Innate and adaptive T cells in influenza disease

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ABSTRACT

Influenza is a major global health problem, causing infections of the respiratory tract, often leading to acute pneumonia, life-threatening complications and even deaths. Over the last seven decades, vaccination strategies have been utilized to protect people from complications of influenza, especially groups at high risk of severe disease. While current vaccination regimens elicit strain-specific antibody responses, they fail to generate cross-protection against seasonal, pandemic and avian viruses. Moreover, vaccines designed to generate influenza-specific T-cell responses are yet to be optimized. During natural infection, viral replication is initially controlled by innate immunity before adaptive immune responses (T-cells and antibody-producing B-cells) achieve viral clearance and host recovery. Adaptive T- and B-cells maintain immunological memory and provide protection against subsequent infections with related influenza viruses. Recent studies also shed light on the role of innate T-cells (MAIT cells, $\gamma\delta$ T-cells and NKT cells) in controlling influenza and linking innate and adaptive immune mechanisms, thus making them attractive targets for vaccination strategies. We summarize the current knowledge on influenza-specific innate MAIT and $\gamma\delta$ T-cells as well as adaptive CD8⁺ and CD4⁺ T-cells, and discuss how these responses can be harnessed by novel vaccine strategies to elicit cross-protective immunity against different influenza strains and subtypes.

INTRODUCTION

Influenza viruses, are enveloped negative-sense RNA viruses with segmented genomes from the family *Orthomyxoviridae* that cause a highly contagious and rapidly spreading acute respiratory infection (reviewed in [1]). Annual seasonal influenza epidemics result in significant morbidity and mortality, especially amongst high-risk groups, including children, the elderly, pregnant women, obese individuals, those with co-morbidities and Indigenous populations. Sporadically, a new influenza virus emerges from an animal host and spreads rapidly in the susceptible population, resulting in a pandemic often associated with significant morbidity and mortality. Influenza viruses replicate in the respiratory mucosa, primarily in respiratory epithelial cells producing large amounts of progeny virions, which then infect alveolar macrophages and resident dendritic cells.

Three types of influenza viruses classified as A, B and C, infect humans [1,2]. While influenza A and B viruses (IAV and IBV, respectively) co-circulate annually during seasonal epidemics, influenza C viruses generally cause mild infections in humans. Of the three, only IAV have established animal reservoirs [2,3], although IBV have been isolated from some animal species [4,5]. Importantly, some swine and avian IAV strains can infect humans. Thus, seasonal IAV/IBV infections present a significant global health problem causing up to 500,000 excess deaths annually due to influenza infection [6,7]. IAVs are further distinguished into subtypes by their surface glycoproteins hemagglutinin (H) and neuraminidase (N) with 18 HA subtypes and 11 NA subtypes identified to date. On the other hand, IBVs are grouped into two antigenically and genetically distinct lineages, called B/Yamagata and B/Victoria [4,5]. Two IAV subtypes (H1N1 and H3N2), along with one or two IBV viruses from the two lineages co-circulate annually causing influenza epidemics. Under immune pressure, influenza viruses acquire mutations in their HA and NA proteins, which allow immune escape in a process known as antigenic drift. Novel IAVs emerge by reassortment, mostly in animal reservoirs, in a process known as antigenic shift. When/if they acquire the ability to spread from person-to-person, novel IAVs can cause a pandemic [2,3].

The first leukocytes that infiltrate the lungs following influenza virus infection are innate cells that include neutrophils and macrophages [8]. They are followed by influenza-specific B and T cells that mediate the adaptive immune response. While antibody-mediated immunity is mainly strain-specific and can prevent infection, T cells (especially CD8⁺ T cells) provide broadly cross-reactive cellular immunity against different influenza viruses and thus can ameliorate the severity of influenza disease [9-13]. Current vaccination strategies with

inactivated influenza viruses induce strain-specific, B cell-based protective immunity directed against the viral hemagglutinin protein [14]. However, these vaccines offer little cross-protection against antigenic drift variants of seasonal IAVs and IBVs. Therefore, the vaccine formulation needs to be updated annually to cover antigenically drifted IAV and IBV strains. Notably, current seasonal influenza vaccines do not protect against new and unanticipated influenza viruses, such as pandemic strains.

T cells recognize epitopes on conserved internal viral proteins and thus they confer broader protection across IAV subtypes [9,15]. Ideally, vaccination strategies that induce broadly cross-protective T cell responses across antigenic drift variant influenza viruses, and potentially against newly emerging zoonotic IAVs, are required to limit the disease burden of influenza epidemics and pandemics. Here, we summarize the current knowledge on the innate and adaptive T cell responses to influenza viruses and discuss how these responses can be exploited by vaccines to prevent or ameliorate severe influenza infections.

Innate immunity as the first barrier against influenza viruses

The innate immune response involves powerful antiviral activity mediated by interferon (IFN), which limits early viral spread and subsequently shapes the adaptive immune response by their cytokine secretion [16]. Innate immunity to influenza viruses also involves several innate T cell subsets such as mucosal-associated invariant (MAIT), natural killer (NKT) and $\gamma\delta$ T cells, which have been a focus of the recent studies.

Mucosal-associated invariant T (MAIT) cells contribute to immunity against influenza viruses

MAIT cells constitute up to 5% of the total T cell pool in humans and are characterized by the expression of a semi-invariant T cell receptor (TCR) V α 7.2 that recognizes bacterial-derived vitamin B2 derivatives presented by the MHC-like protein 1 (MR1) [17]. On activation, MAIT cells produce proinflammatory cytokines and upregulate cytotoxic granzymes and perforin [18]. Although MAIT cells are predominantly associated with control of bacterial infections, there is increasing evidence of their clinical importance in viral infections, autoimmune diseases and cancers [19-26]. With respect to human IAV infection, van Wilgenburg *et al* showed that the number of human MAIT cells in the peripheral blood of severely ill individuals was reduced by nearly half and they were highly activated, as shown by upregulation of granzyme B [26]. Furthermore, Loh *et al* showed that patients who died from severe H7N9 infection had ~3-5-fold fewer MAIT cells in the blood, compared to patients who recovered

from severe H7N9 infection or were healthy [25]. These studies suggest a loss and/or efflux of MAIT cells from the blood to the site of infection during severe influenza disease. Further, both studies showed that the MAIT cell activation in influenza infection was MR1-TCR independent and was mediated by IL-18 [25,26], mainly produced by CD14⁺ monocytes [25]. Reduction in MAIT cell numbers during acute influenza infection could impair protective anti-bacterial immunity increasing the risk of bacterial co-infection, which would enhance disease severity and mortality [27].

NKT cells modulate both innate and adaptive response mechanisms during influenza infection

Natural killer T cells are innate-T lymphocytes restricted by the glycolipid α -galactosylceramide (α GC) presented on MHC class I-like CD1d molecules. Like conventional T cells, they express $\alpha\beta$ TCRs as well as NK cell markers including CD3, CD56 (humans) or NK1.1 (mice). NKT cells constitute approximately 0.1-0.2% of the total T cell pool and are broadly classified into 2 groups: type I invariant NKT (iNKT) cells, expressing the invariant V α 14-J α 18 TCR α chain paired with either V β 2, V β 7 or V β 8 in mice and V α 24-J α 18/V β 11 in humans, and type II diverse NKT cells, which display very diverse $\alpha\beta$ TCR gene pairings [28,29]. In animals, NKT cells can protect against viral infections and cancer [28,30] by regulating innate and adaptive immune responses via rapid secretion of cytokines [31,32], upregulation of CD40 and activation of dendritic cells [33].

In a mouse model of influenza infection, intraperitoneal (i.p.) administration of α GC along with intranasal (i.n) administration of an influenza virus (H1N1/H3N2 recombinant) activates iNKT cells (without affecting the adaptive T cell responses), consequently reducing body weight loss and viral lung titres [34]. Conversely, CD1d^{-/-} mice lacking the CD1d molecule (and therefore, lacking CD1d-stimulation of NKT cells), developed more severe disease following infection with a sublethal dose of influenza (PR8-H1N1) virus, as evidenced by lower body weight, higher lung viral titres, decreased IFN γ production in the bronchoalveolar lavage (BAL) and impaired NK and T cell activity, as compared to wild type (WT) mice [35]. Similarly, J α 18^{-/-} mice (lacking NKT cells expressing J α 18) infected with the virulent influenza PR8-H1N1 virus displayed increased lung injury and mortality, associated with greater infiltration of Ly6C^{hi}Ly6G⁻ inflammatory monocytes in lungs, compared to J α 18^{-/-} mice receiving α GC-boosted iNKT cells. Moreover, α GC-boosted iNKT cells reduced MCP-1 levels by lysing influenza-infected monocytes in a CD1d-dependent manner [36]. These

findings were consistent with another study, in which a rapid reduction in body weight and 100% mortality by 11 days post-infection (d.p.i.) was observed in $J\alpha 18^{-/-}$ mice. The enhanced pathology in influenza-infected $J\alpha 18^{-/-}$ mice was associated with a ~4-fold decrease in D^bPA_{224–233}-specific CD8⁺ T cells in lungs at 4 and 7 d.p.i. and differential accumulation and maturation of CD103⁺ dendritic cells (DCs) [37]. Moreover, the absence of iNKT cells led to suppression of IAV responses due to increased expression of the arginase and nitric oxide system and this was mitigated by adoptive transfer of iNKT cells that restored antiviral immunity in a CD1d- and CD40-dependent manner [38]. Furthermore, the use of α GC as a vaccine adjuvant improved long-term survival of CD8⁺ cytotoxic T lymphocytes and provided heterologous protection in a mouse model of influenza infection [39]. Similarly, α GC analogues, KBC-007 and KBC-009, administered with inactivated influenza A/PR/8/34 virus boosted humoral and cellular immune responses in mucosal and systemic compartments post immunization and induced protective immune responses when challenged with PR8 virus [40]. Intramuscular administration of inactivated A/California/04/2009 pandemic H1N1 virus with α GC to piglets increased NKT-cell numbers, induced high titres of anti-HA mAbs and boosted IAV-specific CD8⁺ T cells post-vaccination. Virus challenge in piglets vaccinated with α GC/inactivated virus resulted in reduced viral shedding in comparison to piglets vaccinated with whole inactive virus alone [41]. Intranasal immunization with inactivated influenza A/PR/8/34 virus with α GC in BALB/c mice induced IgG and IgA antibodies lasting up to 3 months and boosted cellular immunity in comparison to mice immunized with inactivated PR8 virus alone [42].

Collectively, these animal studies demonstrate the role played by NKT cells in modulating innate and adaptive immune responses during IAV infection. α GC as a vaccine adjuvant boosted NKT numbers and improved protection, further supporting the role of NKT cells in ensuring optimal influenza-specific T and B cell responses in the mucosal and systemic compartments. Despite the large number of animal studies investigating the role of NKT cells during influenza infection, there is a paucity of data supporting their protective role in human influenza, and this warrants further investigation.

Anti-viral potential of $\gamma\delta$ T cells during influenza virus infection.

$\gamma\delta$ T cells (approximately 1-10% of peripheral blood T cells in humans) are important components of both innate and adaptive immunity, displaying a vast array of effector functions, making them attractive targets for therapeutic vaccines. $\gamma\delta$ T cells are characterised by a diverse range of TCRs, with specific V γ -V δ pairings found across different anatomical sites. The

antigen specificity of $\gamma\delta$ T cells remains largely unclear. While butyrophilin (BTN) protein family molecules can regulate immunosurveillance of $\gamma\delta$ T cells (reviewed in [43]), phosphoantigens (pAg, recognized via a mechanism involving BTN3A1), human MutS homologue 2 and F1-ATPase along with apolipoprotein can activate V γ 9V δ 2 T cells, while CD1d or MICA-lipid complexes and endothelial protein C receptor can stimulate non-V δ 2 T cells. $\gamma\delta$ T cells can directly kill virally-infected, cancerous or stressed cells by secreting perforin, granzyme B and granulysin, and can promote inflammation and wound healing, and assist antigen presenting cells in generating optimal, antigen-specific T cell responses (reviewed in [43,44]). Murine studies of influenza infection showed that 15-30% of the lymphocytes in the BAL between 10-15 d.p.i were $\gamma\delta$ T cells, compared to very few at 5-7 d.p.i. This suggests that $\gamma\delta$ T cells may play a pivotal role in recovery from influenza infection [45]. Moreover, in a secondary influenza infection model, mice that were challenged with an H1N1 virus a month following H3N2 virus infection, $\gamma\delta$ T cells isolated from the BAL acquired an NK1.1 phenotype and displayed potent cytotoxic functions, as shown by killing of H3N2 virus-infected target cells in the presence of PHA [46.]. Moreover, $\gamma\delta$ T cell hybridomas, derived from TCR β ^{-/-} mouse, cleared IAV and IBV infections efficiently via Hsp60 upregulation in infected cells and not by any viral proteins [47]. In humans, *ex-vivo* $\gamma\delta$ T cells rapidly produced IFN γ and upregulated CD25 and CD69 following IAV infection. The activation of $\gamma\delta$ T cells is dependent on the mevalonate pathway, as shown by a potent reduction in antiviral responses on addition of mevastatin, a mevalonate pathway inhibitor [48]. Furthermore, *in vitro* studies with human peripheral blood mononuclear cells (PBMC) showed that pAg-activated $\gamma\delta$ T cells were capable of killing macrophages and lung alveolar epithelial cells infected with both human (H1N1) and avian (H5N1) influenza viruses [49-52]. Moreover, pAg isopentenyl pyrophosphate (IPP)-expanded human V γ 9V δ 2 T cells displayed cytolytic activity (as measured by % target cell death), increased production of IFN γ against pandemic H1N1 virus-infected monocyte-derived macrophages and expressed the inflammatory chemokine receptors CCR1, CCR5, and CXCR5 [50]. pAg-reactive V γ 9V δ 2 T subsets harbour a semi-invariant V γ 9 pairing with diverse V δ 2 TCRs [53]. V γ 9V δ 2 T cells are innate in nature as they get stimulated prior to exposure with environmental antigens, as exemplified by their potent activation following pAg exposure by fetal blood-derived V γ 9V δ 2 T cells [54]. Overall, these human studies implicate that the pAg-reactive V γ 9V δ 2 T subsets harbour innate-like activity towards influenza infection. These findings highlight the anti-viral potential of $\gamma\delta$ T

cells during influenza infection and suggest that enhancement of $\gamma\delta$ T cell responses might be beneficial in protection against influenza viruses.

Adaptive T cells are crucial for recovery and long-term protection from influenza infection

Following the initial wave of innate immunity, the adaptive immune system becomes activated and plays an important role in viral clearance, host recovery and establishment of immunological memory. The adaptive immune system consists of humoral (virus-specific antibodies) and cellular ($CD4^+$ and $CD8^+$ T cells) components. Here, we highlight the cellular aspects of influenza-specific adaptive immunity and discuss how the current vaccine strategies target memory T cells.

CD8⁺ T cells confer cross-protection across different influenza subtypes

Cytotoxic $CD8^+$ T cells promote efficient elimination of the virus and subsequent host recovery, via the production of pro-inflammatory cytokines and direct killing of virus-infected cells [55,56]. Influenza-specific $CD8^+$ T cells play a key role in broadly cross-reactive immunity to influenza viruses because they recognise 8-11 amino acid long peptide fragments derived from highly conserved internal viral proteins, presented in the context of MHC class-I molecules. $CD8^+$ T cell-mediated cross-protection against different influenza subtypes has been well established in animal studies, including delayed virus clearance in mice lacking $CD8^+$ T cells [55], generation of influenza virus escape mutants in influenza-infected transgenic mice expressing influenza-specific $CD8^+$ TCR [57], or even more strikingly, in wild-type MHC-I-matched (but not MHC-I mismatched) mice during acute influenza infection [58]. When mice that have recovered from a primary influenza infection are re-infected with a heterologous IAV (a strain with different surface glycoproteins but the same internal proteins) that is resistant to neutralization by antibody induced by the primary infection, influenza-specific $CD8^+$ T cells are responsible for accelerated viral clearance and reduced disease burden [13,59-61]. Studies in guinea pigs also demonstrated that priming with the 2009 pandemic H1N1 virus confers protection against the 2013 avian H7N9 IAV [62]. Recently influenza-specific tissue-resident memory (T_{RM}) $CD8^+$ T cells in the upper respiratory tract of mice were shown to be important in mediating protection from pulmonary influenza virus infection [63]. The presence of protective $CD8^+$ T_{RM} populations in the nasal tissue implies that these cytotoxic effectors are in place to contribute to the early control of infection. Indeed, in mouse studies the establishment of $CD8^+$ T_{RM} cells in the upper respiratory tract reduced viral

replication in the nose as early as day 3 and day 5 after intranasal challenge, in a CD8⁺ T cell-dependent manner [63].

CD8⁺ T cells are also associated with protection from disease during human IAV infection. In a seminal paper by Andrew McMichael and colleagues [9], healthy volunteers were experimentally infected with the influenza A/Munich/1/79 (H1N1) virus. Individuals with demonstrable influenza-specific CD8⁺ T cell responses cleared the virus more efficiently, irrespective of the presence of pre-existing antibodies [9]. Subsequently, a retrospective report showed that individuals previously infected with H1N1 IAV in the 1950s were less susceptible to the 1957 H2N2 pandemic [10], further supporting the idea of heterosubtypic immunity that is presumed to be conferred by cellular immunity because antibodies elicited by H1N1 viruses do not protect against H2N2 IAV infection. The advent of the 2009 H1N1 pandemic provided further opportunities to determine the role of CD8⁺ T cells in protection against IAV infection. Indeed, in a household cohort study in the UK, pre-existing IFN γ -producing CD8⁺ T cell responses were associated with lower disease severity [12]. Furthermore, the Flu Watch study, recruiting influenza-infected patients in England between 2006 and 2010, showed a positive correlation between pre-existing IAV-specific CD8⁺ T cells and symptomatic, PCR-confirmed IAV infection during 3 seasonal epidemics and the 2009 H1N1 pandemic [64]. These studies provide compelling evidence that cytotoxic CD8⁺ T cells can confer some level of protection during infection with human influenza viruses. Recently, our group has extended these observations to infection with the avian H7N9 IAV that emerged in China in 2013 in a study of longitudinal samples from hospitalized patients during the first wave of H7N9 infections in China [13]. Patients with most rapid recovery (discharged within 2-3 weeks after hospitalisation) had early and robust H7N9-specific CD8⁺ T cell responses, followed later by neutralising antibody responses. Conversely, patients who succumbed to infection had delayed and/or minimal cytotoxic CD8⁺ T cell responses. Thus, recovery from severe H7N9 infection was associated with robust early CD8⁺ T cell responses.

Longevity and antigen-specificity of influenza-specific CD8⁺ T cells

The ability of CD8⁺ T cells to provide cross-strain protection against distinct influenza viruses makes them promising and attractive vaccine targets. However, one needs to also consider the longevity and breadth of the CD8⁺ T cell response. Indeed, influenza-specific CD8⁺ T cells were stable in the peripheral blood of healthy adults over a period of 13 years, showing that memory CD8⁺ T cells are long-lived [65]. The breadth of CD8⁺ T cell responses results from the high degree of conservation of immunogenic epitopes; recent human studies showed that

CD8⁺ T cells can confer cross-reactivity across multiple IAVs [66-68], including H7N9 [69,70] and H5N1 [71,72] viruses, as well as the two lineages of IBV [73]. Our group has recently identified a set of universal and immunodominant CD8⁺ T cell epitopes (HLA-A*02:01/M1₅₈₋₆₆, HLA-A*03:01/NP₂₆₅₋₂₇₃, HLA-B*08:01/NP₂₂₅₋₂₃₃, HLA-B*18:01/NP₂₁₉₋₂₂₆, HLA-B*57:01/NP₁₉₉₋₂₀₇ and HLA-B*27:05/NP₃₈₃₋₃₉₁), which are conserved across 100% of known human IAVs (from the 1918 pandemic to the 2013 H7N9 strain) [69]. These epitopes encompass various HLA molecules that are differentially prevalent across ethnicities, being particularly rare in the Alaskan and Australian Indigenous populations [74]. It is pertinent to note, that cross-reactivity may occur even when new variant epitopes emerge, depending on the amino acid substitution and/or position of the mutation and its impact on TCR recognition of the peptide/MHCI complex as well the individual's TCR repertoire of the cognate CD8⁺ T cell population. For instance, mutations in anchor residues can allow for escape variants, as it was the case with HLA-B*27:05/NP₃₈₃₋₃₉₁, whereby mutations in position 2 (R384G) abrogated binding of the peptide to the MHCI molecule [75]. Furthermore, for the HLA-B*07:02 or HLA-B*35:01 presented NP₄₁₈₋₄₂₆ peptide, while the anchor residues are maintained, cross-reactivity depends on the position of the mutation in the peptide and the nature of the substitution [68,76]. Conversely, in the context of HLA-A*02:01/M1₅₈₋₆₆, for which sporadic mutations have arisen over the last century, the public αβ TCR clonotype (TRBV19/TRAV27) is able to robustly cross-react with all the variants through induced-fit molecular mimicry, although in some instances with lower affinity [67]. These studies highlight the importance of the TCR repertoire in determining the breadth and protective capacity of the cytotoxic CD8⁺ T cell responses.

While CD8⁺ T cells are crucial in conferring protection, excessive CD8⁺ T cell responses can lead to immunopathology, as recently reviewed by Duan and Thomas [77]. However, the exact molecular and cellular pathways that lead to regulated and effective CD8⁺ T cell responses versus exuberant and pathogenic responses are not entirely clear [78]. Thus, future research is needed to understand how protective memory can be established without immunopathology. It is also important to note that CD8⁺ T cells do not provide sterilizing immunity against infection but promote rapid host recovery and thereby provide protection from disease symptoms and death. This is why CD8⁺ T cells play a pivotal role in reducing disease severity and preventing fatal infections especially during an outbreak of a novel antigenically-shifted influenza strain, to which there is no pre-existing neutralizing immunity in the general population.

Thus, it is evident that CD8⁺ T cells can confer broad protective immunity to antigenically drifted and shifted influenza viruses. The HLA genotype and the TCR repertoire are two key factors that need to be considered in the design of T cell-targeted vaccine strategies to provide wide population coverage, while limiting immune escape.

New insights into the role of CD4⁺ T cells during influenza virus infection

CD4⁺ T cells (T helper cells, Th cells) commonly recognise viral peptide fragments that are 12-16 amino acids in length, presented on major histocompatibility complex class II (MHC-II) molecules. They play an important role in promoting optimal CD8⁺ T cell and B cell responses during influenza infection. Following activation in the draining lymph nodes during influenza infection, naïve CD4⁺ T cells differentiate into effector cells capable of producing cytokines such as IFN γ and IL-2, and migrate to the lung. This is accompanied by the loss of the homing receptor CD62L, downregulation of CCR7 and upregulation of CD44 and CD49d, with transient expression of CCR5 [79]. CD4⁺ T cells, especially T follicular helper cells (Tfh) [80,81], help B cells to generate antibodies by providing co-stimulatory signals. They are important for the priming and maintenance of CD8⁺ T cell responses to generate efficient memory [82,83].

Circulating T follicular helper cells (cTfh; CXR5⁺ PD1⁺ CD4⁺ T cells) have recently been identified during influenza infection and vaccination [84] as a prominent CD4⁺ T cell population in peripheral blood on day 7 after vaccination [85]. These cTfh cells express the Th1 phenotype (CXCR3⁺CCR6⁻) and upregulate markers of activation ICOS and PD-1. Importantly, cTfh cells can provide help in the form of cytokines and co-stimulation signals, to memory B cells for development into antibody-secreting cells (ASCs) *in vitro* [85]. As a result, the presence of cTfh cells correlates with the magnitude and quality of the antibody response and the development of ASCs following vaccination with trivalent inactivated vaccines (TIV) [85,86]. The ontogeny and specificity of cTfh cells and their relationship to memory Tfh reservoirs in lymphoid tissues remain unclear. Recently, clonotypic analysis of cTfh cells revealed the recruitment of recurrent clonotypes following vaccination in multiple years, strongly indicating that the cTfh population that appears following vaccination is memory-derived [87]. Further understanding of the specificity and function of circulating and non-circulating Tfh cells in humans and the role of these cells in influenza infection may inform the development of improved vaccines.

CD4⁺ T cells assist CD8⁺ T cells in becoming activated by licensing Ag-presenting DCs via the CD154 (CD40L)/CD40 signalling pathway [88,89]. However, CD4⁺ T cell-mediated

help for primary activation of naïve CD8⁺ T cells appears to be pathogen-dependent, as it was redundant during influenza virus or LCMV infections [90]. Efficient primary CD8⁺ T cell responses could be established in the absence of CD4⁺ T cell help, as shown by in mice depleted of CD4⁺ T cells or in MHC-II knock out mice [91,92]. Nevertheless, co-stimulatory signalling via CD28 is needed for primary CD8⁺ T cell activation [93] and is required to sustain CD8⁺ T cell responses after activation during the effector phase [94]. Furthermore, virus-specific CD4⁺ T cells have a beneficial impact on primary and secondary responses for themselves, as CD4⁺ T cell licensing of dendritic cells results in enhanced proliferation and differentiation of the cells in influenza infected mice [95].

Regardless of whether CD4⁺ T cell help is required for primary influenza-specific CD8⁺ T cell responses, they are beneficial for generation and recall of CD8⁺ T cell memory in terms of numbers of antigen-specific CD8⁺ T cells after the secondary influenza virus infection [82,96]. Furthermore, CD4⁺ T cell help is needed to localise tissue resident memory CD8⁺ T cells to epithelial cells in the lung, as influenza infection in mice lacking help led to less expression of the integrin CD103 and a dislocation of T_{RM}s at 45 dpi [97]. CD4⁺ T cell help during influenza priming of CD8⁺ T cells led to greater production of Granzyme B (GzmB) in the T_{RM}s after secondary challenge [97].

In addition to their helper function for B cell antibody class switching [98] and licensing of DCs for CD8⁺ T cell activation, CD4⁺ T cells can acquire cytotoxic functions [99,100]. Notably, acquisition of cytotoxic functions after influenza virus infection in mice was strongly tissue-dependent. Therefore, in adoptive transfer studies, cytotoxic CD4⁺ T cells expressing GzmB and Perforin could be found preferentially in mucosal tissue such as the lungs [101] of influenza (PR8-Ova) infected mice but less often in draining mediastinal lymph nodes and spleen at 7 dpi [101]. Furthermore, the route of administration of the virus appears to be important: intranasal infection of mice with PR8 IAV resulted in recruitment of the cytolytic CD4⁺ T cells in the lungs, while intramuscular administration led to diminished lung cytolytic CD4⁺ T cells and sterilizing immunity [102]. Thus, the route of the administration is an important consideration for future vaccine approaches designed to elicit a protective immune barrier at the site of infection.

Ex vivo analysis of influenza-specific CD4⁺ T cells has been recently facilitated by the development of HLA class II tetramers, generally characterised by lower TCR-binding avidity to peptide/MHCII compared to tetramer-specific CD8⁺ T cells. When cross-reactivity to four strains, including the pandemic H1N1 virus was analysed in humans, antigen-specific CD4⁺ T cells were detected against several influenza viral proteins, including the haemagglutinin (H1

and H3), neuraminidase (N1 and N2), nucleoprotein (NP), and Matrix 1 (M1) [15,72,103]. Cross-reactivity of influenza-specific memory CD4⁺ T cells was also shown using overlapping peptides derived from influenza A/Vietnam/CL26/2005 (H5N1) and influenza A/New York/232/2004 (H3N2) in healthy volunteers from the United Kingdom and Vietnam. The viral M1 and NP proteins were the immunodominant targets of this CD4⁺ T cell cross-recognition [72]. Further research, is needed to understand how CD4⁺ T cell help leads to the generation of efficient CD4⁺ and CD8⁺ T cell memory.

Vaccine strategies for induction of broadly cross-reactive adaptive T cell responses.

Currently, inactivated influenza vaccines (IIV) remain the predominant strategy for inducing protective immunity for upcoming epidemics. The IIV, either in a trivalent (H1N1, H3N2 and one IBV from either lineage) or quadrivalent (H1N1, H3N2 and two IBV strains, one from each lineage) formulation, is prepared annually based on recommendations by the WHO. Although IIV induces a B cell and antibody response in primed individuals, it is a weak inducer of T cell immunity, with the exception of CD4⁺ T cells.

Promising approaches for targeting CD4⁺ T cells by vaccination include the use of oil-in-water adjuvants such as MF59® adjuvant [104]. Another approach is the live-attenuated influenza virus vaccine (LAIV), which in contrast to injectable IIV, are able generate long-term and virus-specific lung tissue resident memory CD4⁺ and CD8⁺ T cells in mice [105]. However, human studies on LAIV show minimal increases in CD8⁺ T cell responses after vaccination, as measured by IFN γ production [106,107]. Nevertheless, it remains unclear whether the poor results observed in these human LAIV trials are due to the fact that CD8⁺ T cells are being assessed within the peripheral blood, while memory T cell responses generated after LAIV in mice are established at the site of infection [108].

Given the prominent role of T cells in inducing protective and universal immunity, novel T cell-mediated vaccination strategies are required to complement the induction of antibodies and memory B cells. One novel vaccine formulation is the modified vaccinia virus Ankara (MVA) expressing the influenza viral proteins NP and M1. The MVA-NP+M1 vaccine can induce increases in the number of influenza-specific, IFN γ -secreting CD8⁺ T cells in adults (<0.5% on d56 over d0, n=8) [109]. Additionally, the number of tetramer⁺ HLA-A*02:01/M1₅₈₋₆₆-specific CD8⁺ T cells were shown to increase modestly and upregulate the effector molecules granzyme A and perforin [110]. Importantly, compared to unvaccinated controls, the MVA-NP+M1 vaccine provided modest protection from clinical symptoms upon intranasal challenge with influenza A/Wisconsin/67/2005 (H3N2) [111]. Other vaccine

formulations have incorporated influenza peptides into their formulations, such as Flu-v [112,113] and FP-0.01 [113], or the Multimeric-001 vaccine, which consists of a protein encompassing 9 highly conserved peptides from the M1, NP and HA proteins [114]. Individuals vaccinated with Flu-v showed a modest increase in IFN γ ⁺ T cell responses following *in vitro* stimulation with peptides [112,115], and these responses correlated with reduced viral titres following challenge with A/Wisconsin/67/2005 (H3N2). However, there was no correlation between IFN γ ⁺ T cell responses and clinical symptoms. Consistently, there were no significant differences in viral shedding or symptom score between vaccinated and unvaccinated volunteers following experimental challenge [115]. The FP-0.01 and Multimeric-001 vaccines only show small increases in T cell responses following restimulation of PBMCs from vaccinated individuals and have yet to be tested for the protective efficacy against experimental IAV challenge [113,114]. Table 1 shows an overview of recent trials that target innate and adaptive T cells for influenza vaccination [39-42,104,105,107,109,111-116].

Overall, there are currently no vaccines that induce robust and protective CD8⁺ T cell responses against influenza viruses. Novel vaccination strategies that can establish broadly cross-reactive and long-lived memory CD8⁺ T cells are needed in order to complement antibody responses in the prevention of seasonal influenza and newly emerging influenza viruses.

Concluding remarks

Published studies show clear evidence that both the adaptive (T and B cells) and innate (MAIT cells, $\gamma\delta$ T cells and NKT cells) immune responses play important roles in controlling influenza virus infection. Thus, vaccination strategies that link innate and adaptive cellular immune responses would be attractive. Currently licensed vaccine strategies do not induce robust and protective T cell responses. The development of a vaccine that induces cross-protective cellular immunity against different influenza strains and subtypes, including IAVs with pandemic potential, would be an excellent achievement.

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Compliance with ethics guidelines

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Table 1. Novel vaccine strategies targeting T cells

Host	Immunisation type	Administration route	Viral challenge	Findings	Reference
NKT cells					
C57BL/6J mice	α GC adjuvant + iIAV PR8 (H1N1)	subcutaneous	HKx31 (H3N2)	Faster viral clearance; Improved secondary responses and long term survival of CD8 ⁺ T cells.	Guillonneau (2009) [39]
BALB/C mice	α GC analogue KBC-007 KBC-009 + formalin-inactivated PR8	intranasal	PR8	Significantly higher Ab titers 4 wks after immunization compared to PR8 alone with higher neutralizing activity; Rescue from otherwise lethal PR8 challenge in vaccine + adjuvant treated mice.	Lee (2011) [40]
pigs	UV-inactivated H1N1 A/California/04/2009 (kCA04) SI virus + α GC	intramuscular	CA04	Increased NKT-cell numbers, high titres of anti-HA Abs and boosted IAV-specific CD8 ⁺ T cells, reduced viral shedding.	Artiaga (2016) [41]
BALB/c mice	Formalin-inactivated PR8 + α GC	intranasal	PR8	IgG and IgA antibodies lasting up to 3 months; reduced viral titers; improved survival from lethal challenge.	Youn (2007) [42]
BALB/c mice	LAIV (PR8) (rNS1 1-73)) + α GC derivate (alpha-C-GalCer)	intranasal	PR8	Reduced morbidity and mortality; increased amount of IA Ab (IgG, IgG1, and IgG2a); increased IFN γ secreting CD8 ⁺ T cells.	Kopecky-Bromberg (2009) [113]
T cells					
Human	Monovalent H5N1 (A/Vietnam/1194/2004) subunit vaccine + MF59® adjuvant	intramuscular	<i>In vitro</i> peptide stimulation: H5 A/Vietnam/1194/2004 (clade 1), A/Indonesia/5/05 (clade 2.1), and A/duck/Singapore/97 (clade 0-like)	High titers of neutralizing; long lasting Ab (>6 months), increased pool of memory B cells and H5-CD4 ⁺ T cells; <i>in vitro</i> responses to non-conserved regions of H5.	Galli (2009) [101]
C57BL/6 mice	LAIV (FluMist) (Quadrivalent (A/California/7/2009 (H1N1)pdm09, A/Texas/50/2012 (H3N2), B/Massachusetts/2/2012, or B/Brisbane/60/2008) (2014-2015) and Quadrivalent (A/California/7/2009 (H1N1)pdm09, A/Switzerland/9715293/2013 (H3N2), B/Phuket/3073/2013, and B/Brisbane/60/2008) (2015-2016)	intranasal	PR8 or X31 or heterosubtypic challenge PR8 or X31	localized CD4 ⁺ and virus specific CD8 ⁺ T cells in the lung; long-term responses (45 weeks) and tissue resident memory T cells; heterosubtypic protection.	Zens (2016) [102]
Human	H5N1 pLAIV VN2004	intranasal	<i>In vitro</i> peptide stimulation: H5N1; nonconserved HA proteins of relevant seasonal H1N1 and H3N2 viruses	Increased IFN γ responses of PBMCs to conserved internal proteins M and NP; temporary increase of influenza specific CD8 ⁺ T cells.	Peng (2015) [104]
Human	Modified Vaccinia Virus Ankara (MVA)-NP+M1 (A/Panama/2007/99)	intradermal	<i>In vitro</i> peptide stimulation NP + M1-	Slight increase of IFN γ producing PBMCs and CD8 ⁺ T cells responses to NP and M1.	Berthoud (2011) [106]
Human	Ankara (MVA)-NP+M1	intramuscular	A/Wisconsin/67/2005 (H3N2)	Reduced influenza symptoms after infection.	Lillie (2012) [108]
Human	Synthetic poly-peptide vaccine FLU-v w/wo adjuvant ISA-51	subcutaneous	<i>In vitro</i> stimulation with FLU-v	Increased IFN γ response in PBMCs after vaccination with adjuvant.	Pleguezuelos (2012) [109]
Human	Synthetic poly-peptide vaccine FLU-v + adjuvant ISA-51		A/Wisconsin/67/2005 (H3N2)	Flu-v specific IFN γ responses of CD8 ⁺ T cells 19 days after vaccination; no significant differences in total viral shedding or total symptom score between placebo and FLU-v vaccinated	Pleguezuelos (2015) [112]

Figure 1. Activation of the innate and adaptive arms of the immune system during influenza virus infection. Influenza virus infection leads to activation of innate cells, such as macrophages and neutrophils, to secrete pro-inflammatory cytokines resulting in the recruitment of other leukocytes. Innate T cells play an important role in dampening the effects of influenza infection by the secretion of cytokines and cytotoxic molecules. The exact mechanisms of how these innate T cells are activated (TCR-dependent or indirectly by soluble factors) during influenza virus infection remain controversial. In addition, innate T cells provide important signal molecules to enhance APC activity, which in turn present influenza antigens to CD8⁺ T cells and CD4⁺ T cells for effective viral clearance. CD4⁺ T cells can also provide help to CD8⁺ T cells and antibody-producing B cells to enhance the antiviral response.