemics (with increased morbidity and mortality) occur when a novel, seasonal A/H3N2 drifted strain emerges (16, 20, 21).

Multi-component influenza vaccines are designed to elicit serum antibodies against the HAs of one A/H1N1 strain, one A/H3N2 strain and one (or two) influenza B viruses (Yamagata or Victoria) (22). Increased antibody titres induced by vaccination decrease the risk for infection caused by any strains antigenically similar to those included in the vaccine (23, 24), although they confer limited or no protection against other types or subtypes (including drifted variants) of influenza (25). The global WHO network closely monitors the circulation of influenza viruses in humans and other species, including birds, across the northern and southern hemispheres, whereby information derived from the antigenic and genetic characterization of these strains, along with epidemiological data, is used to select the strains to be incorporated into an upcoming seasonal vaccine (26). This strategy can fail, at least in part, as vaccine preparation takes at least 6 months and the product may no longer match all 3 (or 4) circulating viruses by the time it is released (27). Moreover, pre-existing immunity in humans can be highly variable due to age and prior exposures via infection and/or vaccination (28–34). The level of pre-existing human immunity is considered but often difficult to interpret due to high heterogeneity. First-infection ferret antisera is used to identify and characterize new influenza strains, yet repeated exposures to A/H3N2 variants affect Ab quantity and quality, which makes vaccine-strain selection even more challenging (35). Both immunological responses to influenza viruses and influenza vaccine effectiveness are undoubtedly affected by the combination of antigenic drift and prior immunity. Influenza virus evolution has been widely

studied, yet it is still largely unknown how cross-reactive B cell memory impacts on Ab responses to new strains.

B Cell Memory and Imprinting Against Prior Strains

The idea that immunological memory could impact negatively on Ab responses to novel influenza strains first emerged in the early 1950s, when Francis and Davenport observed that the exposure to a new influenza strain induced higher titres of Abs against variants encountered in childhood than against the prevailing strain (36–39). They proposed the colorfully named concept of “original antigenic sin” (OAS), which states that Abs generated against the first antigen (Ag) encountered in childhood would be repeatedly and preferentially induced at every exposure, even if the epitope remained as a minor secondary antigen. This was considered to be sinful, i.e., detrimental for protection against following influenza infections, since the Abs induced poorly neutralized the most recent strain that had actually triggered them (40, 41). Molecular level analyses of B cell receptor usage have since confirmed that memory B cells elicited by a priming Ag can participate in the immune response toward a structurally related, boosting Ag (42, 43). While it is clear that somatic mutation of the immunoglobulin (Ig) variable (V) region takes place, the extent to which this leads to increased affinity for the priming vs. boosting variant remains controversial (42). These molecular analyses are consistent with more recent observations that Ab boosting is broad, and greatest against more similar viruses, differing somewhat from the OAS concept that centers on the initial antigen encountered (44, 45). Efforts to understand why prior vaccination enhances vaccine effectiveness in some influenza seasons, yet attenuates it in others, has led to further refinements to the OAS hypothesis, namely that imprinted B cell memory responses are not inevitably “sinful” i.e., ineffectual (31). Hensley et al. propose that Ab become focused on selected epitopes which are relatively conserved between successive strains due to a form of competitive dominance by memory B cells and that while this may result in high Ab titres and clinical benefit it may, alternatively, compromise protection if the epitope is altered in future strains. This hypothesis is based on molecular and serological analyses that document focused HI Ab responses in selected individuals (29, 30, 46–49).

At the cellular level, it is clear that memory B cells respond more rapidly than their naïve precursors. Hence, antibody responses may become focused on epitopes that were present in earlier strains because memory B cells specific for those epitopes become rapidly activated at the expense of naïve B cells, which need a higher threshold to respond (50, 51). Memory B cells that bear affinity matured antigen receptors may also be better able to compete with existing Abs for inducing antigen than naïve B cells (52). Several strategies have the potential to promote naïve B cell activation and broaden the Ig response. These include giving repeated vaccine doses (39), increasing the amount and concentration of antigen (53), and adding adjuvants (54). Another suggested mechanism that may promote the enhanced engagement of memory (vs. naïve) B cells is that T regulatory cells (T_{regs}) induced by the initial encounter reduce the amount of

antigen presented on dendritic cells, thus diminishing the antigen availability for naïve B cells, promoting a memory B cell boost at the expense of naïve B precursors (55).

Current Strategies to Improve Seasonal Influenza Vaccine Effectiveness

Strategies to increase seasonal influenza vaccine effectiveness (VE), like high-dose or adjuvanted vaccines, are still under evaluation. Pooled analysis of multiple studies showed that high-dose vaccines significantly reduce the risk of laboratory-confirmed influenza cases in the elderly when the vaccine and the circulating strains are well-matched, but not when they are mismatched. The HAI geometric mean titres after vaccinating with the high-dose vaccine were significantly higher compared to the standard-dose vaccine for the H3 component. However, the proportion of participants with seroprotective HAI Ab levels (HAI titer $\geq 1:40$ or $1:32$) was the same using both vaccines (56). Similarly, high-dose vaccines showed significant increases in VE with a reduction in mortality among the elderly by 36.4% in the 2012–2013 season, when H3N2 viruses were predominantly circulating (57). Nonetheless, seasonal VE on that season was only of 11% for that particular age group (58), indicating that a high-dose vaccine, despite increasing VE, did not induce an epidemiologically significant improve in overall H3N2 VE. Alternatively, the use of a standard-dose influenza vaccine with the MF59 adjuvant (Novartis) can reduce laboratory-confirmed influenza cases as well as hospitalizations due to influenza in the elderly (59) and seasonal trivalent vaccines formulated with this adjuvant are now available for those >65 years old (FluAd, Sequrix).

In addition to MF59, other adjuvants licensed for use with inactivated or sub-unit-based influenza vaccines include Alum-containing formulations (AlPO_4 or $\text{Al}[\text{OH}]_3$) and oil-in-water emulsions, AS03 (GSK) and AF03 (Sanofi Pasteur). The benefits of using these adjuvants to increase seroprotective antibody titres are widely reported in a number of clinical studies, including in individuals who are most susceptible to influenza-related illness. Compared to non-adjuvanted vaccine responses, formulation of mono- and multi-valent influenza vaccines with MF59 induces substantially higher HAI titres and seroconversion rates in children (60–63) with similar improvements observed in the young and elderly using AS03 (64). These formulations are generally well-tolerated and safe, however, incidences of narcolepsy associated with the use of an AS03-adjuvanted A/H1N1pdm2009 vaccine (Pandemrix) limits the use of this adjuvant in the young. Nevertheless, both MF59 and AS03 have been shown to accelerate the induction of vaccine-mediated responses as demonstrated by the use of adjuvanted vaccines in healthy adults (65, 66), children (67) and in the elderly (68), wherein a single vaccination dose is sufficient to induce seroprotective levels of antibody within as little as 3 weeks. In this regard, these adjuvants, along with AF03 or Alum, provide dose-sparing capabilities for mass vaccination of the wider population; similar levels of protection attained with unadjuvanted vaccines can be achieved with using substantially smaller amounts of HA antigen or less vaccination doses when formulated with adjuvant

(69–72). Several studies have also demonstrated the ability of MF59 to induce cross-reactive antibodies against non-vaccine matched strains in prime-boost regimens. Priming of subjects with a clade 0 H5N3 vaccine formulated with MF59 followed by a boost with a clade 1 H5N1 vaccine containing the same adjuvant results in high titres of cross-neutralizing antibody against H5N1 clade 0, 1 and 2 viruses (73–75). These results thus highlight the role that adjuvants can play in generating and broadening the cross-specificity of naïve and pre-existing B cell memory, the possible underlying mechanisms of which are discussed further in subsequent sections below.

Influenza vaccines designed to target Abs toward the conserved epitopes in the HA stem are also under intense study. While heterosubtypic protection with group 1 HA stem vaccines (i.e., H1 and H5 viruses) lacking the highly variable HA head has been demonstrated in animal models (76), studies on group 2 HA stem vaccines (i.e., H3 and H7 viruses) are more limited. Although promising results are observed when immunizing mice with conserved HA stem epitopes from the H3 subtype, by way of cross-clade neutralizing activity (77, 78), immunogenicity and protection are not maintained when using larger animal models like ferrets (78). Therefore, further studies are needed to develop a human B cell-based universal influenza vaccine, with consideration into the potential for influenza viruses to escape from HA-stem targeted Abs (79).

Dissecting the B Cell Response

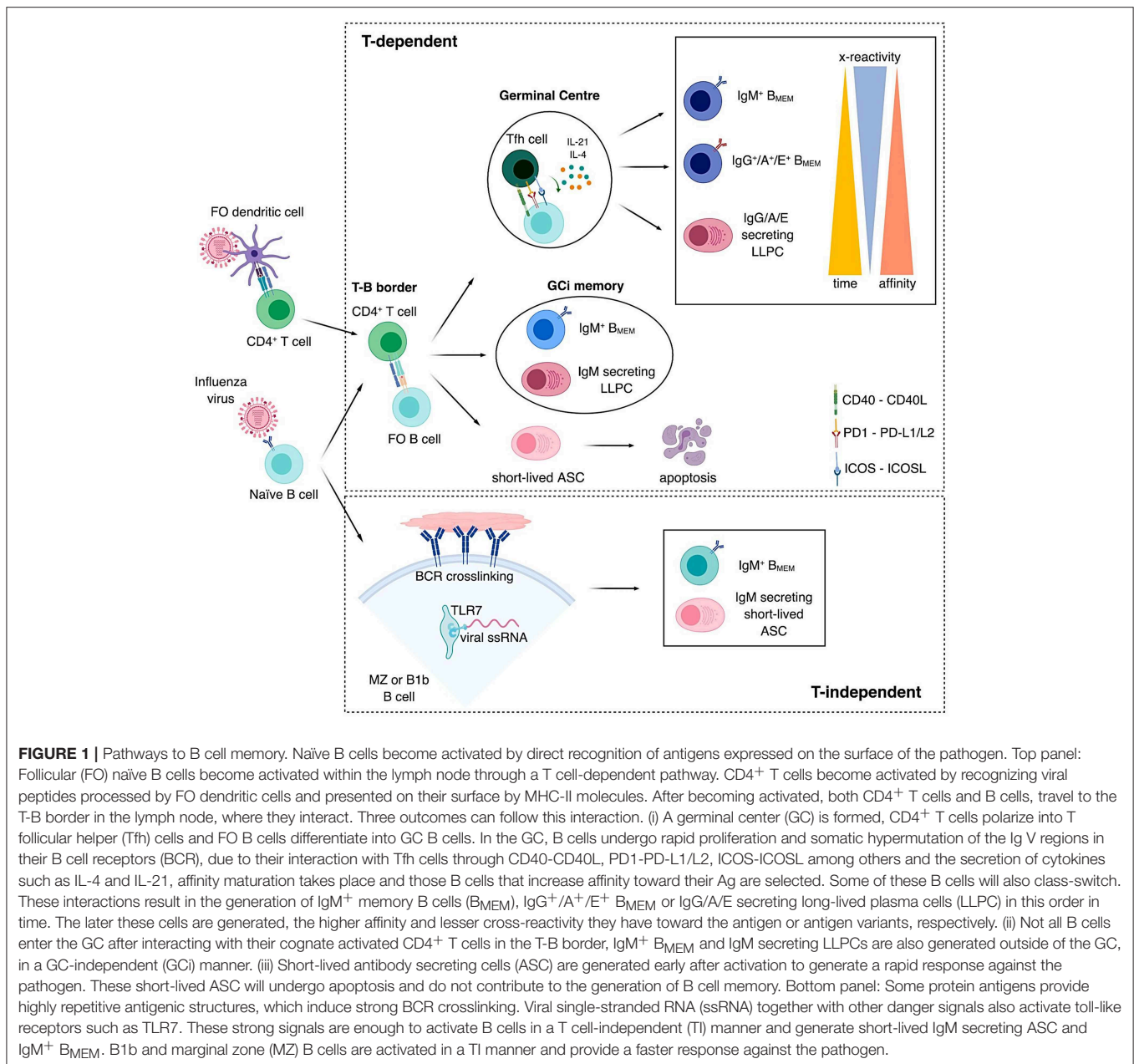
Activation of naïve B cells can elicit short-lived ASCs (also called plasmablasts), long-lived antibody-secreting plasma cells (LLPCs), and memory B cells. The fate of B cells is considered to be highly orchestrated, depending on the mode of stimulation, the affinity of their B cell receptors (BCR, or surface Ig) for antigen and their location (80–82). In the periphery, within secondary lymphoid organs (SLO), naïve B cells are activated by BCR/Ag binding and, depending on whether T cell help is provided, they will continue the response in a T cell-dependent (TD) or T cell-independent (TI) manner. B cell memory resulting from a TI response expresses and produces IgM capable of engaging at broadly low affinity with antigens via multivalent BCR engagement, plus toll-like receptor (TLR), and/or complement engagement (83). In TD responses, B and T cell interaction occurs when antigen is captured through the BCR of specific naïve B cells and presented via cell-surface MHC-II glycoproteins to CD4⁺ helper T cells specific for peptides from the same antigen (84, 85). All B cells activated in this manner either move into lymph node follicles and generate germinal centers (GCs) or differentiate into extrafollicular plasmablasts (86, 87). Through this array of processes, different classes of memory B cells are generated, which can be distinguished by their passage through the GC, location and Ig isotype (81).

In the GC, B cells undergo intense proliferation and broaden their BCR diversity through somatic hypermutation, a process whereby point mutations, insertions, and deletions are introduced within Ig V gene hotspots to generate a broad array of B cell clones with a broad spectrum of affinities for the immunizing Ag (88). This process results in the generation of memory B cells with high-affinity surface Igs and surface

Ig⁺/– plasma cells that maintain serum immunoglobulin levels against the foreign invader. The GC is also the site where a large proportion of BCR-defined clones undergo class switch recombination (CSR), exchanging the Ig isotypes originally expressed (IgM and IgD) for IgG, IgA, or IgE (88–90). The sequential generation of long-lived memory B cells in the GC starts from unswitched memory B cells, followed by class-switched memory B cells and, finally, by LLPC that travel to the bone marrow and other sites (91). The later each B cell population appears, the higher its affinity for Ag (92). Hence, B cells with lower affinity BCRs have a greater propensity to enter, and persist in, the memory pool. Intriguingly, such memory-directed B cells show enhanced Bach2 transcription factor expression when compared to their counterparts with higher BCR affinity, and Bach2 expression inversely correlates with the strength of the B-T follicular helper (Tfh) cell interaction. This suggests that B cells with lower affinity receive weaker T cell help and express higher levels of Bach2, which is clearly a key factor in memory B cell fate determination (92). In addition, expression levels of Blimp-1, the key regulator of plasma cell differentiation and CSR, are regulated by Bach2. Higher Bach2 levels decrease Blimp-1, promoting B cell differentiation toward an unswitched memory fate. The aryl hydrocarbon receptor (AhR), a ligand-induced nuclear transcription factor, is highly induced in B cells upon BCR engagement. AhR promotes Bach2 expression, which in turn suppresses Blimp-1 and therefore the B-Tfh cell interaction becomes weaker and B cell CSR and differentiation into plasma cells are suppressed (93), indicating that it may be a potential target in promoting the generation of low-affinity IgM⁺ B cell memory upon vaccination. This is of particular relevance for the design of the next generation influenza vaccines since, as discussed below, as there is an increasing body of evidence suggesting that low-affinity IgM⁺ memory B cells capable of identifying a broad range of epitopes should be targeted by influenza vaccination.

Heterogenous Memory B Cell Phenotypes Have Different Roles in Secondary Responses

The various modes of TD and TI B cell activation generate memory B cells with varying isotypes and affinities (summarized in **Figure 1**), some bearing highly mutated Igs generated via the GC reaction and others maintaining germline, less specific and more cross-reactive Abs (52, 81, 94). While it is generally accepted that memory B cells show an enhanced capacity for terminal differentiation into ASC, regardless of phenotype and affinity, there is less consensus regarding their propensity to (re)-enter GC reactions. Contrary to early thinking, it is now generally accepted that both IgG⁺ and IgM⁺ memory B cells can re-enter GC reactions, albeit they are more predisposed to differentiate into ASC during recall responses (52, 95–97). Similarly, whether or not GCs form during recall responses together with the character of the memory B cell subsets that participate may depend on the type and amount of antigen, inflammatory signals and the availability and quality of cognate Tfh cells (98). There is evidence that unswitched memory B cells bearing germline BCRs have a greater propensity to enter the GC reaction (99). In particular, IgM⁺ cells with the least mutated V genes were



more prominent within GCs during the recall response to a variant viral protein antigen rather than to the original inducing antigen when sequentially immunizing mice with variant Dengue envelope proteins with 63% amino acid identity (100). However, when using HAs from more closely related influenza viruses, with ~82% sequence identity, the GC response was dominated by highly mutated memory B cells, which led to a worsened antibody response as compared to the primary encounters, even in the presence of an adjuvant (101). In the elderly, a poor adaptive capacity of B cells toward the drifted influenza epitopes has also been demonstrated. This resulted in the expansion of B cell memory targeting mostly conserved but less potent epitopes (102). In contrast, memory B cell expansion after H3N2 infection reflected imprinting toward strains encountered early

in life but also adaptation to the infecting virus (103). These studies suggest that a certain degree of an antigenic difference is needed to induce a protective secondary antibody response by stimulating broadly cross-reactive low-affinity IgM⁺ memory B cells. High-dose and adjuvanted vaccines may improve VE when influenza vaccine strains are antigenically-different. The propensity for IgM⁺ memory B cells to dominate recall GC responses may be further determined by pre-existing antibodies that may outcompete the BCRs from low affinity naïve and IgM⁺ memory B cells, but not high affinity IgG⁺ memory B cells, for antigen (52, 96, 104).

In consideration of the potential for influenza Ab responses to become focused on epitopes present in successive vaccine strains to the detriment of recognizing future variants, it seems

appropriate to think in terms of future vaccines that maintain plasticity and heterogeneity within the B cell response. For example, vaccination strategies that recall IgM⁺ memory B cells with less-mutated BCR repertoires, while also inducing naïve populations together with cognate Tfh cell memory to facilitate memory GC formation (98), may tend to skew the overall response toward the generation of more cross-reactive Abs against variant epitopes.

T Cell-Independent B Cell Responses Against Influenza

In contrast to TD Ags, which are generally proteins that cannot induce cross-linking of multiple BCRs, TI Ags are generally multivalent polysaccharides or other molecules that contain a repetitive array of antigenic epitopes that have that BCR-polymerization propensity. This paradigm is, however, challenged by the finding that high doses of a monomeric protein Ag can also elicit an exclusive TI B cell response (105, 106). In mouse experiments, both TD and TI B cells give rise to short-lived plasma cells and memory B cells (107–109) and contribute not only to resolving primary influenza virus infection, but also to more effective control of virus replication and symptoms after secondary challenge (110). The recall capacity of TI memory B cells is largely a result of Ag driven clonal expansion, however, like other memory B cells, TI memory B cells are able to respond more readily to Ag than their naïve counterparts.

The capacity of inactivated whole (vs. split) virion vaccines to induce superior influenza virus-specific antibody responses (111–113) may in part be due to the greater induction of TI B cell responses (114). Notably, when TI B cell responses were induced Ab affinity and neutralizing activity was enhanced. The ability of inactivated whole virions to induce TI B cell responses is linked to the presence of single-stranded RNAs that activate B cells via a TLR7-dependent mechanism (114), hence TLR7/8 agonists should be considered as potential adjuvants for seasonal influenza vaccines.

Importance of Location for Influenza-Specific Memory B Cells

Unlike LLPCs, memory B cells persist as tissue-resident or circulating among the SLO (115). Memory B cells resulting from a local infection also localize in the affected organs. This occurs following influenza virus infection when influenza-specific memory B cells can be found, not only in lymphoid organs, but also in the lungs. Moreover, memory B cells are also differentially distributed among the lymphoid tissues, indicating that trafficking is influenced by local tissue factors (116, 117). After influenza re-exposure, lung-resident memory B cells differentiate into plasmablasts, providing IgG and IgA *in situ* that quickly neutralizes the virus (117, 118). In general, IgA⁺ memory B cells seem to localize preferentially to the blood and to tissue sites of pathology, while IgG⁺ memory B cells are broadly distributed among tissues that may, or may not, be directly involved in the disease process (116, 117). B cell memory and secreted IgA located in the lungs are essential to provide a quick and effective response against influenza viruses upon exposure, yet current influenza vaccines fail to strongly boost

IgA responses (119). Antigen reaching the mucosa of the lung is required to potentially induce stronger IgA responses and for the generation of lung-resident memory B cells, which establish early after infection. The varied location of memory B cells according to their isotype, together with the fact that different environments drive B cell class-switching to a specific isotype, are of particular interest for vaccine design, particularly where (as in influenza) mucosal surfaces are the primary site of infection.

T Follicular Helper Cell Memory: Recent Advances in Influenza Vaccination

When the GC contracts, the GC Tfh cells exit and develop into resting memory Tfh cells with a less polarized Tfh phenotype (120–125). Tfh cells with a resting memory phenotype both recirculate in blood and can be found in BM, spleen, and lymph nodes (126–128). Circulating Tfh (cTfh) cells are the most accessible subset in humans. Of increasing research interest, cTfh cells are heterogeneous and can be classified into different subsets based on surface marker expression. Resting cTfh cells express CCR7, which differentiates them from their GC counterparts. When cTfh cells become stimulated, they downregulate CCR7 to traffic to the GC (129). Three different subsets of cTfh cells can be distinguished according to the surface expression of the chemokine receptors CXCR3 and CCR6, which are involved in inflammatory-homing and epithelial and mucosal site-homing, respectively (130, 131). The Tfh1 cells are CXCR3⁺ CCR6[−], express the T-bet transcription factor and secrete the Th1 cytokine IFN γ . Conversely, the CXCR3[−] CCR6[−] Tfh2 set expresses the transcription factor GATA3 and produces the Th2 cytokines IL-4, IL-5, and IL-13. Then the Tfh17 cells CXCR3[−] CCR6⁺ cells express the transcription factor ROR γ T and secrete the Th17 cytokines IL-17A and IL-22 (132).

An overall consensus on the functional implications of the different Tfh subsets regarding B cell help is yet to emerge. While the Tfh1 cells are thought not to be efficient B cell helpers, the opposite is true for the Tfh2 and Tfh17 populations (132, 133). However, human studies on the cTfh response following influenza vaccination demonstrate an increase of circulating, activated cTfh1 cells peaking on day 7 after vaccination that positively correlates with the generation of protective Ab responses and the presence of ASCs in blood (115, 134). In the context of influenza immunization, when culturing human cTfh1 cells isolated at day 7 after priming with either naïve or memory B cells, the cTfh1 cells stimulate memory B cell differentiation into plasmablasts, while naïve B cells remain resting. Yet, naïve B cells cultured with Tfh2 and Tfh17 cells can differentiate into plasmablasts (134). Because Tfh cells are essential to induce a proper B cell response and we speculate that naïve B cells are not being sufficiently stimulated due to epitope masking by pre-existing Abs and memory B cells, it could be possible that mainly Tfh1 cells are stimulated after influenza vaccination at the expense of Tfh2 and Tfh17.

Anti-viral CD8⁺ T Cell Responses

Seasonal influenza vaccines are designed to elicit an Ab response. However, the natural influenza virus infection additionally elicits cellular immunity (CD8⁺ T cells, CD4⁺ T cells, MAIT cells,

NK cells) to eliminate the infection. Because influenza viruses are under constant selective pressure, the long-term protective value of any vaccine that targets a specific HA and/or NA will inevitably be compromised with time, immune CD8⁺ T cells are critical for recovery and provide some protection against severe influenza disease, including that resulting from infection with a previously unencountered avian strain. This likely reflects that influenza-specific CD8⁺ T cells tend to recognize HLA-bound peptides derived from more conserved, internal virus proteins. The question is whether vaccines that promote such CD8⁺ T cell memory can, when combined with the classical products that induce virus-specific Ig response, provide better protection against, in particular, a newly invasive pandemic strain. An overview comparison between B and T cell responses after influenza virus drift and shift and how they complement each other is shown in **Table 1**.

Adaptive T cell immunity is mediated primarily by T cells, expressing the CD4 or CD8 co-receptors, respectively. During influenza virus infection, viral proteins are degraded by the proteasome and processed into smaller peptide fragments. These fragments are bound to MHC molecules and carried to the cell surface for presentation. These peptide/MHC complexes (pMHC) are recognized by clonally expressed TCRs on CD4⁺ or CD8⁺ T cells, leading to their activation and recruitment into the virus-specific immune response. The CD8⁺ cytotoxic T lymphocytes act as sentinels, recognizing and killing virus-infected targets, an essential step for virus clearance. Following activation, CD8⁺ T cells also secrete anti-viral cytokines (especially IL-2, IFN- γ , and TNF- α) which further recruit innate and adaptive immune cells into sites of influenza virus-induced pathology and induce anti-viral responses in infected cells (141, 142). When it comes to CTL killing, the secretion of perforin, granzymes and FAS ligand can all be involved in the process of inducing the apoptosis of virus-infected cells (143, 144). Additionally, the expression of TRAIL on CTLs can lead to the elimination of influenza virus infected cells, with a resultant decrease in mortality (145).

T Cell Fate: to Die or Become Memory

Formation of memory CD8⁺ T cells is essential for the protection against re-encountered pathogens. Our understanding of key factors determining the fate of CD8⁺ T cells during influenza is still limited but crucial for the development of a CD8⁺ T cell activating vaccine. During differentiation from naïve to effector, to memory status, CD8⁺ T cells transiently express cell surface molecules that are considered to be predictive of cellular fate and function. Surface expression of IL-7R and KLGR1 on effector CD8⁺ T cells can, at least in some situations, differentiate between CD8⁺ T cells designated as memory precursor effector cells and short-lived effector cells (146). Compared to the IL-7R^{lo}KLGR1^{hi} set, CD8⁺ T cells expressing high levels of IL-7R and low levels of KLGR1 are 10-fold more likely to survive (147) in mice infected with lymphocytic choriomeningitis virus (LCMV). However, it should be noted that these profiles may not be exclusive, as KLGR1⁺ CD8⁺ T cells are detectable after LCMV infection is cleared (148), and the survival value associated with the IL-7R^{hi}KLGR1^{lo} set for LCMV is less obvious for influenza

virus infection (149). Additionally, the discovery of other early markers of memory formation during *Listeria monocytogenes* and vesicular stomatitis virus infection, including expression of ID3 transcription factor (150) and IL-2R α cytokine receptor, showed that CD8⁺ T cell memory generation is certainly multi-factorial (151, 152). Identifying markers of successful memory formation is crucial for evaluation of novel influenza vaccine responses and should be considered in future influenza vaccine studies. More recently, high-throughput sequencing is facilitating the emergence of a broader picture for CD8⁺ T cell differentiation. Single-cell RNAseq of CD8⁺ T cells at the acute phase of LCMV infection indicates that there may be two distinct populations of antigen-induced CD8⁺ T cells that share genes either with “terminal effector” or “memory” cells (153). Compared to naïve CD8⁺ T cells, the “terminal effector-like” set can be shown to have upregulated more than 900 different genes, while the “memory-like” cells only upregulated 27 genes (153). This suggests that the differentiation of “terminal effector” CD8⁺ T cells mandates the upregulation of hundreds of genes involved in both clonal expansion and the mediation of a spectrum of effector functions, while the establishment of CD8⁺ T cell memory requires only the involvement of a few key genes to maintain lymphocyte quiescence. Although the exact factors mediating distinct CD8⁺ T cell fates during early division following viral infection are still in the process of elucidation, experiments with TCR-transgenic mouse models indicate that TCR signaling strength (154), as reflected in IL-2R, IFN- γ R, and mTOR levels during mitosis and asymmetrical division (155–157) is key to the generation of anti-viral CD8⁺ T cell memory. This is an exciting area of research that should, as it unfolds, give a much better understanding of both the molecular basis of CTL memory formation, and provide key measurement parameters that will allow us to skew early vaccine responses so that they provide optimal memory that gives long-lasting protection when recalled by further pathogen challenge.

Importance of Generating Long-Term T Cell Memory

As mentioned above, memory CD8⁺ T cells are important for eliciting long-term, broadly cross-reactive immunity to influenza viruses, and are thought to mediate the protective function mainly via the killing of virus-infected targets (158). Virus-specific CD8⁺ effector T cells also produce proinflammatory cytokines, and the breadth of cytokine production (termed polyfunctionality) often correlates with efficient protection against pathogens, including influenza viruses (159). Polyfunctional memory CD8⁺ T cells (producing IFN- γ , TNF, IL-2, and MIP-1 β) (160) are thought to operate via augmented cytolytic activity via dual IFN- γ /TNF expression (161), IL-2-mediated enhancement of CD8⁺ T cell memory function (162) and increased IFN- γ secretion on a per cell basis (163). One example of the protective capacity of these polyfunctional memory CD8⁺ T cells is the induction of long-lasting memory CD8⁺ T cells against variola (smallpox) virus induced by the Vaccinia vaccine Ankara (164). When CD8⁺ T cells were primed with influenza virus nucleoprotein (NP) expressed by either a recombinant Vaccinia virus or in *Listeria monocytogenes*, the more polyfunctional NP-specific CD8⁺ T

TABLE 1 | The clinical outcome and the B and T cell memory responses after exposure to influenza viruses are summarized below.

	Influenza antigenic site change			
	Antigenic drift <i>Genetic changes in Ag sites alter Ab binding</i>			Antigenic shift
	None	Minimal	Major	Exchange of surface Glycoproteins
Clinical outcome	Little to no symptoms		Unpredictable (135)	Dependent of CD8 ⁺ T cell response - Limited by HLA alleles (136, 197) - Prior exposure to influenza (137–139) T cell memory pool and quality of T cell response (137–139) ⇒ Severe to fatal outcome with prolonged hospitalizations (137)
B cell response	Robust memory B cell response and protective Ab production (135)	Dominated by memory B cells against preserved antigenic sites, yielding a protective but focused Ab response that may not protect against future drift.	Cross-reactive memory B cells produce an early unadapted Ab response to limit virus replication and symptoms, and enter GC reactions to generate updated memory and PCs If enough Ag available, naïve B cells react and generate updated B cell memory	Very limited (if any) protection by memory B cells (31, 140) Response driven by naïve B cells
CD8 ⁺ T cell response	Cross-reactive Not responsive if B cells neutralize the virus			Cross-reactive but not neutralizing immunity Host-specific differences

cells were generated following Vaccinia virus exposure. Mice vaccinated with the Vaccinia virus showed also a greater level of protection against a normally lethal IAV challenge compared to the *Listeria monocytogenes* vaccine group counterparts (165). This indicates, that not only the quantity of memory CD8⁺ T cells is critical for the protection but also their quality. Insights into key factors inducing these polyfunctional CD8⁺ T cells could improve a T cell-based vaccine therefore vastly.

Memory CD8⁺ T cells can be divided conceptually into central and effector T cell memory sets, based on their expression profiles for the CD62L and CCR7 surface proteins (166) that are known to affect cell localization and function (167). The CD62L^{hi}CCR7^{hi} “central memory” CD8⁺ T cells (T_{CM}) can be found in the spleen, blood and lymph nodes, and display superior functions compared to their CD62L^{lo}CCR7^{lo} effector memory CD8⁺ T cell (T_{EM}) counterparts, mainly in terms of their proliferative capacity and IL-2 production profiles (168). In addition, a highly specialized population of tissue-resident (T_{RM}) memory CD8⁺ T cells expressing CD103⁺CD69⁺ can persist in sites of pathology subsequent to virus clearance (169). Following the secondary challenge, CD103⁺CD69⁺ T_{RM} set is able to expand and secrete cytokines, including IFN-γ and TNF, as well as generate more polyfunctional progeny (69% of cells capable of secreting three cytokines), when compared to CD103[−]CD69⁺ (21%) and CD103[−]CD69[−] (16%) parent subsets (160, 169). In the context of influenza, persistence of influenza-specific

CD8⁺ T_{RM}s correlates strongly with protection when mice are challenged with a serologically distinct IAV that shares common internal proteins (170). The T_{RM} population develops from precursors lacking KLRG1 (171, 172) and further studies on T cell receptor (TCR) repertoires suggest that they arise from the same naïve pool as T_{CM} set (173). T_{RM} generation is largely regulated by a series of transcription factors (174), such as Runx3 which is crucial for T_{RM} establishment across a range of tissues (175), and Bach2 which is recognized to restrain the terminal differentiation of effector T cells and help with formation of long-term memory T cells (176). The differentiated T_{RM} phenotype is associated with changes in key transcription factors, including downregulation of Kruppel-like factor 2 (KLF2), TCF1 (177), T-bet, and Eomes (178, 179) as well as upregulation of Hobit, Blimp1 (177) and AhR (180), Nur77 (181), and Notch (182), required for the maintenance of T_{RM}s. While the previously named transcription factors are universal hallmarks of T_{RM} formation, T_{RM} heterogeneity among cells generated at different tissue sites suggest that microenvironmental cues are important for site-specific T_{RM} differentiation. Indeed, generation of the lung T_{RM} set is influenced by transforming growth factor β (TGF-β) along with the presence of IFN-γ-secreting CD4⁺ T cells following influenza virus infection (183, 184). While the generation of influenza-specific T_{RM}s has recently been shown to be vital for robust protection, unlike T_{RM}s generated within the skin or gut (185–187), lung-resident T_{RM}s do not offer long-term

protection, rather they require a constant supply of circulating T_{EM} s cells to replenish the niche over time (188) (summarized in **Figure 2**). In humans, influenza-specific lung-resident T_{RM} cells show a high degree of TCR-sharing with other influenza-specific lung T_{EM} cells, suggesting that both memory cell subsets originate from the same precursors (160). Our understanding of the protective role of memory $CD8^+$ T cells in influenza virus infection also comes from experiments with a C57BL/6 mouse model lacking antibodies, where increased numbers of influenza-specific memory $CD8^+$ T cells and T_{RM} cells led to markedly reduced influenza-induced morbidity (189). Similarly, primary vaccination with a single-cycle, non-replicative H3N2 IAV induced $CD8^+$ T cells capable of protecting against a heterologous (H1N1) lethal challenge (190), an effect that was diminished for mice that had been depleted of $CD8^+$ T cells after vaccination. These studies highlight the potential of long-term memory $CD8^+$ T cells protecting against severe influenza virus infections. A potential that is not harnessed in the current vaccine strategy.

$CD8^+$ T Cells Recognize Highly Conserved Influenza Epitopes

$CD8^+$ T cells can confer broad cross-protection across different seasonal, pandemic and avian influenza IAV strains due to their ability to recognize relatively conserved viral peptides derived from internal influenza components (NP, M1 and PB1, PB2).

The best defined human $CD8^+$ T cell influenza epitope is the immunodominant M1_{58–66} peptide bound to the HLA-A*02:01 molecule (191–193). This peptide is highly conserved within different influenza A subtypes spanning 100+ years (136), including the 1918 and 2009 pandemic H1N1 strains as well as highly pathogenic H5N1 avian viruses (194). Analysis of immunogenic peptide profiles for the avian H7N9 influenza virus established that it shared six universal $CD8^+$ T cell epitopes conserved at ~100% prevalence in human influenza A viruses circulating since the catastrophic Spanish 1918 influenza. These universal human influenza-specific $CD8^+$ T cells epitopes include HLA-A*02:01/M1_{58–66}, HLA-A*03:01/NP_{265–273}, HLA-B*08:01/NP_{225–233}, HLA-B*18:01/NP_{219–226}, HLA-B*27:05/NP_{383–391} (although mutants were found in some H3N2 strains) and HLA-B*57:01/NP_{199–207} (136). The population coverage by the universal HLAs varies greatly across ethnicities. Fifty-six percent of Caucasians displaying at least one universal HLA, while such coverage reached only 16% in the Alaskan and Australian Indigenous populations (136), highlighting the vulnerability of Indigenous populations toward newly-emerged influenza viruses. Additionally, our recent studies found broadly cross-reactive $CD8^+$ T cell responses directed toward the HLA-B37-restricted NP₃₃₈ epitope across IAVs (195), and excitingly, for the HLA-A*02:01-restricted PB1-derived epitope across influenza A, B and C viruses (196). The latter introduces a new paradigm

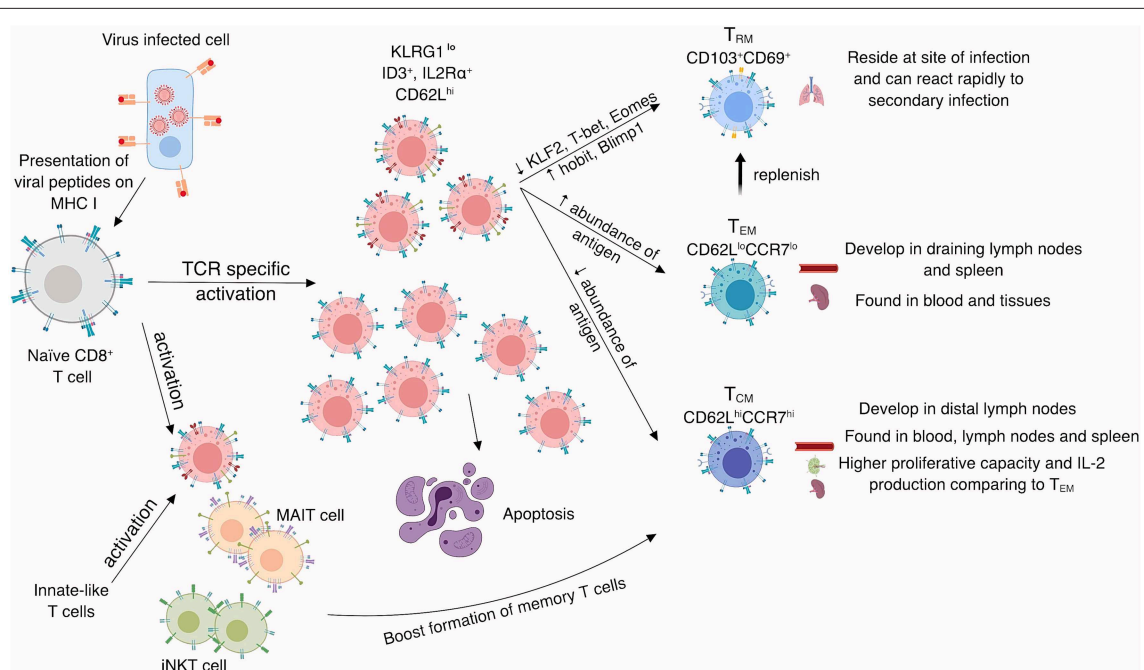


FIGURE 2 | $CD8^+$ T cell memory formation. Naïve $CD8^+$ T cells become activated by recognition of viral peptides presented in the context of MHC-I molecules on the surface of virally-infected APCs. Activated $CD8^+$ T cells divide and differentiate into effector $CD8^+$ T cells, which kill virus-infected cells and secrete cytokines to induce an anti-viral milieu. After viral clearance, mainly $KLRG1^{lo}$, $ID3^+$, $IL2R\alpha^+$, and $CD62L^{hi}$ $CD8^+$ T cells develop into $CD8^+$ memory T cells, while the remaining ~90–95% of $CD8^+$ T cells undergo apoptosis. Memory formation can be augmented by innate-like T cells (iNKT and MAIT cells). Memory $CD8^+$ T cells are divided based on surface marker expression, known to impact their localization. While T_{CM} and T_{EM} can be found in blood and tissues, T_{RM} reside at the site of infection where they can rapidly respond towards a secondary infection. T_{CM} can also be found in lymph nodes and display higher proliferative capacity and IL-2 production compared to their T_{EM} counterparts.

whereby CD8⁺ T cells can potentially confer a measure of previously unrecognized cross-reactivity across all human influenza A, B and C viruses, a key finding for the design of universal vaccines.

Influenza-induced morbidity and mortality can correlate with the expression of certain HLAs, including HLA-A*24:02, A*68:01 or B*39:01 alleles, as shown during the 2009 H1N1 pandemic (197). Analysis of peptide scores demonstrated that HLA-A*24:02 is more likely to bind variable (rather than conserved) viral regions (197). Similarly, we have previously shown that some HLA alleles, including HLA-A*24:02 and A*68:01, are less able to elicit robust immune responses toward the highly conserved NP and M1 peptides (136). Both HLA-A*24:02 and A*68:01, in particular, are found at higher frequencies for Indigenous populations world-wide (136, 197), which may explain the disproportionate impact of pandemic influenza viruses on Indigenous peoples during both (otherwise mild) 2009 pH1N1 pandemic and 1918–1919 (H1N1) Spanish “flu catastrophe” (198–202).

Thus, given the broad potential for cross-protective capacity mediated by CD8⁺ T cells, along with more recent evidence that this effect may indeed be operating in nature to protect people, this aspect of immunity is of considerable interest in terms of developing improved influenza vaccines. However, it is important to note that designing peptide-based T cell vaccines that only cover the major HLA types would clearly be disadvantageous for Indigenous populations globally (203). Further research on CD8⁺ T cell epitopes found in high risk populations is therefore of highest importance to protect people of highest vulnerability.

CD8⁺ T Cells Can Confer Broad Cross-Protection for Heterologous IAV Strains

In the context of newly emerging influenza virus infections in people, correlative studies suggest that established CD8⁺ T cell memory confers cross-reactive immunity against severe influenza disease, as observed during the 2009 pandemic H1N1 (pH1N1) outbreak (139, 204). The high (~70%) conservation of CD8⁺ and CD4⁺ T cell epitopes contributing to pre-existing memory may have been a significant factor in the generally mild outcomes of the 2009 H1N1 pandemic (138). Sridhar et al. showed that individuals with higher numbers of CD8⁺ T cells recognizing conserved influenza epitopes fared better following natural infection with the 2009 H1N1 virus (139). The importance of CD8⁺ T cell-mediated immunity was further highlighted in 2013 following the emergence of the novel avian H7N9 strain (205, 206), which killed ~40% of the infected patients. In H7N9-infected individuals, rapid recovery from hospitalization was associated with the presence of significantly more IFN- γ -secreting CD8⁺ T cells when compared to the situation for those who died (207) and recovered (206).

Development of CTL-Based Vaccines Lessons Learned From the Yellow Fever Vaccine

While the initial experience of IAV infection generally occurs in the first 6 years of life (208), our understanding of both the primary IAV-specific CD8⁺ CTL response and the transition

to influenza-specific T cell memory is very limited for humans. Though one paper by Mbawuike et al. reported on primary infection in infants as early as 6 to 13 months of age (209), studies of such influenza exposures in infants are rare, and have not been performed using contemporary approaches for the analysis of T cell-mediated immunity. The closest we have for humans of any age when it comes to the formation of memory CD8⁺ T cells following first virus encounter is for the live-attenuated 17D yellow fever (YF) vaccine. As might be expected from a plethora of mouse experiments, recent YF vaccination studies showed that deuterium-labeled, epitope-specific CD8⁺ CTLs expanded initially following vaccination, before undergoing a contraction phase characteristic of CD8⁺ T cell memory. These vaccine-induced YF-specific memory CD8⁺ T cells persisted in the blood for at least 2 years after YF vaccination, with an average deuterium half-life decay rate of 493 days (210). A similar YF vaccination study in mice demonstrated that, after initial contraction, the long-lived CD8⁺ T cell memory pool remained consistent in size (211), indicating a potential advantage of a CD8⁺ T cell that would need fewer revaccinations compared to the annual recommendation necessary for the seasonal influenza vaccine. Unfortunately for influenza vaccination, the current IIV used in humans does not induce any CD8⁺ T cell responses that can be targeted for such a longevity analysis (115).

Vaccination Approaches to Induce Memory CD8⁺ T Cells

Different influenza vaccination approaches are currently being investigated in order to induce long-lasting cross-protective immunity. The only licensed vaccines capable of inducing CD8⁺ T cell immunity, such as the YF vaccine, use live-attenuated pathogens. These are not recommended for influenza “high-risk” groups such as pregnant women, immunosuppressed individuals and the elderly. Therefore, new vaccination strategies need to be developed if we are to protect such vulnerable people. Vogt et al. showed that changing the route of vaccine administration of a quadrivalent inactivated influenza vaccine from intramuscular (i.m.) to transcutaneous induces the expansion of vaccine component-reactive CD8⁺ T cells. Interestingly, the vaccine was also able to induce M1_{58–66}-specific responses in a HLA-A*02:01-positive donor, although this was only observed in one individual (212). Another approach currently in development is the *Flu-v* CD8⁺ T cell-activating vaccine (213) containing four 21–35 amino acid-long peptides from internal influenza proteins, which can potentially bind to multiple HLA allelic forms, including the highly prominent HLA-A*02:01. This approach was protective for HLA-A2 transgenic mice and was also capable of inducing IFN- γ -expressing CD8⁺ T cells across all the participants ($n = 15$) in a phase 1b vaccine trial (213, 214). The *Flu-v* product showed that the vaccine reduces both the viral titer and the symptom score after H3N2 virus challenge in humans (215). However, due to the unknown HLA-restriction of the immunogenic epitopes, the HLA coverage of this vaccine is still to be determined. To circumvent the need for prior knowledge of HLA-restricted epitopes to be included in a universal T cell-based vaccine, particularly for less common HLA allelic variants, full-length influenza proteins have been expressed in Vaccinia virus

Ankara vaccine vectors. Berthoud et al. showed that a viral vector encoding for the two internal proteins NP and M1 could induce some CD8⁺ T cell responses (216).

Overall, development of an effective, long-lasting, cross-reactive influenza vaccine relies on an individuals' capacity to generate polyfunctional lung-resident CD8⁺ T cells. However, difficulties in identifying cross-reactive epitopes caused a bottleneck in the development of a universal influenza vaccine. Due to the propensity of IAV to trigger severe outbreaks with pandemic potential, murine models have thus far been developed to test the effectiveness of IAV vaccines based on conserved internal proteins (217–219). While mice immunized with these vaccines can elicit protective CD8⁺ T cell responses, the molecular mechanisms which govern formation of protective memory responses still require further validation in mice, and ultimately in humans.

Innate and Bystander T Cell Activation During Influenza Virus Infection

In addition to the activation and proliferation of CD8⁺ T cells in a peptide-MHC dependent manner, T cells can also become activated via antigen-independent mechanisms, resulting in proliferation of polyclonal T cells (220). In an influenza mouse model, adoptive transfer of TCR-transgenic OT-I CD8⁺ T cells, which recognizes the ovalbumin peptide, into influenza-infected mice, showed that these OT-I cells can non-specifically expand in the lungs of influenza-infected mice. This suggests that CD8⁺ T cells can become activated independently of their TCRs during primary influenza virus infection (221). Similarly, highly activated CD38⁺HLA-DR⁺ CD8⁺ T cells, numerically greatly exceeding influenza-specific CD8⁺ T cell pools, were found in patients hospitalized with severe H7N9 disease (137), suggesting bystander activation of at least some CD38⁺HLA-DR⁺ CD8⁺ T cells. Despite the evidence that bystander CD8⁺ T cell activation occurs during influenza virus infection, the importance of these cells in terms of viral clearance and the induction of long-term memory is poorly understood. To date, the most solid evidence for the role of bystander activation has been observed in innate-like T cells. These cells, unlike conventional CD8⁺ T cells, recognize non-peptide antigens presented by orthologous MHC I-like molecules. They rapidly secrete cytokines following activation and can mediate some level of protection before adaptive immunity is sufficiently advanced (222). Recently, we demonstrated that mucosal-associated invariant T (MAIT) cells become activated during IAV infections in humans and mice (223, 224). These MAIT cells recognize riboflavin-derivative antigens produced by microbial pathogens (225), but can be variously activated by IL-12/IL-18 (224), IL-15, or type I interferons (226). Using a murine model, we showed that MAIT cells rapidly accumulate and become activated in the infected lung and contribute to protection against IAV infection (223). Similarly, invariant Natural Killer T (iNKT) cells, which recognize lipid antigens presented by CD1d, can protect against murine IAV (227–229). In addition, iNKT cells induced by inactivated influenza A virus vaccination in conjunction with alpha-galactosylceramide, an iNKT cell

antigen, can boost influenza-specific memory CD8⁺ T cells and protective immunity in mice (230). The exact contribution of innate T cells vs. conventional CD8⁺ T cell-mediated immunity against influenza viruses is a subject of further investigation. These new insights help to understand the wider range of vaccine responses thus offering us opportunities to generate better strategies to fight against influenza.

CONCLUDING REMARKS

Although current seasonal influenza vaccines can promote the induction of highly specific, long-term memory B cells that produce antibodies against the viral HA1 domain, these antibodies are generally unable to combat newly emerging influenza viruses, including novel pandemic strains and antibody-selected “seasonal” variants that have accumulated mutations in those epitopes surrounding the receptor binding pocket. Generation of high-affinity neutralizing Abs against conserved surface epitopes remains a constant challenge to provide long-lasting and cross-protective B cell memory, and as such, more work is needed to better understand B cell responses against natural infection vs. vaccination, in order to design better B cell- or antibody-based universal vaccines. On the other hand, an influenza vaccine capable of stimulating CD8⁺ T cell responses would generate long-term T cell memory against conserved epitopes without the need for annual vaccination. In addition, a role for innate-like T cells in influenza protection is increasingly emerging, which could potentially be important both for the development of novel therapeutics and for boosting (or maintaining) long-term memory. As a consequence, substantial efforts are being made globally to exploit both innate and adaptive immune components for the development of novel influenza vaccines that induce long-lasting B cell/antibody and/or cross-reactive T cell immune memory populations.

AUTHOR CONTRIBUTIONS

MA and AF wrote the sections on influenza and humoral immunity. LH and XJ wrote the sections on cellular immunity. BC wrote the section on vaccine adjuvants. TN, KK, and PD wrote and modified the original manuscript, and the revised versions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL ARTICLE

Robust correlations across six SARS-CoV-2 serology assays detecting distinct antibody features

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Abstract

Objectives. As the world transitions into a new era of the COVID-19 pandemic in which vaccines become available, there is an increasing demand for rapid reliable serological testing to identify individuals with levels of immunity considered protective by infection or vaccination. **Methods.** We used 34 SARS-CoV-2 samples to perform a rapid surrogate virus neutralisation test (sVNT), applicable to many laboratories as it circumvents the need for biosafety level-3 containment. We correlated results from the sVNT with five additional commonly used SARS-CoV-2 serology techniques: the microneutralisation test (MNT), in-house ELISAs, commercial Euroimmun- and Wantai-based ELISAs (RBD, spike and nucleoprotein; IgG, IgA and IgM), antigen-binding avidity, and high-throughput multiplex analyses to profile isotype, subclass and Fc effector binding potential. We correlated antibody levels with antibody-secreting cell (ASC) and circulatory T follicular helper (cTfh) cell numbers. **Results.** Antibody data obtained with commercial ELISAs closely reflected results using in-house ELISAs against RBD and spike. A correlation matrix across ten measured

ELISA parameters revealed positive correlations for all factors. The frequency of inhibition by rapid sVNT strongly correlated with spike-specific IgG and IgA titres detected by both commercial and in-house ELISAs, and MNT titres. Multiplex analyses revealed strongest correlations between IgG, IgG1, FcR and C1q specific to spike and RBD. Acute cTfh-type 1 cell numbers correlated with spike and RBD-specific IgG antibodies measured by ELISAs and sVNT. **Conclusion.** Our comprehensive analyses provide important insights into SARS-CoV-2 humoral immunity across distinct serology assays and their applicability for specific research and/or diagnostic questions to assess SARS-CoV-2-specific humoral responses.

Keywords: antibody-secreting cells, ELISA, neutralisation assay, SARS-CoV-2 antibodies, T follicular helper cells

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected > 100 million individuals, caused over 2 million deaths¹ and greatly disrupted economies worldwide. While awaiting global vaccination programmes, countries have focused on rapid testing, isolating and contact tracing of SARS-CoV-2-infected individuals using the nucleic acid amplification test (NAAT; RT-PCR),^{2,3} while implementing social distancing and lockdown measures to reduce virus transmission and prevent healthcare systems from being over-whelmed with COVID-19 patients.^{4,5} Concurrently, scientists have endeavoured to unravel the correlates of protective immunity^{2,6,7} and there has been an unprecedented effort to develop and produce vaccines.⁸ The world is now transitioning into a new stage of the pandemic, in which vaccines should become available globally. It is likely that identifying individuals with protective immunity induced by infection or vaccination, and whether previously induced immune responses are protective against potential new variants, will assume increasing importance.⁹ Furthermore, from a scientific standpoint, one of the key knowledge gaps is related to the duration of protective immunity in the population after infection and/or vaccination. This will be closely monitored in the years to come. Thus, from both a diagnostic and scientific standpoint, there will be an increasing demand for reliable rapid serological tests.

In general, virus neutralising antibodies measured by a plaque-reduction neutralisation test (PRNT)¹⁰ or a virus microneutralisation test

(MNT)^{11,12} are considered the gold standard correlate of protection,¹³ although it is currently unclear what titre is required for absolute protection from SARS-CoV-2 infection. These assays detect antibodies that can inhibit viral entry and thus can prevent reinfection with the same virus.¹³ However, SARS-CoV-2-specific PRNT and MNT can only be performed in biosafety level (BSL) 3 containment laboratories by specially trained personnel and take up to 5 days to perform. Furthermore, as they are biological assays, samples must be run in the same assay for accurate comparisons between samples. These assays are also limited by their inability to discriminate different antibody isotypes, and to detect antibodies directed against other non-neutralising epitopes known to contribute to protection against severe disease.¹⁴

Alternative serology assays are required to gain a rapid and comprehensive profile of humoral responses against SARS-CoV-2. Recent advances include the use of (1) the surrogate virus neutralisation test (sVNT), a competition enzyme-linked immunosorbent assay (ELISA) which uses purified receptor binding domain (RBD) of the spike (S) protein and host cell receptor angiotensin-converting enzyme 2 (ACE2) to mimic the virus–host interaction^{15,16} and allows detection of antibodies that block RBD binding to ACE2 without the need for infectious virus or cells and therefore only requiring BSL 2 containment, (2) ELISAs which detect SARS-CoV-2-specific antibodies of different isotypes directed towards viral proteins such as the full S protein, RBD and nucleocapsid (N) protein,¹¹ (3) multiplex-based serology allowing detection of different antibody isotypes against a large set of viral antigens and provides information

on Fc-receptor specificity, while requiring small sera or plasma volumes,^{14,17} (4) chaotropic-based dissociation assays to measure antigen-binding avidity, and (5) commercial semi-quantitative ELISA systems, including assays developed by Euroimmun (Lübeck, Germany) and Wantai (Beijing, China) to detect the level of SARS-CoV-2-specific antibodies in patient samples.^{18–21} These assays are rapid, circumvent the use of infectious virus and cells, and detect responses against a broader range of viral epitopes, thus revealing and identifying different antibody features beyond virus neutralisation that contribute to the prevention of infection and/or severe disease.

Although some studies have compared the specificity and sensitivity of different diagnostic tests,^{22–26} relatively limited data are available on correlations between distinct serology assays and understanding how they relate to the classical MNT readout. Such comparisons are needed to understand the applicability of SARS-CoV-2 serology assays for specific research questions. In our study, we used a cohort of 34 serum/plasma samples from 15 COVID-19 patients to perform a rapid sVNT which is highly applicable to many laboratories as it circumvents the need for BSL 3 containment. We further correlated the antibody data obtained from the sVNT to five additional commonly used SARS-CoV-2 serology techniques as described above for the same COVID-19 patient samples. These included a MNT, in-house and commercial Euroimmun- and Wantai-based ELISAs (RBD, S and N; IgG, IgA and IgM), antigen-binding avidity assays and serology multiplex analysis.^{6,11,14} Furthermore, we correlated the antibody levels obtained from these assays with cellular immune responses important for antibody production, antibody-secreting cells (ASC) and circulatory T follicular helper (cTfh) cells. Our comprehensive analyses provide important insights into SARS-CoV-2 humoral immunity across distinct serology assays as well as their applicability for specific research questions to assess humoral immunity in COVID-19 clinical samples.

RESULTS

COVID-19 patient IgM, IgG and IgA titres correlate between commercial and in-house ELISAs

Our study included 34 SARS-CoV-2 samples from 15 PCR-confirmed COVID-19 cases. Seven patients

were recruited in hospital during acute infection (with three being on supplemental oxygen), and eight patients from the community during convalescence. The median age of the COVID-19 patients was 57 (range 25–74 years) and 67% were females. Patient blood was longitudinally sampled up to three times, between days 2 and 188 post-symptom onset (Supplementary table 1). Acute samples were defined as those obtained within 14 days of symptom onset (range 2–14 days), while convalescent samples were obtained more than 5 weeks post-symptom onset (range 38–188 days). We also recruited 27 pre-pandemic healthy non-exposed controls, with a median age of 52 (range 24–75 years) and 67% were female.

To determine isotype-specific antibody responses against a range of SARS-CoV-2 antigens in COVID-19 patients, we used commercial ELISAs detecting IgM against RBD (Wantai), IgG and IgA against S (Euroimmun) and IgG against N (Euroimmun). Antibody levels against these antigens were significantly higher in acute (median index value IgM (RBD); 13.791, IgG (S); 3.883, IgA (S), IgG (N); 2.633) and convalescent COVID-19 samples (median index value IgM (RBD); 12.986, IgG (S); 4.369, IgA (S); 3.791, IgG (N); 2.378), when compared to healthy non-exposed individuals (median index value IgM (RBD); 0.184, IgG (S); 0.132, IgA (S); 0.260, IgG (N); 0.188) (Figure 1a). Across all isotypes and specificities, the antibody levels were maintained between acute and convalescent samples, although IgM (RBD) and IgA (S) started to decrease at about day 40, while IgG (S and N) were maintained for slightly longer decreasing at about day 80 (Figure 1b). Antibody data obtained with the commercial ELISAs closely reflected the antibody profiles detected with the in-house performed ELISAs against RBD and S (Figure 1c),⁶ in which all COVID-19 patients seroconverted by convalescence as measured by the in-house IgG (RBD) ELISA. Longitudinally, titres for both RBD and S specificities trended towards decreased levels with time post-symptom onset (Figure 1d), which was similar to our observation using the commercial ELISAs.

IgG (S) antibody levels measured by the commercial ELISA correlated strongly across isotypes (IgM, IgA) and specificities (RBD, N) ($P < 0.0001$) (Figure 1e). Similarly, IgG (RBD, S) titres measured by the in-house ELISA positivity correlated with IgM and IgA with matched

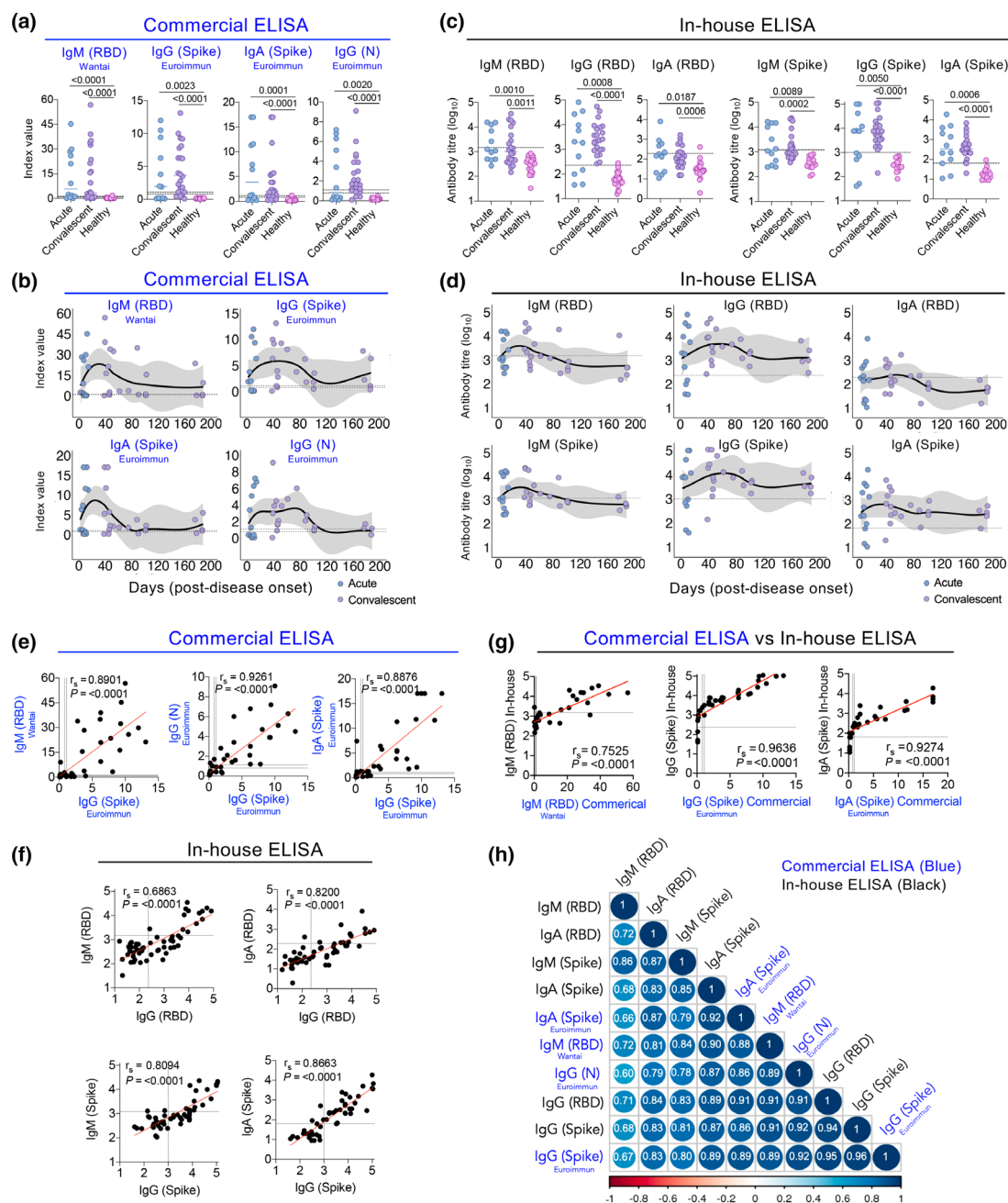


Figure 1. Antibody signatures in COVID-19 patients determined by commercial and in-house ELISAs. **(a)** Antibody levels against SARS-CoV-2 proteins for IgM (RBD), IgG (S and N) and IgA (S) in acute ($n = 7$) and convalescent ($n = 14$) COVID-19 donors and non-exposed healthy individuals ($n = 25$) were measured by commercial ELISA. Grey dotted lines indicate borderline antibody levels as determined by the manufacturer. **(b)** Kinetics of antibody levels determined by commercial ELISA from days post-symptom onset for IgM, IgG and IgA. **(c)** In-house ELISA end-point titres of SARS-CoV-2 RBD and S antibodies where the dotted line indicates the seroconversion cut-off. **(d)** Kinetics of antibody levels determined by in-house ELISA from days post-symptom onset for IgM, IgG and IgA. **(e)** Correlation between commercial ELISA antibody isotopes and specificities ($n = 59$ samples per isotype). **(f)** Correlation between IgG and IgM/IgA for RBD ($n = 58$ samples) and S ($n = 49$ samples) specificities as measured by in-house ELISA. **(g)** Correlation between matched isotype and specificities for commercial and in-house ELISAs ($n = 34$ samples). **(h)** Correlation matrix of commercial and in-house ELISA antibody levels in all acute and convalescent COVID-19 samples. Spearman's ranked correlation coefficients (r_s) are depicted for each paired correlation, with positive correlations shown in shades of blue and negative correlations in shades of red. **(a, c)** Median is shown, and statistical significance was determined by a Kruskal–Wallis multiple comparisons test. **(b, d)** LOESS regression lines with 95% confidence intervals shaded in grey are shown. **(e–g)** Spearman's correlation coefficients and P-values shown.

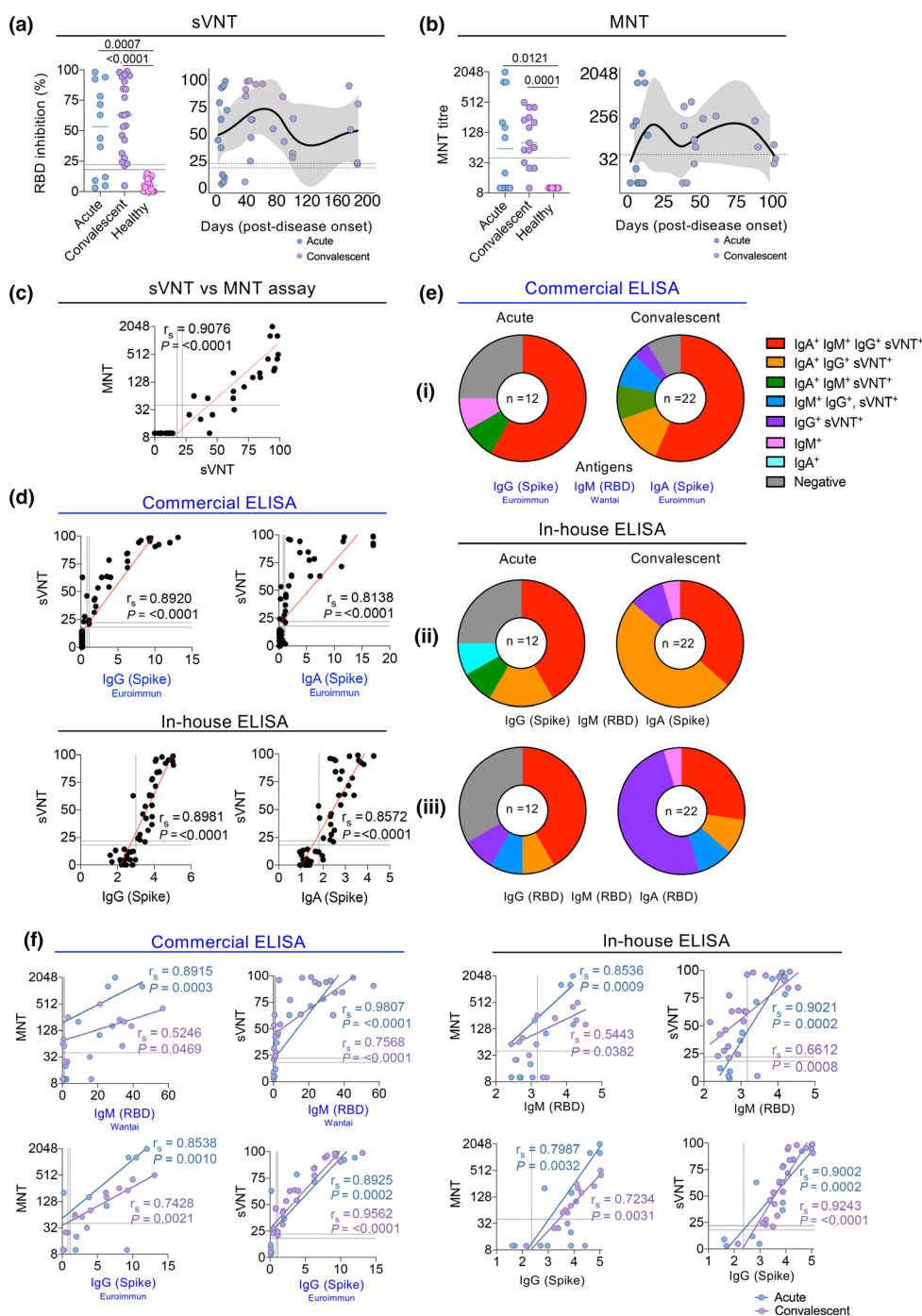


Figure 2. ACE2-RBD binding inhibition correlates with MNT, IgG and IgA titres. **(a)** Percentage inhibition of ACE2 and RBD binding by neutralising antibodies in acute ($n = 12$) and convalescent ($n = 22$) COVID-19 samples and non-exposed healthy individuals ($n = 25$). **(b)** MNT titres are shown in a subset of donors (12 acute, 15 convalescent and 12 non-exposed healthy serum samples) as previously described ⁶. **(a, b)** Medians are shown, and statistical significance was determined by a Kruskal–Wallis multiple comparisons test (left panels). Kinetics of neutralising antibodies from days post-symptom onset (right panels). LOESS regression lines with 95% confidence intervals shaded in grey are shown. **(c, d)** Correlation between sVNT percentage inhibition and **(c)** MNTs (titre log₂), and **(d, top)** commercial ($n = 59$ samples per isotype) and **(d, bottom)** in-house ($n = 49$ samples per isotype) ELISA for S-specific IgG and IgA antibody levels. Spearman's correlation coefficients and P -values shown. **(e)** Isotype and neutralisation profiles for RBD and Spike-specific IgM, IgG and IgA at acute and convalescent timepoints, measured by sVNT and **(i)** commercial and **(ii, iii)** in-house ELISAs. **(f)** Correlation between commercial and in-house IgM (RBD) and IgG (S) ELISAs with sVNT and MNT divided into acute and convalescent phases. Spearman's correlation coefficients and P -values for each phase are shown.

antigen specificities ($P < 0.0001$) (Figure 1f). To confirm that the antibody levels determined via the two different ELISA methodologies were comparable, we analysed paired antigen specificities and found strong positive correlations across all three isotypes ($r_s = 0.7525$ – 0.9636 , $P < 0.0001$) (Figure 1g). We also generated a correlation matrix across the 10 parameters derived from the commercial and in-house ELISAs, revealing positive correlations between all factors. The weakest correlates ($r_s < 0.7$) were between the in-house IgM-RBD versus the commercial IgA-S, IgG-N, IgG-S and in-house IgA-S, IgG-S and IgA-S, whereas the strongest correlates were between commercial IgG-S versus in-house IgG-S ($r_s = 0.96$) and in-house IgG-RBD ($r_s = 0.95$) (Figure 1h).

ACE2-RBD binding inhibition correlates with IgG titres

The rapid surrogate neutralising assay measures neutralising activity of SARS-CoV-2-specific antibodies in COVID-19 patients at a lower biosafety level (BSL 2). The sVNT used in this study was based on antibody-mediated blockade of the interaction between the ACE2 receptor protein and RBD,¹⁵ and unlike the conventional MNT, does not require infectious virus, cells or a BSL 3 laboratory. The sVNT detected neutralising antibodies in all COVID-19-infected individuals except one by convalescence (despite a positive PCR test, this individual was also negative by sVNT and MNT). Both acute and convalescent COVID-19 groups displayed significantly higher ACE2-RBD inhibition than healthy non-exposed individuals ($P = 0.0007$ and $P < 0.0001$, respectively) (Figure 2a). Although we observed a decrease in neutralising antibody titres over time, neutralising activity was maintained into convalescence, with detectable neutralising antibodies found in 3 out of 5 donors at ~180 days post-symptom onset, with the remaining two donors displaying borderline inhibition (21% and 22.9%).

Since the sVNT has been developed relatively recently, we next asked how the ACE2-RBD inhibition detected by sVNT compared to classical MNT titres, as detected on a subset of donors described previously.⁶ Indeed, very similar patterns of neutralisation were observed with the MNT compared to the sVNT, with MNT antibodies detected at significantly higher levels in acute ($P = 0.0121$) and convalescent ($P = 0.0001$) COVID-19 donors when compared to healthy non-

exposed individuals (Figure 2b). Antibody neutralisation activity strongly correlated between the two tests ($r_s = 0.9076$; $P < 0.0001$) (Figure 2c). Importantly, results obtained using the RBD in-house ELISA also correlated with the classical MNT, as shown previously.⁶

Having shown that the frequency of ACE2-RBD inhibition measured by the sVNT is comparable to the neutralisation activity determined by MNT, we tested whether the ACE2-RBD inhibition correlated with the ELISA antibody levels. We observed the frequency of ACE2-RBD inhibition strongly correlated with the S-specific IgG and IgA titres measured by both commercial (IgG (S) $r_s = 0.8920$ $P < 0.0001$, IgA (S) $r_s = 0.8138$ $P < 0.0001$) and in-house ELISAs (IgG (S) $r_s = 0.8981$ $P < 0.0001$, IgA (S) $r_s = 0.8572$ $P < 0.0001$) (Figure 2d). Analysis of antibody titres and ACE2-RBD inhibition revealed that the proportion of COVID-19 samples with undetectable IgM-RBD, IgG-S and IgA-S antibodies (measured in the commercial ELISA) and low neutralising activity (below 18%) decreased from 25% at the acute phase of infection to 4.5% during convalescence (Figure 2ei). Using the in-house ELISA, this trend in antibody responses (against the same antigens used in the commercial ELISAs) and neutralisation activity was also observed, decreasing from 25% at the acute phase to 0% at convalescence as all the recovered individuals had at least one positive isotype response (Figure 2eii). This suggests that a combined detection of IgM, IgG and IgA S or RBD-specific antibody isotypes via the in-house ELISA might be more sensitive and/or has a lower threshold of detection than the commercial ELISA. Interestingly, when antibody levels were measured by commercial ELISA, more than half (59%) of the convalescent donors were positive for all antibody isotypes (IgG-S, IgM-RBD and IgA-S) and ACE2-RBD inhibition (Figure 2ei). In contrast, when titres were measured by in-house ELISA, a majority of the participants were positive for neutralisation activity featuring either all isotypes (IgG-S, IgM-RBD and IgA-S) (36%) or only IgA-S and IgG-S (50%) (Figure 2eii), indicating potentially a lower sensitivity for IgM in the in-house assay. However, when only RBD-specificity was considered, unresponsive frequencies were higher at the acute phase (33%). Moreover, half (50%) of convalescent donors exhibiting neutralising activity had only antibodies with IgG isotype (Figure 2eiii), indicating that a proportion

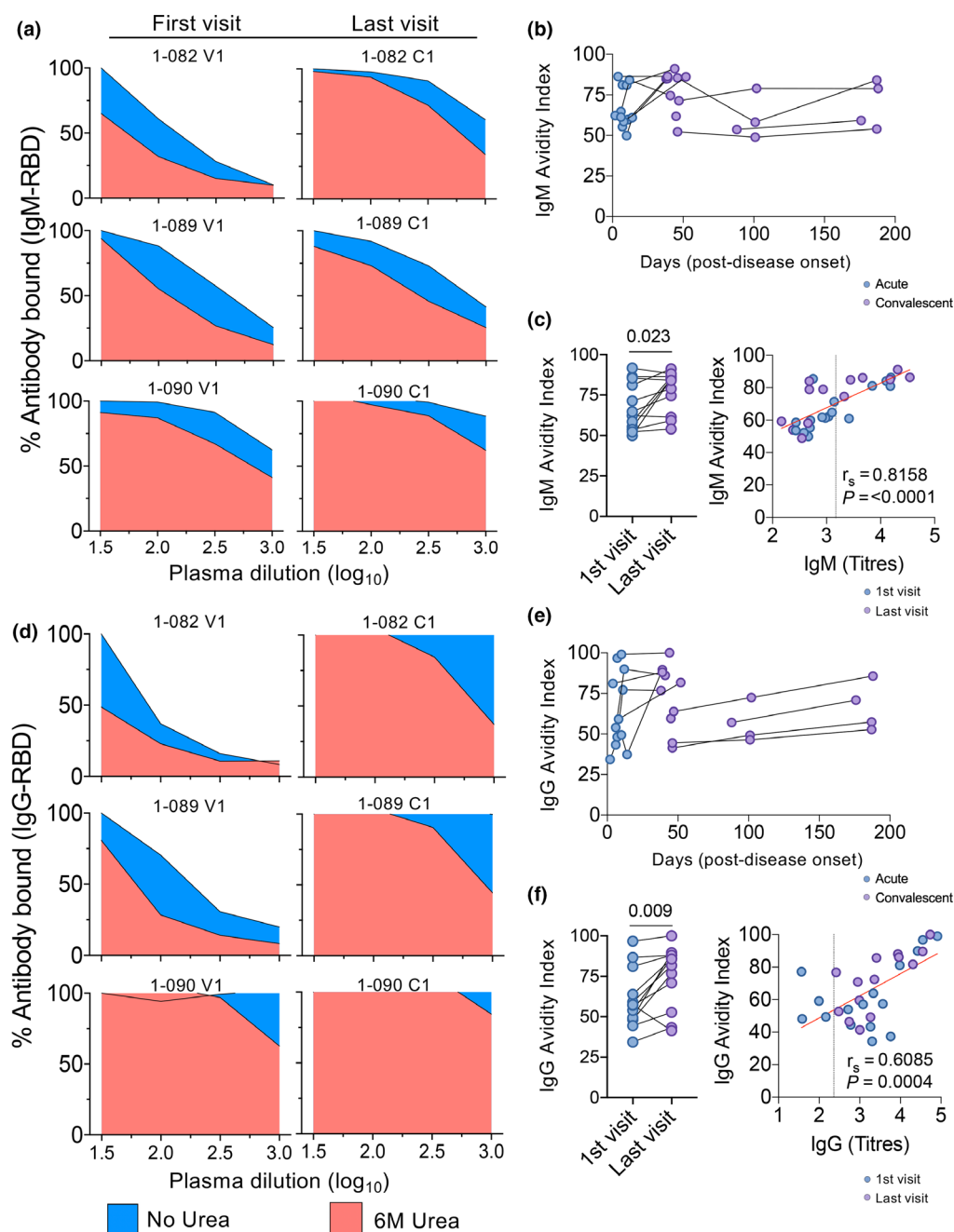


Figure 3. IgG antibody avidity increases at convalescence. **(a)** Representative avidity analysis for IgM RBD-specific antibodies. Plasma was diluted across antigen-coated wells before treatment in the presence (red) or absence (blue) of 6 M Urea. Shown is the percentage of antibody bound at each dilution relative to the amount detected at the lowest dilution (1:31.6) in the absence of urea (100%). **(b)** Longitudinal antibody avidity levels of acute ($n = 7$) and convalescent ($n = 13$) COVID-19 patient samples for RBD-specific IgM. The avidity index reflects the percentage of antibody remaining by comparing the AUC of each antibody titration curve with or without urea treatment. **(c)** Avidity analysis for IgM RBD-specific antibodies in paired samples (left panel). Correlation between in-house IgM (RBD) ELISA titres and IgM avidity scores (right panel). **(d)** Representative avidity analysis for IgG RBD-specific antibodies. **(e)** Longitudinal antibody avidity levels of acute ($n = 7$) and convalescent ($n = 13$) COVID-19 patient samples for RBD-specific IgG. **(f)** Avidity analysis for IgG RBD-specific antibodies in paired samples (left panel). Correlation between IgG (RBD) ELISA titres and IgG avidity scores (right panel). **(c, f, left panels)** First and last samples were collected between 4 and 141 days apart. Statistical significance was assessed by the Wilcoxon matched-pairs signed rank test, $n = 12$. **(c, f, right panels)** Spearman's correlation coefficients and P -values are shown.

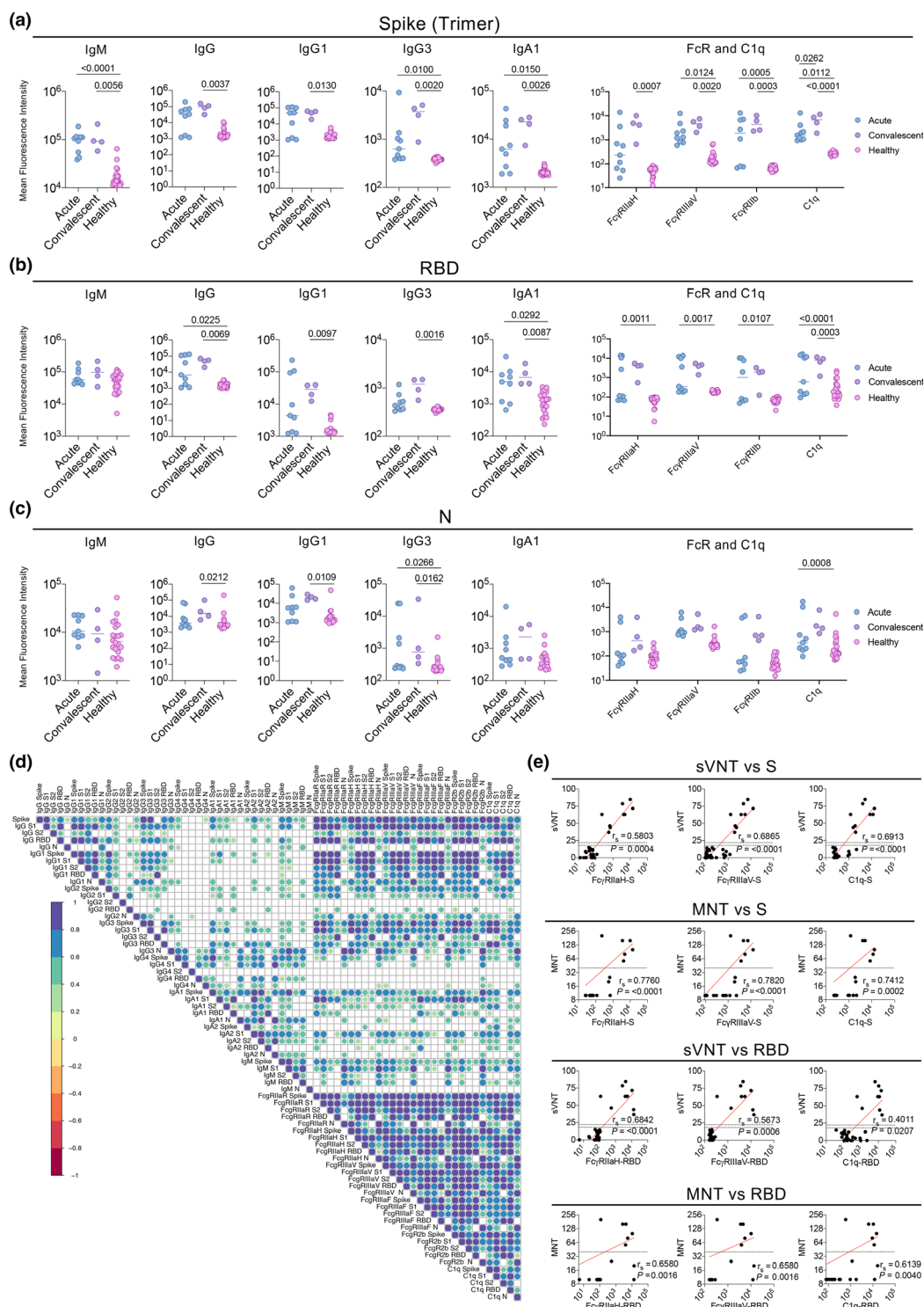


Figure 4. Multiplex analysis of COVID-19 patients. Median fluorescence intensity of selected Ig isotypes, FcR and C1q against (a) S trimer, (b) RBD and (c) N protein within acute ($n = 6$), convalescent ($n = 4$) and non-exposed healthy ($n = 22$) plasma samples. (d) Correlation heatmap of MFI's measured across all 14 detectors (IgM, IgG, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, FcγR1aH, FcγR1aR, FcγR1aV, FcγR1aF, FcγR1b and C1q) against the 5 SARS-CoV-2 antigens (trimeric S, S1, S2, RBD and N). Only significant correlations (FDR corrected $P < 0.05$) are shown. (e) Correlations of sVNT percentage inhibition ($n = 33$ COVID-19 positive and healthy samples) or MNT ($n = 20$ samples) between S- or RBD-specific FcγR1aH, FcγR1aV, or C1q MFI are shown. Spearman's correlation coefficients and P-values are shown.

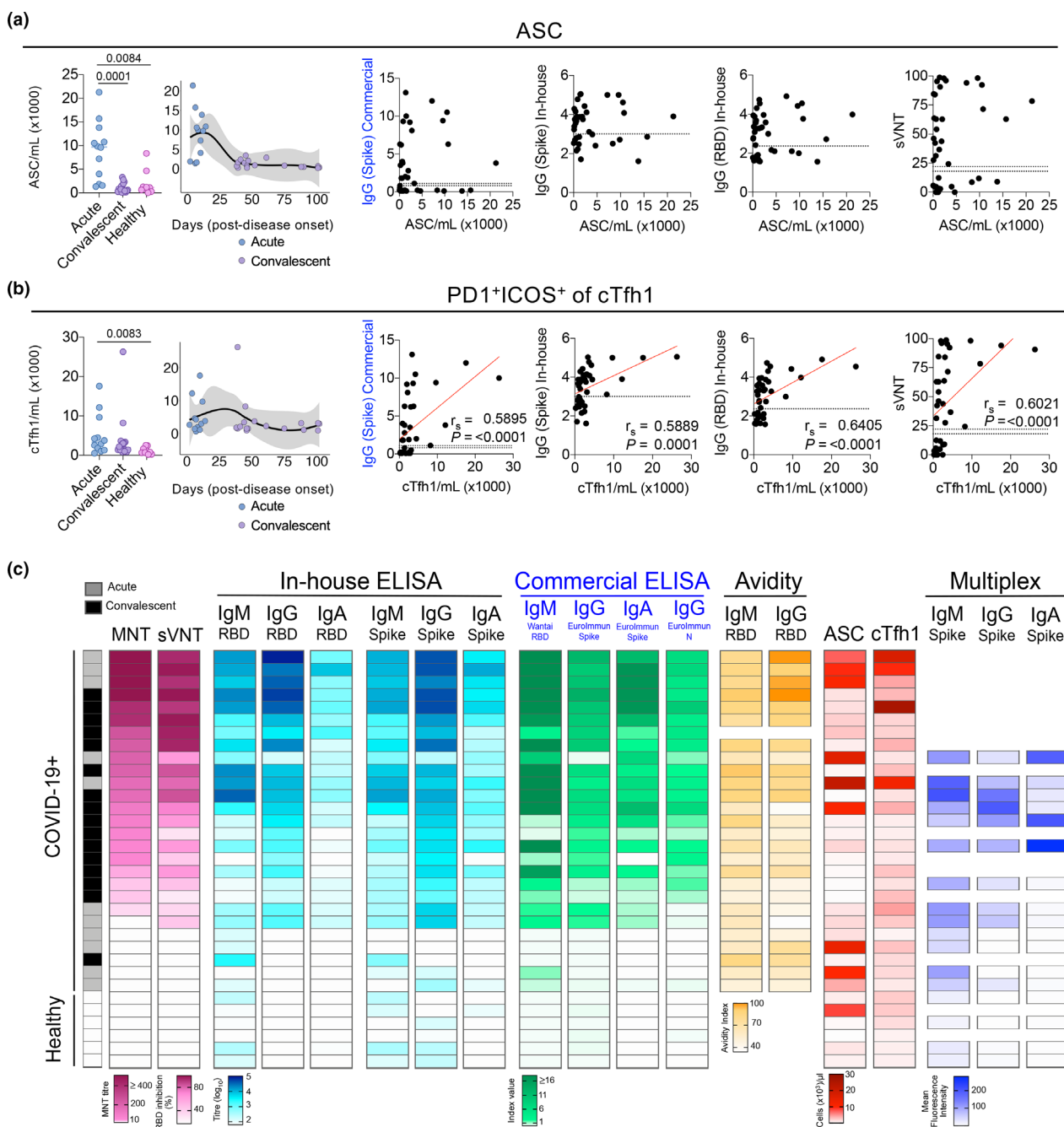


Figure 5. Contribution of cellular factors to antibody-mediated immunity. **(a, b)** Absolute numbers and longitudinal kinetics of **(a)** ASCs and **(b)** cTfh1 cells have previously been described.⁶ Correlations of cellular subsets between commercial and in-house IgG-S, in-house IgG-RBD and sVNT % inhibition are shown in the right panels. **(c)** Heatmap of neutralisation (MNT, sVNT), antibody levels (in-house and commercial ELISA), antibody avidity, ASC and cTfh1 cells and multiplex analyses of acute ($n =$ up to 11), convalescent ($n =$ up to 16) and non-exposed healthy ($n =$ up to 6). Each row represents a different sample with their matched measurements (where available) in each column.

of antibody responses observed in the commercial (Figure 2ei) and in-house ELISA (Figure 2eii) against the S protein are directed to non-RBD regions of the protein.

Given that IgM can mediate a significant proportion of the early neutralisation activity,²⁷ we correlated commercial and in-house ELISA IgM and IgG antibody levels with neutralisation

Table 1. Antibody assay comparison

	MNT	sVNT	Genscript	ELISA Euroimmun (IgG/ IgA; Spike & IgG; N)	ELISA Wantai (IgM; RBD)	In-house ELISA (IgM/ IgA/IgG; RBD/Spike)	Chaotropic-based dissociation assays	Multiplex
Test time	5 days	65 min		120 min (S); 105 min (N)	75 min	48 h	48 h	24 h per detector (antibody isotype or FcR)
Hands-on user time	7 h	30 min		30 min	30 min	~2.5 h (6 plates)	~3 h (6 plates)	~4 h per detector
Capacity, samples/run	96 samples	96 samples		96 samples	96 samples	10 samples per plate	5 samples per plate	384 samples per detector
Volume of sample per test	28 µL	10 µL		10 µL	10 µL	7.3 µL	7.3 µL	1 µL per detector
Plasma vs serum	Either ^a	Either		Either	Either	Either	Either	Either
Samples run in duplicate or singularly	Duplicate in independent assays	Singularly (duplicate for samples within the equivocal zone)		Singularly (duplicate for samples within the equivocal zone)	Singularly (duplicate for samples within the equivocal zone)	Singularly	Singularly	Duplicate in independent assays
Cost per sample (AUD)	–	~\$14		~\$8	~\$8	~\$1.60–\$2.20	~\$1.80–\$2.40	~\$16
TGA approval ⁴²	–	Emergency access scheme		Emergency access scheme	Emergency access scheme	–	–	–
FDA approval ⁴³	–	Emergency use authorisation		Emergency use authorisation (IgG)	–	–	–	–
Biosafety level	BSL 3	BSL 2		BSL 2	BSL 2	BSL 2	BSL 2	BSL 2
Antigen specificity	Whole virus	RBD		S or N	RBD	S, RBD	S, RBD	S, S1, S2, RBD, N
Class switching	No	No		Yes	Yes	Yes	No	Yes
Antibody avidity	No	No		No	No	No	Yes	Yes
Level of expertise	High	Moderate		Moderate	Moderate	Moderate	Moderate	High
Standardisation	Run specific	Yes		Yes	Yes	Yes	Yes	Bead set specific
Result readout	End-point titre	% inhibition		Index value	Index value	End-point titre	Avidity Index	MFI

^aSerum preferred.

measured by sVNT and MNT. We observed that the IgM levels at acute timepoints correlated more strongly with neutralisation activity (commercial IgM with MNT $r_s = 0.8915$ $P = 0.0003$, sVNT $r_s = 0.9807$ $P < 0.0001$; in-house IgM with MNT $r_s = 0.8536$ $P = 0.0009$, sVNT $r_s = 0.9021$ $P = 0.0002$) than IgM levels at convalescence (commercial IgM with MNT $r_s = 0.5246$ $P = 0.0469$, sVNT $r_s = 0.7568$ $P < 0.0001$; in-house IgM with MNT $r_s = 0.5443$ $P = 0.0382$, sVNT $r_s = 0.6612$ $P = 0.0008$) (Figure 2f). In comparison, the strong correlations between IgG levels and neutralisation activity were maintained across both acute and convalescent phases (acute $r_s = 0.7987$ – 0.9002 ; convalescence $r_s = 0.7234$ – 0.9562). These correlations are thus in line with the view that both IgM and IgG contribute to protection during the acute phase; however, IgG responses may have a greater contribution to the overall neutralisation activity over time.

Avidity of IgG antibodies increases at convalescence

Data obtained using the sVNT and the commercial and in-house ELISA assays, as well as our previous MNT data,⁶ clearly demonstrated that the majority of COVID-19 patients had robust antibody titres and neutralising activity, but provided little information on how these parameters were related to the qualitative features of humoral immunity. Since antibody neutralisation quality and potency is often correlated with antibody avidity, a urea dissociation assay (Figure 3a and d, representative donors) was used to determine the proportion of antigen-bound IgM and IgG antibody remaining after 6 M urea treatment (expressed as an avidity index).^{6,14} The avidity of IgM was greater than 50% at all timepoints for all COVID-19 donors tested (Figure 3b). In paired COVID-19 plasma samples ($n = 12$) obtained 4 to 141 days apart (median 40.5 days between first and last visits), we found a significant increase ($P = 0.023$) in IgM avidity on the second visit (Figure 3c), with avidity from day 40 post-symptom onset apparently maintained to at least day ~188 (Figure 3b). IgM avidity also correlated with the IgM titres from in-house ELISAs ($r_s = 0.8158$, $P < 0.0001$) (Figure 3c), an indication of the presence of IgM at high levels after initial pathogen exposure and its pentameric conformation providing antigen binding strength.

In contrast, RBD-specific IgG antibodies in COVID-19 donors ($n = 12$) exhibited lower avidity than IgM at the first visit during the acute phase of disease, which then increased during convalescence (Figure 3e). Paired analysis of IgG avidity during first and last visits, however, showed a more significant increase by the second visit ($P = 0.009$) compared to the IgM (Figure 3f). The fact that IgG avidity did not correlate as strongly with the IgG titre ($r_s = 0.6085$, $P = 0.0004$) indicates that despite high initial titres, IgG responses require time to undergo somatic hypermutation to acquire stronger avidity, or alternatively that the IgG titre and avidity are less interconnected than their IgM counterparts.

Coronavirus multiplex array reveals a range of key antibody features beyond neutralisation

Apart from neutralisation activity exerted by a variety of antibody isotypes, antibodies also have the capacity to engage Fc Receptors (FcRs) or complement as a mechanism to mediate Fc effector functions; they are not limited to targeting the RBD. The coronavirus multiplex array that has previously been applied to systems serology studies,¹⁴ allows detection of a broad range of antibody levels directed against a range of antigen specificities (including trimeric S, spike 1 (S1) and 2 (S2), RBD, N and antigens from seasonal coronaviruses to test cross-reactivity),¹⁴ isotypes (IgG, IgA, IgM) and subclasses (IgG1, IgG2, IgG3, IgG4, IgA1, IgA2), along with functions like C1q [a predictor of classical Antibody-Dependent Complement Activation (ADCA)] and soluble dimer engagement via FcRs (FcγRIIa, FcγRIIb and FcγRIIIa), which have previously been shown to correlate with a range of effector functions.²⁸

A subset of donor samples was examined using the multiplex approach (COVID-19 donors $n = 9$, non-exposed healthy individuals $n = 22$) across five SARS-CoV-2 antigens (trimeric S, S1, S2, RBD and N) and 14 detectors (IgM, IgG, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, FcγRIIaH, FcγRIIaR, FcγRIIIaV, FcγRIIIaF, FcγRIIb and C1q), as previously described.¹⁴ Significantly higher levels of S-specific IgM ($P = 0.0056$), IgG ($P = 0.0037$), IgG1 ($P = 0.013$), IgG2 ($P = 0.017$), IgG3 ($P = 0.002$), IgA1 ($P = 0.0026$) and IgA2 ($P = 0.002$) antibodies were detected in convalescent COVID-19 donors compared to healthy controls, whereas only IgM

($P < 0.0001$), IgG2 ($P = 0.0076$), IgG3 ($P = 0.01$), IgA1 ($P = 0.015$) and IgA2 ($P = 0.0317$) showed significantly higher levels in acute donors (Figure 4a and Supplementary figure 1a). Significant differences between convalescent COVID-19 donors and healthy individuals were maintained when only the RBD-specific antibody response was considered for all antibody isotypes apart from IgM and IgG2, with significantly higher antibodies in acute donors observed only for IgG ($P = 0.0225$) and IgA1 ($P = 0.0292$) (Figure 4b). In comparison, significant differences in N-specific antibodies were limited to convalescent IgG ($P = 0.0212$), IgG1 ($P = 0.0109$) and IgG3 (convalescent $P = 0.0162$ and acute $P = 0.0266$) (Figure 4c). When FcRs and C1q were examined across antigen specificities, similar to the antibody isotypes, the acute and convalescent COVID-19 donors had significantly higher levels than non-exposed individuals for S, S1 and RBD, but less so for N and S2 (Figure 4a–c and Supplementary figure 1b). The increased binding to high avidity FcγRIIIaH131 and FcγRIIIaV158 soluble dimers for the S and RBD-specific antibodies may suggest that, in addition to the potential neutralising activity, antibody-mediated activity such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) could potentially contribute to viral clearance.¹⁴ Furthermore, we observed a trend towards increasing FcR and C1q engagement over time, suggesting that the Fc function is being regulated or modulated in COVID-19 donors during and possibly even after infection. Significant binding to C1q in acute (S $P = 0.0112$, S1 $P = 0.0013$, S2 $P = 0.0002$, RBD $P < 0.0001$, N $P = 0.0008$) and convalescent (S $P < 0.0001$, S1 $P < 0.0001$, S2 $P = 0.0124$, RBD $P = 0.0033$) COVID-19 donors compared to non-exposed healthy individuals also indicates that viral infection induces antibodies that could clear the virus infected cells via ADCA. Overall, the multiplex data positively correlated with each other, with the strongest correlations observed for IgG, IgG1, FcR and C1q detectors against S, S1 and RBD antigens (Figure 4d). In context with the neutralising data, the high avidity FcR and C1q detectors against S and RBD were found to positively correlate with neutralising activity determined by both sVNT and MNT, with the S-specific antibodies generally displaying a stronger correlation than the RBD antibodies (Figure 4e), indicating a potential contribution of neutralising

antibody directed against epitopes outside the RBD.

Acute cTfh-type 1 numbers are indicative of antibody-mediated immunity

We and others have recently shown that SARS-CoV-2 antibody responses correlate with the rise in ASCs and cTfh-type 1 (cTfh1) responses in COVID-19 patients.^{2,6,12,29} In our previous study,⁶ CD38^{hi}CD27^{hi} ASCs peaked rapidly and transiently post infection, with the number of ASCs in a subset of acute COVID-19 donors included in this comparative study ($n = 15$) also being significantly higher than convalescent COVID-19 donors ($P = 0.001$) and non-exposed healthy individuals ($P = 0.0084$) (Figure 5a). In contrast, total activated PD1⁺ICOS⁺ CXCR3⁺CXCR5⁺CD4⁺ cTfh1 cells peaked later post-symptom onset,⁶ with significantly higher levels in acute COVID-19 donors compared to healthy individuals ($P = 0.0083$) included in this study (Figure 5b). The early and temporary increase in ASC did not correlate with antibody levels measured by commercial or in-house ELISA (IgG-S/RBD) or sVNT. However, the number of cTfh1 cells correlated positively with levels of S and RBD-specific IgG antibodies measured by both the commercial and in-house ELISA, as well as ACE2-RBD inhibition by sVNT.

To provide insights into SARS-CoV-2 humoral immunity across the distinct serological assays, we collated a heatmap of neutralisation (MNT, sVNT), antibody levels (in-house and commercial ELISA), antibody avidity, ASC and cTfh1 cells and multiplex analyses from a subset of patients (acute $n = 11$, convalescent $n = 15$) and non-exposed healthy individuals ($n = 6$). When we ranked the COVID-19 patients according to their MNT neutralising titres, COVID-19 patients with the highest neutralising titres also had a high frequency of ACE2-RBD inhibition detected by sVNT. Interestingly, patients with high neutralising activity also had high levels of all antibody isotypes, specificities, avidity, as well as cTfh1 cells (Figure 5c), suggesting a broad range of antibodies likely leads to improved virus neutralisation. This was particularly true for donors with high IgG antibodies levels against S and RBD (as RBD is captured within the S-specific response) also having high sVNT and MNT, suggesting a pivotal role for IgG in neutralisation, although higher levels of neutralising IgA could be expected in the mucosa.¹⁷ While the multiplex

assay lacked donors with very strong MNT and sVNT scores, S-specific antibody levels based on MFI decreased with lower neutralisation activity, particularly for IgG and IgA. Finally, an extended correlation heatmap of ASC, cTfh1 and antibody responses across the six different serology assays evaluated in this study displayed mostly positive correlations, with 2277 positive correlations from a total of 2279 significant correlations (Supplementary figure 2). The exception was IgG avidity with IgG1-S and IgG2-S MFI, as determined by multiplex analyses, which may be due to limited convalescent sampling in this assay ($n = 4$), rather than biological effects as we showed that IgG avidity increases at the later timepoints. Although positive correlations were observed earlier between the FcR and C1q MFIs (S and RBD) with the sVNT and MNT (Figure 4e), these correlations were not significant for MNT after correcting for multiple comparisons in the extended heatmap (Supplementary figure 2). However, clusters of very strong positive correlations were observed as expected between (1) the ELISAs, both in-house and commercial, (2) the ELISAs to the multiplex FcR detectors, (3) multiplex IgG with FcR and (4) within the different the FcR detectors. Therefore, our comparative analysis showed that antibody levels obtained from the above six assays were highly correlated with each other, and with the cTfh1 response.

DISCUSSION

The COVID-19 pandemic is expected to gradually transition to a new phase as vaccines become available that will lead to a rapid increase in protective immune responses within the population. Such increased immunity at the population level can exert immune pressure on the virus and induce escape mutations, which could coincide with lower viral fitness. It is likely that the virus will shift from being a pandemic strain to a seasonally circulating strain, which like other seasonal coronaviruses, will cause repetitive and (hopefully) mild infections,³⁰ as observed for pandemic influenza virus strains, including the deadly 1918 influenza pandemic strain.³¹ Introduction of viral variants, which could potentially evade protective immune responses is also a possibility, as happens frequently with influenza viruses.^{32,33} Thus, there is a need and an increasing demand for rapid reliable serological

testing to identify individuals with protective immunity induced by infection or vaccination. Furthermore, from a scientific standpoint, these assays will be useful to monitor the longevity of humoral immunity, especially in high-risk groups, in order to re-vaccinate before protective immune responses wane.³⁴ Serological testing can also be used to assess whether previously induced SARS-CoV-2 specific antibodies recognise viral variants that have mutated within known neutralising antibody binding sites. Virus neutralisation assays, including the MNT, are considered the gold standard to detect protective antibody responses. However, this assay may not be suitable for every situation and/or laboratory. The MNT needs to be performed under BSL 3 containment, requires highly qualified personnel, needs a relatively large volume of serum, is labour intensive and does not provide information on antibody isotypes or potential protective features beyond virus neutralisation. Another technical limitation is that the current MNT assay is a biological assay, so all samples need to be performed in the same assay for direct comparison. To circumvent these limitations, several alternative assays have been developed (Table 1). However, knowledge on how distinct serology assays correlate with each other and understanding how they relate to the classical MNT readout is limited. In addition, it will be of increasing importance to compare data generated by different laboratories using different serological assays. Our comprehensive analyses provided important insights into SARS-CoV-2 humoral immunity across distinct serology assays, which will enable scientists to select the best assay to answer their research questions.

We demonstrated that all evaluated serological assays strongly correlated with the classical MNT and with each other. Furthermore, all assays had the capability to detect the longevity of antibody responses over time. The decision to choose one of the six assays studied here will therefore greatly depend on alternative factors such as biosafety level, labour (hands-on and total duration of the assay), costs, approval by Therapeutic Goods Administration (TGA; Australia) or Food and Drug Administration (FDA; USA) for diagnostic use, the type of readout and level of expertise (Table 1), and the research question at hand. Access to BSL 3 containment for the MNT, or the high level of expertise and relatively high costs of the multiplex assay means that these assays may not be suitable for most laboratories,

and that the other antibody assays such as the in-house and/or commercial ELISAs are likely to be sufficient if the research aim is to detect protective antibody responses.

However, to understand whether SARS-CoV-2 vaccines can induce similar or even stronger/broader protective immune responses than natural infection, additional serological features beyond virus neutralisation need to be studied. These include class-switching, antibody maturation, recognition of (non-)neutralising epitopes and/or their ability to engage FcRs or complement, both contributing to protection from severe viral infections via ADCC, ADCP and ADCA.^{35–39} When it comes to class-switching, either one of the commercial or in-house ELISAs evaluated herein can be used, as the isotype data of all three assays strongly correlated with each other. However, compared to the commercial ELISAs, the in-house ELISA might be slightly more sensitive for the detection of IgG and IgA, but slightly less sensitive for IgM. Furthermore, the Euroimmun ELISAs have the additional advantage of not only detecting spike or RBD-specific antibodies but also those directed against the nucleocapsid protein. Detection of nucleocapsid-specific antibodies could potentially help differentiate vaccinated individuals (S-based vaccines such as those manufactured by Pfizer, Moderna or AstraZeneca) from naturally infected individuals (who would theoretically have both S and N-specific antibodies). Affinity maturation, for which antibody avidity could be used as a surrogate measure, could best be studied using the chaotropic-based dissociation assay for both IgG and IgM. However, given that IgA RBD titres were often lower than IgM and IgG, this affected the detection range of the assay, limiting its ability to measure accurate differences in antibody binding following urea treatment. Interestingly, when we correlated IgG titres with the IgG avidity data, we showed that, during the course of the immune response, the initial increase in IgG titres was followed by enhanced avidity, which indicates that despite initial high titres, time is needed to acquire strong avidity.

Although multiplex analysis allows one to study isotype switching, the strength lies in its ability to simultaneously study antibody responses at the subclass level to a broad range of viral epitopes, and additionally provide an in-depth understanding of their ability to engage FcRs or

complement. Here, we enlisted 14 detectors against 5 antigens, which totalled to measuring 70 different antibody features in a single assay but could easily be further expanded, as described previously.¹⁴ This makes the multiplex analysis of great interest for an in-depth systems serology comparison of antibody responses induced by natural infection or vaccination. However, this assay is technically challenging, and requires specialised equipment and bioinformatic expertise to grasp the complexity of the large data sets, which makes the assay less suitable for most laboratories.

Germinal centre derived ASCs and Tfh1 cells play an important role in the induction and maintenance of antibody responses. However, since germinal centres are largely inaccessible for most immune and vaccination studies, we and others^{2,6,12,29,40} correlated our serological findings with previously acquired circulating cellular immune responses. We observed an increase of ASCs early during the acute phase of infection, potentially driving the initial increase in SARS-CoV-2 specific antibodies; however, long-lived plasma cells rather than ASCs likely support the production of long-term serological memory. This response was followed by an increase in cTfh1 numbers in the late acute phase. Interestingly, only acute cTfh1 cell numbers, but not ASCs, correlated with spike and RBD-specific IgG antibodies measured by ELISAs and ACE2-RBD inhibition. These results are in agreement with our recent findings that acute cTfh1 cells correlate with and predict antibody titres, especially towards spike, in convalescence as measured by in-house ELISA.⁶ These data may suggest a role for cTfh1 cells in the establishment, class-switching and longevity of humoral immunity in SARS-CoV-2 infection. The caveat there is that only ASC and cTfh1 cells measured in the acute phase can be used as an indicator for humoral immunity, since this is the only timepoint where they can be accurately detected in peripheral blood.

Overall, the scientific community is well equipped with a broad range of serological assays to answer a variety of biologically relevant questions on SARS-CoV-2-specific antibody responses that will arise over the coming months and years. Our study establishes that strong correlations exist between different assays despite the fact that the assays are designed to detect distinct antibody features, thus allowing

laboratories with different technologies to compare their findings to a certain extent.

METHODS

Study participants and ethics statement

Our study enrolled 15 SARS-CoV-2 PCR-positive participants, seven of whom were hospitalised acute patients and eight of whom were sampled at the convalescent phase 38–188 days post-symptom onset (Supplementary table 1). We included 27 pre-pandemic individuals as healthy non-exposed controls. Experiments conformed to the Declaration of Helsinki Principles and the Australian National Health and Medical Research Council Code of Practice. Written informed consents were obtained from all blood donors prior to the study. The study was approved by the University of Melbourne (#2056761, #1955465, #1442952, #1443389), the Alfred Hospital (#280/14) and James Cook University (H7886) Human Research Ethics Committees.

Commercial enzyme-linked immunosorbent assays (ELISAs)

ELISA testing was performed according to manufacturers' 'Instructions For Use' (IFU), with results reported semi-quantitatively as either a signal/cut-off ratio (for Euroimmun and Wantai assays) or percentage inhibition (for surrogate virus neutralisation assay).

Euroimmun anti-SARS-CoV-2 S IgG, S IgA and N IgG (Euroimmun Medizinische Labordiagnostika, Lubeck, Germany); indirect ELISAs for detection of IgG or IgA against SARS-CoV-2 antigens. Microtitre plate wells were coated with either (1) S1-domain or (2) modified N protein. SARS-CoV-2-binding antibodies were detected using enzyme-labelled anti-human-IgG or anti-human-IgA conjugates and a colourimetric substrate. The results were read spectrophotometrically.

Wantai IgM (Beijing Wantai Biological Pharmacy Enterprise, Beijing China); Wantai SARS-CoV-2 IgM is a capture ELISA for detection of IgM-class antibodies to SARS-CoV-2 virus. Anti- μ chain antibodies on the microtitre plate captured any patient IgM-class antibodies, and detection was achieved by recombinant SARS-CoV-2-antigen-horseradish peroxidase (HRP) conjugate followed by a colourimetric substrate. The results were read spectrophotometrically.

SARS-CoV-2 surrogate virus neutralisation test (sVNT)

Manufactured by GenScript (NJ, USA), the sVNT is a blocking ELISA which mimics the virus neutralisation process, detecting circulating neutralising SARS-CoV-2 antibodies that block the interaction between RBD and ACE2 on the cell surface receptor of the host. The test is species and isotype independent. HRP-conjugated recombinant SARS-CoV-2 RBD fragment bound to any circulating neutralising antibodies to RBD preventing

capture by the human ACE2 protein in the well, which was subsequently removed in the following wash step. Substrate reaction incubation time was determined by temperature. The IFU reported that the ideal reaction temperature and time were 25°C for 15 min but that for temperatures lower than 25°C, the time could be extended. Since our control values did not meet the assay validity criteria at 15 min, but fell within the acceptable ranges at 20 min, we used, a 20-min substrate incubation time and results were read spectrophotometrically. Colour intensity was inversely dependent on the titre of anti-SARS-CoV-2 neutralising antibodies.

RBD and Spike protein in-house enzyme-linked immunosorbent assays (ELISAs)

RBD- and S-specific ELISAs for detection of IgM, IgG and IgA antibodies were performed as described,^{6,11,41} with some modifications: Nunc MaxiSorp flat bottom 96-well plates (Thermo Fisher Scientific, Carlsbad, CA, USA) were used for antigen coating, blocking with PBS (containing w/v 1% BSA) and serial dilutions performed in PBS (containing v/v 0.05% Tween and w/v 0.5% BSA). For detection of IgG and IgA, peroxidase-conjugated goat anti-human IgG (Fc γ fragment specific; Jackson ImmunoResearch, West Grove, PA, USA) or alkaline phosphate-conjugated rat anti-human IgA (mAb MT20; MabTech, Stockholm, Sweden) was used and developed with TMB (Sigma-Aldrich, St Louis, MO, USA) substrate for IgG or pNPP (Sigma-Aldrich) for IgA. For IgM, biotinylated mAb MT22 and peroxidase-conjugated streptavidin (Pierce; Thermo Fisher Scientific) was used in conjunction with TMB. Peroxidase reactions were stopped using 1 M H₃PO₄ and plates read on a Multiskan plate reader (LabSystems, Vantaa, Finland). Inter- and intra-experimental measurements were normalised using a positive control plasma from a COVID-19 patient (#1-073) run on each plate. End-point titres were determined by interpolation from a sigmoidal curve fit (all R-squared values > 0.95; GraphPad Prism 8, San Diego, CA, USA) as the reciprocal dilution of plasma that produced $\geq 15\%$ (for IgA and IgG) or $\geq 30\%$ (for IgM) absorbance of the positive control at a 1:31.6 (IgG and IgM) or 1:10 dilution (IgA). Seroconversion was defined when titres were above the mean titres plus 2 standard deviations of healthy non-COVID-19 donors (24 for RBD and 15 for Spike).

Antibody avidity assay

The avidity of RBD-specific IgG and IgM antibodies in plasma samples was measured using urea as the chaotropic agent. Plasma was titrated in half-log dilutions (1:31.6 to 1:1000) onto antigen-coated wells and incubated for 2 h. Wells were washed and 6 M urea added and incubated for a further 15 min at room temperature. Bound antibodies were then detected using the respective secondary detection reagents as described above in the in-house ELISAs. The amount (in percentage) of antibody remaining was determined by comparing the total area of the antibody titration curve (across 4 dilutions) in the presence and absence of urea treatment and is expressed as the avidity index.

Microneutralisation assay (MNTs)

Microneutralisation activity of serum samples was previously described.⁶ Briefly, SARS-CoV-2 isolate CoV/Australia/VIC01/2020³ was propagated in Vero cells and stored at -80°C . Sera were heat-inactivated at 56°C for 30 min. Samples were serially diluted in two-fold dilutions starting at 1:20 and 100 TCID₅₀ of SARS-CoV-2 in MEM/0.5% BSA were added and incubated at room temperature for 1 h. Residual virus infectivity in the serum/virus mixtures was assessed in quadruplicate wells of Vero cells incubated in serum-free media containing $1\ \mu\text{g mL}^{-1}$ of TPCK trypsin at 37°C and 5% CO₂; viral cytopathic effect was read on day 5. The neutralising antibody titre was calculated using the Reed–Muench method, as previously described.¹²

Coronavirus multiplex

A coronavirus multiplex array was previously described.¹⁴ Briefly, SARS-CoV-2 antigens were covalently coupled to magnetic carboxylated beads using a two-step carbodiimide reaction. Antigen-coupled beads were pooled and combined with diluted plasma overnight before washing and staining with detectors (PE-conjugated anti-human IgM, IgG, IgA (and their respective subclasses) antibodies or soluble dimeric FcR or C1q protein followed by streptavidin PE conjugate). Plates were washed and read by the FlexMap 3D, with the binding of the PE detectors measured to calculate the median fluorescence intensity (MFI).

ASCs and cT_{fh}1 analysis

Absolute numbers of ASCs and activated cT_{fh}1 cells have been previously described.⁶ Briefly, whole blood was freshly stained with antibody cocktails, RBC lysed then fixed with 1% PFA before acquiring on a LSRII Fortessa (BD Biosciences, San Jose, CA, USA) and analysed using FlowJo v10 software (FlowJo, Ashland, OR, USA).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

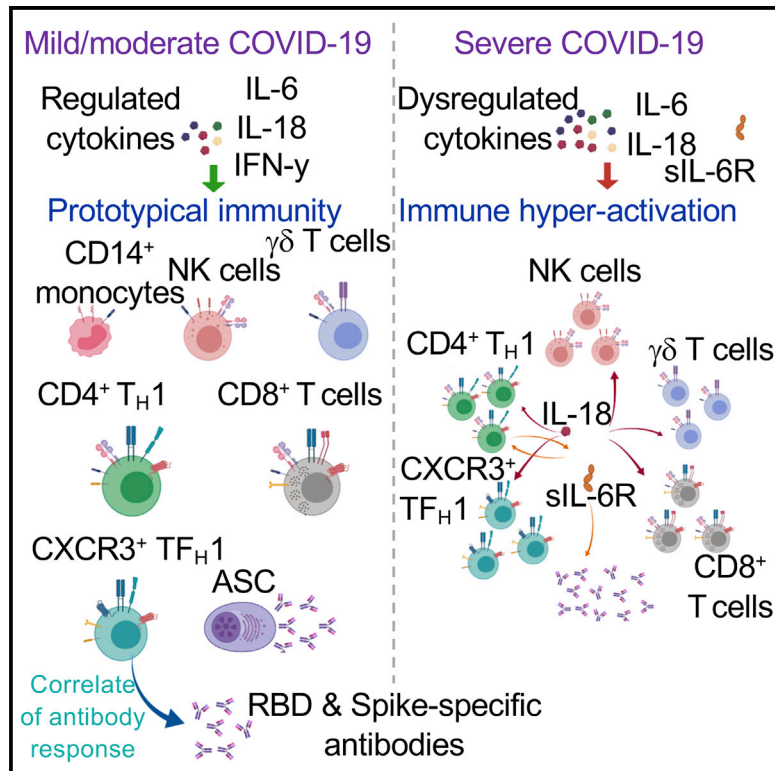
Additional supporting information may be found online in the Supporting Information section at the end of the article.



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Integrated immune dynamics define correlates of COVID-19 severity and antibody responses

Graphical Abstract



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In brief

Koutsakos et al. perform a broad analysis of 184 immune features using blood samples from 85 COVID-19 cases across time and severity groups. The study defines circulating T_{FH1} cells as a correlate of antibody responses and sIL-6R, IL-6, and IL-18 as correlates of disease severity.

Highlights

- Analyses of 184 immune features define kinetics of immune responses to SARS-CoV-2
- Circulating T_{FH1} cells in acute COVID-19 correlate with antibodies
- sIL-6R levels are elevated in severe COVID-19 but do not correlate with IL-6
- Elevated IL-6 and IL-18 correlate with immune cell hyperactivation

Article

Integrated immune dynamics define correlates of COVID-19 severity and antibody responses

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SUMMARY

SARS-CoV-2 causes a spectrum of COVID-19 disease, the immunological basis of which remains ill defined. We analyzed 85 SARS-CoV-2-infected individuals at acute and/or convalescent time points, up to 102 days after symptom onset, quantifying 184 immunological parameters. Acute COVID-19 presented with high levels of IL-6, IL-18, and IL-10 and broad activation marked by the upregulation of CD38 on innate and adaptive lymphocytes and myeloid cells. Importantly, activated CXCR3⁺cT_{FH}1 cells in acute COVID-19 significantly correlate with and predict antibody levels and their avidity at convalescence as well as acute neutralization activity. Strikingly, intensive care unit (ICU) patients with severe COVID-19 display higher levels of soluble IL-6, IL-6R, and IL-18, and hyperactivation of innate, adaptive, and myeloid compartments than patients with moderate disease. Our analyses provide a comprehensive map of longitudinal immunological responses in COVID-19 patients and integrate key cellular pathways of complex immune networks underpinning severe COVID-19, providing important insights into potential biomarkers and immunotherapies.

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has caused >101 million infections and 2.18 million deaths worldwide (as of January 28, 2021). Infection with SARS-CoV-2 results in a spectrum of clinical presentations, called coronavirus disease 2019 (COVID-19), ranging from asymptomatic to fatal disease. Disease severity has been associated with risk factors, including age, gender, and preexisting comorbidities,^{1,2} that correlate with immune responses during acute infection. While robust, broad, and transient immune responses precede patients' recovery in non-severe cases,^{3–6} severe COVID-19 can be associated with exuberant cytokine responses, hyperactivation of innate immune cells and T cells,^{4,6–8} and high titers of SARS-CoV-2-specific antibodies.⁸ At convalescence, the majority of individuals has SARS-CoV-2-specific antibodies and B cell and T cell responses.^{9–13} CD4⁺

T cell responses appear more prominent than CD8⁺ T cell responses in primary SARS-CoV2 infection.^{13,14}

Our understanding of immune responses to SARS-CoV-2 informed the development and clinical evaluation of immunomodulatory therapies, including monoclonal antibodies that target the interleukin-6 (IL-6) signaling pathway and corticosteroids such as dexamethasone.¹⁵ However, results from clinical trials examining the efficacies of current therapeutic approaches are inconsistent.¹⁶ In parallel, global accelerated efforts are focused on the development of safe vaccines against SARS-CoV-2, with the main goal of eliciting neutralizing antibodies against the Spike protein.¹⁷ Efforts are needed to understand integrated immune correlates of recovery and protection from COVID-19, and the complexity of innate and adaptive immune perturbations underpinning severe and fatal COVID-19 to inform the rational design of vaccines and identify predictors of protective immunity.

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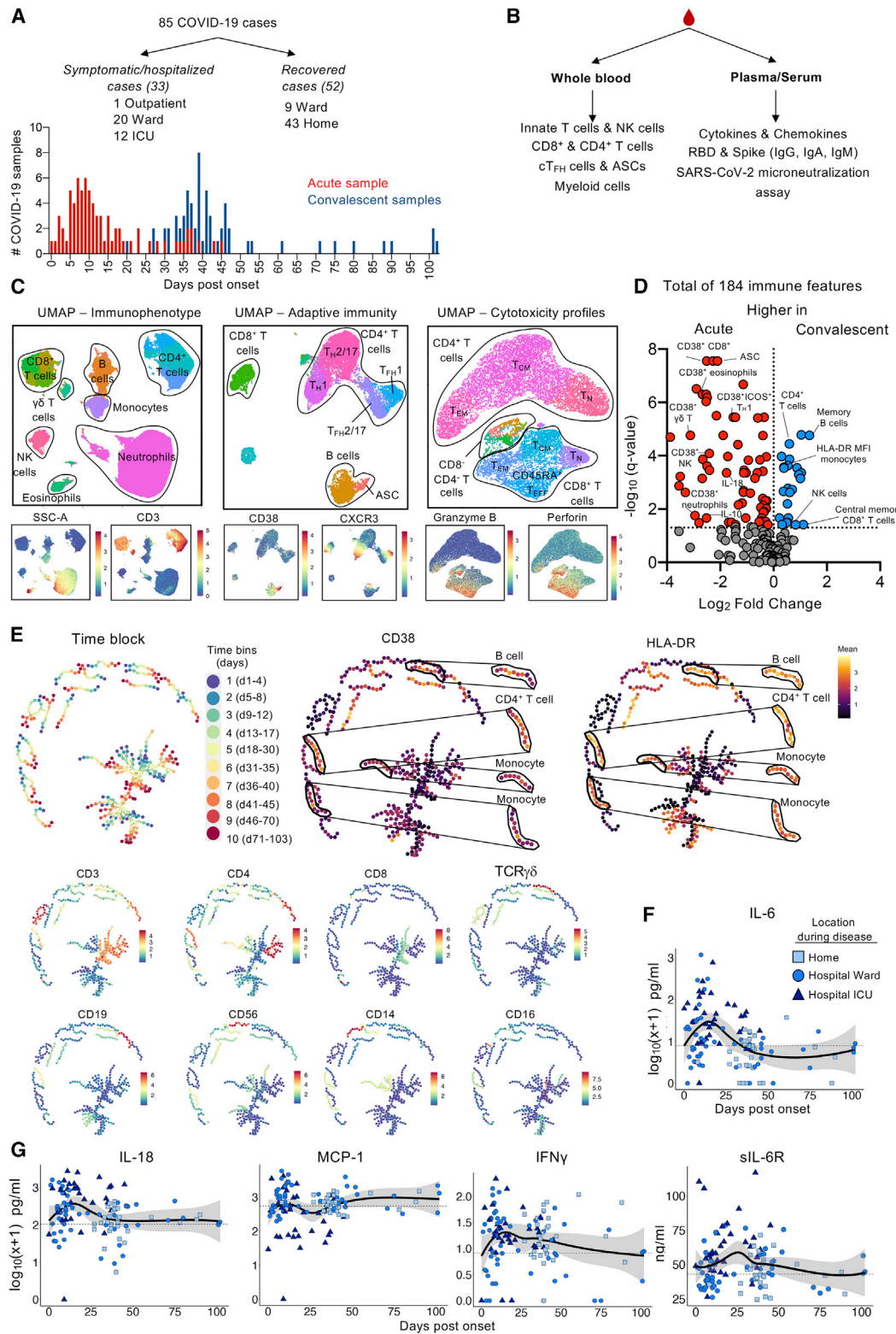
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We performed in-depth analyses of broad innate and adaptive immune responses in longitudinal acute and convalescent blood samples from SARS-CoV-2-infected individuals. Our study revealed integrated innate and adaptive immune dynamics during COVID-19 up to 102 days post-disease symptom onset, with circulating T follicular helper (cT_{FH}) cells strongly correlating with levels of SARS-CoV-2-specific antibodies. We identified IL-18, soluble IL-6 receptor (sIL-6R), and hyperactivated immune responses depicted with high CD38 expression (CD4⁺, CD8⁺, $\gamma\delta$ T cells, innate cells) and human leukocyte antigen-DR isotype (HLA-DR) expression (natural killer [NK] cells) as correlates of COVID-19 severity, as well as correlations between dysregulation of cytokines and immune hyperactivation, providing important insights into potential biomarkers and immunotherapies.

RESULTS

COVID-19 cohort study design

We recruited 85 PCR-confirmed COVID-19 cases. A total of 33 symptomatic/hospitalized individuals were recruited, including 20 ward patients (4 requiring supplemental oxygen support), 12 intensive care unit (ICU) patients (8 requiring invasive ventilation, 3 requiring non-invasive oxygen support), and 1 outpatient. Twenty-three donors were longitudinally sampled up to 6 times while in the hospital and 13 of the donors were sampled into convalescence, between days 20 and 80 post-symptom onset. An additional 52 COVID-19-recovered individuals were recruited after discharge from the hospital ($n = 9$) or after recovering at home ($n = 43$) (Figure 1A; Tables S1 and S2), with 4 being longitudinally sampled twice between days 26 and 102 post-



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symptom onset for immune profiling and serological analyses (Figure 1B). Acute samples were obtained either during the hospital stay in hospitalized patients or within 2 weeks post-symptom onset for the non-hospitalized individuals (outpatient) (Figure 1A, in red). Convalescent samples were obtained either after hospital discharge or at the earliest day 26 post-symptom onset for non-hospitalized individuals (Figure 1A, in blue). Disease severity was determined according to whether the patients recovered at home (mild disease), the hospital ward (moderate disease), or the ICU (severe disease). A total of 66 healthy individuals and 11 non-COVID-19 hospitalized patients were assessed as controls. Our study included 1 ICU patient, no. 1-088, with a hematological malignancy treated with rituximab medication, thus lacking B cells. As this donor had no detectable SARS-CoV-2 antibodies, only cellular and cytokine data were included in the statistical analyses.

Breadth of innate, adaptive, and myeloid immune responses in COVID-19

To understand the dynamics of immune responses to SARS-CoV-2 over time, we analyzed blood from acute and convalescent individuals using 3 multi-parameter flow cytometric panels. The acute group included moderate (ward) and severe (ICU) samples. Using a computational pipeline in the Spectre R package, encompassing the FlowSOM and UMAP (uniform manifold approximation and projection) algorithms,¹⁸ we identified clusters representing major lymphocyte and myeloid lineages and their activation phenotype from the first panel, clusters of adaptive B cell and T cell subsets from the second panel, and cytotoxicity profiles of CD8⁺ and CD4⁺ T cells from the third panel (Figure 1C). The UMAP analysis revealed different subsets of B helper and T_H cells according to CD38 and CXCR3, respectively (second panel), and preferentially high expression of granzyme B and perforin on CD8⁺ effector memory (T_{EM}) and effector memory CD45RA⁺ (T_{EMRA}) T cell subsets (third panel) (Figure 1C). To assess immune profiles between acute and convalescent COVID-19 samples, we combined our FlowSOM clustered data with the data from 14 soluble mediators and plasma receptor binding domain (RBD)-specific immunoglobulin G (IgG), IgM, and IgA antibodies to generate a total of 184 immune features. During acute COVID-19 in hospitalized patients, we observed significant changes in immune compositions compared to healthy controls and/or convalescent samples, particularly a greater proportion of neutrophils, neutrophil-to-lymphocyte and neutrophil-to-T cell ratios, higher proportions of activated populations of CD38⁺ neutrophils, CD38⁺ eosinophils, CD38 expression on CD14⁺ and CD16⁺ monocytes, CD38⁺CD56^{dim} NK cells, CD38⁺γδ T cells, antibody-secreting cells (ASCs), PD-1⁺ICOS⁺ cT_{FH} cells, CD38⁺CD4⁺ T cells, HLA-DR⁺CD4⁺

T cells, and higher proportions of T_{EMRA}-like CD27⁺CD45RA⁺, and CD38⁺CD8⁺ T cells (Figures 1D, S1, and S2). During convalescence, we found higher proportions of T_{CM} CD8⁺ T cells, NK cells, T_{CM}, and T_{EM} CD4⁺ T cells (Figures 1D and S1; Table S4).

To assess immune activation over time, we implemented a time-series algorithm called TrackSOM on the first immunophenotyping panel. TrackSOM is a time series-based clustering and cluster evolution tracking algorithm that combines the single time point clustering capacity of FlowSOM with the tracking by ChronoClust¹⁹ of cluster developments over time. TrackSOM clusters data from all time points using FlowSOM, and thus determines the temporal evolutions of the resulting metaclusters and clusters using the tracking mechanism of ChronoClust. With TrackSOM, we categorized, or “binned,” samples into 10 time intervals (“bins”) post-disease onset (i.e., bin 1 = 1–4, bin 2 = 5–8, bin 3 = 9–12 days, and so on) to allow for at least 4 samples (n = 4–15) per time bin (Figures 1E and S3; Table S3). Strikingly, the tracking pattern of the CD38 activation marker indicated dynamic activation of both innate and adaptive cells peaking within weeks 2–4 of disease onset (bins 4–6) before declining at later time bins (Figure 1E). These patterns were still apparent when we excluded ICU patient no. 1-088, who had continuously high CD38 expression over 4 time points. The expression of HLA-DR was also dynamic across time points, but to a lesser extent. Overall, our analyses revealed differences in immune profiles across the time between acute and convalescent phases, with broad immune activation of innate, adaptive, and myeloid cells.

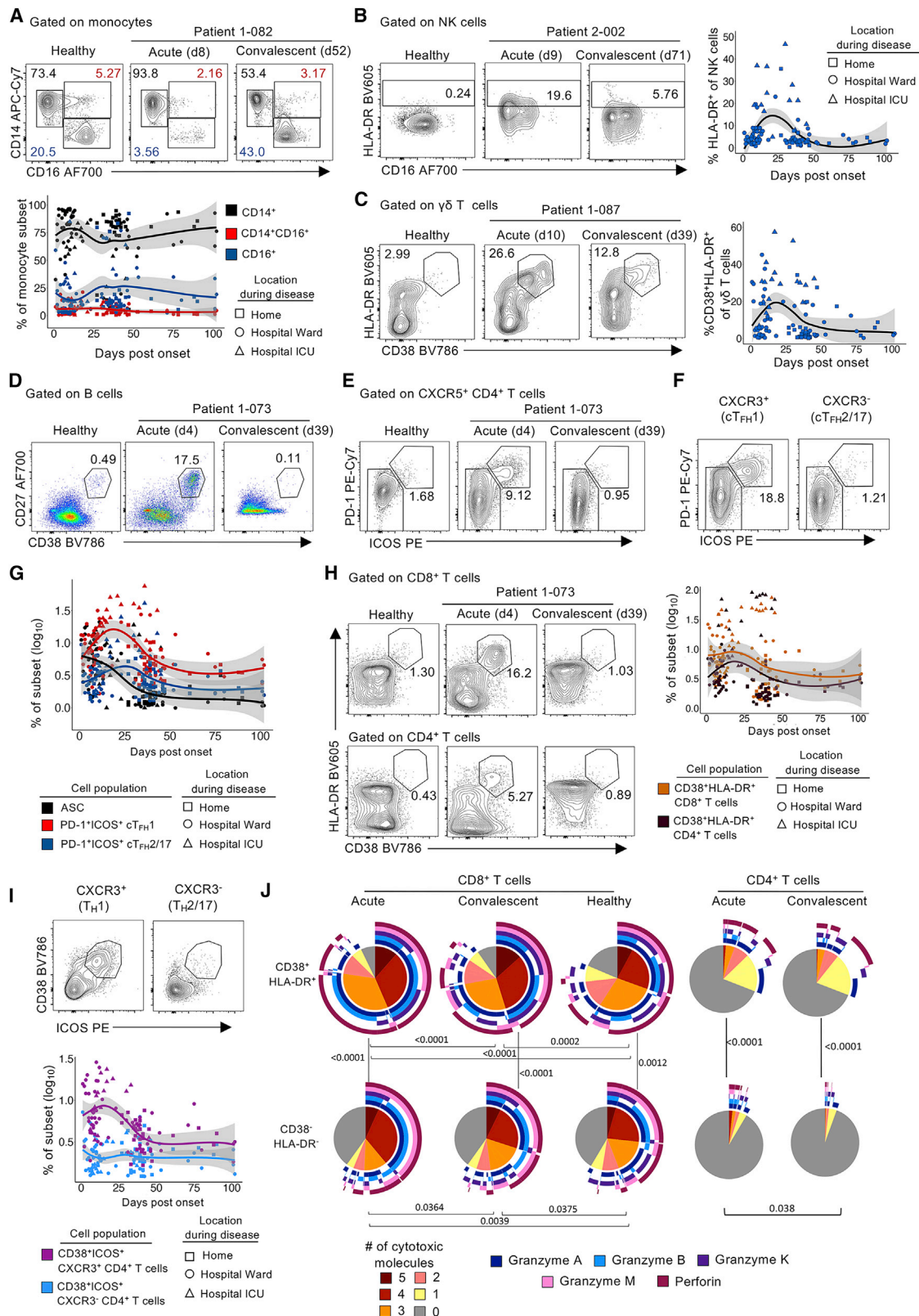
Rapid and transient activation of innate immune cells during COVID-19

To define the dynamics of innate responses, we compared 14 cytokines/chemokines in moderate and severe acute and convalescent COVID-19 plasma samples over time, in comparison to healthy controls. As IL-6 is a key driver of inflammation in COVID-19, possibly mediating pathology via engaging sIL-6R on cells expressing the gp130 co-receptor,²⁰ we also measured sIL-6R levels, since the role of sIL-6R in COVID-19 remains unknown. Inflammatory cytokines IL-6, IL-18, and IL-10 were significantly upregulated in acute samples compared to convalescent samples (Figures 1F, 1G, and S4A), consistent with other studies.^{21–23} The levels of monocyte chemoattractant protein-1 (MCP-1), interferon-γ (IFN-γ), and sIL-6R were also higher in acute samples from some patients compared to healthy controls, but these responses were variable (Figures 1G, S4A, and S4B), possibly reflecting the grouping of all ward and ICU patients in this analysis.

To complement our computational analysis, we applied manual gating (Figure S5) for immune subsets that contribute

Figure 1. Broad immune activation in longitudinal COVID-19 samples

(A and B) Overviews of (A) cohort and samples collected and (B) analyses performed.
(C) UMAP plot from FlowSOM analysis for 3 flow-cytometric panels, with representative clusters and expression profiles.
(D) Volcano plot of 184 immune features in acute and convalescent samples, with key representative features labeled.
(E) TrackSOM analysis of samples stained with the immunophenotype panel, with plots showing time bins of lineage-defining markers and the activation markers CD38 and HLA-DR. Time bins were days 1–4, 5–8, 9–12, 13–17, 18–30, 31–35, 36–39, 41–45, 46–53, and 71–102 (Table S3).
(F and G) Levels of cytokines (F) IL-6 and (G) IL-18, MCP-1, IFN-γ, and sIL-6R in COVID-19 plasma across time. Locally estimated scatterplot smoothing (LOESS) regression line and 95% confidence interval (CI) are shown, n = 119.



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to antiviral immunity in COVID-19 and influenza.^{24–27} Manual gating showed significantly higher activation of immune subsets in acute COVID-19 compared to convalescent and healthy donors (Figure S6A). For innate populations, acute samples had significantly higher proportions of activated HLA-DR⁺ NK cells, CD38⁺HLA-DR⁺ $\gamma\delta$ T cells, and effector-like CD27⁺CD45RA⁺ $\gamma\delta$ T cells, but lower levels of non-classical CD16⁺ monocytes, compared to convalescent and/or healthy donors (Figures 2A–2C; S6B, and S6C). The number of monocytes and $\gamma\delta$ T cells were comparable between acute and convalescent samples, while NK cell numbers were significantly lower in the acute samples (Figure S6D). These highly activated innate cell populations were dynamic, peaking within the first 3–4 weeks and declining thereafter (Figures 2A–2C).

Longitudinal dynamics of B cells, cT_{FH} cells, Th1 CD4⁺, and CD8⁺ T cells in COVID-19

We next defined immune populations that underpin the induction of humoral and cellular adaptive immunity, B cells, cT_{FH} cells, Th1 CD4⁺ cells, and CD8⁺ T cells. We observed significantly higher proportions and numbers of CD38^{hi}CD27⁺ ASCs and activated PD-1⁺ICOS⁺CXCR5⁺CD4⁺ cT_{FH} cells (Figures 2D, 2E, and S6E), a subset of lymphoid T_{FH} cells, in the blood²⁸ of acute COVID-19 patients compared to subsets in convalescent or healthy donors. Moreover, the activation of cT_{FH} cells by the co-expression of PD-1 and ICOS was attributed more to CXCR3⁺ cT_{FH}-type 1 (cT_{FH}1) cells than CXCR3[−] cT_{FH}2/cT_{FH}17 cells (Figures 2F and S6E). Proportions of ASCs increased rapidly and transiently at disease onset, while activated cT_{FH}1 cells gradually increased, peaking after 3–4 weeks, similar to activated innate cells, with cT_{FH}1 cell numbers maintained at convalescence (Figures 2G and S6E). There was substantial CD38⁺HLA-DR⁺ CD8⁺ and CD4⁺ T cell activation during the acute phase, as determined by increased proportions and numbers (Figure S6F). Both populations peaked earlier within the first 2–3 weeks and declined thereafter for home and ward patients but remained high until weeks 6–7 for ICU patients (Figure 2H). We also observed greater proportions and numbers of activated PD-1⁺CD38⁺ and CXCR3⁺CD38⁺ CD8⁺ T cells (Figure S6F) and greater proportions of CD27[−]CD45RA[−] T_{EM}RA-like and CD27[−]CD45RA[−] T_{EM}-like CD8⁺ T cells in acute samples

compared to convalescent or healthy controls (Figure S5G). Similarly, greater proportions of CD27[−]CD45RA[−] T_{EM} CD4⁺ T cells were detected in acute samples (Figure S6G). In line with the T_H1 environment of a viral infection, CXCR3⁺ T_H1 cells highly expressed CD38 with ICOS, factors that were absent in CXCR3[−] T_H2/17 cells (Figures 2I and S6F).

To define cytotoxic profiles of CD8⁺ and CD4⁺ T cells, the expression of granzyme A, B, K, and M and perforin was assessed. Activated CD38⁺HLA-DR⁺ CD8⁺ T cells in acute samples expressed higher levels of granzyme A and B and perforin compared to healthy and/or convalescent patients (Figures S7A and S7B). In addition, activated CD38⁺HLA-DR⁺ CD8⁺ T cells in acute samples had higher proportions of cells expressing multiple cytotoxic molecules, with the majority of CD38⁺HLA-DR⁺ CD8⁺ T cells expressing 3 or 4 cytotoxic molecules (Figure 2J). Although the expression of granzyme B and perforin within CD38⁺HLA-DR⁺ CD8⁺ T cells significantly decreased at convalescence, interestingly, granzyme K and M were increased (Figures S7A and S7B). The overall diversity of cytotoxic molecule expression, measured by the different combinations of molecules simultaneously expressed per cell, also differed at convalescence as compared to acute COVID-19 and healthy individuals (Figure 2J).

While modest, activated CD38⁺HLA-DR⁺ CD4⁺ T cells also expressed higher levels of granzyme B in acute samples (Figures 2J, S7C, and S7D). Similar patterns were found when the expression of granzymes and perforin were analyzed according to their T cell differentiation subsets by FlowSOM analysis (Figure S8).

Overall, SARS-CoV-2 infection induced a prototypical antiviral adaptive immune response comprising ASCs, activated cT_{FH}1 cells, activated T_H1 CD4⁺ T cells, and highly cytotoxic CD8⁺ T cells that emerged during acute COVID-19 and contracted during convalescence.

Seroconversion and antibody signatures during acute and convalescent COVID-19

As humoral immunity plays a key role in antiviral responses, we analyzed antibodies in acute and convalescent samples from 25 COVID-19 patients across multiple time points, by comparing RBD-specific IgG, IgM, and IgA antibodies by ELISA⁹ with healthy controls (Figures 3A and S9). Antibody levels were

Figure 2. Dynamic activation of innate cells, B cells, cT_{FH}1 cells, T_H1 CD4⁺ T cells, and CD8⁺ T cells in COVID-19 patients

(A–C) Representative fluorescence-activated cell sorting (FACS) plots of each immune population for a healthy donor and a COVID-19 patient at acute and convalescent time points.

(A) Proportion of monocyte subsets.

(B) Proportion of activated HLA-DR⁺ NK cells.

(C) Proportion of activated CD38⁺HLA-DR⁺ $\gamma\delta$ T cells in COVID-19 samples against time.

(D–F) Representative FACS plots of ASCs (D), PD-1⁺ICOS⁺ cT_{FH} cells (E), and CXCR3⁺ cT_{FH}1 and CXCR3[−] cT_{FH}2/cT_{FH}17 cells for a healthy donor and a COVID-19 patient at acute and convalescent time points (D and E), an acute time point from a COVID-19 patient (F).

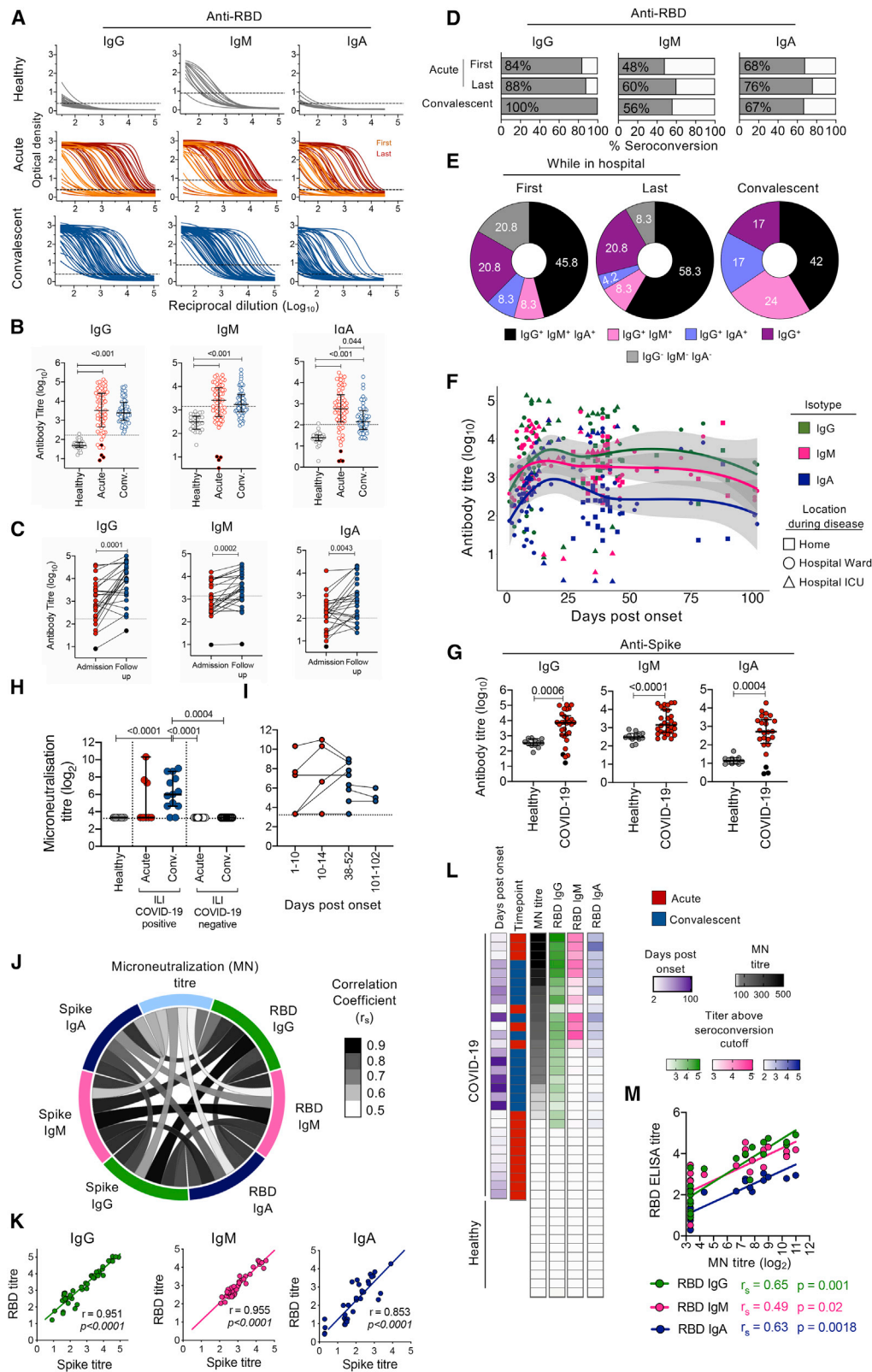
(G) Proportion of ASCs, PD-1⁺ICOS⁺ CXCR3⁺ cT_{FH}1 cells, and PD-1⁺ICOS⁺ CXCR3[−] cT_{FH}2/cT_{FH}17 cells in COVID-19 samples against time.

(H) Proportion of CD38⁺HLA-DR⁺ CD8⁺ and CD4⁺ T cells in COVID-19 samples against time. Representative FACS plots of each immune population for a healthy donor and a COVID-19 patient at an acute and a convalescent time point.

(I) Proportion of CD38⁺ICOS⁺ CXCR3⁺ T_H1 and CXCR3[−] T_H2/17 CD4⁺ cells in COVID-19 samples against time. Representative FACS plots of each population shown for an acute time point from a COVID-19 patient.

(J) Pie charts representing average fractions of CD38⁺HLA-DR⁺, CD38⁺HLA-DR⁺ CD8⁺, and CD4⁺ T cells co-expressing different cytotoxic molecules (slices) and the combinations of granzymes and perforin molecules (arcs). Statistical significance ($p < 0.05$) was determined by permutation tests. Data are based on the manual gating strategy, as per Figure S3.

(A–C and G–I) LOESS regression line and 95% CI are shown, $n = 105$.



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significantly higher in acute ($\log_{10}$ median titer 3.52 IgG, 3.41 IgM, 2.77 IgA) and convalescent COVID-19 samples ($\log_{10}$ median titer 3.38 IgG, 3.24 IgM, 2.15 IgA) compared to those in healthy, non-exposed individuals ($\log_{10}$ median titer 1.68 IgG, 2.49 IgM, 2.15 IgA) (Figure 3B). While IgG and IgM titers were maintained at similar levels between acute and convalescent samples, IgA titers had significantly decreased by convalescence. Paired analysis confirmed a significant increase in antibody titers from the first sampling following admission to just before discharge or at convalescence for all 3 isotypes (Figure 3C). At the first acute blood collection, 84%, 48%, and 68% of individuals seroconverted ($\log_{10}$ antibody titer $>$ mean $+ 2 \times$ SD of healthy individuals) for RBD-specific IgG, IgM, and IgA antibodies, respectively (Figure 3D). IgG seroconversion rates increased to 88% and 100% at the last acute blood collection and at convalescence, respectively, although IgM and IgA were maintained at similar frequencies (Figure 3D). In addition, the proportion of individuals with undetectable RBD-specific IgG, IgM, or IgA antibodies decreased from 20.8% during the acute phase to 0% at convalescence, with 100% of convalescent donors having at least an IgG response to RBD (Figure 3E). Consistently, the levels of RBD-specific IgG, IgM, and IgA antibodies rapidly increased in the first 3 weeks, with IgG and IgM levels being maintained between days 25 and 102 (Figure 3F). We observed a trend toward decreased IgA levels from day 25 onward (Figure 3F), but only in 3/25 patients. In addition, we measured Spike-specific antibodies by ELISA and detected higher IgG, IgM, and IgA antibodies against Spike in pooled acute and convalescent COVID-19 samples compared to healthy donors (Figure 3G).

To determine the neutralizing activity of SARS-CoV-2-specific antibodies, a microneutralization (MN) assay was performed using live SARS-CoV-2 virus infection of Vero cells.¹¹ Neutralizing antibodies were detected at a low frequency in 3/8 acute patients, but were more prevalent in 11/13 convalescent samples, the latter being significantly higher than healthy non-exposed and hospitalized non-COVID patient control groups (Figure 3H). Neutralizing activity increased from the acute to the convales-

cent phase and was still detectable in 3/3 convalescent donors sampled at ~ 100 days post-symptom onset (Figure 3I). Several antibody measurements correlated significantly with each other (Figures 3J–3M). When we ranked COVID-19 patients according to the neutralizing activity of SARS-CoV-2-specific antibodies, COVID-19 patients with the highest MN activity had high levels of RBD-specific IgM, IgG, and IgA, suggesting that engagement of a broad range of antibody isotypes may lead to improved neutralizing activity (Figure 3L). Importantly, antibodies against Spike and neutralizing antibody titers correlated with RBD-specific IgG, IgM, and IgA antibodies (Figures 3J, 3K, and 3M).

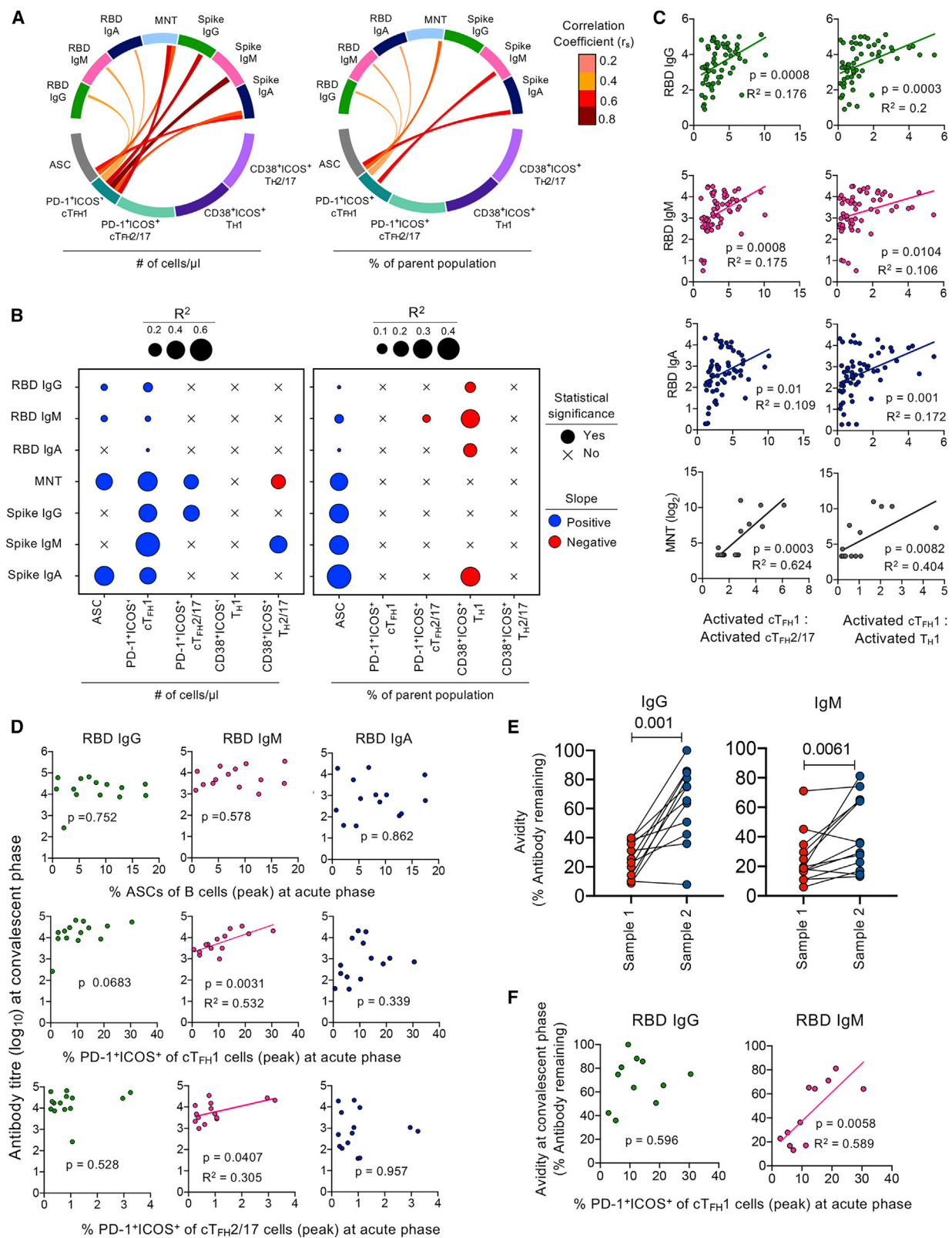
Activated PD-1⁺ICOS⁺ cT_{FH}1 cells correlate with robust humoral immunity

Consistent with previous observations, our analyses of SARS-CoV-2-specific antibodies demonstrated considerable variability among acute COVID-19 patients (Figure 3B). To understand the basis of such variability and identify potential predictors of antibody signatures, we dissected correlations between antibody responses (RBD or Spike, IgG, IgM, or IgA, MN) and immune populations involved in humoral immunity, ASCs, and activated cT_{FH}1, cT_{FH}2/cT_{FH}17, T_H1, and T_H2/T_H17 subsets (Figure 4A). We found significant positive correlations between the number of ASCs and MN titers ($r_s = 0.543$) and Spike-specific IgA antibodies ($r_s = 0.597$). The number of activated cT_{FH}1 cells positively correlated with the levels of RBD- and Spike-specific antibodies of all 3 isotypes ($r_s > 0.288$ for RBD, $r_s > 0.549$ for Spike) and MN titers ($r_s = 0.659$). We observed no significant correlations between CXCR3⁺ cT_{FH}2/cT_{FH}17 cells or activated CD4⁺ T cells not belonging to the CXCR5⁺ T_{FH} lineage and antibody responses.

To evaluate the potential of immune populations as predictors of the acute antibody response, we performed linear regression of each immune cell population against each antibody measurement and consistently found ASCs and activated cT_{FH}1 cells, but not other CD4⁺ T cell subsets, as significant positive predictors of matched RBD-specific, Spike-specific, and MN titers (Figure 4B). While in some instances, the correlations with immune

Figure 3. Antibody signatures against RBD and Spike protein in acute and convalescent COVID-19

- (A) ELISA titration curves against the SARS-CoV-2 RBD for IgG, IgM, and IgA in healthy donors ($n = 25$ – 26), acute ($n = 61$), and convalescent ($n = 63$) COVID-19 patients. Dotted line indicates the cutoff for endpoint titer determination.
- (B) Endpoint titers of SARS-CoV-2 RBD antibodies, in which the dotted line indicates the seroconversion titer.
- (C) Paired endpoint titers of SARS-CoV-2 RBD antibodies from COVID-19 patients ($n = 25$) at admission and follow-up.
- (D and E) Seroconversion rates (D) and (E) isotype profiles for RBD-specific IgG, IgM, and IgA at acute and convalescent time points.
- (F) Kinetics of RBD-specific antibodies for IgG, IgM, and IgA. LOESS regression lines with 95% confidence intervals shaded in gray are shown.
- (G) Endpoint titers of SARS-CoV-2 Spike antibodies for IgG, IgM, and IgA in healthy donors ($n = 10$ – 12) and COVID-19 ($n = 24$ – 30) plasma.
- (H) Microneutralization titers against SARS-CoV-2 in healthy ($n = 21$), COVID-19⁺ (acute $n = 8$, convalescent $n = 13$) and COVID-19[−] (acute $n = 13$, convalescent $n = 7$) sera.
- (I) Longitudinal analysis of microneutralization titers from days postsymptom onset.
- (J) Circos plot depicting correlations (links) between different antibody measurements (edges). Only significant ($p < 0.05$) correlations are shown. The color of the links represents the strength of the correlation based on the Spearman correlation coefficient, $n = 24$ samples for which all antibody measurements were available.
- (K) Correlation between RBD-specific and Spike-specific titers for IgG ($n = 44$), IgM ($n = 41$), and IgA ($n = 34$) samples.
- (L) Heatmap of microneutralization and ELISA titers. Each row represents a different sample with their matched measurements.
- (M) Correlation between microneutralization titers and RBD-specific titers for each isotype ($n = 22$ samples per isotype). Median and interquartile range (IQR) are shown throughout.
- (B and G) Black-filled symbols indicate patient no. 1-088 with rituximab treatment who was not included for statistical analysis. Statistical significance was assessed with a Kruskal-Wallis test with Dunn's correction for multiple comparisons (B and H). (C) Wilcoxon matched-pairs signed rank test or a Mann-Whitney test (G). (K and M) Spearman correlation coefficients and p values shown.



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populations were strong (e.g., activated $cT_{FH}1$ cells and Spike-specific IgM, $R^2 = 0.73$) or modest (e.g., ASC and MN titers [MNTs], $R^2 = 0.36$), they were weak for RBD-specific antibodies ($R^2 < 0.2$). Activation of non- T_{FH} cells was also a negative predictor of RBD-specific antibodies (Figure 4B). We observed that higher ratios of activated $cT_{FH}1$ cells to activated $cT_{FH}2/cT_{FH}17$ cells or activated $cT_{FH}1$ cells to activated T_H1 cells were significant positive predictors of matched MNTs ($R^2 = 0.624$ and $R^2 = 0.404$, respectively) and RBD-specific antibodies for all isotypes, albeit to a lesser extent ($R^2 < 0.2$) (Figure 4C).

As our results are based on immune and antibody analyses from longitudinal samples during acute COVID-19, and are consistent with data in convalescent individuals,¹¹ we determined whether immune populations measured at the acute phase could act as biomarkers for convalescent antibody responses. We analyzed paired longitudinal data from individuals with acute immune and convalescent RBD antibody data ($n = 14$ patients) using the peak value of the immune cell population when >1 acute measurement was available. The levels of activated $cT_{FH}1$ cells were significant positive predictors of convalescent RBD-specific IgM antibodies, by both proportion and number of cells ($R^2 = 0.531$ and $R^2 = 0.37$, respectively) (Figures 4D and S10A). Interestingly, while a similar result was also observed for the proportion of activated $cT_{FH}2/cT_{FH}17$ cells ($p = 0.0407$, $R^2 = 0.305$), this was not observed for cell numbers. Neither the proportion nor the numbers of acute ASCs were significant predictors of convalescent RBD-specific antibodies. Overall, activated $cT_{FH}1$ cells in the acute phase were associated with greater levels of RBD-specific IgM antibodies at convalescence.

Having shown that activated $PD-1^+ICOS^+ cT_{FH}1$ cells correlate with RBD-, Spike-, and MN antibody titers (thus magnitude), we asked whether these $cT_{FH}1$ cells could also be associated with qualitative features of antibodies. We measured the avidity of RBD-specific IgG and IgM antibodies using a urea dissociation assay. In paired COVID-19 plasma samples ($n = 13$) obtained >7 days apart, we found a significant increase in IgG and IgM avidity (percentage of antibody remaining after 6 M urea treatment) in the second plasma sample (Figures 4E and S10B). Avidity also increased with time, where RBD-specific IgG antibodies detected within the first week had significantly lower avidity than samples obtained ≥ 3 weeks later (Figure S10C). The proportion

of $PD-1^+ICOS^+ cT_{FH}1$ cells at the acute time points positively correlated with antibody avidity for RBD IgM ($p = 0.0058$, $R^2 = 0.589$), but not RBD IgG, at convalescence (Figure 4F). Thus, activated $PD-1^+ICOS^+ cT_{FH}1$ cells in acute COVID-19 correlate with robust humoral immunity, in both a quantitative (RBD-specific, S-specific, and MN titers) and qualitative (IgM avidity) sense. Overall, robust antibody responses elicited during SARS-CoV-2 infection were associated with T_{FH} activation; thus, cT_{FH} responses may be valuable as potential biomarkers in vaccine clinical trials.

Hyperactivated $CD38^+HLA-DR^+$ innate and adaptive immune cells, sIL-6R, and IL-18 during severe COVID-19

We selected 19 key innate and adaptive immune subsets identified to be prominent in acute versus convalescent COVID-19 samples (Figure 2) for broader analyses, including acute datasets for the 14 cytokines/chemokines and RBD-specific IgG/IgM/IgA antibodies (total 36 features), while correcting for multiple comparisons (Figure 5A), to elucidate immune mechanisms driving disease severity. In fact, 9/36 (25%) immune features were significantly enhanced in severely ill patients (ICU versus ward), including activated $CD38^+HLA-DR^+CD8^+$, $CD38^+HLA-DR^+CD4^+$, $CD38^+HLA-DR^+ \gamma\delta$ T cells, $PD-1^+ICOS^+ cT_{FH}1$ and $cT_{FH}2/cT_{FH}17$ cells, $HLA-DR^+$ NK cells, patrolling $CD16^+$ monocytes, and higher levels of IL-18 and sIL-6R, reminiscent of prolonged and high expression of $CD38^+HLA-DR^+$ on $CD8^+$ T cells associated with mortality in H7N9-infected patients.²⁶ In the hospitalized moderate patients who did not require ICU care, classical $CD14^+$ monocytes were higher and RBD-specific antibodies were not significantly different (Figure 5A). Nevertheless, the 10 features were sufficient to clearly separate samples from moderate (ward) and severe (ICU) patients by principal-component analysis (PCA), whereby the ward and ICU groups were separated along PC1 (explaining 41.4% of variance) and ICU samples further separated along PC2 (accounting for 18.1% of variance) (Figure 5B).

Hyperactivation of immune cell subsets in ICU patients was further demonstrated by dissecting immune subsets individually. This was evident when analyzing the proportion of activated $CD38^+HLA-DR^+$ immune sets (Figure 5C). Markedly higher proportions of $CD38^+HLA-DR^+CD4^+$ (18.25% versus 2.84%), $CD38^+HLA-DR^+CD8^+$ (28.9% versus 5.55%), $CD38^+HLA-DR^+$

Figure 4. $cT_{FH}1$ cells are associated with acute and convalescent antibody levels

(A) Circos plot depicting correlations (links) between different antibody measurements and various immune cells from acute COVID-19 samples ($n = 61$). Correlations for absolute numbers (left) and proportions of cells (right). Only significant ($p < 0.05$) correlations are shown. The color of the links represents the strength of the correlation based on the Spearman correlation coefficient.

(B) Summary of linear regression analysis between different antibody measurements against various immune cells from acute COVID-19 samples.

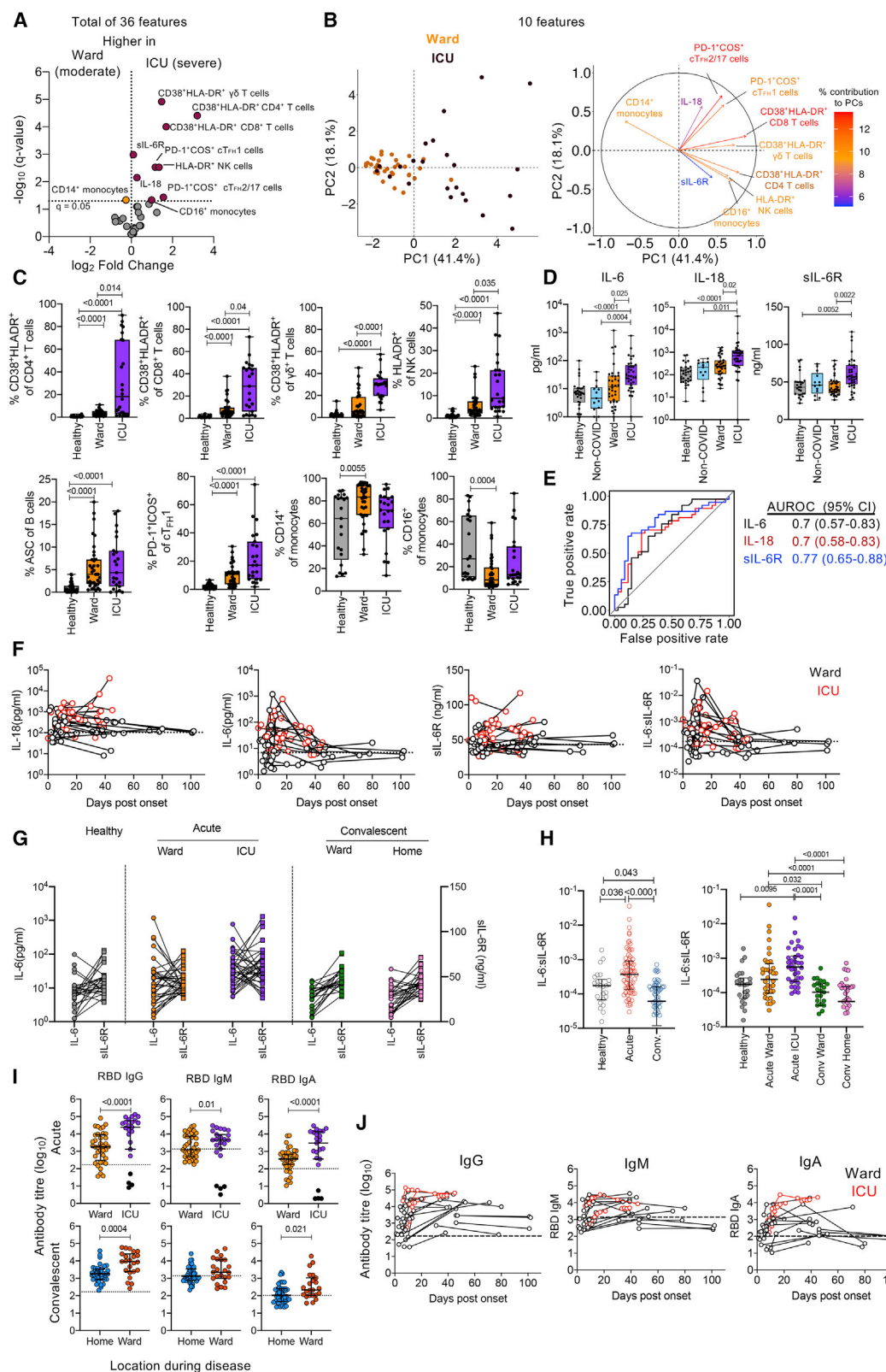
(C) Linear regression analysis of acute RBD-specific titers ($n = 60$ –61) for each isotype or microneutralization titers (MNTs, $n = 16$) and the ratio of acute activated $cT_{FH}1$ cells ($PD-1^+ICOS^+CXCR3^+CXCR5^+CD4^+$ T cells) and activated $cT_{FH}2/cT_{FH}17$ cells ($PD-1^+ICOS^+CXCR3^+CXCR5^+CD4^+$ T cells, left) or activated T_H1 cells ($CD38^+ICOS^+CXCR3^+CXCR5^+CD4^+$ T cells, right).

(D) Linear regression analysis of the proportion of acute ASCs, $PD-1^+ICOS^+ cT_{FH}1$ or $PD-1^+ICOS^+ cT_{FH}2/cT_{FH}17$ cells and paired convalescent RBD-specific titers for each isotype, $n = 14$.

(E) Avidity analysis for IgG and IgM RBD-specific antibodies in paired samples. Frequency of antibody binding after treatment with 6 M urea compared to without treatment is shown. Presented are results from a 1:100 plasma dilution for IgG and a 1:316 dilution for IgM. Samples 1 and 2 were collected 7–70 days apart. Statistical significance was assessed with a Wilcoxon matched-pairs signed rank test, $n = 13$.

(F) Linear regression analysis of the proportion of acute $PD-1^+ICOS^+ cT_{FH}1$ cells and paired convalescent avidity measurements for IgG and IgM RBD-specific antibodies, $n = 11$.

(A and B) RBD IgG/M/A $n = 60$ –61, MNTs $n = 16$, Spike IgG/IgM/IgA $n = 12$ –15.



(legend on next page)

$\gamma\delta$ T cells (30.3% versus 5.48%), HLA-DR⁺ NK cells (8.87% versus 3.63%), and patrolling CD16⁺ monocytes (12.8% versus 8.26%) were found in the ICU compared to the ward group. Strikingly, in some ICU patients, levels of hyperactivated CD38⁺HLA-DR⁺ CD4⁺ T cells constituted ~80% of all blood CD4⁺ T cells, while up to 50% of all blood CD8⁺ T cells, NK cells, and $\gamma\delta$ T cells were found in some ICU patients (Figure 5C), indicating a non-specific bystander mode of activation. Moreover, immune cell activation was clearly higher in ICU patients as the total numbers for each parent population were comparable between severity groups (Figure S11). Immune clustering further identified significant differences between ICU and ward, including higher levels of CD38⁺HLA-DR⁺ neutrophils, eosinophils, NK cells, $\gamma\delta$, CD4⁺, and CD8⁺ T cells in ICU samples (Figure S12). We also found significantly higher neutrophil:lymphocyte and neutrophil:T cell ratios in ICU patients (Figure S12).

Concomitantly, we also observed significantly different levels of IL-6, IL-18, and sIL-6R (Figure 5D). ICU patients had significantly higher levels of IL-18 (ward 238.8 pg/mL, ICU 700.9 pg/mL) and sIL-6R (ward 40.37 ng/mL, ICU 57.9 ng/mL) (Figures 5D and S11C). Importantly, we observed higher IL-6 levels in ICU compared to ward patients (ward 11.95 pg/mL, ICU 28.8 pg/mL, $p = 0.025$). Analysis of the area under the receiver operating characteristic (AUROC) curve indicated that sIL-6R (AUROC = 0.77), IL-18 (AUROC = 0.7), and IL-6 (AUROC = 0.7) are potentially predictive of ward versus ICU cases (Figure 5E).

Longitudinal analyses verified high levels of IL-18, IL-6, and sIL-6R in ICU patients when compared to the ward patients over time, while the ratio of IL-6:sIL-6R was variable among ICU and ward patients (Figures 5F and 5H). When paired IL-6 and sIL-6R data were analyzed, high IL-6 levels in acute COVID-19 did not imply high sIL-6R levels (Figures 5G and 5H). Conversely, it appeared that acute (both ward and ICU) COVID-19 samples with the highest IL-6 plasma concentration had modest levels of sIL-6R. These results have important implications for IL-6/IL-6R immunotherapies and need to be further investigated in larger COVID-19 cohorts.

Further focus on the association of disease severity with SARS-CoV-2 antibody responses revealed significantly higher levels of RBD-specific IgG, IgM, and IgA antibodies in ICU than in ward patients (Figures 5I and 5J), in agreement with previous

reports.⁸ Interestingly, convalescent patients who were originally hospitalized with COVID-19 had significantly higher RBD-specific IgG and IgA antibodies than those who were cared for at home (Figure 5I). ICU patients had higher cT_{FFH}1 responses, albeit insignificant in the proportions and numbers, but comparable ASC responses to those of the ward patients (Figure 5C).

Thus, severe COVID-19 was associated with overwhelming immune activation of innate, adaptive lymphoid and myeloid compartments, higher antibody responses supported by greater cT_{FFH}1 activation, and enhanced levels of IL-6, IL-18, and sIL-6R. Thus, IL-18 and CD38⁺HLA-DR⁺ expression may be useful biomarkers of COVID-19 severity, while sIL-6R levels are relatively low in COVID-19 patients (with the exception of ICU patients), in comparison to the levels detected in cancer patients (up to 195 ng/mL).²⁹

IL-6/sIL-6R and IL-18 hypercytokinemia associated with dysregulation of innate and adaptive immunity

To gain insights into the basis of the immune hyperactivation profiles, we performed correlation analysis of 14 cytokines and 22 immune cell populations (Figures 6A–6C). IL-6 positively correlated with IL-8, IL-18, and IFN- γ (Figure 6A), where elevated levels of IL-6 were reported for severe influenza disease and mortality.^{30,31} Consistent with other studies,² we found that IL-6 negatively correlated with lymphocyte numbers in blood (Figure 6B), possibly reflecting the efflux of immune cells from blood to the site of infection. Importantly, we also found positive correlations between levels of IL-6 or IL-18 with T cells and NK cell hyperactivation (Figures 6B and 6C). IL-6 and IL-18 significantly correlated with the proportion of CD38⁺HLA-DR⁺ $\gamma\delta$ T cells ($r_s = 0.37$, $p = 0.004$ and $r_s = 0.53$, $p < 0.0001$, respectively) and IL-18 significantly correlated with the proportion of CD38⁺HLA-DR⁺ CD4⁺ T cells ($r_s = 0.29$, $p = 0.028$) (Figure 6C). We also found that the proportions of CD14⁺ and CD16⁺ monocytes were positively and negatively associated with the levels of MCP-1, respectively. Significant correlations were detected between the levels of sIL-6R and CD38⁺HLA-DR⁺ $\gamma\delta$ T cells ($r_s = 0.35$, $p = 0.008$), CD38⁺HLA-DR⁺CD8⁺ T cells ($r_s = 0.32$, $p = 0.012$), CD38⁺HLA-DR⁺CD4⁺ T cells ($r_s = 0.42$, $p = 0.0013$), and HLA-DR⁺ NK cells ($r_s = 0.36$, $p = 0.006$). Interestingly, significant correlations were found between sIL-6R with RBD IgG,

Figure 5. sIL-6R and IL-18 are predictors of severe COVID-19

- (A) Volcano plot of differential immune profiles between acute ward and ICU samples based on 19 manually gated immune cell populations, 14 cytokines/chemokine, and 3 RBD-antibody titers.
- (B) PCA analysis of ward and ICU samples using the 10 significant features. Individual samples color-coded based on severity group (left) and the contribution of each feature to the principal components (right) are shown.
- (C) Proportion of acutely activated lymphocytes and monocytes in ward ($n = 36$ –37) and ICU samples ($n = 23$) based on manual gating strategy.
- (D) Acute cytokine levels in healthy controls ($n = 32$), non-COVID-19 influenza-like illness (ILI) ($n = 13$), ward ($n = 36$), and ICU samples ($n = 36$).
- (E) AUROC analysis of IL-6, IL-18, and sIL-6R for discriminating ward versus ICU samples.
- (F) Longitudinal tracking of cytokine levels in ward and ICU patients.
- (G) Matched IL-6 and IL-6R levels in acute ward and ICU patients.
- (H) IL-6:IL-6R ratios in healthy, acute (ward or ICU), and convalescent individuals.
- (I) Endpoint titers of SARS-CoV-2 RBD antibodies for IgG, IgM, and IgA in acute COVID-19 plasma samples from individuals in hospital ward or ICU during the acute phase (top) or in convalescent plasma samples from individuals who were at home ($n = 40$) or in the hospital ward ($n = 24$) during acute COVID-19.
- (J) Longitudinal antibody levels of ward and ICU matched patient samples for RBD-specific IgG, IgM, and IgA. The dotted line represents the seropositivity cutoff value for each isotype (antibody titer > mean + 2 $\times$ SD of healthy individuals).
- (C, D, H, and I) Statistical significance was assessed with a Kruskal-Wallis test with Dunn's correction for multiple comparisons; median and IQR are shown throughout.

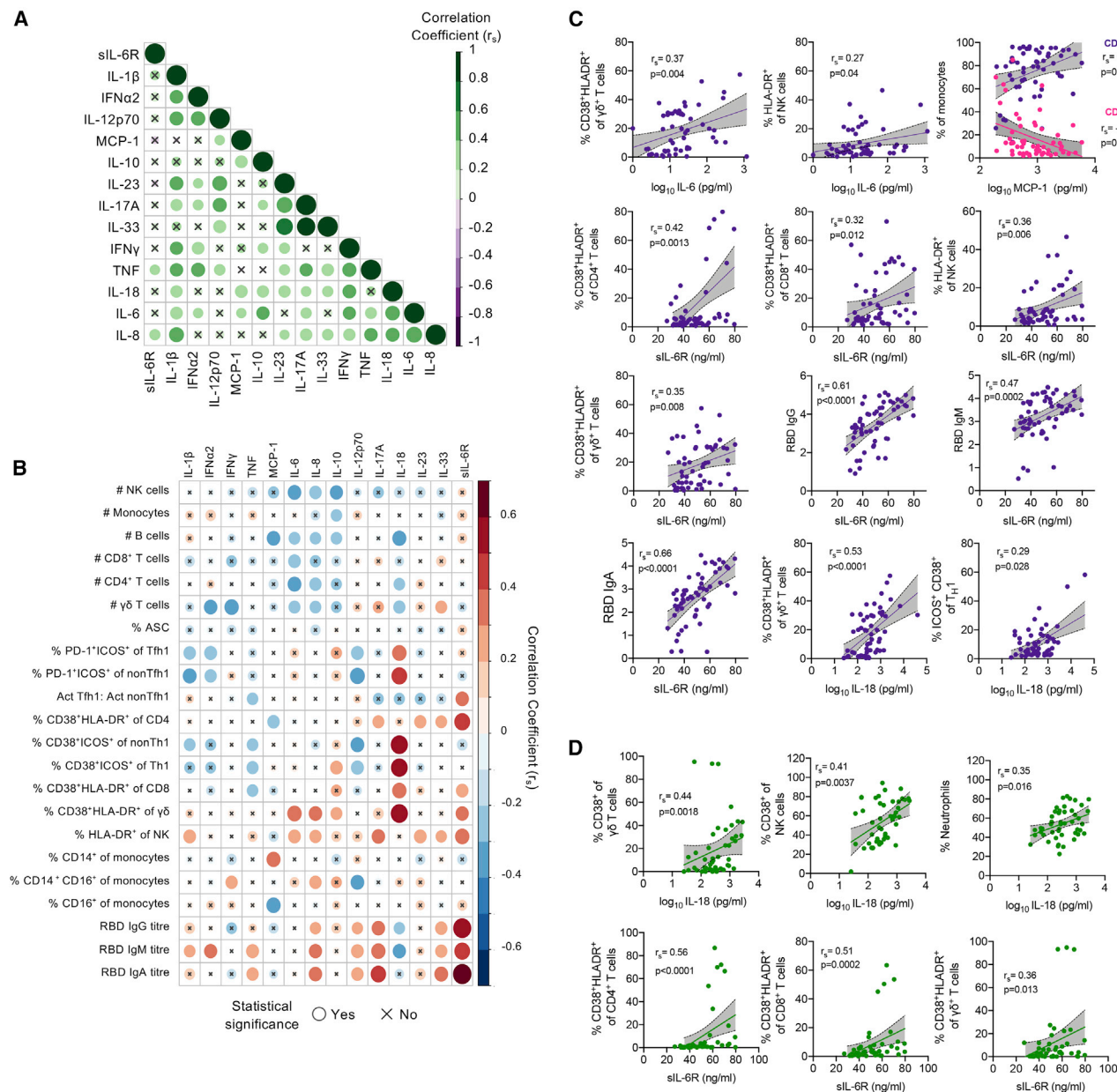


Figure 6. IL-6, sIL-6R, and IL-18 hypercytokinemia are associated with dysregulation of innate and adaptive immunity

(A) Correlation matrix of cytokines in all acute samples. Statistical significance was defined as false discovery rate (FDR)-corrected p value $q < 0.1$.

(B) Heatmap summarizing the correlations between cytokine levels and immune populations in acute COVID-19 based on manual gating strategy. Statistical significance was defined as FDR-corrected p value $q < 0.1$.

(C and D) Representative plots of significant correlations between cytokine levels and immune cell populations from acute samples ($n = 57$) based on manual gating strategy and RBD-antibody titers (C) and FlowSOM analysis (D).

IgM, and IgA titers, with those being higher in ICU samples. We found consistent results when analyzing the proportions of immune cell clusters determined by FlowSOM, with IL-6 and IL-18 positively correlating with activation of myeloid cells, T cells, and NK cells (Figures 6D and S13).

Overall, our study provides a comprehensive map of longitudinal immunological responses in COVID-19 patients at acute and

convalescent phases of SARS-CoV-2 infection (Figure S14) and demonstrates prototypical antiviral immunity in mild/moderate cases, but dysregulated immune hyperactivation in severe COVID-19. Our analyses unify the levels of IL-6, sIL-6R, and IL-18 and their association with severe COVID-19 with the immune hyperactivation profiles at the cellular level characterized by high CD38 expression (Figure 6D).

DISCUSSION

Immune responses toward non-severe SARS-CoV-2 infection resemble a prototypical antiviral immune response, with transient activation of innate myeloid and lymphoid populations at the acute phase. These include cT_{FH}1 cells, T_H1 CD4⁺ T cells, highly cytotoxic CD38⁺HLA-DR⁺CD8⁺ T cells, SARS-CoV-2-specific B cells, and antibodies. This is followed by the subsequent contraction of immune responses after disease resolution, as shown by us and others,^{3,6,11} similar to other acute respiratory infections.^{27,32} Despite prototypical immune responses found in mild/moderate COVID-19 cases, profoundly dysregulated innate and adaptive immunity underlie severe COVID-19, including elevated plasma IL-18 and sIL-6R levels, and hyperactivation of innate, adaptive, and myeloid compartments in ICU COVID-19 patients. Our study provides a comprehensive map of longitudinal immunological responses in COVID-19 patients at acute and convalescent phases and integrates key cellular pathways of complex perturbed immune networks underpinning critical COVID-19, providing important insights into biomarkers and immunotherapies.

Our analysis of humoral immune responses to SARS-CoV-2 demonstrated generation of RBD- and Spike-specific IgG, IgM, and IgA antibodies and their correlation with SARS-CoV-2 neutralization activity. Early activation of ASCs, likely of extrafollicular origin, was associated with Spike-specific IgG, IgM, and IgA antibodies, antibody neutralization activity, and, to a lesser extent, RBD-specific titers. Our data are in agreement with the emergence of IgG, IgM, and IgA ASC in COVID-19 patients, some specific for non-Spike antigens (e.g., nucleocapsid protein), which may explain the lack of a strong correlation between ASCs and RBD-specific antibodies in our cohort and other studies.^{4,6}

Activation of cT_{FH}1 cells is an important correlate of antibody responses following influenza vaccination,²⁵ yellow fever,³³ and hepatitis C virus (HCV).³⁴ Although the lack of Bcl6⁺ T_{FH} cells in lymphoid organs of severely ill COVID-19 patients was shown,³⁵ cT_{FH} response at the convalescent phase was associated with antibody responses to SARS-CoV-2.¹¹ Our analysis extends this observation to the acute phase and importantly associates acute cT_{FH}1 responses with convalescent IgM antibody titers. It also demonstrates an association between cT_{FH}1 responses and an increase in antibody avidity, potentially reflecting affinity maturation in germinal centers. We found that greater activation of cT_{FH}1 cells relative to other CD4⁺ T cell subsets was associated with greater RBD-specific responses and better neutralization activity. Our findings thus support the use of adjuvants that can induce strong T_{FH} responses to improve vaccine efficacies. In humans, GLA-SE in the malaria vaccine induced greater cT_{FH} responses than alum,³⁶ while in non-human primates and mice, MF59 enhanced cT_{FH} activation and antibody responses to HIV-1 and influenza, respectively.^{37,38} Since some of the SARS-CoV-2 vaccines in clinical trials are formulated with alum or MF59,³⁹ it may be important to compare immunogenicity across vaccine formulations.

IL-6 is the key driver of an inflammatory cascade in severe COVID-19;^{1,2,20} thus, therapies targeting sIL-6R are in clinical trials for SARS-CoV-2 infection. Two humanized monoclonal anti-

bodies against IL-6R, tocilizumab (Roche) and sarilumab (Sanofi), safely used for autoimmune diseases, are being evaluated in COVID-19, but neither has met the primary endpoints.^{40,41} There have been limited analyses of sIL-6R in acute and convalescent COVID-19 patients. Surprisingly, although the plasma sIL-6R levels were significantly elevated in severe COVID-19 compared to moderate disease, they remained within the healthy range (up to 80 ng/mL).⁴² Interestingly, while IL-6 was considerably elevated in acute COVID-19 samples, patients with the highest IL-6 concentrations had only modest sIL-6R levels, and levels of sIL-6R did not correlate with IL-6 or any other cytokines. In addition, the elevated levels of IL-6 and IL-18 found in critically ill COVID-19 patients are in the same range as those of patients with non-COVID-19 acute respiratory distress syndrome (ARDS) or sepsis.⁴³ Our results suggest that patients should be screened for both IL-6 and sIL-6R before the initiation of anti-IL-6R therapy, especially since critically ill COVID-19 patients receiving anti-IL-6R therapies may be at higher risk of nosocomial infections.⁴⁴ Interestingly, the levels of IL-6 correlate with lymphocytopenia in our cohort and others,² and treatment with anti-sIL-6R therapies can restore lymphocyte numbers. We also found a correlation between sIL-6R and the activation of CD8⁺, CD4⁺, and $\gamma\delta$ T cells. Since activated T cells are considered a major source of sIL-6R⁴⁵ and severe COVID-19 presents with T cell hyperactivation, this may explain the elevated levels of sIL-6R and support the idea that sIL-6R may be an informative biomarker.

IL-18 was also associated with COVID-19 severity in our analysis and another report.⁷ We found a correlation between IL-18 levels and the activation of innate NK cells and $\gamma\delta$ T cells, as well as CD4⁺ and CD8⁺ T cells, which is consistent with the potential of IL-18/IL-18R signaling to induce bystander activation of innate and adaptive T cells.^{46–48} These observations unify the elevated levels of cytokines, such as IL-6 and IL-18, and their signaling pathways with hallmark observations of severe COVID-19 disease, like hyperactivation of T cells and elevated antibody levels, and thus provide insights into underlying mechanisms and a potential therapeutic role for anti-IL-18 therapies.^{49,50} The use of dexamethasone showed benefits in severely ill COVID-19 patients,¹⁵ consistent with immune hyperactivation observed in those patients. However, neither sIL-6R nor IL-18 were universally higher in ICU patients, nor do they fully explain the immunological differences between ward and ICU samples. Our data further highlight the need for multi-parameter immune profiling of severe COVID-19 patients to identify and appropriately select personalized immune-modulatory treatments.

The hyperactivation of immune cells in severe ICU cases, suggestive of substantial immune dysregulation, was clearly evident from elevated CD38 expression on a broad range of immune cells. The upregulation of CD38 and HLA-DR on T cells in COVID-19 in our study and by others^{4,6,8} is consistent with the high levels of CD38⁺HLA-DR⁺ CD8⁺ T cells observed in HIV,⁵¹ Ebola,⁵² HCV,⁵³ and dengue.⁵⁴ Our data from severe COVID-19 cases support our previous study showing high and prolonged proportion of CD38⁺HLA-DR⁺ CD8⁺ T cells (up to 50% for ~30 days) in patients who died from avian A/H7N9 influenza.²⁶ Numerically, 50%–80% of CD38⁺HLA-DR⁺ T cells found

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in the peripheral blood of severe H7N9 or COVID-19 cases unlikely reflect epitope-specific T cell activation. In fatal H7N9 cases, delayed clonal expansion of H7N9-specific CD8⁺ T cells and distinct transcriptome signatures of CD38⁺HLA-DR⁺ CD8⁺ T cells were indicative of bystander activation. In COVID-19 patients, such T cell hyperactivation was associated with elevated cytokine levels that may drive bystander activation, similar to H7N9 hypercytokinemia.³¹ Thus, while rapid and transient CD38⁺HLA-DR⁺ expression can be detected in patients with relatively rapid recovery,²⁶ prolonged and high expression levels are associated with severe and/or fatal outcomes in COVID-19 and avian H7N9.^{3,26} Given that high CD38 expression is a poor prognostic factor in cancers and HIV,⁵⁵ monitoring CD38 expression on a wide range of immune cells may be a useful marker of immune hyperactivation and disease progression in COVID-19.

Limitations of study

We recognize that some of the analyses performed are limited to a relatively small number of patients available. This is particularly relevant to the analysis of ICU samples, the longevity analysis of antibodies, and the analysis of neutralizing titers. In addition, we could not stratify our cohort based on age groups or the use of immunomodulatory treatments, which should be the focus of future studies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xcrm.2021.100208>.

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AUTHOR CONTRIBUTIONS

K.K. led the study. K.K., T.H.O.N., and A.C.C. supervised the study. K.K., T.H.O.N., M.K., L.C.R., L.H., B.Y.C., C.E.v.d.S., W.Z., and E.B.C. designed the experiments. M.K., L.C.R., L.H., B.Y.C., C.E.v.d.S., J.R.H., W.Z., X.J., L.K., C.Y.W., F.L.M., and T.H.O.N. performed and analyzed the experiments. M.K., T.M.A., G.H.P., F.M.-W., M.N.R., and D.N.E. performed the computational analysis. F.A. and F.K. provided the reagents. J.A.J. and J.A. managed the patient cohorts. J.A.J., N.E.H., C.L.G., O.C.S., J.A.T., C.M.H., J.T.D., S.Y.C.T., D.L.D., T.C.K., A.K.W., S.J.K., J.M., I.T., and A.C.C. recruited the patient cohorts. D.C.J., D.I.G., A.W.C., M.C., N.J.C.K., S.R.L., K.S., T.H.O.N., A.C.C., and K.K. provided intellectual input into the study design and data interpretation. M.K., T.H.O.N., A.C.C., and K.K. wrote the manuscript. All of the authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD45RA HI100 FITC	BD PharMingen	Cat#555488; RRID: AB_395879
CD8a SK1 PerCP-Cy5.5	BD PharMingen	Cat#565310; RRID: AB_2687497
CD56 MEM-188 APC	Biolegend	Cat#304610; RRID: AB_314452
CD16 3G8 AF700	Biolegend	Cat#302026; RRID: AB_2278418
CD14 MΦP9 APC-Cy7	BD PharMingen	Cat#560180; RRID: AB_1645464
CD19 HIB19 BV570	Biolegend	Cat#302236; RRID: AB_2563606
HLA-DR L243 BV605	Biolegend	Cat#307640; RRID: AB_2561913
CD4 SK3 BV650	BD Biosciences	Cat#563875; RRID: AB_2744425
CD27 L128 BV711	BD Horizon	Cat#563167; RRID: AB_2738042
CD38 HIT2 BV786	BD Horizon	Cat#563964; RRID: AB_2738515
CD3 UCHT1 PECF594	BD Biosciences	Cat#562280; RRID: AB_11153674
CD45RA HI100 FITC	BD PharMingen	Cat#555488; RRID: AB_395879
CD8a RPA-T8 BV421	Biolegend	Cat#301036; RRID: AB_10960142
CD14 MΦP9 APC-H7	BD PharMingen	Cat#560180; RRID: AB_1645464
CD19 SJ25C1 APC-H7	Biolegend	Cat#560177; RRID: AB_1645470
HLA-DR L243 BV605	Biolegend	Cat#307640; RRID: AB_2561913
CD4 SK3 BV650	BD Biosciences	Cat#563875; RRID: AB_2744425
CD27 L128 BV711	BD Horizon	Cat#563167; RRID: AB_2738042
CD38 HIT2 BV786	BD Horizon	Cat#563964; RRID: AB_2738515
CD3 UCHT1 PECF594	BD Biosciences	Cat#562280; RRID: AB_11153674
Granzyme A CB9 PE	eBioscience	Cat#12-9177-42; RRID: AB_2572701
Granzyme B GB11 AF700	BD PharMingen	Cat#560213; RRID: AB_1645453
Granzyme K G3H69 PerCP-eFluor710	eBioscience	Cat#46-8897-42; RRID: AB_2573854
Granzyme M 4B2G4 eFluor660	eBioscience	Cat#50-9774-42; RRID: AB_2574374
Perforin B-D48 Pe-Cy7	Biolegend	Cat#353316; RRID: AB_2571973
Peroxidase AffiniPure goat anti-human IgG, Fcγ fragment specific	Jackson ImmunoResearch	Cat#109-035-098; RRID: AB_2337586
Rat anti-human IgA mAb MT20, alkaline phosphate-conjugated	MabTech	Cat#3860-9A; RRID: AB_10736550
Mouse anti-human IgM mAb MT22, biotinylated	MabTech	Cat#3880-6-250
Bacterial and virus strains		
SARS-CoV-2 isolate CoV/Australia/VIC01/2020	Caly et al. ⁵⁶	N/A
Biological samples		
Blood samples (peripheral blood mononuclear cells (PBMCs), serum and plasma samples) from COVID-19 patients and healthy control individuals	Alfred Hospital, Melbourne Health, Monash Health, Austin Health, The University of Melbourne and James Cook University (Australia)	N/A
Chemicals, peptides, and recombinant proteins		
AccuCheck Counting Beads	Thermo Fisher Scientific	Cat#PCB100
3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA, peroxidase substrate	Sigma	Cat#T0440-1L

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alkaline phosphatase yellow (pNPP) liquid substrate for ELISA	Sigma	Cat#P7998-100ML
Pierce High Sensitivity Streptavidin-HRP	Thermo Fisher Scientific	Cat#21130
SARS-CoV-2 RBD protein	Amanat et al. ⁹	N/A
SARS-CoV-2 Spike protein	Juno et al. ⁵⁷	N/A
Critical commercial assays		
eBioscience™ Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat#00-5521-00
LEGENDplex Human Inflammation Panel 1 kit	BioLegend	Cat#740809
Human IL-6R alpha DuoSet ELISA kit	R&D Systems	Cat#RDSY227
Experimental models: cell lines		
Vero C1008, African Green monkey kidney cells	ATCC	Cat#CRL-1586; Lot#3338237; RRID: CVCL_0574
Software and algorithms		
R v3.6.2	The Comprehensive R Archive Network	https://cran.r-project.org
Spectre R package	Ashhurst et al. ⁵⁸	https://github.com/ImmuneDynamics/Spectre
ggplot2 R package	Wickham ⁵⁹	https://cran.r-project.org/web/packages/ggplot2/index.html
circize R package	Gu et al. ⁶⁰	https://cran.r-project.org/web/packages/circize/index.html
corrplot R package	N/A	https://cran.r-project.org/web/packages/corrplot/index.html
pROC R package	Sachs ⁶¹	https://cran.r-project.org/web/packages/pROC/index.html
plotROC package	Sachs ⁶¹	https://cran.r-project.org/web/packages/plotROC/index.html
FlowJo v10.5.3	N/A	https://www.flowjo.com
CytoNorm	Van Gassen et al. ⁶²	https://www.github.com/saeyslab/CytoNorm
FlowSOM	Van Gassen et al. ¹⁸	https://github.com/SofieVG/FlowSOM
UMAP	McInnes and Healy ⁶³	https://arxiv.org/abs/1802.03426
TrackSOM	This paper	https://www.github.com/ghar1821/TrackSOM
Pestle v2 and Spice v6	Roederer et al. ⁶⁴	https://niaid.github.io/spice/
Prism v8.3.1	GraphPad	https://www.graphpad.com
BD FACS Diva v8.0.1	BD Biosciences	https://www.bdbiosciences.com/en-us/instruments/research-instruments/research-software/flow-cytometry-acquisition/facsdiva-software

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Katherine Kedzierska (kkedz@unimelb.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data supporting the findings of this study are available without restriction from the Lead Contact, Katherine Kedzierska (kkedz@unimelb.edu.au) upon request. α code can be downloaded from <https://www.github.com/ghar1821/TrackSOM>.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We enrolled consenting adult patients at Victorian hospitals during the first and second waves of the SARS-CoV-2 pandemic (February–September 2020) with influenza-like illness (ILI), including 33 SARS-CoV-2 PCR-positive patients and 11 patients who presented with ILI but were SARS-CoV-2 PCR negative as negative serological controls. Out of the 33 COVID-19 symptomatic/hospitalized patients, 20 were admitted to the ward, with 4 patients requiring non-invasive oxygen support, 12 were in ICU with 8 requiring mechanical ventilation and 3 requiring non-invasive oxygen support, and 1 was an outpatient (Table S1). For 7 ICU patients only plasma samples were available for cytokine analysis. No patients died during the study. Heparinised blood samples were collected within 24–72 hours of hospital admission (Acute Visit 1, A1) with sequential bleeds every 1–5 days apart until discharge (A2–A6). For 12 COVID-19 patients, a follow-up blood sample was taken approximately 30 days after discharge/recovery (Convalescent Visit 1, C1; median 43 days, 31–80 range). Additional SARS-CoV-2 PCR-confirmed patients ($n = 52$) and pre-pandemic healthy donors ($n = 66$) were recruited through contacts with the investigators and asked to provide a blood sample at the time of enrolment (A1 $n = 1$ and C1 $n = 51$; median 40 days, 26–102 range) and an additional time-point where available (C2). Demographic, clinical and sampling information for COVID-19 patients are described in Table S2.

Human experimental work was conducted according to the Declaration of Helsinki Principles and the Australian National Health and Medical Research Council Code of Practice. All participants provided written informed consent prior to the study. The study was approved by the Alfred Hospital (#280/14), Melbourne Health (HREC/17/MH/53), Monash Health (HREC/15/MonH/64/2016.196), Austin Health (HREC/63201/Austin-2020), The University of Melbourne (#2056689, #2056761, #1442952, #1955465, #2057366.1 and #1443389) and James Cook University (H7886) Human Research Ethics Committees.

METHOD DETAILS

Whole blood flow cytometry

Fresh whole blood was used to measure cell populations, essentially as described,³ using three human antibody panels for enumerating immune cell activation (monocytes and T/B/NK/ $\gamma\delta$ cells), T_{FH} and ASC cell activation, and cytotoxicity profiles of T cell's expressing intracellular granzymes (A, B, K and M) and perforin. Cells were stained, RBC lysed, then fixed in 1% PFA, or intracellularly stained using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Carlsbad, CA, USA), as previously described.³ AccuCheck Counting Beads (Thermo Fisher Scientific) were added to the immune cell activation panel for calculating absolute numbers just prior to acquisition. Samples were acquired on a BD LSRII Fortessa and analyzed using FlowJo v10 software.

RBD and Spike protein ELISAs

Detection of RBD- and Spike-specific antibodies was performed as described^{9,65} with the following modifications; Nunc MaxiSorp flat bottom 96-well plates (Thermo Fisher Scientific) were used for antigen coating, blocking performed with PBS (containing w/v 10% BSA) and serial dilutions performed in PBS (containing v/v 0.05% Tween and w/v 5% BSA). For detection of IgG and IgA, peroxidase-conjugated goat anti-human IgG (Fc γ fragment specific; Jackson ImmunoResearch) or alkaline phosphatase-conjugated rat anti-human IgA (mAb MT20; MabTech), was used and developed with TMB (Sigma) substrate for IgG or pNPP (Sigma) for IgA. For IgM, biotinylated mAb MT22 and peroxidase-conjugated streptavidin (Pierce; Thermo Fisher Scientific) was used. Peroxidase reactions were stopped using 1M H_3PO_4 and plates read on a Multiskan plate reader (Labsystems). Inter- and intra-experimental measurements were normalized using a positive control plasma from a COVID-19 patient (#1-073) run on each plate. Endpoint titers were determined by interpolation from a sigmoidal curve fit (all R-squared values > 0.95 ; GraphPad Prism 8) as the reciprocal dilution of plasma that produced $\geq 15\%$ (for IgA and IgG) or $\geq 30\%$ (for IgM) absorbance of the positive control at a 1:31.6 (IgG and IgM) or 1:10 dilution (IgA). Patients were considered to have seroconverted if titers were above the mean of titers from 16 healthy non-COVID19 donors $+2 \times$ the standard deviation.

Antibody avidity assay

The avidity of RBD-specific IgG and IgM antibodies in plasma samples was measured using urea as the chaotropic agent. Following incubation of plasma at a 1:31.6, 1:100 and 1:316 dilution on antigen-coated plates for 2 h, 6M of urea was added after washing and incubated for 15 min. Bound antibodies were then detected using respective secondary detection reagents in procedures described above. Antibody avidity is expressed as the percentage of remaining antibody bound to antigen following urea treatment compared to the absence of urea. Data of 1:100 diluted plasma for IgG and 1:316 for IgM are reported in Figure 4E, with the rest of the data presented in Figure S7A. Data from 1:100 and 1:316 diluted plasma were used for linear regression analysis of IgG and IgM avidity respectively in Figure 4F.

Microneutralisation assay

Microneutralisation activity of serum samples was assessed as previously described.⁵⁷ SARS-CoV-2 isolate CoV/Australia/VIC01/2020⁵⁶ was propagated in Vero cells and stored at -80°C . Serum samples were heat inactivated at 56°C for 30 min. Samples were serially diluted two-fold starting at 1:20 and 100 TCID₅₀ of SARS-CoV-2 in MEM/0.5% BSA were added and incubated at room temperature for 1 h. Residual virus infectivity in the serum/virus mixtures was assessed in quadruplicate wells of Vero cells incubated in serum-free media containing 1 $\mu\text{g ml}^{-1}$ of TPCK trypsin at 37°C and 5% CO₂; viral cytopathic effect was read on day 5. The neutralising antibody titer was calculated using the Reed–Muench method, as previously described.⁵⁷

Cytokine analysis

Patient's plasma was diluted 1:2 for measuring IL-1 β , IFN- α 2, IFN γ , TNF α , MCP-1 (CCL2), IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, IL-18, IL-23 and IL-33 using the LEGENDplex Human Inflammation Panel 1 kit, according to manufacturer's instructions (BioLegend, San Diego, CA, USA). sIL-6R levels were measured in plasma (diluted 1:300) using the Human IL-6R alpha DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Computational flow cytometry analysis

Computational analysis of data was performed using the Spectre R package⁵⁸ (<https://github.com/ImmuneDynamics/Spectre>). Samples were initially prepared in FlowJo, and populations of interest were exported as CSV files containing scale value (raw) data. In R, data were subject to arcsinh transformation, and data below the limit of detection were compressed to reduce the contribution of noise to the clustering process. Batch alignment was then performed using CytoNorm⁶² as implemented in Spectre. Aligned data were then clustered using FlowSOM and a subset of cells plotted using UMAP.⁶³ Cluster identities were annotated manually. Cellular expression of dynamic markers (CD38, HLA-DR, ICOS, PD-1, granzymes, or perforin) was determined on each population using the non-aligned arcsinh transformed data, and adjusted manually per sample where required. Data from the immune panel were clustered and plotted by UMAP using forward scatter (FSC), side scatter (SSC), CD16, CD14, CD56, CD19, TCR $\gamma\delta$, CD3, CD4, CD8, CD45RA, CD27, CD38, and HLA-DR. Immune cell lineages were manually annotated based on marker expression: eosinophils (FSC^{hi}SSC^{hi}CD16⁺), neutrophils (FSC^{int}SSC^{int}CD16^{hi}), monocytes (FSC^{int}CD14⁺), B cells (CD19⁺), NK cells (CD56⁺), gamma-delta T cells (TCR $\gamma\delta$ CD3⁺), CD4⁺ T cells (CD3⁺CD4⁺), and CD8⁺ T cells (CD3⁺CD8⁺). Subsequently, population subsets were manually annotated based on marker expression: monocytes (classical CD14⁺CD16⁺ and non-classical CD14⁺CD16⁺), B cells (naive CD27⁺CD38⁺, memory CD27⁺CD38⁺, ASC CD27⁺CD38⁺), NK cells (CD56^{bri} and CD56^{dim}), CD4⁺ T cells (naive CD45RA⁺CD27⁺, T_{EMRA} CD45RA⁺CD27⁺, T_{CM} CD45RA⁺CD27⁺, and T_{EM} CD45RA⁺CD27⁺), and CD8⁺ T cells (naive CD45RA⁺CD27⁺, T_{EMRA} CD45RA⁺CD27⁺, T_{CM} CD45RA⁺CD27⁺, and T_{EM} CD45RA⁺CD27⁺). Lymphocytes from the T_{FH} & B cell panel were clustered and plotted by UMAP using on CD45, CD3, CD19, CD4, CD8, CXCR5, CXCR3, CD27, CD38. CD4⁺ T cell subsets were manually annotated based on marker expression: T_H1 cells (CXCR5⁺CXCR3⁺), T_H2/17 cells (CXCR5⁺CXCR3⁺), T_{FH}1 cells (CXCR5⁺CXCR3⁺), and T_{FH}2/17 cells (CXCR5⁺CXCR3⁺). CD3⁺ cells from the cytotoxicity panel were clustered and plotted by UMAP using CD4, CD8, CD45RA, CD27. CD4⁺ and CD8⁺ T cell populations were defined as above for naive CD45RA⁺CD27⁺, T_{EMRA} CD45RA⁺CD27⁺, T_{CM} CD45RA⁺CD27⁺, and T_{EM} CD45RA⁺CD27⁺.

Summary statistics, scatter graphs, volcano plots and PCA plots were created in R, where comparisons were performed using a Wilcoxon rank-sum test (equivalent to the Mann-Whitney test) with the *wilcox.test* function in R. Statistics displayed in scatter graphs were uncorrected, and statistics displayed in volcano plots were corrected with a False Discovery Rate (FDR) adjustment. TrackSOM is a time series-based clustering and cluster evolution tracking algorithm which that combines FlowSOM's single time-point clustering capacity with ChronoClust's¹⁹ tracking of cluster developments over time. TrackSOM was performed on the first immunophenotyping panel by initially clustering data from all time points using FlowSOM, and thereafter tracking the resulting metaclusters and clusters using ChronoClust's tracking mechanism. TrackSOM was using 10x10 grid, producing 40 metaclusters per time. Binned groups are outlined in Table S3.

Statistical analyses

Statistical significance was assessed using Mann-Whitney, Wilcoxon signed-rank test or Kruskal-Wallis test with Dunn's correction for multiple comparisons in Prism 8 (GraphPad) unless stated otherwise. Analysis of immune parameters against days post-onset were visualized in R 3.6.2 using the ggplot2 package⁵⁹ using LOESS fitting with 95% confidence intervals shaded in gray. Correlations were assessed using Spearman's correlation coefficient (r_s) and visualized in R 3.6.2 as circos plots using the circlize package⁶⁰ or heatmaps using the corrplot package and p values of correlations were corrected for multiple comparisons by FDR in R 3.6.2. Cytokine concentrations were $\log_{10}(x+1)$ transformed for correlation analysis. Linear regression analysis was performed in Prism 8 (GraphPad). AUROC analysis was performed in R 3.6.2 using the pROC and plotROC packages.⁶¹ Pie charts in Figure 3 were generated using Pestle v2 and Spice v6 software⁶⁴ and p-values were calculated using a Permutation Test. P-values lower than 0.05 were considered statistically significant.



Breadth of concomitant immune responses prior to patient recovery: a case report of non-severe COVID-19

To the Editor — We report the kinetics of immune responses in relation to clinical and virological features of a patient with mild-to-moderate coronavirus disease 2019 (COVID-19) that required hospitalization. Increased antibody-secreting cells (ASCs), follicular helper T cells (T_{FH} cells), activated $CD4^+$ T cells and $CD8^+$ T cells and immunoglobulin M (IgM) and IgG antibodies that bound the COVID-19-causing coronavirus SARS-CoV-2 were detected in blood before symptomatic recovery. These immunological changes persisted for at least 7 d following full resolution of symptoms.

A 47-year-old woman from Wuhan, Hubei province, China, presented to an emergency department in Melbourne, Australia. Her symptoms commenced 4 d earlier with lethargy, sore throat, dry cough, pleuritic chest pain, mild dyspnea and subjective fevers (Fig. 1a). She traveled from Wuhan to Australia 11 d before presentation. She had no contact with the Huanan seafood market or with known COVID-19 cases. She was otherwise healthy and was a non-smoker taking no medications. Clinical examination revealed a temperature of 38.5 °C, a pulse rate of 120 beats per minute, a blood pressure of 140/80 mm Hg, a respiratory rate of 22 breaths per minute, and oxygen saturation 98% while breathing ambient air. Lung auscultation revealed bi-basal rhonchi. At presentation on day 4, SARS-CoV-2 was detected in a nasopharyngeal swab specimen by real-time reverse-transcriptase PCR. SARS-CoV-2 was again detected at days 5–6 in nasopharyngeal, sputum and fecal samples, but was undetectable from day 7 (Fig. 1a). Blood C-reactive protein was elevated at 83.2, with normal counts of lymphocytes (4.3×10^9 cells per liter (range, 4.0×10^9 to 12.0×10^9 cells per liter)) and neutrophils (6.3×10^9 cells per liter (range, 2.0×10^9 to 8.0×10^9 cells per liter)). No other respiratory pathogens were detected. Her management was intravenous fluid rehydration without supplemental oxygenation. No antibiotics, steroids or antiviral agents were administered. Chest radiography demonstrated bi-basal infiltrates at day 5 that cleared on day 10 (Fig. 1b). She was discharged to home isolation on day 11. Her symptoms resolved

completely by day 13, and she remained well at day 20, with progressive increases in plasma SARS-CoV-2-binding IgM and IgG antibodies from day 7 until day 20 (Fig. 1c and Extended Data Fig. 1). The patient was enrolled through the Sentinel Travelers Research Preparedness Platform for Emerging Infectious Diseases novel coronavirus substudy (SETREP-ID-coV) and provided written informed consent before the study. Patient care and research were conducted in compliance with the Case Report guidelines and the Declaration of Helsinki. Experiments were performed with ethics approvals HREC/17/MH/53, HREC/15/MonH/64/2016.196 and UoM#1442952.1/#1443389.4.

We analyzed the kinetics and breadth of immune responses associated with clinical resolution of COVID-19. As ASCs are key for the rapid production of antibodies following infection with Ebola virus^{1,2} and infection with and vaccination against influenza virus^{2,3}, and activated circulating T_{FH} cells (cT_{FH} cells) are concomitantly induced following vaccination against influenza virus³, we defined the frequency of $CD3^+CD19^+CD27^{hi}CD38^{hi}$ ASC and $CD4^+CXCR5^+ICOS^+PD-1^+cT_{FH}$ cell responses before symptomatic recovery. ASCs appeared in the blood at the time of viral clearance (day 7; 1.48%) and peaked on day 8 (6.91%). The emergence of cT_{FH} cells occurred concurrently in blood at day 7 (1.98%), increasing on day 8 (3.25%) and day 9 (4.46%) (Fig. 1d). The peak of both ASCs and cT_{FH} cells was markedly higher in the patient with COVID-19 than in healthy control participants ($0.61\% \pm 0.40\%$ and $1.83\% \pm 0.77\%$, respectively (average $\pm$ s.d.); $n = 5$). Both ASCs and cT_{FH} cells were prominently present during convalescence (day 20) (4.54% and 7.14%, respectively; Fig. 1d). Thus, our study provides evidence on the recruitment of both ASCs and cT_{FH} cells in this patient's blood while she was still unwell and 3 d before the resolution of symptoms.

Since co-expression of CD38 and HLA-DR is the key phenotype of the activation of $CD8^+$ T cells in response to viral infections, we analyzed co-expression of CD38 and HLA-DR. As per reports for Ebola and influenza^{1,4}, co-expression of CD38 and HLA-DR on

$CD8^+$ T cells (assessed as the frequency of $CD38^+HLA-DR^+CD8^+$ T cells) rapidly increased in this patient from day 7 (3.57%) to day 8 (5.32%) and day 9 (11.8%), then decreased at day 20 (7.05%) (Fig. 1e). Furthermore, the frequency of $CD38^+HLA-DR^+CD8^+$ T cells was much higher in this patient than in healthy individuals ($1.47\% \pm 0.50\%$; $n = 5$). $CD38^+HLA-DR^+$ T cells were also recently documented in a patient with COVID-19 at one time point⁵. Similarly, co-expression of CD38 and HLA-DR on $CD4^+$ T cells (assessed as the frequency of $CD38^+HLA-DR^+CD4^+$ T cells) increased between day 7 (0.55%) and day 9 (3.33%) in this patient, relative to that of healthy donors ($0.63\% \pm 0.28\%$; $n = 5$), although at lower levels than that of $CD8^+$ T cells. $CD38^+HLA-DR^+$ T cells, especially $CD8^+$ T cells, produced larger amounts of granzymes A and B and perforin (~34–54% higher) than did their parent cells ($CD8^+$ or $CD4^+$ populations; Fig. 1e). Thus, the emergence and rapid increase in activated $CD38^+HLA-DR^+$ T cells, especially $CD8^+$ T cells, at days 7–9 preceded the resolution of symptoms. Details on data reproducibility are in the Life Sciences Reporting Summary.

Analysis of $CD16^+CD14^+$ monocytes, which are related to immunopathology, showed lower frequencies of $CD16^+CD14^+$ monocytes in the blood of this patient at days 7, 8 and 9 (1.29%, 0.43% and 1.47%, respectively) than in that of healthy control donors ($9.03\% \pm 4.39\%$; $n = 5$) (Fig. 1f), possibly indicative of the efflux of $CD16^+CD14^+$ monocytes from the blood to the site of infection. No differences in activated $HLA-DR^+CD3^+CD56^+$ natural killer cells were found.

As pro-inflammatory cytokines and chemokines are predictive of severe clinical outcomes for influenza⁶, we quantified 17 pro-inflammatory cytokines and chemokines in plasma. We found low levels of the chemokine MCP-1 (CCL2) in the patient's plasma (Extended Data Fig. 2a), although this was comparable to results obtained for healthy donors (22.15 ± 13.81 ; $n = 5$), patients infected with influenza A virus or influenza B, assessed at days 7–9 (33.85 ± 30.12 ; $n = 5$), and a patient infected with the human coronavirus HCoV-229e (40.56). Thus, in contrast to severe avian

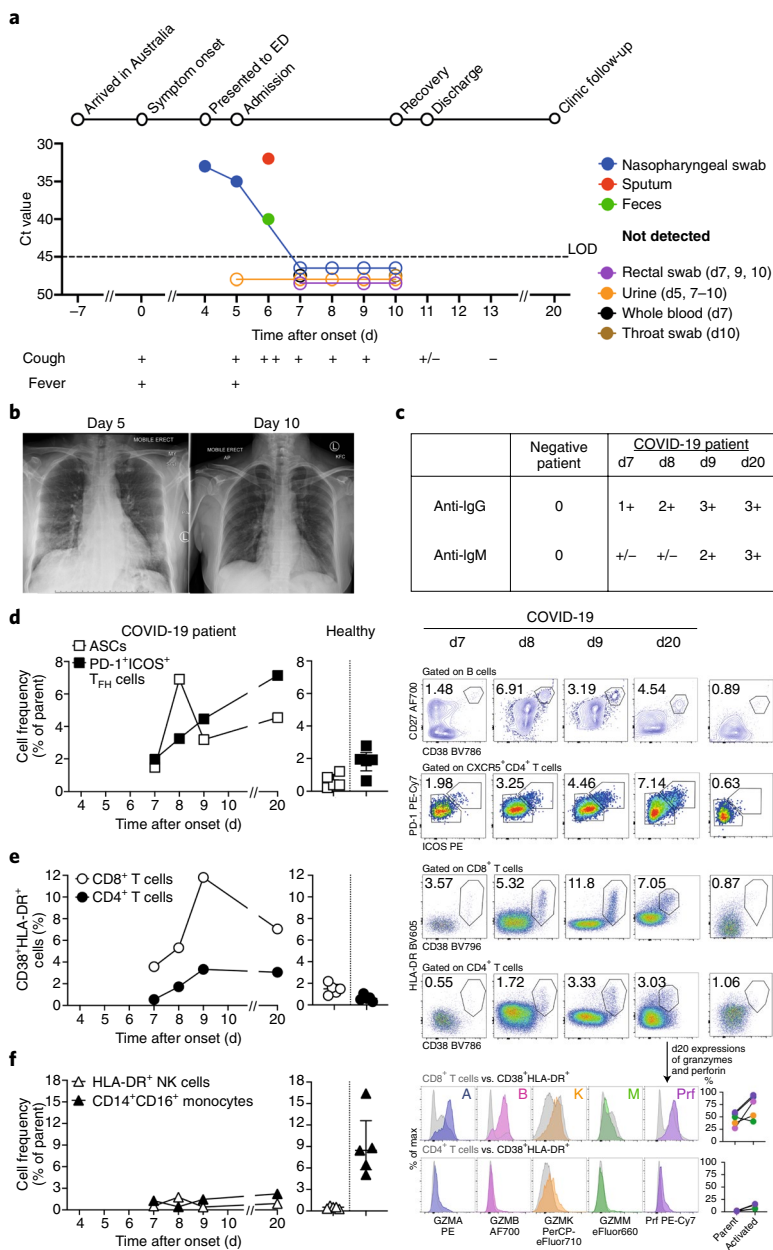


Fig. 1 | Emergence of immune responses during non-severe symptomatic COVID-19. a, Timeline of COVID-19, showing detection of SARS-CoV-2 in sputum, nasopharyngeal aspirates and feces but not urine, rectal swab or whole blood. SARS-CoV-2 was quantified by rRT-PCR; cycle threshold (Ct) is shown. A higher Ct value means lower viral load. Dashed horizontal line indicates limit of detection (LOD) threshold (Ct = 45). Open circles, undetectable SARS-CoV-2. **b,** Anteroposterior chest radiographs on days 5 and 10 following symptom onset, showing radiological improvement from hospital admission to discharge. **c,** Immunofluorescence antibody staining, repeated twice in duplicate, for detection of IgG and IgM bound to SARS-CoV-2-infected Vero cells, assessed with plasma (diluted 1:20) obtained at days 7-9 and 20 following symptom onset. **d-f,** Frequency (left set of plots) of CD27^{hi}CD38^{hi} ASCs (gated on CD3⁺CD19⁺ lymphocytes) and activated ICOS⁺PD-1⁺ T_{FH} cells (gated on CD4⁺CXCR5⁺ lymphocytes) (**d**), activated CD38⁺HLA-DR⁺ CD8⁺ or CD4⁺ T cells (**e**), and CD14⁺CD16⁺ monocytes and activated HLA-DR⁺ natural killer (NK) cells (gated on CD3⁺CD14⁺CD56⁺ cells) (**f**), detected by flow cytometry of blood collected at days 7-9 and 20 following symptom onset in the patient and in healthy donors ($n = 5$; median with interquartile range); gating examples at right. Bottom right histograms and line graphs, staining of granzyme A (GZMA (A)), granzyme B (GZMB (B)), granzyme K (GZMK (K)), granzyme M (GZMM (M)) and perforin (Prf) in parent CD8⁺ and CD4⁺ T cells and activated CD38⁺HLA-DR⁺ CD8⁺ and CD4⁺ T cells. Gating and experimental details are in Extended Data Fig. 3.

H7N9 disease, which had elevated cytokines IL-6, IL-8, IL-10, MIP-1 β and IFN- γ ⁶, minimal pro-inflammatory cytokines and chemokines were found in this patient with COVID-19, even while she was symptomatic at days 7–9.

As the single-nucleotide polymorphism rs12252-C/C in the gene *IFITM3* (which encodes interferon-induced transmembrane protein 3) is linked to severe influenza^{6,7}, we analyzed *IFITM3*-rs12252 in the patient with COVID-19 and found the ‘risk’ *IFITM3*-rs12252-C/C variant (Extended Data Fig. 2b). As the prevalence of *IFITM3*-rs12252-C/C in the Chinese population is 26.5% (the 1000 Genomes Project)⁶, further investigation of the *IFITM3*-rs12252-C/C allele in larger cohorts of people with COVID-19 is worth pursuing.

Collectively, our study provides novel contributions to the understanding of the breadth and kinetics of immune responses during a non-severe case of COVID-19. This patient did not experience complications of respiratory failure or acute respiratory distress syndrome, did not require supplemental oxygenation, and was discharged within a week of hospitalization, consistent with non-severe but symptomatic disease. We have provided evidence on the recruitment of immune cell populations (ASCs, T_{FF} cells and activated CD4⁺ and CD8⁺ T cells), together with IgM and IgG SARS-CoV-2-binding antibodies, in the patient's blood before the resolution of symptoms. We propose that these immune parameters should be characterized in larger cohorts of people with COVID-19 with different disease severities to determine whether they could be used to predict disease outcome and evaluate new interventions that might minimize severity and/or to inform protective vaccine candidates. Furthermore, our study indicates that robust multi-factorial immune responses can be elicited to the newly emerged virus SARS-CoV-2 and, similar to the avian H7N9 disease⁸, early adaptive immune responses might correlate with better clinical outcomes.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of

data and code availability are available at <https://doi.org/10.1038/s41591-020-0819-2>.

Data availability

The source data underlying Fig. 1a,c–f and Extended Data Fig. 2 are provided as Source Data. Raw FACS data are shown in the manuscript. FACS source files are available from the authors upon request. □

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Author contributions

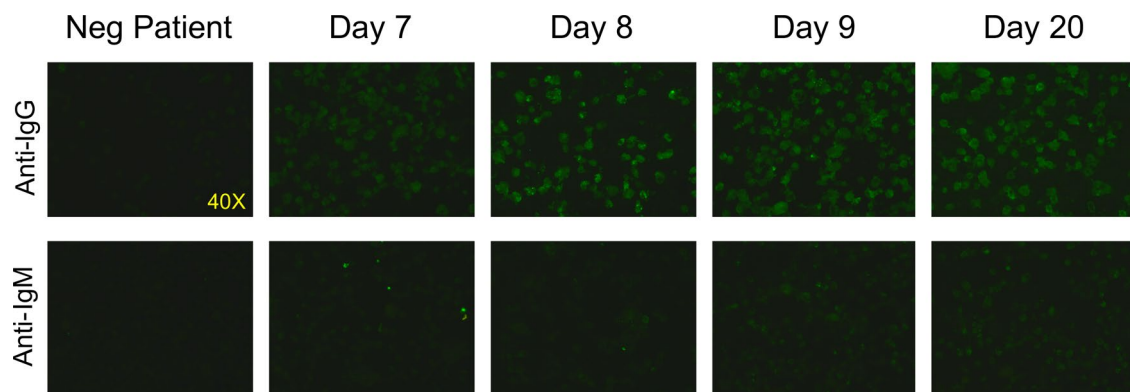
All authors formulated ideas, designed the study and experiments and reviewed the manuscript; T.H.O.N., M.K., J.D., L.C., C.E.S., X.J. and S.N. performed experiments; T.H.O.N., M.K., J.D., L.C. and S.N. analyzed experimental data; and I.T., T.H.O.N., B.C., S.Y.C.T., S.R.L. and K.K. wrote the manuscript.

Competing interests

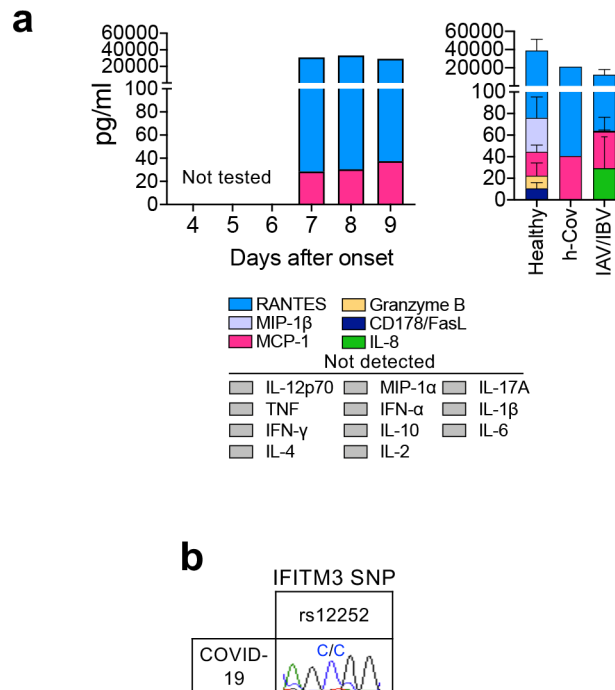
S.R.L.'s institution received funding for investigator-initiated grants from Gilead Sciences, Merck, Viiv Healthcare and Leidos; and honoraria for advisory boards and educational activities (Gilead Sciences, Merck, Viiv Healthcare and Abbvie).

Additional information

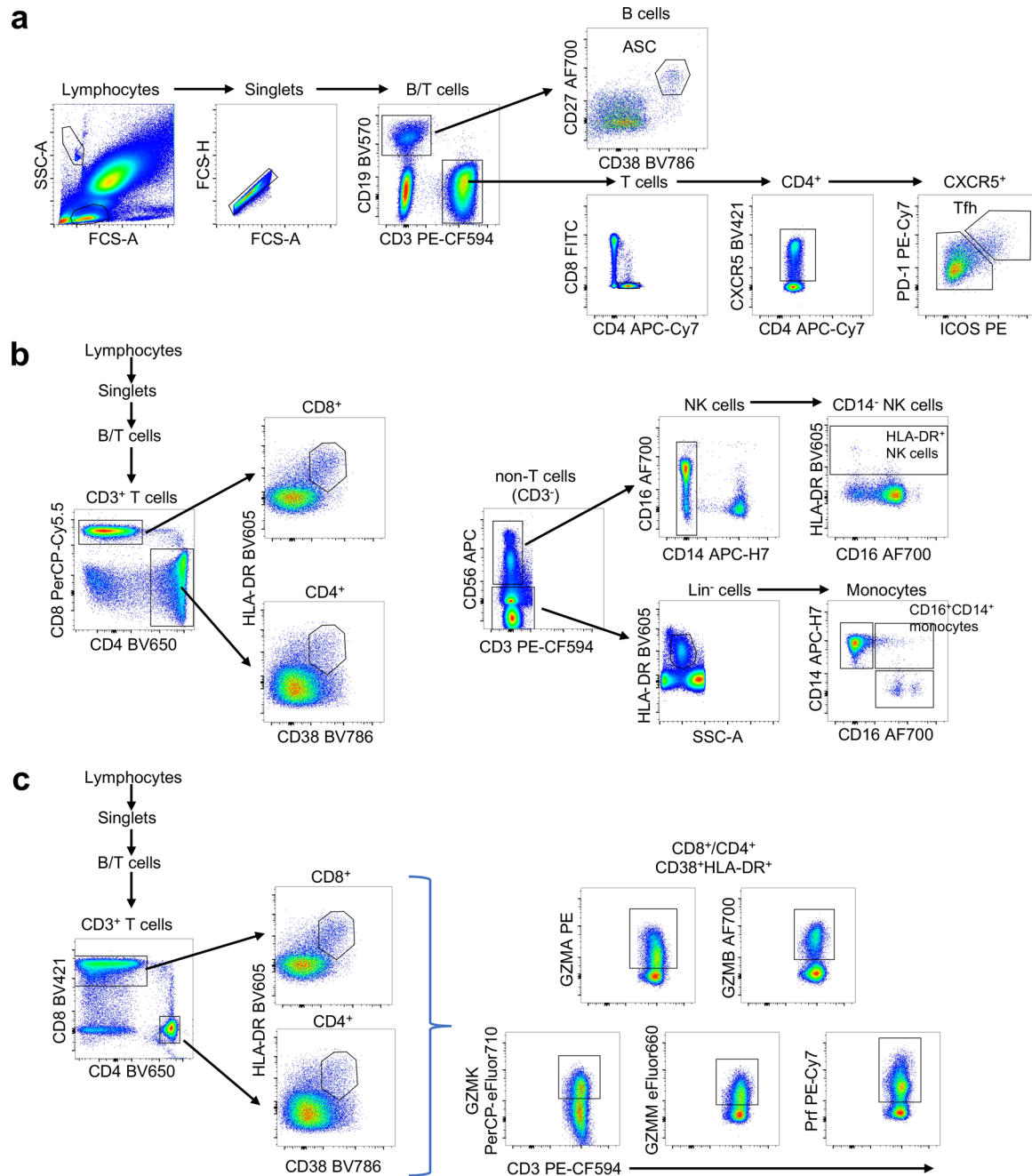
Supplementary information is available for this paper at <https://doi.org/10.1038/s41591-020-0819-2>.



Extended Data Fig. 1 | Detection of IgG and IgM antibodies in SARS-CoV-2-infected Vero cells. Immunofluorescence antibody staining for the detection of IgG and IgM bound to SARS-CoV-2-infected vero cells using plasma (diluted 1:20) collected at d7-9 and d20 following onset of symptoms. Immunofluorescence antibody tests for the detection of IgG and IgM were performed using SARS-CoV-2-infected vero cells washed with PBS and methanol/acetone fixed onto glass slides. SARS-CoV-2 was isolated from another COVID patient, and infectivity at cell harvest was ~80%. 10 μ L of a 1/20 dilution of patient plasma in PBS from days 7, 8, 9 and 20 were incubated on separate wells for 30 mins at 37°C, then washed in PBS and further incubated with 10 μ L of FITC-conjugated goat anti-human IgG and IgM (Euroimmun, Lübeck, Germany) before viewing on a EUROStar III Plus fluorescent microscope (Euroimmun). Prior to detection of IgM antibodies, samples were pre-treated with RF-SorboTech (Alere, Rüsselsheim, Germany) to remove IgG antibodies and rheumatoid factors, which may cause false-negative and false-positive IgM results, respectively.



Extended Data Fig. 2 | Pro-inflammatory cytokines and chemokines and *IFITM3*-rs12252 in a patient with COVID-19. (a) Plasma levels of pro-inflammatory cytokines/chemokines in COVID-19 patient at d7-9, healthy individuals ($n=5$, mean $\pm$ SEM), patient with HCoV-229e and influenza-infected patients ($n=5$). Patient's plasma was diluted 1:4 before measuring cytokine levels (IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-1 β , IFN- α , MIP-1 α , MIP-1 β , MCP-1, CD178/FasL, granzyme B, RANTES, TNF, IFN- γ) using the Human CBA Kit (BD Biosciences, San Jose, California, USA). For RANTES, sera/plasma was also diluted to 1:50. Healthy donors D6-D10 were of a mean age of 32 (range 22-55 years; 40% females). (b) 'risk' *IFITM3*-rs12252 genotyping for the COVID-19 patient. PCR was performed on genomic DNA extracted from patient's granulocytes (using QIAamp DNA Mini Kit, QIAGEN) to amplify the *exon 1* rs12252 region using forward (5'-GGAACTGTTGAGAAACCGAA-3') and reverse (5'-CATACGCACCTTCACGGAGT-3') primers.



Extended Data Fig. 3 | Flow cytometry gating strategy for immune cell subsets. Gating panels are shown for (a) CD27^{hi}CD38^{hi} ASCs and activated ICOS⁺PD1⁺ Tfh cells; (b) activated CD38⁺HLA-DR⁺ CD8⁺ and CD4⁺ T-cells, activated HLA-DR⁺ NK cells and CD14⁺CD16⁺ monocytes; and (c) granzymes (GZM) A/B/K/M and perforin expression on CD8⁺/CD4⁺ T-cells and activated CD38⁺HLA-DR⁺ CD8⁺/CD4⁺ T-cells. Fresh whole blood (200 μ l per stain) was used to measure CD4⁺CXCR5⁺ICOS⁺PD1⁺ follicular T cells (Tfh) and CD3⁺CD19⁺CD27^{hi}CD38^{hi} antibody-secreting B cell (ASC; plasmablast) populations as described³ as well as activated HLA-DR⁺CD38⁺CD8⁺ and HLA-DR⁺CD38⁺CD4⁺ T cells, inflammatory CD14⁺CD16⁺ and conventional CD14⁺ monocytes, activated HLA-DR⁺CD3⁺CD56⁺ NK cells. After the whole blood was stained for 20 mins at room temperature (RT) in the dark, samples were lysed with BD FACS Lysing solution, washed and fixed with 1% PFA. Granzymes/perforin staining (patient d20) was performed using the eBioscience Foxp3/Transcription Factor Staining Buffer Set after the lysis step. All the samples were acquired on a LSRII Fortessa (BD). Flow cytometry data were analyzed using FlowJo v10 software. Healthy donors D1-D5 were of a mean age of 35 (range 24–42 years, 40% females).

Reporting Summary

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- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☒ ☐ The statistical test(s) used AND whether they are one- or two-sided
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Data collection

BD FACS DIVA v8.0.1

Data analysis

FlowJo v10.5.3, Prism v8.3.1,

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- A description of any restrictions on data availability

FACS plots, immunofluorescence and X-rays show raw data. Source data are supplied in "Source Data.xlsx" file

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The sample size was determined by the availability of samples: this is a COVID-2019 patient case report.
Data exclusions	No data were excluded.
Replication	Experiments in figure 1 c were repeated twice in duplicates, with similar results. We have performed repeated flow cytometry stains and analyses, when feasible given the limited clinical material: Staining for COVID patient ASC (Fig 1d) was repeated twice for d7 and d20, and three times for d8, d9 time-points, with similar results. Staining for CD38+HLA-DR+ CD8+ T cells (fig 1e) was repeated twice for d8 and d20, with similar results. Due to a limitation in clinical specimens, the remaining stains for Tfh, NK cells, CD38+HLA-DR+ CD4+ T cells and monocytes was performed once.
Randomization	It is a case study this randomization was not applicable
Blinding	Experiments were not blinded as this was a Case Study

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

We used commercially-available antibodies:
 Antibody/Clone/Fluorochrome/Dilution/Vendor/Cat number:
 CD45RA HI100 FITC 1:50 BD Pharmingen 555488
 CD8a SK1 PerCP-Cy5.5 1:50 BD Pharmingen 565310
 CD56 MEM-188 APC 1:50 Biolegend 304610
 CD16 3G8 AF700 1:50 Biolegend 302026
 CD14 MΦP9 APC-Cy7 1:50 BD Pharmingen 560180
 CD19 HIB19 BV570 1:200 Biolegend 302236
 HLA-DR L243 BV605 1:50 Biolegend 307640
 CD4 SK3 BV650 1:200 BD Biosciences 563875
 CD27 L128 BV711 1:100 BD Horizon 563167
 CD38 HIT2 BV786 1:400 BD Horizon 563964
 CD3 UCHT1 PECF594 1:600 BD Biosciences 562280
 Granzymes/perforin panel:
 Surface
 CD45RA HI100 FITC 1:50 BD Pharmingen 555488
 CD8a RPA-T8 BV421 1:200 BD Pharmingen 301036
 CD14 MΦP9 APC-H7 1:100 BD Pharmingen 560180
 CD19 SJ25C1 APC-H7 1:100 Biolegend 560177
 HLA-DR L243 BV605 1:50 Biolegend 307640
 CD4 SK3 BV650 1:200 BD Biosciences 563875
 CD27 L128 BV711 1:400 BD Horizon 563167
 CD38 HIT2 BV786 1:400 BD Horizon 563964
 CD3 UCHT1 PECF594 1:600 BD Biosciences 562280
 Intracellular
 Granzyme A CB9 PE 1:50 eBioscience 12-9177-42

Granzyme B GB11 AF700 1:50 BD Pharmingen 560213
 Granzyme K G3H69 PerCP-eFluor710 1:50 eBioscience 46-8897-42
 Granzyme M 4B2G4 eFluor660 1:50 eBioscience 50-9774-42
 Perforin B-D48 Pe-Cy7 1:10 Biolegend 353316

Validation

Each antibody used had a validated technical data sheet as per manufacturer's website showing positive staining, and titrated in our laboratory prior to their use.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Vero cells are African Green monkey kidney cells; were used for SARS-CoV-2 infections. Source ATCC – CRL01586 (Vero C1008 Kidney, Monkey). Lot 3338237

Authentication

Cells line was not authenticated

Mycoplasma contamination

Mycoplasma free (tested recently)

Commonly misidentified lines
(See [ICLAC](#) register)

The cell line used is not listed in the ICLAC database

Human research participants

Policy information about studies involving human research participants

Population characteristics

This is a case report:

A 47-year old woman from Wuhan, Hubei province, China presented to an emergency department in Melbourne, Australia. Her symptoms commenced 4-days earlier with lethargy, sore throat, dry cough, pleuritic chest pain, mild dyspnoea and subjective fevers (Fig.1a). She travelled 11 days prior to presentation from Wuhan to Australia. She had no contact with the Huanan seafood market or known COVID-19 cases. She was otherwise healthy, non-smoker, taking no medications. Clinical examination revealed a temperature of 38.5°C, pulse rate 120 beats/minute, blood pressure 140/80 mmHg, respiratory rate 22 breaths/minute, and oxygen saturation 98%, while breathing ambient air. Lung auscultation revealed bibasal rhonchi. At presentation day (d) 4, SARS-CoV-2 was detected by real-time reverse-transcriptase polymerase-chain-reaction (rRT-PCR) from a nasopharyngeal swab specimen. SARS-CoV-2 was again detected at d5-6 from nasopharyngeal, sputum and faecal samples, but undetectable from d7 (Fig.1a). Blood C-reactive protein was elevated at 83.2, with normal lymphocyte (4.3x10⁹/L [range 4.0-12.0x10⁹/L]) and neutrophil counts (6.3x10⁹/L [range 2.0-8.0x10⁹/L]). No other respiratory pathogens were detected. Her management was intravenous fluid rehydration without supplemental oxygenation. No antibiotics, steroids or antiviral agents were administered. Chest radiography demonstrated bibasal infiltrates at d5, which cleared on d10 (Fig.1b). She was discharged to home isolation on d11. Symptoms resolved completely by d13 and she remained well at d20.

Healthy donors (n=10), 4 females, 6 males, mean age of 34.
 Influenza-patients (n=5), 1 female, 4 males, mean age of 55.
 Human HCoV-229e Cov patient (n=1), 1 male, age 27.

Recruitment

Healthy volunteer donors >18 years old were recruited through University of Melbourne (UoM) and virus-infected patients were recruited at Royal Melbourne Hospital (RMH). Signed informed consents were obtained from all blood donors prior to study.

Ethics oversight

Human experimental work was conducted according to the Declaration of Helsinki Principles and according to the Australian National Health and Medical Research Council Code of Practice. Participants provided written informed consent prior to the study. The study was approved by the Royal Melbourne Hospital (HREC Reference number: HREC/17/MH/53 and HREC/15/MonH/64/2016.196) and University of Melbourne (ID #1442952.1 and #1443389.4) Human Research Ethics Committees.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Whole blood staining was performed as outlined in fig legends to Extended Data

Instrument

BD LSR Fortessa was used for acquisition of data

Software

BD FACS Diva v8.0.1, FlowJo v10.5.3

Cell population abundance

We have not sorted the PBMCs

Gating strategy

Gating strategy has been described in methods, figure legends and as Extended Data figure 3

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

TLR2-mediated activation of innate responses in the upper airways confers antiviral protection of the lungs

Georgia Deliyannis, ... , David C. Jackson, Brendon Y. Chua

JCI Insight. 2021;6(5):e140267. <https://doi.org/10.1172/jci.insight.140267>.

Research Article

Infectious disease

Therapeutics

The impact of respiratory virus infections on global health is felt not just during a pandemic, but endemic seasonal infections pose an equal and ongoing risk of severe disease. Moreover, vaccines and antiviral drugs are not always effective or available for many respiratory viruses. We investigated how induction of effective and appropriate antigen-independent innate immunity in the upper airways can prevent the spread of respiratory virus infection to the vulnerable lower airways. Activation of TLR2, when restricted to the nasal turbinates, resulted in prompt induction of innate immune-driven antiviral responses through action of cytokines, chemokines, and cellular activity in the upper but not the lower airways. We have defined how nasal epithelial cells and recruitment of macrophages work in concert and play pivotal roles to limit progression of influenza virus to the lungs and sustain protection for up to 7 days. These results reveal underlying mechanisms of how control of viral infection in the upper airways can occur and support the implementation of strategies that can activate TLR2 in nasal passages to provide rapid protection, especially for at-risk populations, against severe respiratory infection when vaccines and antiviral drugs are not always effective or available.

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TLR2-mediated activation of innate responses in the upper airways confers antiviral protection of the lungs

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Authorship note: GD, CYW, HAM, DCJ, and BYC contributed equally to this work.

Conflict of interest: BYC, DCJ, and NWB are consultants for Ena Respiratory. FM and CD are employees of Ena Therapeutics. FM, CD, and NWB have received income per annum from Ena Therapeutics or Ena Respiratory. The research subject of this publication was funded by Ena Therapeutics or Ena Respiratory. Compounds used for the research in this publication are the subject of the following patent applications owned by Ena Therapeutics: PCT/AU2011/001225 (DCJ), PCT/AU2018/051397 (DCJ, WZ, and CD), PCT/AU2018/051401 (DJ, FM, GD, CW, and CD), and PCT/AU2020/050660 (WZ, DCJ, and CD).

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The impact of respiratory virus infections on global health is felt not just during a pandemic, but endemic seasonal infections pose an equal and ongoing risk of severe disease. Moreover, vaccines and antiviral drugs are not always effective or available for many respiratory viruses. We investigated how induction of effective and appropriate antigen-independent innate immunity in the upper airways can prevent the spread of respiratory virus infection to the vulnerable lower airways. Activation of TLR2, when restricted to the nasal turbinates, resulted in prompt induction of innate immune-driven antiviral responses through action of cytokines, chemokines, and cellular activity in the upper but not the lower airways. We have defined how nasal epithelial cells and recruitment of macrophages work in concert and play pivotal roles to limit progression of influenza virus to the lungs and sustain protection for up to 7 days. These results reveal underlying mechanisms of how control of viral infection in the upper airways can occur and support the implementation of strategies that can activate TLR2 in nasal passages to provide rapid protection, especially for at-risk populations, against severe respiratory infection when vaccines and antiviral drugs are not always effective or available.

Introduction

The onslaught of respiratory pathogens is felt not only during the emergence of novel viruses, such as the pandemic H1N1 influenza virus of 2009, SARS coronavirus, Middle East respiratory syndrome coronavirus, and SARS coronavirus 2 (the causative agent of COVID-19), but endemic seasonal virus infections also pose an equal and ongoing risk of severe disease and death for many individuals. Even when vaccines are in widespread use against viruses, for example influenza, their effectiveness can vary markedly (1) and protection cannot be guaranteed. Similarly, antiviral drugs are not available for all viruses; some that are effective against influenza virus can be used prophylactically (2), but their window of protection is short and ongoing use is not recommended because of the potential for the selection of drug-resistant variants (3). Furthermore, because most antiviral agents are specific to a particular virus family or genus, they may not be appropriate for unrelated endemic or emerging viruses. Clearly, there remains an unmet need for broadly active antiviral prophylactic therapies to prevent virus-induced respiratory complications, especially in highly susceptible individuals.

Effectors of innate immunity can be immediate and of sufficient vigor to limit infection. Although non-specific in the sense that they lack the specificity of adaptive immune responses, innate immune responses can be specifically initiated by activation of TLRs, which exhibit a comprehensive range of recognition capabilities, from signatures of bacterial cell wall components to the RNA and DNA of viruses (4). These pathogenic signatures can alert the host cell to immediately mobilize innate effector mechanisms to combat infection processes, making them prime targets for inducing antiviral responses (5).

In this study, we examine how triggering innate immunity in the upper respiratory tract, an initial site of respiratory virus replication, prevents progression of influenza A virus (IAV) to the lower airways. Influenza virus primarily infects respiratory epithelial cells, beginning with those first encountered in the upper respiratory tract (URT) (6). Alerting epithelial cells occurs through recognition of the viral genome and viral proteins by TLRs, RIG-I-like receptors (RLRs), and NLRs (7, 8). Upon sensing viral invasion, epithelial cells can produce a range of antiviral mediators (9–11) as well as cytokines that promote recruitment of innate immune cells including macrophages, neutrophils, and NK cells to exert effector functions (12, 13). Engagement of the innate immune system prior to viral infection could therefore provide rapid prophylaxis by taking advantage of the multiple mechanisms inherent in innate host defenses.

Synthetic agonists of the viral DNA/RNA-recognizing TLR molecules, TLR3, TLR7/8, and TLR9, in particular, have been found to boost protective innate immune responses against a range of respiratory viruses (14–16). Their success in the clinic, however, could be limited mostly because of short duration of benefit or induction of adverse effects related to activation of the TNF- α and type 1 IFN pathways (17). In our study, we triggered the innate immune system through TLR2 only, which recognizes the S-[2,3-bis(palmitoyloxy)propyl] cysteine (Pam₂Cys) moiety of bacterial lipopeptides and is present on the surfaces of epithelial cells and cells of the immune system. We (18–21) and others (14, 22) have previously studied the stimulatory effects of TLR2 agonists i.n. delivered into the respiratory tract, including the lower airways, to induce protective immunity against bacterial and viral infections.

We have now engineered Pam₂Cys as an immunomodulatory molecule (INNA-X) and here describe an animal model that allows examination of its effects when localized to the URT rather than contacting the lower airways. We demonstrated that targeted delivery of INNA-X to the URT resulted in the rapid induction of a tissue-localized innate immune response characterized by the upregulation of genes associated with, and accompanied by, production of cytokines and the infiltration of innate immune cells. Administration of INNA-X to the URT before exposure to influenza virus reduced the viral burden in nasal epithelia and limited viral progression to the lungs. We also demonstrated that epithelial cells and macrophages work in concert and play pivotal roles in the antiviral defenses. These findings indicate a clear application for use of INNA-X as an immunomodulator to mobilize rapid protection against respiratory tract infection. Because the molecule targets the host and not the pathogen, the method of control is not likely to cause drug resistance and could be effective against currently circulating and emerging respiratory viruses, including those that can cause and/or exacerbate respiratory complications (23).

Results

Delivery of influenza virus and INNA-X to the URT. To model as closely as possible physiological infection of mice with influenza virus and to anatomically restrict treatment with INNA-X, we examined the distribution of i.n. delivered inocula throughout the respiratory tract. Inocula can be delivered to the total respiratory tract (TRT) or restricted to the URT, including nasal passages, by varying the volume administered (24, 25). Inoculation of Evans blue dye in a volume of 50 μ L resulted in staining of the entire respiratory tract, including the nasal turbinates and lungs (Figure 1A). In contrast, administration of 10 μ L restricted dye to the nasal turbinates. With the lesser volume, little or no dye was detected in the lungs and stomach, indicating restriction to the URT (Figure 1B). Similar results to those obtained with 10 μ L were observed using volumes up to 15 μ L (Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.140267DS1>).

To determine whether deposition of a small volume of viral inoculum in the nares is followed by sequential progression of virus throughout the respiratory tract, groups of mice were challenged with Udorn IAV and viral titers in nasal turbinates and lungs measured daily for 5 consecutive days after infection. Virus was detected in the nasal turbinates within 1 day of viral challenge with titers up to 1000-fold greater than the infectious dose administered (Figure 1C). No virus was detected in lungs at this time point, indicating that infection was confined to the URT. Progression of virus from the nose to the lungs was evident from day 2 after the challenge onward and reached a plateau after 3–5 days, concurrent with a decrease in viral titers in the nasal turbinates. We also found that restriction of INNA-X delivery to the URT resulted in containment of cytokine responses to the site of administration (Figure 1D). All subsequent experiments used small volumes of inocula with which to treat or infect mice.

Treatment of the URT with INNA-X limits progression of influenza infection to the lungs. Application of INNA-X to the nasal turbinates 1 or 7 days before delivery of virus to the URT (Figure 2A) significantly

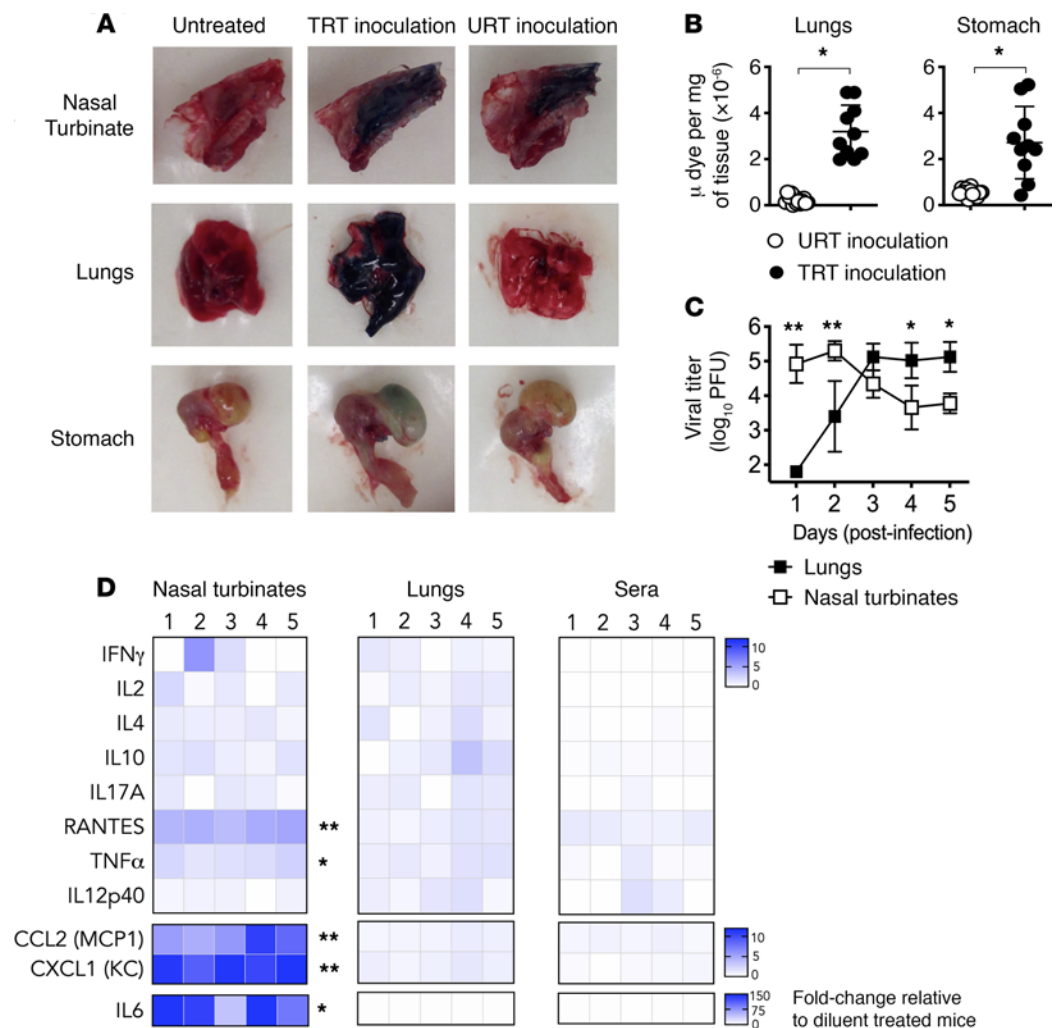


Figure 1. Distribution of inocula after administration to the URT or TRT. (A) Mice ($n = 10$ /group) were i.n. inoculated with 0.125% Evans blue dye in PBS (10 μ L URT inoculation or 50 μ L TRT inoculation). Representative images of nasal turbinates (NT), lungs, and stomach dissected 2 minutes later are shown in comparison with an untreated control. (B) Supernatants from homogenized lungs and stomach were treated with trichloroacetic acid with absorbance measured at 620 nm. Concentrations of dye were interpolated from a standard curve. (C) Mice ($n = 5$ /group) were infected with 500 PFU of Udorn IAV in 10 μ L. Viral titers in NT and lungs were determined in an MDCK plaque assay. (D) The immunostimulatory activity of URT-inoculated INNA-X (5 nmol) was determined in mice ($n = 5$ /group) by measuring cytokine levels by multiplex bead array 1 day after treatment. Statistical analysis was performed using a (B) Welch t test, (C) 2-way ANOVA with a Bonferroni post hoc test (relative to diluent-treated mice) and (D) multiple-comparison Holm-Sidak t test. * $P < 0.01$, ** $P < 0.001$.

reduced the amount of virus recovered from the lower respiratory tract. Lung viral loads, determined at the height of pulmonary viral replication (5 days after infection), were significantly (>80%) reduced in the majority of mice pretreated with INNA-X at either 1 day (Figure 2B) or 7 days (Figure 2C) prior to viral challenge. Viral loads were also significantly reduced in the nasal turbinates, as demonstrated using 5-fold less INNA-X (1 nmol) delivered 1 day prior to viral challenge and measured 1 day after infection (Figure 2, D and E). Similar reductions in lung viral loads were observed 5 days after viral challenge (Figure 2F). These results demonstrated that INNA-X conferred a rapid protective effect at the site of delivery in the URT and limited viral progression from that site, affording protection of the lower respiratory tract. In addition, the therapeutic efficacy of INNA-X treatment was also demonstrated in mice that were treated within 24 hours after exposure to influenza virus (Figure 2G), resulting in more than 80% reduction in lung viral titers in 60% of mice (Figure 2H).

INNA-X induces rapid innate immune responses in nasal turbinates. Because INNA-X-mediated antiviral activity occurred in the URT within 1 day of inoculation, we investigated whether the effects of treatment were mediated by induction of local innate immune responses. Total RNA was harvested from nasal

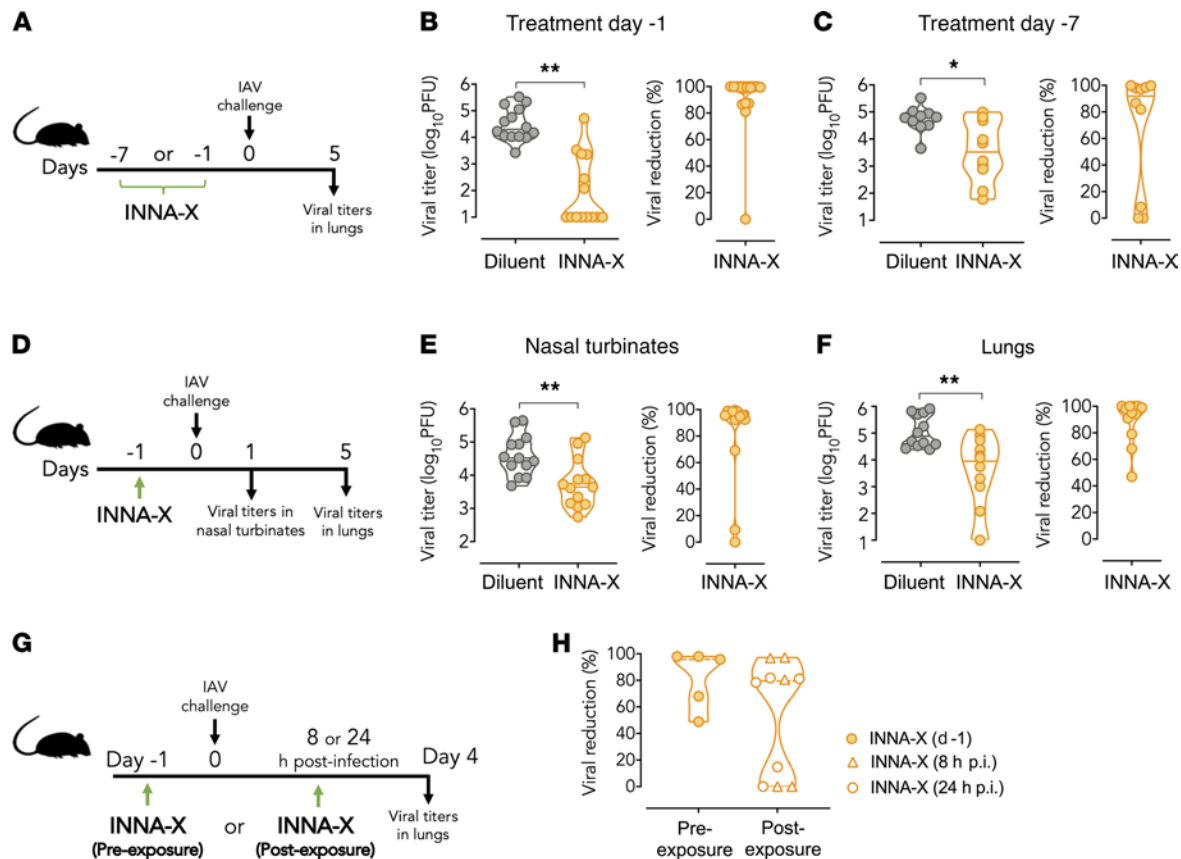


Figure 2. Inhibition of viral replication in the respiratory tract after administration of INNA-X to the URT. (A) Mice ($n = 5$ or 7 /group) were inoculated with 5 nmol of INNA-X prior to challenge with 500 PFU of Udorn IAV. Efficacy of treatment (B) 1 day or (C) 7 days prior to viral challenge was determined by measuring lung viral titers 5 days after infection. The percentage reduction in viral load in each mouse is shown relative to the average viral titer in similarly challenged diluent-treated mice. Results (B and C) are pooled from 2 separate experiments. (D) Mice were inoculated with 1 nmol of INNA-X and challenged 1 day later. Viral titers in (E) nasal turbinates or (F) lungs ($n = 7$ /group) were measured at 1 or 5 days, respectively, after infection. (G) Therapeutic efficacy of treatment with INNA-X was examined by inoculating mice with INNA-X 8 or 24 hours after IAV challenge (postexposure) in comparison to treatment 1 day prior to challenge (preexposure). (H) Reduction in lung viral titers is relative to similarly infected mice treated with diluent at each time point. Statistical analysis was performed using a (B, E, and F) Mann-Whitney or (C) Welch t test. * $P < 0.05$, ** $P < 0.01$.

turbinate tissue 6 and 24 hours after treatment with INNA-X and hybridized to the nCounter mouse immunology panel for analysis of appropriate gene expression. Compared with animals treated with diluent vehicle, a total of 54 genes were differentially expressed, with more than 85% of genes upregulated within 6 hours of treatment (Figure 3A). Upregulated genes included those encoding CXCL1 (KC), CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), and the cytokines TNF- α , IL-1 α , and IL-1 β that are associated with antiviral responses (Figure 3B). These genes, together with a significant number of others, are also typically associated with activation, chemotaxis, and function of immune cells. A smaller number of genes were upregulated after 24 hours (Figure 3A). These were largely representative of a subset of those detected at 6 hours after treatment but were expressed at lower levels, indicating that these genes were rapidly activated after treatment and downregulated shortly thereafter.

To determine the presence of gene products encoded by the upregulated genes in nasal turbinates and in particular IL-6, TNF- α , IL-1 α , and IL-1 β , as well as CXCL1, CCL2, CCL3, and CCL4, we determined their concentrations after treatment. Significantly higher levels of cytokines were detected 6 hours after exposure to INNA-X compared with treatment with diluent (Figure 4A). Whereas concentrations of IL-6, IL-1 α , IL-1 β , CCL2, and CCL4 returned to baseline levels 24 hours after treatment, TNF- α , CXCL1, and CCL3 remained significantly elevated, albeit at lower levels than those determined at 6 hours, corresponding to the downregulation of their respective genes. Notably, with the exception of CXCL1, there were no significant differences in levels of cytokines detected in the lungs between each treatment group at either time point (Figure 4B). In fact, cytokine levels in the lungs of mice remained largely unchanged over the

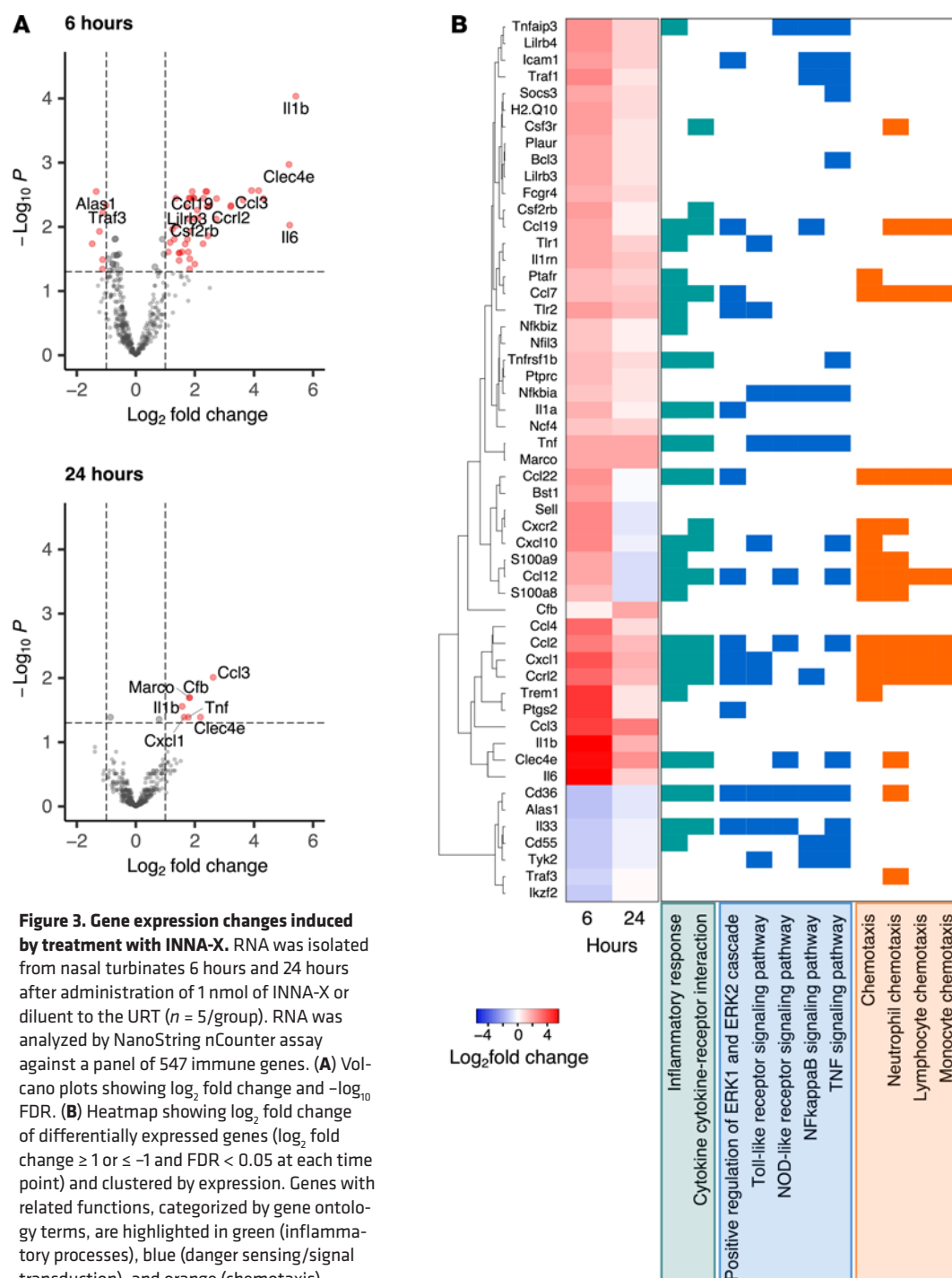


Figure 3. Gene expression changes induced by treatment with INNA-X. RNA was isolated from nasal turbinates 6 hours and 24 hours after administration of 1 nmol of INNA-X or diluent to the URT ($n = 5/\text{group}$). RNA was analyzed by NanoString nCounter assay against a panel of 547 immune genes. **(A)** Volcano plots showing $\log_2$ fold change and $-\log_{10}$ FDR. **(B)** Heatmap showing $\log_2$ fold change of differentially expressed genes ($\log_2$ fold change ≥ 1 or ≤ -1 and FDR < 0.05 at each time point) and clustered by expression. Genes with related functions, categorized by gene ontology terms, are highlighted in green (inflammatory processes), blue (danger sensing/signal transduction), and orange (chemotaxis).

course of 48 hours after INNA-X treatment (Supplemental Figure 2A), confirming that the direct action of INNA-X, when i.n. delivered and in a small volume, was localized to the URT.

The transient increase of cytokines in nasal turbinates of mice treated with INNA-X is also consistent with their function in recruiting immune cells. Phenotyping of cell populations in the nasal turbinates 1 day after treatment revealed an increased number of macrophages, neutrophils, NK cells, and DCs (Figure 4C). Additionally, CD4⁺ T cells were also increased early after treatment compared with animals that received diluent (Figure 4C). Although levels of neutrophils, DCs, and CD4⁺ T cells returned to baseline levels within 3 days, elevated numbers of interstitial macrophages and NK cells were still present at this time but waned over the next 4 days. In contrast, there were no significant changes in the numbers of CD8⁺

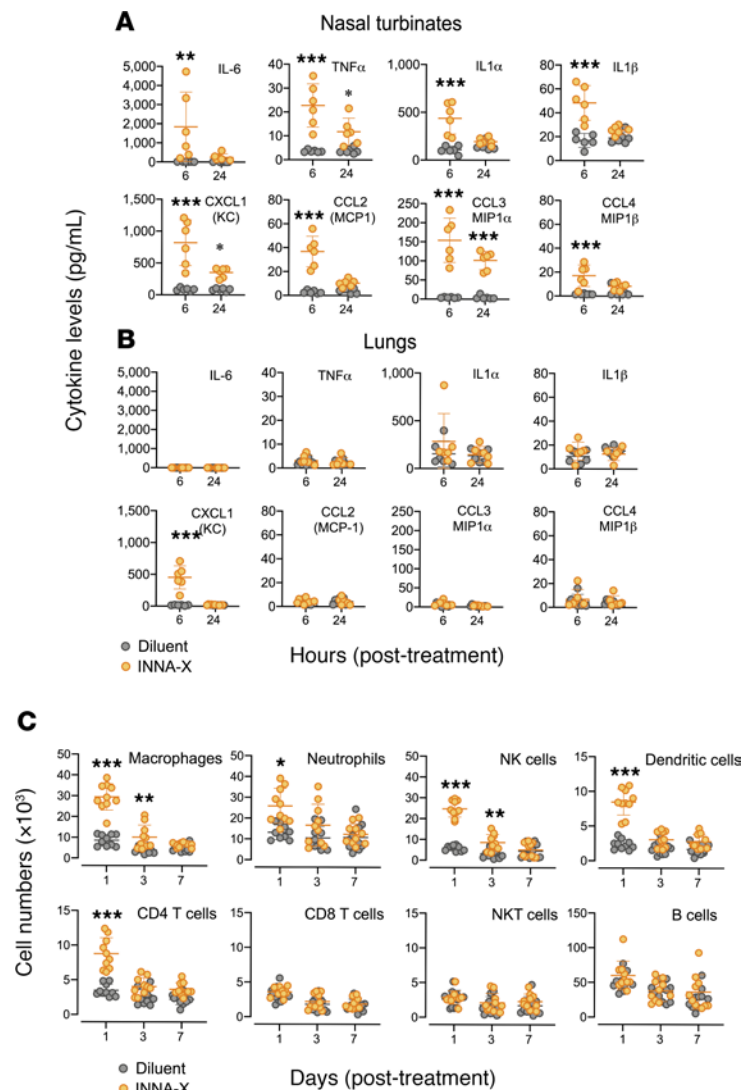


Figure 4. Cytokine levels and cell populations in the respiratory tract of mice after treatment with INNA-X. Mice ($n = 3$ /group) were inoculated with 1 nmol of INNA-X or diluent only and 1 day later cytokine levels in (A) nasal turbinates and (B) lungs were analyzed in a multiplex bead array assay. (C) Nasal turbinates were harvested 1, 3, and 7 days after treatment ($n = 5$ /group) and single-cell suspensions stained with fluorochrome-conjugated antibodies of various specificities and analyzed by flow cytometry. All results are pooled from 2 separate experiments. Statistical analysis was performed by 2-way ANOVA with a Bonferroni post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

T cells, NKT cells, and B cells in animals that received INNA-X compared with diluent treatment at any of the times examined. In contrast, no significant differences in frequencies of these cell populations were observed in the lung (Supplemental Figure 2B). Altogether, these results demonstrated that exposure of the nasal turbinates to INNA-X resulted in rapid local expression of genes and their products, which led to the recruitment of particular populations of immune cells to the delivery site.

Macrophages persist in nasal turbinates of INNA-X-treated mice after infection. Mice were treated with INNA-X and 1 day later challenged with influenza virus and ensuing changes in the number of cell populations followed (Figure 5A). Increased numbers of macrophages, neutrophils, NK cells, DCs, and CD4⁺ T cells were detected in animals 1 day after INNA-X treatment (day 0; Figure 5B) compared with diluent treatment, confirming results described above (Figure 4C). After viral challenge, diluent-treated control mice showed a steady rise in neutrophils and elevated levels of DCs, CD4⁺ T cells, CD8⁺ T cells, and NKT cells on day 7 in response to virus challenge. In INNA-X-treated mice, although most cell types displayed similar kinetics to those observed in control animals, macrophage numbers were significantly elevated throughout the course of infection. This indicates that infection 1 day after treatment did not affect

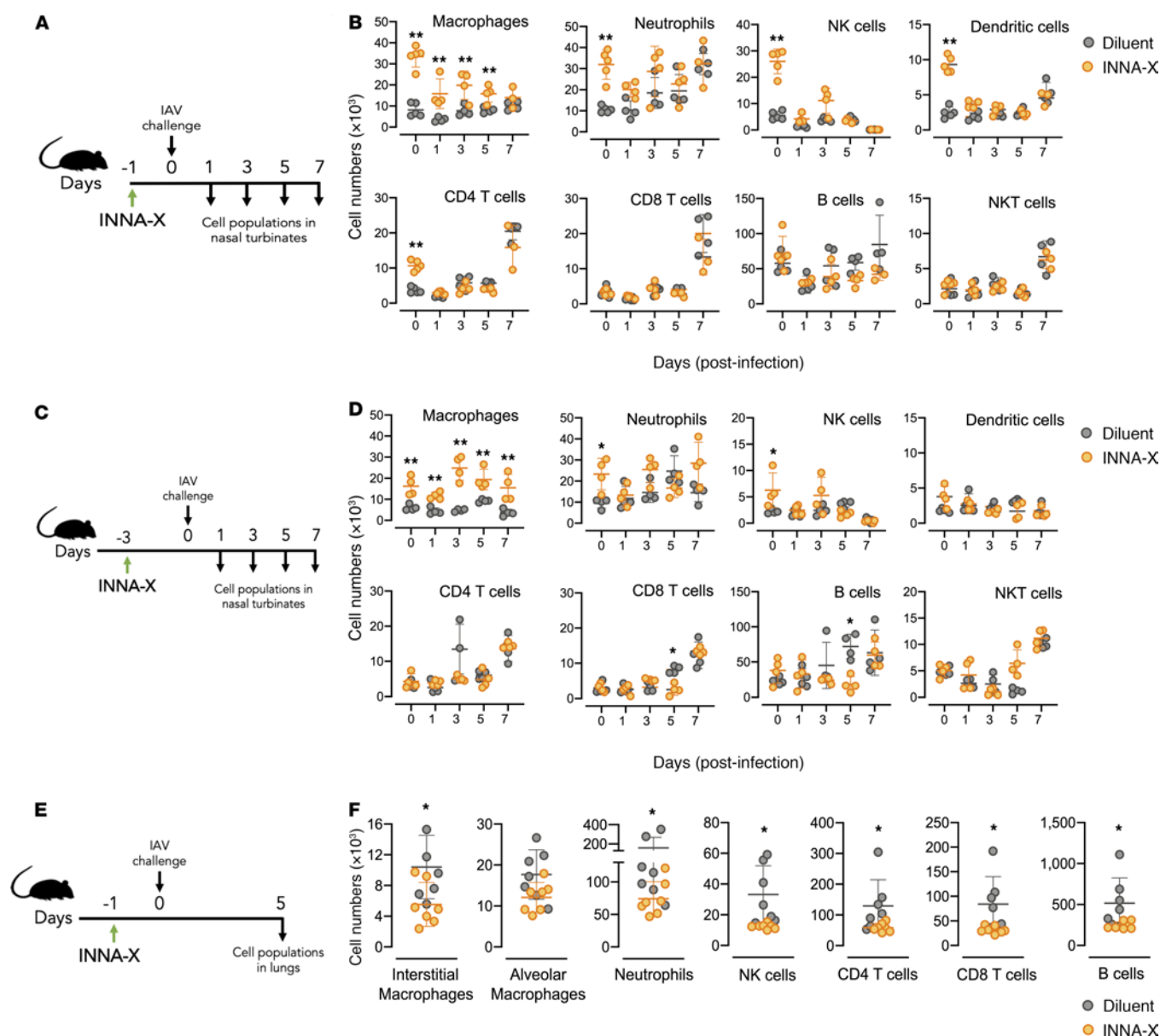


Figure 5. Different cell populations present in the nasal turbinates and lungs after treatment with INNA-X and challenge with influenza virus. (A and B) Mice ($n = 3$ –5/group) were inoculated with 1 nmol of INNA-X or diluent and 1 day later challenged with 500 PFU of Udorn IAV. (B) Frequencies of cell populations in the nasal turbinates on 1, 3, 5, and 7 days after challenge were analyzed. (C and D) Mice ($n = 4$ /group) were treated with 1 nmol of INNA-X and 3 days later challenged with virus to examine cell populations present 1, 3, 5, and 7 days after challenge. Cell populations present 24 hours (B) or 3 days (D) after treatment but prior to challenge with virus are shown in each panel as the day 0 time point. (E and F) Cell populations in the lungs of inoculated mice ($n = 7$ /group) challenged 1 day later were also analyzed at 5 days after infection. Statistical analysis was performed by (B and D) 2-way ANOVA with a Bonferroni post hoc test or (F) a Welch t test. * $P < 0.01$. ** $P < 0.001$.

INNA-X-dependent recruitment of macrophages. When INNA-X was administered 3 days prior to viral challenge (Figure 5C), macrophage levels were again elevated throughout the course of infection (Figure 5D). In contrast to the nasal turbinates, animals pretreated with INNA-X 1 day prior to viral infection had significantly lower numbers of lung immune cells at 5 days after infection (Figure 5, E and F), correlating with decreased viral titers at this site (Figure 2F) and indicating that protection of the URT against infection reduced the need for immune responses to be elicited in the lower respiratory tract.

Epithelial cells in addition to macrophages play a role in INNA-X-mediated virus protection. Because airway epithelial cells can act as a protective barrier against materials, including infectious agents, introduced to the upper airways, we determined whether INNA-X treatment could protect nasal epithelial cells against IAV infection. A clear population of infected cells (intracellular virus nucleoprotein–positive [NP⁺] nasal epithelial [CD45⁺

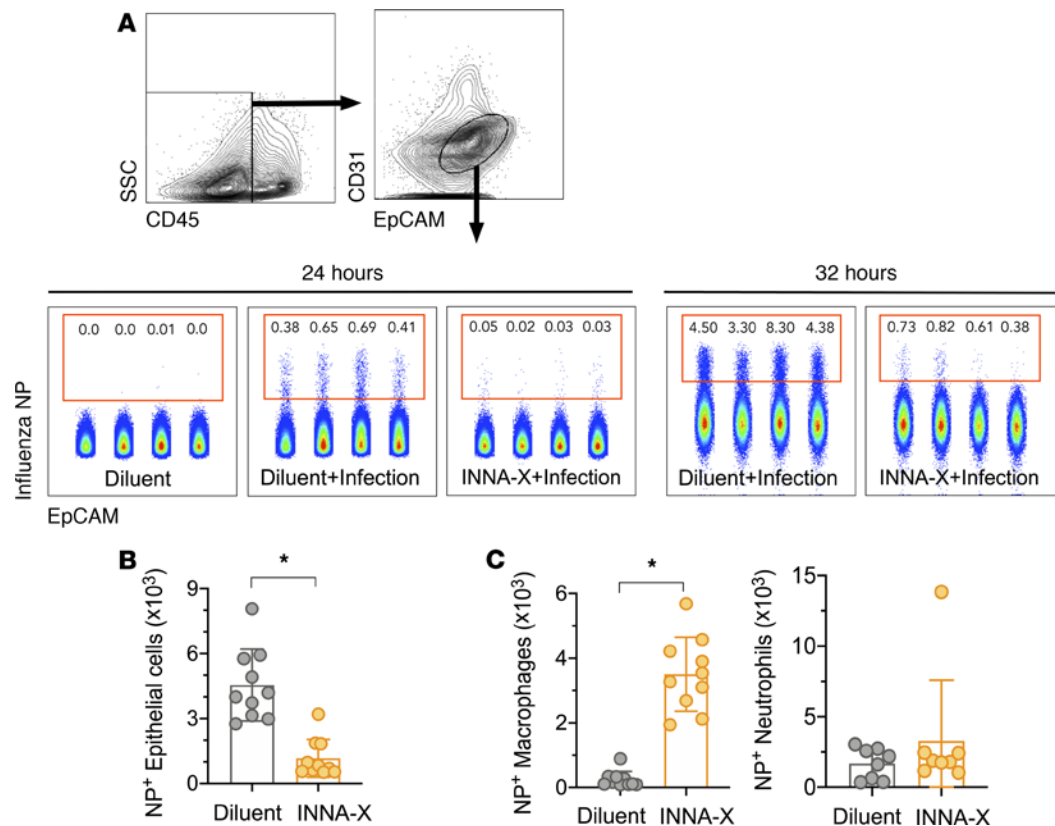


Figure 6. In vivo protection of nasal epithelial cells after viral challenge of mice treated with INNA-X. Mice were inoculated with 1 nmol of INNA-X or diluent and 1 day later challenged with 500 PFU of Udorn IAV. Nasal turbinates were harvested 24 or 32 hours after infection and cell populations analyzed for intracellular influenza virus nucleoprotein (NP) expression. (A) Epithelial cells were distinguished by CD45⁺CD31⁺EpCAM⁺ expression. Center panels depict NP⁺-expressing cell populations from individual animals in a representative experiment. (B) Bar graphs indicate the total number of NP⁺ epithelial cells, (C) NP⁺ macrophages, or NP⁺ neutrophils obtained from 8–10 animals (data pooled from 2 independent experiments). All statistical analysis was performed using a Mann-Whitney *t* test. **P* < 0.001.

CD31⁺EpCAM⁺) was detected in diluent-treated control mice 24–32 hours after viral challenge (Figure 6A). Ex vivo analysis of similar cells in INNA-X-pretreated mice revealed significantly fewer NP⁺ cells as early as 24 hours after infection, demonstrating protection of nasal epithelium (Figure 6B), a result that correlated with significantly lower viral titers observed at this site (Figure 2E). In addition, significantly greater numbers of NP⁺ macrophages but not neutrophils were detected in nasal tissues of infected INNA-X-treated mice (Figure 6C) compared with mice treated with diluent and challenged with virus. Macrophages abortively infected by seasonal influenza virus (26, 27) can act as a “sink” for virus (28). This, together with their ability to limit infection through the phagocytosis of apoptotic virus-infected cells (29), may explain the presence of intracellular NP and highlights the contribution of macrophages to INNA-X-mediated protection of the nasal epithelium.

Dissecting the protective effector roles of nasal epithelial cells and macrophages. We (18) have previously shown that *Tlr2*^{−/−} mice are not responsive to INNA-X treatment, and given that both epithelial cells and macrophages likely play a role in mediating antiviral activity in the nasal turbinates of mice treated with INNA-X, we generated chimeras using TLR2-deficient mice (*Tlr2*^{−/−}) on a C57BL/6 background to assess the relative roles of these cells. Adoptive transfer of donor cells from WT mice into lethally irradiated *Tlr2*^{−/−} recipients (Figure 7A; WT > *Tlr2*^{−/−}) provides a mouse model in which only the immune cells from the WT donor will be responsive to activation by INNA-X. In the inverse (*Tlr2*^{−/−} > WT) model, epithelial cells but not immune cells will be responsive to INNA-X treatment. We also established chimeras where donor bone marrow cells from either WT or *Tlr2*^{−/−} mice were transferred into the same irradiated recipient strains (WT > WT or *Tlr2*^{−/−} > *Tlr2*^{−/−}) as controls.

When the URTs of chimeric mice were examined for their ability to resist viral challenge, infection of nasal epithelial cells was significantly lower in INNA-X-treated and virus-challenged WT > WT chimeras than in similarly treated and challenged *Tlr2*^{−/−} > *Tlr2*^{−/−} chimeras (Figure 7B). This trend supported that

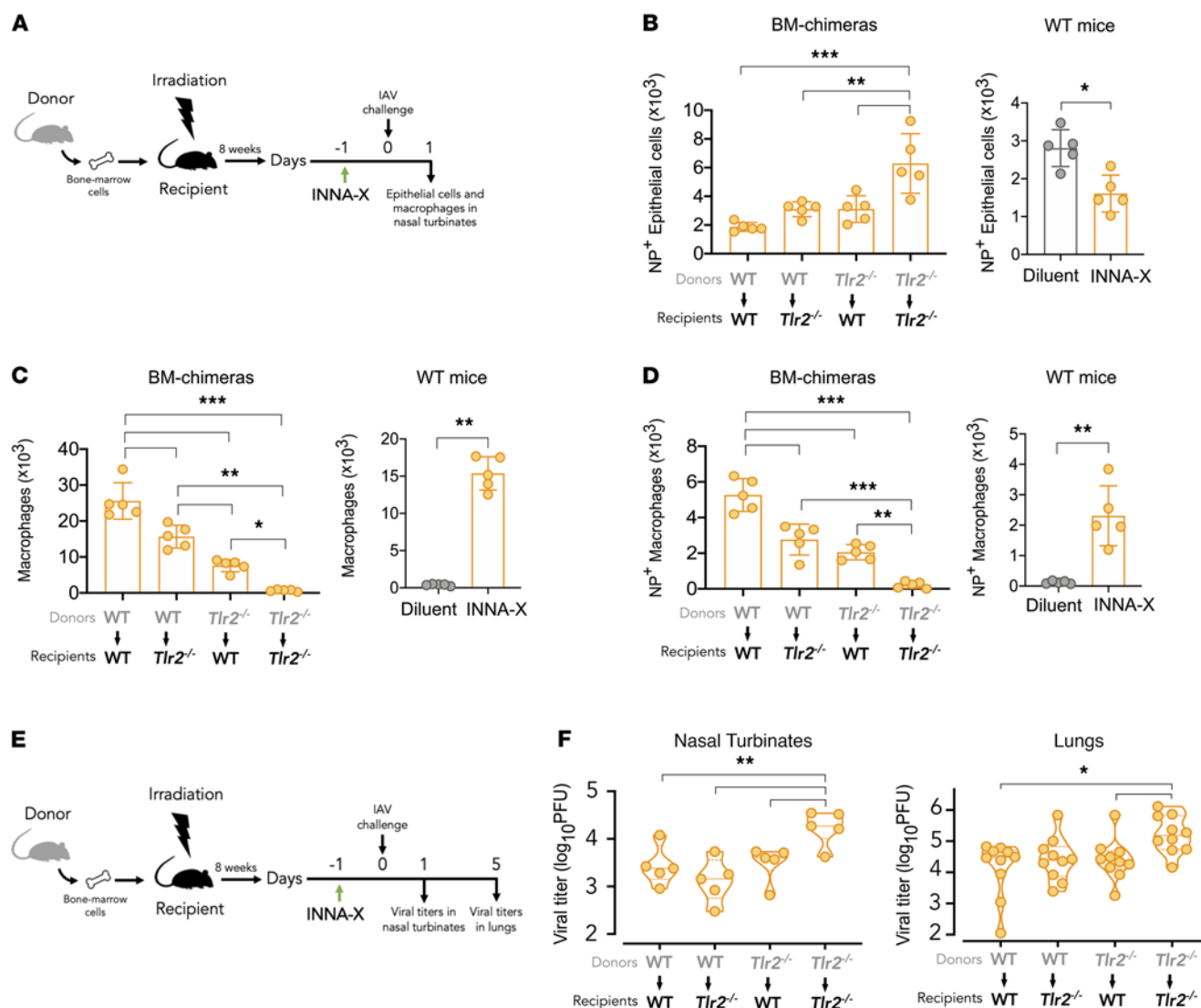


Figure 7. Contribution of immune and nonimmune cells in mediating protection. (A) Bone marrow chimeras were established by adoptive transfer of donor bone marrow cells from WT C57BL/6 or *Tlr2*^{-/-} mice into irradiated recipient mice ($n = 5/\text{group}$). After 8 weeks, animals were inoculated with 1 nmol of INNA-X prior to viral challenge with 500 PFU of Udorn IAV. Separate groups of WT mice were similarly treated with INNA-X and diluent prior to viral challenge. Nasal turbinates were harvested 1 day after challenge and the frequencies of (B) NP⁺-expressing CD45⁺CD31⁺EpCAM⁺ epithelial cells, (C) total macrophages, and (D) NP⁺-expressing macrophages determined. Results representative of 2 experiments conducted independently. (E and F) Viral titers in the nasal turbinates and lungs were also determined 1 and 5 days after infection, respectively. Statistical analysis of data from bone marrow chimeras (B–D and F) were performed by 1-way ANOVA with Tukey's post hoc test and data from WT mice analyzed by a Welch *t* test. Comparison of lung viral titers (F) analyzed by a Kruskal-Wallis multiple-comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

TLR2 expression is required for mediating the antiviral effect of INNA-X. Protection of WT > WT mice was also associated with higher numbers of nasal macrophages (Figure 7C), including populations expressing intracellular viral NP (Figure 7D), recapitulating our observations in WT mice.

Reduced numbers of NP⁺ epithelial cells were observed in WT > *Tlr2*^{-/-} chimeric mice (Figure 7B) compared with *Tlr2*^{-/-} > *Tlr2*^{-/-} chimeras and were accompanied by increased macrophage levels (Figure 7C). This indicates that WT-derived TLR2-expressing immune cells recruited by INNA-X treatment can act to restrict infection of TLR2⁻ epithelial cells. Similar reduction in nasal infected epithelial cells was also observed in *Tlr2*^{-/-} > WT chimeras (Figure 7B). Under the conditions of the experiment, immune cells from *Tlr2*^{-/-} mice are not directly stimulated by INNA-X, leading to the conclusion that INNA-X-treated TLR2⁺ epithelial cells can themselves resist infection. This is supported by the lower macrophage numbers found overall (Figure 7C), although NP-expressing macrophages were still present (Figure 7D). Similarly, there were reduced viral titers in the nasal turbinates of WT > *Tlr2*^{-/-} and *Tlr2*^{-/-} > WT chimeras (Figure 7, E and

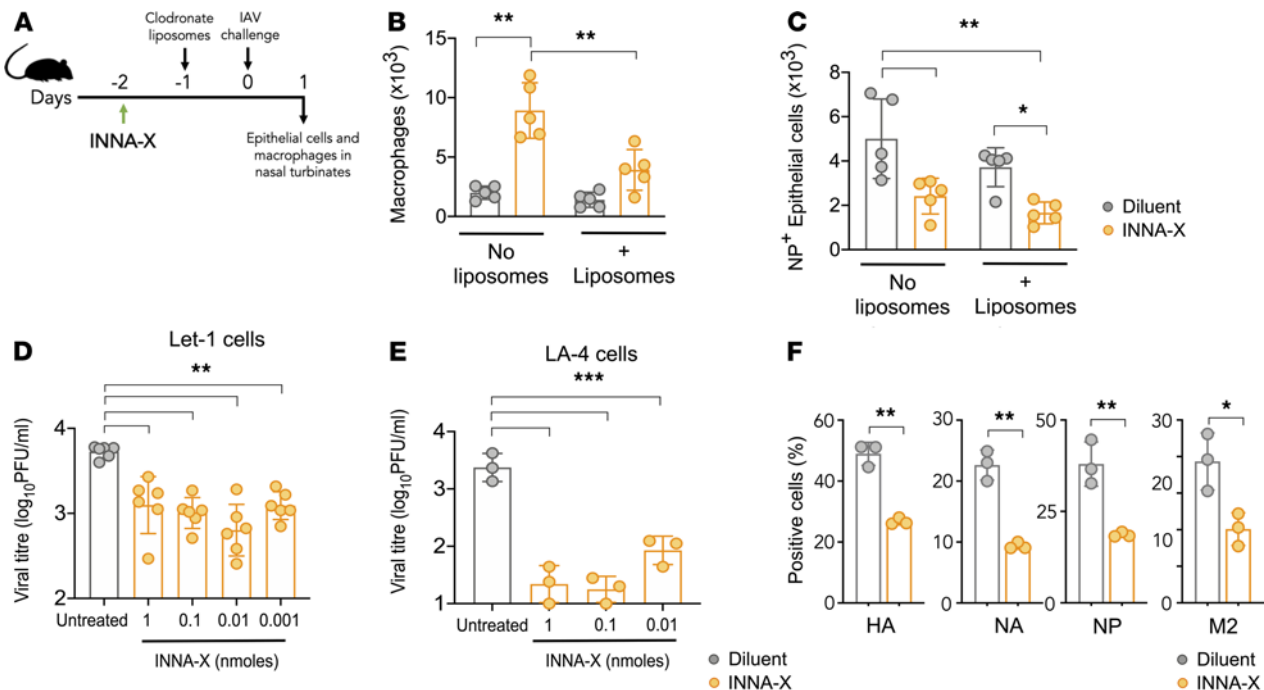


Figure 8. Epithelial cell protection in macrophage-depleted mice and in Let1 and LA-4 cells. (A) Mice ($n = 5/\text{group}$) were inoculated with 1 nmol of INNA-X or diluent and 1 day later received clodronate liposomes via the i.v. (200 μL) and i.n. route (50 μL) or were left untreated. All mice were challenged the next day with 500 PFU of Udorn IAV and 1 day after viral challenge the numbers of (B) macrophages and (C) NP⁺ CD45⁺CD31⁺EpCAM⁺ epithelial cells in nasal turbinates were analyzed. (D and E) 5×10^5 Let1 or 2×10^5 LA-4 cells were incubated in the presence or absence of INNA-X for 24 hours (D) or 18 hours (E) ($n = 3\text{--}5/\text{group}$). Cell monolayers were washed prior to challenge with Udorn IAV (MOI of 0.01). Culture supernatants were harvested 24 hours later and assayed to quantitate viral titers. (F) LA-4 cells were treated with 1 nmol of INNA-X prior to infection. Cells were harvested 8 hours after infection and stained for surface expression of HA, neuraminidase (NA), matrix-2 (M2) influenza viral proteins, and intracellular expression of NP. (G) INNA-X-treated LA-4 cells were lysed and proteins analyzed by reverse-phase liquid chromatography with tandem mass spectrometry (LC-MS/MS) after proteolytic digestion. Sample comparisons were performed using the MaxQuant proteomics platform to determine differentially expressed proteins (red) in INNA-X-treated cells relative to those treated with diluent. Statistical analysis was performed using (B–D and E) 1-way ANOVA with Tukey's post hoc test and (F) Welch t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

F) compared with *Tlr2*^{−/−} > *Tlr2*^{−/−} chimeras. In the case of *Tlr2*^{−/−} > WT chimeras, significantly less virus was also detected in the lung (Figure 7F), suggesting that the effects mediated by TLR2-expressing nonimmune cells extends to protection of the lower respiratory tract.

To further clarify the role of macrophages in mediating epithelial cell protection in vivo, mice inoculated with INNA-X were treated with dichloromethylene diphosphonate-loaded (clodronate-loaded) liposomes to deplete macrophages prior to viral challenge (Figure 8A). Administration of liposomes after INNA-X treatment significantly reduced macrophage numbers in the nasal turbinates but did not affect other cell populations examined (Supplemental Figure 3). Upon viral challenge, compared with animals treated with INNA-X only, a significant reduction in nasal turbinate macrophages was detected in INNA-X-treated mice administered liposomes (Figure 8B). In fact, the numbers of macrophages seen in these animals were not significantly different from those observed in animals that received diluent only. However, irrespective of whether macrophages were depleted, treatment with INNA-X resulted in significantly lower numbers of infected epithelial cells (Figure 8C). Taken together, the results of the bone marrow chimera and clodronate depletion experiments suggest that antiviral effects induced by INNA-X are mediated by both macrophage and epithelial cells.

To investigate the direct protective effects of INNA-X on epithelial cells, we determined whether INNA-X could protect murine airway epithelial cells from infection. Infection of murine airway epithelial cell lines Let1 (30) and LA-4 with influenza virus resulted in more than 3.5 $\log_{10}$ of infectious virus particles released into the cell supernatant. In contrast, significantly reduced titers of infectious virus were recovered from the

supernatants of influenza virus–infected cells pretreated with INNA-X (Figure 8, D and E). These effects also correlate with reduced detection of surface and intracellular viral proteins, including NP, in INNA-X–treated LA-4 cells 8 hours after infection (Figure 8F). Moreover, proteomics analysis of LA-4 cells after INNA-X treatment and prior to infection revealed significant upregulation of mediators involved in apoptosis, chromatin regulation and transcription, leukocyte transendothelial migration, and antiviral activity, most notably pentraxin 3 (PTX3) and caspase-8 (Figure 8G). Taken together, these results indicate that while the influx of macrophages into the nasal turbinates induced by INNA-X treatment can help mediate protection against influenza infection, INNA-X also acts directly on the nasal epithelium to facilitate antiviral resistance.

Discussion

We have demonstrated that administration of INNA-X, when restricted to the upper airways, resulted in a reduction of viral loads in the nasal epithelia and a decline in viral progression to the lungs. Studies on respiratory pathogens in mice typically utilize administration of material to the respiratory tract in a volume that results in deposition throughout the respiratory tract, which can lead to abnormally exaggerated and pathological sequelae. Clearly, the need to limit inflammatory responses in the lower airways and lungs is mandated, and in the case of infectious agents, this method of inoculation may not mimic natural infection because deposition is initially confined to the URT and precedes dissemination to the lower respiratory tract (6, 31, 32). Experimental delivery of material in large volumes may also mask the contribution of immune effectors and mediators that are present in the URT and normally prevent progression to the lower respiratory tract.

Recently, Hua et al. (33) used small delivery volumes to localize murine coronavirus infection to the nasal tissue and showed that mice are protected against severe pneumonia and death when challenged with unrelated viruses, including IAV. We have shown here that reduction in pulmonary virus can also be achieved using INNA-X, rather than by previous infection, to establish protective innate immune–mediated mechanisms. Exposure of nasal turbinates to INNA-X initiated a local proinflammatory effect through upregulation of cytokines, including IL-6, TNF- α , and IL-1 β , exemplifying innate immune responses to influenza infection (8, 34), and the production of chemotactic molecules, including CXCL1 (KC), CCL3 (MIP-1 α), and CCL2 (MCP-1), followed by an influx of macrophages, neutrophils, and other lymphocyte populations. These responses mirror those observed in influenza-infected human epithelial cells (35), which activate genes relating to danger sensing and immune cell migration and support the notion that INNA-X preempts and potentiates the antiviral response in epithelial cells.

Of note, macrophages recruited after INNA-X treatment exhibited a predominantly M2 phenotype as characterized by their low expression of MHC class II, CD86, and inducible NOS (iNOS) and high expression of CD206 and Arginase 1 (Arg1) (36, 37) compared with macrophages from diluent-treated animals (Supplemental Figure 4). These results suggest that they are recruited in response to INNA-X–mediated cytokine production to regulate excessive inflammation and limit tissue damage. Their elevated presence at the site of infection nonetheless still contributes to protection given that a proportion of these macrophages expressed intracellular viral protein, indicating that they had been infected abortively (26, 27) and/or that they had phagocytosed apoptotic virus-infected cells (29), serving to limit viral replication (38–40) before too many rounds of amplification took place.

In this regard, our macrophage depletion experiments and the use of bone marrow chimeras appear to suggest that the protective effects observed are predominantly mediated by epithelial cells and further reinforced by their ability to resist infection in the absence of other cell types *in vitro*. The ability of airway epithelial cells to initiate frontline innate antiviral responses is well known, and while many studies have found the production of IFNs to play a major role (11, 41), our analyses showed no detectable upregulation of gene transcripts for IFNs or significantly increased levels of their products (Supplemental Figure 5) in nasal turbinates after either treatment with INNA-X alone or INNA-X followed by viral challenge when compared with diluent-treated controls. These observations are consistent with our previous findings demonstrating INNA-X treatment of IFN- α receptor–deficient mice (18) are still protected against lethal IAV challenge, thus pointing to other protective mediators at play.

Our analysis of RNA transcripts from nasal turbinates of INNA-X–treated mice detected enrichment of genes involved in the regulation of ERK1/2 signaling cascade, implicating possible involvement of ROS, such as superoxide, hydrogen peroxide, and nitric oxide (14, 42–44). The generation of these molecules forms an important component of host antiviral immune responses in the nasal mucosa and can play a key role in protecting epithelial cells from infection without the requirement for IFN-mediated responses (14). ERK1/2

activity can also be linked to the induction of apoptosis, as evidenced by the detection of caspase-8 activation in INNA-X-treated epithelial cells. In line with this, we have observed the upregulation of genes associated with programmed cell death in INNA-X-treated nasal turbinates, namely those encoding TNF superfamily members, as well as increased expression of Fas (our unpublished observations), thus suggesting treatment primes nasal epithelial cells to undergo apoptosis during the early stages of infection as a countermeasure to limit viral production. In addition, we also detected increased levels of PTX3 produced by INNA-X-treated epithelial cells. PTX3 is an essential mediator of innate resistance to viral pathogens and can be upregulated in the presence of IL-1 β and TNF- α (45), cytokines that were present at elevated levels in the nasal turbinates after treatment. Overall, these findings paint a scenario where INNA-X treatment establishes an immunologically alert state in which activated nasal epithelial cells are prepared for a rapid, broadly protective antiviral response as a first line of defense and aided by the recruitment of macrophages to the nasal tissue.

Our findings from this study have clear relevance for the clinical development of INNA-X as an immunomodulator to mobilize rapid innate immunity against respiratory viruses, including influenza and rhinovirus (18–21). We see a role for this protective strategy in prevention of disease in many contexts, such as frontline health workers, nursing homes, travelers, and individuals who have not been vaccinated, or during outbreaks of virus for which there is no vaccine or treatment and even for those who have been recently exposed. Furthermore, it has not escaped our attention that INNA-X treatment can be implemented as a multidosing regimen to extend and provide continuous protection throughout an outbreak. Of note and highly relevant to COVID-19, our recent studies have since demonstrated its protective efficacy against SARS coronavirus 2 in a ferret model (46), establishing proof of concept that this strategy can be applied in the context of rapidly spreading pandemic viruses to protect immunologically naive global populations.

Methods

Synthesis of INNA-X. The synthesis of a pegylated analog of the diacylated lipopeptide Pam₂Cys, referred to as INNA-X, has been described previously (18). Pam₂Cys is inherently insoluble and has been rendered soluble by others through addition of the amino acid motif Ser-Lys-Lys-Lys-Lys (SK₄) (47). Oligo lysine sequences have, however, been shown to be toxic, albeit at high concentration (48), and even to modulate viral infection processes independent of TLR activation (49). We avoided any off-target effects by incorporating polyethylene glycol as a solubilizing agent into the covalent structure of Pam₂Cys. The final, totally synthetic product contained no detectable lipopolysaccharide (<0.05 endotoxin units/mL) as determined using the Limulus amoebocyte lysate assay (Lonza).

Animals. Female and male C57BL/6 mice expressing the congenic markers CD45.1 or CD45.2 and *Tlr2*^{-/-} mice aged 6–9 weeks were used in this study and were bred and maintained in the Biological Research Facility in the Department of Microbiology and Immunology at the University of Melbourne.

Treatment with INNA-X and viral challenge. Mice were anesthetized with isoflurane and inoculated by i.n. instillation with INNA-X, A/Udorn/307/72 (H3N2) IAV (Udorn IAV), or diluent. The inoculum was confined to the URT by limiting the volume administered dropwise onto the nares to 10–15 μ L; dissemination into the entire respiratory tract was achieved by using an inoculation volume of 40–50 μ L. Udorn IAV was propagated in the allantoic cavity of 10-day-old embryonated hens' eggs; eggs were inoculated with approximately 10³ PFU of virus in 0.1 mL of saline. After 2 days of incubation at 35°C, eggs were chilled at 4°C, and allantoic fluid was harvested, clarified by centrifugation at 720g for 5 minutes, and stored at –80°C until used.

Localization of inoculum determined using Evans blue dye. Anesthetized mice were i.n. inoculated with 10, 15, 20, or 50 μ L of PBS containing 0.125% (w/v) Evans blue dye (MilliporeSigma). After 5 minutes, mice were killed and respiratory tissues (nasal turbinates, trachea, and lung) and stomach tissues harvested. Tissues were rinsed in PBS and homogenized in 1 mL of PBS using a probe homogenizer. Protein was precipitated by addition of 1 mL 100% trichloroacetic acid and centrifugation at 1500g for 1 hour. The optical extinction at 620 nm of the supernatants was compared with a standard curve using a Titertek Multiskan plate reader. Concentrations of dye in tissues were calculated by interpolation from the standard curve using optical density as a function of dye concentration.

Harvesting of tissue and preparation of sera. Viral titers and cytokines were measured in the nasal turbinates and lungs harvested from mice and collected into 1.5 mL of RPMI-1640 medium (MilliporeSigma) containing antibiotics (100 μ g/mL penicillin, 180 μ g/mL streptomycin, and 24 μ g/mL gentamicin). All samples were kept on ice until processed. Tissues were homogenized and clarified supernatants, obtained by centrifugation, were stored at –80°C for subsequent study. Blood, approximately 200 μ L, was obtained from mice

by venipuncture of the submandibular vein. Blood was allowed to clot at 4°C overnight and serum separated by centrifugation at 6000g in a microfuge for 5 minutes and stored at -80°C.

Determination of viral titers. Titers of infectious virus were determined by plaque assay on confluent monolayers of Madin Darby canine kidney cells (MDCK cells; tissue culture cell repository; The Department of Microbiology and Immunology, The University of Melbourne, at the Peter Doherty Institute). Six-well tissue culture plates were seeded with 1.2×10^6 MDCK cells per well in 3 mL of RP10 (RPMI-1640 medium supplemented with 10% [v/v] heat-inactivated FCS, 260 µg/mL glutamine, 200 µg/mL sodium pyruvate, and the antibiotics indicated above). After overnight incubation at 37°C in 5% CO₂, confluent monolayers were washed with RPMI containing antibiotics. Test supernatants serially diluted in RPMI containing antibiotics were added to wells of monolayers and incubated at 37°C in 5% CO₂ for 45 minutes. Monolayers were then overlaid with 3 mL of Leibovitz L15 medium (pH 6.8) containing 0.9% agarose and 2 µg/mL trypsin-TPCK supplemented with antibiotics. Plates were incubated for 3 days at 37°C in 5% CO₂ and PFUs were counted without staining. All assays were carried out in duplicate.

RNA extraction from nasal turbinates. RNA was extracted from nasal turbinates using a RNeasy Mini Kit (Qiagen). Briefly, turbinates were placed in 600 µL of Qiagen RLT buffer containing 1% (v/v) β-mercaptoethanol (Calbiochem) and vortexed for 30 seconds. The lysate was homogenized using QIAshredder columns (Qiagen) before addition of an equal volume of 70% (v/v) ethanol. The RNA was then isolated according to the manufacturer's instructions. RNA quality and concentration were determined using a LabChip microfluidic capillary electrophoresis system (PerkinElmer).

Quantitation of gene expression by NanoString analysis. RNA (50 ng per sample) was hybridized with reporter and capture probe sets from the nCounter mouse immunology panel (XT_PGXMmV1_Immunology; NanoString Technologies) at 65°C for 16 hours in a Bio-Rad thermocycler. Targets were annealed to nCounter cartridges using the nCounter prep station and were imaged using the nCounter digital analyzer set to 280 fields of view (NanoString Technologies).

Analysis of differential gene expression. Quality control of nCounter data and export of raw count data were performed using nSolver version 4.0 software. Differential expression analyses were performed online using the Degust version 3.1.0 tool (50) using the Voom/Limma package. Raw counts were used as input with a minimum count threshold of 10 applied (approximately 2 standard deviations above the mean count of negative control probes). Differential expression was defined as log₂ fold change compared with PBS-treated controls of 1.00 or more or -1.00 or less (i.e., at least 2-fold), with an FDR less than 0.05. Volcano plots were generated using the EnhancedVolcano package (51) in R version 3.5.1 and heatmaps generated using Heatmapper (52) with genes clustered by Euclidian distance. Gene ontology terms were identified using Database for Annotation, Visualization and Integrated Discovery version 6.8 (53) and gene functional annotation tools.

Characterization of cell populations in nasal turbinates. Each turbinate was incubated for 30 minutes at 37°C in a solution of RPMI-1640 containing 1 mg/mL collagenase A and 0.5 mg/mL DNase I (Roche Diagnostics) before being pressed through a metal sieve to collect single-cell suspensions. Red blood cells were lysed by treatment with 0.15 M NH₄Cl and 17 mM Tris-HCl at pH 7.2 for 5 minutes at 37°C. Immune cells were detected using combinations of the fluorochrome-conjugated anti-mouse antibodies for 30 minutes at 4°C. The antibodies used were FITC-NK1.1 (clone PK136), FITC-iNOS (6/iNOS/NOS Type II [RUO]), PE-SiglecF (clone E50-2440), PerCP-Cy5.5-CD8 (clone 53-6.7), PE/Cy7-CD3 (clone 17A2), and AF647-CD64 (clone X54-5/7.1) from BD Biosciences; FITC-CD11b (clone M1/70), Pacific Blue-CD11b (clone M1/70), PE-CD4 (clone GK1.5), PE-CD206 (clone MMR), PerCP-Cy5.5-GR1 (clone RB6-8C5), PerCP-Cy5.5-Ly6G (clone 1A8), APC/Cy7-F480 (clone BM8), and APC-B220 (clone RA3-6B2) from BioLegend; PE-CD16/32 (clone 93), PE/Cy7-CD11c (clone N418), AF700-Arg1 (clone A1exF5), and AF700-CD45 (clone 30-F11) from eBioscience, Thermo Fisher Scientific. CD45⁺ cell populations were classified as follows: interstitial macrophages, Ly6G-F480⁺SiglecF⁺CD64⁺CD11b⁺CD11c⁺; alveolar macrophages, Ly6G-F480⁺SiglecF⁺CD64⁺CD11b⁺CD11c⁺; neutrophils, F480-SiglecF⁺CD11b^{hi}GR1⁺/Ly6G^{hi}; DCs, F480-GR1⁺CD11b^{hi}CD11c^{hi}; CD4⁺ T cells, CD3⁺CD4⁺; CD8⁺ T cells, CD3⁺CD8⁺; B cells, CD3-B220⁺; NK cells, CD3-NK1.1⁺CD16⁺CD32⁺; and NKT cells, CD3⁺NK1.1⁺.

Intracellular detection of Arg1 and iNOS in macrophages was performed after surface marker staining. Cells were fixed, permeabilized, and incubated with fluorochrome-conjugated antibodies using a Cytotfix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer's instructions. Cells were washed extensively, and analysis was carried out using a BD Biosciences LSR II or Canto II flow cytometer and data analyzed using FlowJo software (Tree Star, Inc.).

Epithelial cells were identified by staining with BV421-CD31 (clone 390) and PerCP-CD45 (clone 30-F11) from BD Biosciences and APC-Cy7-EpCAM (clone G8.8) from BioLegend. Detection of intracellular influenza virus NP was performed by permeabilization and fixation of cells using the Foxp3 Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) followed by staining with FITC-conjugated anti-NP antibody (clone 1331, Genetex) according to the manufacturers' instructions.

Analysis of cytokine levels. IFN- γ , IL-2, IL-4, TNF- α , IL-10, IL-6, KC, MCP-1, RANTES, IL-12/IL-23p40, and IL-17A present in homogenate and serum samples were measured using the BD Cytometric Bead Array (CBA) flex kit according to the manufacturer's instructions, with the exception that a total of 0.15 μ L of each capture bead suspension and 0.15 μ L of phycoerythrin detection reagent was used in each 50 μ L sample. For the detection of cytokines not covered in the CBA kit, a LEGENDplex custom panel or a mouse antiviral response mix and match subpanel was used (BioLegend). Samples were analyzed using a BD FACSCanto II flow cytometer, and data were analyzed using FCAP Array software (BD Biosciences) or LEGENDplex version 7.1 software (VigeneTech Inc.). Where data fell below the lower limit of quantitation, data points were displayed at the limit of quantitation for visual and statistical analysis. IFN- λ levels in nasal turbinate samples were measured using a mouse IFN- λ 2/3 (IL-28A/B) ELISA kit according to the manufacturer's instructions (PBL Assay Science).

Generation of bone marrow chimeras. Bone marrow chimeras were established using a method previously described (54). Briefly, CD45.1-expressing C57BL/6 WT and *Tlr2*^{-/-} mice were lethally irradiated with 2 doses of 5.5 Gy 3 hours apart and reconstituted with 5×10^6 T cell-depleted bone marrow cells from CD45.2- or CD45.1-expressing WT or *Tlr2*^{-/-} mice as previously described. Donor and recipient cells were discriminated by isoform expression in vivo. Mice were depleted of radioresistant T cells on the following day by i.p. injection with anti-Thy1 antibody (clone T24; in the form of 100 μ L of cell culture supernatant manufactured in-house). Animals were maintained on antibiotics (neomycin and polymyxin B, MilliporeSigma) contained in drinking water for 6 weeks. Chimeric mice were allowed to reconstitute for at least 8 weeks, by which time they reached an average reconstitution of 93.5%.

In vitro infection of lung epithelial cells. The adherent lung alveolar epithelial type 1-like cell line, Let1, was cultured in DMEM (Invitrogen, Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated FCS, 260 μ g/mL glutamine, 200 μ g/mL sodium pyruvate, and antibiotics (100 μ g/mL penicillin, 180 μ g/mL streptomycin, and 24 μ g/mL gentamicin). LA-4 mouse lung epithelial cells were cultured in Kaighn's modification of Ham's F12 medium (Gibco, Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated FCS, 4 mM L-glutamine, nonessential amino acids (MilliporeSigma), 100 IU of penicillin, 10 μ g of streptomycin/mL, and 50 μ M of 2-mercaptoethanol (Gibco, Thermo Fisher Scientific).

Monolayers were grown to 80%–90% confluence before harvesting using trypsin versine and distributed in 12-well plates at a concentration of 5×10^5 cells (Let1) or 2×10^5 (LA-4) per well in a volume of 1 mL. After establishment of monolayers, medium was replaced with fresh medium containing INNA-X or left untreated and incubated at 37°C in 5% CO₂ for 24 hours. Culture supernatants were then aspirated, and cells were washed prior to the addition of Udorn IAV in 100 μ L (Let1) or 200 μ L (LA-4) at an MOI of 0.01. Cells were incubated for 1 hour, and then virus-containing medium was aspirated and replaced with fresh media or serum-free media with 0.5 μ g/mL of TPCK-treated trypsin (Worthington Biochemical Corporation) for LA-4 cells and incubated for a further 24 hours. Viral titers were determined in harvested cell culture supernatants by plaque titration on MDCK cells.

To analyze viral protein expression in LA-4 cells, cells were infected with X-31 IAV as described above. At 8 hours after infection, infected cells were harvested with a scraper in PBS containing 1 mM EDTA. Surface expression of viral proteins was detected using a goat anti-H3 HA polyclonal antibody (NR-3118, BEI Resources), goat anti-NA polyclonal antibody (NR-3137, BEI Resources), and mouse anti-M2 (clone 14C2, Abcam) in conjunction with anti-goat Ig-Alexa 647 (Invitrogen, Thermo Fisher Scientific, A21447) or anti-mouse Ig-Alexa 647 conjugates (Invitrogen, Thermo Fisher Scientific, A31571), respectively, and analyzed by flow cytometry. Detection of intracellular influenza NP was performed by permeabilization (0.5% Triton X-100 in PBS) and fixation of cells (0.25% Triton X-100, 1 mM EDTA, and 1% FCS in PBS) followed by staining with FITC-conjugated anti-NP antibody (clone 1331, Genetex) prior to flow cytometric analysis.

Proteomics analysis of lung epithelial cells. INNA-X or diluent-treated LA-4 cells were lysed, and proteins were reduced and alkylated in a guanidium chloride lysis buffer (55). Protein quantitation by BCA assay and precipitation were followed by a urea-based trypsin proteolytic digest (56). Samples were desalted using

Empore C18 resin (57), and label-free quantitation analysis was performed using reverse-phase LC-MS/MS using an Orbitrap Elite (Thermo Fisher Scientific). Sample comparisons were performed using the MaxLFQ package in the MaxQuant proteomics platform (58) v1.5.3.30. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (PubMed ID: 30395289) partner repository with the data set identifier PXD023041.

Statistics. All data were tested for normality using a Shapiro-Wilk test to inform the appropriate statistical analysis. For nonparametric analysis between 2 groups, an unpaired 2-tailed Mann-Whitney *t* test was used; for analysis between 3 or more groups, the Holm-Sidak or Kruskal-Wallis test for multiple comparisons was used. For parametric analysis between 2 groups, an unpaired 2-tailed Welch *t* test was used. When 3 or more data sets were compared, 1-way ANOVA with Tukey's post hoc test was used, but when 3 or more groups with 2 or more time points were compared, a 2-way ANOVA with a Bonferroni post hoc test was used. A *P* value less than 0.05 was considered statistically significant. Statistical analyses were performed using Graph-Pad Prism, version 7.0. For the proteomic comparisons, Student's *t* tests were used to compare treatment groups and Benjamini Hochberg multiple hypothesis correction applied using an FDR of 0.05.

Study approval. All animal experimentation was conducted in accordance with institutional regulations following review and approval (permit numbers 1914894 and 1513638) by the University of Melbourne Animal Ethics Committee.

Author contributions

CW, GD, HAQ, SLL, DCJ, and BYC were responsible for the design and conduct of experiments, data analysis, interpretation of results, and drafting of this manuscript. The order of co-first authors reflects the time invested by each author. AB, MC, XJ, and KH performed experiments. WZ, JG, NES, NWB, PCR, and KK provided assistance in the analysis and interpretation of results. LEB provided assistance in the interpretation of results and editing. FM and CD were involved in project management and experimental design. All authors were involved in the editing of this manuscript.

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Suboptimal SARS-CoV-2–specific CD8⁺ T cell response associated with the prominent HLA-A*02:01 phenotype

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An improved understanding of human T cell-mediated immunity in COVID-19 is important for optimizing therapeutic and vaccine strategies. Experience with influenza shows that infection primes CD8⁺ T cell memory to peptides presented by common HLA types like HLA-A2, which enhances recovery and diminishes clinical severity upon reinfection. Stimulating peripheral blood mononuclear cells from COVID-19 convalescent patients with overlapping peptides from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) led to the clonal expansion of SARS-CoV-2–specific CD8⁺ and CD4⁺ T cells in vitro, with CD4⁺ T cells being robust. We identified two HLA-A*02:01-restricted SARS-CoV-2-specific CD8⁺ T cell epitopes, A2/S_{269–277} and A2/Orf1ab_{3183–3191}. Using peptide–HLA tetramer enrichment, direct ex vivo assessment of A2/S₂₆₉+CD8⁺ and A2/Orf1ab₃₁₈₃+CD8⁺ populations indicated that A2/S₂₆₉+CD8⁺ T cells were detected at comparable frequencies ($\sim 1.3 \times 10^{-5}$) in acute and convalescent HLA-A*02:01⁺ patients. These frequencies were higher than those found in uninfected HLA-A*02:01⁺ donors ($\sim 2.5 \times 10^{-6}$), but low when compared to frequencies for influenza-specific (A2/M1₅₈) and Epstein–Barr virus (EBV)-specific (A2/BMLF₁₂₈₀) ($\sim 1.38 \times 10^{-4}$) populations. Phenotyping A2/S₂₆₉+CD8⁺ T cells from COVID-19 convalescents ex vivo showed that A2/S₂₆₉+CD8⁺ T cells were predominantly negative for CD38, HLA-DR, PD-1, and CD71 activation markers, although the majority of total CD8⁺ T cells expressed granzymes and/or perforin. Furthermore, the bias toward naïve, stem cell memory and central memory A2/S₂₆₉+CD8⁺ T cells rather than effector memory populations suggests that SARS-CoV-2 infection may be compromising CD8⁺ T cell activation. Priming with appropriate vaccines may thus be beneficial for optimizing CD8⁺ T cell immunity in COVID-19.

CD8⁺ T cells | COVID-19 | HLA-A*02:01 | SARS-CoV-2 epitopes

The current severe acute respiratory coronavirus 2 (SARS-CoV-2) pandemic has, as of September 2020, infected more than 25 million people, caused at least 850,000 deaths (1), and paralyzed economies globally. Although the majority of infections are mild to moderate and short in duration, ~ 12 to 18% of COVID-19 patients develop severe disease requiring hospitalization, $\sim 5\%$ are critical (2–4), and others who are less severely affected, and even asymptomatic, may still have some underlying pathology (5). These are still early days, and there is much that remains unknown about both the innate and adaptive immune responses in COVID-19. An urgent need is to develop a better understanding so that any

immunopathology can be managed, and vaccine design and immunotherapies optimized.

So far as adaptive immunity is concerned, we do know that SARS-CoV-2–specific antibodies can be found in $\sim 95\%$ of convalescent COVID-19 patients (6, 7) and that titers determined in virus neutralization assays correlate well with spike protein-binding immunoglobulin (Ig) levels measured by ELISA (8, 9). High serum-neutralizing antibody titers tend to be more prominent

Significance

As the recall of CD8⁺ T cell memory promotes rapid recovery in, for example, influenza, we investigated circulating SARS-CoV-2–specific CD8⁺ T cells from COVID-19 patients. For two HLA-A*02:01 SARS-CoV-2–specific CD8⁺ T cell epitopes, we found that, while ex vivo frequencies of responding T cells were approximately fivefold higher than for pre–COVID-19 samples, they were ~ 10 -fold lower than for influenza or EBV-specific memory CD8⁺ T cells. Additionally, SARS-CoV-2–specific CD8⁺ T cells recovered from convalescent COVID-19 patients had an atypically high prevalence of stem cell memory, central memory, and naïve phenotypes. Might this unexpectedly low prevalence of classical effector memory T cells be a negative consequence of the infectious process that could be avoided by prior priming with an appropriately constituted vaccine?

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in severe COVID-19, which could be characteristic of prolonged antigen stimulation due to delayed virus clearance. Otherwise, the duration of SARS-CoV-2–specific IgG persistence in serum is far from clear, and we have much to learn about the CD4⁺ and CD8⁺ T cell responses.

Virus-specific CD8⁺ T cells are generally thought to be involved in the elimination of virus-infected cell “factories” in the acute response to respiratory viruses with, where there is established CD8⁺ T cell memory, that response being enhanced in both rapidity and magnitude to provide a measure of protection against the development of severe disease following secondary virus challenge. Survivors of the 2002–2003 SARS outbreak still maintain CD4⁺ and CD8⁺ T cell populations reactive to the SARS-CoV-1 nucleocapsid protein (10), and evidence of sustained T cell memory has also been found for Middle East respiratory syndrome (MERS) (11). Furthermore, it is possible that there may be some cross-reactive T cell memory for COVID-19 in people who have been infected with these viruses and, perhaps, more broadly, with the previously circulating common cold coronaviruses (12).

For SARS-CoV-2, there is growing evidence that virus-specific T cells are indeed being generated. Our early COVID-19 case study showed that both CD4⁺ T-follicular helper cells and activated CD38⁺HLA-DR⁺CD8⁺ T cells appeared in the patient's blood at 3 d prior to recovery, suggesting that they played a part in the resolution of COVID-19 (13). Recent communications from others also reported the presence SARS-CoV-2–reactive CD4⁺ and CD8⁺ T cells in both acute and convalescent COVID-19 patients (14, 15). More disturbing, however, is an analysis suggesting that at least a proportion of the SARS-CoV-2–specific CD8⁺ T cells recovered from peripheral blood may be showing as an “exhausted” phenotype (16). Clearly, it is a matter of urgency to develop a better understanding of the integrity of the acute CD8⁺ T cell response in COVID-19 and how this impacts disease outcome.

Here, we utilized a combination of peptide prediction and in vitro peptide stimulation with overlapping peptides from the spike (S), nucleocapsid (N), and membrane (M) proteins to identify two SARS-CoV-2 epitopes restricted by HLA-A*02:01 (A2/S₂₆₉ and A2/Orf1ab₃₁₈₃) in individuals with COVID-19. Using peptide-HLA-I tetramers, we performed direct ex vivo tetramer enrichment to define the frequency and activation profiles of the responding SARS-CoV-2–specific CD8⁺ T cells in acute and convalescent COVID-19 patients and in preandemic peripheral blood mononuclear cells (PBMCs), tonsil, and lung tissues from uninfected donors.

Our data establish that HLA-A*02:01–restricted SARS-CoV-2–reactive CD8⁺ T cells can be detected directly ex vivo in both COVID-19 patients and in immunologically naïve individuals. However, while SARS-CoV-2–specific CD4⁺ T cell responses were broadly comparable to those found previously for other viruses, virus-activated CD8⁺ T cells that recognize SARS-CoV-2 peptides presented by the common (at least in Caucasians) HLA-A*02:01 MHC-I glycoprotein both were at low prevalence and express a less than optimal (for virus elimination) phenotype. These findings raise a number of questions. Is this apparent CD8⁺ T cell response defect limited to these particular epitopes? If so, are HLA-A*02:01 individuals at higher relative risk? Alternatively, if this is a general effect, is the SARS-CoV-2 virus in some way subverting CD8⁺ T cell responsiveness? Perhaps COVID-19 may be one disease where an appropriately designed vaccine may do better than nature when it comes to generating a protective CD8⁺ T cell recall response.

Results

COVID-19 Patient Cohort and Uninfected Controls. This study of 18 COVID-19 cases included one person who remained asymptomatic, 10 who were symptomatic but were cared for at home,

and 7 who were admitted to hospital, including 2 requiring supplemental oxygen (*SI Appendix, Table S1*). Control cells were tested from another 17 uninfected individuals who formed a control group (*SI Appendix, Table S2*). All COVID-19 patients (median age 54 y, 55.6% females) seroconverted for SARS-CoV-2 antibodies by receptor-binding domain ELISA (17), and 12 were HLA-A*02:01–expressing individuals. As controls, we analyzed preexisting A2/CD8⁺ T cell responses in preandemic PBMC and tonsil samples from 12 HLA-A*02:01–expressing subjects across three age groups: children (median age 9.5 y), adults (median age 51 y), and the elderly (median age 66.5 y), with 44% being female (*SI Appendix, Table S2*). Additionally, we tested preexisting A2/CD8⁺ T cell populations in lung tissues from five HLA-A2 individuals (median age 42 y).

CD4⁺ and CD8⁺ T Cell Responses to SARS-CoV-2 Overlapping Peptide Pools. We first probed for SARS-CoV-2–specific CD4⁺ and CD8⁺ T cells in convalescent COVID-19 donors using a standard 6-h intracellular cytokine staining (ICS) assay using peptide pools containing 15mers, overlapping by 11 amino acids, which spanned the entire N and M proteins and selected regions of SARS-CoV-2 S protein. The PBMCs were stimulated with one peptide pool and expanded for 10 d before the assessment of SARS-CoV-2–reactive T cells by ICS for intracellular IFN-γ, TNF, and MIP-1β, plus staining for CD107a and perforin (Fig. 1 and *SI Appendix, Fig. S1A*) using individual peptide pools. The responding CD4⁺ T cells all stained for IFN-γ, TNF, MIP-1β, CD107a, and perforin, while the CD8⁺ T cells were predominantly positive for perforin (Fig. 1*A* and *B*). The CD4⁺ T cells showed significant staining for IFN-γ, with five out of six subjects generating IFN-γ⁺CD4⁺ T cell responses to at least one of the SARS-CoV-2 peptide N, M, or S pools, indicating that convalescent COVID-19 patients have solid SARS-CoV-2–specific CD4⁺ T cell immunity. However, while CD8⁺ T cells from 3/6 donors were perforin positive, evidence of modest IFN-γ⁺ activation for the CD8⁺ set was found in only one out of six donors. It thus seems that IFN-γ–producing SARS-CoV-2–specific CD4⁺ T cells expand to a much greater extent than the CD8⁺ set following in vitro peptide stimulation (Fig. 1*C*).

Identification of SARS-CoV-2–Specific HLA-A*02:01–Restricted CD8⁺ T Cell Epitopes. Switching the focus to HLA-specific SARS-CoV-2 CD8⁺ T cell responses, we next identified CD8⁺ T cell specificities for HLA-A*02:01–expressing individuals. Using predicted HLA-A*02:01–binding SARS-CoV-2–derived peptides from the S, N, M, and Polyprotein1ab (Orf1ab) proteins (*SI Appendix, Table S3*; based on two prediction algorithms: NetCTLpan and NetMHCpan; accessed 27 March 2020), PBMCs from five HLA-A*02:01⁺ COVID-19 convalescent individuals were expanded in vitro with a pool of 14 predicted A2/SARS-CoV-2 peptides for 10 d, then restimulated with individual peptides in an ICS assay to determine peptide immunogenicity. Of the 14 peptides screened, S_{269–277} (YLQPRFTLL) generated the strongest CD8⁺IFN-γ⁺ response (mean 0.19%, *n* = 5), with lesser responses being elicited for S_{976–984} (VLNDILSRL) and Orf1ab_{3183–3191} (FLLNKEMYL) (0.07% and 0.08%, respectively, mean, *n* = 5) (Fig. 2 and *SI Appendix, Fig. S1B*). Collectively, we identified one dominant and two subdominant A2/CD8⁺ T cell specificities for SARS-CoV-2.

Peptide sequence conservation analysis for these SARS-CoV-2 immunogenic peptides was extended to previously circulating coronaviruses. Reference protein sequences for SARS-CoV-1 and MERS plus the “common cold” human CoV (hCoV) strains 229E, HKU1, NL63, and OC43 were obtained from National Center for Biotechnology Information. Using the Virus Pathogen Resource (<https://www.viprbrc.org/brc/home.spg?decorator=vipr>), SARS-CoV2 S₂₆₉, S₉₇₆, and Orf1ab₃₁₈₃ peptide sequences were compared to their respective protein sequences within each CoV

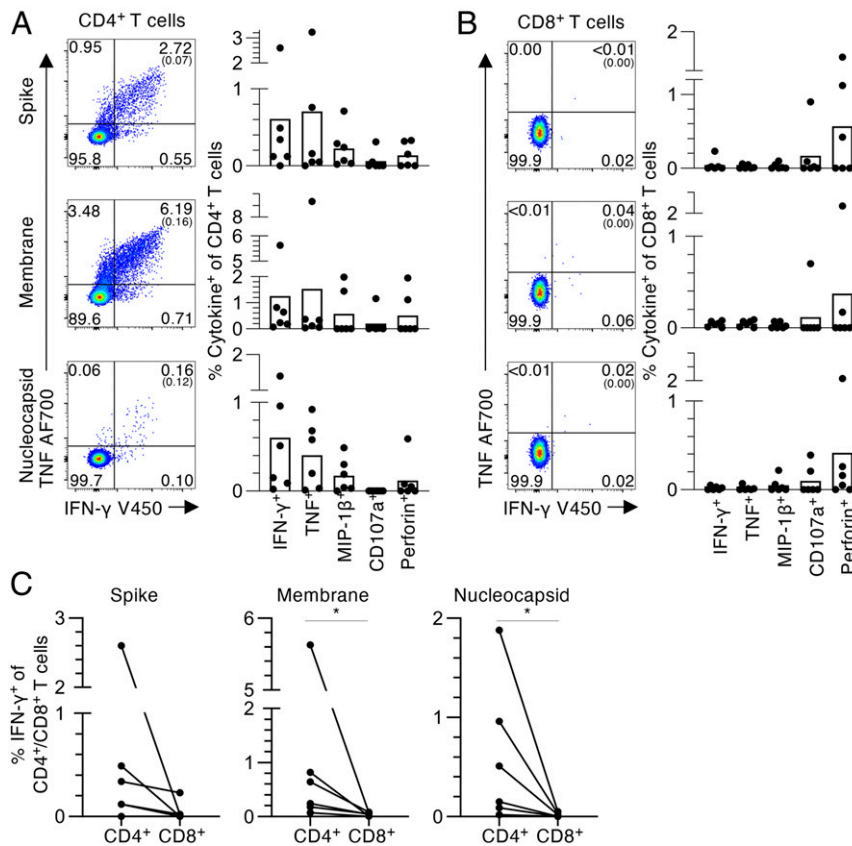


Fig. 1. CD4⁺ and CD8⁺ T cell responses to SARS-CoV-2 overlapping peptide pools. (A) CD4⁺ and (B) CD8⁺ T cell responses to SARS-CoV2 S, M, and N peptide pools in convalescent COVID-19 individuals. (i) Representative fluorescence-activated cell sorter (FACS) plots showing IFN-γ and TNF staining of (A) CD4⁺ or (B) CD8⁺ T cell populations. (ii) Frequencies of IFN-γ⁺, TNF⁺, MIP-1β⁺, CD107a⁺ or perforin⁺ within the (A) CD4⁺ or (B) CD8⁺ T cells, with background staining subtracted ($n = 6$, mean). Background staining values are shown in brackets. Data points show individual COVID-19 convalescent subjects. (C) Paired frequencies of IFN-γ⁺ CD4⁺ and CD8⁺ T cells for S, N, and M peptide pools. Statistical significance was determined with Wilcoxon matched-pairs signed rank test, $*P < 0.05$.

strain (*SI Appendix, Table S3*). Our data showed that SARS-CoV-2/Orf1ab₃₁₈₃ and S₉₇₆ lacked any sequence similarity to hCoV or MERS strains, but each shared 100% sequence identity with SARS-CoV-1/Orf1ab_{3160–3168} (FLLNKEMYL) and S_{958–966} (VLNDILSRL), respectively. SARS-CoV-2/S₂₆₉ shared 78% and 67% sequence identity with MERS/S_{317–325} (KLQPLTFLL) and SARS-CoV-1/S_{256–264} (YLKPTTFML), respectively (positions that differ are underlined). Evidently, the A2/SARS-CoV-2

CD8⁺ T cell epitopes identified may be cross-reactive for SARS-CoV-1 and MERS, while they did not share homology with the common cold hCoVs that circulate in Australia.

SARS-CoV-2–Specific A2/CD8⁺ T Cells Are at Low Frequency in COVID-19 Patients. To further analyze the SARS-CoV-2–specific A2/CD8⁺ populations from COVID-19 patients, we generated tetramers for the A2/S₂₆₉ and A2/Orf1ab₃₁₈₃ epitopes. Tetramer-associated

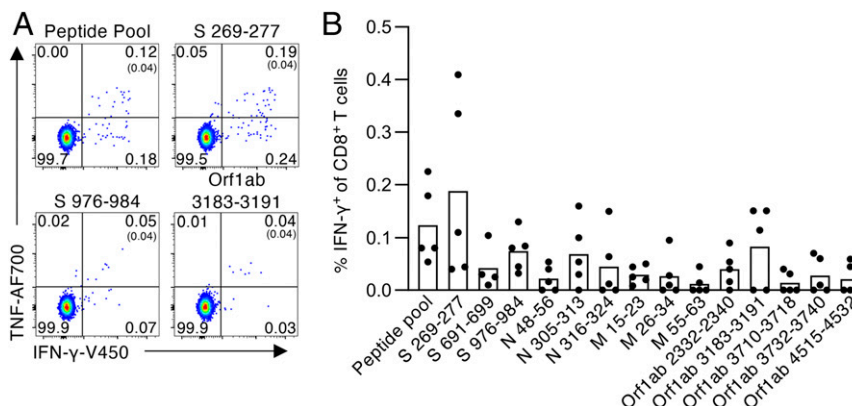


Fig. 2. Identification of SARS-CoV-2-specific HLA-A*02:01-restricted CD8⁺ T cell epitopes. (A) Representative FACS plots of CD8⁺ IFN-γ/TNF staining after stimulation with the SARS-CoV-2 predicted peptide pool and individual S_{269–277}, S_{976–984}, and Orf1ab_{3183–3191} peptides. (B) Frequency of IFN-γ⁺ of CD8⁺ T cells for each SARS-CoV-2 peptide within the predicted peptide pool, with background staining subtracted ($n = 5$, mean). Peptide screen was performed in convalescent COVID-19 PBMCs after 10-d expansion in vitro.

magnetic enrichment (18, 19) was then used to determine the ex vivo frequencies of A2/S₂₆₉⁺CD8⁺ and A2/Orf1ab₃₁₈₃⁺CD8⁺ T cells in acute and convalescent HLA-A*02:01⁺ cases. During the acute phase of COVID-19, A2/S₂₆₉⁺CD8⁺ T cells were readily detected after ex vivo tetramer enrichment at a mean frequency of

1.44×10^{-5} ($n = 3$) in the CD8⁺ set, while the values for the A2/S₂₆₉⁺CD8⁺ and A2/Orf1ab₃₁₈₃⁺CD8⁺ T cells from COVID-19 convalescents were 1.28×10^{-5} ($n = 14$) and 1.77×10^{-6} ($n = 6$), respectively (Fig. 3*A* and *D*). There was no significant difference in the frequency of A2/S₂₆₉⁺CD8⁺ T cells between acute and

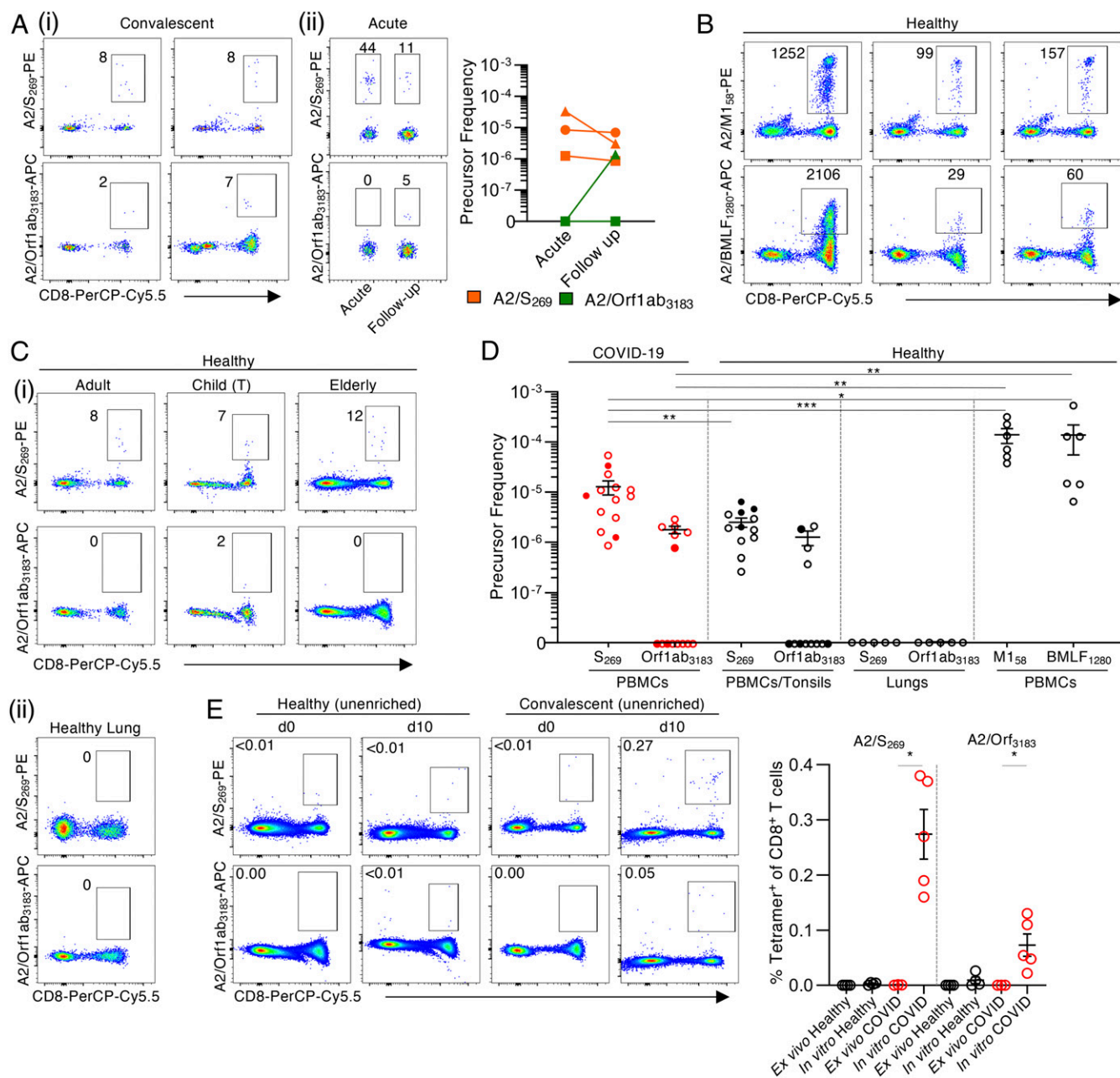


Fig. 3. Low ex vivo frequency of SARS-CoV-2-specific A2/CD8⁺ T cell specificities in acute and convalescent COVID-19 patients. A2/S₂₆₉⁺CD8⁺ and A2/Orf1ab₃₁₈₃⁺CD8⁺ T cells were identified directly ex vivo from healthy (pre-COVID-19) PBMCs, tonsils, and lungs, as well as acute and convalescent COVID-19 PBMCs by tetramer magnetic enrichment. (A) Representative FACS plots of A2/S₂₆₉⁺CD8⁺ and A2/Orf1ab₃₁₈₃⁺CD8⁺ T cells from enriched samples of (i) convalescent and (ii) acute COVID-19 PBMCs. (B) Representative FACS plots of A2/M1₅₈⁺CD8⁺ and A2/BMLF₁₂₈₀⁺CD8⁺ T cells from enriched healthy PBMCs. (C) Representative FACS plots of A2/S₂₆₉⁺CD8⁺ and A2/Orf1ab₃₁₈₃⁺CD8⁺ T cells from (i) enriched adult and elderly PBMCs, and child tonsils (T) and (ii) tetramer staining of human lung tissue. (D) A2/CD8⁺ T cells precursor frequencies were calculated for A2/S₂₆₉⁺CD8⁺, A2/Orf1ab₃₁₈₃⁺CD8⁺, A2/M1₅₈⁺CD8⁺, and A2/BMLF₁₂₈₀⁺CD8⁺ T cells enriched from either PBMCs or tonsils, or stained in lungs. Dots represent individual donors. Means $\pm$ SEM are shown. Red dots are COVID-19 acute (closed circle) and convalescent (open circle) donors. Black dots are healthy adult or elderly PBMCs (open circle), or MNCs from child tonsils (closed circle). Donors with undetectable precursor frequencies of 0 are included on the graph to show the number of donors tested. These donors were not included in statistical analyses. Statistical significance was determined with two-tailed Mann-Whitney *U* test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (E) Representative FACS plots and frequencies of A2/SARS-CoV-2⁺CD8⁺ T cells in the CD8⁺ population in healthy and convalescent donors on d0 and d10 of expansion. Dots represent individual donors. Statistical significance was determined using a two-tailed Mann-Whitney *U* test, **P* < 0.05.

convalescent COVID-19 donors, while minimal A2/S₂₆₉⁺CD8⁺ and A2/Orf1ab₃₁₈₃⁺CD8⁺ T cells were detected in either unenriched or flow-through samples (*SI Appendix, Fig. S2*). Indeed, while too few T cells were available to test other specificities concurrently for the COVID-19 patients, these frequencies of SARS-CoV-2-specific CD8⁺ T cells were significantly lower than those found for influenza A virus (IAV)-specific (1.39×10^{-4} for A2/M1₅₈; $n = 6$) and Epstein-Barr virus (EBV)-specific (1.38×10^{-4} for A2/BMLF1₂₈₀; $n = 6$) memory T cell populations from uninfected controls (Fig. 3 *B* and *D*), and as per previous publications (19, 20).

Are SARS-CoV-2-specific CD8⁺ T cells present in uninfected people? Using ex vivo tetramer enrichment with prepanemic PBMC, tonsil, and lung samples taken from HLA-A*02:01-expressing uninfected individuals (Fig. 3 *C*, *i* and *D*), naïve SARS-CoV-2-specific CD8⁺ T cells directed at A2/S₂₆₉ were detected in all of the PBMC and tonsil samples ($n = 12$), while CD8⁺ T cells directed at A2/Orf1ab₃₁₈₃ were found in only 33% of individuals ($n = 12$), and the lung tissues were uniformly negative (Fig. 3 *C*, *ii* and *D*). Both the A2/S₂₆₉⁺CD8⁺ and A2/Orf1ab₃₁₈₃⁺CD8⁺ were found over a broad range of ages (A2/S₂₆₉: 5 y to 68 y; A2/Orf1ab, 11 y to 65 y). Moreover, the A2/S₂₆₉⁺CD8⁺ T cell frequency of 2.5×10^{-6} (mean, $n = 12$) in pre-COVID-19 healthy individuals was significantly lower than that found for COVID-19-exposed individuals ($P = 0.0064$; Fig. 3*D*). It thus seems that the A2/S₂₆₉⁺CD8⁺ T cells are indeed being activated and clonally expanded during SARS-CoV-2 infection. In contrast, there was no significant difference in frequencies for the A2/Orf1ab₃₁₈₃⁺CD8⁺ T cells from the prepanemic and COVID-19 groups ($P = 0.4121$) (Fig. 3*D*).

To further probe the responsiveness of A2/SARS-CoV-2 CD8⁺ T cells from uninfected versus convalescent COVID-19 donors, PBMCs or tonsil cells were stimulated with the S₂₆₉ and Orf1ab₃₁₈₃ peptides and cultured in vitro for 10 d. In prepanemic “naïve” subjects, no evidence of proliferation in culture was found for the A2/S₂₆₉⁺CD8⁺ or A2/Orf1ab₃₁₈₃⁺CD8⁺ sets (Fig. 3*E*). In contrast, both the A2/S₂₆₉⁺CD8⁺ and A2/Orf1ab₃₁₈₃⁺CD8⁺ T cells from the COVID-19 donors increased significantly in numbers ($P = 0.0357$; Fig. 3*E*). Evidently, the SARS-CoV-2/CD8⁺ T cells from COVID-19 individuals (but not those from SARS-CoV-2 naïve subjects) were primed by SARS-CoV-2 and are thus, at least under in vitro conditions, capable of clonal expansion.

Activation Profiles of SARS-CoV-2-Specific A2/CD8⁺ T Cells Directly Ex Vivo. The activation profiles of A2/S₂₆₉⁺CD8⁺ T cells tested directly ex vivo from acute and convalescent patients were assessed by CD27, CD45RA, and CD95 staining to determine the prevalence of the naïve (T_{Naïve}) (CD27⁺CD45RA⁺CD95⁺), stem cell memory (T_{SCM}) (CD27⁺CD45RA⁺CD95⁺), central memory (T_{CM})-like (CD27⁺CD45RA⁺), effector memory (T_{EM})-like (CD27⁺CD45RA⁺), and effector memory CD45RA (T_{EMRA}) (CD27⁺CD45RA⁺) subsets (Fig. 4*A*). Acute COVID-19 donors displayed the highest proportion (mean of 92%) of T_{CM}-like A2/S₂₆₉⁺CD8⁺ T cells and a low proportion of T_{EM}-like CD8⁺ T cells. The A2/S₂₆₉⁺CD8⁺ T cells from the convalescent versus acute subjects had a lower prevalence of T_{CM}-like (mean of 50%) cells, and larger proportions of the T_{Naïve} (mean of 27%) and T_{SCM} (mean of 15%) sets, indicating that A2/S₂₆₉⁺CD8⁺ T cells expressing the optimally responsive T_{CM} phenotype fall off rapidly in blood sampled after the infection has resolved. Conversely, the majority of A2/S₂₆₉⁺CD8⁺ T cells within prepanemic children and adults were naïve (T_{Naïve}; mean of 68% and 77%, respectively), while this subset was less prominent (mean of 46%) in the elderly. Interestingly, older, uninfected people had a mean of 38% T_{CM}-like A2/S₂₆₉⁺CD8⁺ T cells, similar to the frequency found for COVID-19 convalescents (mean of 50%), but less than that for IAV A2/M1₅₈ (mean of 66%).

The expression profiles for HLA-DR, CD38, PD-1, and CD71 were also determined for tetramer⁺ A2/S₂₆₉⁺CD8⁺ T cells from the COVID-19 patients (Fig. 4*B*). Only T cells from acutely infected donors were positive for these activation markers, with the majority coming from one individual (COVID-19 #2). In contrast, the A2/S₂₆₉⁺CD8⁺ T cells from prepanemic and COVID-19 convalescent subjects were characterized by minimal levels of HLA-DR⁺CD38 and PD-1⁺CD71⁺, suggesting that, while the A2/S₂₆₉⁺CD8⁺ set can be activated during the acute phase of the infection, it does not persist into short-term memory. Overall, our data suggest that naïve A2/SARS-CoV-2-specific CD8⁺ T cells can indeed be expanded approximately fivefold and activated during the acute phase of COVID-19 but that, atypically for what we know for other readily resolved infections like influenza, both the extent of T cell proliferation and the persistence of activated T cells in the blood is low for (days 37 to 101 post disease onset) convalescent individuals.

To further investigate the suboptimal activation of SARS-CoV-2-specific CD8⁺ T cells in COVID-19, the killing capacity of A2/S₂₆₉⁺CD8⁺ T cells was assessed by staining for granzyme A, B, and K, and perforin directly ex vivo. Surprisingly, the majority of A2/S₂₆₉⁺CD8⁺ T cells at both acute (mean of 77.2%) and convalescent (mean of 72.4%) stages of COVID-19 expressed three to four cytotoxic granzymes/perforin (Fig. 4*C* and *SI Appendix, Fig. S3*), indicating their activation status. However, a similarly high expression level of granzymes/perforin was also found on the majority of total CD8⁺ T cells (69 to 82.5%), as per our previous case report (13), but not on non-CD8⁺ T cells (mean of 15 to 21%). As it is highly unlikely that ~80% of all CD8⁺ T cells in the peripheral blood during primary SARS-CoV-2 infection were antigen specific (even if directed at several CD8⁺ T cell epitopes), this suggests that a high proportion of CD8⁺ T cells are activated via some “bystander” mechanism during acute/convalescent COVID-19. The consequences, if any, of this effect for TCR-mediated activation merit further investigation.

Discussion

As the research community drives forward to design and evaluate novel vaccines and immunotherapies for COVID-19, concurrent efforts directed at understanding how immunity works in this disease process are largely focused on patient studies. Applying our established expertise in the analysis of T cell-mediated immunity, we found here that the CD4⁺ “helper” T cell response looks relatively normal when compared with what happens in, for example, people who have been infected with an IAV. However, when it comes to the virus-specific CD8⁺ T cells that play an important role in ameliorating disease severity and driving recovery in other respiratory infections, our findings for COVID-19 are less encouraging. Although we were able to identify two SARS-CoV-2-specific CD8⁺ T cell epitopes associated with the ubiquitous (in Caucasian) HLA-A*02:01 MHC-I glycoprotein (A2/S₂₆₉–277 and A2/Orf1ab₃₁₈₃–3191) and found evidence for T cell responsiveness, the results were not what we expected.

Our findings show that, while “early memory” CD8⁺ T cells can be detected in convalescent HLA-A*02:01 COVID-19 patients at frequencies approximately fivefold higher than those from prepanemic samples, the SARS-CoV-2-specific response was ~10-fold lower than that found regularly for CD8⁺ T cells directed at IAV or EBV epitopes. In general, there was an overrepresentation of SARS-CoV-2-specific tetramer⁺CD8⁺ T cells expressing cell surface phenotypes that are considered to be characteristic of “stem cell memory” and naïve precursor status, suggesting that the infectious process is, in some way, limiting both clonal expansion and differentiation of the “classical” effector and central memory sets. An alternative explanation is, of course, that T cell effectors are being generated but are localized to, and perhaps “consumed in” (driven to apoptosis?) sites of virus-induced pathology.

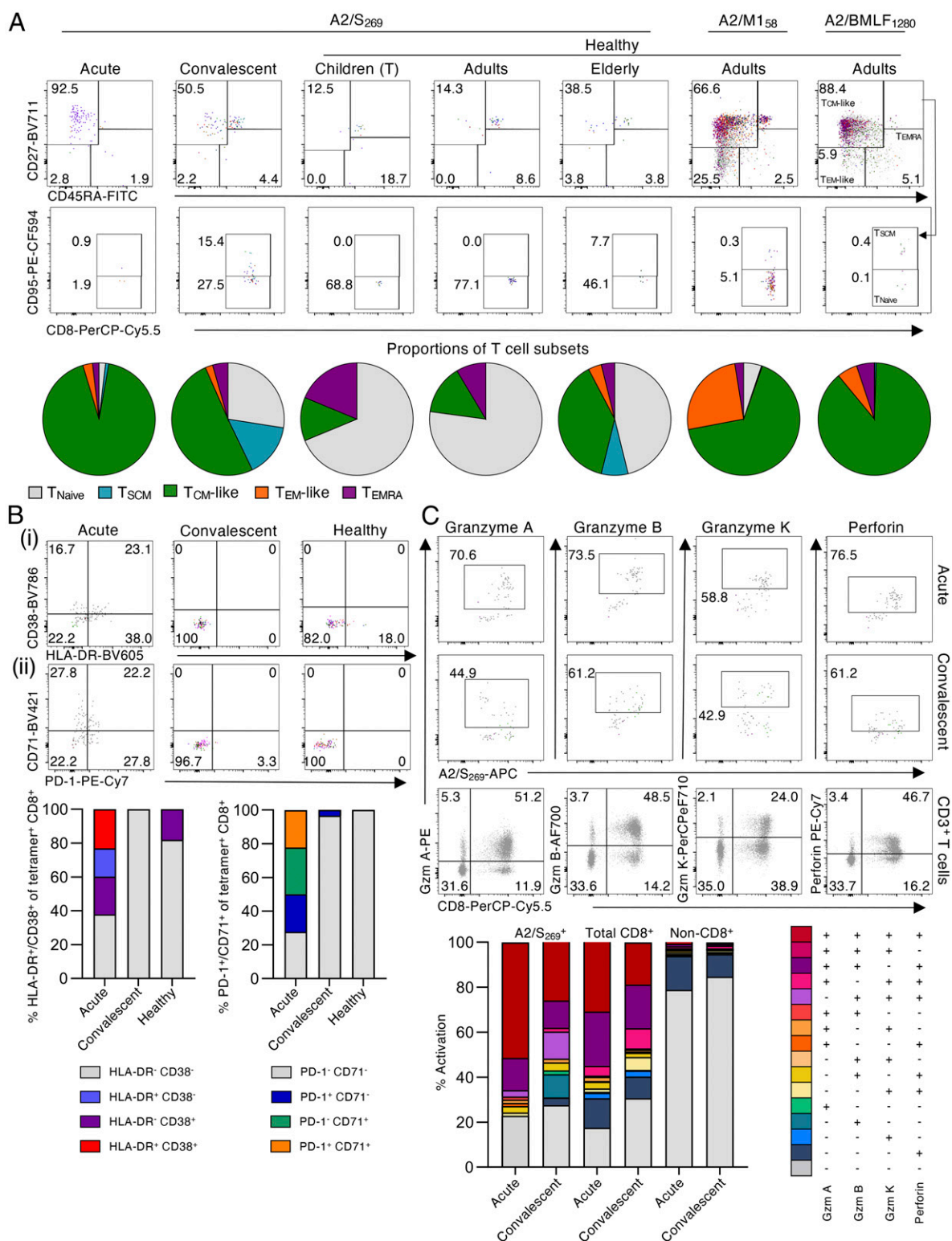


Fig. 4. Ex vivo activation profiles of SARS-CoV-2-specific A2/CD8⁺ T cells in COVID-19 subjects. (A) Overlaid FACS plots of A2/S₂₆₉⁺CD8⁺ T cells from acute COVID-19 ($n = 3$), convalescent COVID-19 ($n = 11$), healthy children (tonsils) ($n = 4$), healthy adults ($n = 4$), or healthy elderly donors ($n = 4$) show T_{Naive} (CD27⁺CD45RA⁺CD95⁺), T_{SCM} (CD27⁺CD45RA⁺CD95⁺), T_{CM-like} (CD27⁺CD45RA⁺), T_{EM-like} (CD27⁺CD45RA⁺), and T_{EMRA} (CD27⁺CD45RA⁺) subsets. Pie charts display the proportion of each phenotype subset based on the combined data per each COVID-19 or healthy donor group. Overlaid FACS plots of A2/M1₅₈⁺CD8⁺ and A2/BMLF₁₂₈₀⁺CD8⁺ T cell memory phenotypes from healthy adults are also shown. (B) Overlaid FACS plots and combined frequencies of A2/S₂₆₉⁺CD8⁺ T cells showing (i) HLA-DR and CD38 or (ii) PD-1 and CD71 activation markers for acute ($n = 3$), convalescent ($n = 11$) and healthy donors ($n = 12$). (C) Overlaid FACS plots and combined frequencies of A2/S₂₆₉⁺CD8⁺ T cells showing granzyme A, B, and K, and perforin staining for acute ($n = 2$) and convalescent ($n = 3$) donors. Representative FACS plots from one donor showing granzymes A, B, and K, and perforin of the total CD3⁺ T cell population. Combination gating was used to determine the frequency of cells with one to four functions for A2/S₂₆₉⁺CD8⁺, total CD8⁺, or non-CD8⁺ T cells. Graphed data across multiple COVID-19 acute, COVID-19 convalescent, or naïve subjects were combined for the activation and phenotypic analyses of A2/S₂₆₉ CD8⁺ T cells.

Even so, it is the case that SARS-CoV-2–specific CD8⁺ T cells were found in all COVID-19 acute and convalescent donors, and in stored pre-pandemic PBMC and tonsil samples (but not lung tissues) from HLA-A*02:01 children, mature adults, and the elderly. As the frequency of these naïve, pre-pandemic SARS-CoV-2–specific CD8⁺ T cells ($\sim 2.5 \times 10^{-6}$) was numerically comparable to that found for naïve HIV (Gag_{77–85}, SLYNTVATL), cancer (Survivin_{96–104}), or Hepatitis C Virus (NS3₁₀₇₃)–specific CD8⁺ T cell populations in healthy HLA-A*02:01⁺ individuals (19–21), both their presence and the fact that they were not readily expanded following in vitro stimulation suggests that they were not a product of prior exposure to some cross-reactive epitope. In fact, these are likely the naïve precursors that would be stimulated by appropriate prime-and-boost vaccine strategies.

Earlier experiments in a mouse model of SARS-CoV-1 showed that a conventional, CD8⁺ T cell-targeted prime-and-boost approach indeed established substantial pools of memory SARS-CoV-1–specific CD8⁺ T cells capable of driving protection against lethal SARS-CoV-1 infection (22). The fact that the frequencies of A2/S₂₆₉⁺CD8⁺ T cells in COVID-19 patients increased approximately fivefold suggests that these SARS-CoV-2–specific CD8⁺ T cells proliferated, to some extent, during primary COVID-19, however not to the level of well-established memory CD8⁺ T cell populations directed at other viral epitopes like IAV-specific A2/M1₅₈ and EBV-specific A2/BMLF₁₂₈₀. Further studies are obviously needed to understand why this is so. In addition, as our acquaintance with this novel CoV continues, we will be able to determine whether there is long-term survival (at least at >1 y) of SARS-CoV-2–specific CD8⁺ memory T cells following primary COVID-19 along with whether, in now healthy survivors, they can be activated and clonally expanded following challenge with an appropriate vaccine.

Surprisingly, the memory A2/S₂₆₉⁺CD8⁺ T cell populations in convalescent subjects were dominated by stem cell memory, central memory, and naïve phenotypes, and lacked expression of the CD38, HLA-DR, PD-1, and CD71 activation markers. This is in stark contrast to the highly activated T_{EM} and T_{EMRA} profiles found ex vivo in both short-term (day 25) and long-term (7 mo) memory A2/M1₅₈⁺CD8⁺ T cells following avian A/H7N9 influenza infection (23, 24). These minimal activation profiles for epitope-specific CD8⁺ T cells in early COVID-19 convalescence could possibly reflect suboptimal priming of A2/S₂₆₉⁺CD8⁺ T cells in primary COVID-19. Furthermore, a recent study by Zhou et al. (25) demonstrated perturbed dendritic cell and T cell function in SARS-CoV2 infection. Impaired dendritic cell function might negatively impact antigen processing and presentation to CD8⁺ T cells, thus at least partially explaining the limited differentiation of SARS-CoV-2–specific CD8⁺ T cells observed here.

It remains unclear whether this is broadly representative of primary CD8⁺ T cell responses in COVID-19 or specific to the epitopes analyzed here. There is a possibility that there are other HLA-A*02:01–restricted immunodominant epitopes, or even immunodominant epitopes restricted by other HLAs in HLA-A*02:01⁺ COVID-19 patients. The A2/S₂₆₉ epitope identified in our study was, however, also independently reported in a recent preprint (26), suggesting it is a common HLA-A*02:01 epitope. Moreover, it is also possible that CD8⁺ T cells directed toward other HLA-A*02:01–restricted epitopes might have expressed high levels of PD-1 and thus had an impaired capacity to expand in vitro due to their exhausted phenotype. Further identification of CD8⁺ T cell epitopes across a broad range of HLA class I alleles and SARS-CoV-2 proteins is needed to provide a more detailed landscape of CD8⁺ T cell responses in COVID-19, their ex vivo frequencies, and activation profiles. In-depth analysis of epitope-specific T cell responses in severe and critical cases is also essential if we are to understand whether the activation profiles of early CD8⁺ T cell memory reflect disease severity.

And, as the range of candidate vaccines that are tested through phase 1 trials expands, it would also be of great benefit to determine whether the characteristics of memory CD8⁺ T cells generated in the absence of active infection look more optimal than those described here.

Stimulation with overlapping peptides led to the expansion of SARS-CoV-2–specific CD8⁺ and CD4⁺ T cells in vitro, although CD4⁺ T cells dominated the response. This might support, at least partially, the previous elegant study showing that CD4⁺ T cells but not CD8⁺ T cells were of a greater importance in primary SARS-CoV-1 infection, as depletion of CD4⁺ T cells (but not CD8⁺ T cells) led to delayed viral clearance from the lungs, associated with reduced neutralizing antibody and cytokine production (27). It is also important to note that the S peptide pool from Miltenyi Biotec used here spans only selected regions (304 to 338, 421 to 475, 492 to 519, 683 to 707, 741 to 770, 785 to 802, and 885 to 1273) rather than the entire protein; thus some CD8⁺ and CD4⁺ T cell responses could have been missed. Recent evidence revealed that Th2 and Th17 cytokine profiles in COVID-19 patients can be associated with differential disease outcomes (28). Our analyses focused on Th1 cytokine responses for CD4⁺ T cells, leaving Th2 and Th17 cytokine responses unknown. Different cytokine profiles of epitope-specific CD4⁺ T cells should be investigated in future studies, especially when SARS-CoV2–specific CD4⁺ T cell epitopes are identified.

Our early report on immunity to COVID-19 in some of Australia's first patients suggested that broad and concomitant immune responses were associated with recovery from mild-to-moderate COVID-19 disease (13). The key immune populations detected included antibody-secreting cells, helper follicular T cells, and activated (CD38⁺HLA-DR⁺) CD8⁺ and CD4⁺ T cells, together with progressive increases in SARS-CoV-2–specific IgM and IgG antibodies. Subsequent studies confirmed the activation of both CD4⁺ and CD8⁺ T cells as indicated by cell-surface marker expression (14, 15). The present much more extensive yet focused analysis does, however, raise questions concerning the integrity of the epitope-specific CD8⁺ T cell response in COVID-19. Given the variation in disease outcome with this infection, that obviously merits much more detailed analysis.

Methods

Study Participants and Ethics Statement. Thirty-five subjects were recruited into this study. Acute and convalescent COVID-19 subjects were recruited via the Alfred Hospital, University of Melbourne, or James Cook University. Seven of the donors were admitted to hospital during their active infection (*SI Appendix, Table S1*). Acute COVID-19 cases were admitted to the hospital ward, with two patients requiring oxygen support (*SI Appendix, Table S1*). Healthy donors were recruited via University of Melbourne or buffy packs obtained from the Australian Red Cross LifeBlood (*SI Appendix, Table S2*). Tonsils were obtained from healthy individuals undergoing tonsillectomy (Tasmania, Australia). Lung samples were obtained prior to the COVID-19 pandemic via the Alfred Hospital's Lung Tissue Biobank. All blood and tonsil donors were HLA typed by Victorian Transplantation and Immunogenetics Service. Peripheral blood was collected in heparinized tubes, and PBMCs were isolated via Ficoll–Paque separation.

Experiments conformed to the Declaration of Helsinki Principles and the Australian National Health and Medical Research Council Code of Practice. Written informed consents were obtained from all blood donors prior to the study. Lung tissues were obtained from deceased organ donors after written informed consents from the next of kin. Written informed consents were obtained from participants' parents or guardians for underage tonsil tissue donors. The study was approved by the Alfred Hospital (#280/14), The University of Melbourne (#2056689, #2056761, #1442952, #1955465, and #1443389), the Australian Red Cross Lifeblood (ID 2015#8), the Tasmanian Health and Medical (ID H0017479), and the James Cook University (H7886) Human Research Ethics Committees.

Cell lines and reagents, ICS, ex vivo tetramer enrichment, and phenotypic analysis are described in *SI Appendix*.

Data Availability. All study data are included in the article and *SI Appendix*.

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MAIT cells contribute to protection against lethal influenza infection in vivo

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Mucosal associated invariant T (MAIT) cells are evolutionarily-conserved, innate-like lymphocytes which are abundant in human lungs and can contribute to protection against pulmonary bacterial infection. MAIT cells are also activated during human viral infections, yet it remains unknown whether MAIT cells play a significant protective or even detrimental role during viral infections in vivo. Using murine experimental challenge with two strains of influenza A virus, we show that MAIT cells accumulate and are activated early in infection, with upregulation of CD25, CD69 and Granzyme B, peaking at 5 days post-infection. Activation is modulated via cytokines independently of MR1. MAIT cell-deficient MR1^{-/-} mice show enhanced weight loss and mortality to severe (H1N1) influenza. This is ameliorated by prior adoptive transfer of pulmonary MAIT cells in both immunocompetent and immunodeficient RAG2^{-/-}γC^{-/-} mice. Thus, MAIT cells contribute to protection during respiratory viral infections, and constitute a potential target for therapeutic manipulation.

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Mucosal associated invariant T (MAIT) cells are an abundant and evolutionarily-conserved¹ class of innate-like lymphocytes², which comprise up to 10% of human peripheral blood³ and respiratory mucosal T cells^{4,5}. MAIT cell development and antigen (Ag)-specific activation are restricted by the monomorphic β 2-microglobulin-associated molecule MHC related protein-1 (MR1)⁶. MAIT cells express a semi-invariant $\alpha\beta$ T cell receptor (TCR)⁷ shown to recognise metabolic derivatives of highly-conserved riboflavin biosynthetic pathways^{8,9}, that are expressed by a wide range of bacteria, mycobacteria and yeasts¹⁰. These molecules are usually short-lived, but are stabilised by MR1, and include 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), which is a potent MAIT cell ligand¹¹, and can be loaded onto MR1 to form specific tetramers to track human and murine MAIT cells^{9,12}. Consistent with these observations, we and others have shown that MAIT cells contribute to protection in vivo against intracellular bacteria including *Francisella tularensis*¹³, *Legionella longbeachae*¹⁴ and mycobacteria¹⁵, whilst human in vitro data suggest they may contribute also to protection against a variety of other respiratory bacterial pathogens, including *Haemophilus influenzae*^{5,16} and *Streptococcus pneumoniae*^{17,18}.

Although this MR1-TCR dependent activation appears specific for riboflavin-expressing prokaryotic pathogens, there is emerging evidence that MAIT cells have the potential also to be involved in immune responses to viruses. MAIT cells have constitutively high surface expression of the interleukin (IL)-18 receptor and IL-12 receptor¹⁹, and we have shown they can be activated by IL-18 synergistically with IL-12²⁰ or IL-15 or type I interferons (IFN)²¹ to induce expression of IFN- γ and Granzyme B²². These responses are MR1 and TCR independent^{20,21}. These phenotypically innate features of MAIT cells, in common with invariant natural killer T cells (iNKT), are believed to be driven by expression of the master transcription factor promyelocytic leukaemia zinc finger protein (PLZF)^{19,23}.

We have previously shown that peripheral MAIT cells are activated in vivo during human infections with influenza A virus (IAV), dengue virus and hepatitis C, whilst in vitro these viruses activate MAIT cells in an IL-18 dependent manner²¹. Moreover, we showed that activation correlated with disease activity in IAV infection²¹ and reduced peripheral blood MAIT cell frequencies were associated with death in severe avian H7N9 IAV disease²². Activation has also been observed by others in hepatitis B²⁴, hepatitis C²⁵ and Zika²⁶ infections, whilst reductions in both peripheral blood MAIT cell frequencies and MAIT cell functional capacity have been observed in a number of clinical studies of viral infections, including HIV²⁷ and HTLV-1²⁶. However, such studies were correlative, have largely been limited to peripheral cells, and have not addressed local activation and function in the lungs. Most importantly, it remains to be shown in vivo whether activation of MAIT cells plays any role—protective or detrimental—during viral infections²⁸.

Here, we show that MAIT cells accumulate in the lungs and are activated during in vivo infection with IAV. MAIT cell activation is modulated via cytokines, independently of MR1. Moreover, animals deficient in MAIT cells show exacerbated disease following IAV challenge, and transfer of MAIT cells to immunodeficient mice ameliorates the severity of IAV-induced disease. Together, these data indicate that MAIT cells are activated and contribute to protection against a lethal viral challenge in vivo.

Results

MAIT cells accumulate and are activated during influenza. First, we sought to determine whether MAIT cells were activated

in vivo during influenza virus infection. For these studies, C57BL/6 (wild-type; WT) mice received an experimental challenge with 100 plaque forming units (PFU) of the mouse-adapted influenza virus strain A/Puerto Rico/8/34/1934 (PR8, H1N1), which causes severe pneumonia in mice, characterised by parenchymal necrosis and infiltrates of macrophages, lymphocytes and neutrophils (Supplementary Fig. 1). We observed rapid accumulation of pulmonary MAIT cells (defined as TCR β ⁺CD45.2⁺CD19[−]MR1-5-OP-RU tetramer⁺ cells, see Supplementary Fig. 2), which peaked around 5 days post-infection (dpi) (Fig. 1a, mean 2.6-fold increase from baseline, Kruskal–Wallis with post hoc Dunn's $P < 0.0001$, $n = 20$, returning to baseline by day 7 (Fig. 1a, b)). By comparison, peak frequencies of non-MAIT ('conventional') CD8⁺ cells occurred later at day 7 post-infection. The proportional increase in MAIT cells was significantly higher than that of CD4⁺ or CD8⁺ cells early during infection at day 5 post-infection ($P < 0.001$), though not by day 7 at which point antigen-specific responses will become dominant over other innate-like cells. In addition to MAIT cell recruitment, IAV infection induced MAIT cell activation, as indicated by increased expression of CD25, CD69 and Granzyme B (Fig. 1c–h). Expression of CD25 and CD69 peaked at day 5 post-infection and was significantly higher in MAIT cells than conventional CD4⁺ or CD8⁺ T cells, persisting until day 13 post-infection. By contrast, CD25 expression on conventional CD4⁺ T cells peaked at 7 dpi, whilst CD69 continued to increase over the course of the experiment and the highest levels were detected on CD4⁺ and CD8⁺ T cells at 13 dpi. Together, these results indicate that MAIT cells accumulate and are activated early in the airways following IAV infection of mice.

Accumulation and activation is modulated via cytokines. We have previously demonstrated that MAIT cells can be activated by viruses independently of MR1, via IL-18 in synergy with IL-12, IL-15 or IFN- α/β ^{20,21}. We therefore investigated the role of these cytokines in the accumulation and activation of MAIT cells in the lungs following IAV infection, using mouse strains deficient in particular cytokines or cytokine receptors. As an additional control for MR1-mediated activation, intravenous injection was used to adoptively transfer C57BL/6 pulmonary MAIT cells into MR1^{−/−} mice (which otherwise lack MAIT cells), as previously described¹⁴. Compared with WT C57BL/6 mice, the magnitude of influenza virus-induced pulmonary MAIT cell accumulation was unaffected by deficiency in IL-12, IL-15, IFN- α/β receptor (IFN α R) or MR1 (Fig. 2a, mean 2.1–2.9-fold increases above baseline at 5 dpi), but was significantly impaired (Kruskal–Wallis with post hoc Dunn's $P < 0.05$) by deficiency in IL-18 (mean 1.3-fold increase) (Fig. 2a).

By contrast, MAIT cell activation (as determined by measurement of CD25 expression) was significantly impaired by deficiency of IL-15, -18 or IFN α R (showing 69%, 70% and 67% reductions in the proportion of MAIT cells expressing CD25 respectively, $P < 0.05$ for each) and most dramatically by deficiency of IL-12 (90% decrease, $P < 0.0001$) (Fig. 2b). Differences in CD69 expression were not statistically significant (Fig. 2c). These results support the concept that in vivo, MR1-independent MAIT cell activation is driven by virus-induced pro-inflammatory cytokines, with a dominant role for IL-18, as previously suggested by in vitro studies²¹.

MAIT cell-deficient mice show enhanced mortality. Given that MAIT cells are activated and accumulate in the lungs following influenza virus infection, we sought to determine whether they might play a protective role or, conversely, enhance the inflammatory cytokine milieu and contribute to exacerbated immune

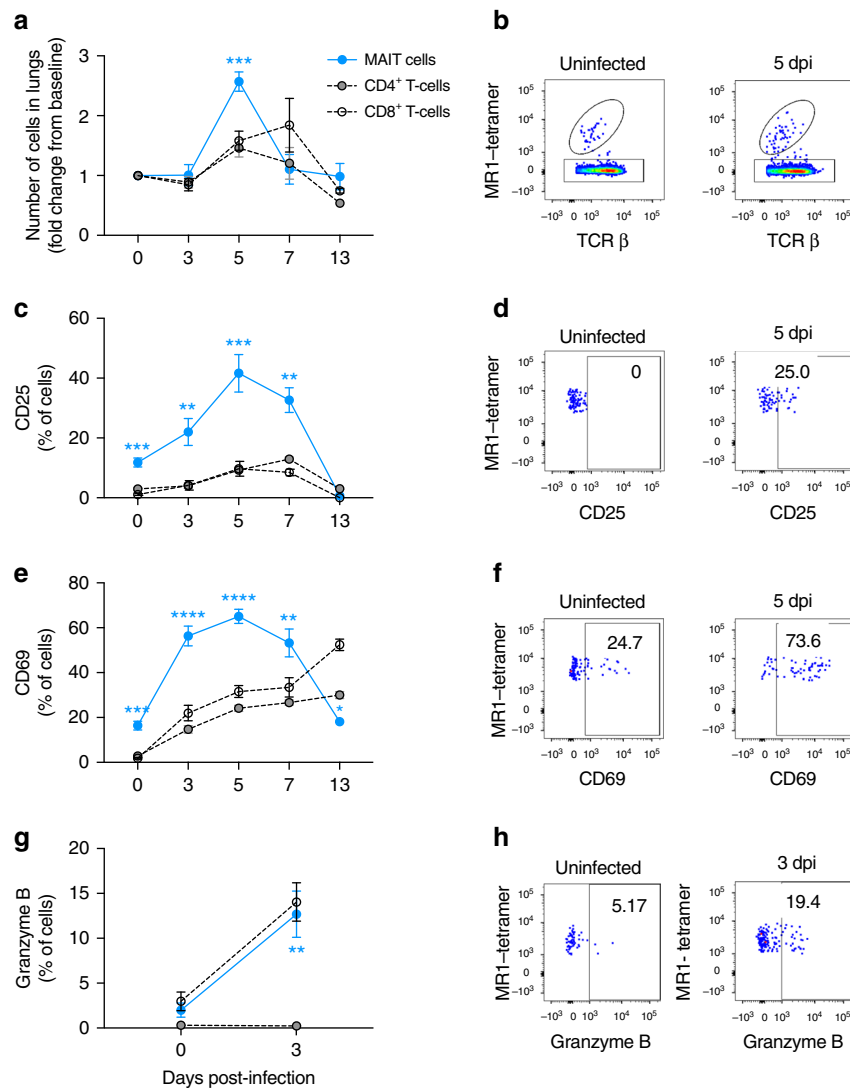


Fig. 1 Early pulmonary MAIT cell accumulation and activation during influenza virus infection. Accumulation and activation of MAIT cells and MR1-5-OP-RU tetramer-negative ‘conventional’ CD4⁺ and CD8⁺ T cells in C57BL/6 mice before and after challenge with 100 plaque-forming units (PFU) PR8 virus. **a** Accumulation of pulmonary MAIT cells, CD4⁺ and CD8⁺ T cells relative to uninfected day 0 controls at 0, 3, 5, 7, 13 days post-infection (dpi) expressed as fold change from baseline, estimated using calibration particles. **b** Representative flow cytometry plots showing TCRβ and MR1-5-OP-RU tetramer staining, gated on live, pulmonary CD45⁺19[−] lymphocytes. Proportion of pulmonary MAIT cells expressing **c** CD25, **e** CD69 and **g** Granzyme B expressed as a percentage of MAIT, CD4⁺ or CD8⁺ T cells. Representative flow cytometry plots show expression of **d** CD25, **f** CD69 and **h** Granzyme B, gated on live pulmonary CD45⁺19[−]TCRβ⁺ MR1-5-OP-RU tetramer[−] lymphocytes. Graphs show combined data (mean ± SEM) from four (days 0, 3, 5), two (day 7) or one (day 13) independent experiments with similar results, with 2–5 mice per group in each replicate. Statistical tests compare MAIT cell subsets with CD4⁺ and CD8⁺ subsets at each time-point by Kruskal–Wallis one-way ANOVA with post hoc Dunn’s tests; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001

pathology. We therefore examined weight loss and survival following influenza virus infection of WT C57BL/6 mice and MR1^{−/−} mice, which have an absolute deficiency of MAIT cells^{3,6,29}. Following challenge with 100 PFU of PR8 virus, MR1^{−/−} mice exhibited greater body weight loss (Fig. 3a) and greater mortality (log-rank Mantel–Cox *P* < 0.0001) (Fig. 3b) in comparison with WT mice. Importantly, the body weight loss and mortality in MR1^{−/−} mice were ameliorated by i.v. adoptive transfer of pulmonary MAIT cells at 1 week prior to influenza virus infection (Fig. 3a, b).

We did not observe significant differences in viral load in lung homogenates prepared from PR8-infected C57BL/6 and MR1^{−/−} mice at days 3 and 5 post-infection (Fig. 3c). However, MR1^{−/−} mice had reduced numbers of SigF⁺CD11b^{int}CD64⁺CD11c⁺ alveolar macrophages at 3 dpi (Fig. 3d, Kruskal–Wallis with post

hoc Dunn’s *P* < 0.05) and reduced pulmonary T cell numbers by 5 dpi (Fig. 3e, *P* < 0.001). The absolute numbers of neutrophils, eosinophils, dendritic cells, NK cells and γδ T cells across the time points post PR8 infection were not statistically significant (Supplementary Fig. 3). Using tetramers for MR1 or to immunodominant epitopes from the nucleoprotein or polymerase acidic protein of influenza virus, we tracked MAIT cells and conventional antigen-specific αβ T cell accumulation during PR8 infection. In MR1^{−/−} mice, we observed a tendency towards decreased accumulation of antigen-specific T cells, at day 9 in splenocytes, and for nucleoprotein at day 7 in the lymph nodes (Supplementary Fig. 4) suggesting MAIT cells may enhance the development of an effective antigen-specific adaptive immune response. Total protein concentrations in cell-free BAL supernatants, a sensitive marker of pulmonary epithelial damage³⁰,

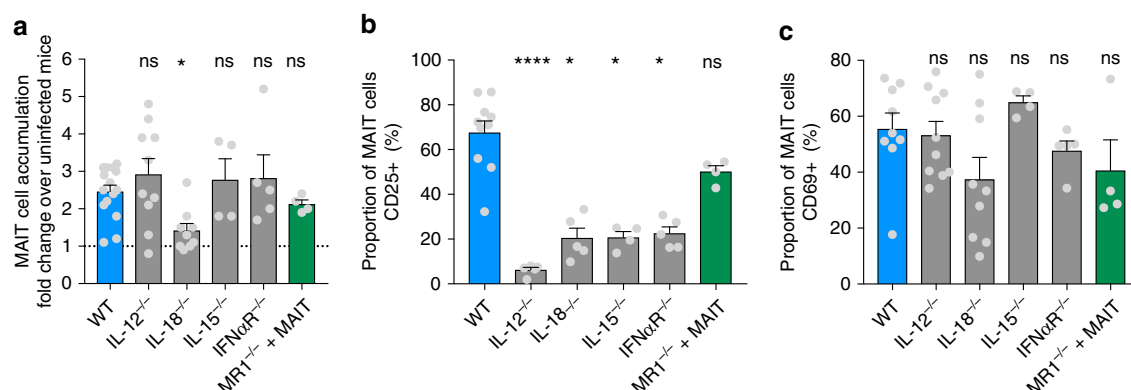


Fig. 2 MAIT cell accumulation and activation are modulated through cytokines, independent of MR1. Accumulation and activation of MAIT cells in wild type (WT) C57BL/6 mice or mice deficient in IL-12, IL18, IL-15, IFN α R or MR1 5 days after challenge with 100 plaque-forming units (PFU) PR8 virus. Mice deficient in MR1 had received adoptive transfer of 1.8×10^5 pulmonary MAIT cells 2 weeks earlier, from WT mice previously infected with 10^6 CFU *S. Typhimurium* BRD509 for 7 days to expand the MAIT cell population. **a** Fold accumulation of pulmonary MAIT cells relative to uninfected controls. **b, c** Proportion of pulmonary MAIT cells expressing CD25 (**b**), and **c** CD69 expressed as a percentage. Graphs show combined data (mean $\pm$ SEM) from one (IL-15 $^{-/-}$, IFN α R $^{-/-}$, MR1 $^{-/-}$) or two (IL-12 $^{-/-}$, IL-18 $^{-/-}$) independent experiments with similar results. Groups compared with WT by Kruskal-Wallis with post hoc Dunn's tests; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$

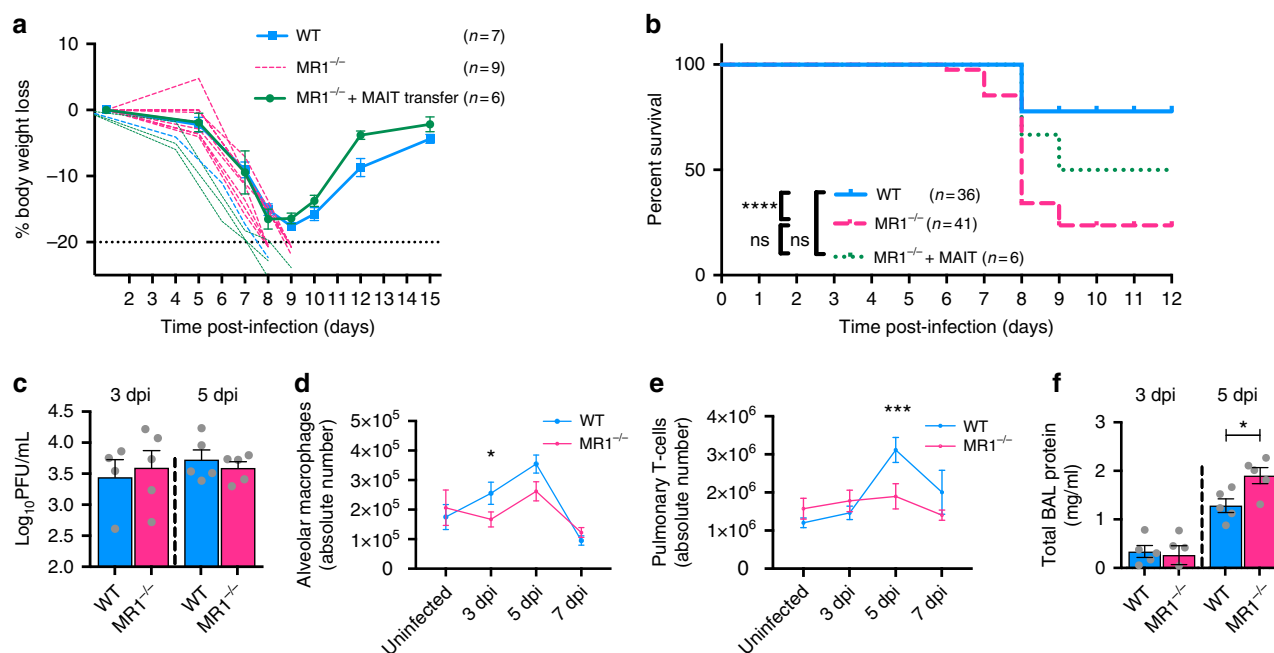


Fig. 3 MR1 $^{-/-}$ mice show enhanced weight loss and mortality in response to severe influenza. **a** Body weight loss expressed as a percentage after infection with 100 PFU PR8 virus. WT ($n = 7$) and MR1 $^{-/-}$ data ($n = 9$) are representative of four experiments with similar results. Adoptive transfer ($n = 6$), performed once, used 1×10^6 pulmonary MAIT cells from WT mice previously infected with 10^6 CFU *S. Typhimurium* BRD509 for 7 days to expand the MAIT cell population. Cells were transferred 1 week prior to influenza virus infection. Graphs show mean weights $\pm$ SEM for surviving mice, with individual plots for animals which succumbed to infection. **b** Survival curves after intranasal infection with 100 PFU of PR8, showing combined data from one (MR1 $^{-/-}$ + MAIT cells, $n = 6$) to four experiments (WT, $n = 36$; MR1 $^{-/-}$, $n = 41$). Survival curves compared using log-rank (Mantel-Cox) tests. **c** Titres of infectious virus in clarified lung homogenates at 3 and 5 dpi, expressed as plaque-forming units (PFU). Data are from a single experiment. **d** Absolute numbers of alveolar macrophages and **e** MR1-5-OP-RU tetramer-negative T cells in single cell lung suspensions prepared from uninfected animals and from animals at 3, 5 and 7 dpi. Data are combined from three experiments with similar results. **f** Total protein concentration in cell-free BAL supernatants at days 3 and 5 post-infection. Graphs show means $\pm$ SEM. *Mann-Whitney $P < 0.05$; *** $P < 0.001$

were also increased in MR1 $^{-/-}$ mice at 5 dpi (Mann-Whitney $P < 0.05$, Fig. 3f). These data indicate that early accumulation of MAIT cells at the site of infection protects against influenza-induced morbidity and mortality associated with excessive lung damage and recruitment of inflammatory cells, but not with viral killing.

As the mouse-adapted PR8 strain is highly virulent in mice, we performed additional experiments using X-31 (H3N2, a

reassortant virus bearing the hemagglutinin and neuraminidase genes from A/Aichi/2/1968 and the remaining six genes from PR8), a strain that shows moderate virulence in mice³⁰. After intranasal infection of WT or MR1 $^{-/-}$ mice with 5000 PFU of X-31, we did not observe significant differences in survival or in titres of infectious virus detected in the lungs (Supplementary Fig. 5a–c), although we did observe a similar virus-induced pulmonary accumulation of MAIT cells as well as virus-induced

MAIT cell activation (2.6-fold increase at 5 dpi, Supplementary Fig. 5d–g). Furthermore, following infection with either PR8 or X-31, we observed reductions in key innate inflammatory cell cytokines in the lungs of MR1^{-/-} compared with wild-type C57BL/6 mice. In particular, in MR1^{-/-} mice we detected significantly lower amounts of monocyte chemotactic protein 1 (MCP-1/CCL2) and IL-6 following infection with 100 PFU of PR8, and of RANTES/CCL5 following 5000 PFU of X-31 with similar trends observed with both virus strains for each of the inflammatory mediators detected (Supplementary Fig. 6). Likewise, there were lower levels of interferon- γ in bronchoalveolar lavage fluid at day 5 post-infection with 100 PFU of PR8 in MR1^{-/-} mice (3.2-fold difference in geometric mean, *t*-test on log-transformed data, *P* = 0.044) (Supplementary Fig. 7).

Together, these data indicate that MAIT cells play a protective role during severe influenza virus infection, enhancing pulmonary T cell accumulation and innate inflammatory cytokine production, and reducing weight loss, mortality and pulmonary epithelial damage.

MAIT cell transfer delays mortality in immunodeficient mice.

To further explore the protective role of MAIT cells during influenza infection, we studied the impact of adoptively-transferred MAIT cells into immunodeficient Rag2^{-/-}γC^{-/-} mice lacking T cell-, B cell- and NK cell-mediated immunity. As previously described^{14,29}, ~0.3 × 10⁶ pulmonary MAIT cells were adoptively transferred into Rag2^{-/-}γC^{-/-} mice. Low numbers of residual contaminating conventional T cells from the donor mice can rapidly expand in these recipient mice. Therefore, residual contaminating conventional T cells were depleted in recipients with anti-CD4 and anti-CD8 mAbs, and the animals rested for 1–2 weeks to allow expansion of the MAIT cell population prior to challenge with PR8 or X-31 strains (Fig. 4a). Adoptive transfer of MAIT cells reduced weight loss (Fig. 4b) and significantly prolonged survival after PR8 infection (Fig. 4c, log-rank Mantel–Cox *P* < 0.05). Furthermore, when we directly compared the protective effect of MAIT, CD8 and NK cells in the same model, MAIT cell transfer was associated with prolonged survival compared with CD8 transfer (Bonferroni-corrected log-rank Mantel–Cox *P* = 0.009), but was not superior to NK cell transfer (*P* = 0.8, Supplementary Fig. 8). Moreover, in this immunocompromised Rag2^{-/-}γC^{-/-} mouse model adoptive transfer of MAIT cells now also provided protection following challenge with the less virulent X-31 strain (Fig. 4d, e, log-rank Mantel–Cox *P* = 0.02).

Human MAIT cells express IFN- γ in response to stimulation of TLR-8 with single-stranded RNA²⁰, and also that human viral activation of MAIT cells can suppress replication of hepatitis C virus via IFN- γ in vitro²¹, we hypothesised that IFN- γ expression by MAIT cells could contribute to their protective role following influenza virus infection. To address whether IFN- γ expression by MAIT cells confers protection against IAV in vivo, MAIT cells from wild-type C57BL/6 and from IFN- γ ^{-/-} donor mice were transferred into Rag2^{-/-}γC^{-/-} mice. As expected, the protective effect of adoptively-transferred MAIT cells was abrogated when the transferred MAIT cells were deficient in IFN- γ (Fig. 4d, e), suggesting that the antiviral effects provided by MAIT cells were mediated, at least in part, by IFN- γ .

Discussion

We have shown in vivo that MAIT cells accumulate in the lungs, are activated and contribute to protection against morbidity and mortality during influenza virus infection in mice. These findings are consistent with our previous human in vitro observations in which we demonstrated MR1-independent MAIT cell activation

in response to exogenous cytokines^{20,21} or to IAV²¹. In those experiments, we co-incubated peripheral blood mononuclear cells (PBMCs) or human CD8⁺ T cells with virus-infected monocytes, macrophages or dendritic cells and observed strong, dose-dependent MAIT cell activation with upregulation of CD69 and IFN- γ and Granzyme B²¹. We also observed virus-induced upregulation of CD69, Granzyme B and IFN- γ on MAIT cells in separate experiments using co-culture of PBMC with IAV infection of a human lung epithelial cell line (A549)²². Here, we observed strong CD25 and CD69 upregulation by MAIT cells in the lungs of IAV-infected mice, which occurred earlier and to a greater extent than in non-MAIT CD3⁺ T cells, and was associated with Granzyme B upregulation, suggesting that these cells are licensed for cytotoxicity early in influenza virus infection.

In the previous in vitro studies, virus-induced responses were unaffected by anti-MR1 blocking antibody^{21,22}, but rather were driven by cytokines. Indeed, this would be consistent with the MAIT cell accumulation (Fig. 2a) and their protective effects observed after adoptively transferring MAIT cells into mice with deletion of MR1 (Fig. 3b). Stimulation with IL-12 and IL-18 is known to induce IFN- γ from CD161⁺⁺ CD8⁺ T cells, of which MAIT cells are the dominant subset in humans²⁰. In both the lung epithelial cell²² and the professional antigen presenting cell²¹ co-culture systems, we found IL-18 to be the dominant activating cytokine in influenza virus infection, acting synergistically with IL-12 or IL-15 or type I interferons²¹. Again, we confirmed these observations in vivo using mice with genetic deletions of these cytokines or their receptors, and found that MAIT cell accumulation and CD69 upregulation were significantly impaired only in the IL-18^{-/-} mice (Fig. 2a, c). Interestingly, CD25 activation was affected to the greatest extent by IL-12 deletion (Fig. 2b), likely because an important general biological function of IL-12 is to induce T cell upregulation of CD25³¹.

Our previous clinical human influenza infection studies showed activation of peripheral blood MAIT cells, measured by Granzyme B upregulation²¹ and a decrease in peripheral blood MAIT cell frequencies, which correlated with disease severity²¹ and with fatal outcomes²². In these studies, it was not possible to determine causal associations nor examine lung tissue. Our murine data recapitulated these findings and revealed activated MAIT cells at the site of infection. In human studies, there are wide inter-individual variations in baseline frequencies of peripheral blood MAIT cells⁴. However, overall the observed clinical association between reduced MAIT cell frequencies and influenza mortality would be consistent with the increased mortality we observed here in MR1^{-/-} mice.

MAIT cells are not unique in their capacity to respond to virus-induced cytokines. Virus-induced bystander activation was first described in conventional CD8⁺ T cells in mice³² but is overall a rare event in these cells³³. However, the high IL-18R expression and IL-12/18 responsiveness appear to be common to a range of innate-like lymphocytes expressing PLZF³⁴, which in humans include iNKT, $\gamma\delta$ T, and NK cells³⁵. Indeed, protective roles for iNKT cells have been shown in murine IAV infection^{36–38}. Nonetheless, whilst iNKT cells are relatively abundant in mice, they are quite rare in humans, constituting approximately 0.1% of peripheral blood T cells^{3,35,39}. In contrast, human MAIT cells are much more abundant comprising 1–10% of blood³ and pulmonary⁴ T cells. Furthermore, at early stages the absolute numbers of these cells in the lungs will markedly exceed the numbers of conventional antigen-specific T cells responding to cognate viral antigens³⁴. Furthermore, as they display a significantly higher TCR-independent upregulation of IFN- γ than conventional T cells²², MAIT cells may have a considerable role in early antiviral protection in humans. Nevertheless, despite much lower relative frequencies of MAIT cells in mice compared

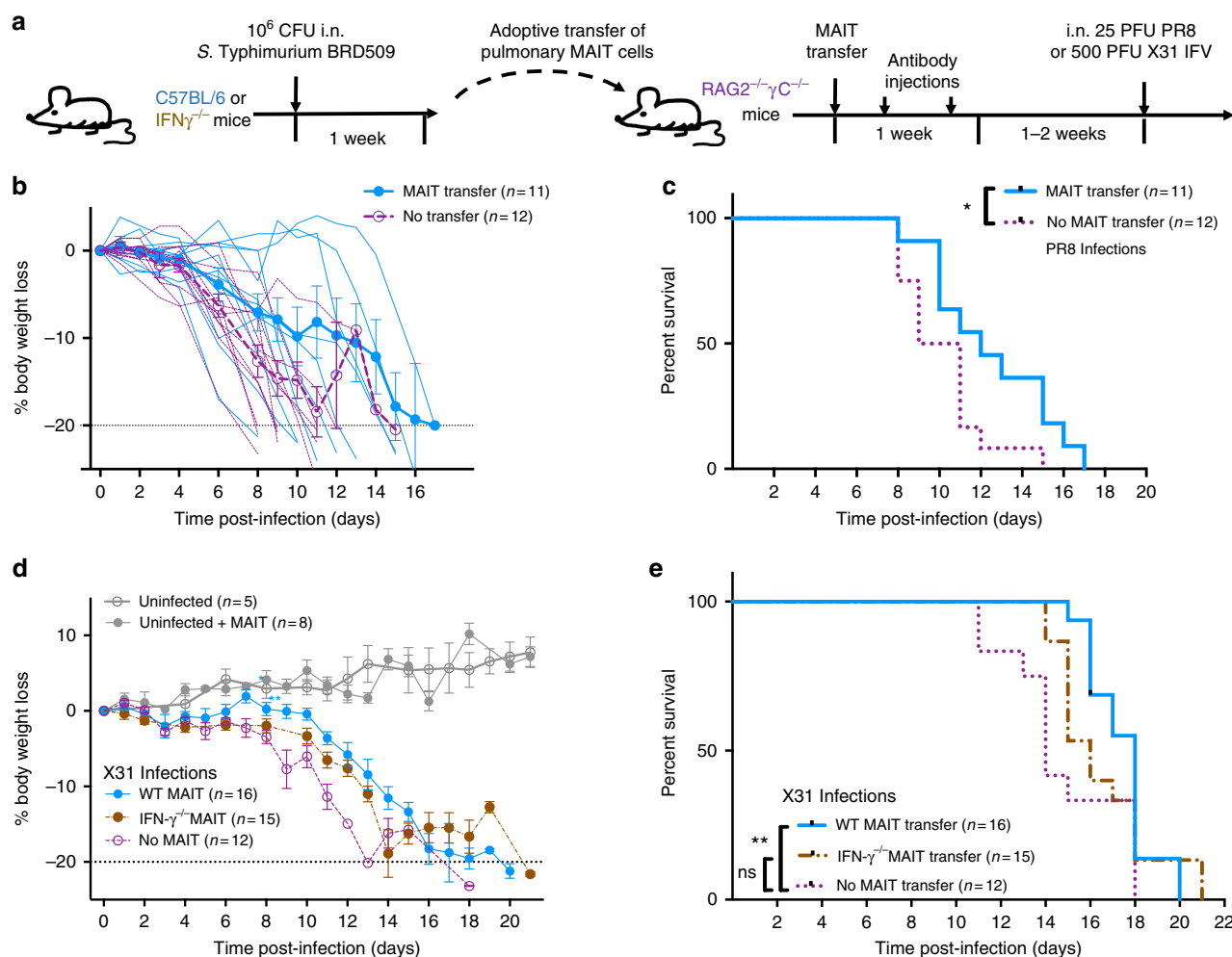


Fig. 4 Adoptive transfer of MAIT cells delays mortality in $Rag2^{-/-}\gamma C^{-/-}$ mice with IAV infection. **a** Schematic of protocol: 3×10^5 pulmonary MAIT cells from C57BL/6 mice (previously infected with 10^6 CFU *S. Typhimurium* BRD509 for 7 days to expand the MAIT cell population) were sorted and transferred intravenously into $Rag2^{-/-}\gamma C^{-/-}$ mice, followed by intraperitoneal anti-CD4 and anti-CD8 antibody injection (0.1 mg each) twice within 1 week to deplete any residual conventional T cells included in the transfer. After 2 weeks, mice were infected i.n. with 25 PFU of PR8 (**b**–**c**) or 500 PFU of X-31 (**d**–**e**). **b** Body weight loss expressed as a percentage (showing mean $\pm$ SEM and individual values for all mice), and **c** survival after infection with 25 PFU PR8 virus. Survival curves compared using log-rank (Mantel-Cox) tests. **d** Body weight loss expressed as a percentage (mean $\pm$ SEM), after infection with 500 PFU X-31 virus. $^{*}P < 0.05$, $^{**}P < 0.01$ denotes significant differences between weights using ANOVA and post hoc Dunnett's comparing against non-transferred $Rag2^{-/-}\gamma C^{-/-}$ mice. **e** Survival curves after infection with 500 PFU of X-31 were compared using log-rank (Mantel-Cox) tests ($P = 0.02$) and Gehan-Breslow-Wilcoxon tests ($P = 0.006$) between WT MAIT and non-transferred $Rag2^{-/-}\gamma C^{-/-}$ mice. Other differences were not significant. Data represent combined data from two (**b**, **c**) or three (**d**, **e**) experiments with similar results

with humans, it is striking that we could observe a protective effect of MAIT cells.

Although MAIT cells protected against influenza virus-induced weight loss and mortality, we did not detect a significant difference in viral titres at the time of peak viral load (i.e. 3–5 dpi). This is not unexpected, as other investigators have also observed significant differences in immunopathology without changes in IAV load³⁸. Furthermore, whilst the cytokine responsiveness of MAIT cells suggests the potential for a possible deleterious role in which they could contribute to the ‘cytokine storm’ effect observed in severe influenza⁴⁰, our data from both C57BL/6 and $Rag2^{-/-}\gamma C^{-/-}$ strains showed no evidence that MAIT cells contributed to enhanced immunopathology. In fact, the opposite effect was seen. This is a critical observation because, although it is known that MAIT cells home to sites of inflammation¹³, are activated by IL-18/-15/-12 to produce IFN- γ ²⁰, and that IFN- γ can be antiviral⁴¹. Only a direct in vivo challenge experiment is able to directly answer the question as to whether, in a complex

host–pathogen interaction within an intact organism, the protective effects of these mechanisms outweigh the detrimental immunopathological consequences.

The importance of MAIT cells to immune protection in humans may be most apparent when MAIT cell frequencies are reduced. MAIT cell frequencies are lowest in early life³ and in the elderly⁴², age groups at the highest risk of influenza mortality⁴³. Influenza mortality is also associated with suppressed cell-mediated immunity, including HIV infection and iatrogenic immunosuppression, conditions in which MAIT cells are also suppressed^{43,44}. Of particular relevance to respiratory viral infections, therapeutic use of inhaled and oral corticosteroids causes marked reductions in airway MAIT cell frequencies in asthma⁴ and chronic obstructive pulmonary disease⁵, and thus may contribute to the increased risk of influenza hospitalisation in these conditions⁴⁵.

Influenza virus is a major human respiratory pathogen, which causes seasonal epidemics resulting in 300–650,000 deaths

annually⁴⁶. IAV can also cause pandemic spread when novel strains emerge⁴³. Although the most effective method for prevention and control of influenza is vaccination, current vaccines show limited efficacy and little heterologous protection between strains, and so require annual reformulation. The TCR-independent nature of the antiviral MAIT cell response therefore offers significant therapeutic potential in at least two ways. Firstly, strategies to target MAIT cell activation, for instance by incorporating MAIT cell ligands and TLR agonists in the formulation of novel adjuvants, could be used to enhance the efficacy of future influenza vaccines. Secondly, we have shown that MAIT cell frequencies can be rapidly 'boosted' through mucosal administration of simple synthetic MAIT cell ligands with TLR agonists^{14,29}, which may prove effective in a number of scenarios including short-term prophylactic vaccination during an epidemic. Such a strategy may also prove beneficial in protecting groups known to show impairments in MAIT cell immunity—such as the elderly or those receiving therapeutic corticosteroids—or even acutely during the early stages of infection.

In summary, we have shown for the first time in vivo evidence of an important contribution of MAIT cells to protective immunity against a major human viral pathogen. As the cytokine-mediated activation of MAIT cells is a mechanism likely to be common to a number of other acute and chronic viral infections, as has already been shown in dengue virus, hepatitis C virus in clinical studies^{21,28}, these findings from an influenza model are likely to be widely applicable to a range of respiratory and systemic viral diseases.

Methods

Animal models. Mice were bred and housed in the Biological Research Facility of the Peter Doherty Institute (Melbourne, Victoria, Australia). MR1^{-/-} mice were generated by breeding Va19iCa^{-/-}MR1^{-/-} mice⁴⁷ (from Susan Gilfillan, Washington University, St. Louis School of Medicine, St. Louis, MO) with C57BL/6 mice and inter-crossing of F1 mice. The genotype was determined by tail DNA PCR at the MR1 locus using the following primers²⁹: Fwd: AGC TGA AGT CTT TCC AGA TCG; Rev (wild type): ACA GTC ACA CCT GAG TGG TTG; Rev (knockout): GAT TCT GTG AAC CCT TGC TTC. IFN- γ knockout mice (B6.129Sv-Irfng^{tm1Ts/J})⁴⁸ are commercially available (Jackson Laboratory). Male mice aged 6–12 weeks, matched for age, sex and weight, without randomisation or blinding, were used in experiments, after approval by, and in accordance with, the requirements of the University of Melbourne Animal Ethics Committee (1513661).

Intranasal virus infection. Intranasal (i.n.) inoculation with 50–1 $\times 10^4$ PFU of X-31 (H3N2; A/Hong Kong/X31) or with 25–150 PFU PR8 (H1N1; A/Puerto Rico/8/34/1934) IAV, or with *S. Typhimurium* BRD509 (10⁶ colony forming units (CFU)) in 50 μ l per nares was performed on isoflurane-anesthetized mice. Virus stocks were grown in the allantoic cavity of 10 day-old embryonated chicken eggs, and the viral titre was determined by a plaque assay on MDCK monolayers, as previously described⁴⁹.

Mice were weighed daily and assessed for visual signs of clinical disease. Animals that lost $\geq 20\%$ of their original body weight and/or displayed evidence of pneumonia were euthanized.

Mice were killed by CO₂ asphyxia, the heart perfused with 10 ml cold RPMI and lungs were taken. To prepare single-cell suspensions, lungs were finely chopped with a scalpel blade and treated with 3 mg ml⁻¹ collagenase III (Worthington, Lakewood, NJ), 5 μ g ml⁻¹ DNase, and 2% foetal calf serum in RPMI for 90 min at 37 °C with gentle shaking. Cells were then filtered (70 μ m) and washed with PBS/2% foetal calf serum. For plaque assays, lungs were placed into RPMI and homogenised using a Polytron System PT 1200 CL 230V (Kinematica, Lucerne, Switzerland). Red blood cells were lysed with hypotonic buffer TAC (Tris-based amino chloride) for 5 min at 37 °C. Approximately 1.5 $\times 10^6$ cells were filtered (40 μ m) and used for flow cytometric analysis. Absolute cell counts were derived by adding to each sample 2.5 $\times 10^4$ blank calibration particles (BD Pharmingen).

Determination of viral load counts in infected lungs. Viral load was determined by counting PFU in MDCK monolayers infected with lung homogenates, at varying dilutions for 45 min at 37 °C, 5% CO₂ before the addition of an Agarose/L15 or MEM overlay containing Trypsin (Worthington Biochemical, NJ, USA), as described⁴⁹. Plates were incubated at 37 °C, 5% CO₂ for 3 days before plaques were counted.

Adoptive transfer. As MAIT cell numbers are low in naive C57BL/6 mice, prior to adoptive transfer experiments MAIT cell populations were expanded by intranasal infection with 10⁶ CFU *S. Typhimurium* BRD509 in 50 μ l PBS for 7 days, as described²⁹. After 7 days, mice were sacrificed, single-cell suspensions prepared and live CD3⁺CD45⁺MR1-5-OP-RU tetramer⁺ cells sorted using a BD FACS Aria III. Simultaneously, from these single cell suspensions, live CD3⁺CD45⁺CD8⁺MR1-5-OP-RU tetramer⁻ were sorted for CD8⁺ T cell adoptive transfer. For the transfer of NK cells, prior to cell sorting, single cell suspensions from naive WT spleens were subjected to magnetic bead-based antibody depletion with anti-CD11b, anti-CD4, anti-CD8 and anti-B220 (reagents kindly provided by Professor Axel Kallies). Live NK1.1+CD3-CD4-CD8-B220-CD11b-CD11c- cells were sorted using a BD FACS Aria III. 3 $\times 10^5$ pulmonary MAIT cells were injected into the tail veins of recipient Rag2^{-/-} γ C^{-/-} mice which then received 0.1 mg each of anti-CD4 (GK.5) and anti-CD8 (53.762) mAb i.p. on days 2 and 5 or 6 to control residual donor-derived conventional T cells. Mice were rested for 2 weeks post transfer to allow full expansion of the MAIT cell population prior to infectious challenge. In a separate set of experiments, 1 $\times 10^5$ pulmonary MAIT or CD8⁺ cells or splenic NK cells were transferred to recipient Rag2^{-/-} γ C^{-/-} mice and rested as described above. Prior to infectious challenge, venous tail blood was stained to confirm the presence of the transferred cell subsets.

Reagents. MR1-5-OP-RU-SA BV421 tetramers were generated as described previously⁹ (now available from the NIH core tetramer facility) and influenza-specific MHC Class I tetramers targeting immunodominant epitopes of nucleoprotein (D^bNP₃₆₆) and polymerase acidic protein (D^bPA₂₂₄) and were utilised conjugated to SA-APC and PE respectively. Antibodies against murine CD3e (clone 145-2C11, catalogue number 561108, fluorochrome PerCP-Cy5.5, dilution 1:200), CD4 (GK1.5, 552051, APC-Cy7, 1:200), CD11b (M1/70, 563015, BV605, 1:600), CD19 (1D3, 561113, PerCP-Cy5.5), CD45.2 (104, 553772, FITC and 563685, BV711, 1:400), CD69 (H1.2F3, 12-0691-81, PE and 70318, FITC), IFN- γ (XMG1.2, 557649, PE-Cy7), Ly6C (AL-21, 561237, AF700, 1:400), Ly6G (IA8, 560601, PE-Cy7, 1:1000), NK1.1 (PK136, 553165, PE, 1:200), SiglecF (E50-2440, 562757, PECF594, 1:1000), TCR β (H57-597, 553174, APC or 553172, PE, 1:500) were purchased from BD (Franklin Lakes, NJ). Antibodies against CD3 (17A2, 56-0032-80, AF700, 1:100) CD8 α (53-6.7, 12-0081-81, PE), CD11c (N418, 17-01114-81, FITC, 1:1000), CD25 (PC61.5, 17-02510-81, APC), F4/80 (BM8, 11-4801-81, FITC, 1:100), Granzyme B (NGZB, 12-8898-80, PE) were purchased from eBioscience (San Diego, CA). Abs against CD19 (6D5, 115546, BV510), F4/80 (BM8, 123116, APC), CD11b (M1/70, 101206, FITC, 1:1000), CD11c (N418, 117336, BV786, 1:400), CD19 (6D5, 115506, FITC, 1:500), CD27 (LG.3A10, 124214, PerCP-Cy5.5, 1:400), CD62L (Mel-14, 104406, FITC, 1:800 and PE-Cy7, 1:500), CD64 (X54-5/71, 139322, AF647, 139311, BV711, 1:400, I-Ab, AF6-120.1, 116422, Pacific Blue, 1:400) were purchased from Biolegend (San Diego, CA). To block non-specific staining, cells were incubated with unlabelled MR1-6-formylpterin tetramer and anti-Fc receptor (2.4G2) for 15 min at room temperature and then incubated at room temperature with Ab/tetramer cocktails in PBS/2% foetal calf serum. 7-aminocaproic acid (5 μ l per sample) was added for the last 10 min or fixable viability stains added according to the manufacturer's instructions: Live/Dead Fixable Aqua (L34957, ThermoFisher, Waltham, MA, 1:800) and ZombieYellow (423104, Biolegend).

Cells were fixed with 1% paraformaldehyde prior to analysis on LSRII or LSR Fortessa or Canto II (BD Biosciences) flow cytometers. For intracellular staining, GolgiPlug (BD Biosciences) was used during all processing steps. Surface staining was performed at 37 °C, and cells were stained for intracellular molecules using the BD Fixation/Permeabilization Kit (BD, Franklin Lakes, NJ) or transcription factors using the transcription buffer staining set (eBioscience) according to the manufacturer's instructions.

The concentration of protein in BAL supernatants was measured by adding Bradford protein dye (BioRad) to diluted BAL supernatant according to the manufacturer's instructions. A standard curve using bovine serum albumin was constructed, and the optical density (OD) was determined at 595 nm.

Cytometric bead analysis. The CBA flex set (BD Bioscience) was used as per manufacturer's instructions to determine the cytokine concentrations of Lung and BAL supernatant⁴⁹. Data were acquired using FACSCantoII and analysis was by FCAP Array software (Soft Flow Inc., Pecs, Hungary).

Statistical analysis. Statistical tests were performed using the Prism GraphPad software (version 7.0, La Jolla, CA). Comparisons were performed using Mann-Whitney tests (two groups) with Bonferroni correction for multiple comparisons, or with Kruskal-Wallis one-way ANOVA with post hoc Dunn's tests (multiple groups). Survival curves were compared using log-rank (Mantel-Cox) tests (two groups) or the Gehan-Breslow-Wilcoxon method for multiple groups. Variance was similar in each case between groups being compared. Sample sizes were estimated based on previous extensive experience in the laboratory with these PR8 and X-31 strains. All statistical tests are two-sided. Flow cytometric analysis was performed with FlowJo10 software (Ashland, OR).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data supporting the findings of this study are available within the paper and its supplementary information files, or are available from the authors upon request.

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Author contributions

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Additional information

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Competing interests: Z.C., S.B.G.E., J.M. and A.J.C. are inventors on patents describing MR1 tetramers. The remaining authors declare no competing interests.

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