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Transcriptional regulation of ILC development and function

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The Walter and Eliza Hall Institute of Medical Research

Department of Medical Research

The University of Melbourne

Abstract

Innate lymphoid cells (ILCs) are a specialized arm of the innate immune system responsible for protecting the mucosal barrier and defending against infection. ILC activation and cytokine production occurs in response to stimuli in the local environment making them an important initiators of the early immune response. However, they also contribute to inflammatory diseases and thus must be tightly regulated. The development and function of ILCs is dependent on the tight regulation of transcription factors. Two transcription factors GFI1 and GFI1B are involved in this differentiation but have been poorly studied and thus how they shape differentiation is not clear.

GFI1 and GFI1B was differentially expressed throughout ILC development. GFI1B expression was limited to the early progenitors whereas GFI1 was expressed in both early progenitors and in all mature ILC subsets in the peripheral organs. GFI1B expression identified the early progenitors in the bone marrow with the capacity to give rise to all ILC subsets. GFI1B expression is also required for the development of progenitors in the bone marrow and lung ILC2. This impacted the lung inflammatory response induced in mice treated with papain as the eosinophil recruitment by ILC2s was impaired. In contrast, GFI1 was highly expressed in mature NK cells and was vital for development and maintenance of these cells. GFI1 regulated multiple NK cell pathways, including proliferation, homing to peripheral organs and effector functions. Defects in these functions impaired the protection mediated by NK cells against metastatic melanoma cells. Together, GFI1 and GFI1B are both key regulators of ILC development, although they regulate different stages of ILC development and function.

ILCs are enriched at the mucosal barrier, particularly at the gastrointestinal tract. They play an important role in protecting against infections but also have the capacity to promote inflammation when dysregulated. Their role in the development of colorectal cancer (CRC) is unclear as individual ILC subsets have been associated with both protecting against or promoting tumorigenesis. Characterisation of the immune infiltrate of mice with CRC showed that there was an accumulation of IL-5-producing ILC2 within the colon and this was strongly correlated to the tumour burden. ILC2-deficient mice developed a higher tumour burden compared to control mice, which indicated a protective role for ILC2s against colorectal cancer.

Declaration

This is to certify that:

1. This thesis comprises my original work towards the degree of Doctor of Philosophy except where indicated in the preface.
2. Due acknowledgements have been made in the text to all other materials used.
3. This thesis is less than 100,000 words in length exclusive of tables, maps, bibliographies and appendices.

Qitong Huang

Preface

The work presented in this thesis was performed at the Walter and Eliza Hall Institute of Medical Research under the supervision of Professor Gabrielle Belz, Dr Lisa Mielke and Dr Tracy Putoczki.

In accordance with the regulations of the University of Melbourne, I assess my contributions in this thesis as follows:

Chapter 3: 95% with assistance from Cyril Seillet for staining and purification of bone marrow progenitor populations

Chapter 4: 90% with contributions from Alexandra Garnham for the bioinformatics analysis of the RNA sequencing data and Nicolas Jacquelot for assistance with the melanoma cell culture.

Chapter 5: 85% with contributions from Adele Preaudet, Paul Nguyen and Tracy Putoczki for endoscopy and monitoring the health of mice with tumours. Text on the pages 141-145 is published in Huang *et. al.*, 2019, *Frontiers in Immunology*, where my contribution to the writing was 50%. Only the published sections that I have 100% written are included.

Therefore, my overall contribution to this thesis is approximately 90%.

List of Publications

Some of the work in this thesis has been presented in the following publication:

Huang Q, Jacquelot N, Preaudet A, Hediye-zadeh S, Souza-Fonseca-Guimaraes F, McKenzie ANJ, Hansbro PM, Davis MJ, Mielke LA, Putoczki TL, Belz GT. (2021). Type 2 Innate Lymphoid Cells Protect against Colorectal Cancer Progression and Predict Improved Patient Survival. *Cancers*. 13(3):559. <https://doi.org/10.3390/cancers13030559>

Other publications during my PhD:

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Huang Q, Belz GT. (2019). Parallel worlds of the adaptive and innate immune cell networks. *Curr Opin Immunol*. 58:53-59. doi: 10.1016/j.coi.2019.04.008.

Huang Q, Belz GT. (2018). Bach2: An Instrument of Heterogeneity for Long-Term Protection. *Immunity*. 48(4):618-620. doi: 10.1016/j.immuni.2018.03.033.

Huang Q, Seillet C, Belz GT. (2017). Shaping Innate Lymphoid Cell Diversity. *Front Immunol*. 8:1569. doi: 10.3389/fimmu.2017.01569

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Abbreviations

AOM	Azoxymethane
BALF	Bronchoalveolar lavage fluid
CAC	Colitis associated cancer
CCL	CC chemokine ligand
CD	Cluster of differentiation
CILP	Common innate lymphoid progenitor
CLP	Common lymphoid progenitor
CoREST	REST co-repressor
CRC	Colorectal cancer
CXCL	Chemokine (C-X-C) ligand
CXCR4	C-X-C chemokine receptor type 4
DNAM-1	DNAX accessory molecule-1
DP	Double-positive
DPBS	Dulbecco's phosphate-buffered saline
DSS	Dextran sodium sulphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EOMES	Eomesodermin
FACS	Fluorescence-activated cell sorting
GFI1	Growth factor independent 1
GFI1B	Growth factor independent 1b
GI	Gastrointestinal
H3K4	Histone 3 lysine 4
H3K9	Histone 3 lysine 9
HDAC	Histone deacetylase
HIFCS	Heat-inactivated foetal calf serum
HP1 α	Heterochromatin protein 1 α
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel disease
ICOS	Inducible T Cell Costimulator
ID2	Inhibitor of DNA binding 2

IEL	Intraepithelial lymphocytes
IFN	Interferon
IL	Interleukin
ILC(s)	Innate lymphoid cell(s)
ILC2p	ILC2 progenitor
ILCp	Innate lymphoid cell progenitor
Imm	Immature
JAK	Janus kinase
KIR	Killer-cell immunoglobulin-like receptors
KLRG1	Killer cell lectin like receptor G1
KO	Knockout
LSD1	Lysine specific demethylase 1
LN	Lymph node
LPL	Lamina propria lymphocytes
LTi	Lymphoid tissue-inducer
M1	Mature 1
M2	Mature 2
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
NCR1	Natural cytotoxicity triggering receptor 1
NK	Natural killer
NKp	Natural killer progenitor
PD-1	Programmed cell death protein 1
PLZF	Promyelocytic leukemia zinc finger
PMA	Phorbol 12-myristate 13-acetate
RCRB	Red Cell Removal Buffer
REST	Repressor element-1 silencing transcription factor
ROR γ t	RAR-related orphan receptor γ
SNAG	SNAIL GFI1
SP	Single-positive
STAT	Signal transducer and activator of transcription
ST2	Interleukin 1 receptor-like 1
T-bet	T-box expressed in T cells

TCR	T cell receptor
Th	T helper cell
WT	Wildtype
Th	T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TNF	Tumour necrosis factor
UC	Ulcerative colitis

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Chapter 1. Introduction

1.1 Overview

Innate lymphoid cells (ILCs) are a specialized family of the innate immune system responsible for protecting against infection and disruption of the mucosal barrier^{1,2}. They are characterised by the classic lymphoid morphology yet lack many of the lineage markers associated with T and B lymphocytes, myeloid cells and granulocytes³. Transcriptional regulation and cytokine production of ILCs parallel the CD4⁺ T helper (Th) family but lack the specificity associated with T cells as ILCs do not express the recombination activating gene (RAG)- dependent rearranged antigen receptors and thus do not undergo antigen-driven clonal selection⁴. Instead, ILC activation and cytokine production occurs through the stimulation by cytokines in the local environment, making them both important sensors and initiators of the early immune response. The appropriate development and function of ILCs is dependent on the tight regulation of transcription factors, which direct their development, and their response to external stimuli.

1.2 Innate lymphoid cell subsets

Based on the transcription factors required for development and cytokines produced, ILCs can be divided into distinct groups. Group 1 ILC (ILC1), comprised of cytotoxic natural killer (NK) cells and non-cytotoxic ILC1, produce interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) to protect against intracellular pathogens and viruses in response to IL-12 and IL-18 stimulation⁵⁻⁷. Group 2 ILC (ILC2) produce IL-5 and IL-13 when stimulated by IL-33 and IL-25 to protect against multicellular pathogens such as helminths⁸⁻¹⁰. Group 3 ILC (ILC3) consists of lymphoid tissue-inducer (LTi) cells, which are responsible for secondary lymphoid organogenesis, and cells that are further divided into natural cytotoxicity receptor (NCR)⁻ and NCR⁺ ILC3 based on NKp46 expression^{11, 12}. ILC3 produce IL-17 and IL-22 in response to IL-23 to fight colonization by extracellular bacteria and for maintaining epithelial cell integrity⁴.

1.3 Developmental stages of ILC

All ILCs originate from the common lymphoid progenitor (CLP) in the foetal liver or adult bone marrow (**Fig 1.1**). The CLP, defined by the expression of IL-7R α ⁺FLT3⁺ckit⁺ among lineage negative cells, has the potential to develop into multiple immune cell lineages,

including T and B cell fate¹³. The progressive expression of different transcription factors sequentially restricts the developmental potential of progenitor cells and induce commitment to the ILC lineage. This results in the transition of CLP to the common innate lymphoid progenitor (CILP) to the innate lymphoid cell progenitor (ILCp), which then differentiates into the precursor of each ILC subset (**Fig. 1.1**).

Downstream of the CLP, the CILP, defined as IL-7R α ⁺ α 4 β 7⁺ckit⁺ cells, lose the potential to develop into B and T cells. The early ILC precursor (EILP) which expresses the transcription factor T cell factor 1 (TCF1) but lacks IL-7R α , in contrast to other stages of development, also develop from the CLP^{14, 15}. Both EILP and CILP have the potential to develop into all ILC subsets, including the LTi and are transcriptionally similar^{1, 14-16}. However, it is not known whether the EILP represents an alternative developmental pathway for ILCs, nor is it understood the functional importance of IL-7R downregulation.

The ILCp, a more lineage-restricted cell population within the CILP, was identified by the expression of the transcription factor promyelocytic leukemia zinc finger (PLZF), and surface receptor programmed cell death-1 (PD-1)¹⁷. Initially, it was thought that ILCp had lost the ability to produce both LTi and NK cells, only retaining the capacity to produce ILC1, ILC2 and ILC3. However, it was recently shown that ILCp also have the capacity to develop into NK cells¹⁸ (**Fig 1.1**). Progenitor cells that are restricted to a specific lineage, such as NK progenitors (NKp) or ILC2 progenitors (ILC2p) have also been identified, however, the transcriptional regulation that drives this developmental divergence between lineages, particularly for ILC1p and ILC3p, is not well understood¹⁹⁻²² (**Fig 1.1**). The ILCs that develop from the progenitors in the bone marrow will then seed the peripheral tissues settling throughout the body to maintain the ILC population within the periphery.

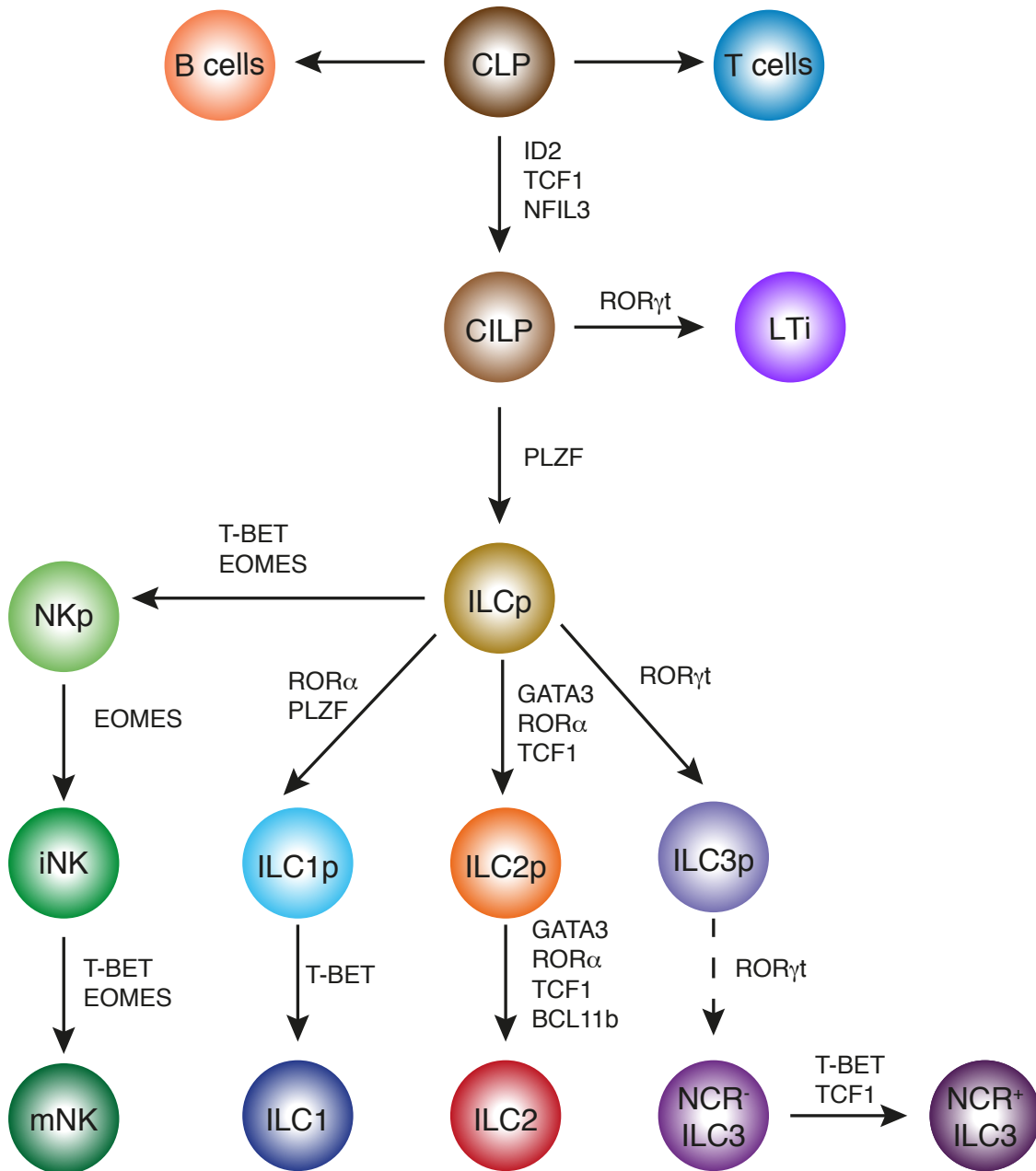


Figure 1.1 Transcription factors involved in ILC development.

Developmental pathway of ILCs from the CLP in the foetal liver or bone marrow to mature ILC in the periphery. Expression of specific transcription factors at different stages of development permits commitment to different lineage restricted ILC progenitors, which eventually develop into mature ILC.

1.4 ILC function in mucosal immunity and cancer

ILCs are enriched at mucosal surfaces and thus play an important role in mediating the early immune response against pathogens, particularly at these sites². ILCs generally lack cytotoxic activity, with NK cells being the exception¹. In response to cytokines in the microenvironment, ILC numbers expand and in turn, produce cytokines to communicate with other immune cells¹. This interaction between immune cells promote infection clearance, tissue repair and influence tumour development¹.

1.4.1 NK cells and ILC1

NK cells protect against intracellular viral infections and mediate anti-tumour protection as patients and animal models with deficient or defective NK cells have recurring viral infections and are at an increased risk of developing cancer²³. In response to viral infection, such as murine cytomegalovirus (MCMV), IL-12 is produced which stimulates IFN- γ and TNF- α production by NK cells²⁴. These pro-inflammatory cytokines inhibit viral reproduction and reduce virus-mediated pathology²⁴. Furthermore, cytotoxic NK cells exocytose granzyme and perforin containing granules to directly mediate lysis of infected cells. NK cells can also recognize tumour cells through the upregulation of ligands such as CD155 or the loss of self-identifying molecules such as the major histocompatibility complex (MHC) class I on the tumour cell. These interactions between the ligands on the tumour cell and NK cell receptors drive NK cell activation and cell-mediated lysis²³.

The main functional difference between NK cells and non-NK ILC1s is that the latter do not have the capacity to mediate cytotoxic killing²⁵. Instead, ILC1s are capable of producing pro-inflammatory cytokines to help drive the inflammatory response. However, their role in antiviral immunity is less understood as it is confounded with NK cells as they are phenotypically similar and both produce IFN- γ and TNF- α ²⁶. Recently, it has been shown that ILC1 are vital for the early immune response against viral infections⁵. ILC1 in the liver, in particular, are responsible for limiting MCMV replication during the early stages of infection⁵. They are an important source of IFN- γ , which is vital in conferring protection and ensuring host survival⁵.

1.4.2 ILC2

ILC2s mediate an immune response required for effective parasite expulsion but they can also be involved in inflammatory allergy responses^{8, 27}. Macrophages, dendritic cells (DC) and epithelial cells damaged during a helminth infection produce the alarmin IL-33 that stimulates ILC2 proliferation and IL-5 and IL-13 cytokine production²⁸⁻³⁰. IL-13 assists with the physical removal of intestinal parasites by stimulating mucus production in goblet cells and increasing the contractility of intestinal smooth muscle cells to push the parasite out^{31, 32}. IL-5 recruits eosinophils to the site of infection where they release cytotoxic granules to damage the helminths^{33, 34}. In response to allergens in the lung, a similar increase in IL-5 cytokine production and eosinophil recruitment is observed. Once the parasite or allergen is cleared, ILC2s play a key role in tissue repair by producing the epidermal growth factor amphiregulin³⁵.

The role of ILC2s in cancer development is less well understood. IL-5 production by ILC2 in the lung has been associated with increased lung cancer metastasis while the growth of the primary tumour was unaffected when lung carcinoma cells were injected into mice³⁶. IL-5 was able to promote metastasis indirectly by recruiting eosinophils, which then in turn recruit regulatory T (Treg) cells, thereby creating an immunosuppressive microenvironment that is suitable for metastasis³⁶. However, it is not fully understood whether ILC2s are involved in the initial tumorigenesis event and whether a similar mechanism operates in gastrointestinal cancers.

1.4.3 ILC3

IL-23 produced during bacterial and fungal infections activate ILC3s to produce IL-17 and IL-22⁴. These cytokines act on intestinal epithelial cells to produce antimicrobial peptides and chemokines to recruit neutrophils to the site of infection^{37, 38}. IL-22 can also act on intestinal stem cells to limit their apoptosis, increase epithelial proliferation and increase mucus production to maintain the intestinal mucosal barrier and prevent further bacterial infection³⁹. In particular, IL-22 produced by NCR⁺ ILC3 during the early stages of *Citrobacter rodentium* infection, prior to T cell activation, is important for protection against infection⁴⁰. The protective role of NCR⁺ ILC3 is even more prominent in immune compromised hosts, indicating a failsafe mechanism to protect against infection when the adaptive immune response is defective⁴⁰.

IL-23-induced production of IL-17 and IL-22 has been identified as a key pathway involved in the development of inflammatory bowel disease (IBD) and colitis-associated cancer (CAC)^{41, 42}. In particular, IL-22 promotes tumour growth by directly inducing epithelial proliferation via phosphorylation of STAT3. Treatment with IL-22 blocking antibodies has been shown to be able to reduce the tumour burden, which provides a new therapeutic option for patients with colorectal cancer³⁷.

1.5 Transcription factors involved in ILC development and function

The development from progenitor cells into mature ILCs, and the function of mature ILCs in the periphery, is in part regulated by the expression of specific transcription factors. The differential expression of key transcription factors such as *Eomes*, *Gata3* and *Ror γ t* dictate the commitment of progenitors to a specific developmental lineage^{11, 20, 43}. In peripheral ILC subsets, these transcription factors also regulate the maturation and expression of cytokines produced by ILCs^{9, 20, 43}. Therefore, accurate expression is required for the development and regulation of an efficient immune response.

1.5.1 Progenitors

The development of CLP into CILP is dependent on the expression of the helix-loop-helix inhibitor of DNA binding 2 (ID2), nuclear factor interleukin 3 (NFIL3) and TCF1. ID2 expression in the CILP population inhibits T and B cell development by preventing the E protein E2A from binding to DNA at E-box enhancers which forms heterodimers that initiates the transcription essential for their development⁴⁴⁻⁴⁶. NFIL3 is also expressed at the CILP stage but unlike ID2 which is expressed continuously, NFIL3 is only expressed transiently which is both necessary and sufficient to drive the development of the ILC progenitors^{47, 48}. Thus, although it is expressed in mature ILCs, it is not required for their function or survival^{17, 49}. TCF1 plays an important role in determining T cell lineage development and differentiation but it is expressed in both the CILP and EILP. It is required to give rise to all ILC subsets^{17, 50-52}. Loss of these three transcription factors individually all result in a reduction of CILP cells in the bone marrow and a subsequent loss of mature ILCs in the periphery when deleted in the early stages of development¹⁷ but it is not clear how these transcription factors are temporally regulated and whether they regulate the expression of each other.

1.5.2 NK cells and ILC1

NK cells and ILC1s are defined by the expression of T-box transcription factors Eomesoderm (EOMES) and T-BET⁴³. EOMES and T-BET are highly homologous proteins that have complementary functions in NK cell development, maturation and function while ILC1s only require T-BET and do not express EOMES⁵³. During early NK cell development in bone marrow, EOMES is required for NK cell progenitors while T-BET is required in peripheral NK cells to drive their maturation as well as maintaining IFN- γ cytokine expression upon IL-12 and IL-18 stimulation⁵⁴. T-BET expression in progenitors plays a role in ILC1 vs NK cell differentiation as T-BET suppresses EOMES expression and inhibits NK cell development, favouring non-NK ILC1 development⁵⁵. However, it is not known which factors specifically mark ILC1 progenitors. Forkhead box protein O1 (FOXO1) is another transcription factor that negatively regulates NK cell activity^{56, 57}. FOXO1 expression has been identified to be inhibit NK cell maturation by regulating T-BET expression as well as control NK cell homing and effector functions⁵⁶. Specifically, FOXO1 inhibits NK cell activation when challenged in a tumour context and this inhibition is removed by phosphorylation of the FOXO1 protein⁵⁶.

1.5.3 ILC2

GATA binding protein 3 (GATA3) is a double zinc finger transcription factor that regulates the development of all ILC subsets⁵⁸ but is particularly vital for the differentiation of progenitors in the bone marrow to the ILC2 lineage and is required for the maintenance of mature ILC2s²⁰. GATA3 also regulates cytokine production through the expression of IL-5 and IL-13 genes and expression of the IL-33 receptor, ST2, on ILC2s⁵⁹. Retinoic acid receptor related orphan receptor (ROR) α is another transcription factor that is involved in ILC2 development^{60, 61}. Loss of ROR α expression results in a decrease in ILC2p in the bone marrow⁶¹. This is also reflected in peripheral tissues with an inability of ILC2 to expand in response to IL-25 stimulation^{60, 61}. Other transcription factors such as BCL11B and TCF1 also regulate ILC2 development and this occurs by regulating GATA3 expression^{51, 52 62}.

1.5.4 ILC3

All ILC3 subsets rely on the nuclear hormone receptor ROR γ t to develop. Loss of expression results in a loss of all ILC3 subsets, including lymphoid tissue-inducer (LTi) cells which is central to the recruitment of lymphocytes, formation of organised lymphoid tissues and isolated lymphoid follicles in the intestine^{11, 63, 64}. ROR γ t directly regulates expression of key ILC3

cytokines IL-17 and IL-22 by binding to the target gene and regulating transcription⁶⁵. In addition of ROR γ t development of the NCR⁺ ILC3 subset also relies on transcription factors T-BET *via* the activation of Notch2⁶⁶ and TCF1 which acts downstream of T-BET and Notch signalling⁵².

1.6 GFI1 and GFI1B

Transcription factors GFI1 and GFI1B regulate the development of hematopoietic lineages⁶⁷. Differential expression of these two transcription factors at the hematopoietic stem cell (HSC) stage of development determines the developmental trajectory of lymphoid and myeloid cells against erythrocytes and megakaryocytes⁶⁸⁻⁷². However, their role in ILC development has not been examined in detail and thus we do not have a complete understanding of their contribution to ILC development.

1.6.1 Structure and function of the domains in GFI1 and GFI1B

GFI1 and GFI1B belong to the GFI1 subfamily of the SNAG (Snail GFI1) family, a group of transcriptional repressors containing a SNAG domain, an intermediate region and a zinc finger domain (**Fig 1.2a**)⁷³. The SNAG domain comprises 20 amino acids at the N terminal region of the protein and contains a nuclear localization motif that is functionally important for the transcriptional function of GFI1⁷⁴. The discovery of a point mutation in the SNAG domain revealed that this change is important for the repressor activity of GFI1, while localization, accumulation or DNA binding activity of the protein were unaffected^{74, 75}. The zinc finger domain at the C terminal region of the protein consists of six zinc fingers (**Fig 1.2a**) which interacts with the specific DNA motif AATC^{73, 74}. Specifically, zinc finger 3, 4 and 5 were vital to the ability of GFI1 to bind to DNA and deletions of this region also affected GFI1's ability to mediate repression, indicating that GFI1 must bind to the DNA in order to mediate repression^{73, 74}. The intermediate domain, the region that spans between the SNAG domain and zinc finger domain. This region is where the majority of the structural difference between GFI1 and GFI1B occurs (**Fig 1.2a**). The SNAG and zinc finger domain shares around 90% similarity between GFI1 and GFI1B while the intermediate region is only 39% similar⁷⁶. The specific function of this intermediate domain is not yet fully understood. It may be involved in protein-protein interactions or it may provide flexibility for the protein to assist with interactions or it is responsible for some other function that is yet to be identified⁷⁷.

The large degree of structural homology between these two proteins implies that there is a degree of redundancy between them during haematopoiesis⁷⁸. The SNAG domain between GFI1 and GFI1B was functionally similar and could be interchanged and still regulate T cell development in the thymus⁷⁸. However, the function of GFI1 and GFI1B also diverges and is non-redundant during the development of other cell types. In the absence of GFI1B, mice are embryonic lethal and do not survive past E15 due to the lack of mature erythrocytes and megakaryocytes^{72, 79}. This phenotype is not seen when GFI1 is deleted. On the other hand, GFI1-deficient mice are deaf due to the role of GFI1 in the development of inner ear hair cells and GFI1B could not compensate for the loss of GFI1 and hearing could not be restored⁷⁸. It is not known how this functional specificity of GFI1 and GFI1B is maintained given that both proteins are structurally similar.

1.6.2 GFI1 interacts with histone modifiers to mediate repression

GFI1 epigenetically regulates transcription through the recruitment of histone modifiers to the target gene to form a protein complex. It has been proposed that the protein complex formation is initiated by GFI1 binding to the DNA through the zinc finger domain, co-factors then interact with either the SNAG domain⁸⁰ or another region of the protein^{81, 82} in a stepwise manner to alter the chromatin structure to facilitate or inhibit transcription (**Fig 1.2b**).

LSD1/CoRest

LSD1 (lysine specific demethylase 1) and CoREST (REST co-repressor) have been shown to interact with the SNAG domain of both GFI1 and GFI1B through immunoprecipitation experiments⁸⁰. LSD1 is a histone demethylase that removes methyl groups from the lysine residue of H3K4 or H3K9⁸³⁻⁸⁷. This modifies the chromatin into either an open configuration to facilitate transcription or a closed configuration to inhibit transcription. CoREST is often required to facilitate this by enhancing the interaction between LSD1 and the nucleosome^{83, 84}. Chromatin immunoprecipitation (ChIP) studies in murine erythroleukemia cells reveal that GFI1B, LSD1 and CoREST all bind the same promoter region of a large percentage of the genes, suggesting that these proteins may form a complex at the site of GFI1B's target genes to mediate function together⁸⁰.

G9a/HDAC

Co-factors can also be recruited to GFI1 target genes independent of the SNAG domain⁸¹. Both G9a and HDAC1 (histone deacetylase 1) require the intermediate region to interact with GFI1⁸¹. G9a, a histone lysine methyltransferase, has been shown to methylate H3K9⁸⁸ and subsequently repress transcription of GFI1 target genes⁸¹. HDAC1 is a histone deacetylase that mediates transcriptional repression through the removal of acetyl groups from histones, thereby inducing a closed chromatin state⁸⁹ and GFI1-mediated repression has been shown to be HDAC1 dependent in a cervical cancer cell line⁹⁰.

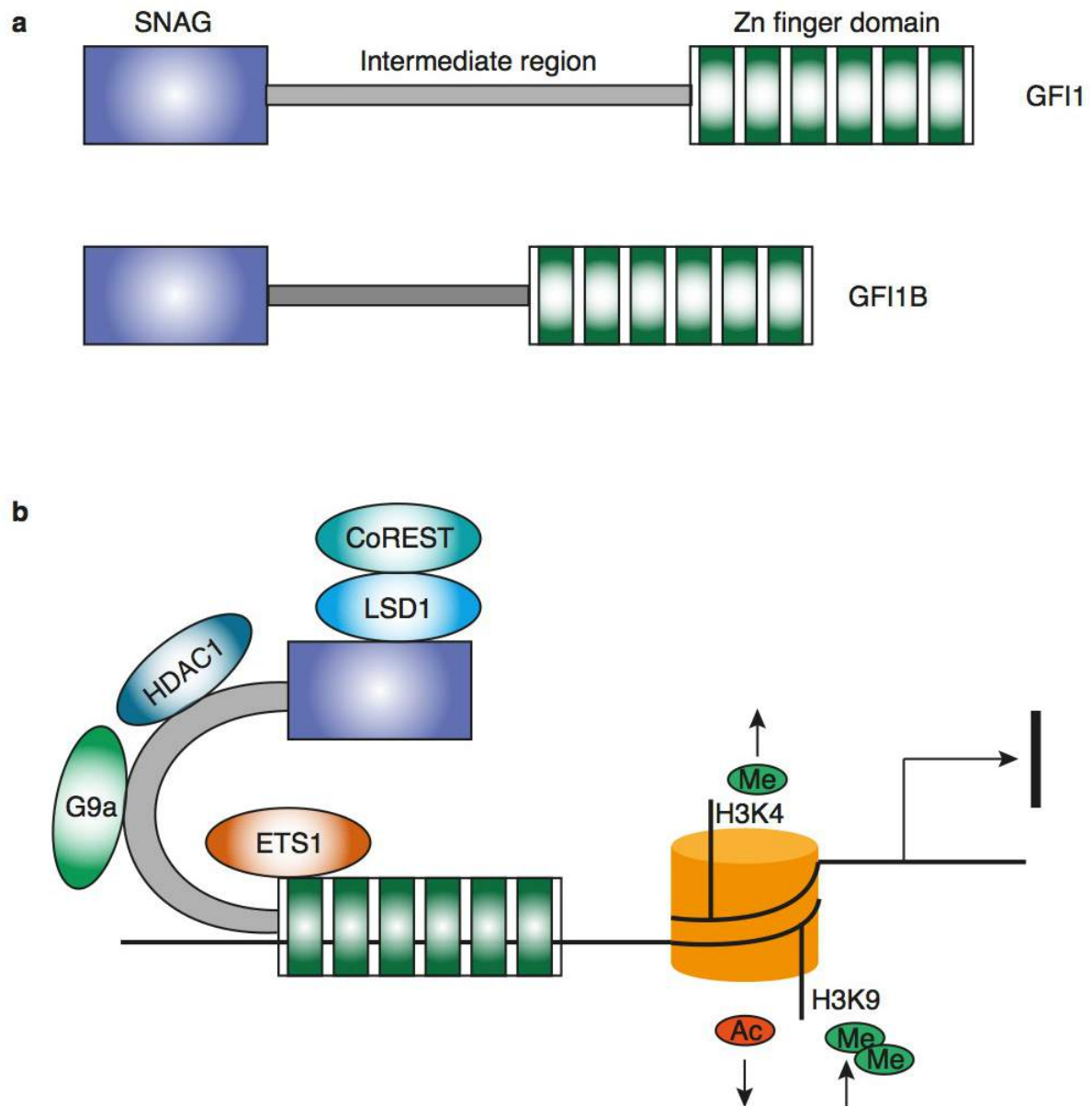


Figure 1.2 GFI1 and GFI1B binds to DNA and modifies chromatin to regulate transcription.

a, The SNAG domain and the six zinc fingers that make up the zinc finger domain are highly homologous between GFI1 and GFI1B. The main difference exists at the intermediate domain.

b, After binding to the DNA of the target gene, GFI1 recruits co-factors to the target gene and mediates histone modifications through the addition or removal of acetyl and methyl groups on H3K4 or H3K9 to mediate transcriptional repression.

1.6.3 Role of GFI1 and GFI1B in hematopoietic cells

Both GFI1 and GFI1B act as transcriptional repressors during haematopoiesis and play complementary roles to regulate the cell fate of HSC⁶⁷ (**Fig. 1.3**). However, as cells become committed to a particular hematopoietic lineage, GFI1 and GFI1B becomes differentially expressed. Both GFI1 and GFI1B are expressed by HSC but they regulate each other's expression^{79, 91, 92}. Loss of GFI1B results in an increase in GFI1 expression in HSC and an expansion of HSC⁹¹. GFI1 is also involved in regulating HSC maintenance and proliferation and the cross regulation of both GFI1 and GFI1B is required to regulate HSC numbers and functional integrity⁹¹⁻⁹³.

GFI1 and GFI1B expression also diverges between the subsequent developmental pathways of HSC. GFI1B is upregulated in erythroid and megakaryocyte lineages but is downregulated in myeloid and lymphoid lineages^{70, 79}. Conversely, GFI1 expression is upregulated in myeloid and lymphoid precursor cells⁹⁴. Germline deletion of GFI1B is embryonic lethal with mice dying at E15 due to defects in erythropoiesis development⁷². Specifically, GFI1B is needed for the development and maturation of erythrocytes^{72, 95}. GFI1B is also involved in regulating the development of megakaryocytes as the loss of GFI1B resulted in an increase in megakaryocyte numbers. However, these megakaryocytes fail to produce platelets^{96, 97}. In granulocyte-myeloid progenitors, GFI1 drives differentiation of neutrophils and DCs while inhibiting the alternative macrophage fate^{98, 99}. GFI1-deficient mice lack mature neutrophils and DCs and these cells fail to differentiate *in vitro* from progenitor cells isolated from GFI1-deficient mice, which indicated a developmental block^{69, 98}. Instead, there is an expansion of abnormal myeloid cells with characteristics of both immature granulocytes and macrophages^{69, 98}.

1.6.4 Role of GFI1 and GFI1B in lymphoid cells

Lymphoid cells primarily express GFI1 throughout their development, although GFI1B expression was also reported in CLP but was absent in mature lymphocytes^{79, 94} (**Fig. 1.3**). The role of these transcription factors in early lymphocyte development is not well understood. The role of GFI1B in CLP has yet to be examined. While mice with germline deletion of GFI1 have reduced CLP numbers, it is unclear whether CLP have a cell intrinsic requirement for GFI1 as HSC depend on GFI1 for maintenance^{92, 93}. In addition, it is not understood if GFI1 or GFI1B plays any role in the development of the subsequent progenitors and mature ILCs in the peripheral organs.

GFI1 is required for the proper development of T cells in the thymus as there is a defect in the development of DN2 T cells when *Gfil* is germline deleted^{100, 101}. The developmental defect from loss of GFI1 only affects the DN subset as targeted deletion of GFI1 in the DP subset using the CD4Cre system¹⁰², results in normal size and cell number within the thymus¹⁰⁰. When GFI1 was conditionally deleted using CD4^{Cre}, there was a reduction in both CD4⁺ and CD8⁺ T cells in the thymus and spleen, with CD4⁺ T cells disproportionately affected compared to CD8⁺ T cells^{100, 103}. The remaining T cells had altered gene expression in pathways regulating in programmed cell death and lymphocyte activation¹⁰³. While the frequency of naïve and effector CD4⁺ T cells did not change, it is unknown whether loss of GFI1 influences the proportions of naïve, effector and memory CD8⁺ T cells¹⁰⁴.

1.6.5 Role of GFI1 in Th cell differentiation

GFI1 influences the polarization of Th cells and maintains lineage stability through the regulation of key transcription factors and cytokines¹⁰⁵⁻¹⁰⁷. This is mediated epigenetically through the recruitment of histone modifiers to the gene loci of lineage defining transcription factors and cytokines to regulate expression¹⁰⁵. Th1 differentiation is increased in the absence of GFI1¹⁰⁵ and is mediated through the histone modifier LSD1, which binds to key transcription factors involved in Th1 differentiation such as T-BET and EOMES. There is increased H3K4 methylation at these gene loci when GFI1 or LSD1 is lost¹⁰⁵. GFI1-deficient Th1 cells also secrete more IFN- γ and TNF- α compared to wild-type Th1 cells¹⁰⁵. This is associated with an increase in H3K9/14 acetylation at the *Ifn γ* gene locus¹⁰⁶. These histone markers are associated with an open chromatin configuration which allow for transcription to occur¹⁰⁸. This suggests that GFI1 suppresses transcription at the locus of key Th1 genes by modifying the chromatin structure. GFI1 also inhibits IL-17 production in Th17 cells by preventing ROR γ t binding to *Il17a* promoter region without directly binding to *Il17a* promoter itself^{107, 109}

GFI1 maintains Th2 lineage stability by regulating GATA3 protein levels post-transcriptionally¹⁰⁶. GATA3 is ubiquitinated and degraded by 26S proteasome and GFI1 blocks this process¹⁰⁶. The SNAG domain of GFI1 is required for the stabilization of GATA3 protein but the specific mechanism is unknown as it is not dependent on the transcriptional repression function of the SNAG domain¹⁰⁶. GFI1 regulates cytokine production in Th2 cells through chromatin modifications. GFI1 increases H3K4 methylation of the IL-5 promoter. This creates

an active chromatin state at the IL-5 gene locus in Th2 cells^{100, 106}. GFI1 is also important for Th2 expansion and survival as it is required for effective STAT5 phosphorylation during IL-2 driven proliferation¹⁰⁰.

1.6.6 Role of GFI1 and GFI1B in ILC

A limited number of studies have investigated the role of the GFI1 family in ILCs. Indeed, only the GFI1 has been examined in ILC2s in detail¹¹⁰. GFI1 expression in ILC2 is very high and this transcription factor has been implicated in (i) the development of ILC2 from the earliest ILC progenitors, (ii) cytokine production by ILC2 and (iii) orchestrating the transcriptional program that maintains ILC2 identity¹¹⁰ (**Fig. 1.3**). The expression and function of GFI1 and GFI1B in other ILC subsets have not yet been reported.

As ILC2 develops from CLP to ILC2p to mature ILC2, GFI1 expression progressively increases. When GFI1 is deleted in all haematopoietic cells, mice fail to develop ILC2p or mature ILC2¹¹⁰. This finding established the crucial importance of GFI1 in the development of ILC2s but this study did not determine which stage(s) of development GFI1 was required. It is plausible that GFI1 might be required at specific developmental stages, or alternately, ILC2 may depend on the continuous expression of GFI1 for the homeostatic maintenance of the lineage. Furthermore, the loss of GFI1 results in reduced numbers of ILC2 in the lung at steady-state but this reduction was not seen in the mLN¹¹⁰. The origin of these ILC2s in the mLN is unknown but it may represent an alternative pathway of ILC2 development.

GFI1 is also required to maintain ILC2 lineage stability by associating with the gene locus of key ILC2 genes such as *Gata3* (which encodes the transcription factor GATA3), *Il5* (cytokine IL-5) and *Il1rl1* (IL-33 receptor, ST2), as well as ILC3-related genes such as *Il17*¹¹⁰. Loss of GFI1 results in the downregulation of ILC2 genes and upregulation of ILC3 related genes and subsequently, GFI1^{-/-} ILC2s become capable of producing IL-17, a cytokine usually restricted to ILC3s¹¹⁰. Collectively, these findings indicate that GFI1 is capable of both promoting and inhibiting gene expression depending on context and the target genes. However, the role of GFI1 in ILC3s has not been examined. In addition to regulating *Gata3* transcript levels, GFI1 may also maintain GATA3 protein stability by preventing proteasome degradation of GATA3, similar to Th2 cells¹⁰⁶. This may provide another mechanism by which GFI1 can maintain GATA3 levels.

Many co-factors involved in regulating ILC2 transcription have also been found to interact with GFI1^{111, 112}. Thus, one possible mechanism of GFI1 regulation of ILC2 may be through the recruitment of various epigenetic co-factors, although this feature has not been directly investigated. These co-factors include G9a and ETS1, both of which have been shown to play a critical role in ILC2 differentiation^{111, 112}. G9a regulates the development of ILC2p in the bone marrow together with the maintenance of the number of ILC2. This is in part due to epigenetic inhibition of genes normally expressed by ILC3 in ILC2¹¹². As G9a is able to recapitulate many of the phenotypes seen when GFI1 was deleted in ILC2, this suggests that GFI1 may recruit G9a to the promoters of genes such as *Rorc* which encodes the transcription factor ROR γ t, and *Il17f* which encodes the cytokine IL-17F and are associated with ILC3 development and function. In this way, GFI1 exerts transcriptional regulation¹¹². ETS1 is another transcription factor that interacts with the zinc finger domain of GFI1 and together, ETS1 and GFI1 co-localise on the promoter region of the target gene to regulate transcription⁸². ETS1 is known to play a role in both NK cell development¹¹³ and ILC2 development¹¹¹ but it is not known if this occurs through GFI1 regulation.

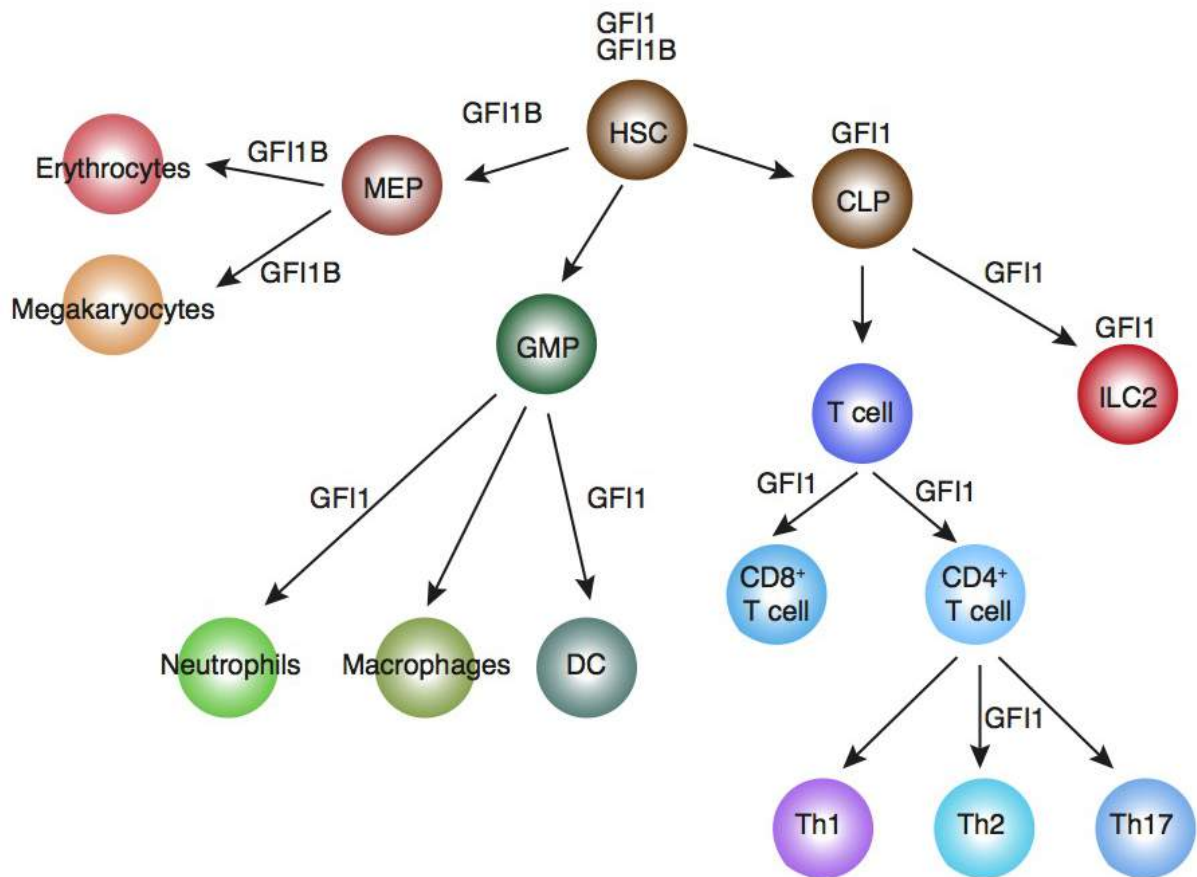


Figure 1.3 GFI1 and GFI1B regulate the development of hematopoietic cells.

GFI1 and GFI1B regulates the development of hematopoietic cells at many stages. GFI1B is primarily involved in erythrocyte and megakaryocyte development, whereas GFI1 is involved in myeloid and lymphoid cell development. HSC hematopoietic stem cell, MEP Megakaryocyte-erythrocyte progenitor, GMP granulocyte-monocyte progenitor, CLP common lymphoid progenitor, Th T helper.

1.7 Thesis aims

Many transcription factors are involved in regulating ILC development and their immune response towards a pathogen. This regulation is important for establishing a diverse immune repertoire and mediating efficient protection of the host.

Transcription factors GFI1 and GFI1B have been previously reported to be involved in multiple stages of hematopoietic cell development including the lymphoid lineage. However, it is unclear at what stage they are involved in mediating ILC development, nor is understood their function in regulating ILC development and function. Finally, it is not known whether their dysregulation would result in an altered immune response when undergoing immune challenge during an infection or development of cancer.

ILCs, particularly ILC3s, have also been implicated in development of specific cancers, including CRC, as they are enriched within the gastrointestinal tract and have been found to accumulate in colitis patients. However, it is not known whether the other ILC subsets play an active role in driving CRC progression.

Therefore, the aims of this thesis were to:

1. Map the expression of GFI1 and GFI1B during ILC development and in the specific ILC subsets.
2. Determine the role of GFI1 and GFI1B in the development of ILC progenitors and individual ILC subsets in the peripheral organs
3. Determine the functional impact of dysregulation of these transcription factors during an immune response.
4. Characterise the immune composition within the colon of mice with CRC.
5. Determine whether ILCs and their associated cytokines are involved in tumorigenesis.

Chapter 2. Materials and Methods

2.1 Media and Solutions

The solutions below were prepared and sterilized by the Walter and Eliza Hall Institute of Medical Research Media Department and supplemented as described.

2.1.1 Media

Hanks Free Balanced Salt Solution (without Calcium and without Magnesium) – pH 7.1 containing 5.4 mM KCl, 0.44mM KH₂PO₄, 136.9mM NaCl, 4.17mM NaHCO₃, 0.338mM Na₂HPO₄·2H₂O, 5.55mM C₆H₁₂O₆ and 0.002% Phenol Red in type II water.

Mouse Tonicity Roswell Memorial Institute (MT RPMI) 1640 – pH 7.15 containing 1 packet of RPMI-1640 powder (Gibco), 23.8mM NaHCO₃, 15.4mM NaCl, 1mM C₃H₃NaO₃, 1x Penicillin-Streptomycin Solution (Sigma Aldrich) in type II water.

Ethylenediaminetetra-acetic acid (EDTA) – pH 7.2 containing 100nM EDTA disodium salt powder (Univar) in type II water.

Red Cell Removal Buffer (RCRB) – pH 7.3 containing 156mM NH₄Cl, 11.9mM NaHCO₃ and 0.097mM EDTA in type II water.

2.1.2 Solutions

Fluorescence-activated cell sorting (FACS) buffer – 2% (v/v) heat-inactivated foetal calf serum (HIFCS) and 0.01% sodium azide in DPBS

Dissociation buffer – 2% (v/v) HIFCS and 5mM EDTA in Hanks Free Balanced Salt Solution (without Calcium and without Magnesium)

Digestion buffer – 2% (v/v) HIFCS, 1mg/mL (w/v) collagenase type III or IV (Worthington), 200µg/mL Dispase (Gibco) and 50µg/mL DNase I (Roche)

MACS Buffer – 2mM EDTA and 2% (v/v) HIFCS in DPBS

Sort collection media – 50% (v/v) HIFCS in DPBS

Supplemented (SC) RPMI – 23.83mg/mL HEPES (Gibco), 6mg/mL L-glutamate, 1.2mg/mL penicillin G (Sigma Aldrich), and 2mg/mL streptomycin (Sigma Aldrich), 1M 2β-Mercaptoethanol (Sigma Aldrich) in MT RPMI-1640.

Complete RPMI – 10% (v/v) HIFCS and 5% (v/v) SC RPMI in MT RPMI-1640

2.2 Animals

Mice were bred and maintained in a specific pathogen free facility at WEHI. All mice were treated or euthanised at 6-12 weeks of age. All animal experiments were performed in accordance with the National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Scientific Purposes guidelines and were approved by the Animal Ethics Committee of the Walter and Eliza Hall Institute of Medical Research (Ethics 2015.003 and 2017.037). Several strains of genetically modified mice were used and are described below.

Table 2.1 Mouse strain names and description

Mouse strain	Reference	Description
<i>Gfi1</i> ^{+/tdTomato}	114-116	BAC (bacterial artificial chromosome) clone containing ~148 kb of the <i>Gfi1</i> locus was modified by homologous recombination to contain the H2B- (Td) Tomato cDNA downstream of the <i>Gfi1</i> start codon, therefore H2B- (Td) Tomato reporter protein is produced instead of GFI1. <i>Gfi1</i> ^{+/tdTomato} mice were backcrossed for at least 10 generations to the C57BL/6 background. Mice were phenotyped by flow cytometry using the blood.
<i>Gfi1b</i> ^{+/GFP}	94, 116	Exon 2 to exon 7 of the <i>Gfi1b</i> gene is replaced with the <i>GFP</i> gene through homologous recombination. <i>Gfi1b</i> ^{GFP/GFP} homozygous mice are deficient for GFI1B and are embryonic lethal. <i>Gfi1b</i> ^{+/GFP} mice were backcrossed for at least 10 generations to the C57BL/6 background. Mice were phenotyped by flow cytometry using the blood.
<i>Gfi1</i> ^{fl/fl}	100	Cre-mediated recombination removes exons 4-5, deleting the DNA-binding domain, subsequently preventing GFI1 from

		binding to DNA and mediating function. Mice were genotyped via PCR using the following primers: F1F-CAGTCCGTGACCCTCCAGCAT F2R-CTGGGAGTGCACCTGCCTTGTGTT The product length of the <i>wt</i> allele is 210bp, 340bp for the <i>floxed</i> allele. Deletion is determined via PCR using the following primers: F1F-CAGTCCGTGACCCTCCAGCAT F3R-CCATCTCTCCTTGTGCTTAAGAT The product length of the deleted allele is 250bp.
<i>Gf1b^{fl/fl}</i>	91	Cre-mediated recombination removes exons 2-4, deleting the SNAG domain. Mice were genotyped via PCR using the following primers: LP5-3s GGTTCCTACCAGTCTGGCCCTGAACTC LP3-3r CTCACCTCTCTGTGGCAGTTTCCTATC LP5-4r TACATTCATGCTTAGAACTTGAGTC The product length of the <i>wt</i> allele is 256bp, 295bp for the <i>floxed</i> allele, and 540bp for the deleted allele.
<i>NCR^{+/iCre}</i>	117	Improved Cre (iCre) recombinase inserted at the 3' end of the <i>Ncr</i> (NKp46) gene. Cre-mediated recombination and conditional deletion of floxed gene in cells expressing NKp46. Mice were genotyped via PCR using the following primers: F_NKP46 GGAAGTGAAGGCAACTCCTG R KI_NKP46 CCCTAGGAATGCTCGTCAAG R WT1_NKP46 TTCCCGGCAACATAAAATAAA The product length of the <i>wt</i> allele is 300bp and 247bp for the knock-in allele.
IL-5TgN	118	IL-5 is overexpressed as IL-5 transcription is under the control of the dominant control region of the <i>hCD2</i> gene, a T cell surface antigen. IL-5 is constitutively expressed in T cells and significantly higher concentrations of IL-5 are found in the serum of these mice.

IL-5 ^{Venus}	119	The <i>Venus</i> reporter gene is knocked in at the <i>Il5</i> gene locus, thereby disrupting IL-5 expression so homozygous reporter mice are IL-5 deficient. The mice are on a BALB/c background.
<i>RORα^{fl/fl}</i>	120	Cre-mediated recombination removes exons 3, creating a frame shift that subsequently disrupts RORα expression. Mice were genotyped via PCR using the following primers: 5'-TGAGTGGTAACACCACGGCACGC-3' 5'-TGGAGCAGAATCATCCAGGAGGCC-3' 5'-CAACGGGTTCTTCTGTAGTCC-3' The product length of the <i>wt</i> allele is 573bp and ~650bp for the <i>floxed</i> allele.
<i>IL7R^{Cre}</i>	121	iCre was inserted into the first exon of the <i>Il7r</i> gene locus. Cre-mediated recombination and conditional deletion of floxed gene in cells expressing IL-7R. Mice were genotyped via PCR using the following primers: IL-R1 CCTGAAACTTTGCCCCCTCCATA IL-R2 CCATAGAATAGTGCAGCCTTGCCTC IL-R3 AGCGAAAGCTCTACCCAGAGC The product length of the <i>wt</i> allele is 600bp and 700bp for the knock-in allele.
<i>Rag2^{-/-}γC^{-/-}</i>	122	The coding regions of <i>Rag2</i> and exons 4 to 8 and part of exon 3 of the common gamma chain gene was deleted, resulting in a lack of T cells, B cells, and ILCs. Mice were phenotyped by flow cytometry using the blood identified by the lack of B220 and TCRβ staining.

2.2.1 Generation of Bone marrow Chimeras

Host mice with the congenic marker CD45.1 were lethally irradiated (two doses of 550 rad). Bone marrow cells, from *Gfi1^{fl/fl}NCR^{iCre}* and CD45.1⁺CD45.2⁺ *NCR^{iCre}* control mice (2.5 x10⁶ mixed at a 1:1 ratio) was intravenously injected into each host mouse and allowed to reconstitute. The efficiency of reconstitution of the BM donors were determined 5-6 weeks post irradiation *via* bleeds.

2.2.2 Adoptive Transfer

Bone marrow from *Gfi1*^{+/Tomato} *Gfi1b*^{+/GFP} mice from the sternum and hip bones was crushed with a mortar and pestle and cells from the tibia and femur were flushed using 3mL sterile DPBS. Cells were then filtered through a 70µm cell strainer. BM progenitors were first labelled the cells with IL-7R-biotin antibody and then positively selected using Anti-Biotin MicroBeads to recover IL-7R/biotin complexed cells (Miltenyi Biotec). Cells positive for various lineage markers were excluded using antibodies for TCRβ, CD3ε, CD19, B220, NK1.1, NKp46, CD11b and Ter119. Common lymphoid progenitors (CLP, Lin⁻IL-7Rα⁺Flt3⁺α4β7⁻c-kit⁺) and common innate lymphoid cell progenitor (CILP, Lin⁻IL-7Rα⁺Flt3⁺α4β7⁺c-kit⁺CD25⁻) cells were sorted based on GFP expression and between 600-700 purified progenitor cells were intravenously injected into each sub-lethally irradiated (one dose of 450rad) host *Rag2*^{-/-} *γC*^{-/-} mouse. Recipient mice were analysed 6 weeks after transfer.

2.3 Isolation of Lymphocytes

2.3.1 Liver, Spleen and Lymph nodes

Single cell suspensions were generated by mashing the organs through 70µm cell strainer. Liver lymphocytes were isolated by using a 33% isotonic Percoll (GE Healthcare) gradient, centrifuged at 2500 rpm for 20 min at room temperature. Red blood cells were lysed from the spleen and liver using RCRB.

2.3.2 Intestinal intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL)

Intestinal lymphocytes were isolated from either the small or large intestine by incubation for 45 min at 37°C in dissociation buffer (see 2.1.2 Solutions) with gentle shaking for removal of intestinal epithelial cells. Peyer's patches were removed prior to dissociation of the small intestines. Supernatant containing the IEL was filtered from the tissue. The tissue was then digested for 45 min at 37°C in Digestion buffer (see 2.1.2 Solutions) with gentle shaking. Supernatant containing the LPL filtered and mononuclear cells from the IEL or LPL were isolated between the interphase of a 40%-80% (v/v) Percoll gradient after centrifugation at 2500 rpm for 20 min at room temperature.

2.3.3 Lung and Salivary glands

Lungs were perfused with PBS to remove blood from the organ. Lung and salivary glands were cut using scissors and digested for 45- 60 mins at 37°C in digestion buffer (see 2.1.2 Solutions) with gentle shaking. The digested lung and salivary gland tissue were then mashed and filtered through 70µm cell strainer. Cells were resuspended in 40% (v/v) Percoll then underlaid with 80% Percoll for lung or 70% (v/v) for salivary glands. Preparations were centrifuged at 2500 rpm for 20 min at room temperature with the brake off. Lymphocytes were isolated at the interphase of a 40%-80% (v/v) or 40%-70% (v/v) Percoll gradient for lung and salivary glands respectively, then washed twice with PBS.

2.3.4 Bone marrow

Bone marrow was flushed from the tibia and femur with 3mL of DPBS and filtered through a 70µm cell strainer. Red blood cells were lysed using RCRB.

2.4 Antibodies and Flow cytometry

Flow cytometry was used to enumerate the cell number and to determine the expression of surface markers and intracellular transcription factors following staining with fluorescently labelled antibodies.

Surface receptors: For analysis of surface molecules, cells were stained with the following antibodies: c-kit (2B8), NKp46 (29A1.4), ST2 (RMST2-2), TCRβ (H57-597), IL-7Rα (A7R34 or SB/199), CD25 (PC61.5), CD3e (145-2C11), Sca-1 (D7), KLRG1 (2F1), CD45.2 (104), CD45 (30-F11), NK1.1 (PK136), CD49a (Ha31/8), CD49b (DX5), CD11b (M1/70), CD122, (TM-b1), α4β7 (DATK32), FLT3 (A2F10), PD-1 (J43), CD27 (LG.7F9 or LG.3A10), CD19 (1D3), Trail (N2B2), CD69 (H1.2F3), CD1d-tetramer, CD62L (MEL-14), F4/80 (BN8), major histocompatibility complex (MHC) II (M5/114.15.2), CD44 (IM7), CD4 (GK1.5), CD8 (53-6.7). The lineage marker mix included antibodies against TCRβ, CD3ε, CD19, B220, NK1.1, NKp46, CD11b and Ter119. Cells were stained on ice for 30 minutes then washed with FACS buffer. Cells were centrifuged at 1500 rpm for 5 minutes at 4°C and the supernatant was discarded.

Apoptosis: For apoptosis analysis, cells were stained with Annexin V (Invitrogen) in KDS BBS buffer with 2mM EDTA and 2% FCS at room temperature for 15 minutes.

Transcription factors: For transcription factor staining, cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and the following antibodies were used: EOMES (Dan1/mg), TCF1 (C63D9), ROR γ t (Q31-378), T-BET (eBio4B10). Cells were stained on ice for 30 minutes then washed with permeabilization buffer (according to the manufacturer's instructions). Cells were centrifuged at 1700 rpm for 7 minutes at 4°C and the supernatant was removed, then discarded.

Cytokines: For cytokine staining, cells were stimulated in RPMI 1640, 5% supplemented (SC) RPMI and 10% HIFCS, with 100ng/mL PMA, 100ng/mL ionomycin, 10ng/mL Golgi plug and 10ng/mL Golgi stop (BD Bioscience) for 4 hours and stained with IFN- γ (XMG1.2) and TNF- α (Mab11).

Cell viability: Live and dead cells were discriminated by a Fixable Viability Dye (BD Bioscience or BioLegend), propidium iodide (PI) or Hydroxystilbamidine, methanesulfonate (Invitrogen).

Phosphorylation markers: For phosphorylation staining, cells were stimulated in hIL-15 (50ng/ml) in RPMI 1640 with 5% supplemented (SC) RPMI and 10% HIFCS for 30 mins and subsequently fixed and permeabilised with Perm Buffer III (BD Phosflow). Cells were then stained with pSTAT5 (pY694).

Acquisition and Analysis: Flow cytometry was performed using a LSRFortessa X-20 and FlowJo analysis software (FlowJo LLC).

2.5 Cell Culture

2.5.1 *In vitro* CTV labelled proliferation

Splenic NK cells were first enriched for using EasySep Mouse NK Isolation Kit (STEMCELL Technologies), then labelled with CellTrace Violet (CTV) (Invitrogen). Immature (Imm, CD11b⁻ KLRG1⁻), M1 (CD11b⁺ KLRG1⁻) and M2 (CD11b⁺ KLRG1⁺) subsets were cell sorted

on the Aria III and cultured in Complete RPMI (10% FCS and 5% SC RPMI) with 50ng/mL of hIL-15. Cells were analysed at day 0, 3 and 5 of culturing.

2.6 Molecular Biology

2.6.1 Preparation and RNA Sequencing

NK cells and ILC1 from the spleen and liver of *Gfi1^{fl/fl} NCR^{iCre}* and *NCR^{iCre}* control mice was enriched for using EasySep Mouse NK Isolation Kit (STEMCELL Technologies). Imm, M1, M2 and ILC1 were stained for using antibodies against NK1.1, NKp46, CD49b, CD49a, CD11b and KLRG1 and sorted by flow cytometry using Aria III (BD Biosciences) into either DPBS with 50% (v/v) HIFCS or RNAlater (Invitrogen). Cells were washed and lysed using the RLT Plus lysis buffer (Qiagen) with 2M Dithiothreitol (DTT, Invitrogen) and stored at -80°C. Samples were later processed using the RNeasy Micro Plus kit (Qiagen) as per the manufacturer's protocol. Library preparation was performed using TruSeq RNA Library Prep Kit v2 (Illumina). Three biological replicates of each sample were prepared and sequenced. Samples were sequenced on an Illumina NextSeq 500 (Illumina) generating 75bp paired-end reads, 20 million reads per sample.

2.6.2 RNA Sequencing Analyses

Analysis was done with the assistance of Alexandra Garnham. All samples were aligned to build mm10 of the mouse genome using Rsubread's (v1.32.0)¹²³ align function. In all cases approximately 89.5% of fragments (read pairs) uniquely mapped to the genome. Gene counts were then obtained using Rsubread's featureCounts function. Genes were identified using RefSeq annotation to the mm10 genome provided by Rsubread. Differential expression analyses between the experimental groups were then undertaken using the edgeR (v3.26.5)¹²⁴ and limma (v3.40.2)¹²⁵ software packages.

Prior to analysis all ribosomal RNAs (rRNAs) were removed together with genes with no current symbol. Expression based filtering was then performed using edgeR's filterByExpr function with default parameters. Libraries were then normalized using the TMM method¹²⁶. Following filtering and normalization the data was transformed to log₂-counts per million reads (log₂CPM). Differential expression between groups was assessed relative to a fold -change threshold of 1.1 using linear models and robust empirical bayes moderated t-statistics with a

trended prior variance (limma-trend pipeline)¹²⁷⁻¹²⁹. To increase precision the linear models incorporated an adjustment for sample replicate number. P-values were adjusted to control the false discovery rate (FDR) below 5% using the Benjamini and Hochberg method.

Over-representation analyses of the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases for differentially expressed genes were performed using limma's goana and kegg functions respectively. Gene set enrichment analyses used the roast method¹³⁰ implemented in limma with 9,999 rotations. Barcode plots illustrating this enrichment were drawn using limma's barcodeplot function.

The Multi-Dimensional Scaling (MDS) and Mean-Difference (MD) plots were drawn using limma's plotMDS and plotMD functions respectively. Heatmaps were generated using the pheatmap software package (v1.0.12). Prior to rendering all heatmaps and MDS plots, the replicate batch effect was adjusted for using limma's removeBatchEffect function.

2.7 Immune challenge

2.7.1 B16F10 Metastatic Melanoma Model

B16F10 murine melanoma cells¹³¹ were maintained in complete RPMI, containing 10% (v/v) HIFCS and 5% (v/v) SC RPMI. Each recipient mice received 2.5×10^5 B16F10 cells injected via the tail vein. Injections were performed by animal technicians of the WEHI mouse facility. Fourteen days after injection the lungs and liver were harvested and fixed in 10% neutral buffered formalin. B16F10 metastases were counted using a dissecting microscope.

2.7.2 Papain-induced lung inflammation

Mice were treated intranasally with PBS or 20 μ l of papain (5 mg/ml diluted in PBS) on three consecutive days. On the fourth day, papain treated mice were administered CD45 antibody (5 μ l in 100 μ l PBS) *via* IV 5 minutes prior to sacrificing the mice. Bronchiole alveolar lavage fluid were collected from the lungs of the mice and the right superior lobe of the lung was fixed in 10% formalin and processed for histology (see Histology). The remaining lobes of the lung, mediastinal LN, mesenteric LN and spleen were taken for flow cytometry analysis.

2.7.3 AOM and DSS treatment

To induce colorectal cancer, azoxymethane (AOM) was intraperitoneally injected (10mg/kg, Sigma-Aldrich) into mice. One week later, the mice were given 2% (w/v) dextran sodium sulphate (DSS, Affimetrix/USB or Alfa Aesar, M.W. = 40-50kDa) drinking water ad libitum for 5 days, followed by normal drinking water without DSS. Mice were weighed daily and monitored for changes in posture, stool quality, peripheral blood flow indicated by foot-pad colour and food intake. After two weeks and once the mice have regained their weight, the mice were given another round of 2% (w/v) DSS water. A total of three rounds of 2% (w/v) DSS water were given to the mice. Tumour area was measured at autopsy.

2.7.4 Endoscopy

Tumours at the distal colon were monitored *via* endoscopy, as previously described¹³² with the assistance of Paul Nguyen, Adele Predaudet and Tracy Putoczki after the second and third round of DSS treatment. Mice were anesthetised by inhalation of 3% isoflurane in 100% O₂ (0.3 L/min), then maintained with 1% isoflurane. The miniature endoscope sheath was inserted into the rectum of mice up to 3cm. Videos from the endoscope were recorded onto a laptop using iMovie (Apple Inc.). Mice were euthanized once tumours were detected in the AOM and DSS treated group.

2.7.5 Histology

Tissues were prepared by fixing in 10% (w/v) formalin for a minimum of 12 hours, before being embedded in paraffin blocks, sectioned and stained with H&E. Embedding, sectioning and staining were performed by the WEHI Histology Facility.

2.8 Statistical Analysis

Unpaired Student's *t*-test with two-tailed distribution for two samples with equal variance was performed to determine whether the difference between the control group and experimental group had reached statistical significance. Data are shown as mean $\pm$ SEM (standard error of the mean) where indicated. SEM is calculated from three or more mice. When less than three mice are used, the individuals data points are shown and no calculations are made. Mann-Whitney test was used to determine the statistical significance of tumour number and burden between either the AOM+DSS treated and untreated group or between mice with different

genotypes. The direction and strength of the correlation between cell counts and tumour burden was determined using Pearson's correlation to calculate r . One-way ANOVA with Tukey's multiple comparison's test was used to determine the statistical difference between the frequency of IL-5R α ⁺ epithelial cells within the colon tissue and tumour of AOM+DSS treated mice and colon tissue of untreated mice. p -values of 0.05 or less were considered statistically significant. Prism (GraphPad, version 7) was used to generate graphs and perform statistical tests.

Table 2.2 Antibodies for Flow Cytometry*Surface Antibodies*

Antibodies	Clone	Fluorochrome	Source/Company	Catalogue #
c-kit (CD117)	2B8	PERCP-eFluoro710	eBioscience	46-1171-80
NKp46 (CD335)	29A1.4	PE	eBioscience	12-3351-82
NKp46 (CD335)	29A1.4	PE-Cy7	eBioscience	25-3351-82
NKp46 (CD335)	29A1.4	Alexa700	BD Pharmigen	561169
ST2 (IL-33R)	RMST2-2	APC	eBioscience	17933582
TCR β	H57-597	APC-eFluoro780	eBioscience	47-5961-82
TCR β	H57-597	PE-Cy7	BioLegend	109222
IL-7R α (CD127)	A7R34	Pac Blue	BioLegend	135024
IL-7R α (CD127)	SB/199	PE-Cy7	BD Pharmigen	560733
IL-7R α (CD127)	A7R34	Biotin	eBioscience	13-1271-85
CD3e	145-2C11	Alexa 700	Antibody Facility	NA
CD3e	145-2C11	BUV395	BD Horizon	563565
Sca-1 (Ly6A/E)	D7	V500	BD Horizon	561228
CD25	PC61.5	Pac Blue	eBioscience	48-0251-82
B220 (CD45R)	RA3	Alexa 700	Antibody Facility	NA
KLRG1	2F1	BV711	BD Horizon	564014
KLRG1	2F1	PE-Cy7	eBioscience	25-5893-82
CD45.2	104	BV786	BD Horizon	563686
CD45.2	104	Pac Blue	BioLegend	109832
CD45	30-F11	BV786	BD Pharmigen	564225
NK1.1	PK136	APC	eBioscience	17-5941-82
NK1.1	PK136	BV650	BD Horizon	564143
NK1.1	PK136	APC-Cy7	BD Pharmigen	560618
NK1.1	PK136	BV510	BD Horizon	563096
CD49a	Ha31/8	APC	BD Pharmigen	562113
CD49a	Ha31/8	BV711	BD Horizon	564863
CD49b	DX5	Pac Blue	Antibody Facility	NA
CD11b (Mac-1)	M1/70	FITC	BD Pharmigen	553310
CD11b (Mac-1)	M1/70	BV510	BD Horizon	562950

CD11b (Mac-1)	M1/70	eFluro450	BD Horizon	560455
CD11b (Mac-1)	M1/70	Alexa 700	Antibody Facility	NA
CD122	TM-b1	PERCP-eFluro 710	eBioscience	46122282
CD122	TM-b1	BV650	BD OptiBuild	740475
$\alpha 4\beta 7$	DATK32	APC	eBioscience	17588780
FLT3 (CD135)	A2F10	Biotin	eBioscience	13-1351-82
FLT3 (CD135)	A2F10	PE-CF594	BD Horizon	562537
PD-1 (CD279)	J43	BV605	BD Pharmigen	563059
CD27	LG.7F9	APC-Cy7	eBioscience	47-0271-82
CD27	LG.3A10	BV510	BioLegend	124229
CD19	ID3	BUV395	BD Horizon	563557
CD19	ID3	APC	BD Pharmigen	550992
CD19	ID3	PERCP-Cy5.5	eBioscience	45-0193-82
Trail	N2B2	PE	eBioscience	12595182
CD69	H1.2F3	Pac Blue	BD Horizon	560690
CD1d-tetramer		Pac Blue	Collaborator	Godfrey Lab
CD62L	MEL-14	Pac Blue	BD Horizon	560507
CD62L	MEL-14	APC	BD Pharmigen	553152
F4/80	BN8	APC-eFluro 780	eBioscience	47-4801-82
MHCII (I-A/I-E)	M5/114.1 5.2	BV605	BD Pharmigen	563413
MHCII (I-A/I-E)	M5/114.1 5.2	BV711	BioLegend	107643
CD44	IM7	BV786	BD Pharmigen	563736
CD4	GK1.5	BUV496	BD Horizon	564667
CD8	53-6.7	BUV737	BD Horizon	564297
Ter119		Alexa 700	Antibody Facility	NA
Ly6G	1A8	BV510	BD OptiBuild	740157
Ly6C	HK1.4	PERCP-eFluro 710	eBioscience	45-5932-82
Siglec F	E50-2440	PE	BD Pharmigen	552126
EpCAM		APC-Cy7	Collaborator	
CD31	390	PE-Cy7	BD Pharmigen	561410

Annexin V		Alexa Fluro 647	Invitrogen	A23204
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Cell Viability

Antibodies	Fluorochrome	Source/Company	Catalogue #
Hydroxystilbamidine, methanesulfonate	Fluorogold	Invitrogen	H22845
Propidium iodide		Antibody facility	
Fixable Viability Dye	eFluor506	eBioscience	65-0866-14
Fixable Viability Dye	Alexa 700	BD Horizon	564997

Intracellular Antibodies

Antibodies	Clone	Fluorochrome	Source/Company	Catalogue #
Ki-67	B56	FITC	BD Pharmingen	556026
pSTAT5	pY694	PE	BD Phosflow	612567

Transcription Factors

Antibodies	Clone	Fluorochrome	Source/Company	Catalogue #
EOMES	Dan1/mg	PERCP-eFluor 710	eBioscience	46487582
TCF1	C63D9	PE	Cell Signalling	07/2017
T-BET	eBio4B10	PERCP-Cy5.5	eBioscience	45-5825-82
T-BET	4B10	PE-Cy7	eBioscience	25-5825-82
T-BET		APC	Miltenyi Biotec	130-098-607
ROR γ t	Q31-378	Pac Blue	BD Horizon	562894
GATA3	TWAJ	PE	eBioscience	12-9966-42
GATA3	L50-823	BV711	BD Horizon	565449
GATA3	TWAJ	PERCP-eFluor 710	eBioscience	46-9966-42

Cytokines

Antibodies	Clone	Fluorochrome	Source/Company	Catalogue #
IFN- γ	XMG1.2	PE	BD Pharmingen	554412
TNF- α	Mab11	APC	BD Pharmingen	554420
IL-5	TRFK5	PE	eBioscience	12-7052-82

IL-13	eBio13A	Alexa488	eBioscience	53-7133-82
IL-17A	TC11-18H10.1	ALEXA488	BioLegend	506910
IL-22	IL22JOP	APC	eBioscience	17-7222-82

Secondary reagents for flow cytometry

Antibodies	Clone	Fluorochrome	Source/Company	Catalogue #
Streptavidin		PE-Cy7	BD Pharmingen	557598

Table 2.3 Reagents

Tumour induction

Reagents	Source/Company	Catalogue #
Azoxymethane (AOM)	Sigma-Aldrich	A5486
Dextran sulfate sodium salt (DSS)	Affymetrix/USB Alfa Aesar	J14489 J63606

In Vitro Stimulation

Reagents	Source/Company	Catalogue #
BD GolgiStop	BD Biosciences	554724
BD GolgiPlug	BD Biosciences	555029
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	P1585
Ionomycin	Sigma-Aldrich	I9657
hIL-15	Peprtech	200-15-50
mIL-12	Peprtech	210-12
mIL-18	Miltenyi Biotec	

Isolation

Reagents	Source/Company	Catalogue #
EasySep Mouse NK cell Isolation Kit	STEMCELL Technologies	19855
Anti-Biotin MicroBeads	Miltenyi Biotec	130-090-485
Percoll	GE Healthcare	17089101

Dissociation and Digestion

Reagents	Source/Company	Catalogue #
Ethylenediaminetetra-acetic acid (EDTA)	Univar	AJA180-500G
Collagenase III	Worthington	LS004182
Collagenase IV	Worthington	LS004188
Dispase	Gibco	17105041
DNase I	Roche	10104159001

Staining

Reagents	Source/Company	Catalogue #
CellTrace Violet	Invitrogen	C34557
Annexin V, Alexa Fluor™ 647 conjugate	Invitrogen	A23204
Foxp3/Transcription Factor Staining Buffer Kit	eBioscience	00-5523-00
Lyse/Fix Buffer 5X	BD Phosflow	558049
Perm Buffer III	BD Phosflow	558050

RNASeq Reagents

Reagents	Source/Company	Catalogue #
RNAlater Solution	Invitrogen	AM7020
RNeasy Plus Micro Kit	Qiagen	74034
Dithiothreitol (DTT)	Invitrogen	D1532
TruSeq RNA Library Prep Kit v2	Illumina	RS-122-2001 RS-122-2002

Chapter 3. Delineating the role of GFI1 and GFI1B in ILC development

3.1 Abstract

GFI1 and GFI1B are both involved in regulating the development of hematopoietic cells but their role specifically in ILC development and function have not been examined. Using a novel reporter mouse to map the expression of GFI1 and GFI1B during ILC development, I identified that GFI1B expression is restricted to the early stages of ILC development whereas GFI1 is expressed in both progenitor and mature cells. Interestingly, expression of GFI1B marked the progenitor cells with greater developmental potential in a stage-specific manner. In the absence of GFI1B, early ILC progenitors in the bone marrow failed to develop, which lead to a reduction of ILC2 in the lung. Collectively, these findings indicate GFI1B is an important transcriptional regulator during early ILC development and the development of mature ILC2 in the lung.

3.2 Introduction

3.2.1 Role of GFI1 and GFI1B in HSC

Both GFI1 and GFI1B play vital and complementary roles to regulate haematopoiesis. Both are expressed by hematopoietic stem cells (HSC) and are involved in regulating its maintenance and proliferation^{79, 92, 93}. But as progenitor cells become committed to an erythrocyte-megakaryocyte lineage, GFI1 expression is downregulated and GFI1B expression is maintained^{67, 79, 95}. Germline deletion of GFI1B is embryonic lethal as it is vital for the development of erythropoiesis⁷⁹. Specifically, it is required for erythrocyte maturation and regulating megakaryocyte development^{72, 96}. In cells committed to a lymphoid-myeloid lineage, GFI1B expression is downregulated and GFI1 expression is maintained^{79, 94}. In granulocyte-monocyte progenitors, GFI1 drive neutrophil and dendritic cell development and suppress the development of macrophages⁶⁹. In the absence of GFI1, there is accumulation of an abnormal myeloid population with an immature phenotype.

In the lymphoid lineage, the common lymphoid progenitor (CLP), which has the capacity to give rise to all lymphoid cells including ILCs, expressed GFI1⁹⁴. Germline deletion of GFI1 resulted in a reduction of hematopoietic progenitors, with almost a complete loss of CLP numbers in the bone marrow and subsequent loss of peripheral lymphocytes^{92, 103}. However, it is not known if GFI1 is intrinsically required by the CLP as it is known to impact HSC maintenance. The use of conditional deletion models showed that GFI1 regulated differentiation of CD4⁺ T cells^{100, 105, 109}. Through the recruitment of epigenetic modifying protein LSD1, GFI1 prevents the methylation of histone H3K4 to regulate the expression of key genes involved in T cell differentiation and function and suppress Th1 and Th17 development and function^{105, 109}. GFI1 also drives Th2 differentiation and cytokine production by preventing the degradation of the GATA3 protein^{100, 106}. Although GFI1B is not highly expressed in the lymphoid lineage, it has been reported to be expressed in the CLP and transiently expressed during T cell development in the thymus⁷⁹. However, it is not known whether GFI1B in these cells is functionally important

3.2.2 Transcriptional regulation of ILC development

Our current understanding of the role of GFI1 and GFI1B in ILCs has been limited by the ability to target deletion at a stage that is specific for ILC development. GFI1 has been identified to be highly expressed by ILC2p in the bone marrow and this expression was upregulated upon IL-33 treatment¹¹⁰. GFI1 played a key role in regulating ILC2 development and differentiation by suppressing ILC3-associated genes and maintaining ILC2 identity¹¹⁰. However, it is not known at which stages of ILC development GFI1 is expressed or is required to regulate development as many of these experiments were performed in mice with germline deletion of GFI1. Furthermore, the expression and function of GFI1 and GFI1B has not been examined in the development and function of other ILC subsets.

3.2.3 Aims and Hypothesis

Gene expression data generated from our lab indicated that *Gfi1* expression was highly expressed throughout development in early progenitors in the bone marrow, as well as mature ILC subsets in the peripheral organs. However, *Gfi1b* expression was restricted to the bone marrow progenitors and was not expressed in mature cells (unpublished). Therefore, I hypothesised that GFI1 and GFI1B may be important regulators of ILC development from bone marrow progenitors. I also investigated whether this regulation of ILC development may influence the functional capacity of ILCs and impact the host immune response to challenges.

The aim of this chapter was to:

- (a) Map the expression of both GFI1 and GFI1B throughout ILC development using a dual reporter mouse model.
- (b) Determine the role of GFI1 and GFI1B in ILC development and function by examining their impact on the developmental potential of bone marrow progenitors and the functional capacity of cells derived from those progenitors.

3.3 Results

3.3.1 Characterisation of GFI1 and GFI1B expression in ILCs during development.

GFI1 and GFI1B are differentially expressed in bone marrow progenitors

Previous studies have established that GFI1 and GFI1B are expressed by the CLP in the bone marrow of adult mice^{79, 92}. To date, however, there has not been a systematic analysis of expression in the subsequent progenitor stages, particularly those that ultimately contribute to the ILC progenitors. Thus, it is not known whether GFI1 or GFI1B is expressed in lineage-restricted ILC progenitors or in mature ILCs in the periphery or how GFI1 and GFI1B are expressed in relation to each other. To simultaneously map the expression of both GFI1 and GFI1B in lymphoid progenitors in the bone marrow, I took advantage of the *Gfi1b*^{+/GFP}*Gfi1*^{+/tdTomato} (*Gfi1b*^{GFP}*Gfi1*^{Tomato}) dual reporter mouse to analyse immune cell lineages^{79, 114-116}. In these reporter mice, GFI1 expression is unaffected as the *Gfi1* promoter drives the expression of the Tomato reporter on a BAC and does not impact the *Gfi1* gene locus¹¹⁴. GFP is expressed by replacing the *Gfi1b* gene locus with the *GFP* gene⁷⁹. However, there is no dose effect of GFI1B as Vassen *et al.* has reported that mice with a single allelic substitution of *Gfi1b* with *GFP* are phenotypically indistinguishable from wildtype mice⁷⁹. Therefore, the addition of these reporter genes does not impact the phenotype of these mice.

I analysed the early bone marrow progenitors CLP, CILP and ILCp for the expression of both transcription factors indicated by fluorescent protein expression (Fig. 3.1a). Within CLP CILP and ILCp populations, a GFP⁺ and GFP⁻ population was found (Fig. 3.1b). In contrast, GFI1-Tomato was expressed by all CLP, CILP and ILCp populations. All CLP were positive for GFI1-Tomato and intermediate and high levels of expression intensity could be observed (Fig. 3.1b). CILP and ILCp predominantly expressed GFI1-Tomato at intermediate intensity (Fig. 3.1b).

Both GFI1 and GFI1B were expressed in the early progenitor cells, but it is not known how these two transcription factors are expressed in relation to each other. Therefore, I looked at the simultaneous expression of both GFI1 and GFI1B and quantified the proportions of cells that expressed them. Bone marrow progenitors could be categorised into three groups based on GFI1 and GFI1B expression. GFI1B⁺ cells are identified as cells that express GFI1-GFP and intermediate levels of GFI1-Tomato. GFI1B-GFP negative cells that expressed intermediate or

high levels of GFI1-Tomato were identified as GFI1^{int} and GFI1^{hi} respectively (Fig. 3.1c). No cells expressed both GFI1B-GFP and high levels of GFI1-Tomato (Fig. 3.1c). In CLP, 60-80% were GFI1B⁺ cells (Fig. 3.1c). This fraction diminished in CILP and ILCp to 40-50% (Fig. 3.1c). The remaining CLP were primarily GFI1^{hi} cells (~20%) and very few (~5%) were GFI1^{int} cells (Fig. 3.1c). The frequency of GFI1^{hi} cells decreased 2.5-fold in CILP compared with CLP and were absent in the ILCp. The frequency of GFI1^{int} cells increased to approximately 40% in CILP and ILCp (Fig. 3.1c). Collectively, this heterogeneous expression of GFI1 and GFI1B within the different progenitor stages suggests that the level of expression may play different roles during ILC developmental.

Lineage committed ILCs primarily express GFI1

Expression of both GFI1 and GFI1B changed as multi-lineage bone marrow progenitors developed. Therefore, I further examined GFI1 and GFI1B expression in mature ILCs in the periphery. Analysis of ILC1s and NK cell progenitors (NKp), immature NK (iNK) and mature NK (mNK) cells in the bone marrow of dual reporter mice revealed that these subsets did not express GFI1B-GFP (Fig. 3.2b-c). However, they all expressed GFI1-Tomato with 70-85% expressing high levels of GFI1-Tomato and the remainder expressing intermediate levels of GFI1 (Fig. 3.2b-c). This pattern of expression was maintained in mature ILC1 and NK cells in the liver as only 3-5% of ILC1 and NK cells expressed GFI1B-GFP, whereas all cells expressed GFI1-Tomato and expression was high in 70% and 90% of NK cells and ILC1, respectively (Fig. 3.2d-f). Although ILC1 and NK cells expressed GFI1, the MFI of ILC1 was ~1.5-fold higher than that of NK cells in the bone marrow, and over 2-fold higher in the liver (Fig. 3.2b,e).

High GFI1 expression had previously been reported in ILC2 progenitors (ILC2p) in the bone marrow¹¹⁰ but the expression of GFI1B has not yet been described. Similarly, the expression of GFI1 and GFI1B in mature ILC2 and ILC3 in peripheral organs has not been mapped. Analyses of these subsets in the *Gfi1b*^{GFP}*Gfi1*^{Tomato} dual reporter mouse strain revealed that 75-87% of ILC2p in the bone marrow were GFI1^{hi}, with the remaining cells expressed intermediate levels of GFI1. GFI1B was not expressed by ILC2p in the bone marrow (Fig. 3.3a-b). Mature ILC2 in the small intestine continued to lack GFI1B expression, but >99% of cells were GFI1^{hi} (Fig 3.3c-e). ILC3 in the small intestine also expressed GFI1 but the MFI was six-fold lower than that of ILC2 (Fig. 3.3d.) Of the ILC3 that expressed NKp46 (NCR⁺ ILC3),

88-92% of cells expressed GFI1^{hi} and 6-8% of cells expressed GFI1^{int} (Fig. 3.3c-e). The remaining ILC3 that did not express NKp46 (NCR⁻ ILC3), which consisted of DN ILC3 and lymphoid tissue-inducer (LTi) cells, 80-85% of cells expressed GFI1^{hi} and 10-15% of cells expressed intermediate levels of GFI1 (Fig. 3.3c-e). ILC3 in the small intestine did not express GFI1B (Fig. 3d-e). Together, these data show that GFI1 is expressed by early ILC progenitors and mature ILC subsets whereas GFI1B expression is primarily expressed in early ILC progenitors but not in mature ILC subsets.

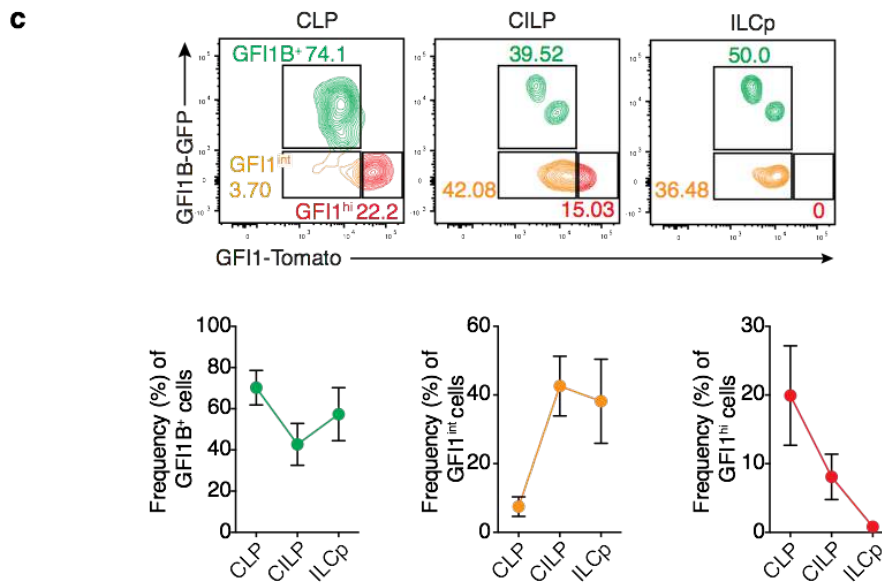
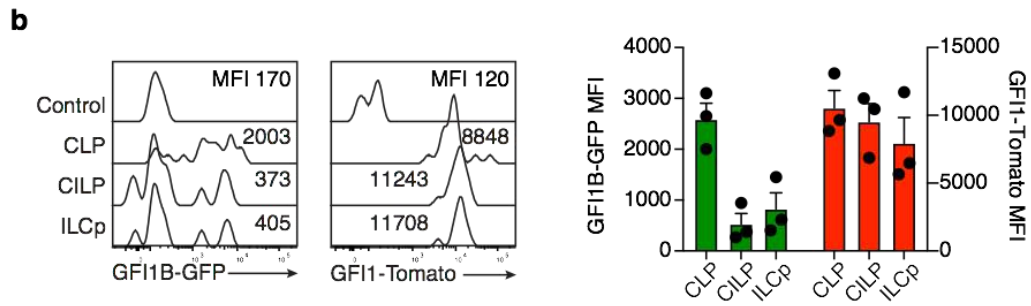
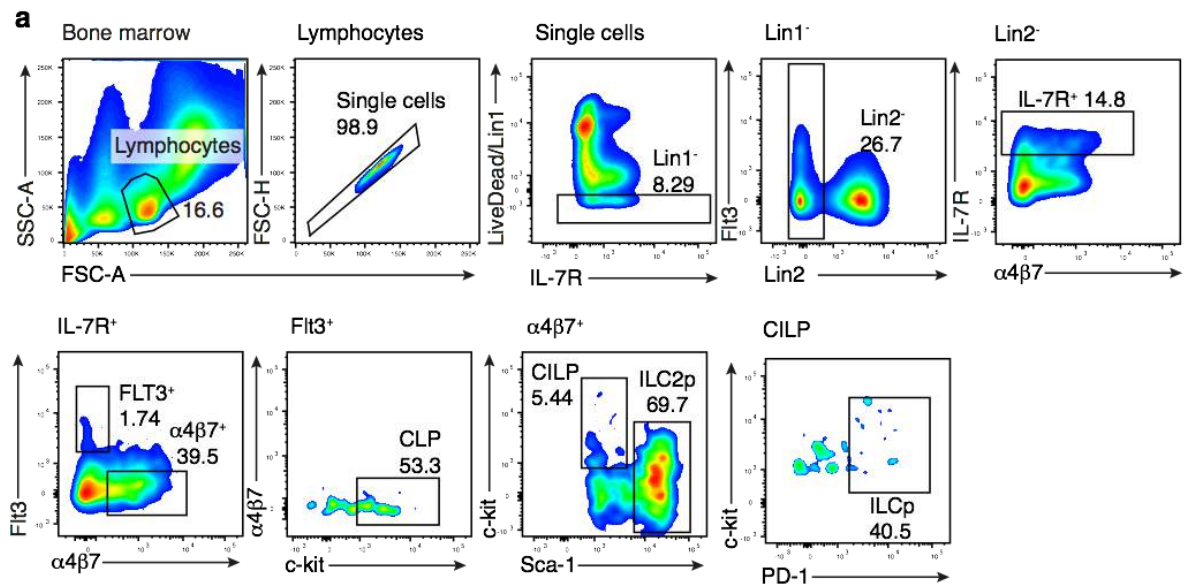


Figure 3.1 GFI1 and GFI1b are differentially expressed in early ILC progenitors in bone marrow.

a, Gating strategy for ILC progenitors isolated from the bone marrow in *Gfi1b^{GFP}Gfi1^{Tomato}* dual reporter mice. Bone marrow progenitors were identified as live lymphocytes that are negative for lineage markers (Lin1: CD3⁺NKp46⁺CD11b⁺CD19⁺Ter-119⁺, Lin2: TCRβ⁺CD19⁺B220⁺NK1.1⁺F4/80⁺). Lymphoid progenitors were defined as CLP (IL-7R⁺α4β7⁻Flt3⁺ckit^{int}), CILP (IL-7R⁺α4β7⁺Sca-1⁻ckit⁺), ILC2p (IL-7R⁺α4β7⁺Sca-1⁺ckit⁻) and ILCp (IL-7R⁺α4β7⁺Sca-1⁻ckit⁺PD-1⁺).

b, Expression of GFI1B-GFP and GFI1-Tomato in CLP, CILP and ILCp from bone marrow isolated from *Gfi1b^{GFP}Gfi1^{Tomato}* dual reporter mice. Control cells were live lin⁻IL-7R⁺GFI1-Tomato⁻GFI1B-GFP⁻ cells. Histograms show MFI for individuals (left) and mean ± SEM (right) of one of two independent experiments (*n* = 2-3 mice/genotype/experiment).

c, Contour plots (upper panels) showing the gating and histograms (lower panels) showing the frequency of GFI1B⁺ (GFI1B-GFP⁺GFI1-Tomato^{hi}, green) GFI1^{int} (GFI1B-GFP⁻GFI1-Tomato^{int}, orange) and GFI1^{high} (GFI1B-GFP⁻GFI1-Tomato^{high}, red) cells in bone marrow progenitor of *Gfi1b^{GFP}Gfi1^{Tomato}* mice. Data show the mean ± SEM pooled from two independent experiments (*n* = 2-3 mice/genotype/experiment).

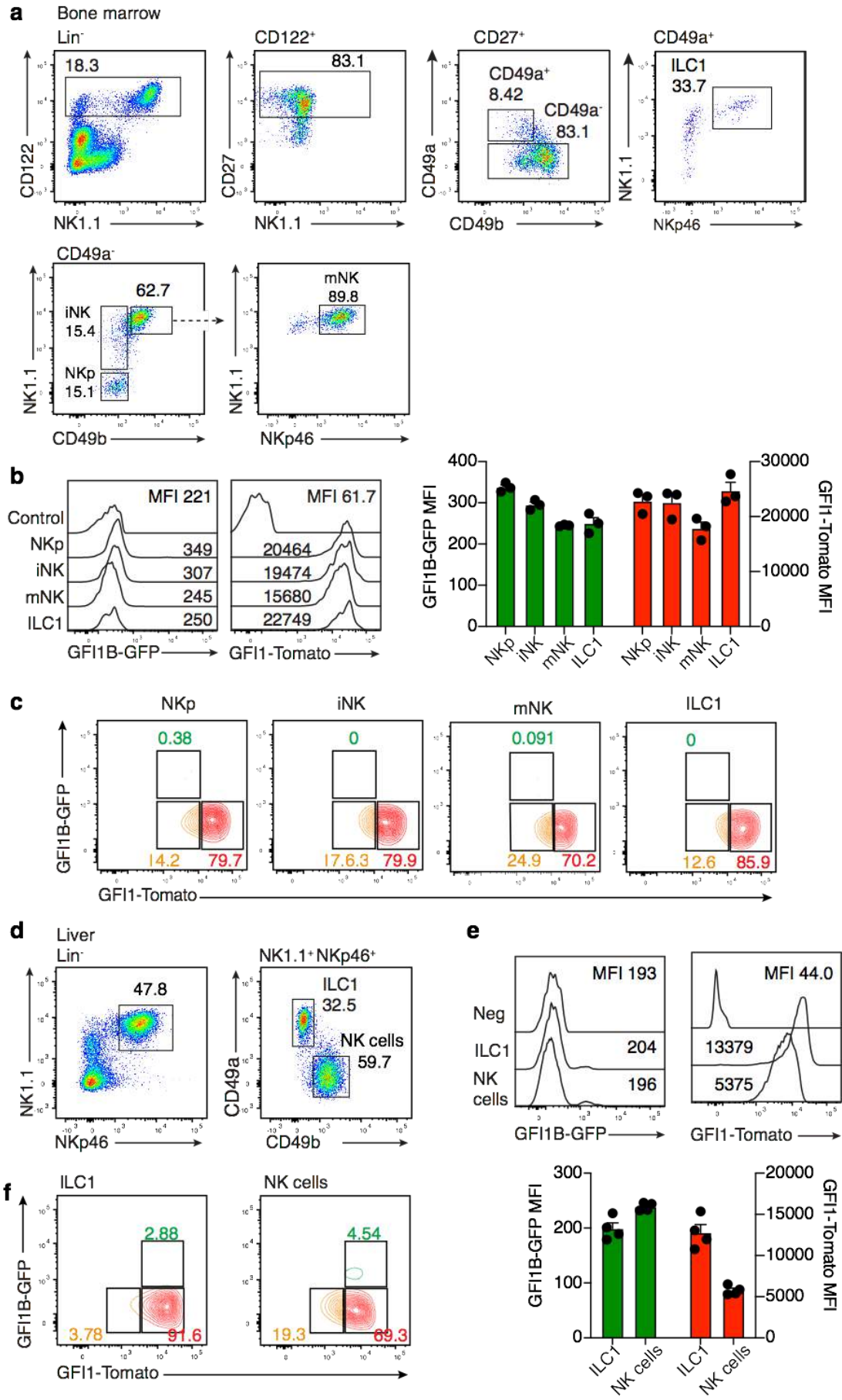


Figure 3.2. NK cells primarily express GFI1 during development in bone marrow and as mature cells in periphery

a. Gating strategy for NK cells progenitors in the bone marrow. Lineage-restricted NK cells were identified by gating on live Lin⁻CD122⁺CD27⁺ that were CD49a⁻ while ILC1 cells expressed CD49a⁺NK1.1⁺NKp46⁺. Within the lineage-restricted NK cells, NK progenitor (NKp) cells were identified as NK1.1⁻CD49b⁻, immature NK (iNK) cells as NK1.1⁺CD49b⁻ and mature NK (mNK) cells as NK1.1⁺CD49b⁺NKp46⁺.

b. Expression of GFI1B-GFP and GFI1-Tomato in NK cell progenitors in the bone marrow as identified above from *Gfi1b*^{GFP}*Gfi1*^{Tomato} dual reporter mice. Control cells are live lin⁻ cells from the bone marrow of *Gfi1b*^{GFP}*Gfi1*^{Tomato} mice that are negative for GFI1B-GFP and GFI1-Tomato expression. Histograms show MFI for individuals (left) and mean ± SEM (right) of one of three independent experiments (*n* = 2-3 mice/genotype/experiment).

c. Contour plots showing the gating of GFI1B⁺ (GFI1b-GFP⁺GFI1-Tomato^{hi}, green) GFI1^{int} (GFI1B-GFP⁻GFI1-Tomato^{int}, orange) and GFI1^{high} (GFI1B-GFP⁻GFI1-Tomato^{high}, red) subsets in bone marrow progenitor of *Gfi1b*^{GFP}*Gfi1*^{Tomato} mice. Data is representative of three independent experiments (*n* = 2-3 mice/genotype/experiment).

d. For liver, conventional CD49b⁺ NK cells and CD49a⁺ ILC1 were identified within the live lin⁻CD3⁻TCRβ⁻CD19⁻NK1.1⁺NKp46⁺ population.

e. Expression of GFI1B-GFP and GFI1-Tomato in ILC1 and NK cell in the liver as identified above from *Gfi1b*^{GFP}*Gfi1*^{Tomato} mice. Control cells are live lin⁻NK1.1⁺ cells from the liver of *Gfi1b*^{GFP}*Gfi1*^{Tomato} mice that are negative for GFI1B-GFP and GFI1-Tomato expression. Histograms show MFI for individuals (top) and mean ± SEM (bottom) pooled from two independent experiments (*n* = 2 mice/genotype/experiment).

c. Contour plots showing the gating of GFI1B⁺ (GFI1b-GFP⁺GFI1-Tomato^{hi}, green) GFI1^{int} (GFI1B-GFP⁻GFI1-Tomato^{int}, orange) and GFI1^{high} (GFI1B-GFP⁺GFI1-Tomato^{high}, red) subsets in liver ILC1 and NK cells of *Gfi1b*^{GFP}*Gfi1*^{Tomato} mice. Data is representative of two independent experiments (*n* = 2 mice/genotype/experiment).

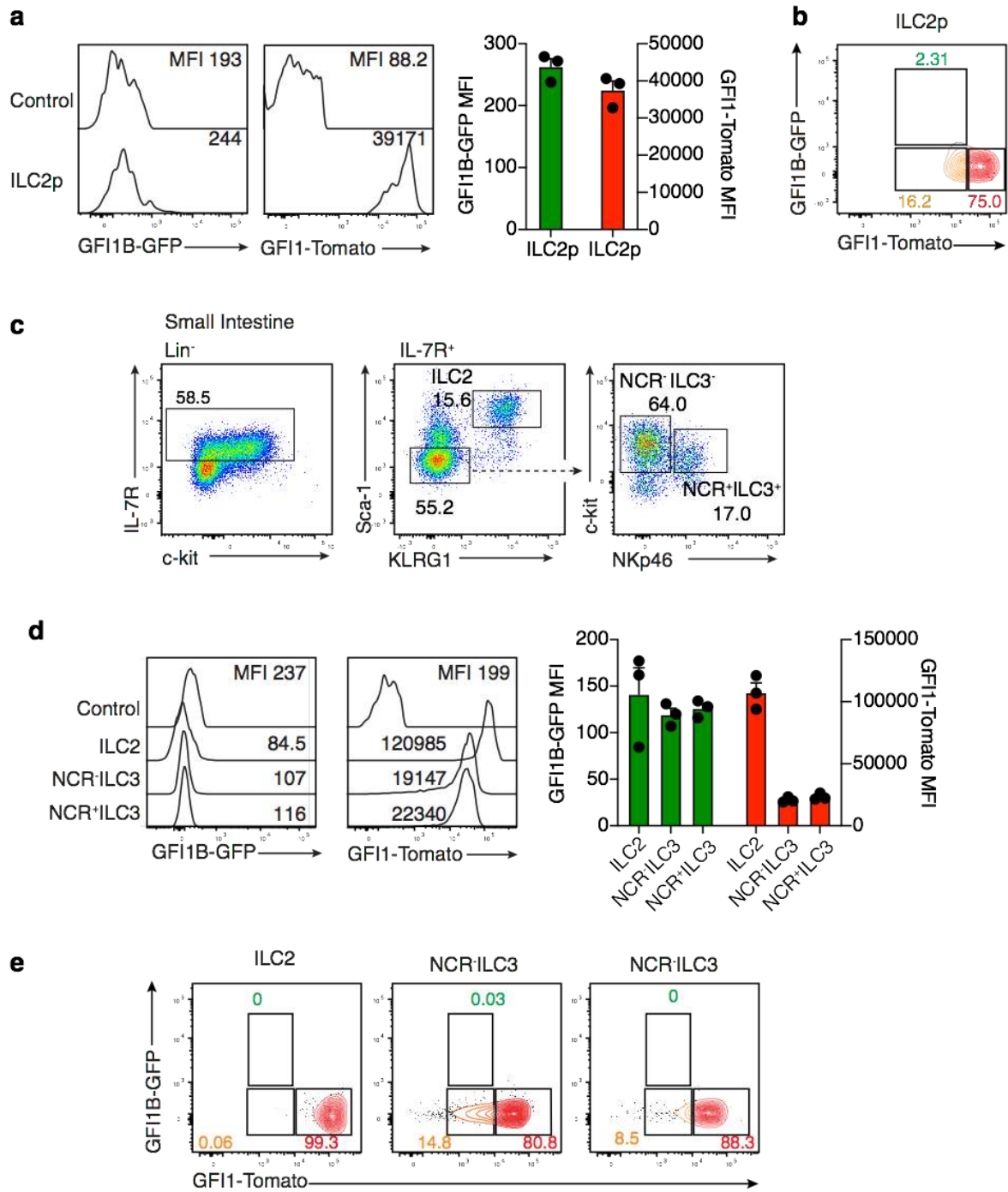


Figure 3.3. Lineage committed ILC2 and peripheral ILC3 express GFI1 but not GFI1B.

- a.** Expression of GFI1B-GFP and GFI1-Tomato in ILC2 progenitors (ILC2p) in the bone marrow as identified in **Fig. 1a** from *Gfi1b^{GFP}Gfi1^{Tomato}* dual reporter mice. Neg cells are live lin^- cells from the bone marrow of *Gfi1b^{GFP}Gfi1^{Tomato}* mice that are negative for GFI1B-GFP and GFI1-Tomato expression. Histograms show MFI for individual mice (left) and mean $\pm$ SEM (right) from one of two independent experiments ($n = 2-3$ mice/genotype/experiment).
- b.** Contour plots showing the gating of GFI1B⁺ (GFI1b-GFP⁺GFI1-Tomato^{hi}, green) GFI1^{int} (GFI1B-GFP⁺GFI1-Tomato^{int}, orange) and GFI1^{high} (GFI1B-GFP⁺GFI1-Tomato^{high}, red) subsets in bone marrow progenitor of *Gfi1b^{GFP}Gfi1^{Tomato}* mice. Data is representative of two independent experiments ($n = 2-3$ mice/genotype/experiment).
- c.** Gating strategy for ILC2s and ILC3s in the small intestine. ILC2s were identified by gating on live lin^- (NK1.1⁻CD3⁻TCR β ⁻CD19⁻ IL-7R⁺ cells that are Sca-1⁺KLRG1⁺, while ILC3s are Sca-1⁺KLRG1⁻ckit⁺. ILC3s were further separated into NCR⁻(NKp46⁻) or NCR⁺(NKp46⁺) subsets.
- d.** Expression of GFI1B-GFP and GFI1-Tomato in ILC2 and ILC3 in the small intestine as identified above from *Gfi1b^{GFP}Gfi1^{Tomato}* mice. Neg cells are live lin^- IL-7R⁺ cells from the small intestine of *Gfi1b^{GFP}Gfi1^{Tomato}* mice that are negative for GFI1B-GFP and GFI1-Tomato expression. Histograms show MFI for individuals (left) and mean $\pm$ SEM (right) of one of two independent experiments ($n = 2-3$ mice/genotype/experiment).
- e.** Contour plots showing the gating of GFI1B⁺ (GFI1b-GFP⁺GFI1-Tomato^{hi}, green) GFI1^{int} (GFI1B-GFP⁺GFI1-Tomato^{int}, orange) and GFI1^{high} (GFI1B-GFP⁺GFI1-Tomato^{high}, red) subsets in ILC2 and ILC3 of *Gfi1b^{GFP}Gfi1^{Tomato}* mice. Data is representative of two independent experiments ($n = 2-3$ mice/genotype/experiment).

GFI1B expression in bone marrow progenitors determine developmental potential and trajectory.

While GFI1 was expressed similarly between the stages of ILC development, GFI1B expression was heterogenous and was only expressed in cells with multi-lineage potential i.e CLP, CILP and ILCp. In progenitor cells that have committed to a specific cell type, i.e NKp and ILC2p, the expression was lost. Therefore, I hypothesised that GFI1B expression reflected the developmental potential of early bone marrow progenitors. To determine whether this was the case, I examined the developmental potential of the different early lymphoid progenitors. To this end, 600 GFI1B-GFP⁺, GFI1B-GFP⁻ CLP and CILP were sorted from the bone marrow of *Gfi1b^{GFP}Gfi1^{Tomato}* dual reporter mice and injected into sub-lethally irradiated *Rag2 γ C* mice. After 6 weeks, the reconstitution of these donor bone marrow progenitors was analysed to determine the lineage trajectory for each ILC subset and assess differences in the developmental capacity in mice that received GFI1B-GFP⁻ or GFI1B-GFP⁺ bone marrow progenitors (Fig. 3.4a).

The proportion of ILC1 and NK cells that developed in the spleen and liver were comparable between *Rag2 γ C* mice that had received either GFI1B-GFP⁺ or GFI1B-GFP⁻ CLP (Fig. 3.4b-c). Although there were large variations in the numbers of NK cells that was recovered between individual mice, there were no significant differences between the number of ILC1 and NK cells that developed either from GFI1B-GFP⁺ or GFI1B-GFP⁻ CLP (Fig. 3.4b-c). This indicated that GFI1B expression at the CLP stage did not reflect any developmental preference between ILC1 and NK cells, nor did it affect the capacity of CLP to generate either ILC1 or NK cells that localize in the spleen and liver.

In *Rag2 γ C* mice that received CILP, only GFI1B-GFP⁺ CILP developed into both ILC1 and NK cells in the spleen and liver while GFI1B-GFP⁻ CILP could only develop into NK cells and did not form ILC1 (Fig. 3.4b-c). Similar numbers of splenic NK cells developed from both GFI1B-GFP⁺ and GFI1B-GFP⁻ CILP, while in the liver, GFI1B-GFP⁺ CILP could give rise to two-fold more NK cells than GFI1B-GFP⁻ CILP (Fig. 3.4b-c). This indicated that there is a difference in developmental potential between GFI1B-GFP⁺ and GFI1B-GFP⁻ cells at the CILP stage of development as GFI1B-GFP⁻ cells lose the ability to develop into ILC1 in the spleen and liver.

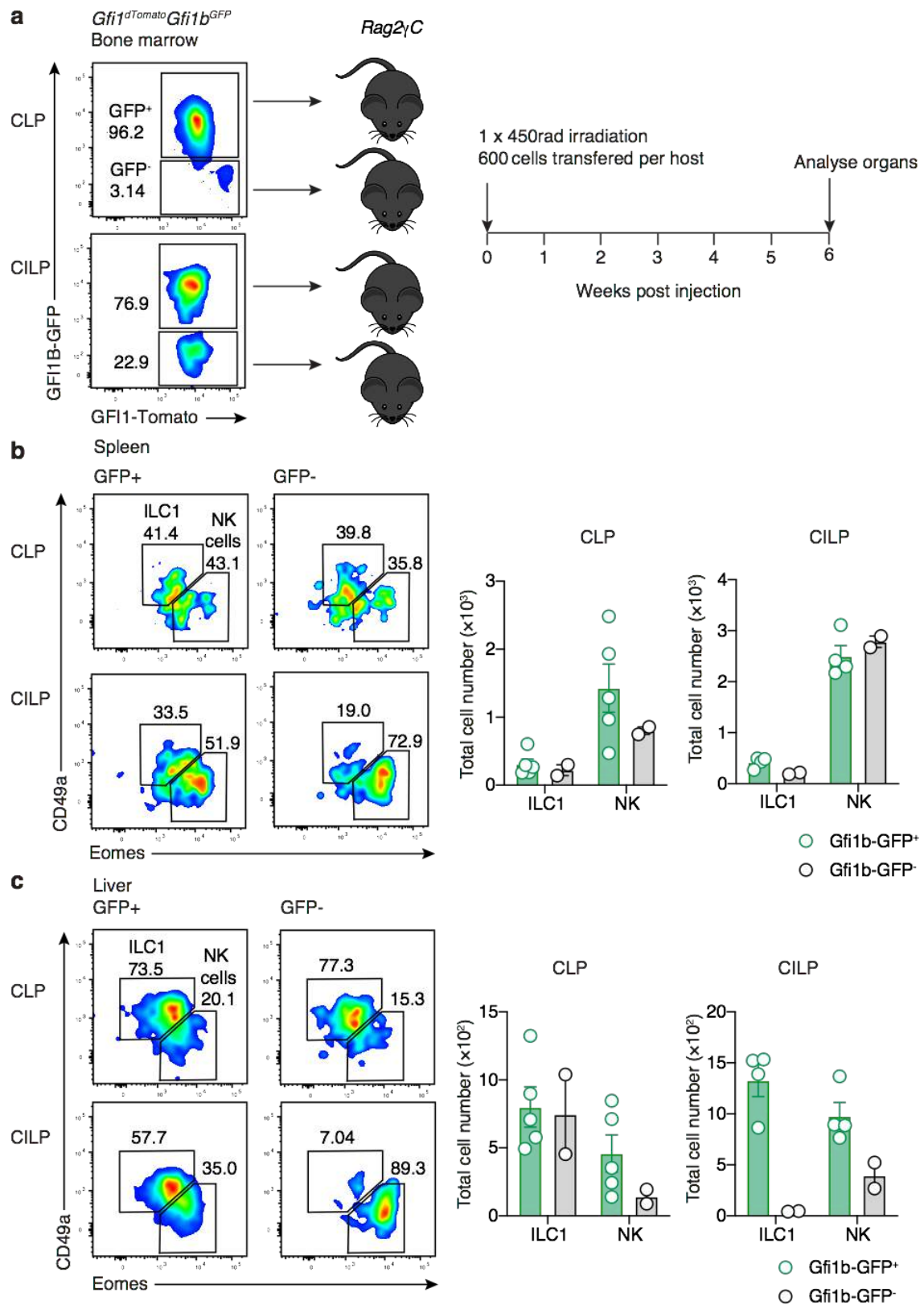


Figure 3.4. GFI1B delineates between ILC1 and NK development in CILP cells.

a. Sorting strategy to isolate and adoptively transfer 600 GFP⁺ and GFP⁻ CLP (lin⁻IL-7R⁺Flt3⁺ckit^{int}) and CILP (lin⁻IL-7R⁺ α 4 β 7⁺ckit^{int}) bone marrow progenitors from *Gfi1b*^{GFP}*Gfi1*^{Tomato} dual reporter mice into sub-lethally irradiated *Rag2* γ ^{-/-} recipient mice. Reconstitution of ILC subsets in peripheral organs was analysed after six weeks. Flow cytometric density plots show the frequency of each population within the CLP and CILP populations.

b-c. Analysis of the development of ILC1 (NK1.1⁺NKp46⁺CD49a⁺) and NK cells (NK1.1⁺NKp46⁺Eomes⁺) from the **(b)** spleen and **(c)** liver of *Rag2* γ ^{-/-} mice six weeks after adoptive transfer of progenitors. Representative density plots (left panels) show the frequency of ILC1 and NK cells of the spleen and liver reconstituted from either GFI1B-GFP⁺ or GFI1B-GFP⁻ CLP and CILP cells. Histograms (right panels) show mean $\pm$ SEM from one of two similar experiments of total ILC1 and NK cells from the spleen and liver of each animal (*n* = 2-5 mice/genotype/experiment).

In the small intestine, GFI1B-GFP⁺ and GFI1B-GFP⁻ CLP generated similar proportions and similar numbers of ILC1 and NK cells, nor was there a difference in the total number of ILC1 recovered between mice that were transferred GFI1B-GFP⁺ and GFI1B-GFP⁻ CILP (Fig. 3.5a). However, there was a two-fold reduction in the number of NK cells recovered from the small intestine of mice that had received GFI1B-GFP⁻ CILP compared to mice that had received GFI1B-GFP⁺ CILP (Fig. 3.5a). This indicated that there was no difference in lineage trajectory or developmental capacity between GFI1B-GFP⁻ and GFI1B-GFP⁺ cells at the CLP stage in the small intestine. However, GFI1B-GFP⁻ CILP have a greater capacity for ILC1 development than GFI1B-GFP⁺ CILP but also have less developmental capacity for NK cells. ILC2 and ILC3 in the small intestine and ILC2 from the lungs had developed from either GFI1B-GFP⁺ or GFI1B-GFP⁻ CLP at similar numbers (Fig. 3.5b-c). While GFI1B-GFP⁺ CILP could successfully generate ILC2 and ILC3 in the small intestine, ILC2 and ILC3 failed to develop from GFI1B-GFP⁻ CILP cells (Fig. 3.5b). Similarly, there was no difference in the number of ILC2 that had developed in the lung between GFI1B-GFP⁻ and GFI1B-GFP⁺ cells at the CLP stage but there was a reduction of ILC2 generated from the GFI1B-GFP⁻ CILP compared to GFI1B-GFP⁺ CILP although this had not yet reached statistical significance (Fig. 3.5c). This indicated that GFI1B expression did not delineate between developmental potential at the CLP stage but CILP that expressed GFI1B had greater developmental capacity than GFI1B⁻ CILP. In summary, there were no differences in developmental lineage or capacity between CLP cells based on GFI1B expression. However, CILP that expressed GFI1B could develop into multiple ILC subsets to generate mature ILCs in the periphery as well as a capacity to generate more ILC numbers.

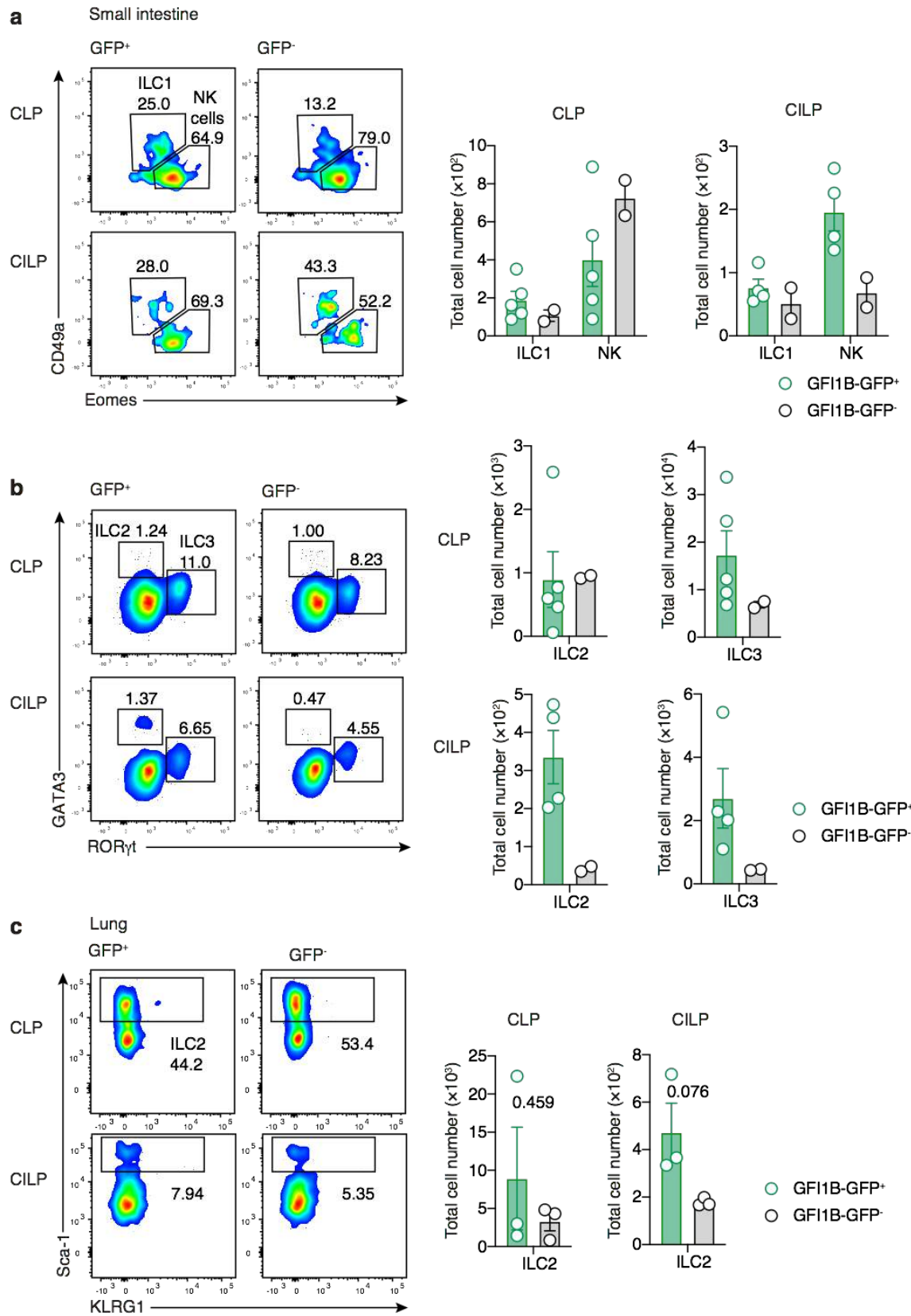


Figure 3.5. GFI1B-expressing CILP have greater developmental capacity.

a-b. Analysis of the development of ILC1 (NK1.1⁺NKp46⁺CD49a⁺), NK cells (NK1.1⁺NKp46⁺Eomes⁺) (**a**) and ILC2 (lin-GATA3⁺) and ILC3 (lin-RORγt⁺) (**b**) from the small intestine of *Rag2* γ ^{-/-} mice six weeks after adoptive transfer of either GFI1B-GFP⁺, GFI1B-GFP⁻ CLP or CILP cells. Representative density plots (left panels) and histograms of mean $\pm$ SEM (right panels) showing total ILC1 and NK cells/animal isolated from the small intestine from one of two similar experiments ($n = 2-5$ mice/genotype/experiment).

c. Analysis of the development of ILC2 (lin-IL-7R⁺Sca-1⁺KLRG1^{+/-}) recovered from the lung of *Rag2* γ ^{-/-} mice six weeks after adoptive transfer of transfer of either GFI1B-GFP⁺, GFI1B-GFP⁻ CLP or CILP cells. Representative density plots (left panels) and histograms of mean $\pm$ SEM (right panels) show total ILC2 from the lung each animal from one experiment of ($n = 3$ mice/genotype/experiment). Statistical significance was calculated using unpaired Student's *t* test.

3.3.2 Role of GFI1 in ILC development at steady-state

Loss of GFI1 in GFI1-deficient mice

While GFI1 was expressed by all cells throughout ILC development, the level of expression varied between developmental stages and between ILC subsets in peripheral organs. Therefore, I hypothesised that GFI1 may regulate the development and/or maintenance of specific ILC subsets. To examine the effect of loss of GFI1 during ILC development, the *Gfi1*^{fl/fl} mouse¹⁰⁰ was crossed to the *Il7ra*^{Cre} mouse¹²¹. Exons 4 and 5 of the *Gfi1* allele is flanked by loxp regions and these were excised by Cre-mediated recombination following IL-7R-driven Cre expression. This deletion introduces a reading frame shift and results in the production of a non-functional protein¹⁰⁰ (Fig 3.6a). IL-7R is expressed early during lymphocyte development in the CLP of the bone marrow. Therefore, the *Il7ra*^{Cre} will drive deletion of *Gfi1* in IL-7R-expressing cells and affect downstream immune cell subsets including CILP, ILCs, T cells and B cells (Fig. 3.6b). To determine the deletion efficiency of GFI1, I examined expression of the *Gfi1* allele in splenic B and T cells from *Gfi1*^{fl/fl} and *Gfi1*^{fl/fl}*Il7ra*^{Cre} mice. PCR targeting the floxed region (340bp) could be detected in cells from *Gfi1*^{fl/fl} mice but not in cells from *Gfi1*^{fl/fl}*Il7ra*^{Cre} mice (Fig. 3.6c). This showed that the *Gfi1* allele was completely deleted in cells that either express or originated from progenitors that express IL-7R.

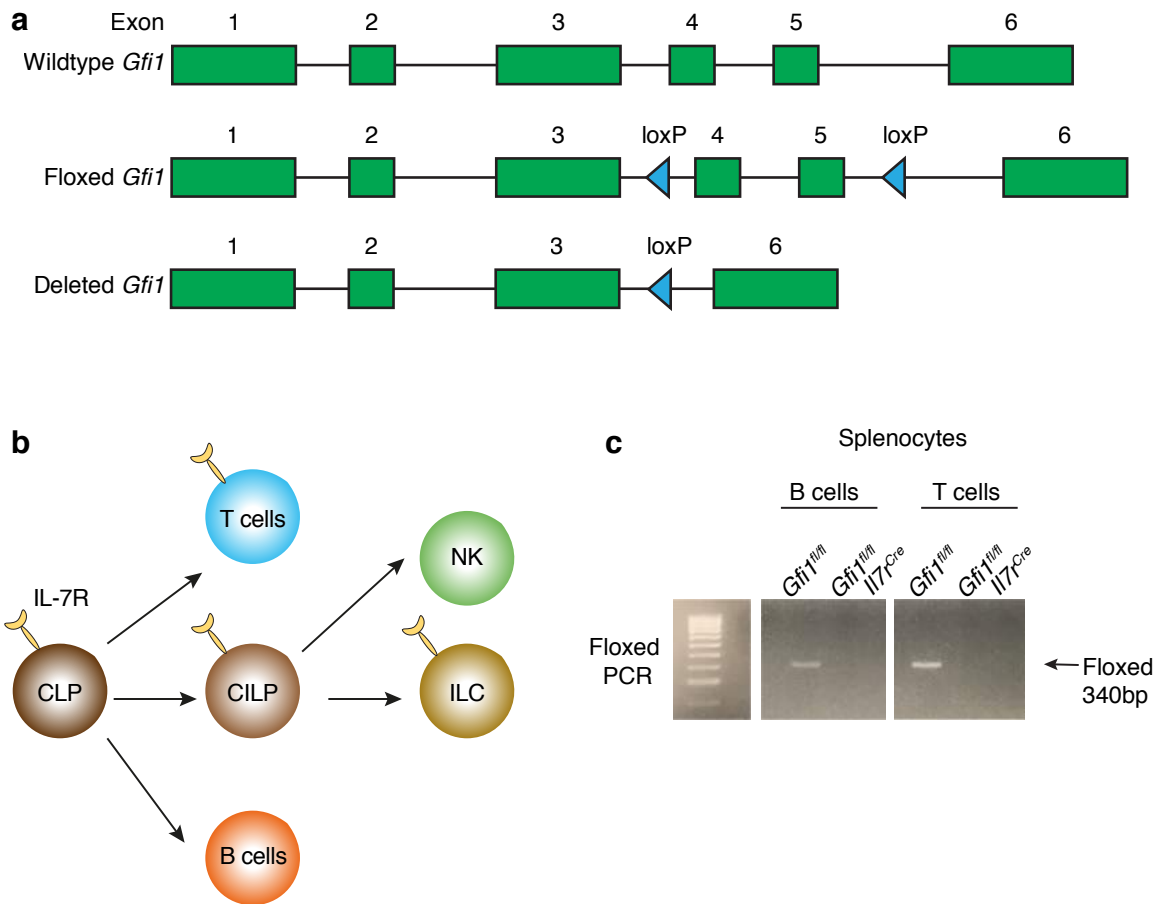


Figure 3.6. Mouse model of *Gfi1* deletion driven by IL7R expression.

a. Schematic of the wildtype, floxed and deleted *Gfi1* allele after Cre-mediated excision. Exons and loxP sites as indicated.

b. Developmental pathway of CLP developing in T cells, B cells, NK cells or ILCs. IL-7R expression as indicated.

c. Floxed *Gfi1* alleles identified by PCR. PCR was performed with primers targeting the floxed region on B cells and T cells sorted from the spleen of *Gfi1^{fl/fl}* and *Gfi1^{fl/fl} Il7ra^{Cre}* mice ($n=1-2$ mice/genotype from one experiment).

GFI1 is required for bone marrow progenitor and ILC2 development

Germline deletion of GFI1 had reduced CLP numbers⁹² but it was not known if GFI1 is required by CLP or if it reflects the loss of HSC, which also require GFI1. It is also not known whether subsequent ILC progenitors also require GFI1. Progenitor cells in the bone marrow and mature ILC in peripheral organs of *Il7ra^{Cre}Gfi1^{+/+}* and *Gfi1^{fl/fl}Il7ra^{Cre}* mice were examined to determine whether GFI1 is required for the development of these cells. There was a trend of reduced CLP, CILP ILCp and ILC2p frequency and number in *Il7ra^{Cre}Gfi1^{+/+}* (Fig. 3.7a-c), which matched with reduced numbers of CLP and ILC2p in GFI1 germline deleted mice^{92, 110}. In the lineage committed NK progenitors in the bone marrow, there was a trend of reduced NKp and iNK within the bone marrow of GFI1-deficient mice but no significant difference of mNK numbers (Fig. 3.7d). There was a trend of reduced ILC2 in the small intestine and mesenteric LN which is also in accordance with previous reports¹¹⁰ but no significant difference in the number of ILC3 that had developed (Fig. 3.8a,b). Together, these data show that GFI1 is vital for the development and or maintenance of early progenitors, NK progenitors in the bone marrow and ILC2 in the periphery. Despite the defect in the progenitors, normal numbers of mNK in the bone marrow and ILC3 in the gastrointestinal tract could be maintained, suggesting that there may be a compensatory mechanism to restore normal numbers.

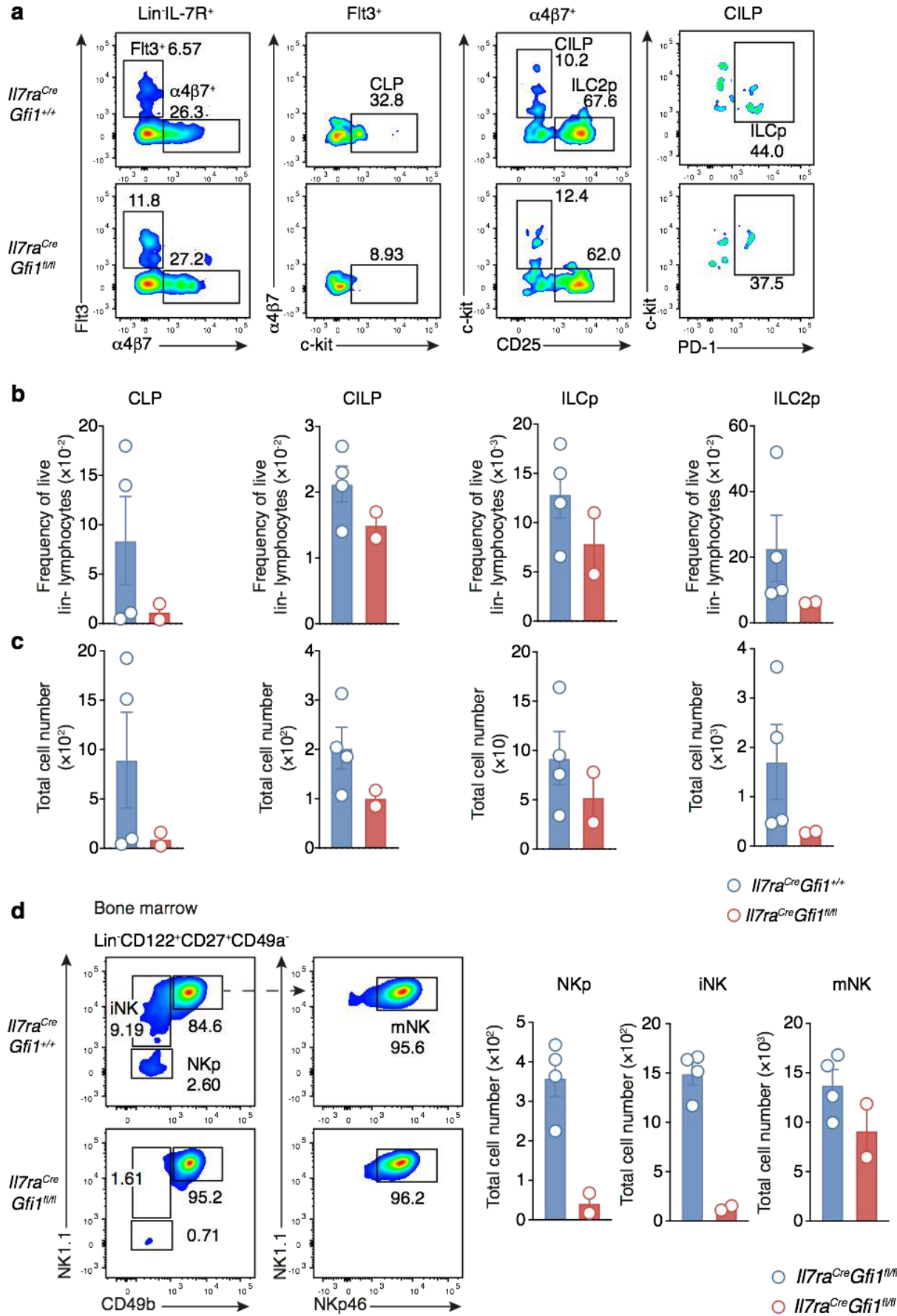


Figure 3.7. Loss of GFI1 reduces ILC progenitor cell numbers in the bone marrow.

a. Flow cytometric profiles of the early progenitors in the bone marrow of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice. Within the live lin⁻ (CD3⁺TCRβ⁺CD11b⁺CD19⁺B220⁺NK1.1⁺NKp46⁺F4/80⁺Ter-119⁺) IL-7R⁺ gate, CLP (Flt3⁺ckit^{int}), CILP (α4β7⁺ckit^{int}), ILCp (α4β7⁺ckit^{int}PD-1⁺) and ILC2p (α4β7⁺CD25⁺) were analysed.

b. Frequency and **c.** total number of bone marrow progenitors per mouse as indicated in **a.** was enumerated. Data show the mean ± SEM from one experiment (*n*=2-4 mice/genotype/experiment).

d. Flow cytometric profiles (left) and histograms enumerating the total cell number (right) of the NK cell lineage committed progenitors in the bone marrow of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice. Data show and mean ± SEM of NKp (NK1.1⁻CD49b⁻ cells), iNK cells (NK1.1⁺CD49b⁻) and mNK cells (NK1.1⁺CD49b⁺NKp46⁺) from one experiment (*n*=2-4 mice/genotype/experiment).

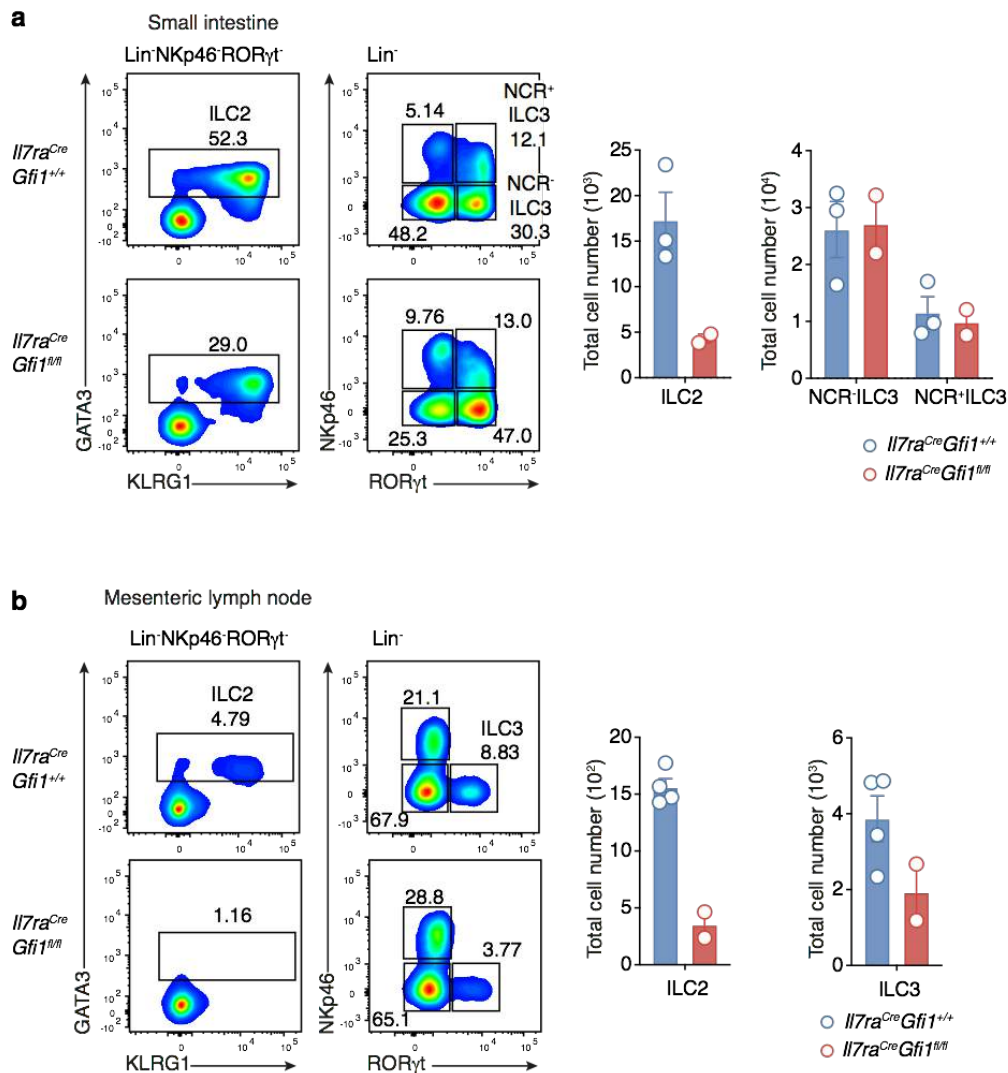


Figure 3.8. Loss of GFI1 alters ILC2 numbers in the gastrointestinal tract.

a. Flow cytometric profiles (upper panel) and histograms enumerating the total cell number (bottom) of ILCs from the small intestine of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice. Within the live lin⁺ cell population, ILC2 (RORγt⁺NKp46⁺GATA3⁺), NCR⁻ (RORγt⁺NKp46⁻) and NCR⁺ (RORγt⁺NKp46⁺) ILC3 cells were identified. Data show the mean ± SEM from one experiment ($n=2-3$ mice/genotype/experiment).

b. Flow cytometric profiles (upper panel) and histograms enumerating the total cell number (bottom) of ILCs from the mesenteric lymph node of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice. Within the live lin⁺ cell population, ILC2 (RORγt⁺NKp46⁺GATA3⁺) and ILC3 (RORγt⁺NKp46⁻) cells were identified. Data show the mean ± SEM from one experiment ($n=2-4$ mice/genotype/experiment).

3.3.3 Role of GFI1B in ILC development at steady-state

GFI1B is required by bone marrow progenitors at steady-state

GFI1B expression was heterogeneous in bone marrow progenitors and may define a population of progenitors that have greater developmental capacity for ILCs than progenitors that did not express GFI1B (Fig. 3.4, 3.5). Furthermore, GFI1B expression is lost in progenitors that have become lineage committed and in mature ILCs in the periphery. I hypothesised that GFI1B may be playing an important role during the early development of ILCs but may not be needed by mature ILCs in the periphery. To identify the role of GFI1B on early bone marrow progenitors, we crossed the *Gfi1b^{fl/fl}* mouse⁷⁹ to the *Il7ra^{Cre}* mouse¹²¹ to generate *Il7ra^{Cre}Gfi1b^{fl/fl}* mice that conditionally delete GFI1B in IL-7R-expressing cells. Exons 2, 3 and 4 of the *Gfi1b* allele is flanked by loxp regions and these are excised by Cre-mediated recombination following IL-7R-driven Cre expression (Fig. 3.9a). This results in loss of GFI1B expression at the early stages of ILC development, in the CLP cells and in subsequent cells that develop from CLP. PCR was performed to check deletion of GFI1B in B cells, T cells and NK cells sorted from the spleen and IL-7R⁺ and IL-7R⁻ cells sorted from the bone marrow of *Gfi1b^{fl/fl}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. The floxed region (295bp) of the *Gfi1b* allele could be detected in B cells, T cells, NK and IL-7R⁺ of *Gfi1b^{fl/fl}* mice, whereas the deleted region (540bp) could be detected in cells from *Il7ra^{Cre}Gfi1b^{fl/fl}* mice (Fig. 3.9b). Both the floxed and deleted regions of the *Gfi1b* allele could be identified in IL-7R⁻ cells in the bone marrow (Fig. 3.9b). This is due to the presence of IL-7R⁻ cells do not express IL-7R and therefore contain the floxed region and cells that had previously expressed IL-7R and therefore had deleted GFI1B, but then downregulated IL-7R expression, reflecting the developmental pathway of these IL-7R⁻ cells.

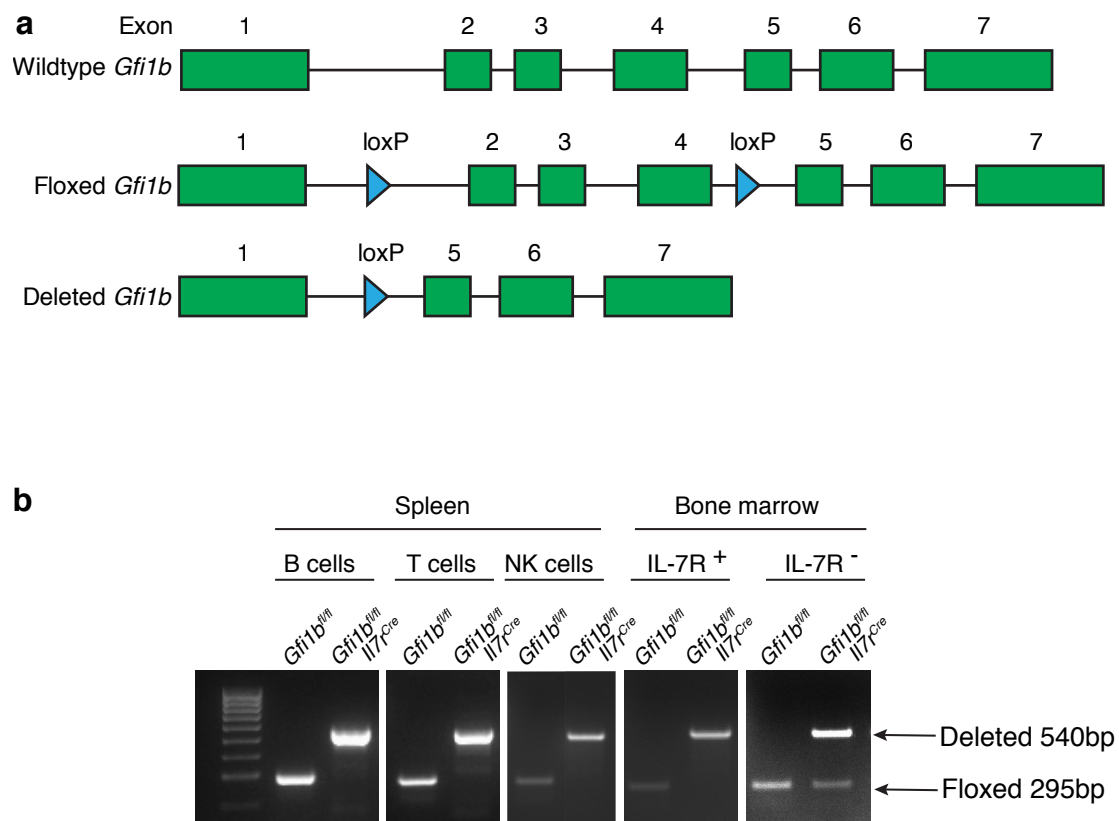


Figure 3.9. Mouse model of *Gfi1b* deletion driven by IL7R expression.

a. Schematic of the wild-type, floxed and deleted *Gfi1b* allele after Cre-mediated excision. Exons and loxP sites as indicated.

b. Floxed and deleted *Gfi1b* alleles identified by PCR. PCR was performed with primers targeting the floxed region (top) and deletion region (bottom) in B cells, T cells and NK cells purified from the spleen, and IL-7R⁺ and IL-7R⁻ cells sorted from the bone marrow of *Gfi1^{fl/fl}* and *Gfi1^{fl/fl} Il7^{Cre}* mice ($n=1-2$ mice/genotype from one experiment).

Next I analysed bone marrow progenitors from *Il7ra^{Cre}Gfi1b^{fl/fl}* mice to determine the impact of loss of GFI1B on their development. There was a significant reduction in both the frequency and total cell number of CLP within the bone marrow of GFI1B-deficient mice (Fig. 3.10a-c). The frequency of the CILP and ILCp within the bone marrow did not significantly decrease, however, a 2 to 3-fold reduction was found in the total cell number (Fig. 3.10a-c). There was also a significant reduction in both the frequency and total cell number of ILC2p (Fig. 3.10a-c). From the NK cell lineage, there was a 3.5 and 1.6 fold reduction in the total cell number of both the NKp and iNK cells, respectively within the bone marrow of GFI1B-deficient mice but by the mNK cell developmental stage, the number of GFI1B-deficient mNK cells is comparable to the number within control mice (Fig. 3.10d). The reduction in the number of early bone marrow progenitors indicated that GFI1B expression was important for either the development and/or maintenance of progenitors within the bone marrow.

GFI1B is not required by mature NK cells

Deletion of GFI1B resulted in a loss in the number of early progenitors but not for mNK cells in the bone marrow. I further examined the impact of this deletion on the development of mature ILC1 and NK cells in the periphery. The total number of ILC1 in the spleen and liver as well as number of splenic NK cells in GFI1B-deficient mice is comparable to the numbers within the control mice (Fig. 3.11a-b). There was an increase in the total number of NK cells within the liver and this was also reflected in an increase in the number of each NK maturation subset within the liver (Fig. 3.11b). This indicated that GFI1B-deficient mature ILC1 and NK cells were not impacted by the developmental defect in the bone marrow and could maintain comparable numbers in the peripheral organs. Furthermore, the frequency of each maturation subset was similar between GFI1B-deficient mice and control mice (Fig. 3.11a-b), which suggested that GFI1B did not play any role in NK cell maturation.

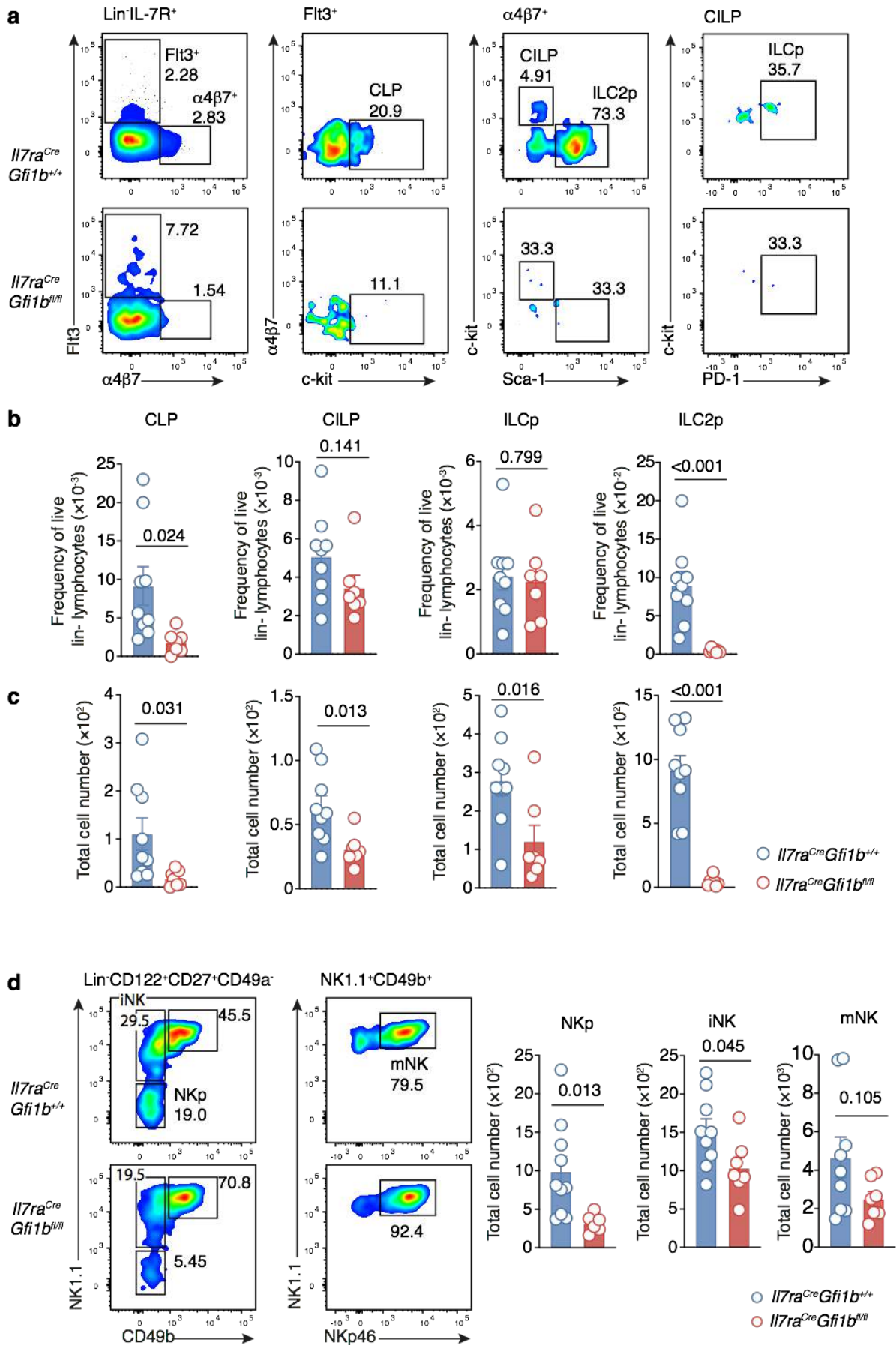


Figure 3.10. GF11B-deficient mice have reduced bone marrow progenitors.

- a.** Flow cytometric profiles of the early progenitors in the bone marrow of *Il7ra^{Cre}Gfi1b^{+/+}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. Within the live lin⁻ (CD3⁺TCRβ⁺CD11b⁺CD19⁺B220⁺NK1.1⁺CD122⁺NKp46⁺F4/80⁺Ter-119⁺) IL-7R⁺ gate, CLP (Flt3⁺ckit^{int}), CILP (α4β7⁺ckit^{int}), ILCp (α4β7⁺ckit^{int}PD-1⁺) and ILC2p (α4β7⁺Sca-1⁺) were identified.
- b.** Frequency and **c.** total number of bone marrow progenitors as indicated above (**a**) was enumerated. Data show the mean ± SEM from each bone marrow progenitor population pooled from two independent experiments (*n*=3-5 mice/genotype/experiment). Statistical significance was calculated using an unpaired Student's *t* test.
- c.** Flow cytometric profiles (left) and histograms enumerating the total cell number (right) of the NK cell lineage committed progenitors in the bone marrow of *Il7ra^{Cre}Gfi1b^{+/+}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. Data show and mean ± SEM of NKp (NK1.1⁻CD49b⁻ cells), iNK cells (NK1.1⁺CD49b⁻) and mNK cells (NK1.1⁺CD49⁺) pooled from two experiments (*n*=3-5 mice/genotype/experiment). Statistical significance was calculated using an unpaired Student's *t* test.

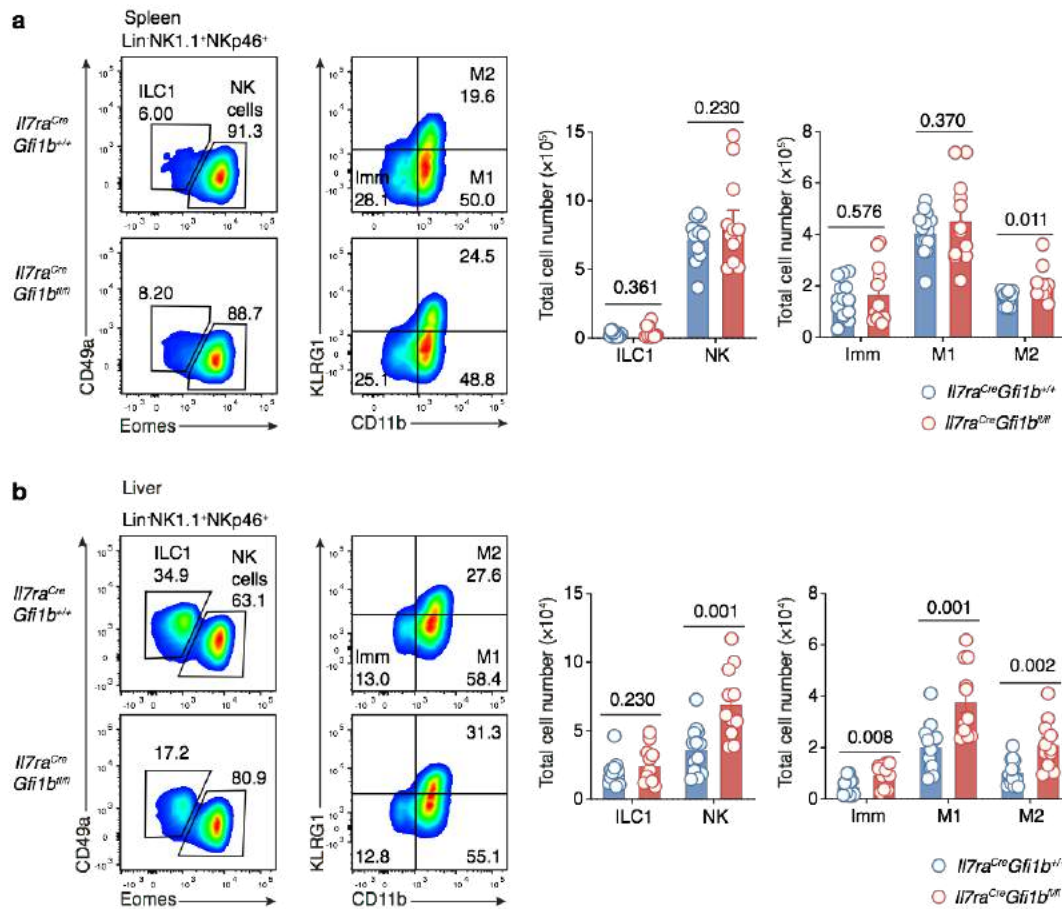


Figure 3.11. Loss of GFI1B impacted mature NK cell localisation in the liver.

a, b. Enumeration of the total number of ILC1 and NK cells from the spleen (**a**) and liver (**b**) of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice. Flow cytometric profiles showing the frequency (left) of ILC1 (CD49a⁺), NK cells (Eomes⁺) from lin⁺ NK1.1⁺NKp46⁺ cell population and each maturation stage (Imm CD11b⁻KLRG1⁻, M1 CD11b⁺KLRG1⁻ and M2 CD11b⁺KLRG1⁺) from NK cells. Histograms (right) show the mean $\pm$ SEM of the total number of ILC1, NK cells and NK cell subsets pooled from three experiments ($n=3-5$ mice/genotype/experiment). Statistical significance was calculated using an unpaired Student's *t* test.

Loss of GFI1B reduced ILC2 in the lung but not in small intestine

To determine the impact of GFI1B deletion on the development of mature ILC2 and ILC3 cells in the periphery, the lung, mesenteric LN and small intestine were analysed. There was a 5.6-fold reduction in the number of ILC2 in the lung of GFI1B-deficient compared to the lungs of wild-type mice (Fig 3.12a). A 1.4-fold reduction of ILC2 numbers was also observed in the mesenteric LN but ILC2 numbers in small intestine were comparable to the numbers within the wild-type mice (Fig 3.12a). No difference was observed in ILC3 numbers within the mesenteric LN and small intestine of GFI1B-proficient and deficient mice (Fig 3.12b). This showed that while ILC2 development within the lung was significantly impaired from loss of GFI1B, the development of other ILC subsets in the periphery were unaffected. This difference implied that there may be organ specific reason for the significant reduction of ILC2 in the lung and to a lesser extend the mesenteric LN but normal numbers within the small intestine.

Despite the significant loss of ILC2p numbers in the bone marrow and ILC2 in the lung, there was no significant difference in the number of ILC2 in the small intestine. Since GFI1B was not expressed in ILC2 in the small intestine and mesenteric LN, I questioned whether ILC2 in the lung may express GFI1B and could be impacted by the loss of GFI1B. Analysis of GFI1B-GFP in *Gfi1b^{GFP}Gfi1^{Tomato}* reporter mice showed that while ILC2 in the mesenteric LN and small intestine did not express GFI1B, 12-16% of ILC2 in the lungs did express GFI1B (Fig. 3.13a). I then examined the expression of the IL-33 receptor, ST2 and the activation marker KLRG1 on ILC2s in these organs and found that there was no significant difference in the expression of these markers on GFI1B-deficient ILC2 compared wild-type mice (Fig 3.13b-c). This indicated that the GFI1B-deficient ILC2s are phenotypically similar to their wild-type counterparts in the lung, mesenteric LN and small intestine and the loss of ILC2 numbers in the lung is unlikely to be due to a signalling defect.

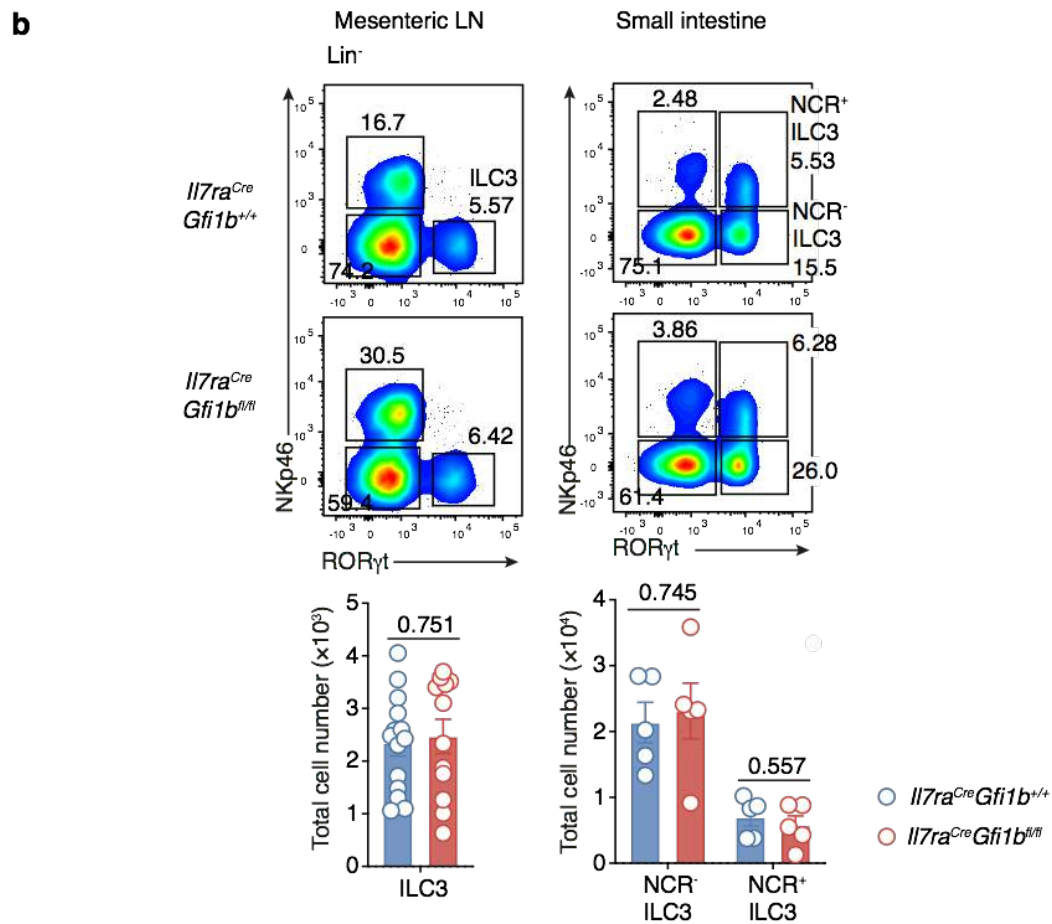
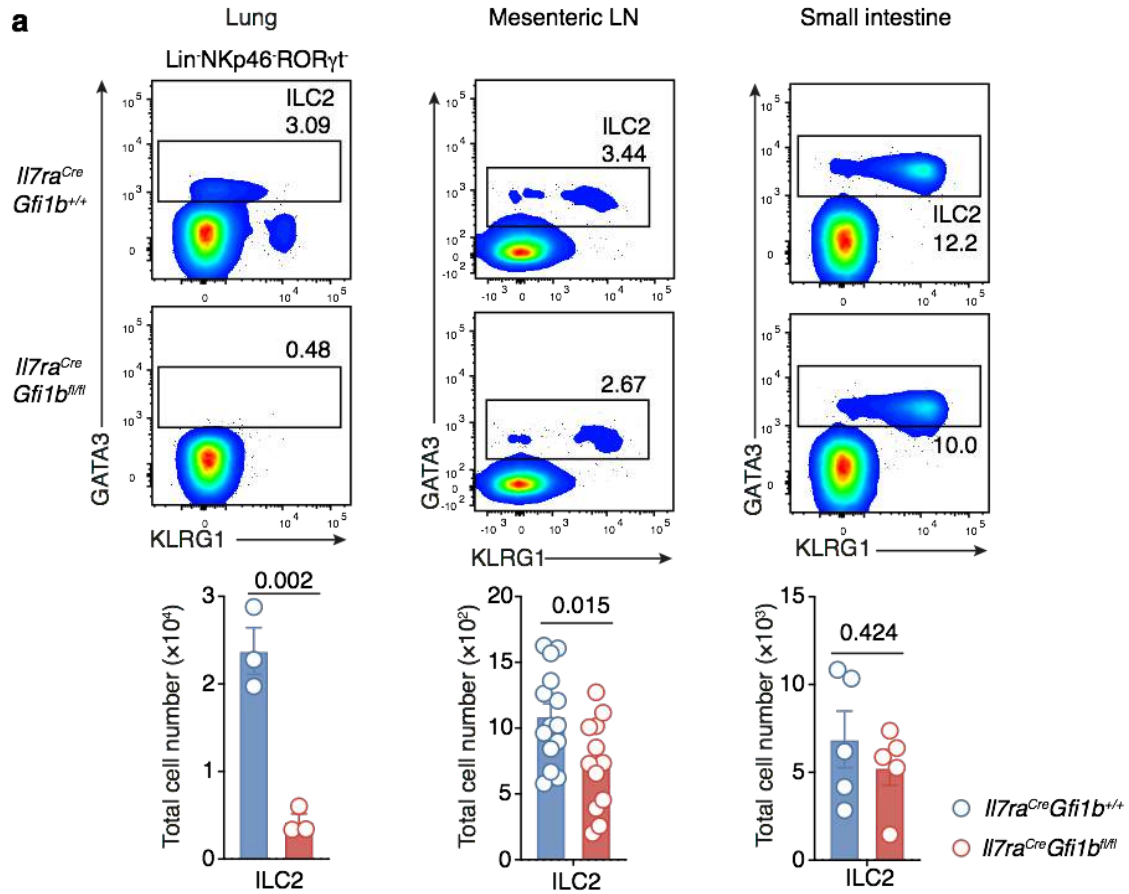


Figure 3.12. Loss of GFI1B alters ILC2 numbers in the lung.

a. Enumeration of the total cell number of ILC2s from the lung, mesenteric LN and small intestine of *Il7ra^{Cre}Gfi1b^{+/+}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. Flow cytometric plot showing frequency of ILC2 (ROR γ t⁺NKp46⁻GATA3⁺) from the live lin⁻ROR γ t⁺NKp46⁻ cell population. Histograms show the mean $\pm$ SEM of total cell number of each organ/animal from one experiment for the lung, pooled from three experiments for the mesenteric LN and one of three independent experiments for the small intestine ($n = 3$ -5 mice/genotype/experiment). Statistical significance was calculated using unpaired Student's *t* test.

b. Enumeration of the total cell number of ILC3s from the mesenteric LN and small intestine of *Il7ra^{Cre}Gfi1b^{+/+}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. Flow cytometric plot (top) show frequency of ILC3 (ROR γ t⁺NKp46⁻) in the mesenteric LN and NCR⁻ and NCR⁺ ILC3 in the small intestine () of the live lin⁻ cell population. Histograms show the mean $\pm$ SEM of total cell number (bottom) pooled from three experiments for the mesenteric LN and one of three independent experiments for the small intestine ($n=3$ -5 mice/genotype/experiment). Statistical significance was calculated using unpaired Student's *t* test.

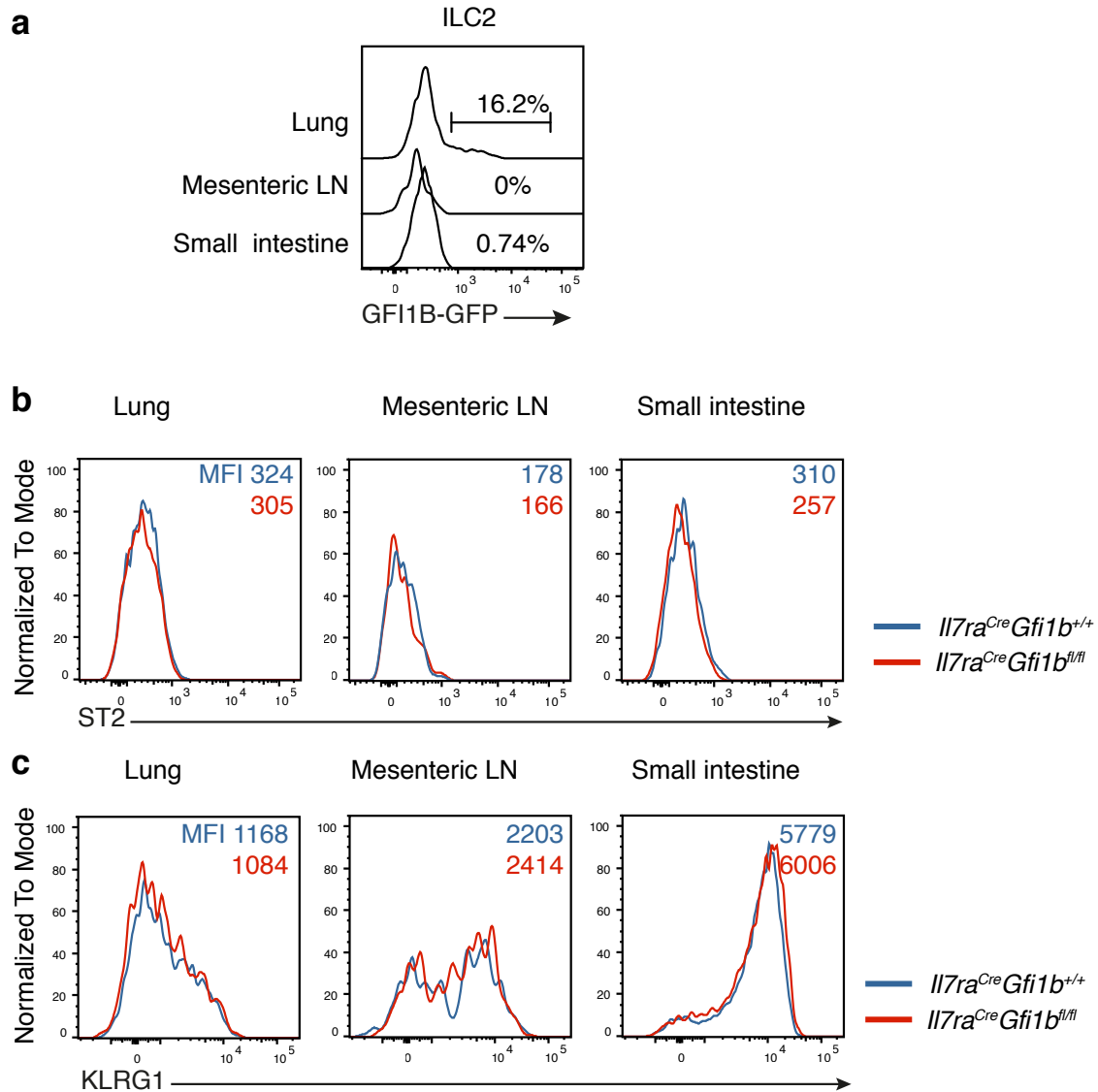


Figure 3.13. GFI1B is expressed by ILC2 in the lung but not in mesenteric LN or small intestine.

a. Expression of GFI1B-GFP in ILC2 in the lung, mesenteric LN and small intestine from *Gfi1b^{GFP}Gfi1^{Tomato}* mice. Data show representative histograms of organs from one mouse from one experiment ($n = 2-4$ mice/organ). Frequency of GFP⁺ ILC2 of each individual organ is indicated.

b-c. Expression of surface receptors **b**, ST2 (IL-33R) and **c**, KLRG1 on ILC2 from the lung mesenteric lymph node and small intestine of *Il7ra^{Cre}Gfi1b^{+/+}* (blue line) and *Il7ra^{Cre}Gfi1b^{fl/fl}* (red line) mice. Geometric MFI of ILC2s from each genotype is indicated. Data show representative histograms of one mouse from one experiment ($n=3$ mice/genotype/experiment).

GFI1B-deficient bone marrow progenitors have impaired development of lung ILC2

The loss of GFI1B had significantly affected ILC2 numbers in the lung but development of other ILC subsets were not affected to the same extent in other organs. I hypothesised that the other ILC subsets had originated from an alternative progenitor source that was not impacted by loss of GFI1B. To test the capacity of ILCs to develop specifically from progenitors from the bone marrow, bulk bone marrow cells from either GFI1B-deficient or GFI1B-proficient mice were transferred into sub-lethally irradiated Rag2 γ C mice (Fig. 3.14a). Rag2 γ C mice lack ILCs so all ILCs recovered from these mice five weeks post transfer would have originated from the donor bone marrow cells and would not be subjected to competition with host ILCs. GFI1B-deficient bone marrow cells were able to reconstitute ILC1 and NK cells to comparable numbers to wild-type bone marrow cells in the spleen, liver, lung and small intestine (Fig. 3.14b). These cells also had similar frequencies of Ki67 expression (Fig. 3.14c), indicating that they had similar proliferative capacity to wild-type cells.

ILC2p in the bone marrow was reduced in GFI1B-deficient mice compared to wild-type mice. This was also reflected in the reduction of ILC2p in the bone marrow of host mice transferred with GFI1B-deficient bone marrow cells (Fig 3.15a). Similarly, less ILC2 had reconstituted in the lung and small intestine from the donor GFI1B-deficient bone marrow cells compared to mice which received wildtype bone marrow although the latter had not yet reached statistical significance (Fig. 3.15b,c). There was no statistically significant difference in the number of ILC3 reconstituted in the small intestine, nor was there a difference in the expression of Ki67 in ILC2 and ILC3 that had developed in the lung and small intestine (Fig. 3.15c,d). Together, this showed that GFI1B-deficient bone marrow cells had the capacity to generate the ILC1, NK cells and ILC3 in the periphery at comparable numbers to those that had developed from wildtype bone marrow progenitors but not for ILC2.

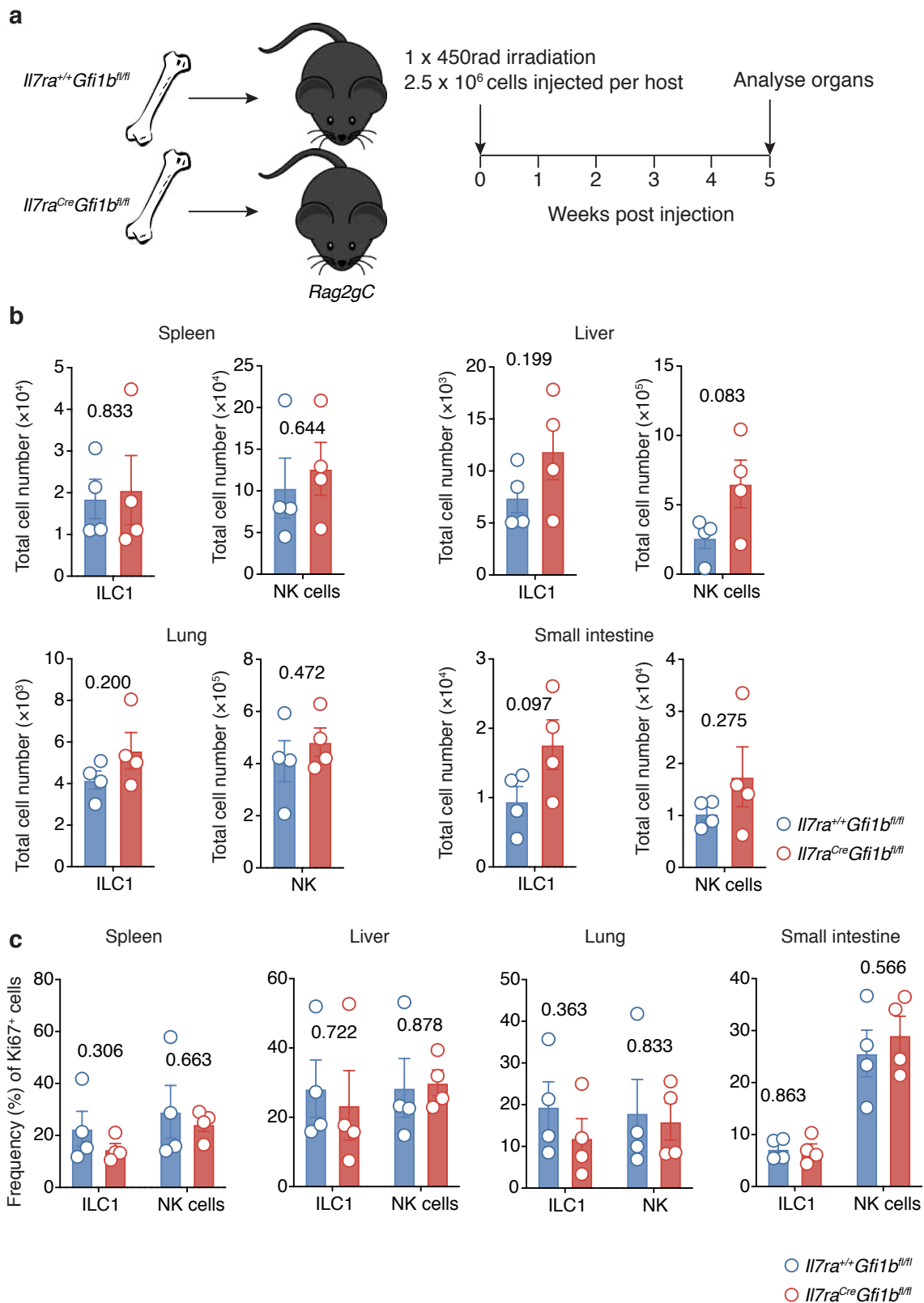


Figure 3.14. GFI1B-deficient bone marrow has full potential to generate ILC1 and NK cells in periphery.

- a.** Schematic showing the bulk transfer of bone marrow from *Il7ra*^{+/+}*Gfi1b*^{fl/fl} and *Il7ra*^{Cre}*Gfi1b*^{fl/fl} mice into sub-lethally irradiated *Rag2*^{γ^{-/-}} recipient mice. Reconstitution of ILC subsets in peripheral organs was analysed five weeks later.
- b.** Enumeration of total ILC1 and NK cell numbers from the spleen, liver, lung and small intestine of *Rag2*^{γ^{-/-}} mice reconstituted with either *Il7ra*^{+/+}*Gfi1b*^{fl/fl} or *Il7ra*^{Cre}*Gfi1b*^{fl/fl} bone marrow.
- c.** Frequency of Ki67⁺ cells in ILC1 and NK cells in the spleen, liver, lung and small intestine reconstituted from either *Il7ra*^{+/+}*Gfi1b*^{fl/fl} or *Il7ra*^{Cre}*Gfi1b*^{fl/fl} bone marrow.
- b-c.** Histograms show the mean ± SEM from one experiment (*n*=4 mice/genotype /experiment). Statistical significance was calculated using unpaired Student's *t* test.

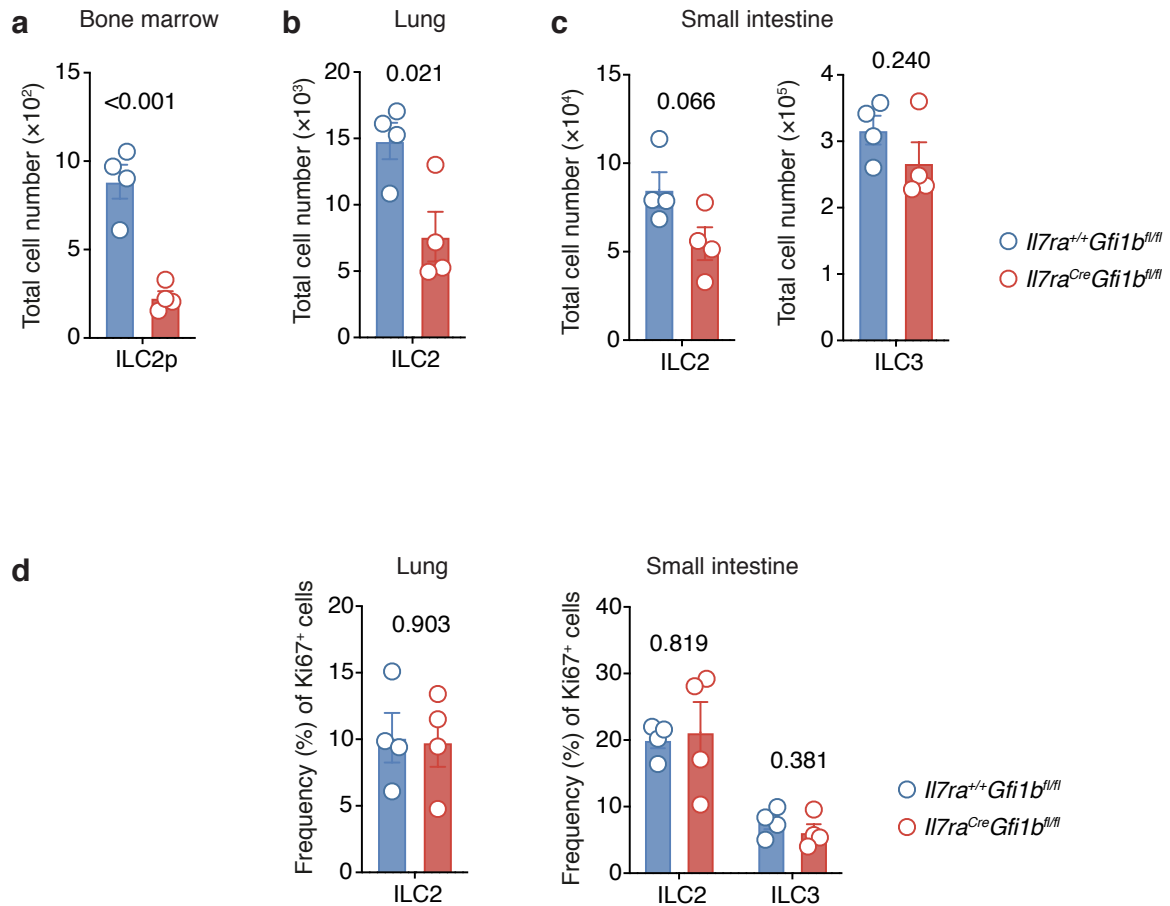


Figure 3.15. Gfi1b deficient BM have reduced capacity to reconstitute ILC2 in periphery.

a-c. Enumeration of total ILC2p from the bone marrow (**a**), ILC2 from the lung (**b**) and ILC2 and ILC3 from the small intestine (**c**) of *Rag2 γ ^{-/-}* mice reconstituted with either *Il7ra*^{+/+}*Gfi1b*^{fl/fl} or *Il7ra*^{Cre}*Gfi1b*^{fl/fl} bone marrow. Histograms show the mean $\pm$ SEM from one experiment ($n=4$ mice/genotype /experiment). Statistical significance was calculated using unpaired Student's *t* test.

d. Frequency of Ki67⁺ cells in ILC2 from the lung and ILC2 and ILC3 from the small intestine of *Rag2 γ ^{-/-}* mice reconstituted with either *Il7ra*^{+/+}*Gfi1b*^{fl/fl} or *Il7ra*^{Cre}*Gfi1b*^{fl/fl} bone marrow. Histograms show the mean $\pm$ SEM from one experiment ($n=4$ mice/genotype /experiment). Statistical significance was calculated using unpaired Student's *t* test.

3.3.4 The functional capacity of activated GFI1B-deficient lung ILC2

Lung inflammation is impacted by loss of GFI1B during papain challenge

At steady-state, the loss of GFI1B affected the ability to generate adequate numbers of lung ILC2. This suggested that GFI1B-deficient mice may respond differently to being challenged with an agent to induce lung inflammation that is dependent on a robust ILC2 response. To test this, both *Il7ra^{Cre}Gfi1b^{fl/fl}* and *Il7ra^{+/+}Gfi1b^{fl/fl}* control mice were administered intranasally with either PBS or papain for three consecutive days to induce lung inflammation. On the fourth day, the mice were taken for histology and flow cytometric analysis (Fig. 3.16a). Histological examination of the lung of mice given PBS or papain was shown by H&E staining (Fig. 3.16b). Wild-type and GFI1B-deficient mice that received PBS showed thin-walled alveolar with no minimal infiltration of polymorphonuclear (PMN) cells into the alveolar spaces, whereas papain treated wild-type mice had thickened alveolar septum and increased infiltration of PMN and eosinophils within the alveolar spaces, indicative of peribronchiolar inflammation (Fig. 3.16b). GFI1B-deficient mice treated with papain also had increased infiltration of PMN and eosinophils in the alveolar spaces compared to PBS treated mice but less than the papain treated control mice (Fig. 3.16b). This indicated that a lower inflammatory response was triggered in GFI1B-deficient mice treated with papain compared with control mice.

Enumeration of ILC2 within the lungs by flow cytometry showed that at steady state, the loss of ILC2 in the lungs of GFI1B-deficient mice was again observed (Fig. 3.16c). In papain treated mice, there was a two-fold reduction in the number of GFI1B-deficient ILC2 in the lung compared to wild-type ILC2 numbers (Fig. 3.16c). While there was no significant difference in the total ILC2 numbers in the mediastinal LN of GFI1B-deficient mice compared to control mice at steady state, there was a trend of increase in the number of ILC2 in the mediastinal LN of papain-treated GFI1B-deficient mice. There were no statistically significant differences in the number of ILC2 in the mesenteric LN of GFI1B-deficient mice compared with the control at steady-state, nor after papain treatment (Fig. 3.17c).

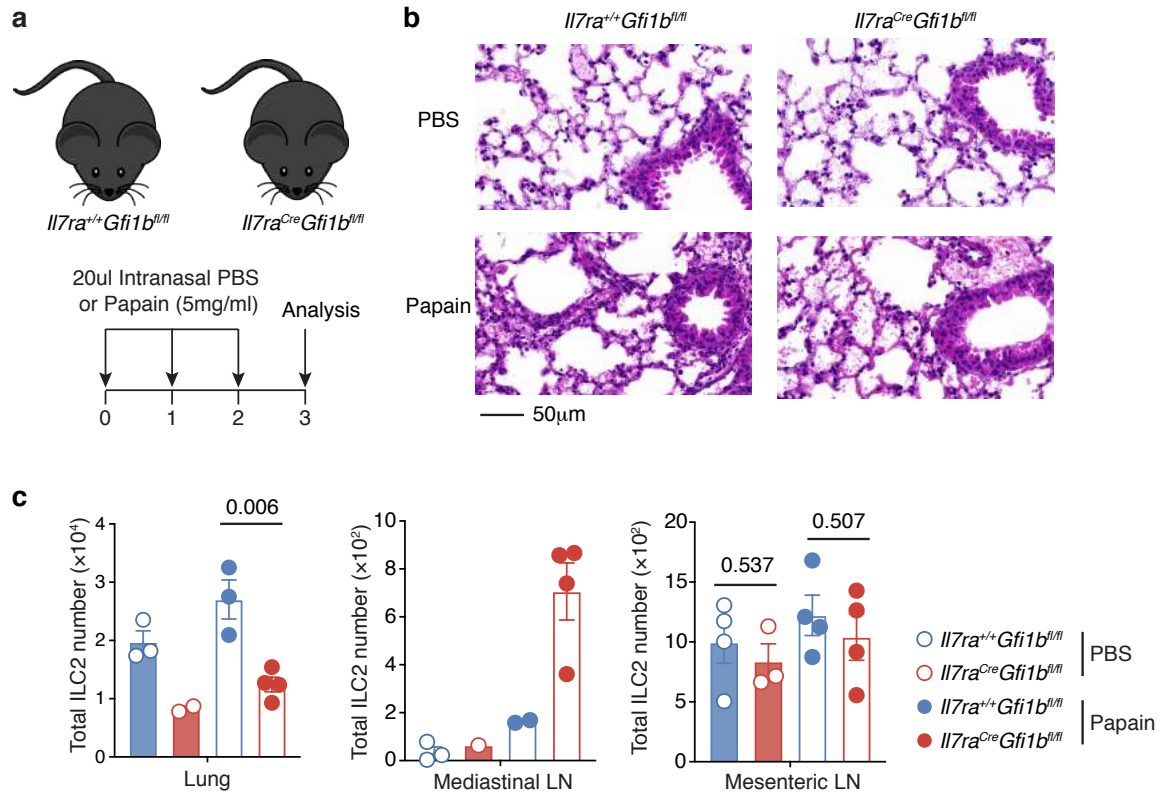


Figure 3.16. ILC2 numbers reduced in the lung but accumulate in the mediastinal LN of GFI1B-deficient mice during papain challenge.

a. Schematic of the experimental setup. *Il7ra^{+/+}Gfi1b^{fl/fl}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice received either PBS or papain administered intranasally for three consecutive days. On the fourth day, papain treated mice were intravenously injected with CD45 antibody five minutes prior to sacrificing the mice and harvesting the organs.

b. Representative histology images of the right superior lobe of the lung of PBS and papain treated *Il7ra^{+/+}Gfi1b^{fl/fl}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice, 40x magnification, 50μm scale bar as indicated.

c. Enumeration of ILC2 (lin⁻IL-7R⁺ RORγt⁺ GATA3⁺) numbers in the lung, mediastinal LN and mesenteric LN of *Il7ra^{+/+}Gfi1b^{fl/fl}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice treated with either PBS or papain. Histograms show the mean ± SEM from one experiment (n=1-4 mice/genotype/treatment/experiment). Statistical significance was calculated using unpaired Student's *t* test.

ILC2 effector functions not impacted by GFI1B deletion

During inflammation, ILC2s can migrate from organs such as the small intestine, into the circulation to be trafficked to the inflamed organ and can proliferate locally¹³³⁻¹³⁵. Therefore, the functionality of GFI1B-deficient ILC2s during papain treatment was assessed. To determine if there were defects in the recruitment of GFI1B-deficient ILC2 to the lungs during papain treatment, CD45 fluorescent antibody was injected into the circulation of the mice five minutes prior to sacrificing them. This was used to identify the CD45 IV⁺ cells that were within the circulation, compared to cells that deep within the tissue, that would be CD45 IV⁻. There was no difference in the frequency of ILC2 that were CD45⁺ in the lungs of GFI1B-deficient mice compared to the control mice (Fig. 3.17a), indicating that there is no intrinsic defect in the ability of GFI1B-deficient ILC2 to migrate. To measure the proportion of ILC2s undergoing cell cycling, Ki67 was used to identify proliferating cells. While there was an increase in the proportion of Ki67⁺ ILC2 in papain treated mice compared to PBS treated mice, there was no statistically significant difference in the frequency of Ki67⁺ ILC2 within the lung tissue (CD45 IV⁻ cells) between GFI1B-deficient and GFI1B-proficient ILC2 at both steady-state and after papain treatment, nor was there a significant difference in ILC2s that were in the circulation (CD45 IV⁺ cells) (Fig. 3.17b).

Expression of KLRG1, a marker of activation, was also examined on lung ILC2 and was found to have increased upon challenge with papain. However, there was no difference in KLRG1 expression between GFI1B-deficient and GFI1B-proficient ILC2 in the tissue and in the circulation (Fig. 3.17c). This suggested that GFI1B is not required for ILC2 activation. When lung lymphocytes were re-stimulated with PMA and ionomycin, there was no statistically significant difference in the frequency of IL-5 or IL-13-producing cytokines between GFI1B-deficient and GFI1B-proficient ILC2 within the tissue at steady state nor when challenged with papain (Fig 3.17d). There was also no statistically significant difference in the frequency of cytokine producing ILC2 within the circulation (Fig 3.17d). Together, this shows that the reduction of ILC2 numbers in the lungs of GFI1B-deficient mice remained during lung inflammation, but there was no intrinsic defect of activation and function of GFI1B-deficient ILC2 when challenged with papain. However, this experiment will require more repeats to confirm this.

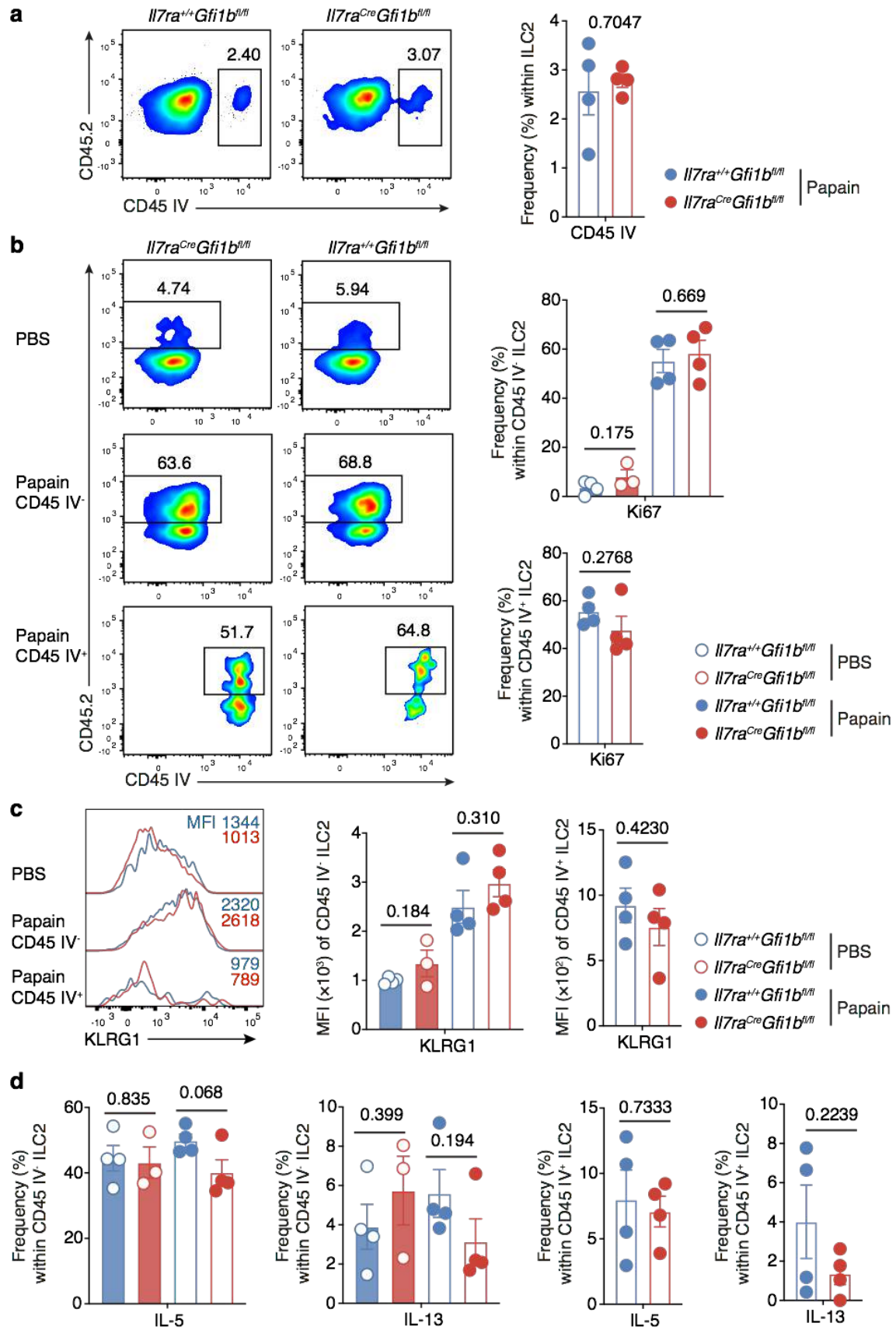


Figure 3.17. GFI1B does not regulate ILC2 cytokine production and localization.

- a.** Flow cytometric density plots (right) and histograms (left) showing the frequency of lung ILC2 from papain treated *Il7ra*^{+/+}*Gfi1b*^{fl/fl} and *Il7ra*^{Cre}*Gfi1b*^{fl/fl} mice that are positive from intravenously injected CD45 antibody (CD45 IV⁺) to identify leukocytes that are present in the vasculature of the lungs compared to those that are deep within the organ.
- b.** Flow cytometric density plots (right) and histograms (left) showing the frequency of Ki67 expression within CD45 IV⁻ (top) and CD45 IV⁺ (bottom) lung ILC2 from PBS and papain treated *Il7ra*^{+/+}*Gfi1b*^{fl/fl} and *Il7ra*^{Cre}*Gfi1b*^{fl/fl} mice
- c.** Histograms of one representative mouse (left) and histograms of the MFI (left) of KLRG1 expression within CD45 IV⁻ and CD45 IV⁺ lung ILC2 from PBS and papain treated *Il7ra*^{+/+}*Gfi1b*^{fl/fl} and *Il7ra*^{Cre}*Gfi1b*^{fl/fl} mice.
- d.** Frequency of IL-5 and IL-13 producing cells within CD45 IV⁻ (left) and CD45 IV⁺ (right) lung ILC2 from PBS and papain treated *Il7ra*^{+/+}*Gfi1b*^{fl/fl} and *Il7ra*^{Cre}*Gfi1b*^{fl/fl} mice.
- a-f,** Histograms show the mean $\pm$ SEM from one experiment ($n=3-4$ mice/genotype/treatment/experiment). Statistical significance was calculated using unpaired Student's *t* test.

Inflammatory response does not drive ILC2p development

The expansion of ILC2 in the lungs from papain challenge is in part, due to local proliferation, as evident by the increase in Ki67 (Fig. 3.17b). But the pro-inflammatory state in the lung may also drive ILC2 development from progenitor in the bone marrow, which then migrate to the lung^{136, 137}. ILC2p in the bone marrow was examined and there was an 11-fold decrease in the number of GFI1B-deficient ILC2p compared to control ILC2p in bone marrow at steady state and a 5.6-fold reduction in papain-treated mice (Fig. 3.18a). This indicated that GFI1B-deficient ILC2p in bone marrow was unable to expand and compensate for the reduced cell numbers when challenged with papain. Recently, an IL-18R⁺ ILC2 precursor was identified in the lung to have the capacity to give rise to ILC2s¹³⁸. When these ILC2p in the lung was examined in GFI1B-deficient mice, there was no statistically significant difference in the number of ILC2p in the lung of GFI1B-deficient mice compared to control mice at steady-state and in mice treated with papain (Fig. 3.18b) indicating that these ILC2p in the lung are unlikely to be impacted by the loss of GFI1B.

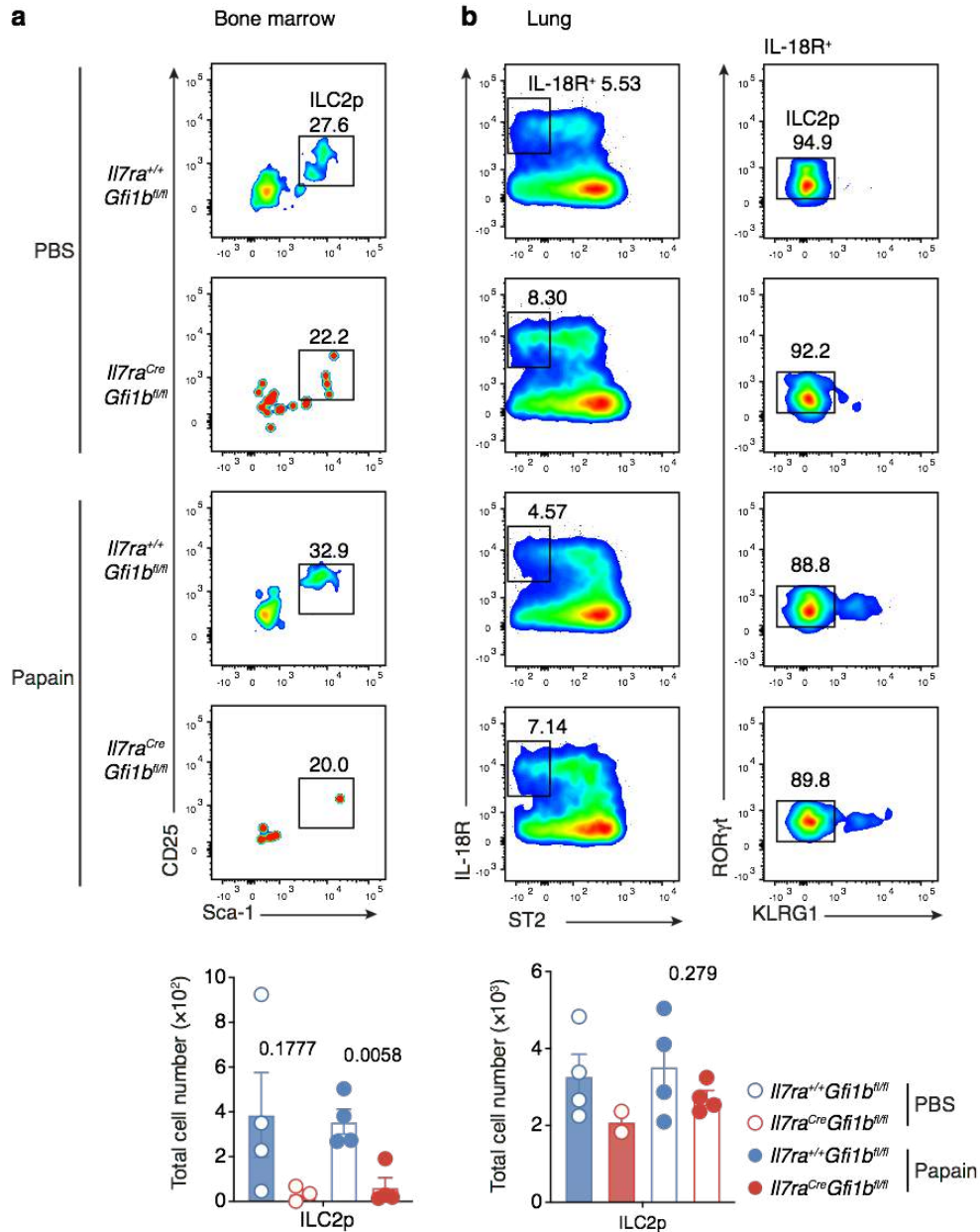


Figure 3.18. Loss of GFI1B impacted ILC2p in the bone marrow but not in the lung.

a. Flow cytometry density plots (top) and total cell number (bottom) of bone marrow ILC2p (lin⁺IL-7R⁺α4β7⁺Sca-1⁺CD25⁺) of PBS or papain treated *Il7ra^{+/+}Gfi1b^{fl/fl}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. Histograms show mean ± SEM from one experiment ($n=3-4$ mice/genotype/treatment). Statistical significance was calculated using unpaired Student's t test.

b. Flow cytometry density plots (top) and total cell number (bottom) lung ILC2p (lin⁺IL-7R⁺IL-18R⁺ST2⁻RORγt⁻KLRG1⁻) of PBS or papain treated *Il7ra^{+/+}Gfi1b^{fl/fl}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. Histograms show mean ± SEM from one experiment ($n=2-4$ mice/genotype/treatment). Statistical significance was calculated using unpaired Student's t test.

GFI1B-deficient mice have impaired eosinophil activation and recruitment

ILC2 play an important role in eosinophil recruitment and activation by producing cytokines such as IL-5²⁷. To assess the capacity of GFI1B-deficient ILC2 to recruit eosinophils, the eosinophil number within the lung, bronchiole alveolar lavage fluid (BALF), mediastinal LN and mesenteric LN was enumerated. Treatment with papain induced an accumulation of eosinophils within the lungs and BALF of wild-type mice compared to PBS treated mice (Fig 3.19a-b). There was no statistically significant difference in the number of eosinophils in the lungs of GFI1B-deficient mice compared to control mice challenged with papain, but there was a reduction in the eosinophil numbers in the BALF of GFI1B-deficient mice although this had not reached statistical significance (Fig. 3.19a-b). Conversely, there was a trend of increased eosinophils in the mediastinal LN of GFI1B-deficient mice compared to control mice when treated with papain (Fig, 3.19a-b). Papain treatment did not result in an increase in eosinophil numbers in the mesenteric LN and no significant difference was observed in eosinophil numbers in the between GFI1B-deficient and proficient mice (Fig. 3.19a-b), indicating immune cells in the mesenteric LN do not seem to be involved during lung inflammation, nor were the eosinophils impacted by loss of GFI1B in ILC2s. However, eosinophil recruitment and accumulation were impacted in the BALF and mediastinal LN of papain treated mice with GFI1B-deficient ILC2.

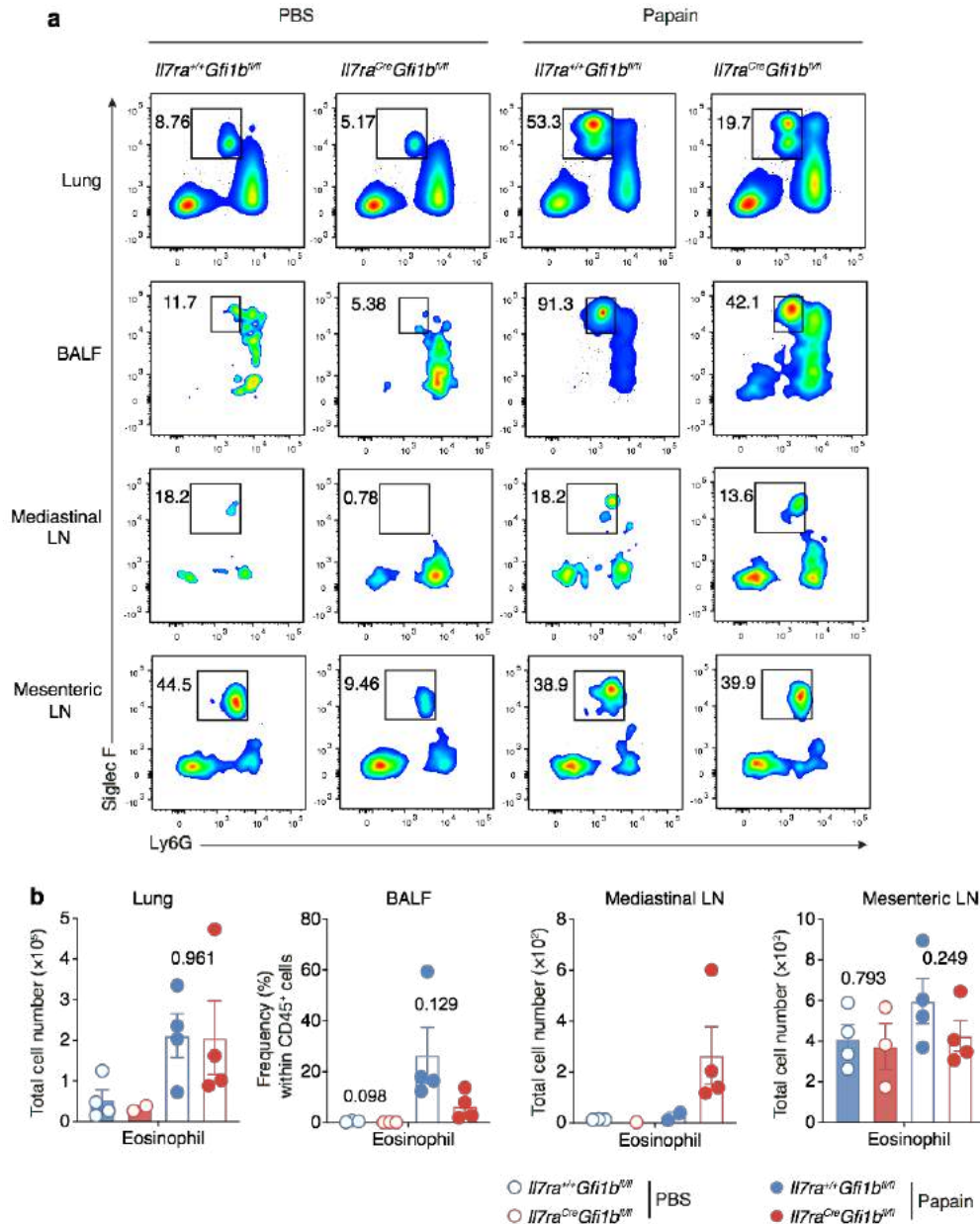


Figure 3.19. Eosinophil activation and localisation impacted by GFI1B-deficient ILC2 during papain challenge.

a. Flow cytometry density plots showing eosinophils (CD11b⁺MHCII⁺SiglecF⁺SSC^{hi}) from the lung, bronchiole alveolar lavage fluid (BALF), mediastinal LN and mesenteric LN of PBS or papain treated *Il7ra^{+/+}Gfi1b^{fl/fl}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice.

b. Enumeration of total eosinophil cell number in the lung, mediastinal LN and mesenteric LN and frequency of eosinophils within live CD45.2⁺ cells of the BALF of PBS or papain treated *Il7ra^{+/+}Gfi1b^{fl/fl}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. Histograms show the mean $\pm$ SEM from one experiment ($n=1-4$ mice/genotype/treatment/experiment). Statistical significance was calculated using unpaired Student's *t* test.

3.4 Discussion

Previously, the role of GFI1 and GFI1B in ILCs, especially during development of early progenitors, was not well understood. This work shows, with greater resolution, that GFI1 and GFI1B expression is heterogeneous and changes dynamically throughout ILC development in the bone marrow. While GFI1 was expressed by progenitors in the bone marrow and mature ILCs in the peripheral organs, GFI1B expression was restricted to the CLP, CILP and ILCp developmental stages of bone marrow progenitors. Expression of GFI1B, specifically at the CILP developmental stage, marks the progenitor cells that have multilineage developmental potential of all ILC subsets, while the CILP that do not express GFI1B primarily develop into NK cells and have a reduced capacity to develop into other ILC subsets. This identifies GFI1B⁺ CILP as the progenitor stage where ILC1 and NK cell development is delineated. This also indicates that CILP is a heterogeneous progenitor population with GFI1B marking the cells with greater developmental capacity to generate ILC1, ILC2 and ILC3 subsets in the periphery. The transcriptional regulation behind this increased developmental potential is currently unknown. To identify the transcriptional regulatory pathways involved to give multilineage capacity and developmental potency, GFI1B⁺ and GFI1B⁻ CILP cells would be sorted and sequenced to identify genes that are differentially expressed between these two populations.

3.4.1 GFI1B regulates early ILC progenitor development

By using the *Il7ra*^{Cre}*Gfi1*^{fl/fl} and *Il7ra*^{Cre}*Gfi1b*^{fl/fl} mouse models, GFI1 and GFI1B were shown to be required for the development of early ILC progenitors in the bone marrow. Although these mice are heterozygous for IL-7R, as the Cre construct replaces the first 70 nucleotides of exon 1, the *Il7ra*^{Cre} control mice are phenotypically normal since of the *Il7r* gene sequences upstream and downstream of exon 1 are preserved¹²¹. GFI1 was also required for the development ILC2, which is in accordance with previous reports, but is also important of NK cell progenitors in the bone marrow, which has not been reported. Further examination of the role of GFI1 in NK cells is addressed in chapter 4. Despite the loss of GFI1B impairing the development of bone marrow progenitors, mature ILCs were able to overcome this defect and achieve normal numbers in the periphery, with the exception of ILC2 in the lungs. These ILC1, NK and ILC3 in the periphery can be derived from progenitors in the bone marrow, and there was no difference in the proliferation between cells that developed from GFI1B-deficient or

GFI1B-proficient bone marrow progenitors. This ruled out the idea that mature ILCs from GFI1B-deficient mice relied on foetal liver progenitors independent of GFI1B to develop and did not originate from the bone marrow. However, bone marrow progenitors were unable to fully reconstitute ILC2 in the lungs and small intestine. There was no difference in the proliferative capacities of ILC2s that were generated from GFI1B-deficient bone marrow cells. This suggested that ILC2 from the lung develops from progenitors in the bone marrow whereas ILC2 in the small intestine may develop from an alternative progenitor.

ILC2p numbers were significantly reduced when GFI1B was deleted, despite not expressing GFI1B itself. This may reflect a defect in the developmental capacity of an earlier progenitor that relies on GFI1B for efficient ILC2p development. Furthermore, the transfer of bulk bone marrow cells did not take into account the initial reduction of progenitors within the bone marrow of GFI1B-deficient mice, especially the reduction in ILC2p. Therefore, it is also possible that the intrinsic capacity for ILC2p in the bone marrow to develop into ILC2 in the lung is normal, it was only impaired by the reduced numbers of progenitors available for development. To determine whether GFI1B affects the developmental potential of ILC2p in the bone marrow, equal numbers of bone marrow ILC2p would be transferred into ILC deficient host mice and evaluate the numbers of ILC2p that can develop. This will determine whether the reduction in mature ILC2 is merely due to a reduction in progenitor numbers and the developmental capacity of ILC2s are normal, or whether there is an intrinsic developmental defect due to the loss of GFI1B.

3.4.2 ILC2 immune response during inflammation

The reduction in ILC2 in the lungs of GFI1B-deficient mice impacted on the inflammatory response in papain treated mice. There was a reduced inflammatory response in the lungs of GFI1B-deficient mice as there was less eosinophil infiltration within the bronchiole alveolar space. While there was no significant reduction in the number of eosinophils recruited to the lung of GFI1B-deficient, this may be due to under sampling and a clearer difference may be observed with repeated experiments. This inability to recruit eosinophils was predominantly due to reduced ILC2s present in the lung as there was no difference in ILC2 activation and cytokine production in GFI1B-deficient mice. In the *Il7ra^{Cre}Gfi1b^{fl/fl}* mice, T cells are also deficient for GFI1B. However, there was no difference in the number of CD4⁺ or CD8⁺ T cells

in the periphery of GFI1B-deficient mice at steady-state (data not shown). While T cell function was not examined, papain challenge drives an inflammatory response that is predominantly mediated by ILC2 and is independent of the adaptive immune system. Therefore, it is unlikely that the changes observed in the inflammatory response are related to T cell dysfunction from the loss of GFI1B.

Papain treatment had also induced an accumulation of ILC2 and eosinophils in the mediastinal LN of GFI1B-deficient mice. No increase in ILC2 number was observed in the mesenteric LN, suggesting that this is specific to the draining LN during an inflammatory response. This increase of ILC2 could be due to increased local proliferation, either reflecting an intrinsic capacity for proliferation or due to increased stimulation from molecules drained from the lungs. An alternative explanation of the increase in GFI1B-deficient ILC2 in the mediastinal LN is due to the dysregulation in the balance between ILC2 ingress and egress through the LN. It is possible that GFI1B-deficient ILC2 fail to egress from the mediastinal LN during papain challenge and subsequently accumulate within the lymph node. ILC2s are tissue-resident at steady-state and do not migrate¹³⁹. However, ILC2s from the intestine can migrate to the lung or mesenteric LN in an S1P-dependent manner during inflammation¹⁴⁰. Specific ILC subsets express specific homing receptors in a tissue specific manner. CCR7, CCR9 and $\alpha 4\beta 7$ have been identified to be expressed by ILCs, including ILC2, throughout the gastrointestinal tract and mesenteric LN^{141, 142}. CCR9 and $\alpha 4\beta 7$ but not CCR7 was required for normal ILC2 numbers in the gastrointestinal tract¹⁴¹. It is not known if these homing receptors are expressed by ILC2 in the lung or if they play a similar role in homing to the lung and associated draining LN during inflammation. More precise examination of ILC2 trafficking between lung and mediastinal LN can be done using *Kaede* transgenic mice¹⁴³, which contain a photoconvertible fluorescent protein to directly identify either migratory or tissue resident cells within a LN.

In summary, this data has identified a novel role of GFI1B in bone marrow progenitors during the early development ILCs. GFI1B-expressing CILP represent a subset of progenitor cells with greater multi-lineage developmental potential. Expression of GFI1B in bone marrow progenitors is vital for their developing in the bone marrow and also for ILC2s in the lung. Understanding the role of GFI1B in the development of ILCs and in particular, its role in ILC2 in the lung will be important in understanding the tissue-specific transcriptional regulation of these cells.

Chapter 4. Understanding the role of GFI1 in NK cells

3.1 Abstract

Natural killer (NK) cells are innate lymphocytes that protect against virus infections and cancerous cells. To ensure this process occurs effectively, NK cell maturation and function must be tightly regulated by transcription factors. Growth factor independent 1 (GFI1) is a transcription factor that is critical for the regulation of CD4⁺ T cells and Group 2 innate lymphoid cell development and function but has not been implicated in other cell types. RNASeq data showed differential expression of GFI1 in NK cells, therefore, I hypothesised that GFI1 may be involved in NK cell regulation.

Using *Gfi1*^{Tomato} reporter mice, I mapped GFI1 expression and demonstrated that it is upregulated during NK cell maturation. Given this high expression, to determine the requirement for GFI1 in peripheral NK cells, I examined how deletion affected NK cell subsets and observed a significant reduction in mature NK cell numbers. Using RNASeq analyses, several dysregulated pathways were identified, including cell proliferation, migration and activation. Defects in these pathways suggest that NK cell mediated protection may be impaired. Therefore, I challenged mice with B16F10 melanoma cells and found a striking increase in the metastatic burden in the lungs of mice with GFI1-deficient NK cells, compared to the wildtype mice. Collectively, these results indicate that GFI1 regulates key processes of NK cell maturation and function, which are required to mediate effective anti-tumour protection.

3.2 Introduction

3.2.1 Transcriptional regulation of NK cell maturation

NK cells are ILCs that provide immune surveillance and mediate protection against virus infected and cancerous cells¹⁴⁴. NK cells undergo three stages of maturation, immature (Imm), mature 1 (M1) and mature 2 (M2) and tight transcriptional regulation is required for the efficient maturation and function of NK cells⁵³. Initially, immature NK cells were identified by the expression of CD27 and as they mature, they gain expression of the integrin CD11b and lose CD27 expression¹⁴⁵. Subsequently, killer cell lectin like receptor G1 (KLRG1) was identified as a marker of mature NK cells and the three sequential stages of NK cell maturation are defined as CD11b⁻KLRG1⁻ Imm NK cells, CD11b⁺KLRG1⁻ M1 NK cells and CD11b⁺KLRG1⁺ M2 NK cells¹⁴⁶. This maturation process is regulated through the interplay between transcription factors T-box expressed in T cells (T-bet) and Eomesodermin (Eomes)⁴³. T-bet expression is required for early development of immature NK cells but is downregulated by Eomes as NK cells mature⁵⁵. At each stage of maturation, NK cells acquire different functions^{145, 147}. Immature NK cells are the most proliferative subset, whereas mature NK cells gain effector functions such as IFN- γ and granzyme production^{145, 147}. This specificity of NK cell immune response at each maturation stage means that proper maturation is required to mediate effective protection against pathogens.

4.2.2 Activation and function of NK cells mediated via surface receptors

NK cell development and function is tightly regulated by the expression of transcription factors, which then in turn dictate NK cell response to external signaling via surface receptors⁵³. During development, NK cells egress from the bone marrow through the downregulation of C-X-C chemokine receptor type 4 (CXCR4), mediated by GATA3, and migrate throughout the body via the circulation^{148, 149}. NK cells can also express L-selectin (CD62L) to home to the regional lymph node (LN) where they mediate protection in the secondary lymphoid organs¹⁵⁰.

In the periphery, the capacity of NK cells to activate and exert effector functions is controlled by signaling via activating receptors such as natural cytotoxicity triggering receptor 1 (NCR1, also known as NKp46), DNAX accessory molecule-1 (DNAM-1) and inhibiting receptors such as T cell immunoreceptor with Ig and ITIM domains (TIGIT) and CD96^{151, 152}. For example,

interactions between the poliovirus receptor CD155, which is often overexpressed on tumour cells, and DNAM-1 drives NK cell anti-tumour activity, whereas engagement with TIGIT suppresses NK cell effector functions¹⁵³⁻¹⁵⁵. Once activated, NK cells can produce pro-inflammatory cytokines such as IFN- γ to modulate the adaptive immune response, and to facilitate direct lysis of target cells through the production of cytotoxic molecules such as perforin and granzyme B¹⁵⁶.

4.2.3 Potential for GFI1 as a regulator on NK cell fate

Growth factor independent 1 (GFI1) is a transcriptional regulator that can act by recruiting histone modifying proteins to regulate CD4⁺ T cell development and function. GFI1 can directly regulate gene expression of *Tbx21*, *Eomes* and *Runx2* by changing histone methylation in Th1 cells and also prevent GATA3 protein degradation in Th2 cells to promote a type-2 immune response^{105, 106, 15}. GFI1 is also an important regulator in ILC2 by suppressing genes associated with ILC3 development and function¹¹⁰. However, it is not known whether GFI1 plays a role in NK cell development or function. One transcription factor that has been identified as a key regulator of NK cell maturation and function is FOXO1⁵⁶. FOXO1 suppresses NK cell maturation by binding to the *Tbx21* promoter region and regulating its expression⁵⁶. FOXO1 also influences NK cell homing and effector functions when challenged with tumour⁵⁶. GFI1 has been shown to be a negative regulator of FOXO1 in DP thymocytes¹⁵⁷ but it is not known if a similar regulation also occurs in NK cells.

3.2.2 Aims and Hypothesis

In the previous chapter, I showed that GFI1 is highly expressed by NK cells throughout their development and that deletion of *Gfi1* early in development impacted the NKp and iNK progenitor stages of NK cells in the bone marrow. Furthermore, gene expression data generated from our laboratory indicated that *Gfi1* expression increases as NK cells mature (unpublished). Therefore, I hypothesised that GFI1 may be an important regulator of NK cell development. Since NK cells play an important role in mediating protection against virus infected or cancerous cells, loss of this regulation may result in loss of homeostasis and impact on immune response against these diseases.

The aim of this chapter was to:

- (a) Map the expression of GFI1 in NK cells using the novel reporter mouse strain.
- (b) Determine the role of GFI1 in NK cells through examining their maturation, proliferation and effector function.
- (c) Determine the molecular pathways regulated by GFI1 using RNASeq.

3.3 Results

3.3.1 Characterisation of GFI1 expression in ILC1 and NK cells

GFI1 is differentially expressed in NK cells and ILC1 during development in multiple tissues.

Using the *Gfi1b^{GFP}Gfi1^{Tomato}* dual reporter mouse strain, I have demonstrated that GFI1 was highly expressed in NK progenitor cells in the bone marrow, as well as mature NK cells and ILC1 in the liver. In contrast, GFI1B expression was low (Fig 3.1-3.3, Chapter 3). This suggests that GFI1B expression was not required by NK cells and ILC1 in the periphery. Therefore, I focused my subsequent analysis on GFI1 expression and function.

To gain a broader understanding of the expression of GFI1 in the group 1 ILCs, ILC1 and NK cells, bone marrow, inguinal LN, spleen, liver, lung and small intestine were analysed in *Gfi1b^{GFP}Gfi1^{Tomato}* dual reporter mice. These different tissues would allow assessment of whether tissue-specific microenvironment may affect GFI1 expression in tissue-resident and circulating ILC1 throughout the body. Expression of GFI1-Tomato in ILC1 in the bone marrow, inguinal LN, spleen and lung was low compared to ILC1 in the liver and small intestine, which was two-fold higher (Fig. 4.1a). By comparison, expression of GFI1-Tomato from NK cells in the bone marrow and inguinal LN was approximately 1.4 fold lower compared to the NK cells in the spleen, liver, lung and small intestine (Fig. 4.1a). This suggests that GFI1 expression may be important for ILC1 in a tissue specific manner, whereas GFI1 may play a similar function within NK cells, regardless of the organ.

Within the NK cell populations in the spleen, GFI1 expression was higher in the mature M1 and M2 subsets compared with the Imm subset (Fig. 4.1b). This was also observed in NK cell subsets in the liver and the lung (not shown). This differential expression of GFI1 during NK cell maturation suggests that GFI1 may regulate different processes at the different stages of NK cell maturation.

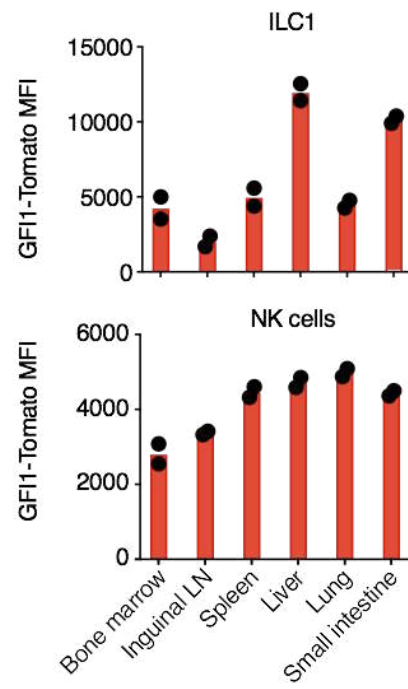
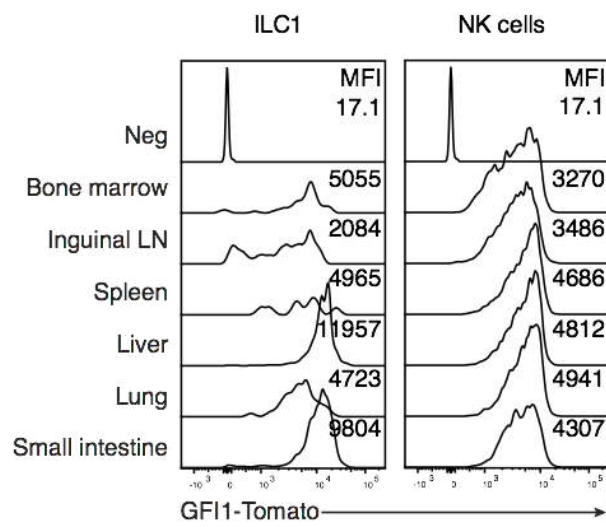
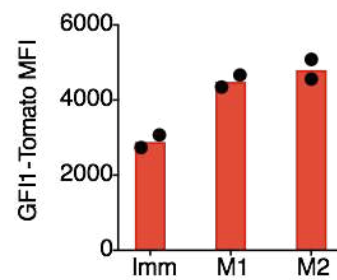
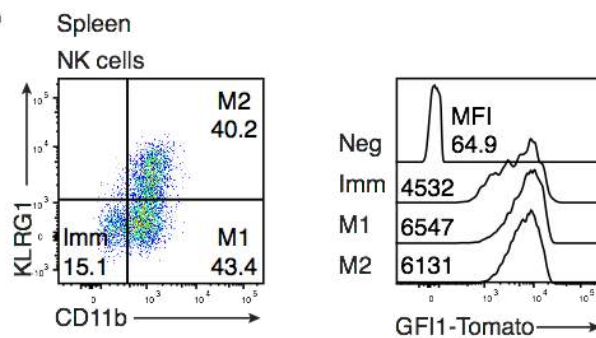
a**b**

Figure 4.1. Differential GFI1 expression in NK cells and ILC1 is development and tissue specific.

a, Histograms showing the expression of GFI1-Tomato from ILC1 (live CD3⁻TCRβ⁻CD19⁻NK1.1⁺NKp46⁺CD49a⁺) and NK cells (live CD3⁻TCRβ⁻CD19⁻NK1.1⁺NKp46⁺CD49b⁺) isolated from the bone marrow, inguinal LN, spleen, liver, lung and small intestine of *Gfi1*^{+/Tomato} mice, showing the mean fluorescent intensity (MFI) of GFI1-Tomato expression. Negative control (Neg) is gated on live lymphocytes from the bone marrow of *Gfi1*^{+/Tomato} mice that are negative for GFI1 expression. Data show representative histograms from the organs of one mouse (left) and the mean and individual data points from one representative experiment (bone marrow *n*=8, spleen *n*=8 and liver *n*=8 from four independent experiments, small intestine *n*=2, inguinal LN *n*=2, lung *n*=2 from one experiment).

b, Gating strategy showing flow cytometric dot plots of Imm (CD11b⁻KLRG1⁻), M1 (CD11b⁺KLRG1⁻) and M2 (CD11b⁺KLRG1⁺) NK cell maturation subsets (left panel) and histograms (middle and right panel) showing GFI1-Tomato expression. Cells were isolated from the spleen of *Gfi1*^{+/Tomato} mice and show the MFI of GFI1-Tomato expression. Negative control (Neg) is gated on live lymphocytes from the spleen of *Gfi1*^{+/Tomato} mice that are negative for GFI1 expression. Data show representative flow cytometry plots (left) histograms from one mouse (middle) and the mean and individual data points (right) from one of five similar experiments (*n*=2-3 mice/genotype/experiment).

Role of GFI1 in group 1 ILCs

Loss of GFI1 corresponds to a reduction in mature NK cells

Given that GFI1 was highly expressed in ILC1 and NK cell subsets (Fig. 4.1) I explored whether this GFI1 expression was important for the development and/or maintenance of these cells in peripheral organs at steady-state. To determine this, the *Gfi1^{fl/fl}* mouse¹⁰⁰ was crossed with the *NCR^{iCre+/T}* mouse¹¹⁷. Exons 4 and 5 of the *Gfi1* allele is flanked by loxp regions and these are excised by Cre-mediated recombination following NKp46-driven Cre expression. This deletion introduces a reading frame shift and results in the production of a non-functional protein¹⁰⁰ (Fig. 4.2a). NKp46 is expressed during the later stages on NK cell development and not at progenitor stages in the bone marrow. Therefore, deletion of GFI1 does not affect the progenitor stages of NK cell development and is restricted to NK cells in late stages of development.

To determine the deletion efficiency of GFI1 in NK cells, I examined expression of the *Gfi1* allele in Imm, M1 and M2 NK cell subsets from *NCR^{iCre+/T}* and *Gfi1^{fl/fl}NCR^{iCre+/T}* mice. Splenic NK cells of each maturation stage were sorted and RNA of each cell subset was converted into cDNA and sequenced. There was a loss of exons 4 and 5 in the *Gfi1* allele at each maturation stage of splenic NK cells (Fig. 4.2b). This was also observed in hepatic NK cell subsets (data not shown). This corresponds to the flanked loxP regions that are deleted on Cre-mediated recombination. We also found that other elements of the allele showed an increase in the level of expression of the *Gfi1* transcripts (Fig. 4.2b). This effect has previously been reported in T cells and is thought to reflect a self-regulatory mechanism utilized by this gene¹⁵⁸.

I used flow cytometry to analyse the group 1 ILC populations isolated from GFI1-deficient mice and observed that there was a two-fold reduction in the frequency of NK1.1⁺NKp46⁺ cells in the spleen and a five-fold reduction in the liver of GFI1-deficient *Gfi1^{fl/fl} NCR^{iCre+/T}* mice compared to *NCR^{iCre+/T}* control mice (Fig. 4.3a-b). This corresponded to a 1.5 and two-fold reduction in the total number of NK cells in the spleen and liver. The number of ILC1 in the spleen were not altered by the loss of GFI1. However hepatic ILC1s were almost completely depleted (Fig. 4.3b). Further analysis of NK cell maturation showed that while the number of Imm NK cells within the spleen and liver were unaffected, there was a progressive loss of M1 cells, followed by a substantial loss of M2 cells when GFI1 was deleted (Fig. 4.3a-b).

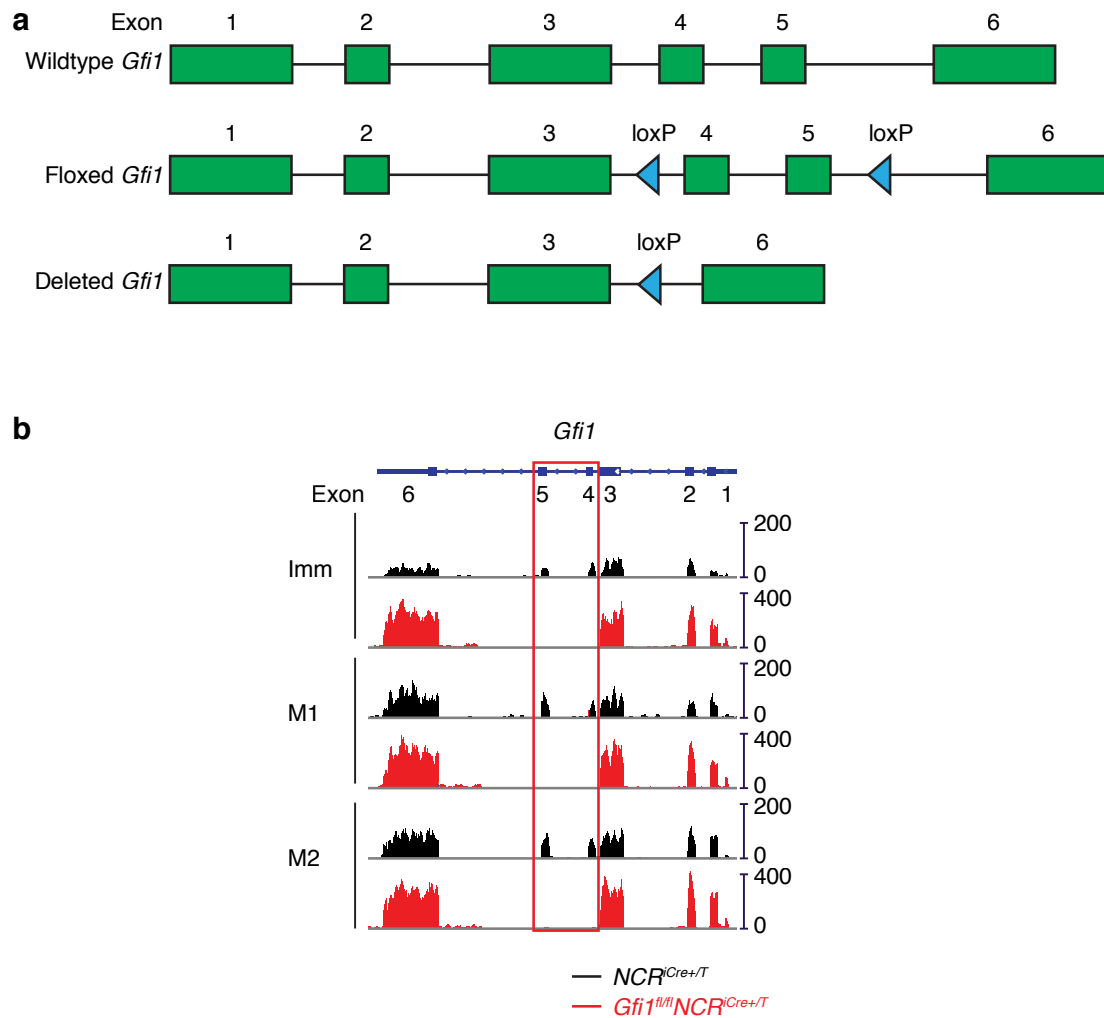


Figure 4.2 Mouse model of GFI1 deletion in NK cell maturation.

a, Schematic of the wildtype, floxed and deleted *Gfi1* allele after Cre-mediated excision. Exons and loxP sites as indicated.

b, Genome browser of normalised RNASeq transcript of reads across the *Gfi1* locus from *Gfi1*^{fl/fl}*NCR*^{Cre+/T} (red) and *NCR*^{Cre+/T} (black) NK cell subsets from the spleen. Deleted regions of exon 4 and 5 as indicated by the red box.

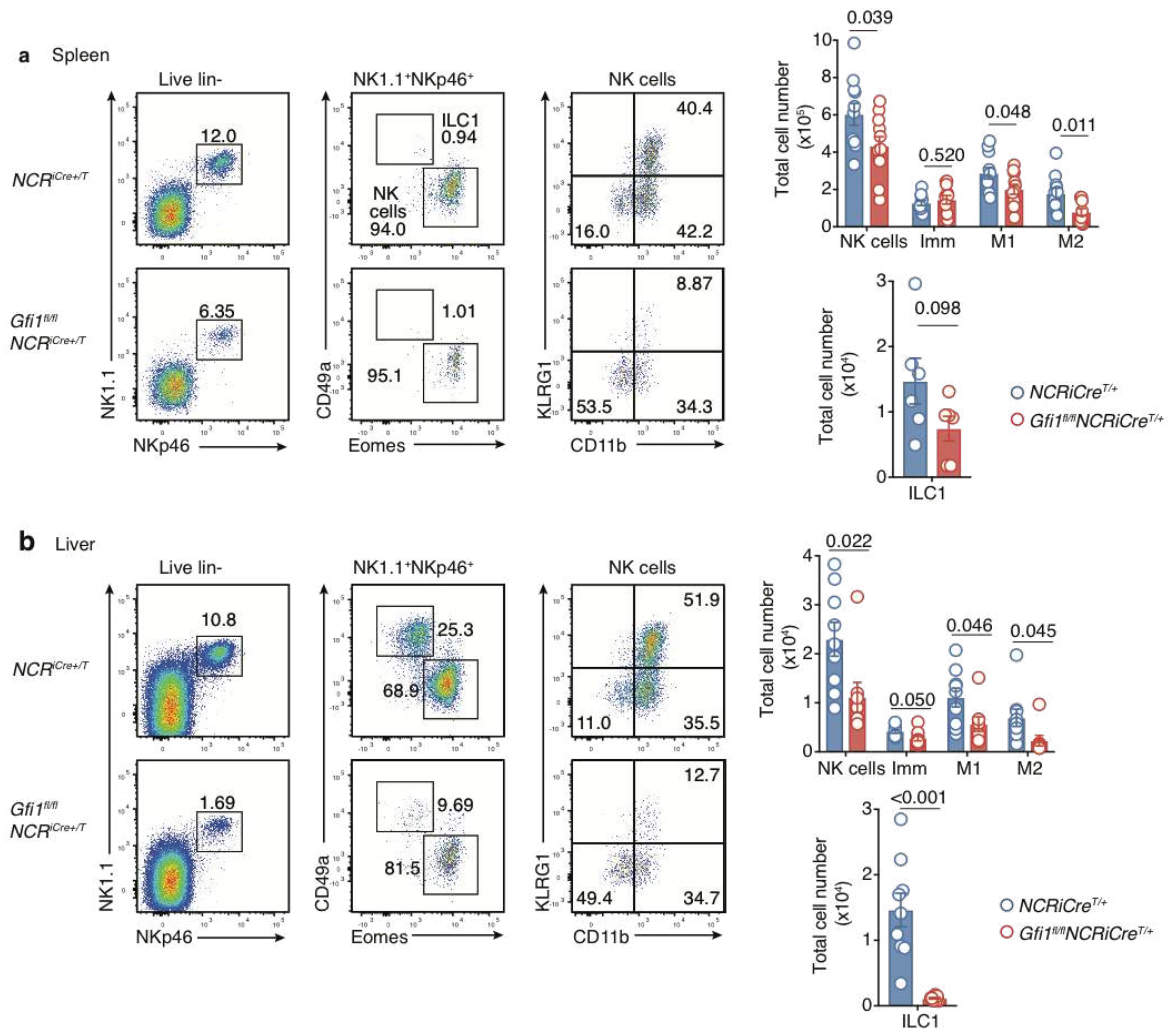


Figure 4.3. Late stage deletion of GF11 impairs NK cell maturation and drives tissue-specific loss of ILC1.

a-b, Prevalence of ILC1 (live lin-NK1.1⁺NKp46⁺CD49a⁺), NK cells (live lin-NK1.1⁺NKp46⁺Eomes⁺), and each NK cell subset; Imm (CD11b⁻KLRG1⁻), M1 (CD11b⁺KLRG1⁻) and M2 (CD11b⁺KLRG1⁺) isolated from the **a**, spleen and **b**, liver of *Gfi1^{fl/fl}NCRiCre^{+/T}* and *NCRiCre^{+/T}* mice. The left panels show representative flow cytometric dot plots, with the gating strategy and frequencies of each cell subset. The right panels show the histograms with the mean $\pm$ SEM of each subset of each organ pooled from three to four independent experiments (spleen $n=10-12$ and liver $n=9$). Statistical significance was calculated using a Student's *t* test with multiple comparisons.

Eomes expression increased in the absence of GFI1 in NK cells

I further analysed the expression of key group 1 ILC surface markers CD49a and CD49b to confirm the identify of NK cell and ILC1. I also analysed receptors CD122 (IL-2R β , used for IL-2 and IL-15 signalling) and IL-7R, which are required for NK cell and ILC1 development. Expression of CD122, IL-7R, CD49b and CD49a in GFI1-deficient splenic NK cell subsets and ILC1 were similar to the levels found in wild-type cells (Fig. 4.4a). GFI1-deficient NK cell subsets in the liver also had similar expression of IL-7R, CD49b and CD49a to their wild-type counterparts, whereas increased expression of CD122 was observed (Fig. 4.4b). This indicates that GFI1 deletion did not result in the loss of markers used to define ILC1 and NK cells.

The reduction of mature NK cells suggested that NK cell maturation was impacted by the loss of GFI1. Therefore, I analysed the expression of Eomes, a transcription factor involved in NK cell maturation but not expressed by ILC1^{43, 55}. Eomes expression was downregulated as NK cells mature. However, deletion of GFI1 in NK cells resulted in an increase in the expression of Eomes in M1 and M2 NK cell subsets in the spleen, while a dramatic increase in Eomes expression was observed in all NK cell subsets and ILC1 in the liver compared to wild-type control cells (Fig. 4.4c). These results suggest that GFI1 suppresses Eomes expression. T-bet is another NK transcription factor involved in NK cell development and maturation^{43, 55}. T-bet acts in balance with Eomes and is upregulated during NK cell maturation⁵³. Analysis of T-bet expression showed that T-bet expression was not significantly changed in in GFI1-deficient M2 NK cells (Fig. 4.4d). This suggests that GFI1 may regulate NK cell maturation by affecting Eomes expression but not T-bet, however, T-bet expression will need to be confirmed with more experiments.

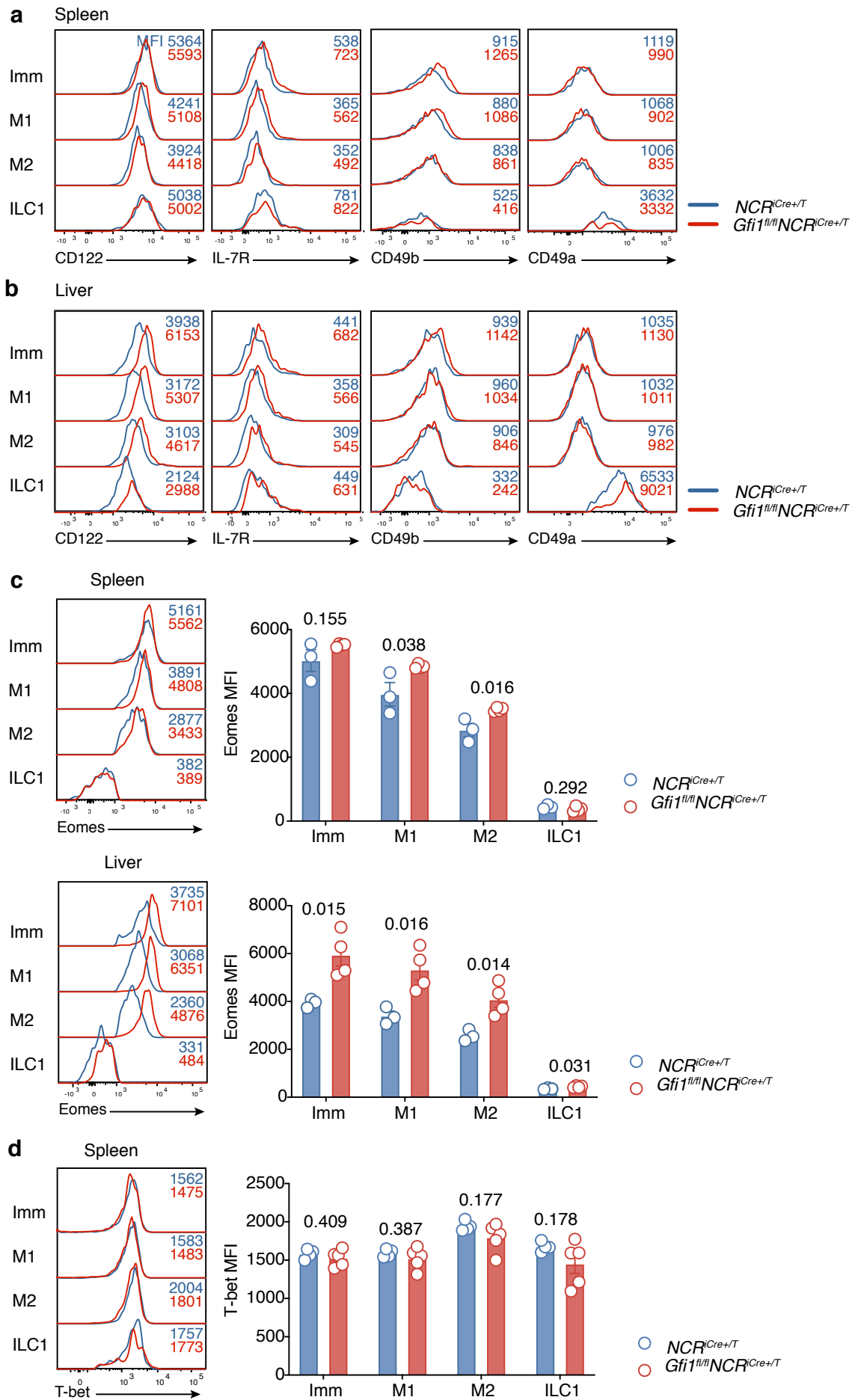


Figure 4.4. GF11 regulates the expression of key NK receptors and transcription factors.

a-b, Expression of CD122, IL-7R, CD49b and CD49a on live $\text{lin}^{-}\text{NK1.1}^{+}\text{NKp46}^{+}\text{Eomes}^{+}$ NK cell subsets Imm ($\text{CD11b}^{-}\text{KLRG1}^{-}$), M1 ($\text{CD11b}^{+}\text{KLRG1}^{-}$), M2 ($\text{CD11b}^{+}\text{KLRG1}^{+}$) and ILC1 (live $\text{lin}^{-}\text{NK1.1}^{+}\text{NKp46}^{+}\text{CD49a}^{+}$) isolated from the **a**, spleen and **b**, liver of $Gfi1^{fl/fl} NCR^{iCre+/T}$ (red line) and $NCR^{iCre+/T}$ (blue line). Geometric MFI of cells isolated from genotype is indicated. Data show representative histograms from one representative mouse from two similar experiments ($n=1-4$ mice/genotype/experiment).

c, Expression of Eomes on NK subsets and ILC1 isolated from the spleen (top panel) and liver (bottom panel) of $Gfi1^{fl/fl} NCR^{iCre+/T}$ (red line) and $NCR^{iCre+/T}$ (blue line). Geometric MFI of cells isolated from genotype is indicated. Data show representative histograms from one representative mouse (right) and the mean $\pm$ SEM (left) of the MFI of each subset from one of three similar experiments ($n=3$ mice/genotype/experiment). Statistical differences were calculated using an unpaired Student's t test.

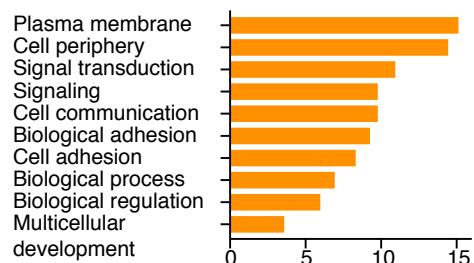
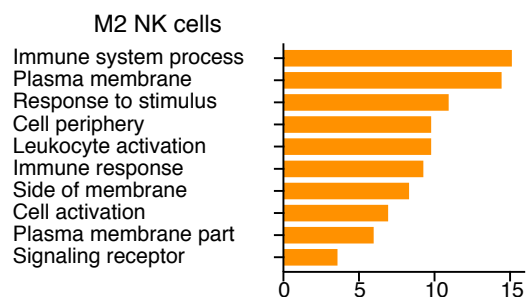
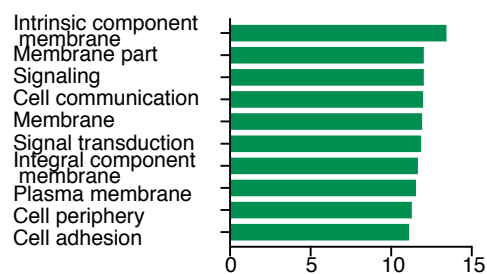
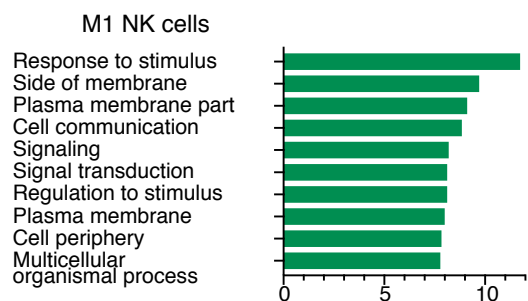
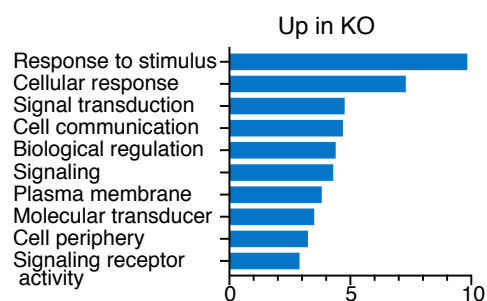
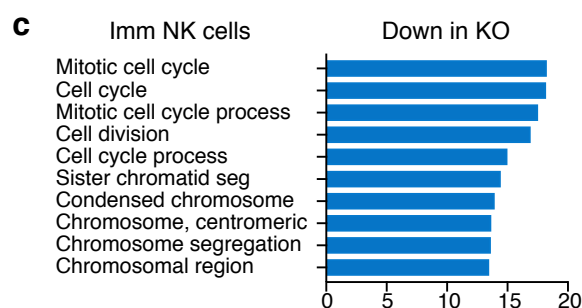
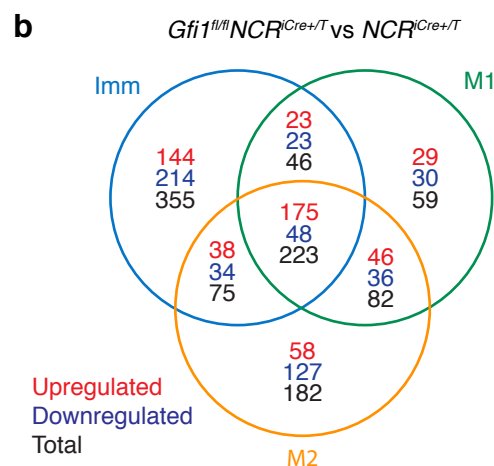
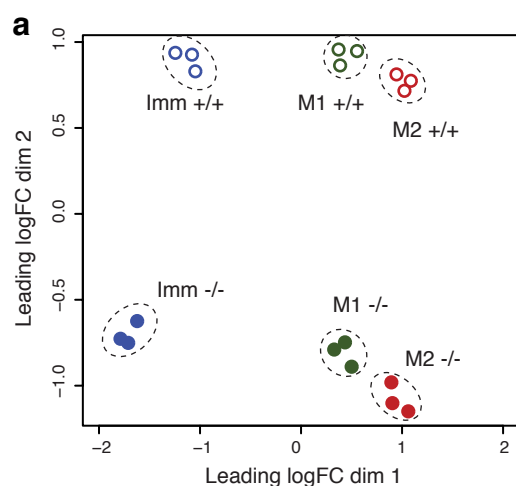
d, Expression of T-bet on NK subsets and ILC1 isolated from the spleen of $Gfi1^{fl/fl} NCR^{iCre+/T}$ (red line) and $NCR^{iCre+/T}$ (blue line). Geometric MFI of cells isolated from genotype is indicated. Data show representative histograms from one representative mouse (right) and the mean $\pm$ SEM (left) of the MFI of each subset from one experiment ($n=4-5$ mice/genotype). Statistical differences were calculated using an unpaired Student's t test.

3.3.2 Molecular regulation of NK cells by GFI1

Delineation of pathways regulated in different NK cell subsets

Given the high expression of GFI1 in mature NK cells and their subsequent loss when GFI1 is deleted, I hypothesised that GFI1 may regulate cell cycling or survival pathways involved in homeostatic maintenance of mature NK cells. To determine the regulatory pathways affected by the loss of GFI1 expression and the specific genes that are dysregulated in these pathways, Imm, M1 and M2 splenic NK cells were purified by flow cytometric sorting from *NCR^{iCre+/T}* and *Gfi1^{fl/fl}NCR^{iCre+/T}* mice and analysed by RNA sequencing. Multi-dimensional scaling analysis revealed that Imm, M1 and M2 NK cells each exhibited a unique transcriptional signature. In addition, the transcriptional profile of Imm, M1 and M2 GFI1-deficient NK cells were distinct compared with wild-type NK cell subsets (Fig. 4.5a). Differential gene expression analysis identified a total of 699 genes in Imm NK cells, 410 genes in M1 and 562 genes in M2 cells that were either up- or down-regulated between wild-type and GFI1-deleted NK cells. Of these differentially expressed genes, 233 genes were identified in all three maturation stages (Fig. 4.5b).

To determine whether the up- and down-regulated genes were enriched for specific functions or gene products in each NK cell subset, we performed GO analyses. Of the 319 down-regulated genes in GFI1-deficient Imm NK cells, genes associated with cell cycling were over-represented, whereas in the 380 up-regulated genes, cell signaling genes were enriched (Fig. 4.5c). In M1 and M2 cells, down-regulated genes were enriched with genes involved in signaling and immune cell activation, while the 273 and 317 up-regulated genes in M1 and M2 cells enriched for genes involved in cell adhesion (Fig. 4.5c). This indicates that the role of GFI1 in regulating NK cells is different for each maturation stage and GFI1 may promote cell cycling in Imm NK cells and cell signaling or immune cell activation in M1 and M2 cells.



-Log₁₀ (P)

Figure 4.5. GFI1 promotes a transcriptional profile associated with cell cycling and immune cell activation in NK cells.

a, Multidimensional scaling (MDS) plot of clustering of Imm, M1 and M2 RNASeq analyses from *Gfi1^{fl/fl} NCR^{iCre+/T}* and *NCR^{iCre+/T}* spleen NK cells. Data show individual biological samples with the distance calculated using the top 500 most variable genes between each triplicate set of samples.

b, Venn diagram analysis of common and unique up- and down-regulated genes between *Gfi1^{fl/fl} NCR^{iCre+/T}* and *NCR^{iCre+/T}* of each NK cell subset.

c, GO analyses showing the 10 most highly ranked (via *p* value) up- and down-regulated gene family categories between *Gfi1^{fl/fl} NCR^{iCre+/T}* (KO) and *NCR^{iCre+/T}* Imm, M1 and M2 NK cell subsets.

Global dysregulation of migration and activation pathways in GFI1-deficient NK cells

To identify the global molecular pathways controlled by GFI1 in NK cells, I compared genes that were differentially expressed in the absence of GFI1 across all NK cell subsets. Of the 223 genes identified to be changed and shared across subsets in GFI1^{-/-} cells, the majority of these genes (175 genes) had increased in expression when *Gfi1* was deleted, while another 48 genes were decreased (Fig. 4.5b). Expression of key transcription factors regulating NK cell development and maintenance were upregulated all subsets of GFI1-deficient NK cells. This included *Foxo1*, *Klf4* and *Lef1* (Fig. 4.6a). Genes encoding integrins and receptors involved in NK cell migration and activation were also dysregulated in GFI1-deficient NK cells. This included cytokine receptors *Il18r* and *Il7r* and chemokine receptor *Cxcr4* which had increased expression when GFI1 was deleted, while NK cell-related inhibitory and activating receptors *Cd96* and *Cd226* were reduced (Fig. 4.6a). These differentially expressed genes were then compared against the KEGG database to determine which pathways are may be impacted by the loss of GFI1 in all NK cell subsets. KEGG pathways associated with biological and cellular regulation as well as cell signaling and adhesion were found to be over-represented in the genes that were differentially expressed by all NK cell subsets (Fig. 4.6b). This indicates that GFI1 may regulate NK cell development and its capacity for immunosurveillance and effector functioning.

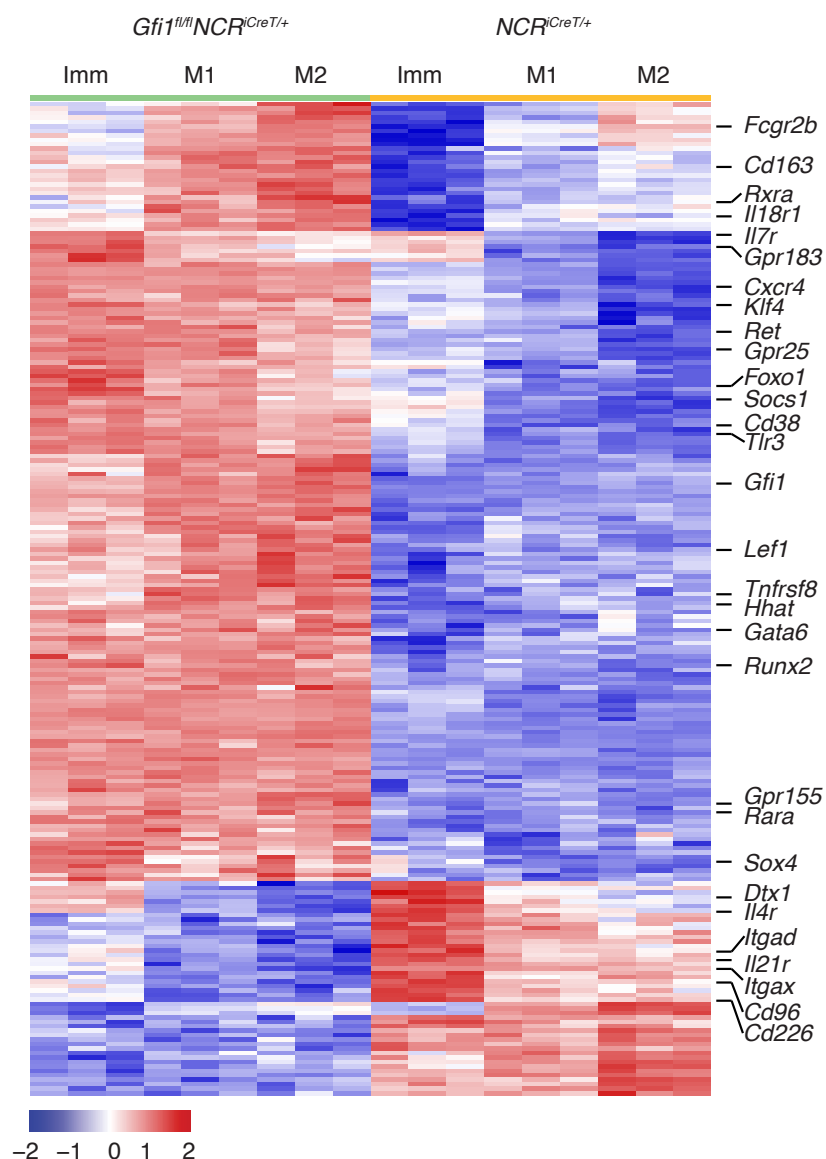
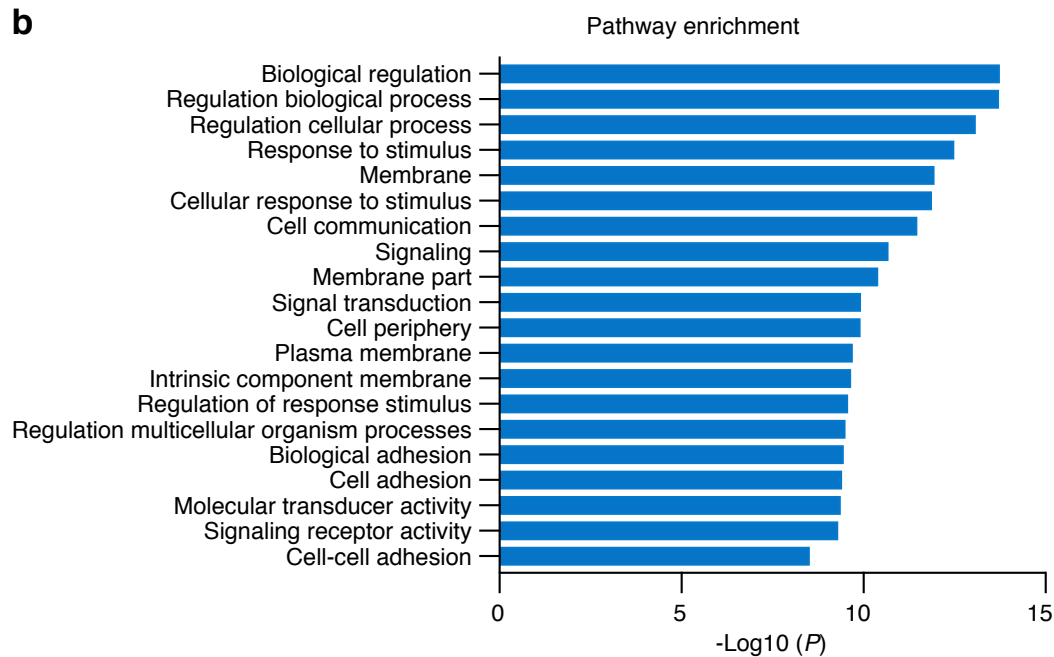
a**b**

Figure 4.6. A core molecular network is regulated by GFI1 across NK cell subsets.

a, Heatmap analysis showing the expression of genes that were identified to be differentially expressed between *Gfi1^{fl/fl} NCR^{iCre+/T}* and *NCR^{iCre+/T}* NK cells for each maturation stage. Data show expression for each gene across three independent biological replicates for each NK cell subset.

b, Cellular pathways defined by KEGG analysis ranked by P value (Log10) for differentially expressed genes that were up regulated in all three maturation stages of *Gfi1^{fl/fl} NCR^{iCre+/T}* spleen NK cells compared with *NCR^{iCre+/T}* NK cells. (<https://www.genome.jp/kegg/>).

3.3.3 Role of GFI1 in cell cycling and survival

GFI1-deficient NK cells exhibit delayed proliferation

My pathway analysis indicated that a number of genes associated with cell cycling in GFI1-deficient Imm NK cells were differentially expressed. Specifically, genes involved in cell cycling such as *Ccna2*, *Ccnb1*, which encodes cyclin proteins and cyclin dependent kinases, *Cdn1ka* and *Cdkn3* were reduced in GFI1-deficient NK cells (Fig. 4.7a). Genes associated with cell survival were also differentially expressed, including *Klf4*, which promotes NK cell survival¹⁵⁹, and the pro-apoptotic gene *Bbc3* (encodes the protein PUMA)^{160, 161} which were increased. To determine whether the loss of GFI1, and the subsequent changes in gene expression, resulted in a change in the ability of NK cells to proliferate, NK cells were stained for Ki67, a protein associated with cells preparing for cell division. At steady-state, the frequency of Ki67⁺ GFI1-deficient Imm NK cells were reduced by 50% in comparison with GFI1-sufficient Imm NK cells. An approximate 40% reduction was also observed in M1 and M2 subsets (Fig. 4.7b). These data indicate that GFI1-deficient NK cells have a reduced capacity to undergo proliferation.

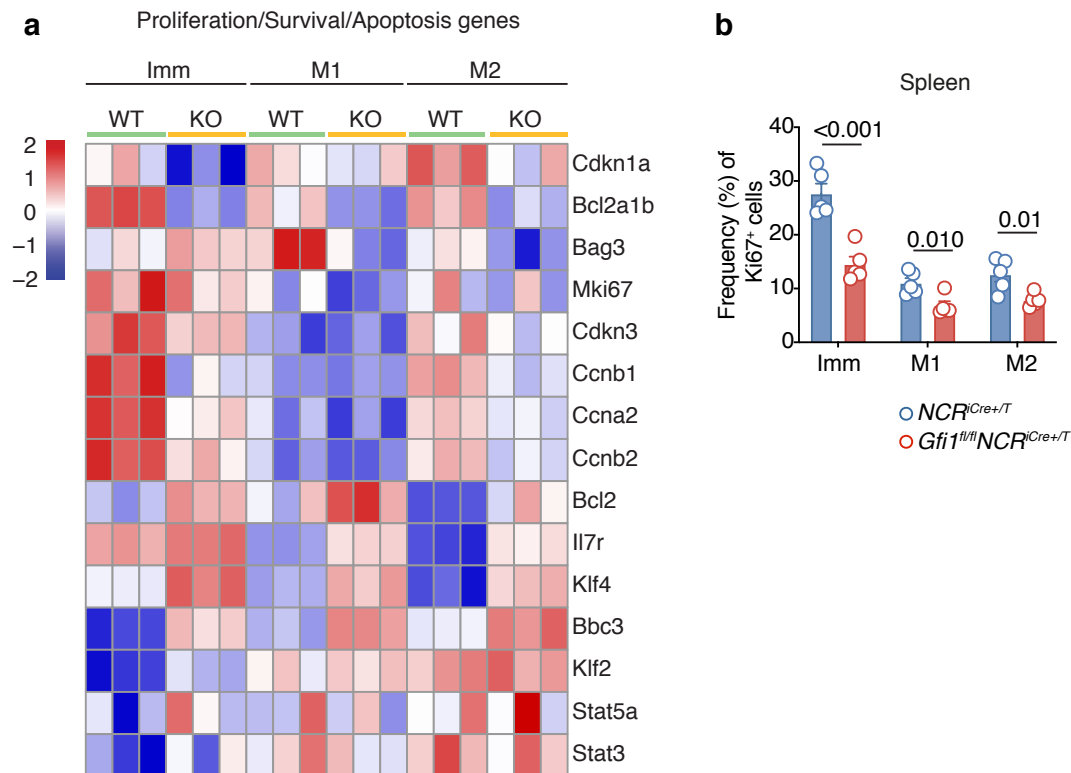


Figure 4.7. GFI1-deficient NK cells exhibit reduced proliferation.

a, Heatmap showing the expression of manually curated genes that are associated with survival and cell cycling from *Gfi1^{fl/fl} NCR^{iCre+/T}* and *NCR^{iCre+/T}* NK cells, each maturation stage is shown. Data show expression for each gene across three independent biological replicates for each NK cell subset.

b, Frequency of Ki67⁺ cells of each NK subset from the spleen of steady-state mice *ex vivo*. Data show the mean $\pm$ s.e.m of each subset from one experiment ($n=4-5$ mice/genotype). Statistical differences were calculated using an unpaired Student's *t* test.

IL-15 is a key cytokine required for NK cell survival and driving proliferation^{160, 162, 163}. Despite the normal expression of *Il2rb*, the gene encoding CD122 (not shown), and CD122 expression (Fig 4.3a) in NK cells, there was a significant reduction in their proliferation. Therefore, I tested whether the defects in proliferation observed in GFI1-deficient NK cells were due to a failure to respond to IL-15 signalling through the CD122 receptor. To test the capacity of GFI1-deficient NK cells to respond to IL-15 stimulation compared to control NK cells, we labelled NK cells with the tracking dye CellTrace Violet (CTV), FACs purified the NK cells of both genotypes into each maturation subset and cultured the cells for 5 days in hIL-15 (Fig. 4.8a). At 5ng/mL and 10ng/mL of hIL-15, NK cells did not divide as indicated by cells retaining a high level of CTV staining (Fig. 4.8b). At 25ng/mL of hIL-15, control Imm and M1 cells have started to divide, with cells having undergone up to two to three cycles of division, whereas GFI1-deficient Imm NK cells have only divided once and M1 did not divide (Fig. 4.8b). M2 cells of either genotypes did not divide at 25ng/ml of IL-15 (Fig. 4.8b). At 50ng of hIL-15, WT Imm and M1 cells have undergone up to four to five rounds of division and only 5-12% of WT Imm cells remain undivided (Fig. 4.8b). The GFI1-deficient Imm NK cells divided up to three times, with 45-52% of cells remaining undivided and GFI1-deficient M1 cells had only undergone one division (Fig. 4.8b). Control M2 cells had divided up to four times, whereas GFI1-deficient M2 cells remain undivided (Fig. 4.8b). Collectively, these results show that a greater proportion of GFI1-deficient NK cells remain undivided and those that do enter division, undergo fewer rounds of division compared with GFI1-sufficient NK cells at all maturation stages when stimulated with hIL-15. Thus, GFI1 is required by NK cells for efficient cell division.

In addition to division, IL-15 is required by NK cells for survival^{160, 162}. To determine whether loss of GFI1 has impacted on the ability of NK cells to survive, the number of cells per well was quantified after 5 days of culturing. At 5ng/mL and 10ng/mL of IL-15, NK cell subsets did not divide. The number of GFI1-deficient NK cells cultured at these concentrations did not differ significantly to the number in control wells (Fig. 4.8c). This suggests that NK cell survival is not impacted by the loss of GFI1. At the higher concentrations of IL-15, NK cells proliferated and therefore increased the total number of NK cells within each well. Therefore, survival cannot be determined by only analysing the cell number. Therefore, NK cells cultured in IL-15 for five days were also stained with Annexin V to identify the cells that are undergoing apoptosis. GFI1-deficient Imm and M1 NK cells cultured at non-proliferative concentrations (5 and 10ng/ml) of IL-15 had a lower proportion of cells undergoing apoptosis compared to

their wild-type counterparts while there was no difference in the frequency of M2 NK cells. At proliferative concentrations (25 and 50ng/ml) of IL-15, all GFI1-deficient NK cells had a lower frequency of apoptotic cells compared to wild-type NK cells (Fig. 4.8d). This suggests that GFI1 may play a role in regulating apoptosis of NK cells, particularly in Imm and M1 subsets and the difference is more pronounced in proliferating M2 cells. However, this survival advantage is small and doesn't result in a significant biological impact on cell number.

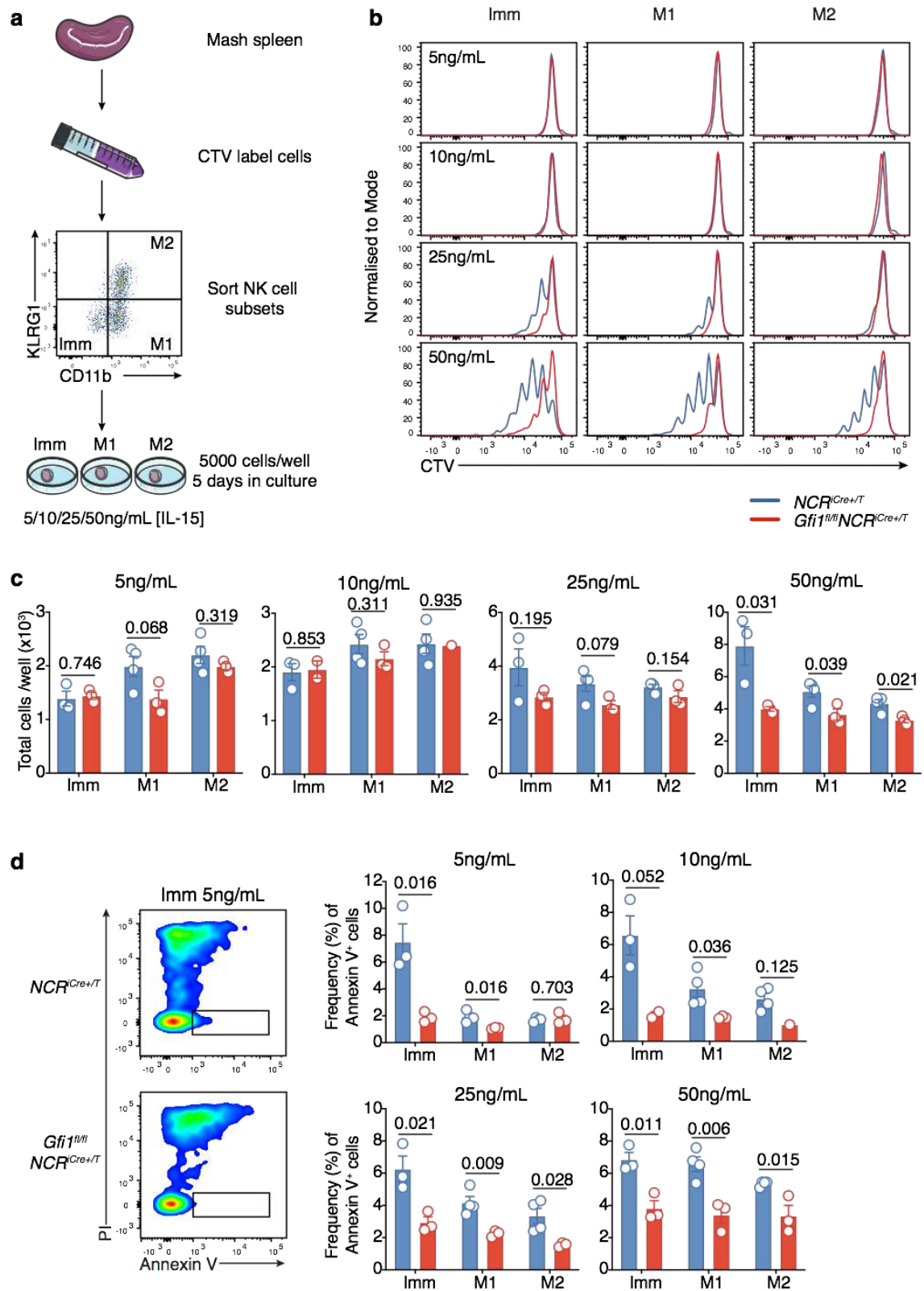


Figure 4.8. GFI1-deficient NK cells fail to respond to IL-15 stimulation.

a, Schematic of CTV labelled *in vitro* cultures of sorted NK cells in titrating concentrations of IL-15 to measure proliferation.

b, Proliferation of Imm (CD11b⁻KLRG1⁻), M1 (CD11b⁺KLRG1⁻) and M2 (CD11b⁺KLRG1⁺) NK cell subsets stimulated with 5ng/ml, 10ng/ml, 25ng/ml and 50ng/ml of recombinant hIL-15 for 5 days *in vitro*. Splenic NK cell subsets of *Gfi1^{fl/fl}NCR^{iCre+/T}* and *NCR^{iCre+/T}* control mice were labelled with CellTrace Violet (CTV) dye, then purified by cell sorting. Representative FACS histograms show proliferation of CTV-labelled *Gfi1^{fl/fl} NCR^{iCre+/T}* (red) and *NCR^{iCre+/T}* (blue) NK cells from one of two similar experiments. (*n*=3-4 mice/genotype/experiment).

c, Number of live NK cells per well after 5 days culture with 5ng/ml, 10ng/ml, 25ng/ml and 50ng/ml of recombinant hIL-15. Data show the mean $\pm$ s.e.m of each subset from one of two similar experiments (*n*=3-4 mice/genotype/experiment). Statistical differences were calculated using an unpaired Student's *t* test.

d, Flow cytometric plots (left) of Imm NK cells that are PI⁻Annexin V⁺ after 5 days of culture in 5ng/ml of hIL-15. Histograms (right) show the mean $\pm$ SEM of the frequency of live NK cell (PI⁻NK1.1⁺) subsets that are Annexin V⁺ after 5 days of culture in 5, 10, 25 and 50ng/ml of hIL-15 from one experiment (*n*=3-4 mice/genotype). **c**, **d** Statistical differences were calculated using an unpaired Student's *t* test.

Loss of GFI1 impairs STAT5 phosphorylation

Due to the defects observed in IL-15 driven proliferation when GFI1 is deleted, we investigated the mechanism behind IL-15 signalling that may be responsible for this defect. IL-15 interactions with its receptor CD122, leads to a signalling cascade where Janus kinase (JAK) 1 and JAK3 activation phosphorylates signal transducer and activator of transcription 5 (STAT5). pSTAT5 then dimerises and translocates to the nucleus to regulate transcription of genes involved in cell cycling and survival ¹⁶⁴. Since expression of the IL-15 receptor CD122 was not reduced when GFI1 was deleted (Fig. 4.4a) and RNASeq analysis indicated that *Stat5* expression was not altered at a gene transcript level (Fig. 4.7a), I analysed the downstream signalling capacity of NK cells by stimulating them *in vitro* with hIL-15 for 30 minutes and measured pSTAT5 by intracellular flow cytometry (Fig. 4.9a,b). The pSTAT5 MFI of stimulated wild-type NK cells increased 2-fold compared to the MFI of unstimulated NK cells, indicating that hIL-15 stimulation successfully induced STAT5 phosphorylation (Fig. 4.9b). Expression of pSTAT5 was reduced in stimulated GFI1-deficient M2 NK cells compared to wildtype NK cells (Fig. 4.9). This suggests that the defect in IL-15 driven proliferation is not due to a change in expression of the CD122 receptor, and that the defect could be due to defects in signalling to induce pSTAT5 within GFI1-deficient NK cells, however further analysis is required to confirm these findings.

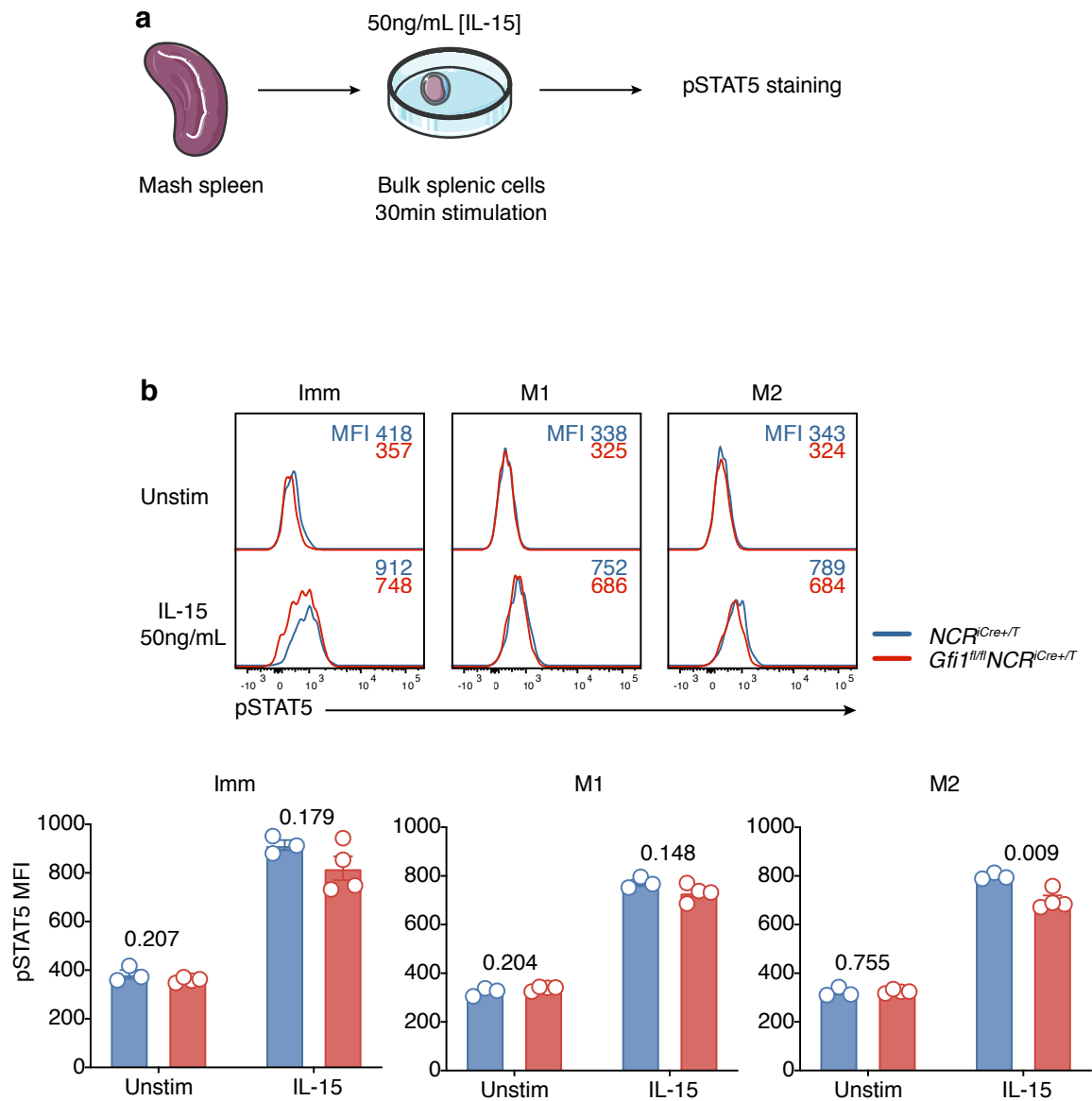


Figure 4.9. GFI1-deficient NK cells have impaired STAT5 phosphorylation.

a, Schematic of *in vitro* stimulation with IL-15 to measure pSTAT5.

b, Expression of pSTAT5 in unstimulated and IL-15 stimulated *Gfi1^{fl/fl} NCR^{iCre+/T}* (red line) and *NCR^{iCre+/T}* (blue line) splenic NK cell subsets. Geometric MFI of each genotype is indicated. Data show representative histograms from one mouse (top panel) and the mean $\pm$ SEM (bottom panel) of the MFI of each subset from one experiment ($n=3-4$ mice/genotype). Statistical significance was calculated using an unpaired Student's *t* test.

GFI1 regulates the localisation of NK cells

NK cells circulate throughout the body and localise in different organs depending on the signals they receive. This ensures that NK cells are correctly positioned to mediate an efficient immune response. In addition to dysregulated cell signalling genes, adhesion genes were also altered across all NK cell maturation stages when GFI1 was deleted in splenic NK cells (Fig. 4.6). In particular, expression of genes involved in NK cell homing were dysregulated in GFI1-deficient NK cells. This included an increase in *Sell* (CD62L) and *Ccr7* expression, which is important for NK cell homing to LN, and *Cxcr4*, which retains NK cells in the bone marrow^{148, 150, 165, 166} (Fig 4.10a). This suggests that potentially, NK cell trafficking to and from the LN and bone marrow could be affected by the loss of GFI1. To determine how GFI1 impacted NK cell trafficking throughout the body, I enumerated the total number of NK cells across multiple organs from *Gfi1^{fl/fl} NCR^{iCre+/T}* and *NCR^{iCre+/T}* mice. I had previously shown that NK cell numbers in GFI1-deficient mice were reduced in spleen and liver (Fig. 4.3). Although there wasn't a significant difference in NK cell numbers in the small intestine, analysis of lung showed a two-fold reduction (Fig. 4.10b). However, the total number of NK cells within the bone marrow and mesenteric LN had increased 1.5 and 2-fold respectively (Fig. 4.10b). The accumulation of NK cells in the LN and the increase in gene expression of *Sell*, which encodes the surface receptor CD62L and is involved in leukocyte tethering and rolling along the endothelium¹⁶⁷, suggested that CD62L expression on NK cells may be responsible for the change in NK cell homing. However, CD62L expression on GFI1-deficient NK cells had a two-fold reduction in the liver compared to GFI1-sufficient NK cells (Fig. 4.10c). There was no significant change in the level of expression in the spleen, mesenteric LN and small intestine (Fig. 4.10c). There was only a 1.14 fold increase of CD62L in NK cells in the lung (Fig. 4.10c). This suggests that the increased number of NK cells in LN is not due to an increase in CD62L expression.

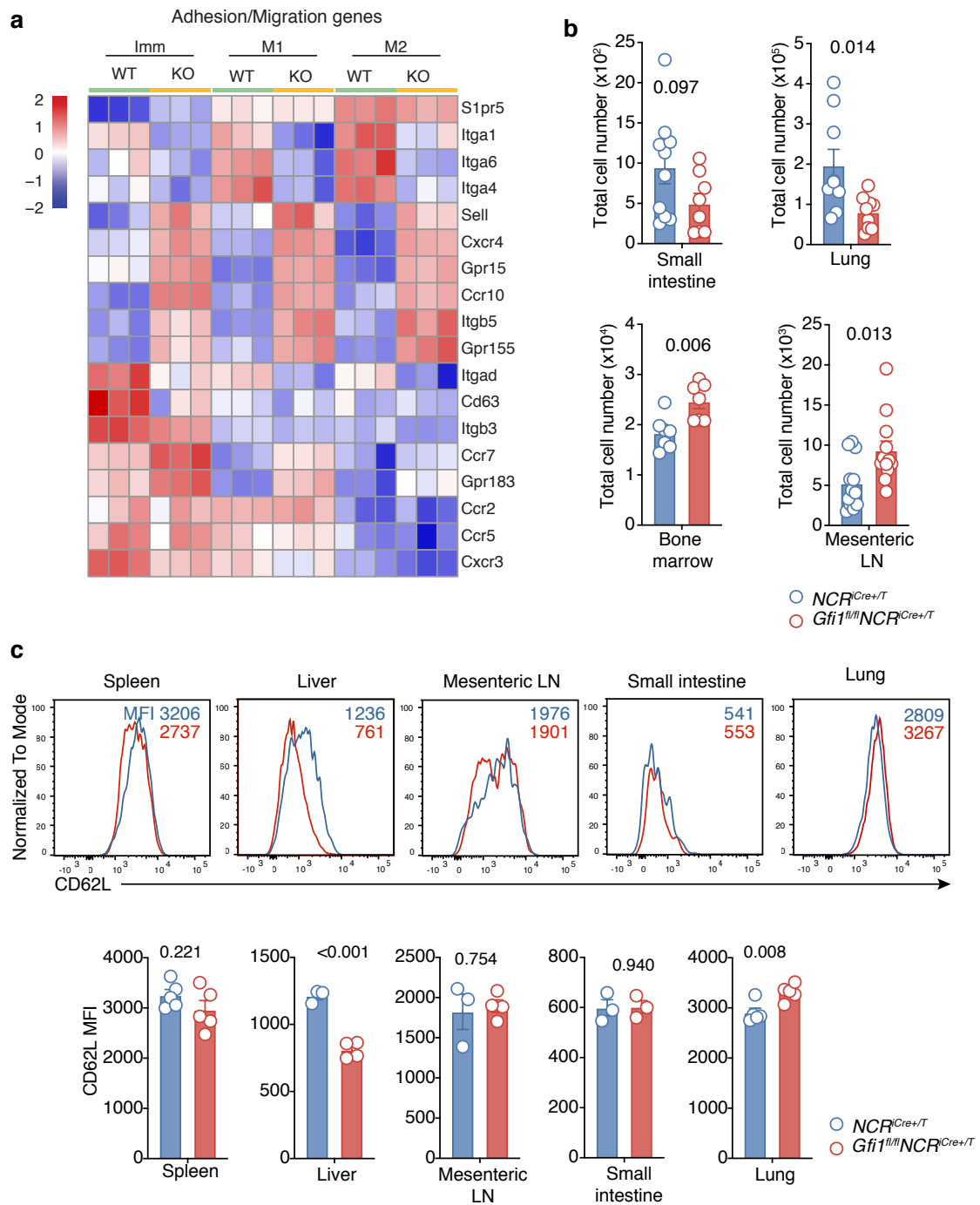


Figure 4.10. GFI1-deficient NK cells accumulate in the bone marrow and LN.

a, Heatmap showing the expression of manually curated genes, that are associated with adhesion and migration between *Gfi1^{fl/fl} NCR^{iCre+/T}* and *NCR^{iCre+/T}* NK cells for each of the maturation stages. Data show expression for each gene across three independent biological replicates for each NK cell subset.

b, Enumeration of total NK cells isolated from the small intestine lung, bone marrow and mesenteric LN of *Gfi1^{fl/fl} NCR^{iCre+/T}* and *NCR^{iCre+/T}* control mice. Data show the mean $\pm$ SEM of each organ pooled from two to four independent experiments (small intestine $n=8-10$, lung $n= 9$, bone marrow $n= 7$ and mesenteric LN $n= 12$), Statistical significance was calculated using a Student's *t* test.

c, Expression of CD62L on *Gfi1^{fl/fl} NCR^{iCre+/T}* (red line) and *NCR^{iCre+/T}* (blue line) NK cells isolated from the spleen, liver, mesenteric LN, small intestine and lung . Geometric MFI of each genotype is indicated. Data show representative histograms of each organ and the mean $\pm$ SEM (bottom panel) of the MFI of each organ from one experiment ($n=3-5$ mouse/genotype). Statistical significance was calculated using an unpaired Student's *t* test.

3.3.4 Role of GFI1 in NK cell activation and effector function

GFI1 regulates effector functions of NK cells

NK cell activity is regulated through the balance of positive and negative signals received through their activating and inhibiting receptors. Activating receptors recognise pathogen-induced signals and initiate an effector response while signalling through inhibitory receptors downregulates this response¹⁶⁸. Pathway analysis indicated that genes involved in immune activation was decreased in GFI1-deficient M1 and M2 NK cell subsets compared with wild-type cells (Fig. 4.5c). Specifically, expression of genes such as *Cd226*, *Tigit*, *Cd96* were differentially expressed and were decreased in GFI1-deficient NK cell subsets (Fig. 4.11a). Protein expression of the activating marker DNAM-1 and inhibitory marker TIGIT was then examined by flow cytometry and I found that at steady-state, GFI1-deficient NK cells in the spleen and lungs had reduced expression of DNAM-1 and TIGIT (Fig. 4.11b). The reduced expression of DNAM-1 by GFI1-deficient NK cells suggests that these cells are predicted to have a reduced cytotoxic capacity and respond poorly to tumour challenge.

NK cells are activated and produce pro-inflammatory cytokines and cytotoxic molecules in response to IL-12 and IL-18 cytokine stimulation^{6, 169}. From the RNASeq data, the receptors for IL-12 and IL-18, *Il12rb2* and *Il18r1*, were both increased when GFI1 was deleted (Fig. 4.12a), therefore I tested whether GFI1-deficient NK cells were more responsive to cytokine stimulation compared to wildtype NK cells. Stimulation of total splenocytes with IL-12 and IL-18 for four hours, revealed that there was no statistically significant difference in the frequency of IFN- γ -producing Imm, M1 and M2 NK cells in the absence of GFI1 (Fig. 4.12b). Stimulation with PMA and ionomycin was also performed to evaluate whether the total capacity of NK cell cytokine production was affected by the loss of GFI1. No difference in the frequency of IFN- γ -producing cells for any of the NK cell subsets was observed (Fig. 4.12c). This indicates that the NK cell response to IL-12 and IL-18 stimulation and their intrinsic capacity for cytokine production was not significantly impacted by loss of GFI1. To confirm these data, additional independent biological replicates will need to be performed.

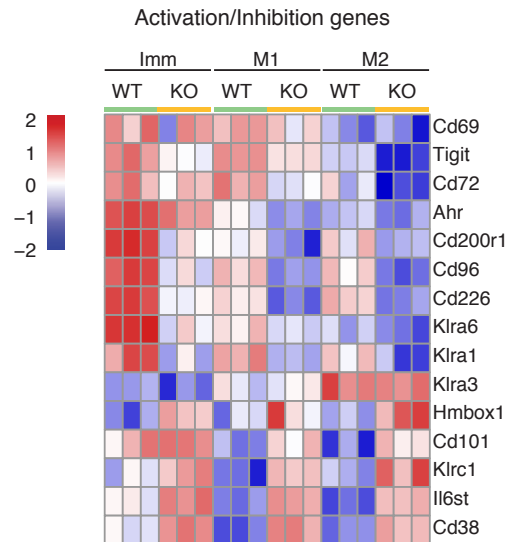
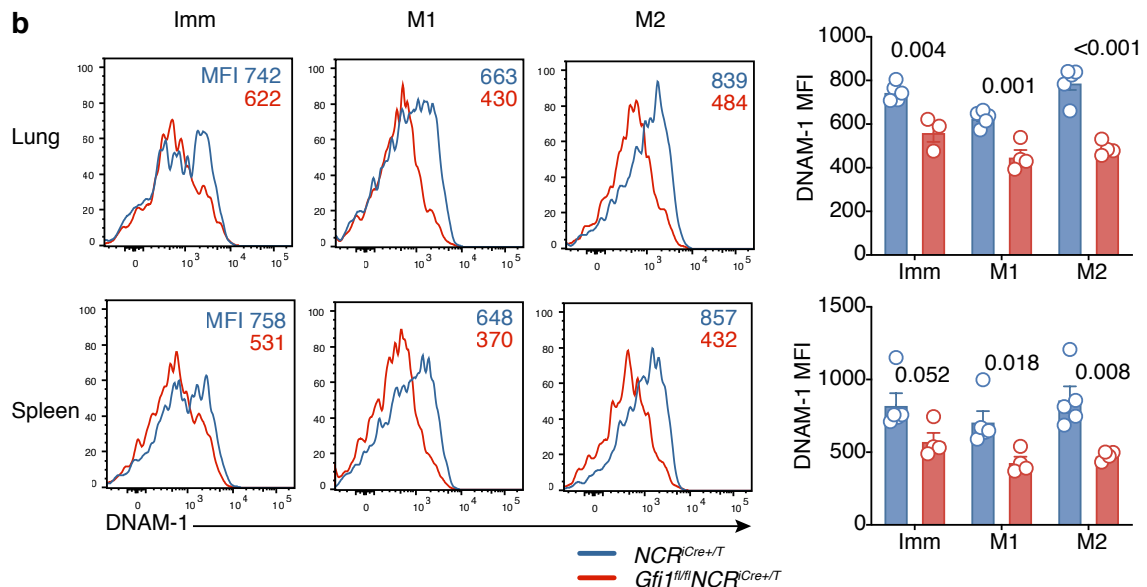
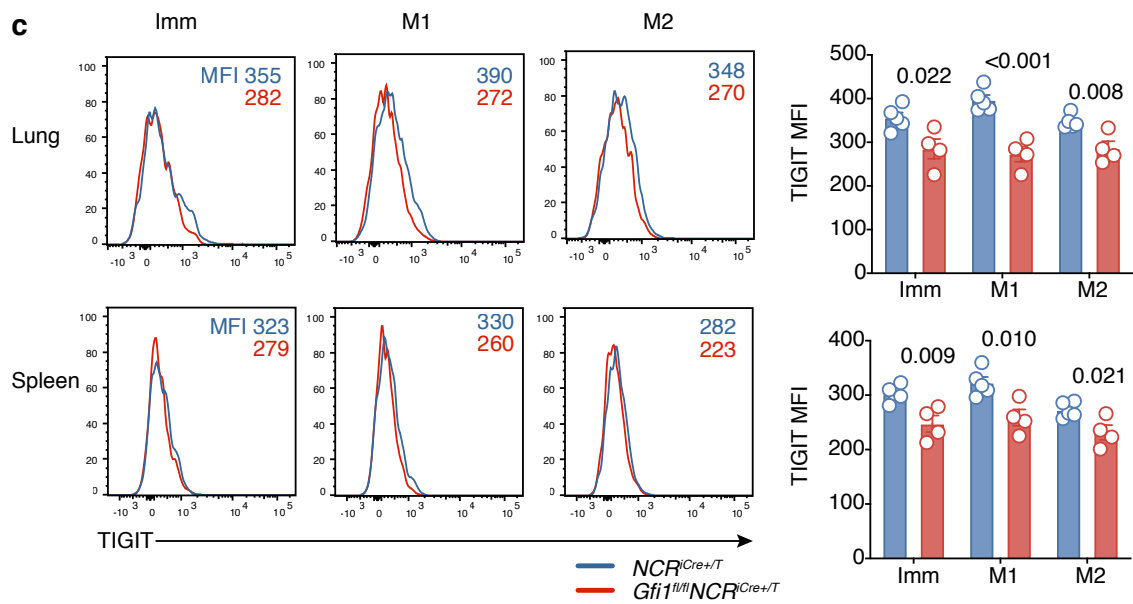
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Figure 4.11. GF11-deficient NK cells have reduced activation at steady state.

a, Heatmap showing the expression of manually curated genes, that are associated with NK cell activation and inhibition between *Gfi1^{fl/fl} NCR^{iCre+/T}* and *NCR^{iCre+/T}* NK cells for each of the maturation stages. Data show expression for each gene across three independent biological replicates for each NK cell subset.

b-c, Expression of **b**, DNAM-1, and **c**, TIGIT on the surface of *Gfi1^{fl/fl} NCR^{iCre+/T}* and control *NCR^{iCre+/T}* NK cells from the lung and spleen. Geometric MFI of each marker on each NK cell subset is shown. Data show representative profiles (left) and the mean $\pm$ SEM of the geometric MFI of each marker of individual mice from one experiment of one experiment and ($n=5$ mice/genotype).

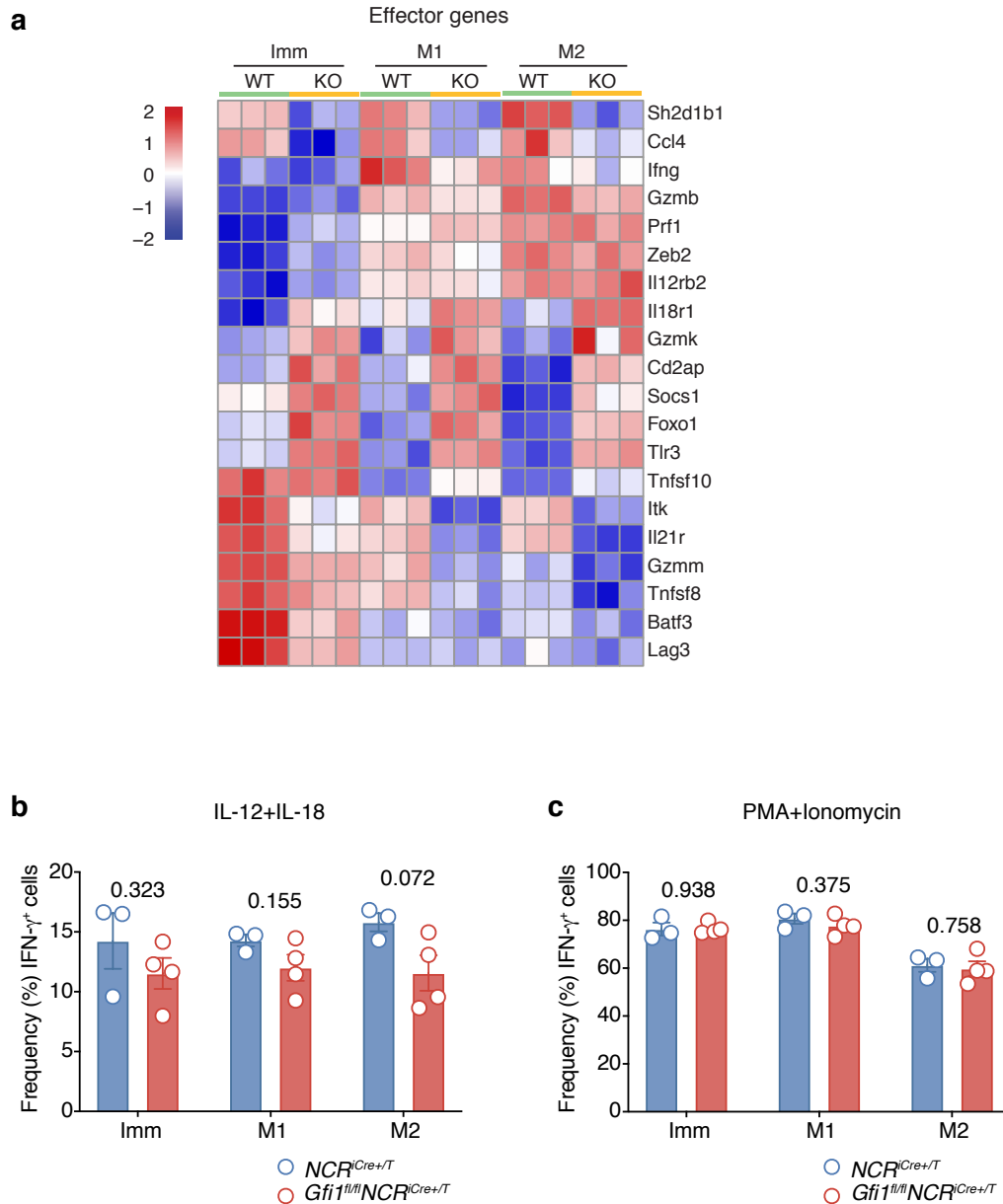


Figure 4.12. GFI1-deficient NK cells have reduced IFN- γ production when stimulated with IL-12 and IL-18.

a, Heatmap showing the expression of manually curated effector genes between $Gfi1^{fl/fl}NCR^{iCre+/T}$ and $NCR^{iCre+/T}$ NK cells for each of the maturation stages. Data show expression for each gene across three independent biological replicates for each NK cell subset.

b-c, Frequency of IFN- γ -producing NK cells for splenic $Gfi1^{fl/fl}NCR^{iCre+/T}$ and control $NCR^{iCre+/T}$ NK cell subsets stimulated with **b**, IL-12 and IL-18 or **c**, PMA and ionomycin for four hours. Data show the mean $\pm$ SEM of individual mice from one experiment ($n=3-4$ mice/genotype/condition). Statistical differences were calculated using unpaired Student's t test.

GFI1-deficient mice fail to control melanoma metastasis

NK cells are important for immune surveillance and protecting against metastasis. In particular, NK cells have been shown to be vital in controlling the tumour burden in the model of B16F10 melanoma cells. In *Mcl1Ncr^{Cre}* mice, which lack NK cells, injected melanoma cells establish a significant tumour burden in the lungs and had metastasised to other organs such as kidney, liver, LN and bone marrow, whereas melanoma cells were only found in the lung and at lower tumour burden by comparison¹⁶². Given the defects in activation and proliferation when GFI1 is deleted and the known function of NK cells in immunosurveillance, I investigated whether the dysregulation of these attributes affected the protection against the establishment of tumours in GFI1-deficient mice. To test this, GFI1-deficient NK cells were injected intravenously with B16F10 melanoma cells into *NCR^{iCre+/T}* and *Gfi1^{fl/fl}NCR^{iCre+/T}* mice and quantified the tumour burden two weeks later (Fig. 4.13a). In control mice, blackened metastatic tumours were primarily found in the lungs, with an average of 42 tumours per lung, and one tumour per liver. By comparison, GFI1-deficient mice had three-fold more tumours in the lung and liver (Fig. 4.13b,c). Analysis of NK cells within the lung revealed that there was a significant reduction in the number of mature M2 NK cells (Fig. 4.13d). Loss of these mature NK cells are likely to contribute to the increased tumour burden observed within GFI1-deficient mice.

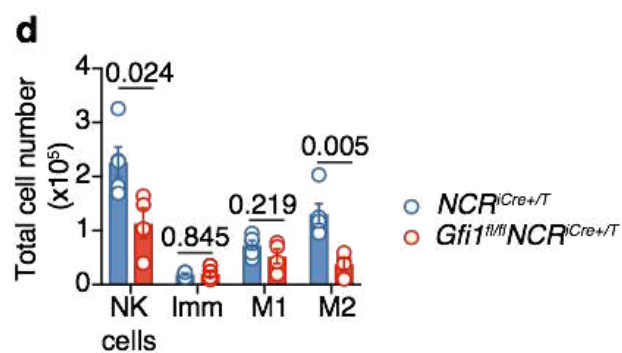
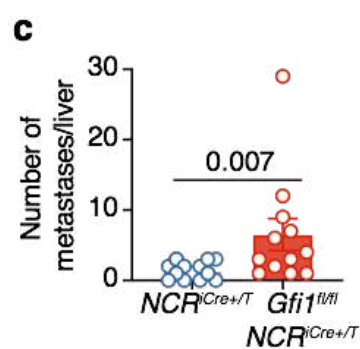
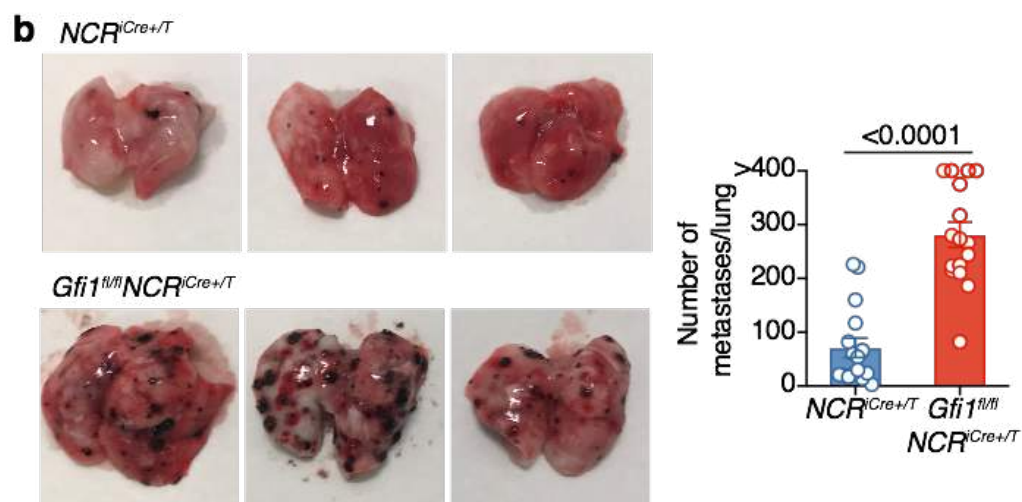
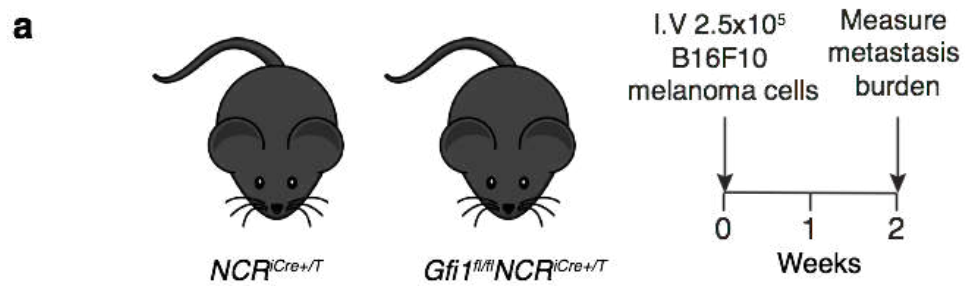


Figure 4.13. GF11-deficient NK cells fail to control B16F10 melanoma tumour metastasis.

a, Schematic of experimental set up. *Gfi1^{fl/fl}NCR^{iCre+/T}* and *NCR^{iCre+/T}* control mice were injected iv with 2.5×10^5 B16F10 melanoma cells. Tumour burden was counted 12-14 days later.

b, Infiltration of B16F10 tumours in the lungs of *Gfi1^{fl/fl}NCR^{iCre+/T}* and *NCR^{iCre+/T}* control mice. Representative lung images are shown (left panels). Histograms (right panel) show the mean number of metastases per lung $\pm$ s.e.m pooled from three independent experiments ($n=3-9$ mice/genotype/experiment). Statistical significance was calculated using the Mann-Whitney *U* test.

c, Infiltration of B16F10 tumours in the liver of *Gfi1^{fl/fl}NCR^{iCre+/T}* and *NCR^{iCre+/T}* control mice. Histograms show the mean number of metastases per lung $\pm$ s.e.m pooled from two independent experiments ($n=3-9$ mice/genotype/experiment). Statistical significance was calculated using the Mann-Whitney *U* test.

d, Enumeration of NK cells and NK cell subsets isolated from the lung of mice injected with B16F10 melanoma cells. Data show mean $\pm$ SEM of one of two similar experiments ($n=4-5$ mice/genotype/experiment). Statistical significance was calculated using an unpaired Student's *t* test.

Loss of GFI1 disadvantages NK cells in a competitive setting

The challenge with B16 melanoma cells established that mice with GFI1-deficient NK cells are unable to control the melanoma burden. It was unclear whether this reflected a failure of function of NK cells, or a failure of recruitment of cells. To test the intrinsic functional capacity of wildtype and GFI1-deficient NK cells in the same environment where both cell types receive the same signal, mixed bone marrow chimeras were generated. Host CD45.1 congenic mice were lethally irradiated and reconstituted with a 1:1 ratio of bone marrow from *NCR^{iCre+/T}* CD45.1⁺CD45.2⁺ and *Gfi1^{fl/fl}**NCR^{iCre+/T}* CD45.2⁺ mice. After six weeks reconstitution, mice were injected with B16F10 melanoma cells and the immune response analysed at two weeks (Fig. 4.14a). Development of immune cells under a competitive environment during tumour challenge, showed that GFI1-deficient CD45.2⁺ NK cells reconstituted four-fold less cells than their CD45.1⁺CD45.2⁺ wildtype counterparts, while B cells, which were not impacted by the loss of GFI1, developed in similar proportions regardless of host origin (Fig. 4.14b). Detailed analysis of the NK cells subsets showed that while there was a 1.5 fold reduction in the frequency of Imm GFI1-deficient CD45.2⁺ NK cells compared to CD45.1⁺CD45.2⁺ wildtype Imm NK cells, the frequency of GFI1-deficient CD45.2⁺ M1 NK cells were reduced by 50% and M2 NK cells showed a four-fold reduction in frequency compared with CD45.1⁺CD45.2⁺ wild-type NK cells (Fig. 4.14c). This indicates that GFI1-deficient NK cells develop less efficiently compared to wild-type cells in a competitive environment and also recapitulates the observation of reduced GFI1-deficient NK cells in the lungs of intact mice challenged with melanoma cells.

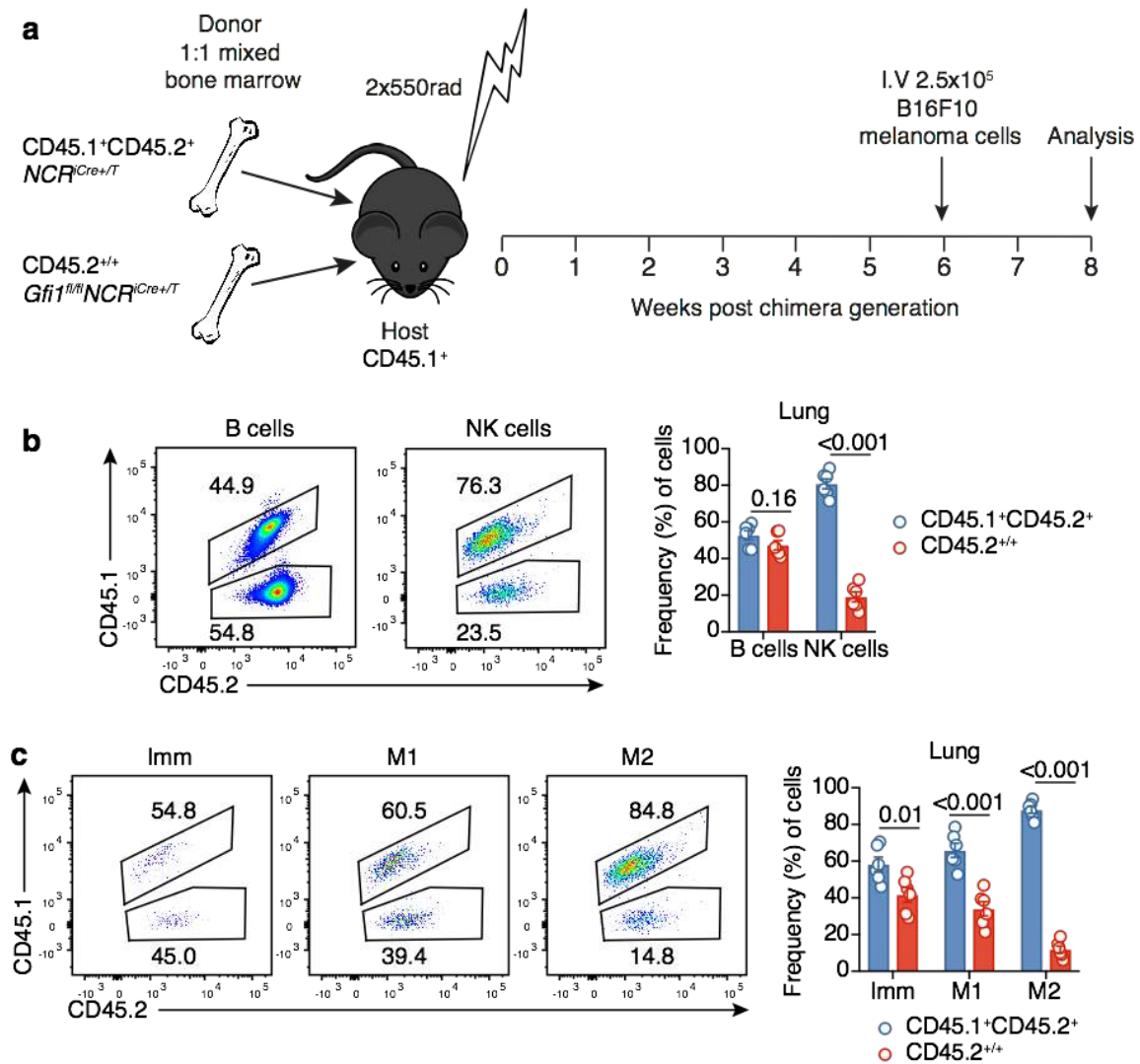


Figure 4.14. GFI1-deficient NK cells are outcompeted by wildtype NK cells.

a, Schematic of generation of mixed bone marrow chimeras. A 1:1 ratio of *Gfi1*^{fl/fl}*NCR*^{iCre+/T} (CD45.2^{+/+}) and *NCR*^{iCre+/T} (CD45.1⁺CD45.2⁺) bone marrow cells was injected into irradiated (2x550rad) host mouse (CD45.1⁺). After six weeks of reconstitution, the host mouse was inoculated with 2.5×10^5 B16F10 melanoma cells and the lungs were taken for flow cytometry analysis after 14 days.

b-c, Representative dot plots of flow cytometry analysis (left panels) and frequency (right panels) of each donor population within **b**, B cells (CD19⁺), NK cells (lin⁻NK1.1⁺NKp46⁺CD49b⁺) and **c**, NK cell subsets (based on CD11b and KLRG1 expression) isolated from the lung. Data show mean $\pm$ SEM of CD45.2^{+/+} and CD45.1⁺CD45.2⁺ cells of each subset from one of three experiments ($n=6$ mice/genotype/experiment). Statistical significance was calculated using an unpaired Student's *t* test.

GFI1-deficient NK cells have reduced effector function in tumour setting

In this environment where GFI1-deficient and proficient NK cells receive the same level of signalling, the functional capacity of GFI1-deficient NK cells when activated by tumour cells were examined. Ki67 expression was measured in each NK cell subset to determine whether the proliferative capacity of GFI1-deficient NK cells was affected in a tumour setting. The frequency of Ki67⁺ GFI1-deficient NK cells was reduced compared to wildtype NK cells (Fig. 4.15a). These results indicate that GFI1-deficient NK cells could not restore their proliferative capacity when activated by tumour cells. Consistent with expression of DNAM-1 at steady state conditions, there was a reduction in DNAM-1, in addition to the inhibitory receptor TIGIT in GFI1-deleted NK cells when challenged by tumour cells. However, expression of the apoptosis-inducing ligand TRAIL did not change (Fig. 4.15b). The full capacity of these NK cells to produce cytokines was examined by stimulation with PMA and ionomycin. I found that the GFI1-deficient M1 cells had a reduction in the frequency of IFN- γ producing cells. (Fig. 4.15c). The intensity of granzyme B production was also reduced in M2 NK cells when GFI1 was lost (Fig. 4.15d). This indicated that loss of GFI1 affected both the number of mature NK cells within the lung and the capacity of NK cells to mediate effector functions to facilitate tumour elimination.

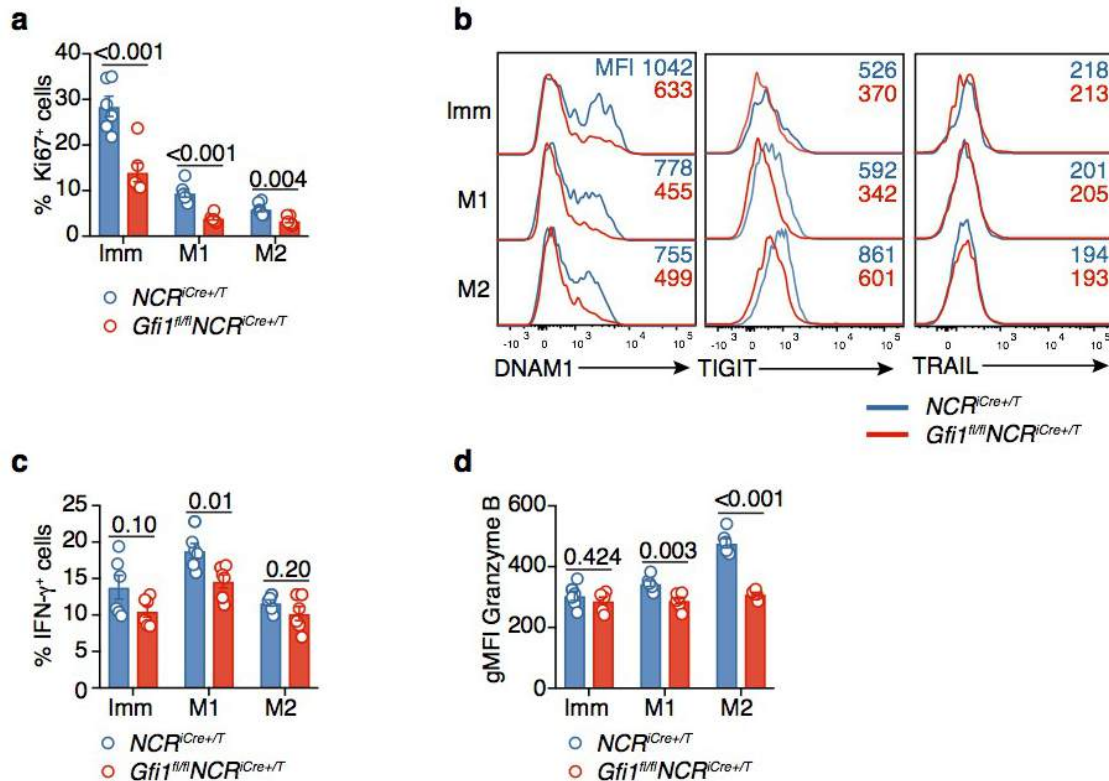


Figure 4.15. Loss of GFI1 reduced activation of NK cells.

a, Frequency of Ki67⁺ cells in *Gfi1*^{fl/fl}*NCR*^{Cre+/T} (CD45.2^{+/+}) and *NCR*^{Cre+/T} (CD45.1⁺CD45.2⁺) NK cell subsets isolated from the lungs of mixed bone marrow chimeras 14 days after inoculation with B16F10 melanoma cells.

b, Expression of DNAM-1, TIGIT and TRAIL on the surface of CD45.2^{+/+} and CD45.1⁺CD45.2⁺ NK cell subsets from the lungs of mixed bone marrow chimeras 14 days after inoculation with B16F10 melanoma cells. Geometric MFI of each marker on CD45.2^{+/+} and CD45.1⁺CD45.2⁺ NK cell subsets is shown. Data show representative profiles of one of three similar experiments ($n=6$ mice/genotype/experiment).

c, Frequency of IFN- γ -producing cells in *Gfi1*^{fl/fl}*NCR*^{Cre+/T} and *NCR*^{Cre+/T} NK cell subsets isolated from the lung of mixed bone marrow chimeras inoculated with B16F10 14 days previously. Cells were stimulated with PMA and ionomycin for 4 hours prior to intracellular staining.

d, Expression of granzyme B produced by *Gfi1*^{fl/fl}*NCR*^{Cre+/T} and *NCR*^{Cre+/T} NK cell subsets isolated from the lungs of mixed bone marrow chimeras inoculated with B16F10 14 days previously. **a,c,d**, Histograms show mean $\pm$ SEM of each donor population from one of three independent experiments ($n=6$ mice/genotype/experiment). Statistical significance was calculated using an unpaired Student's t test.

Discussion

GFI1 is a transcriptional regulator of ILC2 and CD4⁺ T cell development and function^{100, 105, 110} but it has not been reported whether GFI1 is also involved in the regulation of other lymphocytes, such as ILCs. The experiments performed in this study show a previously unknown requirement of GFI1 by group 1 ILCs, with a focus on identifying the multiple pathways involved in regulating NK cell development and function.

3.3.5 Homeostatic regulation of NK cells

NK cells undergo homeostatic proliferation at steady-state to occupy the NK cell niche and maintain sufficient numbers of NK cells to mediate immunosurveillance and to also ramp up proliferation during an immune response to a disease challenge. In particular, the Imm NK cell subset has a greater proliferative capacity compared to the mature subsets¹⁴⁷. My studies show that loss of GFI1 disrupts the NK cell capacity to proliferate (Fig. 4.7-8). This defect is most evident in Imm NK cells in which deletion of GFI1 resulted in downregulation of cell cycling genes. NK cells depend on IL-15 signalling to drive proliferation and survival^{160, 162, 163}. Following IL-15 binding to the IL-15R on NK cells, JAK1 activation subsequently induces STAT5 phosphorylation and dimerization to initiate transcription of genes involved in cell cycling¹⁷⁰. This defect in the absence of GFI1 is not due to a loss of IL-15R expression, therefore, we investigated the JAK-STAT downstream signalling pathway. While RNASeq results did not show any differences in the expression of *Stat5a* or *Stat5b*, preliminary results indicated that there was a reduction of phosphorylation of STAT5 in GFI1-deficient NK cells (Fig. 4.9). Further analyses will be required to confirm these findings. In the absence of STAT5 phosphorylation, JAK/STAT signalling would be disrupted, resulting in a diminished capacity for GFI1-deficient NK cells to undergo proliferation and upregulate cytolytic functions^{171, 172}. Alternatively, GFI1 could also regulate cell cycle gene. It has been shown in HeLa cells that the cell cycle regulator *p21^{Cip/WAF1}* (also known as *Cdkn1a*) is a direct target of GFI⁸¹. GFI1 recruits G9a and HDAC1 to the promoter region of *p21^{Cip/WAF1}* and increases H3K9 methylation, thereby suppressing *p21^{Cip/WAF1}* expression⁸¹. As RNASeq data also indicated that *p21^{Cip/WAF1}* was downregulated in GFI1-deficient Imm NK cells (Fig. 4.7a), it is possible GFI1 is also directly regulating NK cells via a similar mechanism.

3.3.6 NK cell mediated protection in metastasis

Using the B16F10 melanoma model, I show that mice with GFI1-deficient NK cells are unable to effectively control tumour progression (Fig. 4.13). This loss of protection correlated with a substantial loss of mature NK cells, the cells mainly responsible for mediating effector functions through cytokine production and cytotoxic killing. The reduction in NK cell numbers is, in part, due to a failure of GFI1-deficient NK cells to respond to the IL-15 that are produced within the tumour microenvironment¹⁷³ and therefore cannot proliferate efficiently (Fig 4.15a).

The function of mature NK cells is also compromised as GFI1-deficient NK cells had reduced expression of DNAM-1, which plays an important role to drive anti-tumour activity in NK cells (Fig. 4.15b-d). Subsequently, GFI1-deficient NK cells with low DNAM-1 expression also had reduced IFN- γ and granzyme B production compared to GFI1-proficient NK cells. Since no significant difference in cytokine production was observed when steady-state NK cells were stimulated with IL-12 and IL-18, this indicated that GFI1's role in regulating cytokine production may be specific to activation in response to tumour cells, other factors *in vivo* or when the cells are in a competitive environment. As the functional defects in GFI1-deficient NK cells were established in a chimera setting with melanoma tumours, additional experiments are required to assess mixed bone marrow chimeras without tumours. These experiments would show the capacity of cytokine production in GFI1-deficient NK cells when they are required to compete with wild-type NK cells. Furthermore, it is not known if this decline in NK cell activation and effector functioning is significant enough to directly impact tumour clearance. Therefore, an *in vitro* killing assay should be performed where equal numbers of either GFI1-proficient or deficient NK cell subsets are co-cultured with B16F10 melanoma cells and death of melanoma cells are quantified. This will determine the direct killing capacity of GFI1-deficient NK cells and whether the reduced activation is sufficient to result in a greater metastatic burden on the host.

3.3.7 GFI1-mediated transcriptional regulation

Mechanistically, it not yet known how GFI1 regulates these processes. However, RNASeq has allowed me to identify a number of transcription factors that are differentially expressed when GFI1 was deleted, including *Foxo1*, *Tox*, *Id2*, *Eomes*, *Klf2*, *Mef* and *Zeb2*. These transcription

factors are involved in regulating NK cell development, localisation and activation and my data indicate that these processes are also disrupted by loss of GFI1. This is a good indication that GFI1 is a key regulator of NK cells by targeting the expression of these transcription factors. Future experiments performing CUT&Tag will allow me to identify the direct binding partners of GFI1¹⁷⁴. This process is preferable compared to ChIP as it is sensitive enough to utilise low cell numbers, whereas ChIP will require NK cells to be cultured in vitro to expand their numbers and may alter the epigenetic profile.

FOXO1, a negative regulator of many NK cell processes including maturation, migration and cytotoxic functions⁵⁶, may be a possible candidate for GFI1 targeted regulation. Since *Foxo1* expression had increased in all NK cell subsets following GFI1 deletion, this suggested that GFI1 negatively regulates *Foxo1* expression but will need to be confirmed on a protein level with antibody staining. FOXO1 has been proposed to bind directly to T-bet to downregulate expression and thereby control NK cell maturation⁵⁶. However, there was no significant difference in T-bet expression in GFI1-deficient NK cells, therefore, FOXO1 may also regulate NK cell development in a T-bet-independent pathway. FOXO1 has also been shown to be a negative regulator of NK cell cytokine production and cytotoxic killing^{56, 57}. During NK cell activation, FOXO1 is phosphorylated and sequestered in the cytoplasm, where it is ubiquitinated and degraded. This phosphorylation process is DNAM-1 dependent¹⁷⁵. As expression of the *Foxo1* transcript had increased in GFI1-deficient NK cells and mice with GFI1-deficient NK cells had higher tumour burdens, I hypothesis that GFI1-mediated inhibition of *Foxo1* transcription may be an alternative mechanism to regulate FOXO1 activity during NK cell activation. If GFI1 does negatively regulate FOXO1 expression, this presents an interesting potential treatment strategy for metastatic tumours that respond well to NK targeting. Whether increased GFI1 expression has the potential to improve NK cell responses against metastatic tumours may be worth investigating.

Chapter 5. Role of ILCs and associated cytokines in colorectal cancer

5.1 Abstract

Chronic inflammation of the gastrointestinal tract contributes to colorectal cancer (CRC) progression. While the role of adaptive T cells in CRC is now well established, the role of innate immune cells, specifically innate lymphoid cells (ILCs), is not well understood. To define the role of ILCs in CRC I employed a chemically-induced CRC mouse model and identified an accumulation of ILCs, particularly IL-5-producing ILC2, within the colon of mice with CRC. This strongly correlated with an increase in tumour burden as well as a concomitant increase in eosinophil and neutrophil numbers. ILC2s mediate a protective role against the development of CRC as ILC2-deficient mice developed a higher tumour burden compared with littermate wild-type controls. This suggests a critical role for ILC2 in CRC, suggesting a potential new avenue to improve clinical outcomes through ILC2-agonist based therapeutic approaches.

4.2 Introduction ¹

4.2.1 Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer diagnosed worldwide, with Australia and New Zealand leading in incidence rates compared to the rest of the world¹⁷⁶. Implementing screening programs to assist with early diagnosis can improve patient outcomes¹⁷⁷. Yet CRC mortality still remains the second highest cause of cancer related deaths, with more than 900,000 deaths every year worldwide¹⁷⁶. The risk of developing CRC is associated with age, sex and environmental factors such as exposure to smoking, alcohol and poor diet¹⁷⁸⁻¹⁸¹. In addition, inflammatory bowel disease (IBD) and chronic colitis have also been associated with an increased risk of CRC development¹⁸²⁻¹⁸⁴.

4.2.2 The role of immune cells in CRC

Most colorectal tumours develop from glandular epithelial cells of the colon or rectum¹⁸⁵ into benign lesions called polyps¹⁸⁴. These polyps progressively acquire genetic and epigenetic modifications to form malignant tumours^{179, 180, 184, 186, 187}. Interactions with local immune cells strikes a critical balance between pro- and anti-tumour immune responses, which determine either the eradication of nascent lesions or the development and progression of transformed cells that form malignant tumours^{184, 188}. In particular, gene expression of cytokines associated with CD4⁺ T cells have been found to be elevated in primary colorectal tumours compared to normal mucosa¹⁸⁹. While expression of Th2-associated genes did not positively or negatively correlate with any clinical outcomes, low expression of Th1 and high expression of Th17-associated genes were associated with a poor clinical outcome^{189, 190}. It is important to note that T cells are not the only source of these cytokines. ILCs are also key producers of pro-inflammatory cytokines and their gene signatures can parallel those of CD4⁺ T cells^{191, 192}. However, the impact of ILCs on the development of CRC is not well understood. It has not been explicitly examined whether the source of these cytokines is T cells or ILCs were so it is possible that ILCs also contribute to CRC development¹⁸⁴.

¹ A portion of the Introduction has been published (Huang *et al.*, 2019, *Frontiers in Immunology*), where my contribution to the writing was 50%. In this Introduction chapter, only the published sections that I have 100% written are included.

Type 1 innate lymphoid cells

The role of ILCs is only beginning to be examined in CRC, however, they have been reported to be capable of both driving and protecting against the development of CRC (Fig. 5.1). Analysis of human colorectal tumours have revealed that they are infiltrated by NK cells and intraepithelial-like ILC1^{193, 194}. These cells secrete cytotoxic molecules such as granzymes and perforin^{193, 194} and cytokines such as IFN- γ following stimulation^{184, 193}. Studies tracking intraepithelial ILC1s show that they accumulate in the intestine of patients with Crohn's Disease and in CRC^{184, 193, 195, 196}. These intraepithelial ILC1s can be activated by cytokines such as IL-12 and IL-18, which can be produced by intestinal epithelial cells following activation, to produce IFN- γ and exacerbates colitis^{184, 195, 197}. Further studies are required to define whether intraepithelial ILC1s are driving the development of CRC, or whether they also have the capacity to be key players in the cytotoxic anti-tumour response.

NK cells are known to mediate anti-tumourigenic activity¹⁹⁸. The loss of class I major histocompatibility complex (MHC) on cancer cells lowers the inhibitory signal to NK cells and the high expression of ligands on tumour cells activate NK cell, marking them for NK cell-mediated lysis, providing a mechanism to potentially prevent tumour cell re-emergence or CRC metastasis^{184, 199, 200}. However, tumour cells can circumvent NK cell recognition by upregulating MHC class I expression to suppress NK cell activation or by reducing the expression of NCR ligands to escape NK cell-targeted killing^{184, 199}. Furthermore, NK cell infiltration can be impaired despite elevated levels of chemokines found in CRC tumours^{184, 201}. Thus, circulating NK cells in CRC patients are often phenotypically altered reflecting such induced changes^{184, 202}. These patients exhibit reduced NKp44 expression on their CD56^{dim} cells²⁰², a key subset that normally exhibit potent effector function. In addition, high NKp30 expression on circulating CD56^{bright} cells has been associated with a shortened disease-free survival period in CRC patients^{184, 202}. Other receptors and ligands expressed on NK cells of CRC patients such as activating and inhibitory KIRs²⁰³ or TIGIT¹⁵¹ have also been suggested to play distinct roles in the development, progression and metastasis of CRC tumours¹⁸⁴. In mice, neutralizing TIGIT markedly prolongs the survival of tumour bearing animals^{151, 184}. However, when NK cells are depleted in this setting, the effect was completely abrogated highlighting the critical role of NK cells. Collectively, it is clear that NK cells are involved in the control of CRC progression and metastasis but are also susceptible to tumour-directed

dysregulation^{184, 204}. To circumvent such events, current NK cell-based therapies aim to boost NK cell function in an effort to strategically improve patient outcomes^{184, 205-208}.

Type 2 innate lymphoid cells

The role of ILC2 has been examined in a number of different cancers including lung and gastric cancer^{119, 184, 209}. Nevertheless, their involvement in colorectal cancer is, to date, only poorly understood¹⁸⁴. ILC2 frequency is increased in mucosal samples from IBD patients compared with healthy individuals^{184, 210}. In CRC tumours, ILC2s are activated and express higher levels of ICOS and CD69 compared with ILC2 found in normal colon tissue^{184, 193, 194}.

Activated ILC2s are prominent producers of cytokines such as IL-4, IL-5 and IL-13 and are potentially responsible for the elevated levels of cytokines found in the serum of CRC bearing mice²¹¹. However, the subsequent downstream signalling of these cytokines in CRC are not clear as they have been differently correlated to prognosis in different studies. In CRC, high expression of the IL-13 receptor subunit, IL-13R α 2 on epithelial cells is associated with more advanced disease stages, lymph node involvement, the presence of metastases and reduced survival^{184, 212}. In contrast, Saigusa²¹³ *et al* found that low preoperative serum IL-13 was a predictive marker for poorer prognosis of CRC patients¹⁸⁴. The role of IL-5 has not been studied in detail in CRC. Patients suffering from UC, who have a higher risk of CRC development, were found to have elevated IL-5 transcript levels^{184, 214}. While the impact of having elevated IL-5 levels in CRC patients is not known, one possible downstream target are eosinophils. IL-5 has been found to be important for eosinophil recruitment, expansion and activation^{215, 216}. In turn, eosinophils secrete chemoattractants such as CXCL9, CXCL10 and CCL5 which promote the accumulation of CD8⁺ T cells in the tumour^{184, 217}. The presence of peritumoral eosinophils is a prognostic factor has also been associated with favourable progression free survival²¹⁸. However, neutralizing IL-5 in mice and reducing eosinophil numbers have been reported to both diminish the inflammation associated with DSS-induced colitis and promote CRC growth of subcutaneously injected MC38 CRC cell lines *in vivo*^{219, 220}. Therefore, the exact role of ILC2 produced cytokines on CRC development or patient survival is currently unknown¹⁸⁴.

Type 3 innate lymphoid cells

Inflammation is a key driver of cancer progression and the link between IBD and the development of CRC is well established^{184, 221}. This inflammation can be exacerbated by the loss of barrier protection occurring early during CRC development^{184, 222}, allowing for the infiltration of microbes and their associated products into the tumour microenvironment. This activates dendritic cells and macrophages to produce IL-23²²³, a key cytokine regulating ILC3 function^{184, 224}. IL-23 has been found to be overexpressed in both mouse and human CRC tumours^{37, 222, 225} and IL-23 deficiency has been shown to protect against tumour development in a number of mouse models of cancer^{184, 225}. Conversely, chronic IL-23 injection induces ILC3-dependent duodenal adenoma development in mice^{184, 226}. Furthermore, local IL-23 expression drives ILC3 accumulation and activation within the colonic lamina propria^{184, 227, 228}. These intestinal ILC3 express higher levels of the activating receptors ICOS and CD69^{193, 194}, regulators of tumour responses CD244 and CD39¹⁹³, and exhibit enhanced production of the downstream signalling cytokines IL-17 and IL-22 that contribute to the pathogenesis of CRC^{37, 184}.

Several studies have reported high IL-17 expression in both mouse and human CRC compared with normal adjacent tissues^{37, 184, 229-231}. ILC3s are a major producer of IL-17 but other immune and non-immune intestinal cell types can also express this cytokine, including Th17 cells, $\gamma\delta$ T cells and Paneth cells^{184, 232}. Importantly, IL-17 transcripts are increased during progression of tumours from the adenoma to carcinoma stages in CRC patients^{184, 233}. Furthermore, IL-17 can alleviate cell cycle inhibition, thereby promoting epithelial proliferation and intestinal inflammation²³⁴ and increase angiogenesis by inducing VEGF expression^{184, 231}. Administration of anti-IL-17 antibodies have been shown to ameliorate inflammation severity³⁷ while genetic ablation of IL-17 was able to reduce tumour burden^{184, 222, 228, 234, 235}. Although, these studies show that IL-17 is a pro-inflammatory cytokine involved in CRC pathogenesis, IL-17 may also play a protective role. Lin and colleagues²³⁰ reported better patient overall survival associated with high IL-17 levels²³⁰ and neutralizing IL-17 exacerbated Crohn's disease and DSS-mediated colitis by increasing gut permeability^{184, 236, 237}.

In a similar pattern to IL-17, the ILC3-driven IL-22 pathway plays both protective and pathogenic roles during colitis and tumorigenesis¹⁸⁴. Under normal conditions, IL-22 is

required for homeostatic epithelial cell proliferation through STAT3 activation^{184, 238, 239}. Loss of IL-22 results in delayed tissue repair and exacerbated inflammation²⁴⁰. Conversely, sustained IL-22-dependent epithelial proliferation leads to tumour progression and neutralizing IL-22 reduced CRC development^{37, 184}. In addition to this, loss of IL-22 binding protein, a soluble decoy receptor neutralizing IL-22-driven cell activation, has been shown to increase epithelial cell proliferation and was associated with a higher tumour burden^{184, 241}.

These studies suggest that IL-23 signalling in ILC3 is central in driving CRC development¹⁸⁴. Indeed, mice lacking ILC3 are protected from tumour development when treated with IL-23²²⁶. However, the downstream IL-17 and IL-22 signalling pathways seem to play a dual role in CRC¹⁸⁴. These findings suggest that initial activation of the IL-23/ILC3 pathway is sufficient to trigger tumour development but additional mechanisms influence CRC progression and outcomes¹⁸⁴. Collectively, further investigations are warranted to unravel the role of these cytokines in CRC pathogenesis¹⁸⁴.

5.1.1 Aims and hypothesis

Analysis of immune subsets in CRC patient samples have identified tissue-resident ILCs and are thought to be involved in anti-tumour responses¹⁹⁴. However, key cytokines produced by ILCs, with the exception of IL-17 and IL-22, have not been extensively examined in CRC development. Furthermore, many cytokines have the capacity to play dual roles in promoting or suppressing inflammation and tumour development. Therefore, careful and detailed analysis of the role of ILCs in CRC and the cytokines they produce are required to fully dissect their role in disease development and progression.

I hypothesised that ILCs contribute to the progression of inflammation-associated cancer and represent a novel therapeutic opportunity.

The aim of this chapter was to:

- (a) Catalogue the expression of ILCs and their associated cytokines in a murine model of CRC.
- (b) Identify a candidate cytokine that may contribute to tumour progression.
- (c) Investigate a causative role in CRC progression.

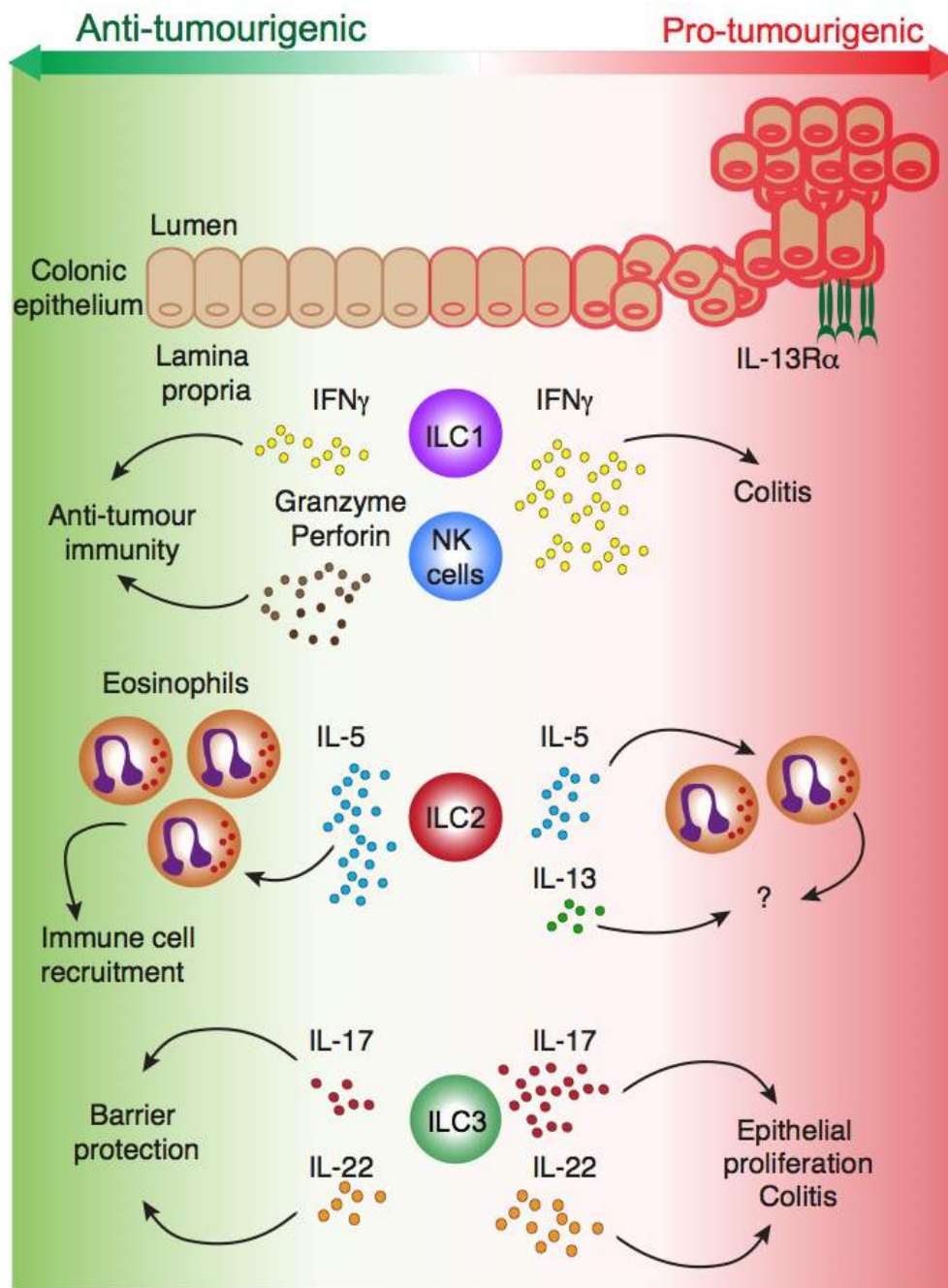


Figure 5.1. Summary of the role of intestinal ILCs in the development of CRC.

Through the secretion of cytokines and cytotoxic molecules, ILCs can modulate the tumour microenvironment to either control or promote CRC development and progression

4.3 Results

4.3.1 Characterisation of the source of IL-5 in CAC

The AOM-DSS model of CAC induces multiple tumours in the colon

To investigate whether ILCs are involved in colitis-associated colorectal cancer (CAC), an inducible CAC model, referred to as the AOM+DSS model was used (Fig. 5.2A). Azoxymethane (AOM) was injected i.p. into C57BL/6 mice. AOM has been reported to be processed into methylazoxymethanol by enzymes in the liver, transported to the intestine and further degraded, resulting in DNA alkylation and mutations which accumulate in epithelial cells¹³². Colonic tumours induced by AOM mimic the aberrant activation of the Wnt-signalling pathway often found in patient tumours²⁴². In these tumours, the β -catenin gene contains missense mutations and the protein becomes resistant to proteasomal degradation. This results in increased activation of Wnt target genes, and cell proliferation²⁴³. In the experimental setting, multiple rounds of the pro-inflammatory agent DSS (dextran sodium sulfate) were subsequently administered in the drinking water to disrupt the epithelial barrier of the colon, stimulating a pro-inflammatory response^{244, 245}. The combination of AOM and DSS accelerates tumour growth and accurately recapitulates the inflammation present in CRC patients²⁴⁶.

Endoscopy of mice between 7-10 weeks after the commencement of the AOM-DSS treatment revealed that these mice had inflamed colonic lining, increased mucus production, loose stools and developed macroscopic tumours, whereas the untreated mice did not show these symptoms (Fig. 5.2B). Tumours were primarily located at the mid and distal regions of the colon (Fig. 5.2C). The majority of the mice had accumulated between 2-8 tumours per colon with a combined tumour area of between 10-45mm² per colon (Fig. 5.2C).

H&E staining of the colon was performed to show the histopathology induced by AOM+DSS treatment. In the distal region of the colon, AOM+DSS treated mice showed increased immune cell infiltration, and macroscopic regions of adenocarcinoma growth. Untreated colons had crypts of uniform length, whereas AOM+DSS treated colon had darkly stained aberrant crypt structures (Fig 5.2D). These features mimic the development of CRC in humans²⁴⁶.

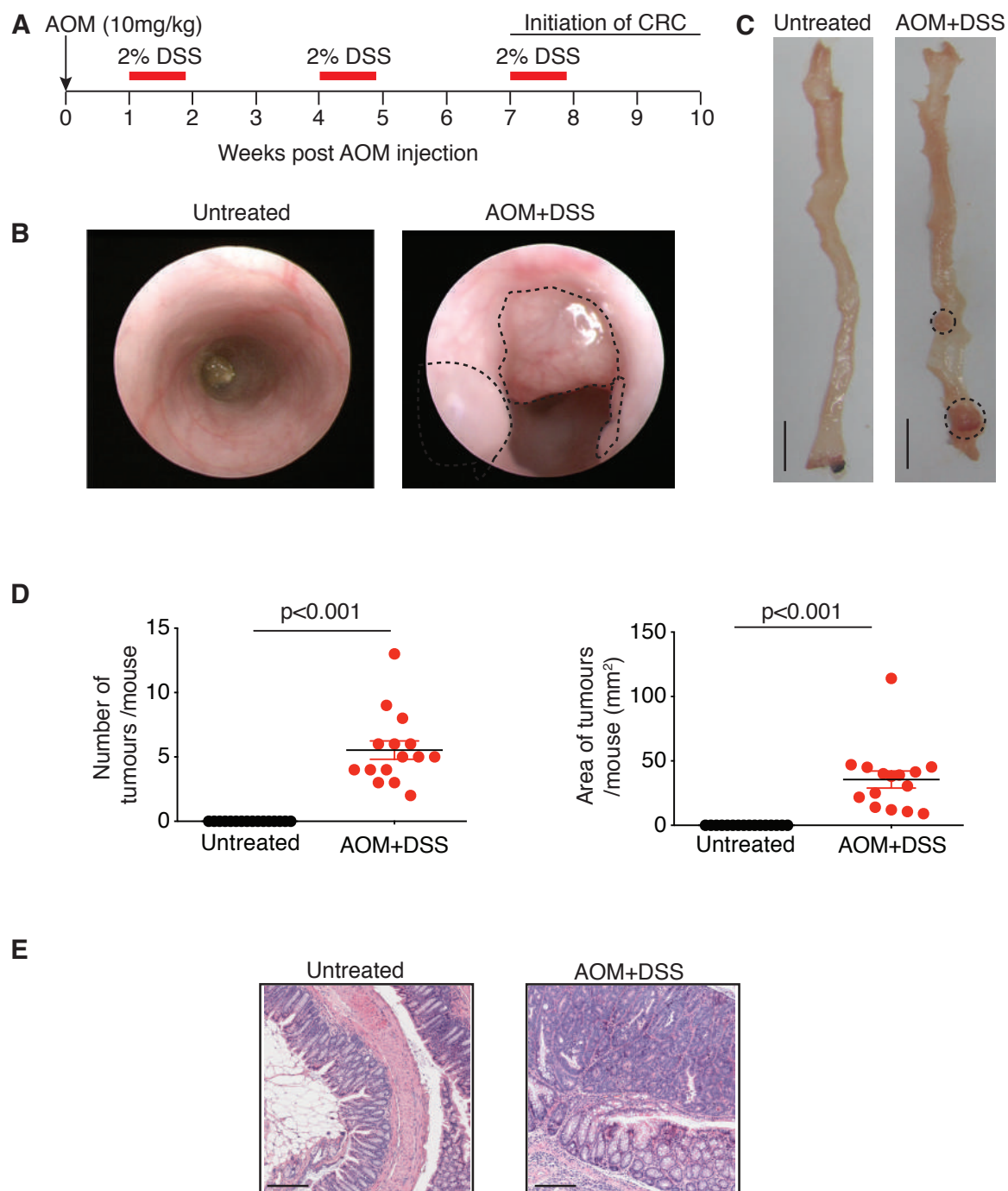


Figure 5.2. AOM+DSS treatment induces development of colitis-associated colorectal cancer.

A. Schematic illustration of the AOM/DSS treatment protocol for C57BL/6 mice. AOM (10mg/kg) was administered I.P to mice. This was followed by three cycles of 2% (w/v) DSS in the drinking water *ad libitum* for five days, then two weeks of normal water between each cycle. Colorectal cancer developed between 7-10 weeks after initiation of first treatment.

B. Representative endoscopy images of untreated (left panel) and AOM/DSS treated mice (right panel). Images are representative of 2-3 mice analysed in four separate experiments. Dotted line indicates tumour growth. Endoscopy was performed by Adele Preaudet.

C. Representative whole mount images of colons from an untreated (left panel) and an AOM+DSS treated (right panel) mouse. Images are representative of four separate experiments with 2-3 mice in each treatment group per experiment. Dotted lines indicate the position of tumour in colon. Scale bar, 1 cm.

D. Enumeration of the total number of tumours (left panel) and area of tumours (mm², right panel) per mouse in untreated and AOM+DSS treatment groups. Shown are the mean $\pm$ s.e.m of data pooled from four experiments (2-3 mice/treatment/experiment). Statistics analyses was performed using the Mann-Whitney nonparametric test; exact *p*-values are shown.

E. Representative H&E staining of the distal colon of untreated and AOM+DSS treated mice. Images are representative of 10< mice analysed. Scale bar, 200 μ m.

Total ILC1 and ILC3 are increased in AOM-DSS treatment

The ILC population within the colon, mesenteric LN and spleen of mice treated with AOM+DSS and untreated naïve mice was analysed using flow cytometry (Fig. 5.3A). In untreated mice, the most prevalent ILC subset within the colon was ILC2, while NK cells were the most abundant subset in the mesenteric LN and spleen (Fig. 5.3B). In mice treated with AOM+DSS, there was a 2-fold, 6.7-fold and 3.8-fold increase in the number of ILC1, NK cells and ILC3 respectively in the colon compared with untreated mice (Fig. 5.3B). ILC1, NK cells and ILC2 had also increased in the mesenteric LN of AOM+DSS treated mice whereas there was no change in the number of ILCs in the spleen. This indicates that this is a localised inflammatory response in the colon and corresponding draining lymph node, but there is no systemic effect by AOM+DSS.

To determine if the accumulation of ILC subsets was correlated with the tumour burden, the total area of tumours per colon was compared to the number of each ILC subset. Most immune cell subsets did not demonstrate a significant correlation with tumour induction. The number of NK cells showed a moderate positive correlation with the tumour burden ($r=0.642$) (Fig. 5.3C). While the correlation with other ILC subsets was weak (Fig. 5.3C).

IL-5-producing ILC2 are prominent in CAC model

Different cytokines have been implicated in modulating the tumour microenvironment. It has been shown that IL-22 produced by ILC3s were responsible for driving tumorigenesis in immune-deficient mice³⁷ while IL-5 can facilitate metastasis of tumour cells, including colon adenocarcinoma cells to the lung³⁶. Therefore, I assessed whether the ILCs, in an immune competent setting, were a potential source of cytokines that influenced CAC progression, particularly IL-5 produced by ILC2.

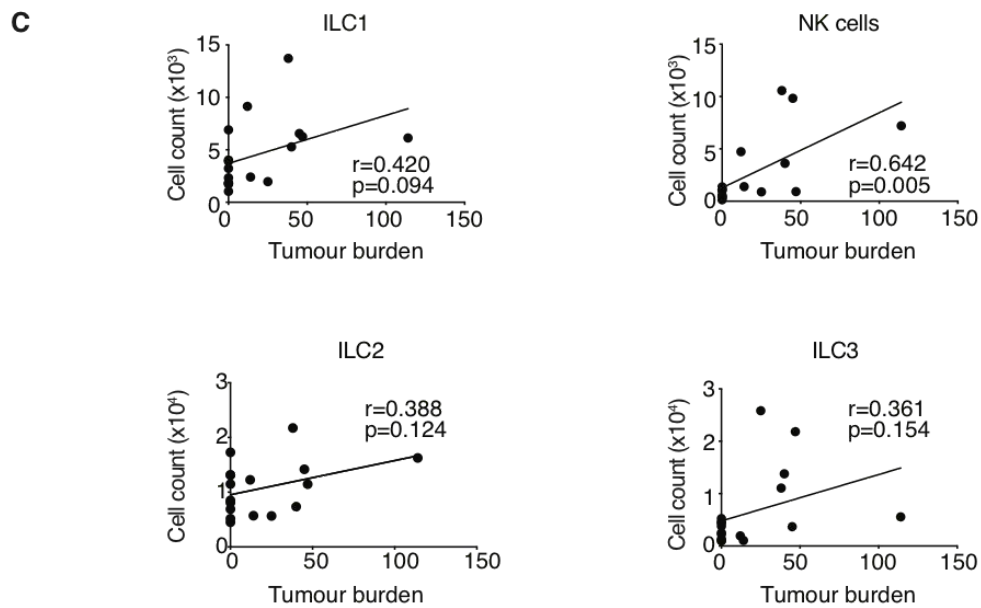
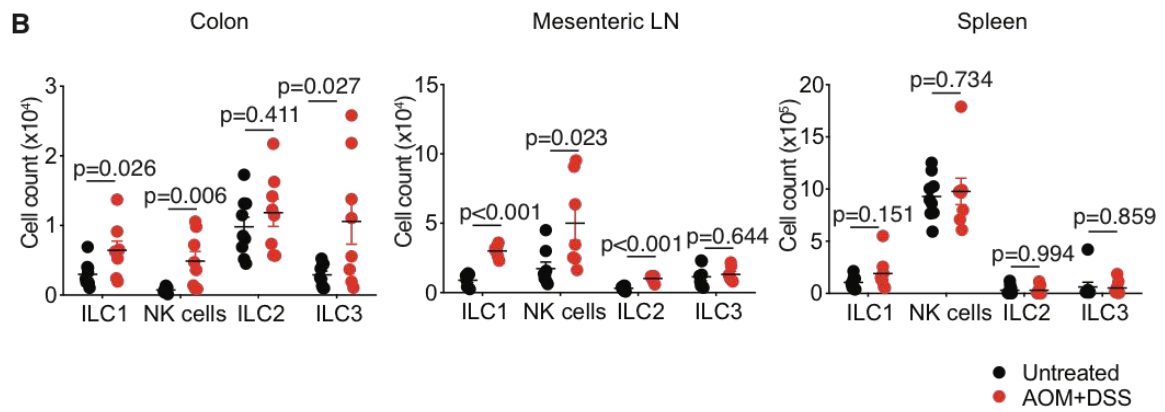
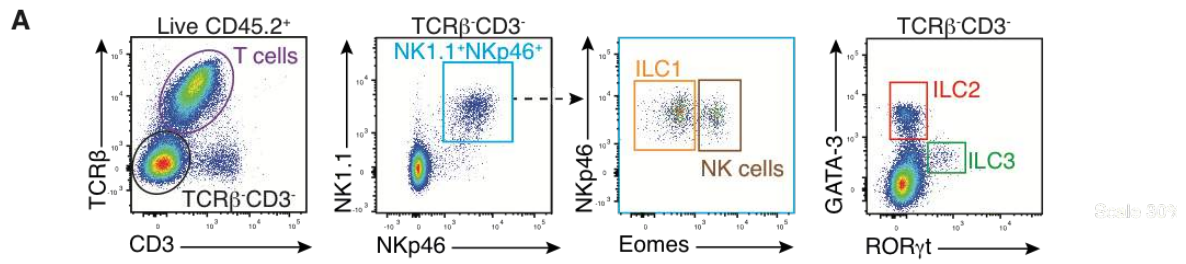


Figure 5.3. ILCs accumulate in the colon following AOM+DSS treatment.

A. Gating strategy for flow cytometric analysis of ILCs and T cells. T cells are defined as live CD45.2⁺ TCRβ⁺CD3⁺ cells; ILCs defined as CD45.2⁺TCRβ⁻CD3⁻ cells with group 1 ILCs (NK1.1⁺NKp46⁺) divided into ILC1s (Eomes⁻) and NK cells (Eomes⁺). ILC2s are defined as TCRβ⁻CD3⁻ cells that are GATA-3⁺ while ILC3s are RORγt⁺.

B. Enumeration of ILC subsets isolated from the colon, mesenteric lymph node (MLN) and spleen of untreated and AOM+DSS-treated mice between 7-10 weeks after treatment the initiation of treatment. Data show the mean ± SEM of data pooled from four independent experiments (*n*=2-3 mice/treatment/experiment). Statistical analyses was undertaken using an unpaired Student's *t* test, exact *p*-values are shown.

C. Correlation of total ILC for each subset with tumour burden (area of tumour mm²) per colon calculated using a linear regression model. The correlation (*r*) and *p*-value as indicated.

The colons of AOM+DSS treated and untreated mice were dissociated and digested to isolate lymphocytes from the lamina propria and subsequently restimulated with PMA and ionomycin in the presence of monensin and brefeldin A to assess their capacity to produce cytokines (Fig. 5.4A). Flow cytometric analysis showed that there was an increase in the frequency of TNF- α -producing ILC1 and NK cells, IL-5-producing and IL-13-producing ILC2, and IL-17-producing ILC3 in the colon of AOM+DSS treated mice compared to untreated mice (Fig. 5.4B). While there was no change in the frequency of IFN- γ -producing ILC1 and NK cells and IL-22-producing ILC3s, the total number of cytokine-producing cells for all ILC subsets had increased in tumour-bearing mice (Fig. 5.4C). IL-5-producing ILC2s were numerically the largest population of cytokine-producing ILC present in this environment (Fig. 5.4C).

The total area of the cumulative tumour mass was then compared against the number of cytokine-producing ILCs. This showed that the number of IFN- γ and TNF- α -producing ILC1 only had a weak positive correlation while IFN- γ and TNF- α -producing NK cells and IL-17-producing ILC3 had a moderate positive correlation with the tumour burden. These cytokine-producing ILCs may not directly affect the size of tumour growth. In contrast, the number of IL-5- ($r=0.819$) and IL-13- ($r=0.922$) producing ILC2 and IL-22-producing ILC3 ($r=0.830$) had a strong positive correlation with the tumour burden and is therefore may be involved in tumour development (Fig. 5.4D). As the role of IL-22 and IL-13 have been extensively investigated in CAC, I focussed my investigations on IL-5 by ILC2 in CAC development.

T cells predominantly produce IFN- γ , TNF- α and IL-17 in CAC model

ILCs are not the only source of cytokines in the colon microenvironment, T cells also have the ability to produce cytokines such as IL-17 which are in CRC patients^{229, 231}. Therefore, T cells and their capacity to produce cytokines during CAC was also examined. There was an increase in the number of T cells within the colon and mesenteric LN of mice with CAC but no difference was observed in the spleen (Fig. 5.5A). The number of T cells within the colon was also strongly correlated with the tumour burden ($r=0.862$) (Fig. 5.5B). Following restimulation, there was an increase in the frequency of TNF- α - and IL-17-producing T cells in the colon of AOM+DSS treated mice compared to untreated mice (Fig. 5.5C). The number of IFN- γ -, TNF- α - and IL-17-producing T cells also increased in the colon of mice with CAC and these are the predominant cytokines produced by T cells (Fig. 5.5D). In addition, IL-5- and IL-22-producing

producing T cells were significantly increased in CAC, although these did not represent a major population of cells (Fig. 5.5D). There was also a strong positive correlation between the number of TNF- α -, IL-13- and IL-22-producing T cells and the tumour burden, whereas the number of IFN- γ -, IL-5- and IL-17 only had a moderate positive correlation (Fig. 5.5E). Cytokine-producing T cells are likely to play a role in influencing tumour development. However, ILC2s, and not T cells, are the main producers of IL-5 in the CAC microenvironment (Fig. 5.4C, 5.5D) and therefore, I focused on examining the role of IL-5-producing ILC2s in the regulation of tumour development.

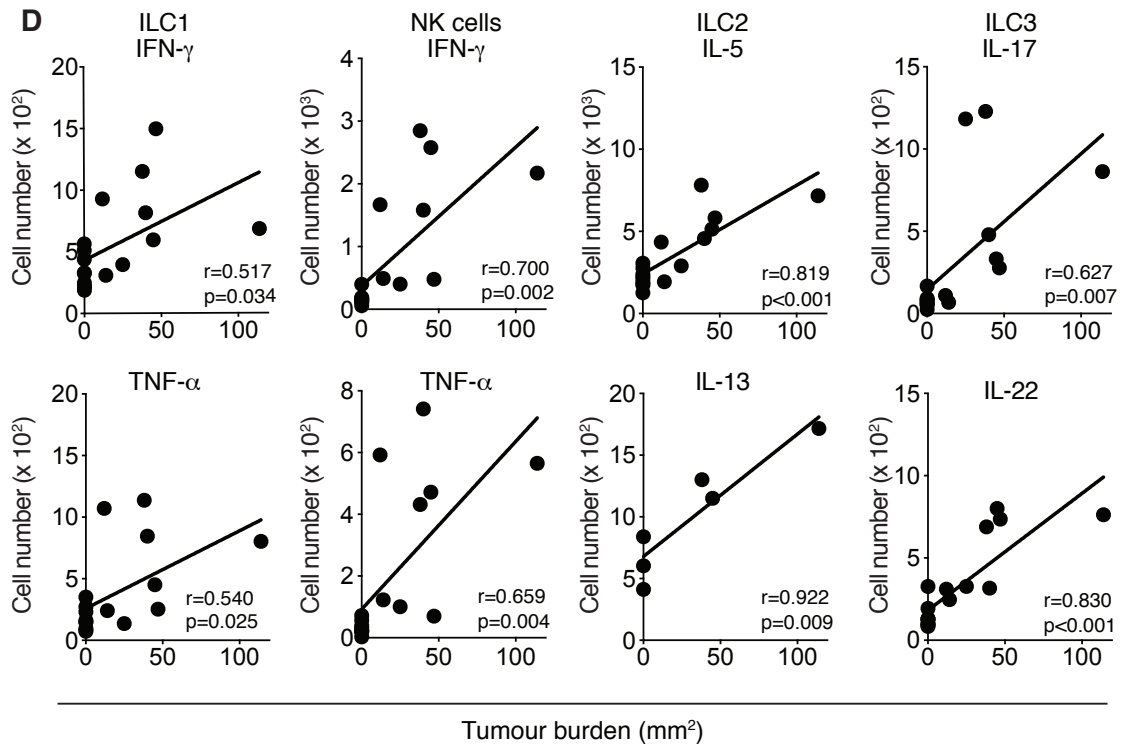
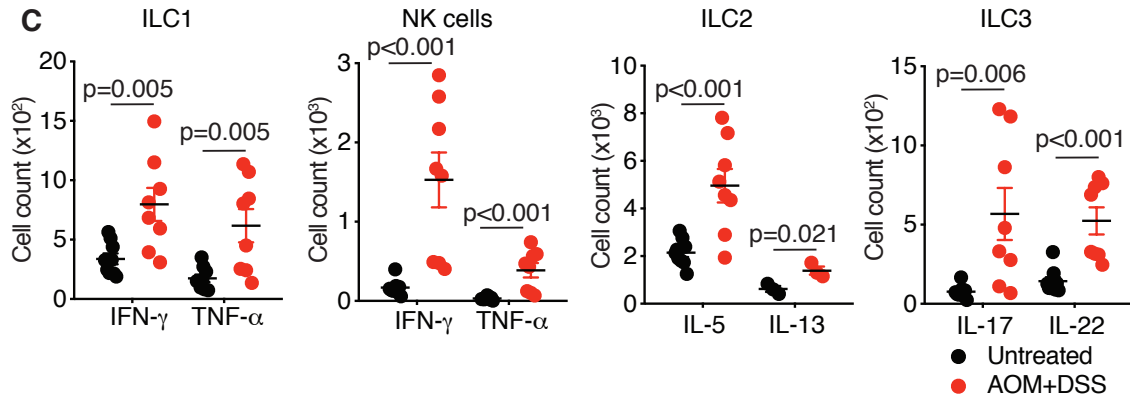
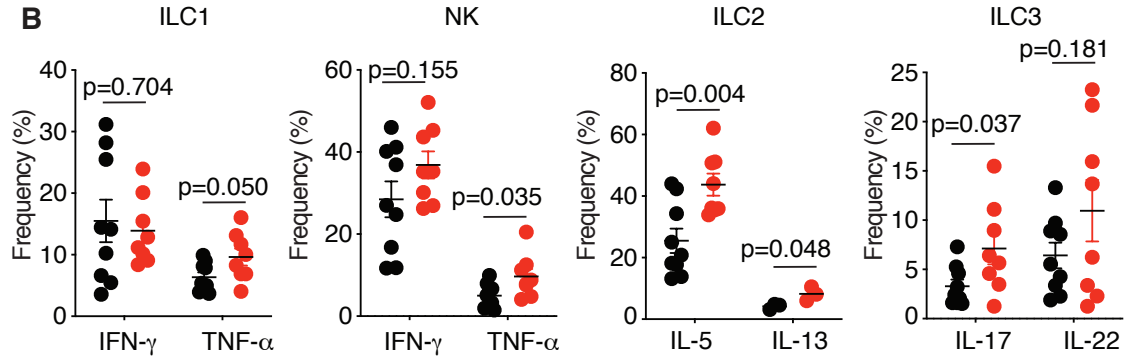
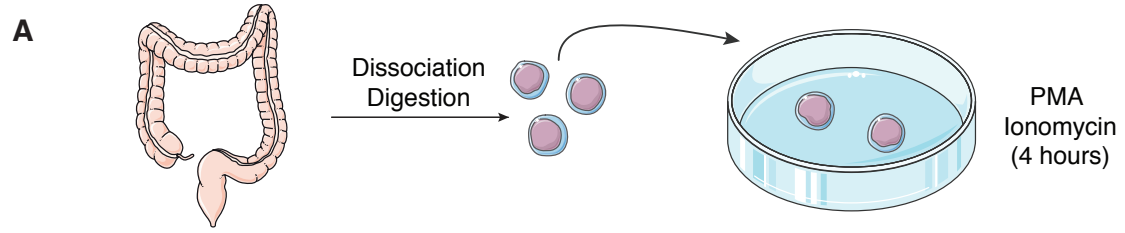


Figure 5.4. IL-5-producing ILC2s accumulate in the colon upon induction of CAC.

A. Schematic representation of the process to isolate and restimulate colonic lymphocytes from untreated and AOM+DSS treated mice. Single cell suspensions of lymphocytes isolated from the colon after dissociation and digestion were restimulated with PMA and ionomycin in the presence of monensin and brefeldin A to induce cytokine production.

B-C. Frequency of cytokine-producing cells within each ILC subset (**B**) and total number of cytokine-producing ILC subsets (**C**) isolated from the colon of untreated and AOM+DSS treated mice 7-10 weeks after treatment initiation. Data show the mean $\pm$ SEM of data pooled from 1-4 independent experiments ($n=2-3$ mice/treatment/experiment). Analyses of IL-13 was performed once; all other data show analyses of four pooled experiments. Statistics were calculated using an unpaired Student's *t* test and show exact *p*-value.

D. Correlation of cell count of cytokine-producing ILC subsets with tumour burden (area of tumour mm²) per colon using the linear regression model. The correlation (*r*) and *p*-value as indicated.

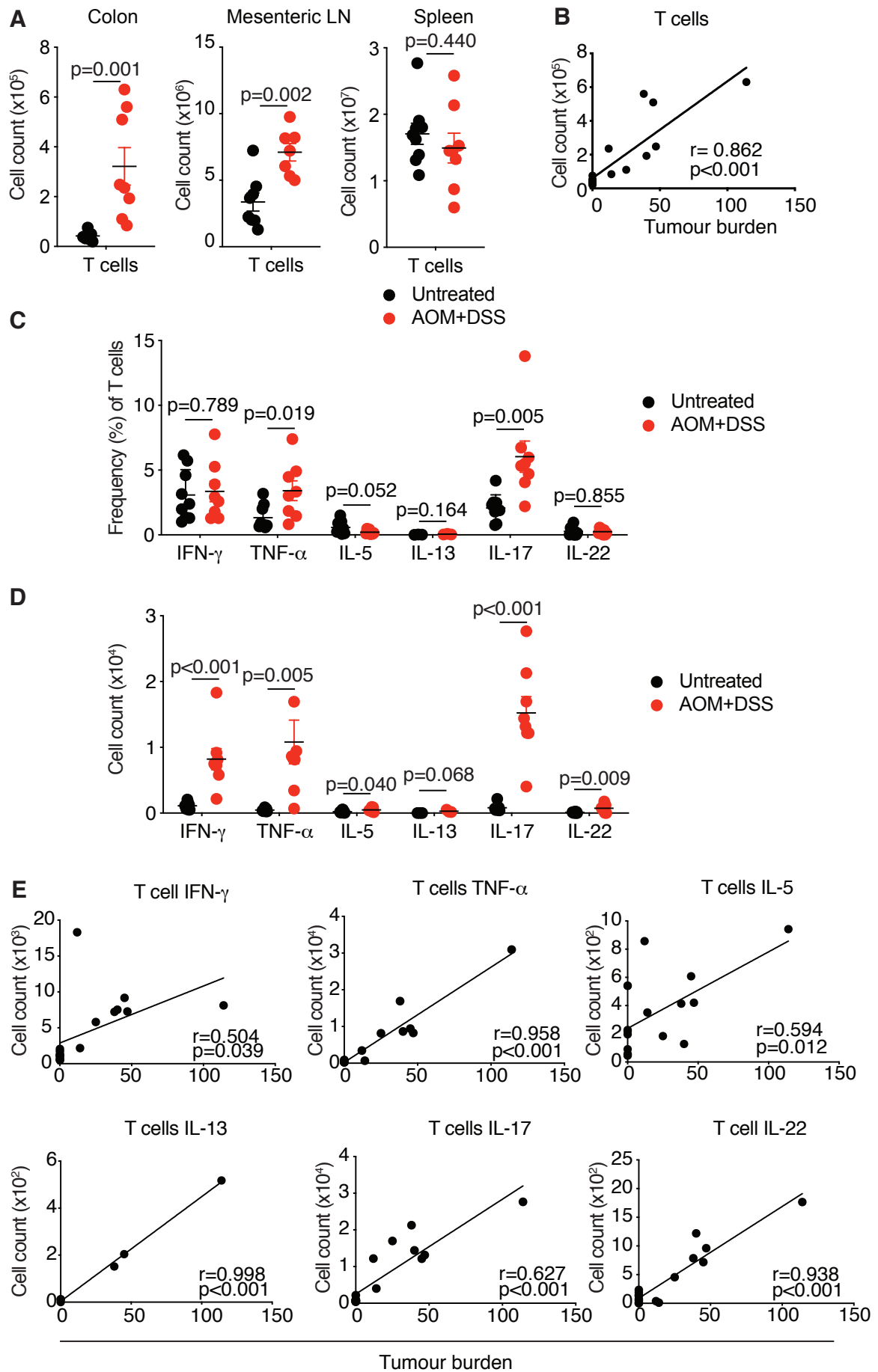


Figure 5.5. Cytokine-producing T cells accumulate in the colon during development of CAC.

A. Enumeration of T-cells isolated from the colon of untreated and AOM+DSS treated mice 7-10 weeks post treatment initiation. Data show the mean $\pm$ SEM of data pooled from 1-4 independent experiments ($n=2-3$ mice/treatment/experiment). Statistics were calculated using an unpaired Student's t test and show exact p -values.

B. Correlation of total T cells with the tumour burden (area of tumour mm^2) per colon using the linear regression model. The correlation (r) and p -value as indicated.

C-D. Frequency (**C**) and total number (**D**) of cytokine-producing T cells isolated from the colon of untreated and AOM+DSS treated mice 7-10 weeks post treatment initiation. Data show the mean $\pm$ SEM of data pooled from 1-4 independent experiments ($n=2-3$ mice/treatment/experiment). Experiment with IL-13 data performed once, all other data originated from experiments performed four times. Statistics were calculated using an unpaired Student's t test, p -value as indicated.

E. Correlation of cytokine-producing T cells with the tumour burden (area of tumour mm^2) per colon using the linear regression model. The correlation (r) and p -value as indicated.

Eosinophils, neutrophils epithelial cells express the IL-5R during CAC tumorigenesis

Based on the significant increase in the number of IL-5-producing ILC2s in the colons of mice from the CAC model, and the role of IL-5 in the induction of eosinophil-mediated inflammation and epithelial cell hypertrophy in the airways²⁴⁷, I speculated that IL-5 produced by ILC2 may recruit eosinophils or signal to other cells create a pro-inflammatory environment and impact on CAC development.

To determine whether the epithelial cells of the tumour and non-tumourous colon tissue expressed the IL-5R subunit, IL-5R α , the tumours were processed separately from the colon tissue of mice treated with AOM+DSS and this was compared to colon tissue of untreated mice (Fig. 5.6A). IL-5 produced by ILC2 generally acts via short range interactions, therefore, to identify potential targets of IL-5 signalling, eosinophils and neutrophils were also analysed (Fig. 5.6B). Flow cytometry analysis showed eosinophils, neutrophils and epithelial cells in the colon of untreated mice, non-tumourous colon tissue of AOM+DSS mice and within tumours expressed IL-5R α (Fig. 5.6C). Furthermore, the frequency of IL-5R α expressing epithelial cells in non-tumourous colon tissue of AOM+DSS mice and within tumours was higher than epithelial cells from untreated mice (Fig. 5.6D). This suggests that epithelial cells of the colon of mice with CAC could potentially respond to IL-5 secreted by ILC2 to influence tumorigenesis. There was also a trend of increased of eosinophils and neutrophil numbers in the colon of AOM+DSS treated mice compared to untreated colons (Fig. 5.6E), which is consistent with their known recruitment in settings of high IL-5 production¹¹⁸. Therefore, it is possible that the recruitment of these cells into the colon by IL-5 may also influence CAC development.

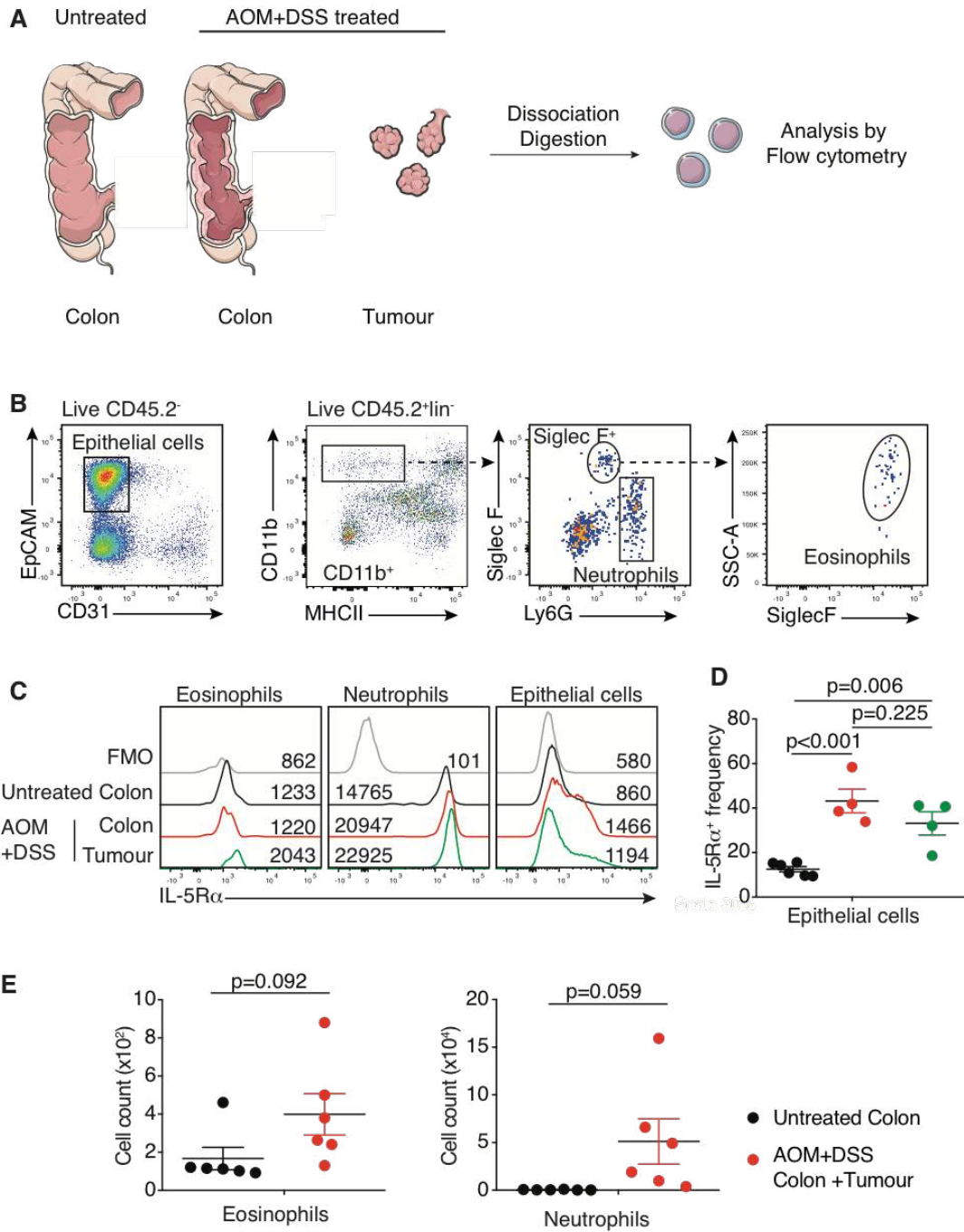


Figure 5.6. IL-5R is expressed on epithelial cells, eosinophils and neutrophils.

A. Schematic representation of protocol for tissues analysis. Tumours (AOM+DSS tumour) from the colon of AOM+DSS treated mice were excised and processed separately from the rest of the non-tumour regions of the colon (AOM+DSS colon). Both the colon and tumours of AOM+DSS treated mice were compared with the colon of untreated mice (untreated colon).

B. Gating strategy to identify epithelial cells, eosinophils and neutrophils in the colon. Within the live CD45.2⁻ population, epithelial cells are defined as the EpCAM⁺CD31⁻ cells. Within the live CD45.2⁺ population, lineage markers were excluded (TCRβ⁻CD19⁻) and CD11b⁺MHCII⁻ were gated on. Neutrophils were defined within this population as Ly6G⁺, while eosinophils were Siglec F⁺ that are SSC-A^{hi}.

C. Expression of IL-5Rα on eosinophils, neutrophils and epithelial cells from colons and tumours of mice 7-10 weeks post initiation of AOM+DSS treatment and colon of untreated mice. Fluorescence minus one (FMO) of IL-5Rα staining is used as negative control. Mean fluorescence intensity (MFI) of IL-5Rα expression as indicated. Data show one representative histogram from one of two independent experiments with similar results (*n*=2-3 mice/treatment/experiment).

D. Comparison of the frequency of IL-5Rα⁺ epithelial cells within the colon tissue and tumour of AOM+DSS treated mice and colon tissue of untreated mice. Data show the mean ± SEM of data pooled from two independent experiments (*n*=2-3 mice/treatment/experiment). Statistics was calculated using One-way ANOVA with Tukey's multiple comparison's test, *p*-value as indicated.

E. Comparison between the number of eosinophils and neutrophils isolated from the colon of untreated mice and the pooled colon and tumour of AOM+DSS treated mice. Data show the mean ± SEM of data pooled from two independent experiments (*n*=3 mice/treatment/experiment). Statistics were calculated using an unpaired Student's *t* test, *p*-value as indicated.

4.3.2 Role of IL-5 overexpression in the development of CAC

IL-5TgN mice overexpress IL-5 and had increased recruitment of eosinophils in the colon but not neutrophils.

Due to the increase in IL-5-producing ILC2s in the colon and the expression of the IL-5R on cells in the microenvironment with the potential to respond during development of CAC, I hypothesised that the increased IL-5 produced by ILC2 may be playing a role in the development of CAC.

To assess this, I first established that IL-5TgN mice, an overexpression mouse model of IL-5 was an appropriate model to use. Transcription of IL-5 in these mice is regulated by the dominant control region of the human CD2 gene¹¹⁸. These mice have been reported to have constitutive IL-5 expression and increased eosinophils and neutrophils in the blood¹¹⁸. The colons of C57BL/6 and IL-5TgN mice were processed and stimulated with PMA and ionomycin (Fig. 5.7A). The immune cell number and cytokine production of these mice were compared to establish a baseline immune environment in the colon when CAC is induced. The overexpression of IL-5 did not impact on the number of ILCs within the colon, nor did it impact the number of CD4⁺ and CD8⁺ T cells (Fig. 5.7B). Consistent with previous reports, the number of IL-5-producing T cells and IL-5-producing ILC2s were increased although the latter did not reach statistical significance. The number of IL-17- and IL-22-producing ILC3 and T cells appeared to be unaffected by the increased IL-5 production (Fig. 5.7C). In C57BL/6 mice, ILC2, but not T cells, are the major producer of IL-5 (Fig 5.7D). Whereas in IL-5TgN mice, both ILC2 and T cells are major producers of IL-5 (Fig. 5.7D).

The IL-5TgN mice had in a significant increase in the number of eosinophils within the colon as well as in the MLN and spleen (Fig. 5.7E, not shown), which is in accordance with previous reports¹¹⁸. However, while neutrophils were reported to be increased in the blood¹¹⁸ and my analysis indicated an increase in the MLN and spleen, there was no difference in neutrophil numbers in the colon of IL-5TgN mice compared to control C57BL/6 mice (Fig. 5.7E, not shown). This indicates that while neutrophils do express the IL-5R and can be recruited by IL-5 in selective peripheral organs, it is not affected by IL-5 overexpression in the colon at steady state. Whether neutrophil recruitment to the colon is regulated by IL-5 during CAC is still unknown.

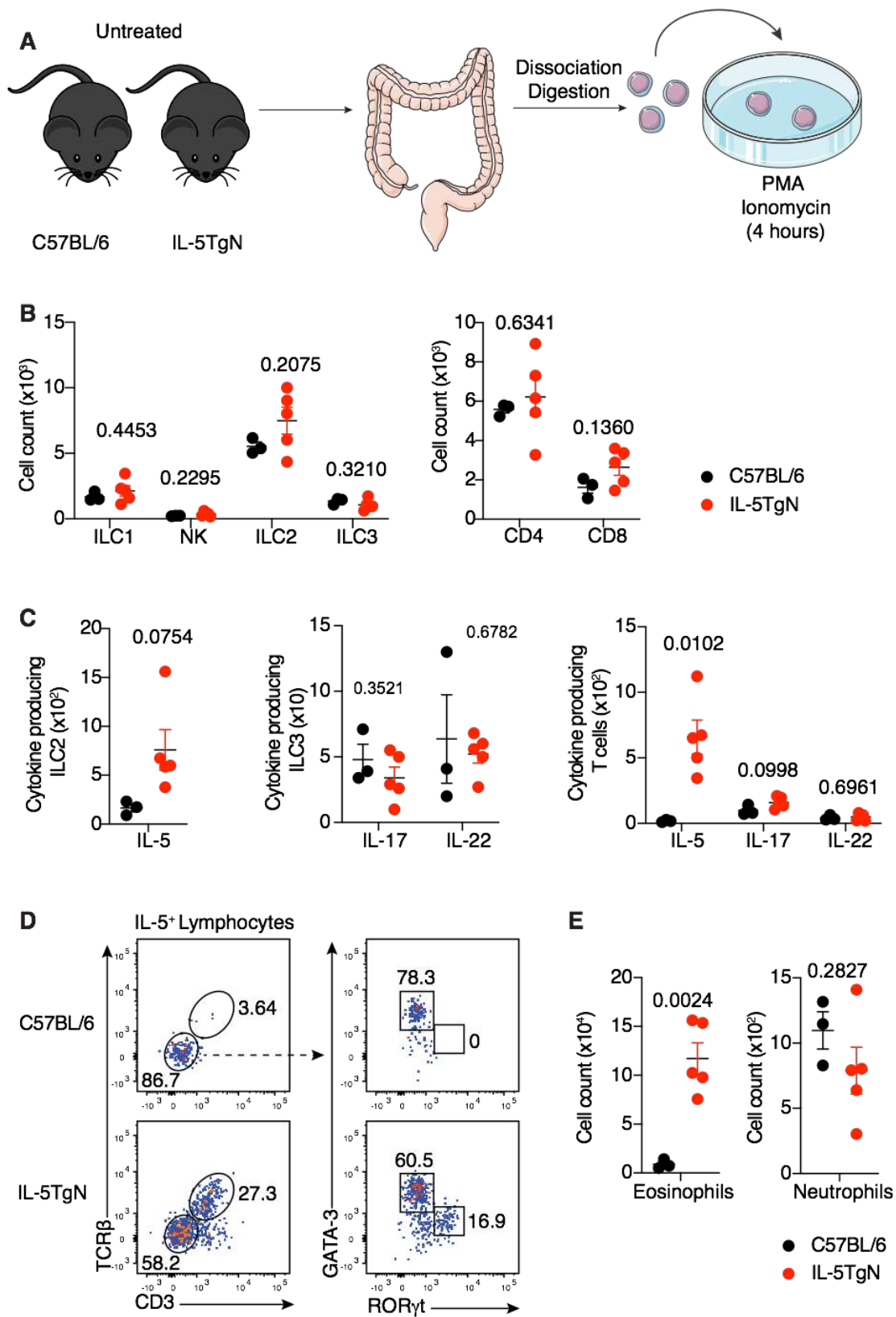


Figure 5.7. IL-5TgN mice have increased IL-5 production and eosinophils in the colon.

A. Schematic representation of the process to isolate and restimulate colonic lymphocytes from untreated C57BL/6 and IL-5TgN mice. Single cell suspension of lymphocytes isolated following colon dissociation and digestion were restimulated with PMA and ionomycin to induce cytokine production.

B. Enumeration of ILC (left) and T cell (right) subsets isolated from the colon of untreated C57BL/6 and IL-5TgN mice. Data show the mean $\pm$ SEM of data representative of two experiments ($n=3-4$ mice). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

C. Enumeration of cytokine producing ILC2s (left) ILC3 (centre) and CD4⁺ T cells (right) isolated from the colon of untreated C57BL/6 and IL-5TgN mice. Data show the mean $\pm$ SEM of data representative of two experiments ($n=3-4$ mice). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

D. Representative flow cytometry plots showing the proportion of cells expressing IL-5 within the colon of untreated C57BL/6 and IL-5TgN mice.

E. Enumeration of eosinophils (left) and neutrophils (right) isolated from the colon of untreated C57BL/6 and IL-5TgN mice. Data show the mean $\pm$ SEM of data representative of two experiments ($n=3-5$ mice). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

Overexpression of IL-5 does not impact CAC development

To address the role of increased IL-5 expression in CAC, C57BL/6 and IL-5TgN mice were treated with AOM+DSS (Fig. 5.8A) and endoscoped to monitor the development of tumours. Macroscopic tumours were detected from the endoscopy in majority of mice from both the C57BL6 and IL-5TgN groups by the end of three rounds of DSS treatment (Fig. 5.8B). Mice that did not develop macroscopic tumours had loose stools and increased mucus production, indicative of colitis (not shown). Tumours developed primarily in the distal regions of the colons of both C57BL/6 and IL-5TgN mice (Fig. 5.8C) with no significant difference macroscopically between the control colon and those with overexpression of IL-5. When the tumour burden of colons were measured to assess the impact of IL-5 overexpression on tumour development, both groups had on average two tumours per colon and a similar tumour burden of on average 12mm² combined tumour area for control colons and 9mm² for IL-5TgN colons (Fig. 5.8D). This indicates that the overexpression of IL-5 in the colon microenvironment does not significantly impact on the development of macroscopic tumours in CAC.

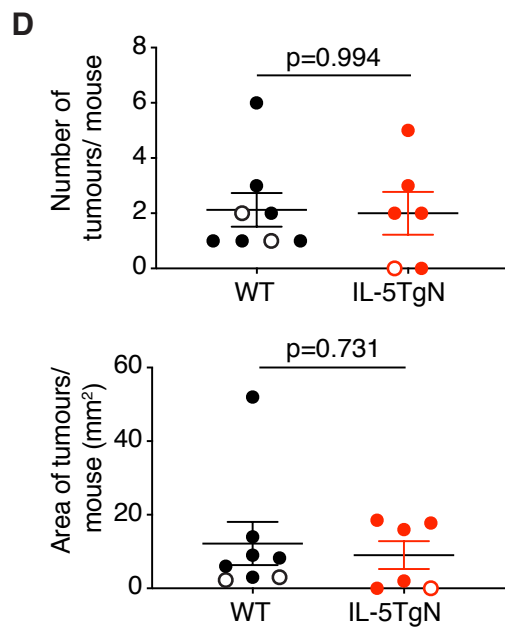
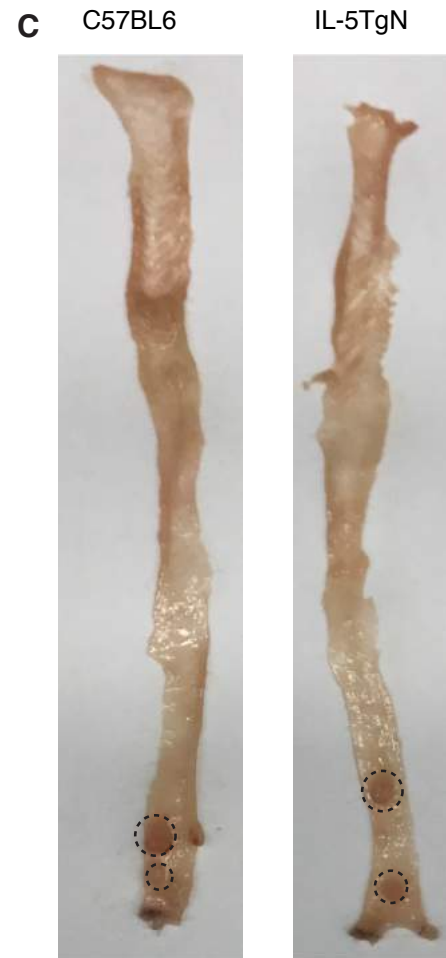
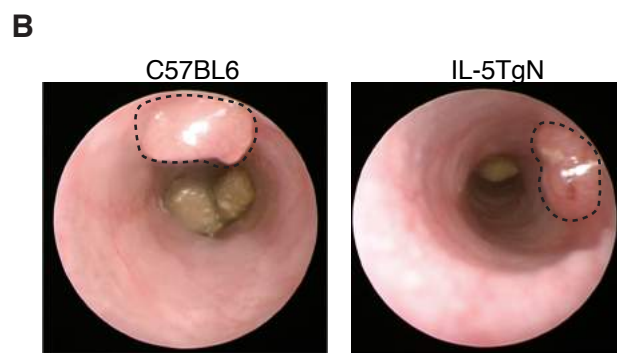
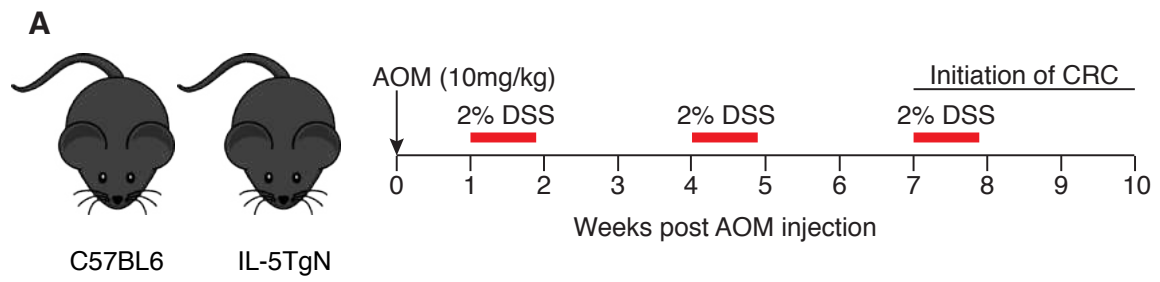


Figure 5.8. Overexpression of IL-5 does not impact on CAC tumour burden within the colon.

A. Schematic illustration of the AOM/DSS treatment protocol for C57BL/6 mice. AOM (10mg/kg) was administered I.P to mice, followed by three cycles of 2% (w/v) DSS *ad libitum* in their drinking water for five days with two weeks of normal water between each cycle. Colorectal cancer development occurred between 7-10 weeks post initiation of treatment.

B. Representative endoscopy images of untreated (left panel) and AOM/DSS treated mice (right panel). Images are representative of 6-8 mice analysed per experiment from three independent experiments. Dotted lines indicate tumour growth.

C. Representative whole mount images of colons from an untreated (left panel) and an AOM+DSS treated (right panel) mouse. Images are representative of 2-3 mice/genotype analysed in three separate experiments. Dotted lines indicate tumour growth. Scale bar, 1 cm.

D. Comparison of the total number of tumours (top) and area of tumours (mm², bottom) and per mouse in untreated and AOM+DSS treated mice. Data show the mean $\pm$ standard error of mean (SEM) of data representative of three independent experiments (6-8 mice/treatment/experiment). Statistics were calculated using the Mann-Whitney nonparametric test with p-values as indicated. Open circles represent males, closed circles represent females.

Tumour burden strongly correlated with neutrophil numbers but not due to IL-5 overexpression

Further analysis of colonic immune cells in the CAC context revealed that there was no difference in ILC or T cell numbers between C57BL/6 and IL-5TgN mice treated with AOM+DSS (Fig. 5.9A). One independent experiment revealed a reduction in the number of ILC3s within the colon of IL-5TgN mice, but this was not present in the two other repeats (Fig. 5.9A).

Following PMA and ionomycin stimulation, there was an increase in the frequency and number of IL-5-producing ILC2 and T cells, but no difference in the frequency of IL-17- or IL-22-producing ILC3s and T cells was observed (Fig. 5.9B-C). This indicated that IL-5 overexpression did not affect the cell number and cytokine production of other ILC subsets and T cells. Similar to IL-5 overexpression at steady-state, IL-5 overexpression in CAC resulted in an increase in eosinophil numbers in the colon but no change in the number of neutrophils nor the frequency of IL-5R expression on colonic epithelial cells (Fig. 5.9D, E).

While there was no difference between the groups of control and IL-5 overexpression colons, I examined whether the lack of a significant difference in tumour burden may be explained by the variation observed in the potential targets of IL-5 signalling. Therefore, I correlated the number of eosinophils, neutrophils and frequency of IL-5R expression on colonic epithelial cells with the tumour burden in each colon. While the number of eosinophils and IL-5R expression did not correlate with the tumour burden, there was a strong positive correlation between the number of neutrophils per colon and the tumour burden, regardless of IL-5 expression (Fig 5.9F). This indicated that IL-5 overexpression recruited eosinophils in the colon but this did not affect the tumour development in CAC. The strong correlation between neutrophil numbers and tumour burden suggested a potential role for neutrophil recruitment in driving CAC tumorigenesis, but this occurred independently of IL-5 signalling.

4.3.3 Role of IL-5 deletion in the development of CAC

IL-5 expression is lost in IL-5Venus mice and associated with reduced eosinophils in the colon

Overexpression of IL-5 and increased eosinophil numbers did not significantly alter the development of CAC (Fig. 5.8, 5.9). However, this overexpression model has a number of limitations, including IL-5 production being driven by CD2 promoter in T cells, which is not the predominant source of IL-5 in the colon. Therefore, a second hypothesis was tested, that loss of IL-5 would impact on the development of CAC. To do this, I acquired IL-5Venus mice, where the *Venus* gene was knocked into the IL-5 gene locus, meaning that mice homozygous for the Venus reporter no longer expressed IL-5¹¹⁹.

As the IL-5Venus mice were on a BALBC background, the colons of BALBC and IL-5Venus mice at steady-state were processed and stimulated with PMA and ionomycin (Fig. 5.10A) to establish the immunological baseline, as BALBC mice are immunologically different from C57BL6 mice. C57BL6 mice are thought to have a Th1 biased immune response which makes them more resistant to certain infections whereas BALBC mice have a Th2 dominant immune response²⁴⁸. Furthermore, in DSS-induced acute colitis, while both C57BL6 and BALBC mice showed signs of colonic mucosal injury and intestinal inflammatory response, the mucosal damage has been shown to be worse in C57BL6 mice²⁴⁹. Both strains of mice also have elevated cytokine production by T cells when treated with DSS but C57BL6 had a more Th1 dominant immune profile whereas BALBC had a Th2/Th17 biased response²⁴⁹.

There was no difference in the number of ILC and T cell subsets within the colon of IL-5Venus mice compared to BALBC mice, nor was there a difference in the frequency of type 3 cytokines produced by ILC3 and T cells (Fig. 5.10B,C). As expected, there was a loss in the frequency of IL-5 producing ILC2s and T cells, and a subsequent loss of eosinophils within the colon (Fig. 5.10C,D). However, neutrophil numbers remain unchanged (Fig. 5.10D).

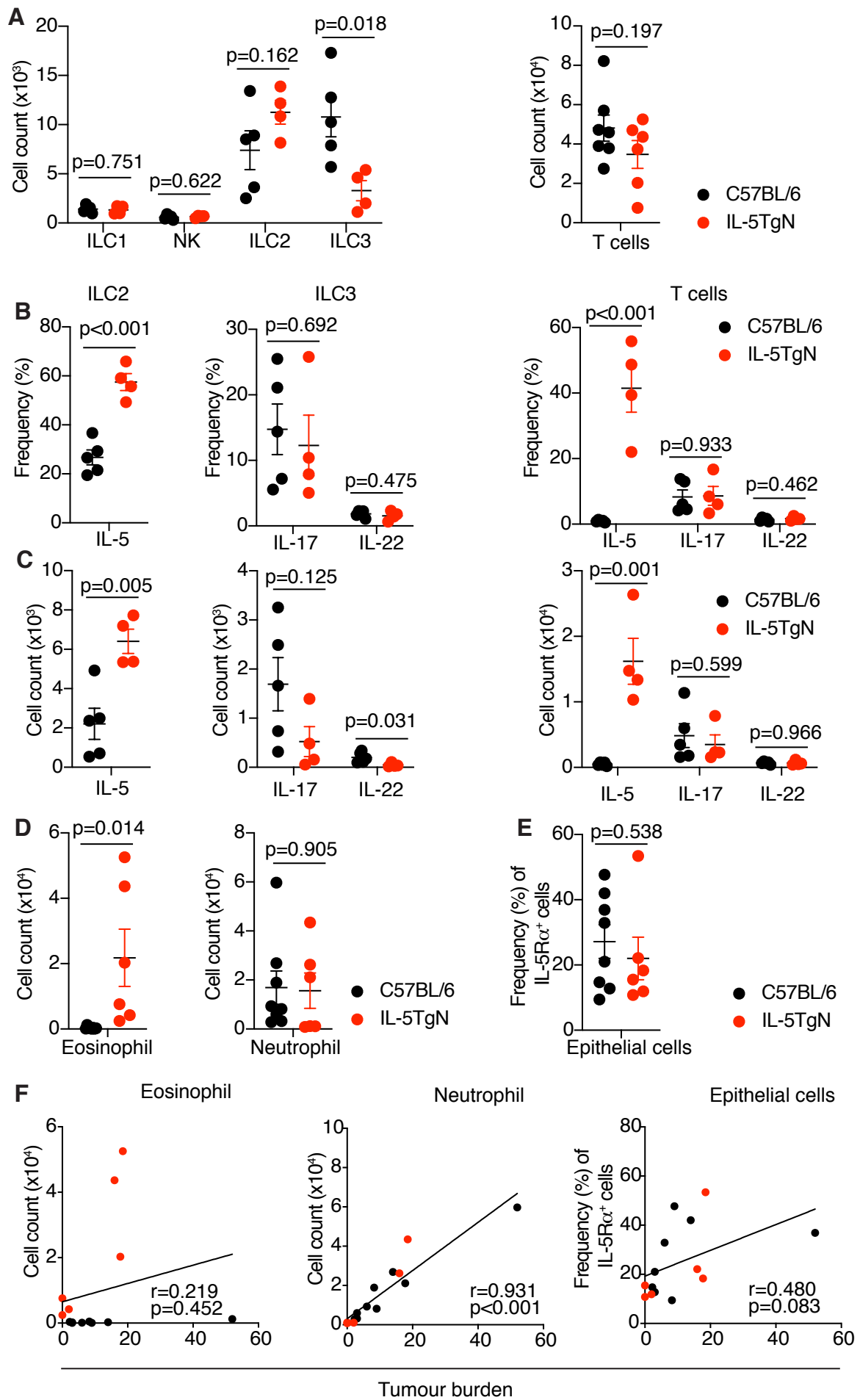


Figure 5.9. Overexpression of IL-5 is associated with eosinophil influx but has no correlation with tumour burden.

A. Enumeration of ILC subsets and T cells isolated from the colon of untreated and AOM+DSS treated mice 7-10 weeks post treatment initiation. Data show the mean $\pm$ SEM of data pooled from two independent experiments for ILCs and three independent experiments for T cells ($n=2-3$ mice/treatment/experiment). Statistics were calculated using an unpaired Student's t test, p -value as indicated.

B-C. Frequency (**B**) and number (**C**) of cytokine-producing ILC2s (left panel) ILC3s (centre panel) and T cells (right panel) isolated from the colon and stimulation with PMA and ionomycin for four hours.

D. Comparison between the number of eosinophils and neutrophils isolated from the colon of untreated mice and the pooled colon and tumour of AOM+DSS treated mice. Data show the mean $\pm$ SEM of data pooled from two independent experiments ($n=3$ mice/treatment/experiment). Statistics were calculated using an unpaired Student's t test, p -value as indicated.

E. Comparison of the frequency of IL-5R α^+ epithelial cells within the colon tissue and tumour of AOM+DSS treated mice and colon tissue of untreated mice. Data show the mean $\pm$ SEM of data pooled from two independent experiments ($n=2-3$ mice/treatment/experiment).

F. Correlation of the number of eosinophils (left panel), neutrophils (centre panel) and frequency of IL-5R α^+ epithelial cells (right panel) with the tumour burden (area of tumour mm²) per colon using the linear regression model. The correlation (r) and p -value as indicated.

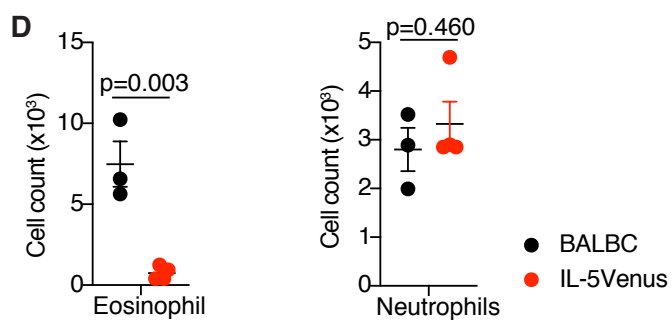
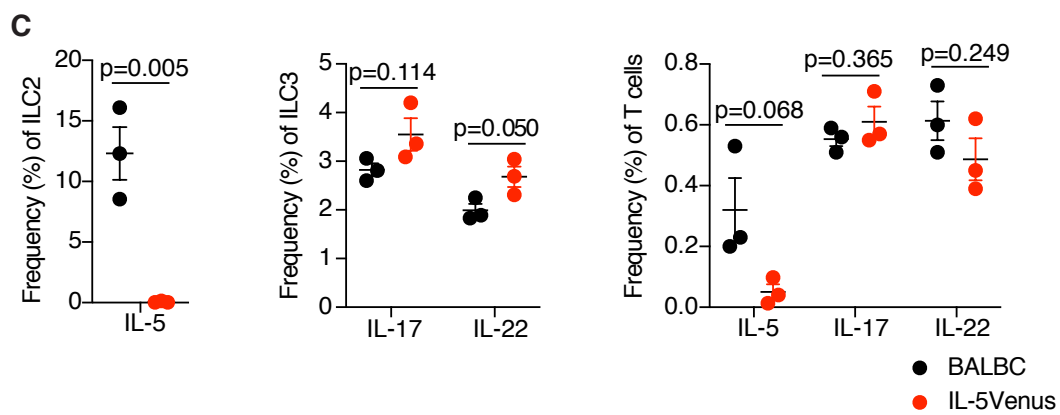
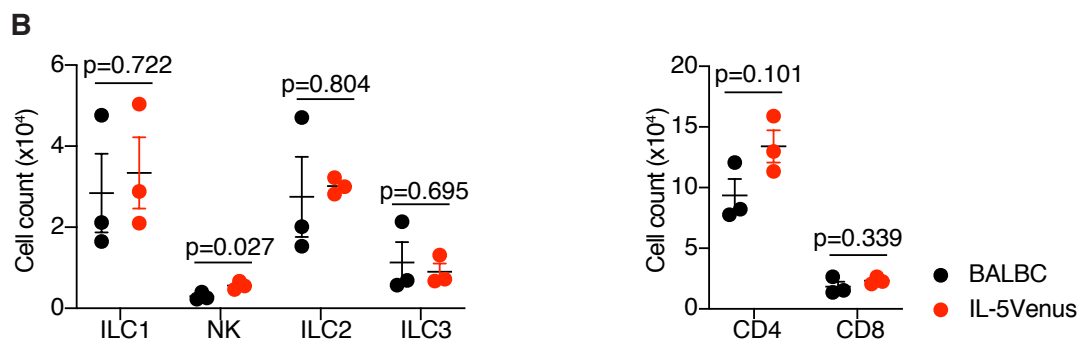
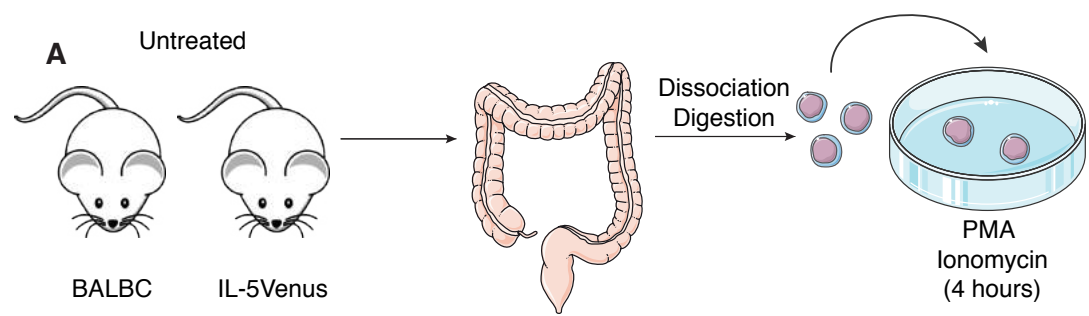


Figure 5.10. IL-5Venus mice have reduced IL-5 production and less eosinophils in the colon.

A. Schematic representation of the process to isolate and restimulate colonic lymphocytes from untreated BALBC and IL-5Venus mice. Single cell suspension of lymphocytes isolated following colon dissociation and digestion were restimulated with PMA and ionomycin to induce cytokine production.

B. Enumeration of ILC (left panel) and T cell (right panel) subsets isolated from the colon of untreated BALBC and IL-5Venus mice. Data show the mean $\pm$ SEM of data representative of three independent experiments ($n=3$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

C. Enumeration of cytokine producing ILC2s (left) ILC3 (centre) and CD4⁺ T cells (right) isolated from the colon of untreated BALBC and IL-5Venus mice. Data show the mean $\pm$ SEM of data representative of three independent experiment ($n=3-4$ mice). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

D. Enumeration of eosinophils (left panel) and neutrophils (right panel) isolated from the colon of untreated BALBC and IL-5Venus mice. Data show the mean $\pm$ SEM of data representative of three independent experiments ($n=3$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

AOM+DSS treatment induces CAC in BALBC mice with eosinophilia and neutrophilia in the colon

To determine if the increase of IL-5-producing ILC2 is also seen in BALBC mice that develop CAC, I compared the colon of BALBC mice treated with AOM+DSS to the colon of untreated mice (Fig. 5.11A.) After AOM+DSS treatment, BALBC mice successfully developed between two to nine macroscopic tumours per colon with an average total area of approximately 23mm² (Fig. 5.11B). Similar to AOM+DSS treated C56BL6 mice, AOM+DSS treated BALBC mice had a similar increased T cells, NK cells, eosinophils and neutrophils while ILC2 numbers remained unchanged (Fig. 5.11C). However, ILC1 and ILC3 numbers in BALBC mice did not increase as they did in C57BL6 mice (Fig. 5.11C). Unexpectedly, the frequency and number of IL-5-producing ILC2s in AOM+DSS treated BALBC mice did not change (Fig. 5.11D,E). There was an increase in the frequency and number of IL-17-producing ILC3 but no difference in IL-22 (Fig. 5.11D,E). IL-17 was the predominant cytokine produced by T cells during CAC and the frequency and number of IL-17-producing T cells had increased compared to untreated mice, although the former had not reached statistical significance (Fig. 5.11D). This indicated that immune cells, in particular ILC2s, may respond differently during the development of CAC in BALBC mice. Nevertheless, eosinophils and neutrophils had increased, similar to in C57BL6 mice. Therefore, I examined whether the loss of IL-5 would impact these cells and alter the development of CAC.

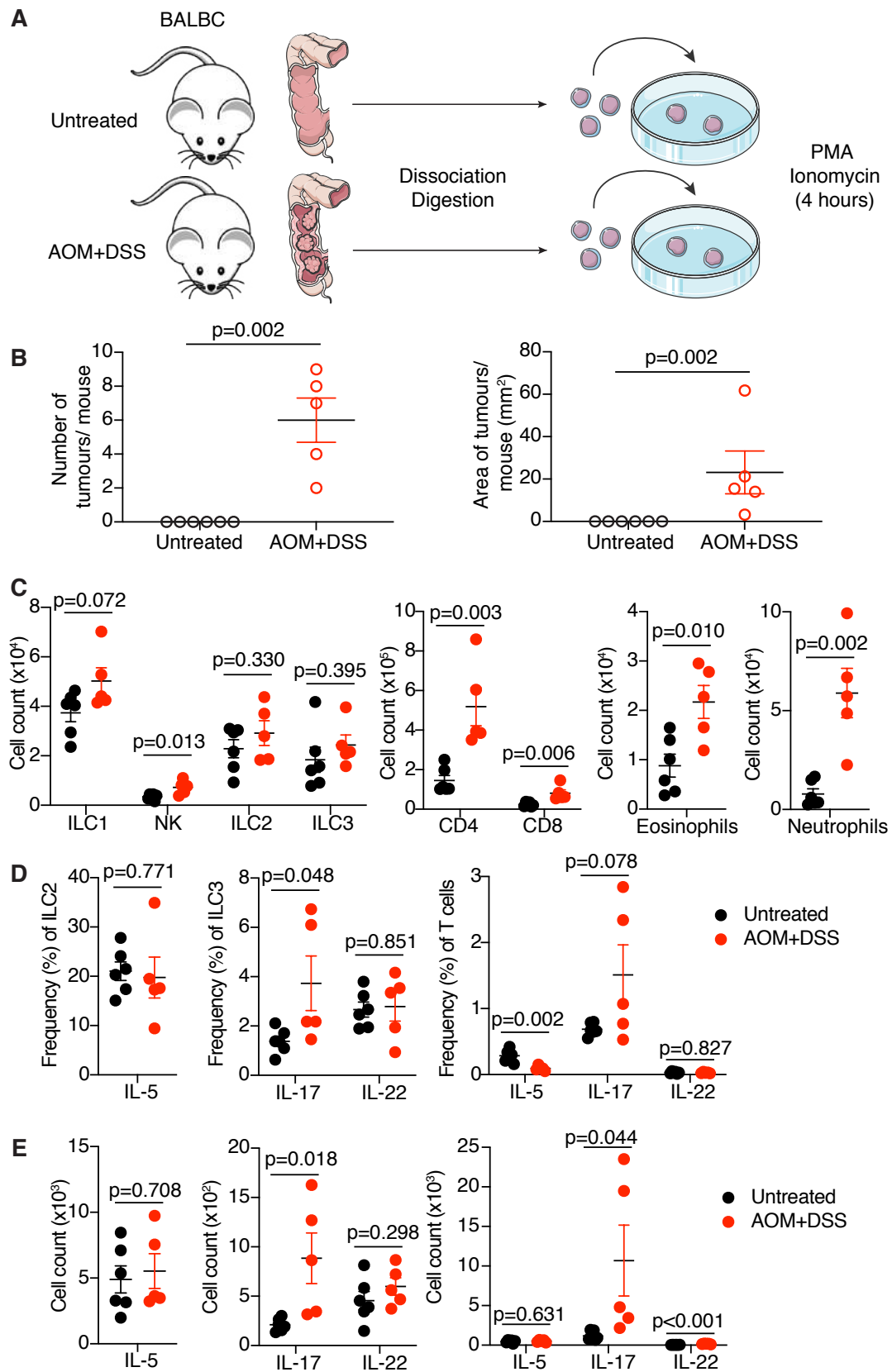


Figure 5.11. AOM+DSS treatment in BALBC mice induces CAC and eosinophilia.

A. Schematic representation of the process to isolate and restimulate colonic lymphocytes from untreated and AOM+DSS treated BALBC mice. Single cell suspension of lymphocytes isolated following colon dissociation and digestion were restimulated with PMA and ionomycin to induce cytokine production.

B. Comparison of the total number of tumours (left panel) and area of tumours (mm², right panel) per mouse in untreated and AOM+DSS treated mice. The mean $\pm$ standard error of mean (SEM) of data pooled from two experiments (2-3 mice/treatment/experiment) is shown.

Statistics were calculated using the Mann-Whitney nonparametric test with p-values as indicated.

C. Enumeration of ILC subsets, T cell subsets, eosinophils and neutrophils isolated from the colon of untreated and AOM+DSS treated BALBC mice. Data show the mean $\pm$ SEM of data pooled from two separate experiments ($n=2-3$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

D-E. Frequency (**D**) and number (**E**) of cytokine producing ILC2s (left panel) ILC3s (centre panel) and T cells (right panel) isolated from the colon of untreated and AOM+DSS treated BALBC mice. . Data show the mean $\pm$ SEM of data pooled from two separate experiments ($n=2-3$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

Loss of IL-5 did not impact CAC development

To analyse the impact of loss of IL-5 expression in CAC, BALBC and IL-5Venus mice were treated with AOM+DSS (Fig. 5.12A) and endoscoped to monitor the development of tumours. Macroscopic tumours were detected via endoscopy imaging in the majority of mice from both the BALBC and IL-5Venus mice by the end of three rounds of DSS treatment (Fig. 5.12B). Mice that did not develop macroscopic tumours had loose stools and increased mucus production, indicative of colitis (not shown). A difference in colon length, potentially indicative of differences in severity of colonic inflammation, was initially observed in the first experiment between IL-5Venus mice bred in-house and BALBC mice ordered from a different animal facility (Fig. 5.12C); however, this difference was not seen in later experiments when littermate controls were used, indicating that microflora variation between animal facilities impacted the original experiment. Tumours developed primarily between the mid to distal regions of the colons of both BALBC and IL-5Venus mice with BALBC mice developing between 2- 20 tumours per colon with a total tumour area of between 7-209mm² whereas IL-5Venus mice developed between 7-9 tumours per colon with a total tumour area of between 13-46mm² (Fig. 5.12C,D). There was no statistical difference in the tumour burden between BALBC and IL-5Venus mice (Fig 5.12D). Due to the large variation of tumour number and tumour area that was observed in BALBC mice, it was difficult to conclude whether loss of IL-5 had any impact on the development of CAC. Therefore, this experiment was repeated three more times. The second repeat had a similar degree of variation in BALBC mice but in the third and fourth repeat, AOM+DSS could not successfully induce tumour development in female mice of both BALBC and IL-5Venus genotypes. Within the groups of males, there was insufficient mice numbers to make conclusions.

Figure 5.12. Loss of IL-5 does not impact on CAC tumour burden.

A. Schematic illustration of the AOM/DSS treatment protocol for BALBC and IL-5Venus mice. AOM (10mg/kg) was administered I.P to mice, followed by three cycles of 2% (w/v) DSS in their drinking water *ad libitum* for five days with two weeks of normal water between each cycle. Colorectal cancer development occurred between 7-10 weeks post initiation of treatment.

B. Representative endoscopy images of BALBC (left panel) and IL-5Venus mice (right panel) treated with AOM+DSS. Images are representative of 3-8 mice analysed per experiment from three independent experiments. Dotted line indicate tumour growth.

C. Representative whole mount images of colons from BALBC (left panel) and IL-5Venus mice (right panel) treated with AOM+DSS. Images are from one experiment, and representative of three separate experiments. Dotted lines indicate tumour growth. Scale bar, 1 cm.

D. Comparison of the total number of tumours (left) and area of tumours (mm², right) and per mouse in BALBC and IL-5Venus mice treated with AOM+DSS. Data show the mean $\pm$ SEM of data pooled from three separate experiments (1-3 mice/treatment/experiment). Statistics were calculated using the Mann-Whitney nonparametric test with *p*-values as indicated. Open circles represent males, closed circles represent females.

4.3.4 Role of ROR α in the development of CAC

ROR α deletion impacts ILC1 and ILC2 globally but has no effect on T cell number

As I could not conclude whether loss of IL-5 impacted development of CAC in BALBC mice, I used an alternative mouse model, the *ROR α ^{fl/fl} IL-7RCre* mice on the C57BL6 background, to address whether loss of ILC2 impacted CAC development. Previous studies using ROR α deficient staggerer mice show that ROR α is important in ILC2 development and type 2 dependent responses^{60, 61}, loss of ROR α in IL-7R expressing cells should result in loss of ILC2s. ROR α has also been reported to play a role in Th17 development and IL-17 expression²⁵⁰ and since ILCs and T cells share a common progenitor, common lymphoid progenitor (CLP), which expresses IL-7R, ROR α will be deleted in all ILC and T cells subsets. Therefore, I examined the effects of ROR α deletion on the immune composition and cytokine production within the colon of *ROR α ^{fl/fl} IL-7RCre* mice compared with that of *IL-7RCre* mice (Fig. 5.13A).

At steady-state, there was a complete loss of ILC2 number and reduction in the frequency of IL-5-producing ILC2s within the colon of ROR α deficient mice (Fig. 5.13B,C). Neither the NK cells, ILC3s CD4⁺ and CD8⁺ T cell subset numbers, nor the frequency of cytokine-producing ILC3s and CD4⁺ T cells were affected by the deletion of ROR α (Fig. 5.13B-C). Surprisingly, there was also a loss of ILC1 numbers within the colon. As ROR α has not yet been reported to play a role in ILC1 development, I analysed the number of ILC1 and ILC2 within the lung, small intestine and mesenteric lymph nodes as ILCs are enriched within these organs. I found that ILC1 and ILC2s were reduced in all the organs analysed in ROR α deficient mice (Fig. 5.13D). This indicates in addition to being important of ILC2, ROR α is also a key regulator of ILC1 development, which is a novel finding.

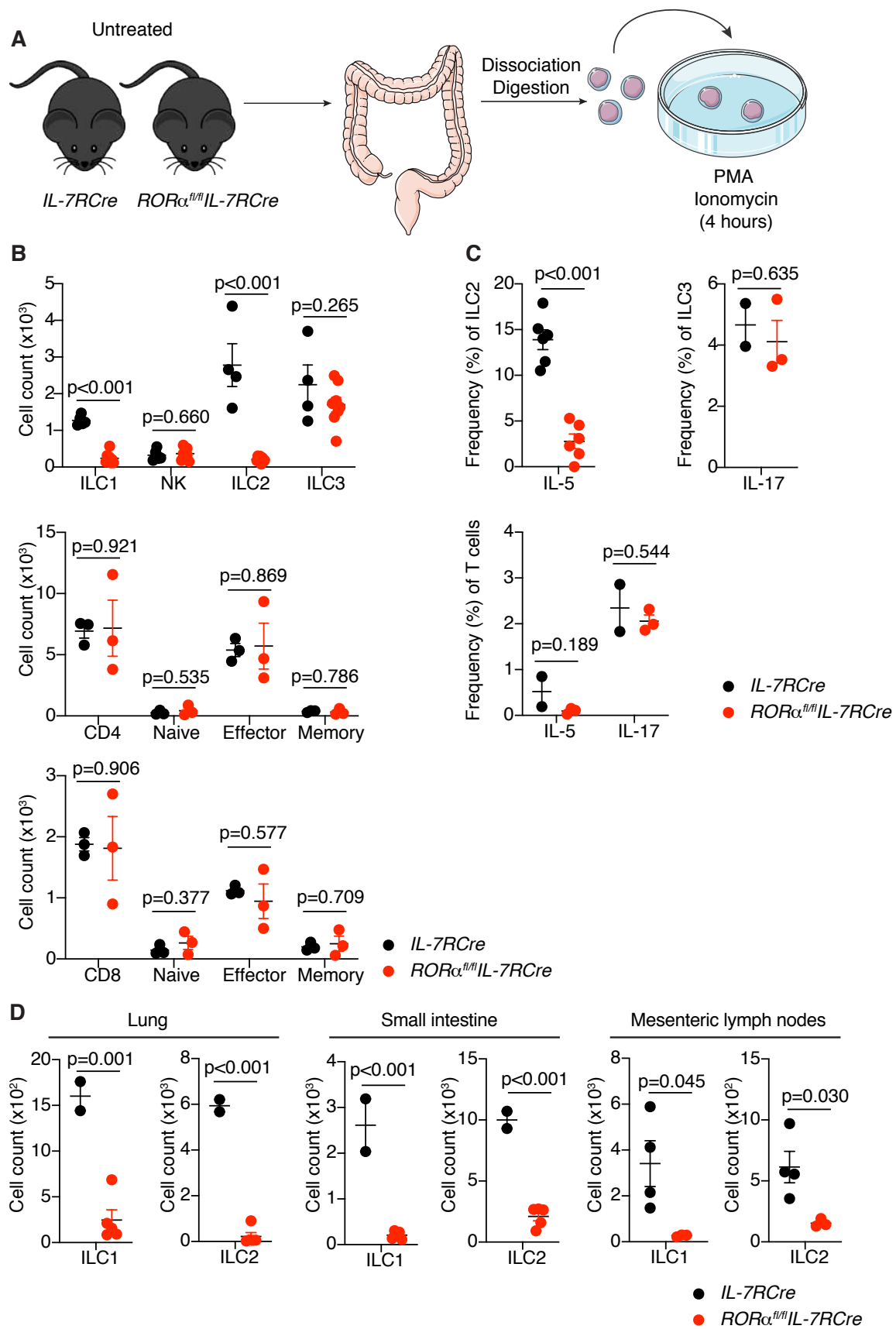


Figure 5.13. ROR α deletion affects global ILC1 and ILC2 numbers but not T cell number.

A. Schematic representation of the process to isolate and restimulate colonic lymphocytes from untreated *IL-7RCre* and *ROR α ^{fl/fl}IL-7RCre* mice. Single cell suspension of lymphocytes isolated following colon dissociation and digestion were restimulated with PMA and ionomycin to induce cytokine production.

B. Enumeration of ILC subsets (top), CD4⁺ (centre) and CD8⁺ (bottom) T cell numbers isolated from the colon *IL-7RCre* and *ROR α ^{fl/fl}IL-7RCre* mice. Data show the mean $\pm$ SEM of ILC number pooled from two experiments and CD4⁺ and CD8⁺ T cell numbers from one experiment ($n=2-4$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

C. Frequency of cytokine producing ILC2, ILC3 and CD4⁺ T cells isolated from the colon of *IL-7RCre* and *ROR α ^{fl/fl}IL-7RCre* mice. Data show the mean $\pm$ SEM of the frequency of cytokine producing ILC2 pooled from two experiments and ILC3 and CD4⁺ T cell frequencies from one experiment ($n=2-4$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

D. Enumeration of ILC1 and ILC2 numbers from the lung, small intestine and mesenteric lymph node from *IL-7RCre* and *ROR α ^{fl/fl}IL-7RCre* mice. Data show the mean $\pm$ SEM of data from one experiment ($n=2-4$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, p-value as indicated.

ROR α deficiency promotes tumour development in CAC

To determine the effect of ROR α deficiency on tumour development, *IL-7RCre* and *ROR α ^{fl/fl}* *IL-7RCre* mice were treated with AOM+DSS (Fig. 5.14A) and endoscoped to monitor the development of macroscopic tumours. The colons of both *IL-7RCre* and *ROR α ^{fl/fl}* *IL-7RCre* mice had a significant amount of mucus, loose stools, uneven colon lining but *ROR α ^{fl/fl}* *IL-7RCre* had larger, more occlusive tumours that blocked the colon (Fig 5.14B). Both *IL-7RCre* and *ROR α ^{fl/fl}* *IL-7RCre* mice developed macroscopic tumours at the mid and distal regions of the colon but ROR α deficient mice developed twice as many tumours per mouse compared to control mice had a 2.8-fold increase in the total area of tumours compared to control mice (Fig. 5.14D). These results suggested that ROR α deletion affected immune cells that are involved in protecting against CAC development.

ROR α -deficient mice with CAC have reduced ILC1 and ILC2 numbers and reduced cytokine production capacity

To identify the defective immune cells potentially responsible for the increase in tumour burden when ROR α is deleted, I analysed the immune cells within the colons with CAC and found that similar to colons at steady-state, ROR α -deficient ILC1 and ILC2 numbers were reduced while NK and ILC3 numbers were not affected compared to control colons (Fig. 5.15A). There was a significant reduction in the frequency and total number of IL-5-producing ILC2 in ROR α -deficient mice but no difference in the frequency and number of IFN- γ -producing NK cells and IL-22-producing ILC3s (Fig. 5.15B-C). The frequency of IFN- γ -producing ILC1 did not change but there was a reduction in the total number of IFN- γ -producing ILC1, whereas the frequency of IL-17-producing ILC3 was reduced but no change in total number of cells (Fig. 5.15B-C). While the number of CD4⁺ and CD8⁺ T cells were unchanged, there was a reduction in the frequency of cytokine producing CD4⁺ T cells for IFN- γ , IL-5 and IL-17 (Fig. 5.15C,D). This also corresponded to a reduction in the number of IL-5-producing CD4⁺ T cells, but no change in the number of IFN- γ - or IL-17-producing CD4⁺ T cells (Fig. 5.15F). This shows that CAC tumours that develop in ROR α -deficient mice with CAC are in an immune deficient environment. In particular there is an absence of ILC1 and ILC2 cells and the cytokines they produce, which suggests that they may facilitate tumour growth.

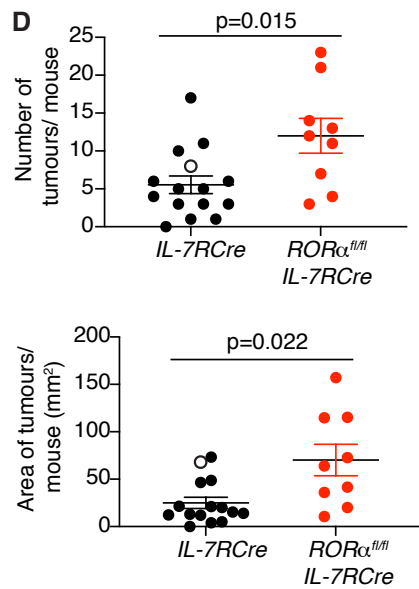
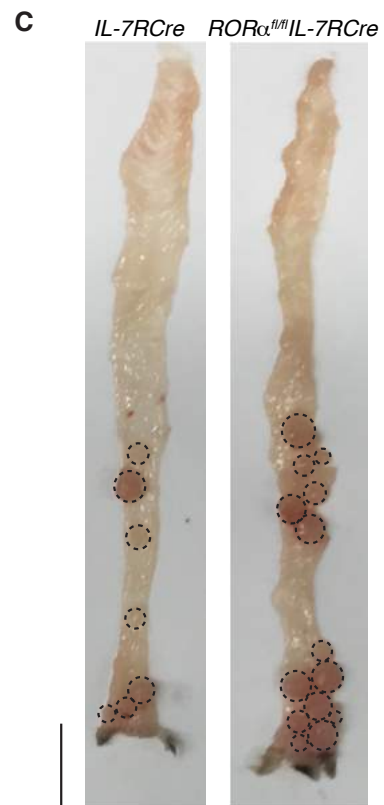
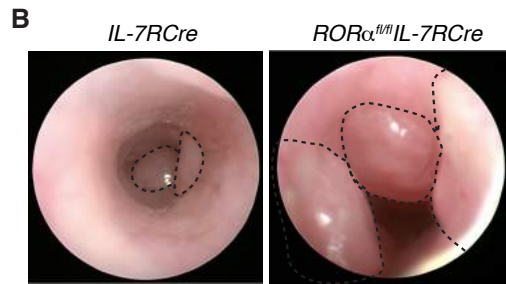
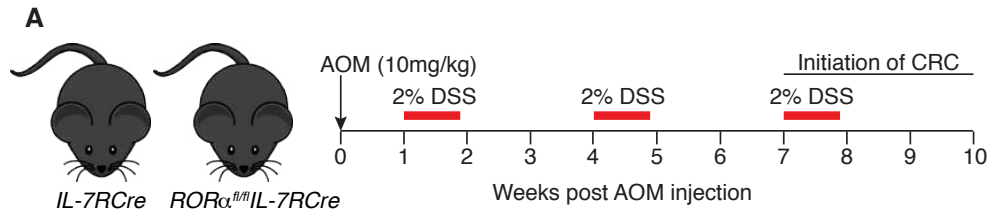


Figure 5.14. Loss of ROR α promotes tumour development in the CAC model.

A. Schematic illustration of the AOM/DSS treatment protocol for control *IL-7RCre* and *ROR $\alpha^{fl/fl}$ IL-7RCre* mice. AOM (10mg/kg) was administered I.P. to mice, followed by three cycles of 2% (w/v) DSS *ad libitum* in their drinking water for five days with two weeks of normal water between each cycle. Colorectal cancer development occurred between 7-10 weeks post initiation of treatment.

B. Representative endoscopy images of *IL-7RCre* (left panel) and *ROR $\alpha^{fl/fl}$ IL-7RCre* (right panel) treated with AOM+DSS. Images are representative of 9-15 mice/genotype/experiment from two independent experiments. Dotted lines indicate tumour growth.

C. Representative whole mount images of colons from *IL-7RCre* and *ROR $\alpha^{fl/fl}$ IL-7RCre* mice treated with AOM+DSS. Images are representative of 2-5 mice/genotype analysed in three separate experiments. Dotted lines indicate tumour growth. Scale bar, 1 cm.

D. Comparison of the total number of tumours (top) and area of tumours (mm², bottom) per mouse in *IL-7RCre* and *ROR $\alpha^{fl/fl}$ IL-7RCre* mice treated with AOM+DSS. Data show the mean $\pm$ standard error of mean (SEM) of data pooled from three separate experiments (2-5 mice/genotype/experiment). Statistics were calculated using the Mann-Whitney nonparametric test with p-values as indicated. Open circles represent males, closed circles represent females.

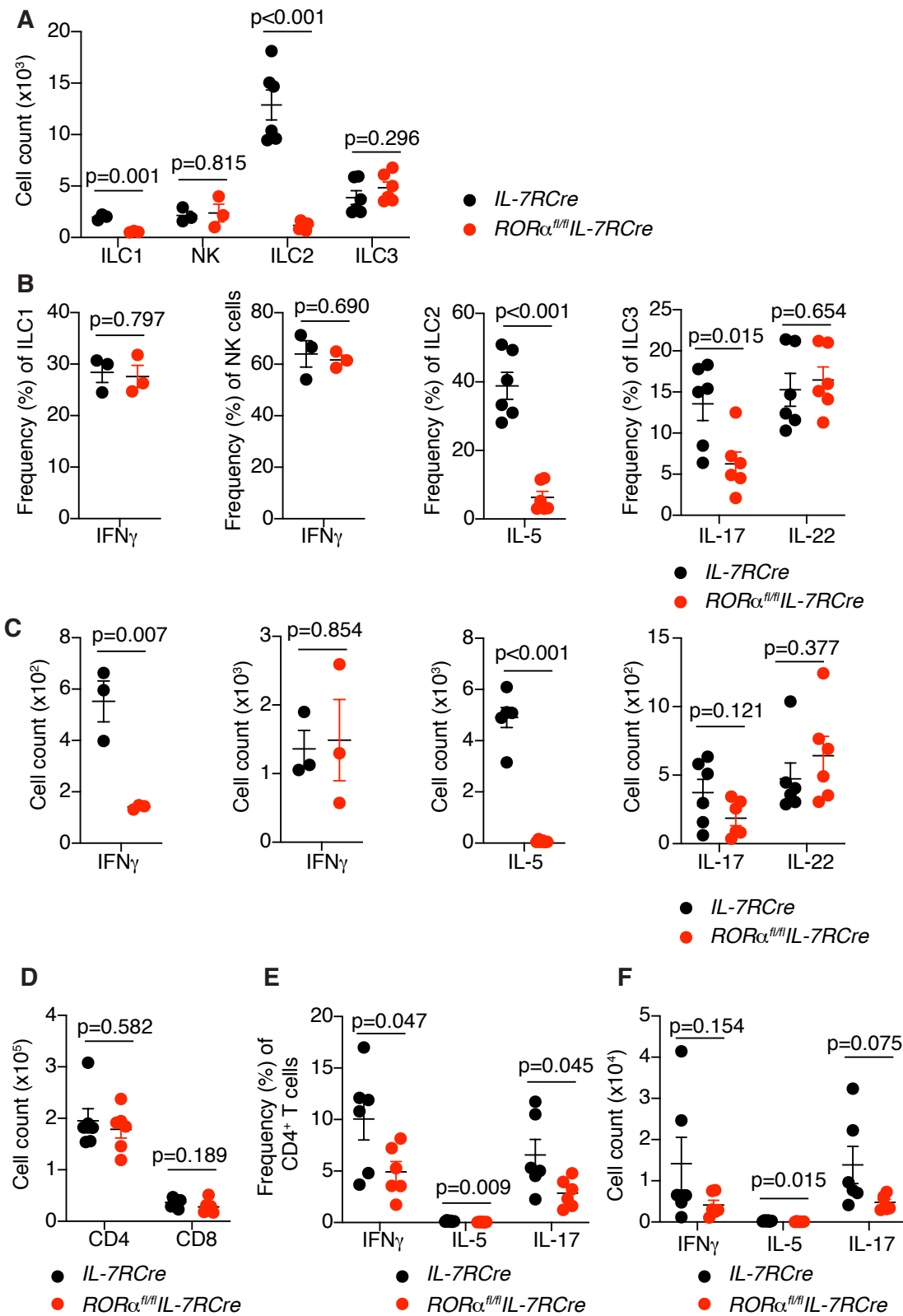


Figure 5.15. ROR α -deficient mice with CAC have reduced ILC numbers and reduced cytokine producing capacity of ILC and CD4⁺ T cells.

A. Enumeration of ILC subsets isolated from the colon of AOM+DSS treated *IL-7RCre* and *ROR $\alpha^{fl/fl}$ IL-7RCre* mice. Data show the mean $\pm$ SEM of ILC1 and NK data from one experiment and ILC2 and ILC3 data pooled from two separate experiments ($n=2-3$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, *p*-value as indicated.

B-C. Frequency (**B**) and number (**C**) of cytokine-producing ILCs isolated from the colon of AOM+DSS treated *IL-7RCre* and *ROR $\alpha^{fl/fl}$ IL-7RCre* mice. Data show the mean $\pm$ SEM of ILC1 and NK data from one experiment and ILC2 and ILC3 data pooled from two separate experiments ($n=2-3$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, *p*-value as indicated.

D. Enumeration of CD4⁺ and CD8⁺ T cell subsets isolated from the colon of AOM+DSS treated *IL-7RCre* and *ROR $\alpha^{fl/fl}$ IL-7RCre* mice. Data show the mean $\pm$ SEM of data pooled from two separate experiments ($n=2-3$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, *p*-value as indicated.

E-F. Frequency (**E**) and number (**F**) of cytokine producing CD4⁺ T cell isolated from the colon of AOM+DSS treated *IL-7RCre* and *ROR $\alpha^{fl/fl}$ IL-7RCre*. Data show the mean $\pm$ SEM of data pooled from two separate experiments ($n=2-3$ mice/genotype/experiment). Statistics were calculated using an unpaired Student's *t* test, *p*-value as indicated.

4.4 Discussion

Immune cells play a key role in mediating protection against cancer; however, they are also at risk of being dysregulated and can promote cancer development²⁵¹. Recently it has become apparent that ILCs are a subset of immune cells that play an important role in regulating the development of many cancers²⁵² one of them being CRC. ILC3 and its associated cytokines IL-17 and IL-22 have been implicated in tumour development but both beneficial and detrimental roles have been attributed to their influence on tumour development^{37, 230, 231, 234}. Furthermore, analysis of patient CRC samples has shown that the other ILC subsets and their associated cytokines and signalling pathway were altered¹⁹³, but it is not known whether they influence tumour development. In these studies, I set out to investigate whether ILCs are involved in CRC development. I show that cytokine-producing ILCs accumulate in the colon of mice with CRC and that IL-5-producing ILC2 are the main subset, which not previously been known. Mice deficient for ILC2 and ILC1 developed higher tumour burdens, which suggested that these cells may play a protective role in tumour development.

4.4.1 Infiltration of immune cells in CRC potentially driven by ILC2

Patients with CRC have an altered immune composition within the colon compared with healthy individuals^{193, 194}. Often there is an influx of immune cells being recruited and the types of immune cells recruited and their functional capacity can impact the patient prognosis²⁵³. While ILC2s with a more activated phenotype were found in patient CRC samples¹⁹³, the role of ILC2 in CRC has not been examined. My experiments show that the capacity of ILC2 to produce cytokine increases in the mice with CAC. this indicates that these ILC2s may be of a more activated phenotype. This is in accordance with the expression of activation markers such as ICOS and CD69 observed in CRC patient samples^{193, 194}.

The phenotype of ILC2s also has the potential to change as CRC progresses. It was recently reported that ILC2 in the colon of mice in the early stages of CRC had a low expression of PD-1 and HS3ST1, while ILC2 in late stage of CRC had high expression²⁵⁴. It is not known how the changes in the expression of these markers will affect the functioning of ILC2 and how this drives CRC development.

The increase in ILC2 activation during CRC may be due to the increase in cytokines such as IL-33, which is seen in patient samples and in mouse models^{255, 256}. During inflammation, epithelial cells are damaged and release IL-33 to remodel the stroma and promote angiogenesis^{255, 257}. However; these pathways may be altered to drive tumour development. IL-33 is also known to recruit immune suppressive cells such as Tregs to create a pro-tumourigenic environment²⁵⁸ but it is not known whether modulation of ILC2 also contributes to this pathogenesis. IL-25, another ILC2 activating cytokine, has been shown to be protective against CRC. Blockade with IL-25 antibody increased tumour burden in AOM+DSS induced CAC and it is thought that this occurs through the suppression of the type 2 response, while promoting Th17 pathway cytokines²⁵⁹. Although IL-25 blockade did not alter the transcript level of IL-5, IL-4 and IL-13, there was a reduction in the frequency of eosinophils in the colon with CAC. Therefore, it is likely that altering IL-5 does not impact the development of CAC but activated ILC2 may also produce other effector molecules that are protective against CAC.

4.4.2 Modulating IL-5 expression failed to alter tumour burden

IL-5 has been implicated to be important in lung metastasis but conflicting results have been reported, indicating that IL-5 may involve complicated regulatory pathways. Ikutani *et al.* reported that IL-5 was protective against lung tumour metastasis of B16F10 melanoma cells and is important for eosinophil migration towards tumour cells¹¹⁹ but it was not clear if eosinophils were responsible for mediating the protection against tumour cells, or whether IL-5 was acting directly on tumour epithelial cells to mediate protection. Conversely, it has also been reported that IL-5 deletion was able to significantly protect against metastasis to the lungs³⁶. They also show similar protective effects of lung metastasis with IV injections of B16F10 melanoma cells and MC38 colon adenocarcinoma cells. This is thought to occur through IL-5 modulation of the microenvironment, specifically eosinophils which can then secrete the chemokine CCL22 to modulate Tregs cells³⁶.

In my experiments, I did not observe a significant difference in the tumour development when IL-5 expression was modulated whereas Arnold *et al.* recently showed that IL-5 did impact tumour growth by regulating eosinophils to drive Th1 responses²²⁰. However, there were a number of differences between our experiments which may explain the difference in our findings. Arnold *et al.* showed that IL-5 overexpression was protective against subcutaneously

injected MC38 cells in IL-5TgN mice compared to control mice, whereas my data shows that IL-5 overexpression did not change the tumour burden in AOM+DSS treated mice. It was previously reported that IL-5TgN mice also had increased neutrophil numbers in the blood ¹¹⁸. However, IL-5 overexpression had no impact on neutrophil numbers in the colon (Fig. 5.6E), indicating potentially a tissue specific response required for neutrophil recruitment to the colon. Therefore, it is possible that the protection against MC38 subcutaneous tumour development may in part be due to the increase of circulating neutrophils in the periphery but is not infiltrating into the colon to mediate tumour protection. Furthermore, in the context of IL-5 overexpression, it is not well understood what the role of IL-5 signalling on epithelial cells is, especially in a tumour context. It has been reported that human airway epithelial cells may express IL-5R and stimulation with IL-5 had resulted in increases in signalling molecules with cell growth and proliferation²⁶⁰. My experiments identify IL-5R expression on colorectal epithelial cells, but it is not known whether IL-5R on these epithelial cells are functionally responsive to IL-5 signalling. It is possible that IL-5 stimulates epithelial proliferation and negates the protective effect of increased eosinophil infiltration and ultimately there is no significant difference in the tumour burden. To test the proliferative capacity of epithelial cells under IL-5 stimulation in isolation, without the influence of external cells, CRC cell lines such as MC38 could be cultured with or without IL-5 and their proliferation measured by the increase of cultured cells.

Arnold *et al.* also showed that APC^{Min/+} mice, a model of spontaneous CRC development, treated with anti-IL-5 antibodies had developed more adenomas, whereas the mouse model of IL-5-deficient mice on a BALBC background that I used did not conclusively determine whether IL-5 regulated the tumour burden of CRC. BALBC mice have been reported to develop less severe inflammation and tumour burdens compared to C57BL6 ²⁴⁹. There was also no difference in the expression of IL-5-producing ILC2 in BALBC mice therefore, loss of IL-5 in this model may not significantly impact tumour development. This difference in disease outcome between BALB/c and C57BL/6 mice highlights that differences in baseline immune systems for individual patients may result in different degrees of immune responses against the same pathology and in turn, may respond differently to the same treatment. Nevertheless, eosinophils seem to play a role in protecting against CRC development and ILC2, as the key IL-5 producer in the colon, could potentially be responsible for mediating this protection. This would be a novel role of ILC2, to be able to mediate protection in CRC. Alternative methods

of IL-5 deletion, such as anti-IL-5 treatments should be tested to determine whether it can impact CAC development in AOM+DSS treated mice.

4.4.3 ILC1s contribute to the development of CAC

My results suggest that ILC1s in the colon may also be involved in protecting against tumour development as ROR α mice are also ILC1-deficient (Fig. 5.13). This lack of ILC1 was unexpected as ROR α -deficient mice had previously not been reported to have reduced ILC1 numbers. Either ROR α expression is important for ILC1s in the periphery, or it is important for a progenitor subset involved in ILC1 development.

The role of ILC1s during CRC tumour development has not been examined previously. ILC1s were shown to accumulate in patients with Crohn's disease and in CRC patients^{194, 196}. But it is not known if they drive inflammation or if they mediate anti-tumour immunity. Based on the low correlation between ILC1 and tumour burden (Fig. 5.3C, 4C), the impact of ILC1 on tumour development may be small. To address the role of ILC1 specifically, *ROR α ^{fl/fl} NCRCre* mice can be bred to selectively delete ROR α in NKp46 expressing cells. Loss of ROR α does not appear to affect NK cell numbers, ILC3s in the colon are predominantly NKp46⁻ and only ILC1 numbers should be reduced.

The observation that ROR α expression in immune cells are responsible for the protection against tumour development is a novel finding. Future experiments dissecting the mechanism of the role of ROR α , ILC1 and ILC2 will have important implications for understanding how the gastrointestinal immune system contributes to tumour development and disease progression. Uncovering these mechanisms could lead to new therapeutic targets in the treatment of CRC.

Chapter 6. Discussion

5.1 Transcriptional regulation of early ILC development

There has been great interest in understanding the transcriptional regulatory network that govern ILC development and function. The development of new technologies enabling the examinations of immune populations on a single cell level has revealed the heterogeneity of tissue-specific ILCs and the transcriptional regulation required to produce this diversity. Elucidating the developmental pathways that drive this variation will enable a greater understanding of how immune protection is mediated against a wide variety of pathologies. Our lab had previously identified a number of transcription factors that shape ILC development. Specifically, expression of transcription factors ID2, NFIL3 and TCF1 is required and temporally regulated, for CLP to develop into the CILP¹⁷. NFIL3 is only transiently expressed before TCF1 and ID2 is expressed in CILP and is absent in progenitor populations that subsequently develop¹⁷. Furthermore, this expression of NFIL3 is vital for the development of CILP but is dispensable for the development of downstream progenitors and mature cells after the onset of ID2 expression¹⁷. However, more work is required to establish precisely how this core group of transcription factors are regulated, in relation to each other, to drive early progenitor development. Even less is known the molecular cues that drive the early specification of different ILC subsets. In particular, progenitor populations are highly heterogeneous with different capacities for the development of different ILC subsets. The identity of progenitor populations that diverge between the development of ILC subsets is currently unknown.

The role of two transcription factors, GFI1 and GFI1B, were assessed to discover how they shaped development and function of individual ILC subsets. Despite their structural homology, GFI1 and GFI1B were differentially expressed throughout ILC development and played significant roles in regulating the development of ILC progenitors in the bone marrow as well as the development and function mature ILCs in the periphery. Previously, GFI1B had only been described in the development of erythrocytes and megakaryocytes⁶⁷. However, I have identified that is much more broadly involved in haematopoiesis as it is also a key regulator of the development of ILC progenitors in the bone marrow and ILC2 in the lung. Thus, these findings reframe the model in which GFI1B is restricted to erythrocyte and megakaryocyte development, to also include the lymphoid lineages, in particular ILCs.

Using reporter mice that marked the expression of GFI1B by GFP, I showed that GFI1B expression at the CILP stage has been shown to define the IL-7R⁺ progenitor cells with the greater developmental potential. It is these GFI1B⁺ CILP cells that had the capacity to generate the full range of ILC subsets that could localize in peripheral tissues, whereas GFI1B⁻ CILP predominantly generated NK cells (Fig. 6.1). This marks a bifurcation in the developmental pathway between NK cells and other ILC subsets that has not been previously identified. Indeed, GFI1B appears to be a vital for the development of lymphoid progenitors in the bone marrow and further examination of the molecular regulation involved via RNASeq would be valuable in identifying the transcription factors that drive ILC development.

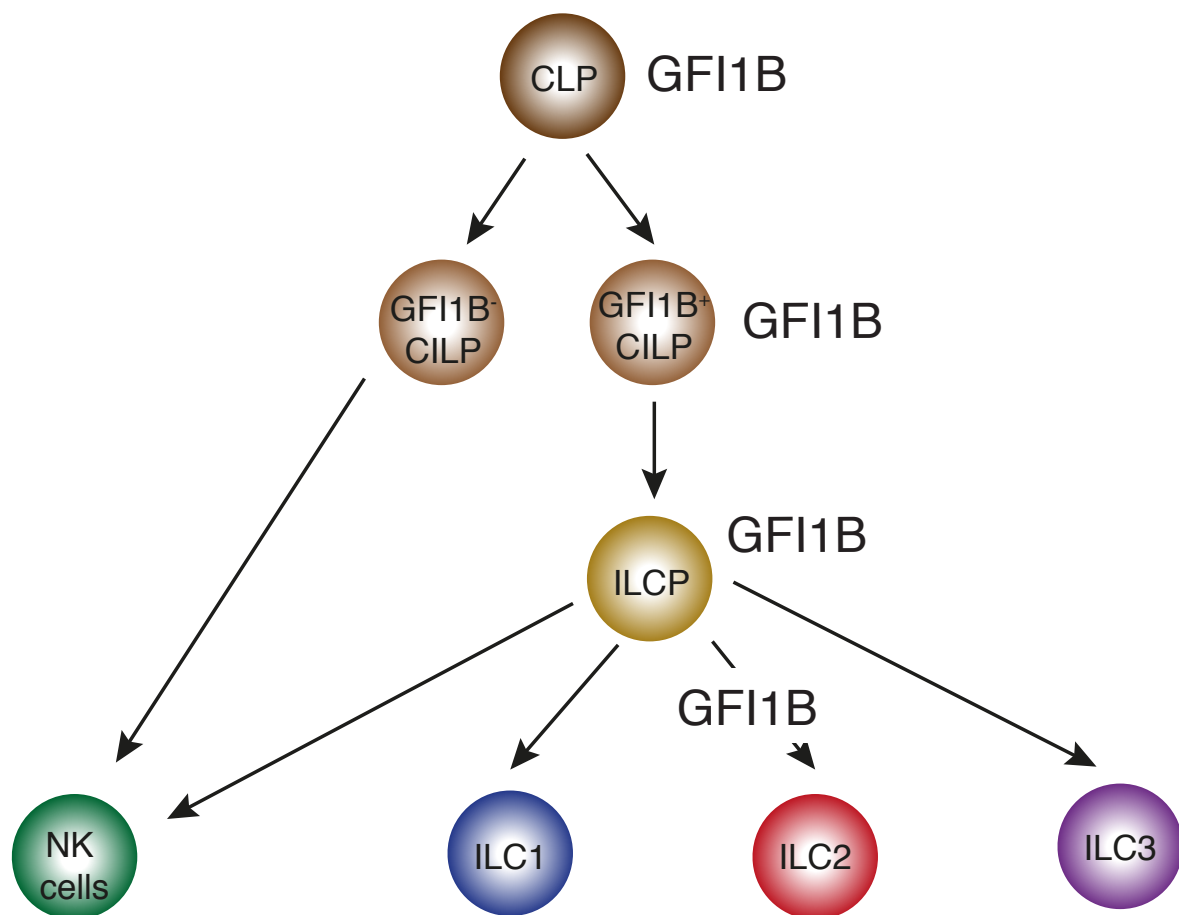


Figure. 6.1 Updated model of ILC development.

GFI1B expression is restricted to lymphoid progenitors and is not expressed by mature ILCs. In the bone marrow, GFI1B is required for progenitors to develop. This in turn impacts the development of mature ILC2 in the lung. Furthermore, GFI1B expression also demarcates the CILP cells with the greater developmental potential to give rise to all ILC subsets.

5.2 Tissue specificity of ILC2 development and function

GFI1, but not GFI1B, has previously been implicated in the development of ILC2¹¹⁰. Examination of the role of GFI1B in regulating ILC2 uncovered that GFI1B specifically regulated tissue-specific ILC2 development. GFI1B-deficient mice failed to develop ILC2 in the lung, although ILC2 did develop in the small intestine. This could have been due to a failure of recruitment of progenitors from the bone marrow to the tissues. I found that, in part, this could be due to fewer ILC2p in the bone marrow in GFI1B-deficient mice, but an alternative explanation could also be due to a more direct impact in regulation a potential tissue-resident ILC2 progenitor. Progenitors have been identified in the foetal intestine and adult lung, marked by expression of arginase-1 and IL-18R1 respectively, which have the capacity to differentiate locally to give rise to ILC2s^{138, 261}. Preliminary experiments indicated that GFI1B did not regulate the number of IL-18R1⁺ progenitor in the lung (Fig. 3.18b) but it is not known whether progenitors in the foetal intestine were affected

This potential difference in developmental origins also raises the question of the source of ILC2s during an inflammatory response. In other studies, sequencing analysis of developmental trajectories as well as experimental observations indicate that both recruited and resident ILC2s contribute the ILC2 population within the lung during infection¹³⁸. My data showed that, similar to this study, there was both local proliferation of ILC2 in the lung and circulating ILC2 following papain treatment (Fig. 3.18) although it is not known where the circulating ILC2 had originated from. These recruited ILC2 could have originated from the bone marrow, following IL-33 stimulation and have a homeostatic or ‘natural’ phenotype, or they could be derived from the small intestine following responses to IL-25 and have an inflammatory phenotype^{133, 137}. Transcriptional heterogeneity between ILC2s of different organs have been identified with single cell RNASeq and tissue-specific signals was thought to have contributed to the differences in activation states²⁶². Further analysis on the capacity of GFI1B-deficient ILC2 within different tissues to respond to different cytokine stimulation may give an indication on their developmental origin and identify the molecular pathways driving ILC2-mediated immunity.

5.3 Transcriptional regulation of ILC1

Despite both ILC1 and NK cells belonging to group 1 ILCs, they are developmentally distinct. NK cell expression of EOMES differentiates them from ILC1, although there are exceptions as ILC1 in the salivary gland also express EOMES^{263, 264}. I found that ROR α , previously indicated to be restricted to ILC2, was also required for ILC1 in all tissues but was not required by NK cells or ILC3. Thus, a role for ROR α in ILC1 is new. Recently, it has been reported that ROR α was specifically expressed by ILC1, ILC2 and ILC3 but was not expressed by NK cells²². This group also showed that loss of ROR α did not impact the number of ILC3 in naive mice⁶⁰, suggesting that either ILC1 and ILC2 share a common developmental progenitor that is impacted by the loss of ROR α or ILC1 was intrinsically regulated by ROR α . It is unknown whether ROR α impacts progenitor populations. Analysis of this feature may enable the identification of a progenitor that is specific for ILC1 development distinct from NK cells.

I found that GFI1 also regulated ILC1 but this was specific to the liver (Fig. 4.3). Although the mechanism of this regulation is not yet known, a similar phenotype was seen in mice deficient for the transcription factor HOBIT²⁶⁵. It has previously shown that ILC1 in the liver are critical to mediating early responses against MCMV infections providing a key source of pro-inflammatory cytokines⁵. It is not known whether ILC1s are involved in regulating other diseases particularly in the liver even though large population of ILC1s are resident in this tissue. Whether ILC1 play an important role in other diseases such as hepatitis virus infection or alcoholic liver disease remains to be investigated. However, the identification of GFI1 and ROR α as transcription factors that regulate ILC1 presents a new opportunity to generate mouse models to study the role of ILC1 without disrupting other cell types.

5.4 Human pathologies associated with GFI1 mutations

Gain of function mutations in GFI1 have been reported in patients with acute myeloid leukemia, whereas loss of function mutations have been reported in colorectal cancer patients^{266, 267}. However, the impact of these mutations in GFI1 have primarily been assessed in the context of the cancer cell itself as GFI1 acts as a suppressor of tumour cell proliferation. Less is known about the impact of GFI1 mutations on the immune system. GFI1 mutations

have been reported in patients with neutropenia and lymphopenia which was linked to a disruption of their transcriptional repressor activity²⁶⁸ but it is not known if this immune deficiency puts patients at risk of developing recurring infections or cancer.

I made the novel discovery that GFI1 expression in murine NK cells is vital for mediating protection against tumour cells. NK cells that are deficient for GFI1 fail to localise to peripheral organs, and they could not proliferate effectively to maintain normal NK cell numbers. Expression of GFI1 in NK cells was particularly important during tumour challenge for the recruitment and activation of NK cells to mediate tumour protection. There was reduced NK cell numbers in GFI1-deficient mice challenged with tumour cells and the NK cells present showed reduced production of pro-inflammatory cytokines such as IFN- γ and cytotoxic molecules such as granzyme B. Accurate transcriptional regulation of NK cells to induce effector function is therefore important as NK cells play a vital role in immunosurveillance and mediating protection against tumour development. NK cell function may be dysregulated by inhibitory molecules present in an immunosuppressive tumour microenvironment, but patients mutations that result in NK cell defects may also be at increased risk of developing cancers²⁶⁹. It is not known whether individuals with GFI1 mutations also have defects in their NK cell population but given the similarities of phenotypes between individuals with GFI1 mutations and GFI1-deficient mice, it is likely that GFI1 also regulates NK cell functions in humans. Given the integral relationship between immune system and regulation of cancer development, GFI1 mutations in NK cells are also likely to be associated with human pathologies. Screening patients with NK cell immunodeficiency may identify GFI1 mutations which could lead to a greater understanding of the pathogenesis within patients.

5.5 Role of ILC2 in CRC

ILCs respond rapidly during the initial gastrointestinal immune response to a breach in epithelial barrier integrity and communicate to the adaptive arm of the immune response²⁷⁰. However, the role of ILC family members in CRC appears to be divergent. NK cells have been reported to be required for anti-tumor immunity in CRC^{194, 199}, while ILC1 and ILC3 have been linked to the promotion of CRC^{37, 228, 271}.

In my analyses of CRC, I show that ILC2 is the predominant ILC member in the inflamed colon of tumor-bearing mice and demonstrate that ILC2 has a role in restraint of tumor progression. The mechanisms regulating ILC2-mediated protection during CRC are not yet fully described. Single cell RNASeq of ILCs during the early and late stages of CRC development in mice identified changes in ILC phenotype and function as tumors developed²⁵⁴. In this study, they showed that the PD-1⁻ ILC2 subset was present during the early stages of CRC and this was largely replaced by PD-1⁺ ILC2 which then promoted tumor development²⁵⁴. These observations suggest that the role of ILC2 may change as tumors progress. Recently, it has been shown that the ILC2-derived cytokines, IL-5 and GM-CSF, both have the capacity to control tumor development as the genetic ablation of either IL-5 or GM-CSF increased the tumor burden in the MC38 murine CRC model, while overproduction of IL-5 facilitated protection against tumor development²²⁰. The protection against the development of CRC was thought to be mediated by eosinophils that depend on IL-5 and GM-CSF to drive migration to immune cell migration to the tumor microenvironment, which in turn, drives Th1 cell responses against tumors²²⁰. In my studies, I examined the role of IL-5 but could not delineate a clear role in affecting CRC development. This could be due to differences in the mouse background or differences in the site of tumour growth as the role of IL-5 was demonstrated in an ectopic model of CRC with MC38 cell lines injected subcutaneously and treated with anti-IL-5²²⁰, whereas I induced development of CRC in the colon with a carcinogenic and pro-inflammatory agent in IL-5-deficient mice. Differences in immune composition and signaling molecules in the microenvironment between the gastrointestinal tract and the subcutis may impact on tumour growth. However, my findings identify ILC2s as the main IL-5 producer in the colon tumor microenvironment. Through this function, ILC2s could be key players in mediating tumor protection through the activation of eosinophils. Establishment of this will require further investigation.

5.6 Conclusion

Work in this thesis has contributed to our understanding of the transcriptional regulation involved in ILC development and function. I have identified GFI1 and GFI1B as key regulators of ILC distinguishing the differential temporal regulation by these transcription factors. GFI1B played a key role in early ILC development and in mature ILC2 in the lung, whereas GFI1 was important for NK cell maturation and function. The efficient functioning of ILCs is critical

in mediating protection against pathologies and immunodeficient mice have increased tumour burdens seen in both metastatic melanoma and colorectal cancer models. Further work identifying the mechanisms behind the transcriptional regulation of ILCs will provide a greater understanding of the transcriptional network regulating ILC development and function and how immune protection is mediated.

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Appendix

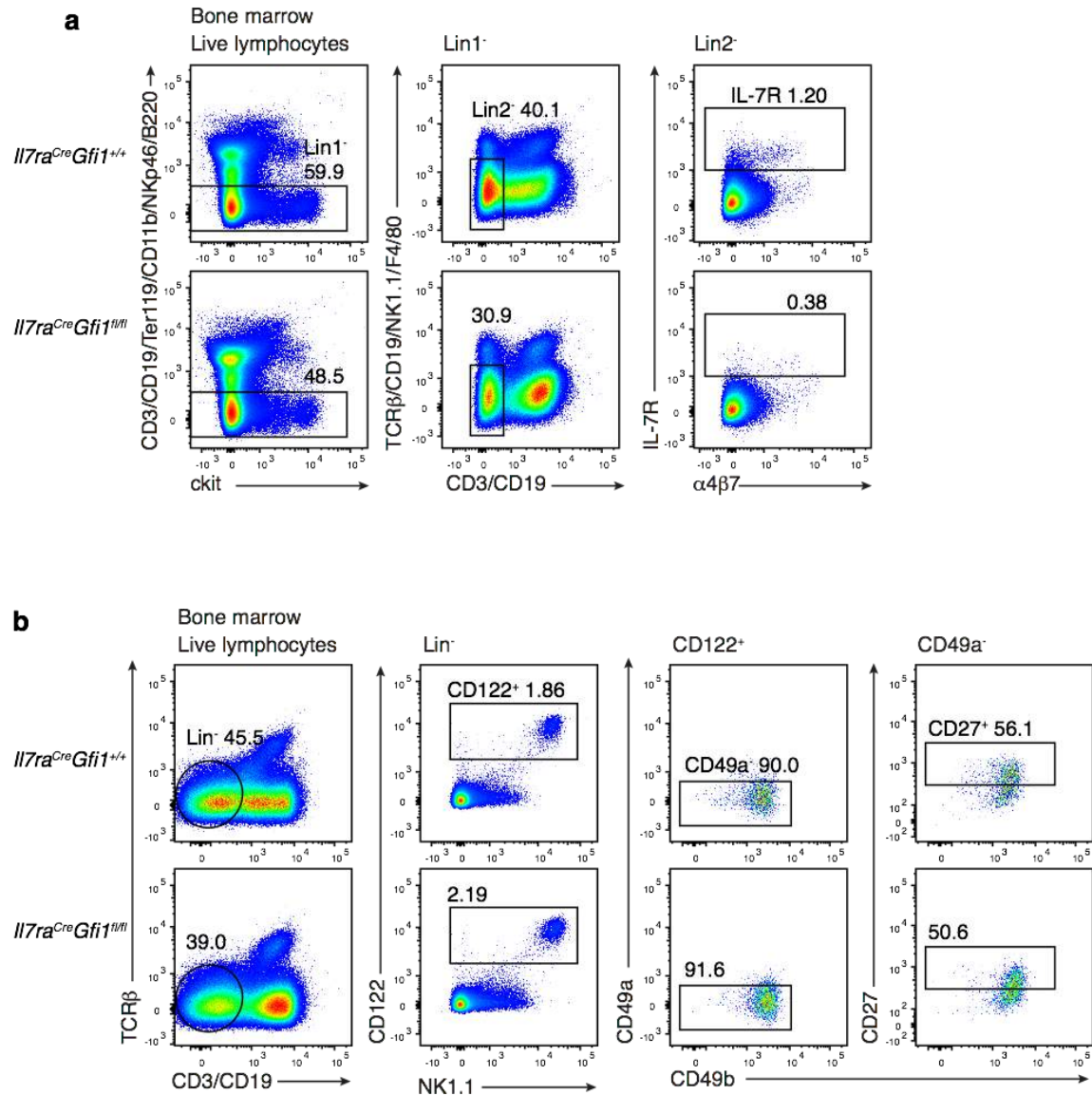


Figure 7.1 Gating strategy for bone marrow progenitors in *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice.

a. Flow cytometric profiles of the gating strategy used to identify early progenitors in the bone marrow of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice. Within the live lymphocyte gate, lineage cells (CD3⁺TCRβ⁺CD11b⁺CD19⁺B220⁺NK1.1⁺NKp46⁺F4/80⁺Ter-119⁺) were excluded and IL-7R⁺ cells were gated on.

b. Flow cytometric profiles of the NK progenitors in the bone marrow of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice. Within the live lymphocyte gate, lineage cells (CD3⁺TCRβ⁺CD19⁺) were excluded and CD122⁺CD49a⁻CD27⁺ cells were gated on.

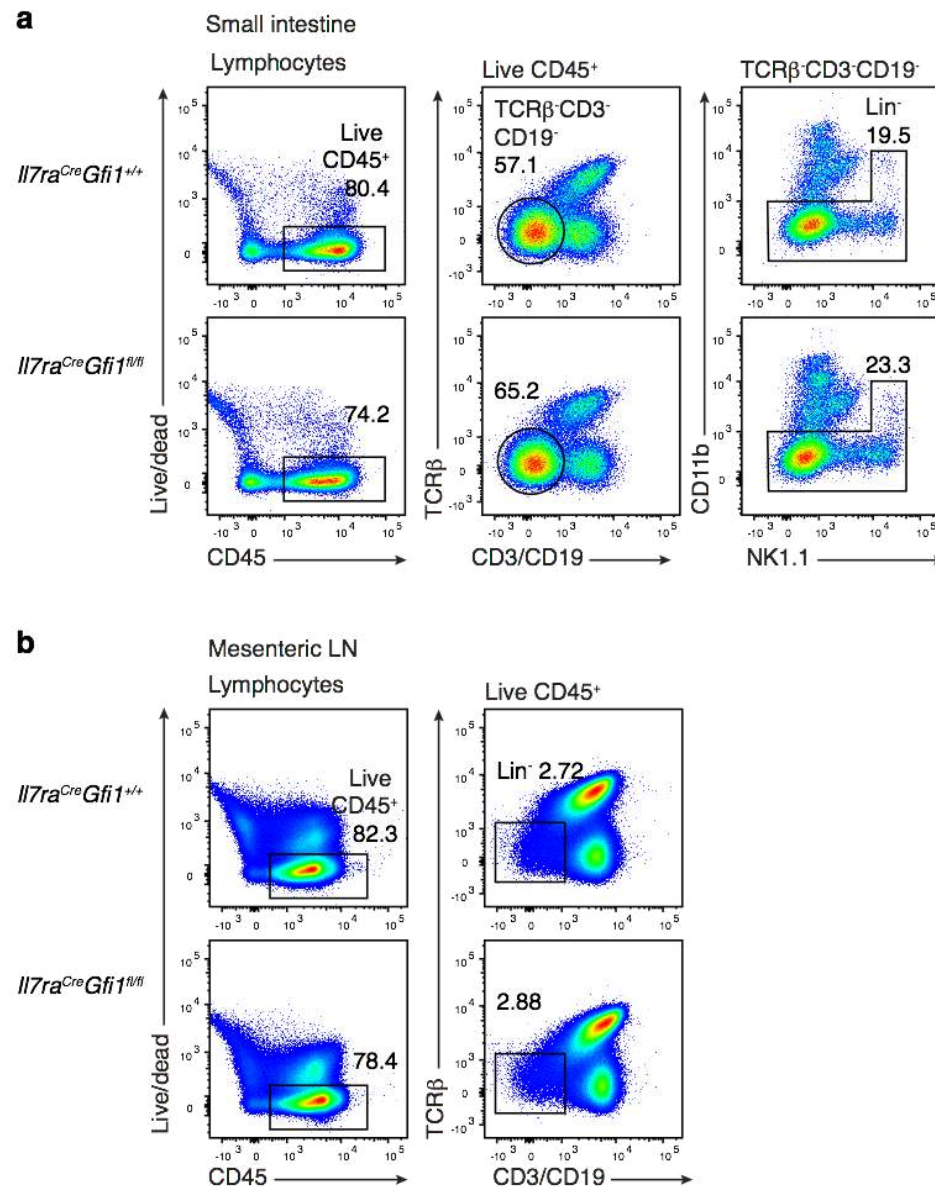


Figure 7.2 Gating strategy for ILC2 and ILC3 in the small intestine and mesenteric LN of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice.

a. Flow cytometric profiles of the gating strategy used to identify ILCs from the small intestine of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice. Within the lymphocyte population, live CD45⁺ cells were gated and lineage cells (CD3⁺TCRβ⁺CD19⁺CD11b⁺) were excluded.

b. Flow cytometric profiles of the gating strategy used to identify ILCs from the mesenteric lymph node of *Il7ra^{Cre}Gfi1^{+/+}* and *Il7ra^{Cre}Gfi1^{fl/fl}* mice. Within the lymphocyte population, live CD45⁺ cells were gated and lineage cells (CD3⁺TCRβ⁺CD19⁺) were excluded.

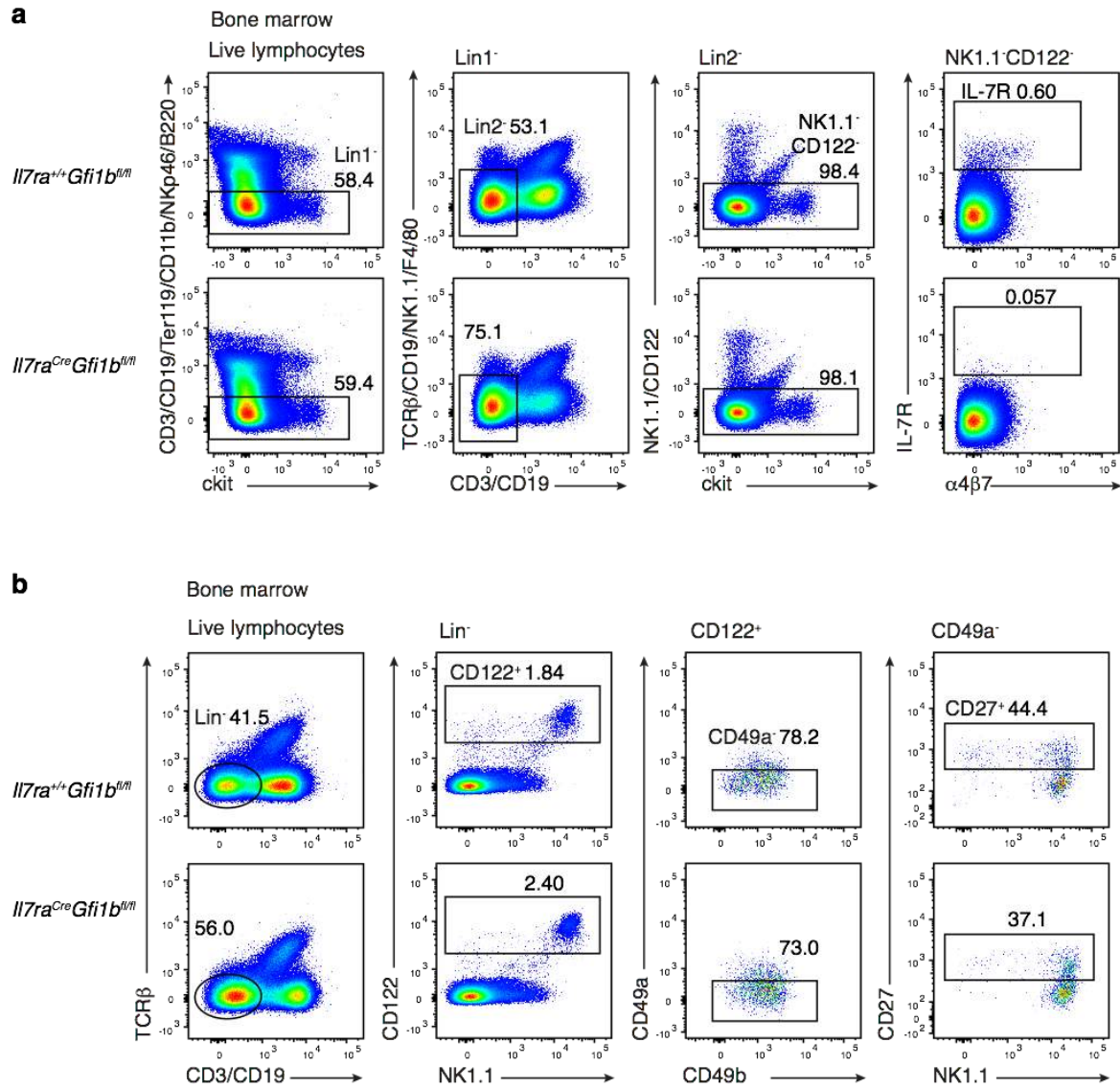


Figure 7.3 Gating strategy for bone marrow progenitors in *Il7ra^{Cre}Gfi1b^{+/+}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice

a. Flow cytometric profiles of the gating strategy used to identify early progenitors in the bone marrow of *Il7ra^{Cre}Gfi1b^{+/+}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. Within the live lymphocyte gate, lineage cells (CD3⁺TCRβ⁺CD11b⁺CD19⁺B220⁺NK1.1⁺CD122⁺NKp46⁺F4/80⁺Ter-119⁺) were excluded and IL-7R⁺ cells were gated on.

b. Flow cytometric profiles of the gating strategy used to identify NK progenitors in the bone marrow of *Il7ra^{Cre}Gfi1b^{+/+}* and *Il7ra^{Cre}Gfi1b^{fl/fl}* mice. Within the live lymphocyte gate, lineage cells (CD3⁺TCRβ⁺CD19⁺) were excluded and CD122⁺CD49a⁻CD27⁺ cells were gated on.

Article

Type 2 Innate Lymphoid Cells Protect against Colorectal Cancer Progression and Predict Improved Patient Survival

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Simple Summary: Colorectal cancer is the second leading cause of cancer-related death worldwide. The immune system plays a key role in controlling tumour onset and development. However, our immune system is complex and includes many different cell types which differently impact colorectal cancer outcomes. In this study, we investigated the function of the specialised type 2 innate lymphoid cells (ILC2) in colorectal cancer development and progression. We found that ILC2 infiltrate colorectal tumours and their presence was associated with reduced tumour burden in mice. In patients, this infiltration correlated with improved overall survival. Collectively, our work reveals that ILC2s are beneficial to colorectal cancer outcomes.

Abstract: Chronic inflammation of the gastrointestinal (GI) tract contributes to colorectal cancer (CRC) progression. While the role of adaptive T cells in CRC is now well established, the role of innate immune cells, specifically innate lymphoid cells (ILCs), is not well understood. To define the role of ILCs in CRC we employed complementary heterotopic and chemically-induced CRC mouse models. We discovered that ILCs were abundant in CRC tumours and contributed to anti-tumour immunity. We focused on ILC2 and showed that ILC2-deficient mice developed a higher tumour burden compared with littermate wild-type controls. We generated an ILC2 gene signature and using machine learning models revealed that CRC patients with a high intratumour ILC2 gene signature had a favourable clinical prognosis. Collectively, our results highlight a critical role for ILC2 in CRC, suggesting a potential new avenue to improve clinical outcomes through ILC2-agonist based therapeutic approaches.

Keywords: colon cancer; colitis-associated cancer; ILC2; IL-5; IL-13; inflammation

1. Introduction

The GI tract is lined with a single layer of epithelial cells that create a physical barrier to assist with the prevention of trillions of microorganisms translocating from the intestinal lumen into the systemic circulation, which could otherwise lead to serious complications,

including sepsis. Millions of tissue-resident immune cells patrol the GI tract, making it the largest component of immune surveillance within the body [1]. Consequently, a fine balance between pro- and anti-inflammatory immune responses is required to maintain GI homeostasis [1]. Chronic dysregulation of these immune responses has been shown to contribute to CRC development and progression in mice [2,3], mirroring the observation that inflammatory bowel disease (IBD) patients have an increased risk of CRC development [4].

While the contribution of the adaptive immune response to CRC onset and progression is well described [5,6], much less is known about the role of the innate immune response [7,8]. Innate lymphoid cells (ILCs) comprise a major component of GI inflammation, and have been implicated in murine CRC models [3,9,10] and found within human CRC tumours [11,12]. ILCs are evolutionarily conserved [13], mimicking key functions of adaptive T lymphocytes [14]. They are grouped into five subsets: natural killer (NK) cells, ILC1, ILC2, ILC3 and lymphoid tissue-inducer (LTi) cells based on their developmental program, transcription factors and cytokine expression [15,16].

NK cells and ILC1 express T-bet, produce interferon-(IFN)- γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) [17] and have well-described roles in responses to viral [18] and bacterial infections [19,20]. Similar to the anti-tumourigenic activity described for other tumour types [21–23], NK cells can effectively induce cell death in cancer cells, including CRC stem cells and cancer-initiating cells [24]. However, due to the selective pressure imposed by the microenvironment, tumour cells may evade NK cell detection and tumour cell lysis by upregulating MHC-Class I and downregulating NCR ligands to escape NK-cell-mediated killing [24]. An intraepithelial-like ILC1 population has been found to infiltrate human CRC and have the capacity for granzyme and perforin secretion as well as IFN- γ production, making them potential key players in the anti-tumour response [11,12].

ILC3s and LTi cells express the transcription factor ROR γ t and produce IL-17A and IL-22, cytokines that are essential to the host response to bacterial infections and maintenance of intestinal homeostasis, although their role in wound healing responses can also lead to augmented tumour progression [9,25–28]. The activity and function of ILC3s are also regulated by IL-23 [29], a cytokine that is produced by activated intestinal dendritic cells and macrophages [30], with IL-23 also shown to promote CRC development [31]. While LTi cells are critical to lymphoid organogenesis, it is not known if they are involved in CRC progression [32].

Our understanding of the role of ILC2s in CRC is limited. ILC2s express ROR α and GATA3 and secrete type-2 cytokines [33–35], including interleukin-(IL)5, IL-13 and GM-CSF that promote the clearance of helminth and parasitic infections [36,37]. In addition, ILC2s produce amphiregulin, a protein necessary for wound healing and tissue repair [38]. While IL-13 signalling in CRC [39] and other tumour types [40,41] has been associated with a poor prognosis, the role and function of ILC2-derived IL-5 are still poorly understood. IL-5 is critical for the development, recruitment, activation and survival of eosinophils [42,43] which have been associated with anti-tumour responses and favourable CRC outcomes [44–47]. Thus, ILC2s may convey different prognostic value according to the level of cytokines that they secrete. This complicates the interpretation of the role of ILC2s in CRC.

Here, we utilised complementary CRC murine models to investigate the role of different ILC subsets in CRC progression. We demonstrate that ILC2-deficient mice developed increased tumour burden compared with littermate wild-type animals. Using an ILC2 gene signature, we reveal that patients with an elevated tumour-derived ILC2 gene signature had increased survival, irrespective of patient age, gender, lymph node status or tumour stage. Collectively, our results demonstrate that ILC2s restrain tumour progression and are associated with favourable disease outcomes, highlighting that they are important potential candidates for therapeutic modulation to boost anti-tumour immunity and improve patient survival.

2. Results

2.1. ILC Are Present in a Murine Model of Colon Cancer and Implicated in Tumour Growth

In order to understand the role of ILCs in CRC, we first established that ILCs contributed to CRC progression. To this end, we generated subcutaneous MC38 murine colorectal carcinoma syngeneic xenografts in wild-type (C57BL/6) mice, T and B cell-deficient *Rag1*^{−/−} mice and severely immunocompromised *Rag2*^{−/−}*γc*^{−/−} mice, which lack both adaptive and innate lymphoid cells, and compared the tumour growth rates (Figure S1A,B, Table S1). We found that *Rag1*^{−/−} and *Rag2*^{−/−}*γc*^{−/−} mice had increased tumour growth and size, and reduced survival compared with wild-type controls, in accordance with previous reports [48,49] (Figure S1B, Table S1). However, we also observed that tumours grew even faster in *Rag2*^{−/−}*γc*^{−/−} mice compared to *Rag1*^{−/−} mice (Figure S1B, Table S1). Thus, while adaptive immune cells exert a strong influence in controlling the growth of MC38 tumour cells, these data suggest a role for the innate compartment of the immune system in the anti-tumour response.

We then determined whether ILCs had the capacity to infiltrate within the MC38 subcutaneous tumour in wild-type (C57BL/6) mice. After 16 days of tumour growth, we found that all ILC subsets, including NK cells, ILC1, ILC2 and ILC3 infiltrate MC38 tumours (Figure S1C) and represented 5–10% of tumour-infiltrating leukocytes (Figure S1C,D). Interestingly, we also observed an increase in the number of all ILC subsets in tumour-draining lymph nodes (dLN) compared with the non-draining lymph nodes (cLN) (Figure S1E–G), suggesting ILC trafficking within the tumour microenvironment. As all ILC subsets had the capacity to infiltrate into the MC38 tumour, we further examined ILCs during the development of colitis-associated cancer (CAC) in the colon microenvironment.

2.2. ILCs Are Increased in Inflammation-Associated Colon Cancer

We next employed a mouse model of CAC to validate our observations in a model more representative of human disease. In the CAC model, animals received the colonic mutagen azoxymethane (AOM), followed by three cycles of the luminal irritant dextran sodium sulphate (DSS) resulting in the formation of tumours in the distal colon (Figure 1A; Figure S2A–C). Tumours in this model are associated with increased inflammatory cell infiltration, which occurs in parallel with the development of regions of adenocarcinoma characterised by disorganised crypt structures (Figure S2D).

We characterised ILC subsets within colons of age- and sex-matched C57BL/6 untreated mice, or mice that developed CAC (Figure 1A,B). Our results demonstrate that although ILC2 numbers were unchanged (Figure 1C), they were the most prevalent ILC subset in the colon at a steady-state (Figure S3, Table S2). In contrast, the number of NK cells, ILC1 and ILC3 increased significantly in tumour-bearing mice (1.9-fold, 3.2-fold and 1.9-fold, respectively; Figure 1C). We also examined the mesenteric lymph node (MLN) and found that ILC1, ILC2 and NK cells accumulated in tumour-bearing mice, while ILC3 numbers did not change, suggesting that ILC3 responses may be localised to the colon (Figure 1C). There were no changes in ILC numbers within the spleen (Figure 1C). These results highlight that ILC responses to CAC are predominantly localised to the GI tract.

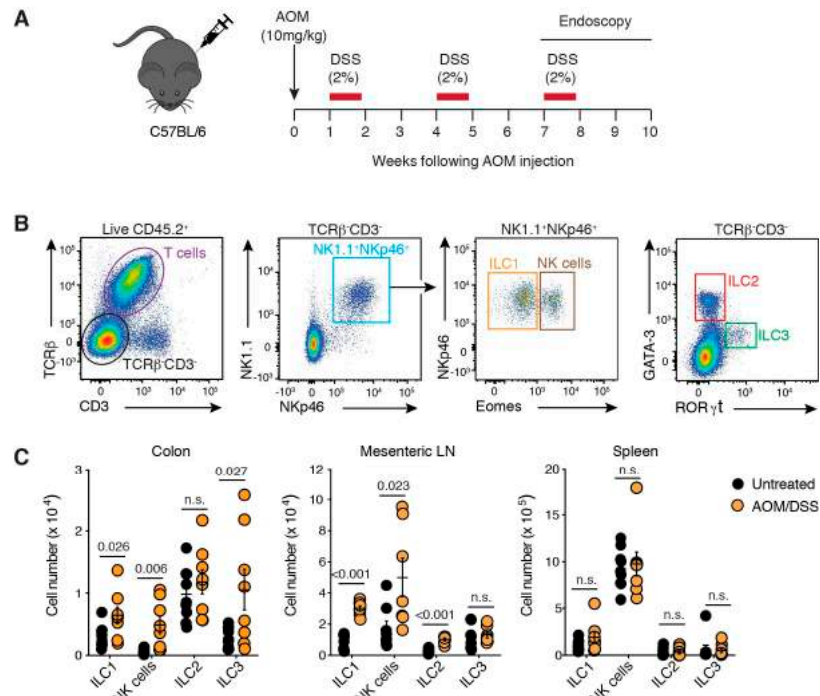


Figure 1. ILCs infiltrate colorectal tumours. **(A)** Schematic illustration of the AOM/DSS treatment protocol. Naïve C57BL/6 mice injected with AOM (10 mg/kg, i.p.) followed by three five-day cycles of 2% (*w/v*) DSS ad libitum in their drinking water separated by two weeks of normal water between each cycle. Tumours developed in the distal colon between 7–10 weeks after the commencement of treatment. **(B)** Flow cytometric analysis of ILCs within lamina propria isolated from the colon. Live CD45.2⁺ lymphocytes were segregated into T cells (TCRβ⁺CD3⁺) and ILCs (TCRβ[−]CD3[−]). ILC subsets were further delimited into NK1.1⁺NKp46⁺ ILC1 (Eomes[−]) and NK cells (Eomes⁺); ILC2s (GATA-3⁺) and ILC3 (RORγt⁺). **(C)** Enumeration of ILC subsets isolated from the colon, mesenteric lymph node (LN) and spleen of untreated and AOM/DSS-treated mice 7–10 weeks after initial treatment. Each dot represents one mouse. Data show mean ± s.e.m. of results pooled from four independent experiments (*n* = 2–3 mice/treatment/experiment). Statistics were calculated using an unpaired Student's *t*-test, *p*-value as indicated. n.s.: not significant.

2.3. The ILC2-Derived Cytokines, IL-5 and IL-13, Are Elevated in CAC

We next sought to determine if there were changes in the expression level of prototypic ILC cytokines in CAC. Following short-term restimulation of colonic ILC subsets isolated from tumour-bearing CAC mice with phorbol myristate acetate and ionomycin we observed significant increases in TNF-α (ILC1), IL-5 and IL-13 (ILC2) and IL-17 (ILC3) production in tumour-bearing mice (Figure 2A). Among these cytokines, we found that IL-5 expression by colon-derived ILC2s was most highly upregulated in tumour-bearing mice, whereas expression was not altered in the MLN or spleen compared to untreated mice (Figure S4). Interestingly, the frequency of IL-5- and IL-13-expressing ILC2s and IL-17A-producing ILC3s were positively correlated with tumour burden (Figure 2B), suggesting that CAC development may be augmented by the local accumulation of these cytokines in the colon.

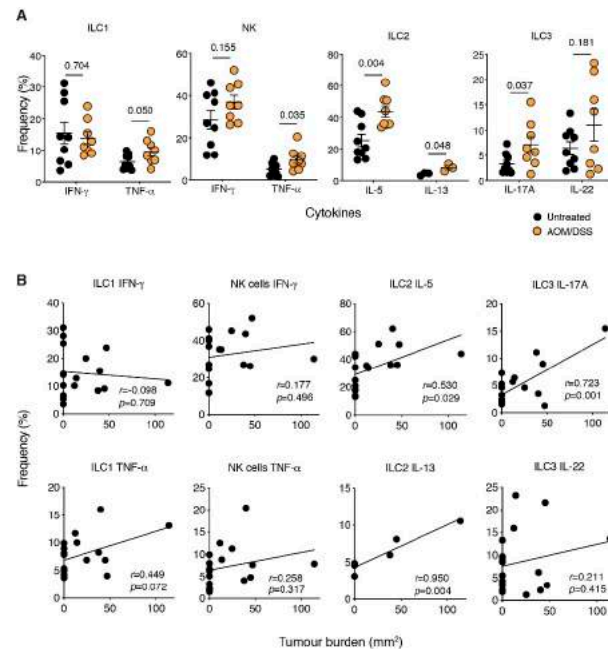


Figure 2. Increased cytokine expression during CAC. (A,B) Intracellular flow-cytometric analyses of TNF- α , IFN- γ , IL-5, IL-13, IL-17A and IL-22 cytokine production by colonic ILC1s, NK cells, ILC2s and ILC3s after short-term restimulation with PMA, ionomycin in the presence of Golgi Stop and Golgi Plug. Cells were stimulated for 4 h before staining. Frequency and correlation with tumour burden of cytokine-producing ILC isolated from the lamina propria of untreated and AOM DSS-treated mice. Each dot represents one mouse. Data show the mean $\pm$ s.e.m. IL-13 data is derived from one experiment and all other data are pooled from four independent experiments ($n = 2$ –3 mice/genotype/experiment). Statistical differences were analysed using the Mann–Whitney nonparametric test. The correlation between frequencies and tumour burden was determined using Pearson’s correlation coefficient to calculate r . Both the r coefficient and p -values are indicated.

2.4. Loss of ILC2 Results in Increased Tumour Development in CAC

To determine whether ILC2s have a role in CAC, we crossed the *Rora*^{fl/fl} mouse strain with the *IL-7R*^{Cre/+} strain to inactivate the *Rora* gene by Cre recombinase in IL-7R expressing cells [35] (Figure 3A). *Rora* is necessary for ILC2s development [35] and is highly expressed in mature ILC2s across all organs, including GI ILC2s [50]. Age- and gender-matched *Rora*^{fl/fl} *IL7r*^{CreT/+} and littermate control *Rora*^{+/+} *IL7r*^{CreT/+} mice underwent the CAC protocol, and tumour development was monitored by endoscopy (Figure 3A,B). While both genotypes developed macroscopic tumours at the mid and distal regions of the colon (Figure 3C), the *Rora*^{fl/fl} *IL7r*^{CreT/+} mice had more and significantly larger tumours (Figure 3B–D). Collectively, these results indicate that the *Rora* expressing IL-7R-positive immune cells limit CRC progression, as their loss augments tumour growth.

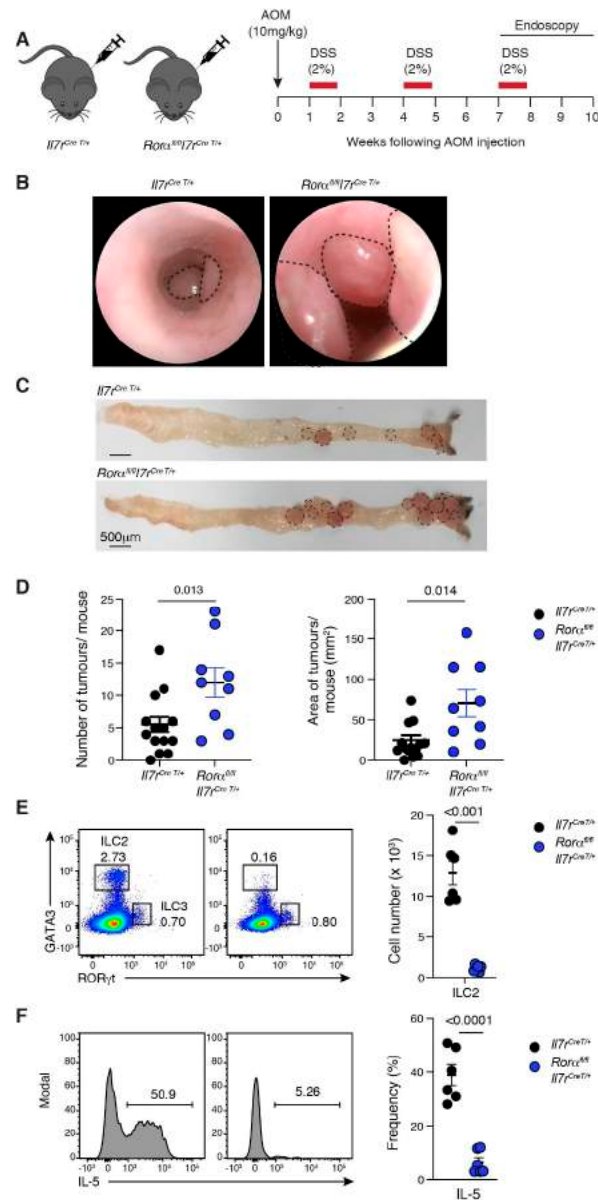


Figure 3. Loss of ILC2 augments CAC. (A) Schematic illustration of the AOM/DSS treatment protocol. *Il7r^{Cre} T/+* and *Rorα^{fl/fl} Il7r^{Cre} T/+* mice injected with AOM (10 mg/kg, i.p.) followed by three five-day cycles of 2% (w/v) DSS ad libitum in their drinking water separated by two weeks of normal water between each cycle. Colonic tumours developed between 7–10 weeks after the commencement of treatment. (B) Endoscopic images of AOM/DSS-treated *Il7r^{Cre} T/+* and *Rorα^{fl/fl} Il7r^{Cre} T/+* mice (right panel). Images are representative of two independent experiments (*n* = 9–15 mice/genotype/experiment).

Dotted lines indicate colonic tumours. (C) Representative whole mount images of colons from $Il7r^{CreT/+}$ and $Rora^{fl/fl}Il7r^{CreT/+}$ mice treated with AOM+DSS. Images are representative of two to five mice/genotype analysed in two separate experiments. Dotted lines indicate tumour size and location. Scale bar: 500 μ m. (D) Number (left panel) and area of tumours (mm^2 , right panel) per mouse in $Il7r^{CreT/+}$ and $Rora^{fl/fl}Il7r^{CreT/+}$ mice treated with AOM+DSS. Each dot represents one mouse—closed circle for female. Data show the mean $\pm$ s.e.m ($n = 2\text{--}5$ mice/genotype/experiment) pooled from two independent experiments. Statistical differences were analysed using the Mann–Whitney nonparametric test. p -values are indicated. (E) Representative flow cytometric colour plots (left panels) and enumeration (right panels) of ILC2 subsets isolated from the colon of AOM+DSS-treated $Il7r^{CreT/+}$ and $Rora^{fl/fl}Il7r^{CreT/+}$ mice. Each dot represents one mouse. Data show the mean $\pm$ s.e.m. pooled from two independent experiments ($n = 3$ mice/genotype/experiment). Statistical differences were calculated using an unpaired Student's t -test. p -values are indicated. (F) Representative histograms (left panels) and frequency (right panel) of cytokine-producing ILC2 isolated from the colon of AOM+DSS-treated $Il7r^{CreT/+}$ and $Rora^{fl/fl}Il7r^{CreT/+}$ mice. Each dot represents one mouse. Data show the mean $\pm$ s.e.m. pooled from two independent experiments ($n = 3$ mice/genotype/experiment). Statistical differences were calculated using an unpaired Student's t -test, p -values are indicated.

In order to confirm that $ROR\alpha$ deletion results in ablation of ILC2s, we analysed the number and function of T cells and ILCs within the colons of untreated (Figure S5) $Rora^{fl/fl}Il7r^{CreT/+}$ and $Rora^{+/+}Il7r^{CreT/+}$ mice, compared to tumour-bearing mice (Figure S6 and Figure 3E,F). We observed no changes in the cell numbers of NK cells, ILC3, $CD4^+$ and $CD8^+$ T cells or their capacity to produce subset-specific cytokines at either a steady-state or during CAC development (Figures S5 and S6). We did, however, find a significant loss of ILC2s, consistent with earlier reports [36,51] (Figure 3E). $ROR\alpha$ -deficient mice also showed a reduction in ILC1 numbers within the colon. However, ILC1-deficient $Mcl1^{fl/fl}NCR^{CreT/+}$ mice treated with AOM+DSS developed a comparable tumour burden to C57BL6 mice (Figure S7), indicating no protective role in CAC. Collectively, our data demonstrate that ILC2 play a crucial role in preventing CAC tumour progression. Of the few remaining ILC2s in $ROR\alpha$ -deficient mice, there was also a reduction in IL-5-producing cells within the colon (Figure 3E,F and Figure S5), suggesting that ILC2-derived cytokines may suppress CRC progression.

2.5. An ILC2 Gene Signature Is Associated with Increased Overall Survival in CRC

In order to ascertain the potential relevance of ILC2 and their associated cytokines for the clinic, we generated an ILC2 gene signature using machine learning (Table S3). This gene signature notably includes GATA3 and KLRG1, two genes highly expressed by ILC2 (Table S3). We applied this signature to human colorectal adenocarcinoma RNAseq datasets available within the TCGA database [52] and found that patients with an elevated ILC2 gene signature, indicative of high ILC2 infiltration, had increased overall survival compared with patients with low ILC2 infiltration (Figure 4A). Using multivariate analyses, we found that the favourable prognosis conferred by a high tumour ILC2 gene signature was independent of other covariates including age at diagnosis, sex, the presence of metastatic lymph nodes or the stage of the disease already shown to influence CRC prognosis [6,53,54]. While the age of CRC patients (hazard ratio: 1.65, range 1.328–2.1) and the presence of tumour-invaded lymph nodes (hazard ratio: 2.51, range 1.687–3.7) both significantly negatively impacted cancer prognosis, the presence of ILC2 in tumours (hazard ratio: 0.72, range 0.447–1.1) was independently associated with a better outcome (Figure 4B). Our results suggest that a high ILC2 gene signature may be a useful clinical tool to inform patient treatment strategies in the future.

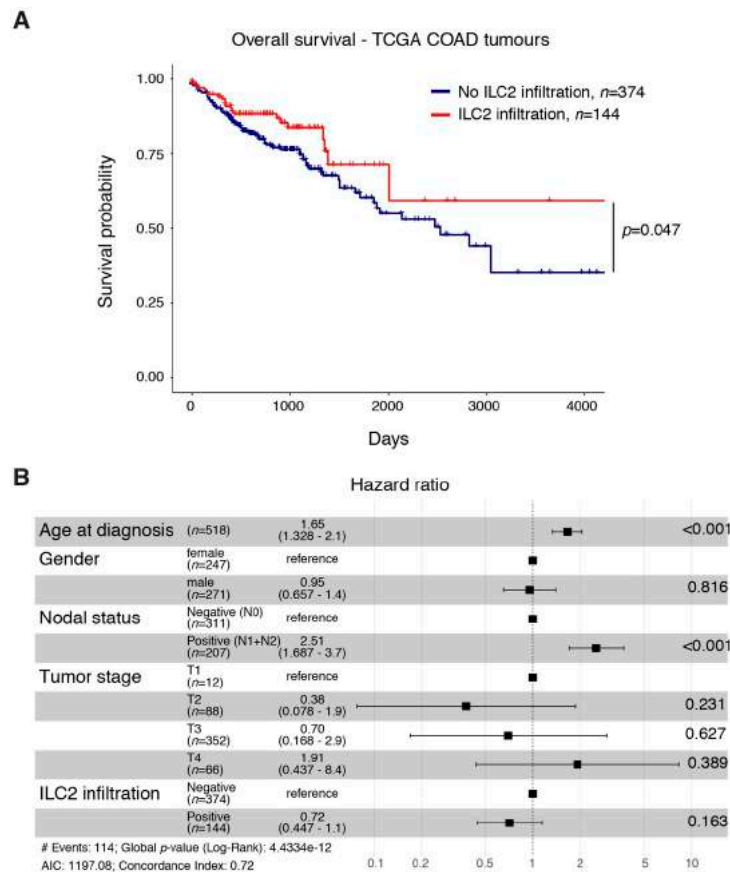


Figure 4. An ILC2 gene signature identifier is associated with improved overall survival for CRC patients. (A) Analysis of the impact of tumour ILC2 infiltration on colorectal cancer patient survival using the publicly available TCGA database. (B) Multivariate analyses showing hazard ratio of the impact of the age at diagnosis, gender, the presence of metastatic lymph nodes, tumour stage and ILC2 infiltration probability on CRC patient survival.

3. Discussion

ILCs were originally discovered over 40 years ago, however, the diversity of the ILC family members has only recently been recognised [15], and the unique features they contribute to the immune response and disease outcomes are only beginning to be unravelled. ILCs respond rapidly during the initial GI immune response to a breach in epithelial barrier integrity and communicate to the adaptive arm of the immune response [55]. However, the role of ILC family members in CRC appears to be divergent, with NK cells required for anti-tumour immunity [12,24], while ILC1 and ILC3 are commonly linked to the promotion of CRC [9,21,29]. Here we show that ILC2s are the predominant ILC member in the inflamed colon of tumour-bearing mice and demonstrate that ILC2s have a role in the restraint of tumour progression.

IL-33 and IL-25 are two well-known ILC2 stimulatory cytokines that are released by damaged epithelial cells. These cytokines induce tissue repair, in part, by activating

ILC2s, which have been reported to be important to maintain intestinal tissue integrity [56]. In our study, we found that ILC2s infiltrate CRC tumours and produced higher levels of cytokines than was found in ILC2 from naïve mice and this potentially drove anti-tumour responses. These observations suggest that these tumour-infiltrating ILC2s are likely activated by locally produced ligands such as IL-33 and IL-25 and play a protective role in CRC progression. However, these ligands also have the capacity to promote epithelial cell growth and regulatory T cell activation that can lead to increased tumour development and progression [57,58]. Thus, during the development of tumour cells in the intestinal mucosa, competition for cytokine availability between different cell types can occur and may drive divergent CRC outcomes. This may explain some of the conflicting results previously described [57,59].

The downstream mechanisms of ILC2-mediated protection during CRC are not yet known. Single-cell RNAseq of ILCs during the early and late stages of CRC development in mice identified changes in ILC phenotype and function as tumours developed [3]. In particular, the PD-1[−] ILC2 subset present during the early stages of CRC was replaced by a predominantly PD-1⁺ ILC2 subset that was capable of promoting tumour development [3]. These observations suggest that the role of ILC2s may change as tumours progress. Recently, it has been shown that the ILC2-derived cytokines, IL-5 and GM-CSF, both have the capacity to control tumour development as the genetic ablation of either IL-5 or GM-CSF increased the tumour burden in the MC38 murine CRC model, while overproduction of IL-5 facilitated protection against tumour development [45]. The protection against the development of CRC was thought to be mediated by eosinophils that depend on IL-5 and GM-CSF to drive migration to the tumour microenvironment, which in turn, promotes Th1 cell responses against tumours [45]. Our findings identify ILC2s as the main IL-5 producer in the colon tumour microenvironment, suggesting that ILC2s may be a key player in mediating tumour protection through the activation of eosinophils, although this remains to be established. We also found increased IL-13 expression by colonic ILC2s isolated from CRC-bearing mice. IL-13-expressing ILC2s are often associated with increased MDSC differentiation and pro-tumorigenic functions in bladder cancer [41], prostate cancer and acute promyelocytic leukemia [40]. RORα-deficient mice would also have reduced IL-13 production due to the absence of ILC2s but it is not known whether this has also contributed to the increase in tumour burden.

Catalogues of the immune cell infiltrate in different genetic and molecular subtypes of CRC are emerging. This information will contribute to the evolution of immunotherapy to treat CRC, beyond the current opportunity for microsatellite instability high/deficient mismatch repair (dMMR-MSI-h) patients [60] and may alter the approach to standard chemotherapy for other patient sub-populations. There is the potential to identify patients who will benefit from targeted boosting of certain immune populations. Thus, an increased definition of the tumour immune infiltrate and an understanding of its prognostic value is urgently needed. As a diagnostic criterion, the tumour immune response and its composition is still largely disregarded in CRC. This is in sharp contrast to the recent findings reported by the Immunoscore® team whose findings strongly advocate for the quantification of CD3⁺ and CD8⁺ T cell infiltration in colorectal cancer [5,6] as prognostic markers to better define the risk of recurrence and survival, and to better guide clinicians in decisions surrounding patient treatment [61,62]. This worldwide taskforce demonstrated that the quality of the immune response in CRC clearly matters and could serve as a strong prognostic indicator for stratifying patients. These analyses pave the way for understanding the contribution of different immune cell subsets such as ILC2s in CRC prognosis and treatment. In the interim, gene signatures that can predict patient outcome are becoming increasingly important in clinical decision making. Since the frequency of ILCs within the tumour microenvironment is low compared with adaptive immune cells, we generated an ILC2 expression signature, which revealed that ILC2s confer a positive clinical outcome, which aligned with our findings in vivo using murine models. Thus, well-modelled machine learning approaches offer resolution beyond the frequency of cell

type infiltration to uncover genetic networks and cell types that may have applications in identifying novel links between the infiltration of rare immune cells and patient prognosis.

Collectively, our study has revealed ILC2s as an immune cell subset, associated with favourable CRC outcomes. Further investigation will be important to uncover the key stimuli together with the cellular and molecular mechanisms that drive ILC2 anti-tumour function in CRC. Further studies are now warranted to confirm these findings aiming at determining new ILC2-related targets that might be used in combination with current therapeutic regimens.

4. Materials and Methods

4.1. Mice

Rag1^{−/−} and *Rag2*^{−/−}*γc*^{−/−} [63], *Rorα*^{fl/fl} [35] and *Il7r*^{Cre} [64] mice were maintained on a C57BL/6 (Ly5.2) background (originally derived from the Jackson Laboratory) and have been described previously. *Rorα*^{fl/fl} mice were crossed to the *Il7r*^{Cre} strain to generate the *Rorα*^{fl/fl}*Il7r*^{Cre} line that lacks *Rorα* expression in all lymphocytes [36]. C57BL/6 mice were bred and maintained in specific pathogen free conditions at the Walter and Eliza Hall Institute of Medical Research. Both females and males were used at 6–12 weeks old. Animals were used according to the guidelines of the Australian code for the care and use of animals of the National Health and Medical Research Council of Australia. Experimental procedures were approved by the Animal Ethics Committee of the Walter and Eliza Hall Institute of Medical Research (AEC 2017.037 and 2018.008).

4.2. Cell Culture and Xenograft Establishment

The MC38 colorectal cancer cell line was maintained at 37 °C, 5% CO₂ in complete RPMI-1640 media containing 10% heat-inactivated foetal calf serum (FCS), 1 mM L-Glutamine, 50 mM β-mercaptoethanol, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells routinely tested negative for *Mycoplasma*. The right flank of mice was shaved and 5 × 10⁵ MC38 cells resuspended in sterile PBS was injected subcutaneously. Tumour size was measured routinely using callipers every two to three days until ethical endpoint. Tumour area was calculated as the length x width of the tumour.

4.3. Isolation of Lymphoid Cells

Intestinal tissues: Single-cell suspensions of lymphocytes were isolated from the stomach, small intestine, caecum and colon following incubation for 40 min at 37 °C in Ca²⁺- and Mg²⁺-free Hanks media containing 2% FCS and 5 mM EDTA with gentle shaking to remove intestinal epithelial cells [27,65]. The supernatant was discarded and tissues were then incubated with gentle shaking in 1 mg/mL (*w/v*) collagenase type IV (Worthington, Lakewood, NJ, USA), 200 µg/mL DNase I (Roche, Basel, Switzerland) and 4 U/mL Dispase (Sigma, Darmstadt, Germany) in RPMI-1640 + 2% (*v/v*) heat-inactivated foetal calf serum (FCS) for 45 min at 37 °C. Preparations were filtered and mononuclear cells were isolated by centrifugation on a 40–80% Percoll gradient. Lymphocytes were recovered from the interface and washed twice.

Spleen and lymph nodes: Single-cell suspensions were generated from lymph nodes and spleen by gently dissociating tissues using 70 µm filters. Red blood cells were lysed from the spleen using Red Cell Removal Buffer and washed with PBS prior to antibody staining.

Tumours: Tumours were weighed, minced and incubated with gentle shaking in 1 mg/mL (*w/v*) collagenase type IV (Worthington, Lakewood, NJ, USA) and 0.05 mg/mL DNase I (Roche, Basel, Switzerland) in RPMI-1640 for 45 min at 37 °C. Preparations were filtered and washed in PBS before resuspension in FACS Buffer for flow-cytometry staining.

4.4. Flow Cytometry

Single-cell suspensions were stained with the following antibodies: TCRβ (H57-597), NKp46 (29A1.4), NK1.1 (PK136) and fixable viability dye from eBioscience (San Diego, CA,

USA); and CD4 (GK1.5), CD8 (53-6.7), CD19 (ID3), CD3 ϵ (145-2C11), CD45 (30-F11), CD45.2 (104), CD90.2 (30-H12) and CD49a (Ha31/8) from BD Biosciences (Franklin Lakes, NJ, USA). Fixation and intracellular staining were performed using the Transcription Factor Staining Buffer Set (eBioscience) and antibodies against GATA-3 (TWAJ, eBioscience), ROR γ t (Q31-378, BD Biosciences) and EOMES (Dan11mag, eBioscience). Cytokine expression was determined by restimulation of cells isolated by digestion from the colon tissue in the presence of 100 ng/mL phorbol-12-myristate-13-acetate (PMA), 100 ng/mL ionomycin and 10 μ g/mL GolgiPlugTM and GolgiStopTM (BD Biosciences) in complete RPMI-1640 media (containing 10% heat-inactivated FCS, 1 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 50 μ M β -mercaptoethanol) for 4 h. Cells were then fixed and stained for intracellular cytokines IFN- γ (XMG1.2, BD Pharmingen), TNF- α (Mab11, BD Pharmingen), IL-5 (TRFK5, eBioscience), IL-13 (eBio13A, eBioscience), IL-17A (TC11-18H10.1, BioLegend) and IL-22 (IL22JOP, eBioscience). Cells were analysed using a Fortessa X20 (BD Biosciences) and FlowJo software (Ashland, OR, USA) was used for the analysis.

4.5. Colitis-Associated Colon Cancer

Azoxymethane (AOM) was injected intraperitoneally (10 mg/kg, Sigma, Darmstadt, Germany) and one week later dextran sodium sulphate (DSS) (molecular mass 36,000–50,000 Da; MP Biomedicals, Santa Ana, CA, USA) was added into drinking water 2% (*w/v*) *ad libitum* for five days followed by two weeks of regular water. This was followed by two additional rounds of treatment with DSS, for a total of three cycles. Tumours at the distal colon were monitored via endomicroscopy using established methods [66] after the second and third round of DSS treatment. Tumour burden, indicated by the total number of tumours and the total area of tumours, was determined on autopsy.

4.6. Histology

Tissues were fixed in 10% neutral buffered formalin and processed for wax embedding and routine Hematoxylin and Eosin (H&E) staining. Slides of tissues were scanned in an Aperio and analysed with the ImageScope v11.2.0.780 software (Leica Microsystems Pty Ltd., Wetzlar, Germany).

4.7. Training an Intestinal ILC2 Classifier

A classifier for gut ILC2 phenotype was developed using RNAseq data from wild-type mouse ILCs extracted from small intestine lamina propria [67], available on Gene Expression Omnibus (GEO) through accession number GSE85154. Raw counts were downloaded from the corresponding GSE accession, logCPM transformed and TMM normalised [68] using edgeR [69]. A set of 241 genes were selected from a panel of 700 immune genes overlapping the top 1000 highly variable genes in single-cell transcriptome profiles of ILCs extracted from tonsils (GSE70580) [70]. An extreme gradient boosting classifier [71] was trained to 70% of the data using linear boosting (booster = “gbliner”) with a binary logistic loss function (objective = “binary:logistic”), three rounds of iteration (nrounds = 3) and a moderate L1 penalty of 0.05 (alpha = 0.05). The classifier was tested on the remaining 30% of the dataset.

4.8. Quantification of Infiltrating ILC2 in the TCGA COAD Cohort Using the Intestinal ILC2 Classifier

Raw RNA sequencing data for TCGA COAD tumours and clinical annotation were downloaded using TCGAAbiolinks Bioconductor package [72]. RNAseq counts were logCPM transformed and TMM normalised as described earlier. The classifier was applied to logCPM TCGA COAD data ($n = 521$) to compute a confidence score for the presence of infiltrating ILC2 for each patient. Since the test data, the TCGA COAD cohort, is inherently different and more heterogeneous than the training data, the confidence scores are likely to be over-estimated. We, therefore, computed the probability that model predictions can be wrong by measuring similarities between TCGA RNAseq profiles and class centroids in the training data. For each sample in TCGA, we computed the Euclidean distance between

the gene expression profile of the sample and class centroids in the training data, based on the 241 genes included in the model. We then computed the similarity of patient profiles to the training data by applying a radial basis function (RBF) kernel to Euclidean distances. We considered any sample in TCGA with prediction confidence greater than 20% and more than 90% similarity to the gene expression distribution in the training data as confident ILC2 infiltrating samples, resulting in 144 identifications (i.e., 144 patients with gut ILC2 infiltration). A Kaplan–Meier plot and survival analysis were done using survival [73] and survminer [74] R packages based on 518 TCGA COAD patient samples with valid survival data.

4.9. Statistical Analysis

All statistical analyses were performed with GraphPad Prism software (Version 7.0 or 8.0, GraphPad Software, San Diego, CA, USA). An unpaired two-tailed Student's *t*-test was used for pairwise comparisons. The Mann–Whitney test was used to determine the statistical significance of tumour number and burden between either the AOM+DSS treated and untreated group or between mice with different genotypes. Data are shown as the mean $\pm$ standard error of the mean (s.e.m). The correlation between cell counts and tumour burden was determined using Pearson's correlation coefficient to calculate *r*. Tumour growth experiments were analysed with TumGrowth software (<https://kroemerlab.shinyapps.io/TumGrowth/>) [75] with default settings at the exception of the original tumour measurements that were log-transformed before linear mixed-effect modelling. Cross-sectional analyses were analysed using ANOVA and Holms adjusted *p*-values are indicated. Cox proportional hazards modelling with Holms adjustments were applied when assessing the impact of the genotype on mouse survival. *p*-values ≤ 0.05 were considered statistically significant. All experiments were performed at least two times with similar results obtained for each time.

5. Conclusions

Colorectal cancer is associated with an increase in cytokine-producing ILC2s in the colon and these cells are protective against the development of colorectal cancer. Furthermore, an ILC2 gene signature in patient samples is associated with increased survival. This is a novel finding, highlighting the protective role of ILC2s in CRC and is a promising new diagnostic tool to help identify patients with a higher probability of survival.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2072-6694/13/3/559/s1>, Figure S1: Enumeration of ILC subsets in MC38 tumour-bearing mice, Figure S2: Incidence of CRC tumours in AOM DSS-treated mice, Figure S3: ILC composition within the intestinal tract, mesenteric lymph nodes and spleen of untreated C57BL/6 mice, Figure S4: The ILC cytokine production in spleen and mesenteric lymph nodes is not influenced during CAC progression, Figure S5: Deletion of ROR α results in loss of ILC1 and ILC2 but not T cells or other ILC subsets at steady-state, Figure S6: Deletion of ROR α results in loss of ILC1 but not cytokine production or other lymphoid subsets during CAC, Figure S7: Loss of ILC1 and NK cells does not impact CAC development, Table S1: Longitudinal, cross-sectional and survival analyses from MC38 tumour-bearing C67BL6, Rag1 $^{-/-}$ and Rag2 $^{-/-}$ γ c $^{-/-}$ for TumGrowth analysis, Table S2: ANOVA analysis of ILC subsets in gastrointestinal organs at steady-state, Table S3: ILC2 gene signature.

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Innate Lymphoid Cells in Colorectal Cancers: A Double-Edged Sword

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The immune system plays a fundamental role at mucosal barriers in maintaining tissue homeostasis. This is particularly true for the gut where cells are flooded with microbial-derived signals and antigens, which constantly challenge the integrity of the intestinal barrier. Multiple immune cell populations equipped with both pro- and anti-inflammatory functions reside in the gut tissue and these cells tightly regulate intestinal health and functions. Dysregulation of this finely tuned system can progressively lead to autoimmune disease and inflammation-driven carcinogenesis. Over the last decade, the contribution of the adaptive immune system in controlling colorectal cancer has been studied in detail, but the role of the innate system, particularly innate lymphoid cells (ILCs), have been largely overlooked. By sensing their microenvironment, ILCs are essential in supporting gut epithelium repair and controlling bacterial- and helminth-mediated intestinal infections, highlighting their important role in maintaining tissue integrity. Accumulating evidence also suggests that they may play an important role in carcinogenesis including intestinal cancers. In this review, we will explore the current knowledge about the pro- and anti-tumor functions of ILCs in colorectal cancer.

Keywords: tumor immunology, adaptive immunity, innate immunity, tumor immunosurveillance, immunotherapy

INTRODUCTION

Colorectal Cancer—Epidemiology, Pathophysiology, and Risk Factors

Colorectal cancer (CRC) is the third most common cancer worldwide with more than 1.8 million patients diagnosed each year (1). Despite intensified screening programs, most patients are diagnosed at the later stages of disease (2). This results in CRC emerging as the second leading cause of death with more than 900,000 deaths every year (1). CRC is a multifactorial and heterogeneous disease (3) with most cases being sporadic and fewer than 5% of patients with CRC have a positive familial history with inherited germline mutations that increase the risk of tumor development (3, 4).

Tumors are widely distributed from the proximal region of the colon to the rectum and each location is associated with different tumor-intrinsic characteristics (5). Most of these tumors arise from benign lesions called polyps, which progressively acquire genetic and epigenetic modifications to form malignant tumors and metastases (3, 4, 6, 7). These include deletions, amplifications, mutations or methylation of the promoter of certain genes such as *APC*, *SMAD4*, *TP53*, *BRAF*, *MYC*, *KRAS*, *PTEN*, or *MLH1* (3, 4, 8). The risk of developing CRC increases with age (4, 5)

and is higher in men than in women (9). Other environmental factors such as exposure to smoking, alcoholic beverages, the presence of visceral fat and poor dietary patterns are all features associated with a higher risk of developing CRC (3, 5). In addition, inflammatory bowel disease (IBD) and chronic colitis have also been associated with an increased risk of CRC development (10, 11). However, improved anti-inflammatory treatments and increased surveillance have been effective in reducing CRC incidence for these patients (12).

Treatment

The most effective first line treatment for patients diagnosed with localized CRC is surgery. In certain cases, neoadjuvant chemotherapy allows early reduction of the tumor burden to increase the chances of complete tumor resection (3, 4). For patients with metastatic CRC, there are increased treatment options available which include chemotherapies and targeted therapies.

Chemotherapy

Tumor recurrence occurs in 15–50% of patients who have undergone complete resection of loco-regional tumor lesions (4). To reduce this risk, patients often receive adjuvant chemotherapy treatment as a first line approach increasing both patient progression free and overall survival (13, 14). More recently, chemotherapy has been combined with targeted therapy to further improve patient overall response rates.

Targeted Therapies

Multiple treatment approaches have been developed in an endeavor to provide curative outcomes for patients. These include targeting oncogenic drivers (RAS, BRAF), receptors of growth factors (EGFR) or pathways involved in angiogenesis such as VEGFR (15). As an example, Bevacizumab, a human IgG1 antibody directed against VEGF-A, increased patient overall and progression-free survival when combined with chemotherapies (14, 16, 17).

Recent breakthroughs in tumor immunology have revolved around the clinical efficacy of therapeutic antibodies that are designed to block critical checkpoint molecules expressed at the membrane of circulating and tumor-infiltrating immune cells (18). Programmed death-1 (PD-1) blocking antibodies have been shown to induce remarkably durable clinical responses in many different cancer types, including CRC (19, 20). These anti-PD-1 sensitive CRC tumors harbor defective mismatch DNA repair mechanism (called microsatellite instability high or MSI high tumors) and is associated with enhanced mutational load, neoantigen formation, T cell infiltration of the tumor, and immune checkpoint expression (4, 8, 20, 21). However, only half of these CRC patients experience durable clinical responses (22) and many others develop resistance during treatment. To understand the underlying mechanisms, Grasso and colleagues performed large scale genomic analyses of more than 1,200 CRC tumors (23). This revealed that MSI high tumors have a higher rate of mutated genes in critical immune-modulating pathways such as antigen presentation that then drive resistance to treatment. In addition, upregulation of the WNT pathway in

both MSI high and microsatellite stable CRC leads to a “cold” tumor microenvironment that is poorly infiltrated by anti-tumor T cells (23). Combined infusion of anti-CTLA-4 and anti-PD-1 blocking antibodies increased patient survival in metastatic CRC compared to anti-PD-1 treatment alone. However, it also induced higher levels of toxicity (24).

Currently, the use of immunotherapy and its integration into strategies for CRC care are growing (25). However, the quality of the immune response has not yet been fully factored into treatment decisions despite significantly influencing CRC patient outcomes. A better understanding of the tumor immune infiltrate would greatly inform treatment decisions and allow more strategic design of future combination therapies.

THE TUMOR IMMUNE CONTEXTURE IN COLORECTAL CANCER—THE SUCCESS OF THE ADAPTIVE IMMUNE SYSTEM

Most colorectal tumors develop from glandular epithelial cells of the colon or rectum (26) and evolve through close interaction with their microenvironment and diverse immune cell types. A critical balance between pro- and anti-tumor immune responses determines either the eradication of nascent lesions or the development and progression of transformed cells that form malignant tumors (27). It has been demonstrated that in patients where initial failure to eradicate emerging tumors occurs, immune cells still play a critical role in dictating the patient response to treatment and outcome (28–32). To account for the quality of the immune infiltrate, a scoring system called Immunoscore™ (33) has been developed by tracking adaptive immune cells such as CD3+CD8+ and memory (CD45RO+) T cells (Table 1). This allows the immune infiltrate and its location within the tumor sample to be integrated to stratify patients and accurately predict dissemination to distant metastasis (34), disease-free and overall survival (30, 31, 35) (Tables 1, 2). However, the impact of innate cells has not been considered. In this review, we will summarize the latest evidence implicating a role for innate lymphoid cells in CRC development and their impact on disease outcome.

TABLE 1 | Parameters examined in Immunoscore™.

Cell type	Cytotoxic lymphocytes (CD3+CD8+) Memory T cells (CD45RO+)
Location	Tumor center Invasive margin
Density	0—low density of both cell populations in both regions 4—high density of both cell populations in both regions
Prognosis-Cox analysis	Disease free survival Overall survival Disease specific survival

TABLE 2 | Immunoscore™ and its association with the risk of relapse.

Immunoscore	Risk of relapse
0—low density of both cell populations in both regions	High
1—one cell population in one region	
2—one or both cell population(s) in one or both region(s)	
3—one or both cell population(s) in one or both region(s)	Low
4—high density of both cell populations in both regions	

ROLE OF THE INNATE LYMPHOID CELLS IN COLORECTAL CANCER

Innate lymphoid cells (ILCs) are the innate counterpart of the T lymphocytes, mirroring key aspects of their phenotype and function. ILCs are divided into five subsets, classified based on their development, transcription factor expression, cytokine production and functions. These are NK cells, ILC1, ILC2, ILC3, and Lymphoid Tissue inducers (LTi) (36). They are enriched in mucosal tissues, including the intestinal tract (37) and respond to signals derived from their micro-environment such as cytokines, alarmins, and other inflammatory and non-inflammatory stimuli (38, 39) to drive appropriate immune responses and maintain tissue homeostasis. In addition, ILCs express particular receptors and ligands at their surface regulating further their function (40, 41). NK cells and ILC1 are mainly involved in the early protection against viruses (42), bacteria (43, 44), and cancer (45) through the secretion of interferon (IFN)- γ and granulocyte-macrophage colony-stimulating factor (46). ILC2 are essential in host protection against helminth and parasites throughout interleukin (IL)-4, IL-5, and IL-13 production (47). ILC3 express mainly IL-17 and IL-22, both cytokines critical to mucosal immune responses and epithelium regeneration (48). Finally, LTi are critical for lymphoid organogenesis (49). Beyond this, significant plasticity occurs between ILC subsets depending on environmental stimuli they receive, thus directly impacting on their effector functions and immune responses (50–55). While ILCs are essential to lymphoid organogenesis and tissue homeostasis, they also participate to the development of autoimmune diseases (56, 57) or inflammation-driven carcinogenesis (58) (Figure 1).

Type 1 Innate Lymphoid Cells

High-dimensional cytometric analyses have revealed that human colorectal tumors are highly infiltrated by NK cells and intraepithelial-like ILC1 (59, 60). They secrete cytotoxic molecules such as granzymes and perforin (59, 60) and cytokines such as IFN- γ following stimulation (59), making them key potential players in cytotoxic anti-tumor responses (Figure 1A). In contrast, a separate study found impaired NK cell infiltration despite elevated levels of chemokines found in CRC tumors (61) (Table 3).

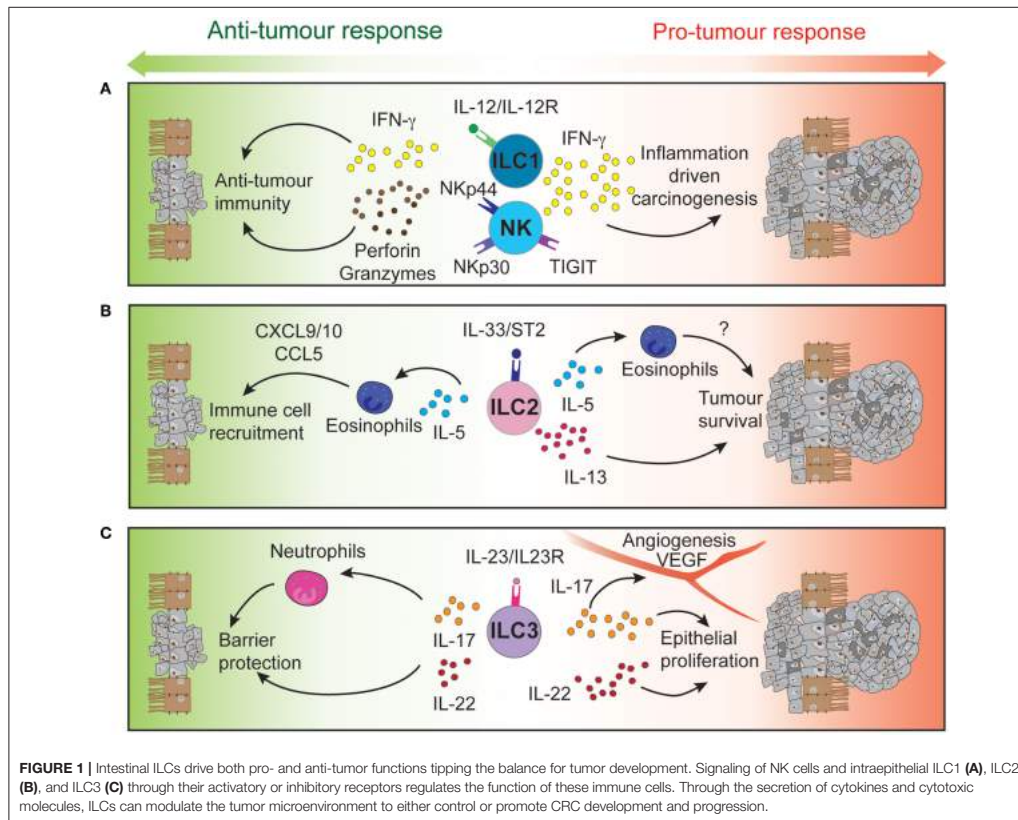
Studies tracking intraepithelial ILC1s have shown that they accumulate in the intestine of patients with Crohn's Disease and in CRC (51, 59, 62). These intraepithelial ILC1s can be activated by chemokines and cytokines such as IL-18 produced

by intestinal epithelial cells (63) or IL-12 from dendritic cells and monocytes (62). This induces IFN- γ production in ILC1 which, in contrast to previously mentioned studies (59, 60), drives the pathogenesis of anti-CD40-induced colitis in *Rag1*^{−/−} mice (62) (Table 3 and Figure 1A). Given these divergent outcomes, further studies are required to analyze the exact role of intraepithelial ILC1, their receptor expression and cytokine production using genetically-modified conditionally-deficient mice and human samples. How intraepithelial ILC1 influence patient outcomes and responses to therapy, is, to date, poorly understood.

NK cells are known to mediate anti-tumorigenic activity (45). In CRC, NK cells effectively eliminate CRC stem cells and cancer-initiating cells, which express lower levels of class I major histocompatibility complex (MHC) but high levels of ligands that bind the natural cytotoxicity activating receptors (NCR) NKP30 and NKP44. Consequently, these cells are “marked” for NK cell mediated lysis (64) providing a mechanism to potentially prevent tumor cell re-emergence or CRC metastasis. However, tumor cells can circumvent this process by differentiating and upregulating MHC class I expression to suppress NK cell activation or by reducing their expression of NCR ligands to escape NK cell-targeted killing (64). Thus, circulating NK cells in CRC patients are often phenotypically altered reflecting these induced changes. These patients exhibit reduced NKP44 expression on their CD56^{dim} cells (65), a key subset that normally exhibit potent effector function. In addition, high NKP30 expression on circulating CD56^{bright} cells has been associated with a shortened disease-free survival period in CRC patients (65). Other receptors and ligands expressed on NK cells of CRC patients such as activating and inhibitory KIRs (66) or TIGIT (67) have also been suggested to play distinct roles in the development, progression, and metastasis of CRC tumors (Table 3 and Figure 1A). In mice, neutralizing TIGIT markedly prolongs the survival of tumor bearing animals. However, when NK cells are depleted, this effect was completely abrogated highlighting the critical role of NK cells in this context. In addition, tumor infiltrating NK cells can transdifferentiate into ILC1-like cells in the presence of transforming growth factor- β (TGF- β) which is particularly abundant in the tumor microenvironment and associated with tumor progression (54). Does TGF- β accumulate in CRC tumors? If so, similar NK cell—ILC1 transdifferentiation could occur in CRC tumors and this would potentially with worsen prognosis. Collectively, it is clear that NK cells are involved in the control of CRC progression and metastasis but are also susceptible to tumor-directed dysregulation (41). To circumvent such events, current NK cell-based therapies are designed to boost NK cell functions in an effort to strategically further improve patient outcomes (68–71).

Type 2 Innate Lymphoid Cells

The role of ILC2 has been examined in a number of different cancers including lung and gastric cancer (72, 73). Nevertheless, their role in colorectal cancer is, to date, only poorly understood. In fact, they are found to a relative lower frequency compared to other ILC subsets that reside in the intestine. Nevertheless, ILC2 frequency is increased in mucosal samples from IBD patients



compared with healthy individuals (74). In CRC tumors, ILC2s express higher levels of ICOS and CD69 compared with ILC2 found in normal colon tissue (59, 60). These phenotypically distinct ILC2s could be a potential source of IL-4, IL-5, and IL-13 as both IL-4 and IL-13 are found at elevated levels in the serum of CRC bearing mice (75).

IL-13 can act directly on tumor epithelial cells in both an autocrine or paracrine manner to promote their survival and tumorigenesis (Figure 1B). In CRC, high expression of the IL-13R α 2 receptor is associated with more advanced disease stages, lymph node involvement, the presence of metastases and reduced survival (76). In contrast, Saigusa et al. found that low preoperative serum IL-13 was a predictive marker for poorer prognosis of colorectal cancer patients (77). These discrepancies indicate that IL-13 expression and its predictive value may be context dependent. The role of IL-5 has not been explicitly studied in the context of CRC. Nevertheless, it is important for eosinophil recruitment, expansion and activation (78, 79). In turn, these cells secrete chemo-attractants such as CXCL9,

CXCL10, and CCL5 which promote the recruitment of CD8⁺ T cells to the tumor site (80) (Table 3 and Figure 1B). The presence of peritumoral eosinophils is an independent prognostic factor associated with favorable progression free survival (81). In contrast, patients suffering from ulcerative colitis, have a higher risk of CRC development and were found to have elevated IL-5 transcript levels (82). Neutralizing IL-5 in mice reduced eosinophil numbers and diminished the inflammation associated with DSS-induced colitis (83).

The role of ILC2s, their activation pathways and subsequent downstream signaling in CRC are highly contentious. This is because the same biomarkers or effector molecules (e.g., IL-5 and IL-13) have been found to exhibit opposing roles in CRC outcome, and thus, differentially impact patient prognosis. A similar effect is seen for the cytokine IL-33, a molecule released by damaged epithelial cells and regulator of ILC2 activation. Several studies have found both the cytokine IL-33 and its receptor ST2, to be elevated in mouse and human CRC tissues (84–86), suggesting a potential role of this IL-33/ST2 pathway

TABLE 3 | Role of ILC in promoting or inhibiting CRC tumorigenesis.

Immune cell type	Prognosis		References
	Anti-tumor	Pro-tumor	
NK cells	- High cytotoxicity - Recognition of cancer initiating cells	- Low number of infiltrating NK cells - Reduced level of activating receptors (e.g., Nkp44, Nkp30) - Increased expression of inhibitory receptors (e.g., TIGIT) - Low cytotoxicity	(61, 64, 65, 67)
ILC1	Produce IFN-γ and cytotoxic molecules associated with anti-tumor immunity*	- Accumulate in inflamed tissue - IFN-γ promotes inflammation*	(58, 62)
ILC2	- High pre-operative serum IL-13 - IL-5 recruits eosinophils	- Local IL-13 promote tumor epithelial survival and proliferation. - Elevated IL-5 in ulcerative colitis, anti-IL-5 reduced eosinophils and colitis*	(76, 77, 81–83)
ILC3	- IL-17 recruits neutrophils to protect tissue barrier* - IL-22 promotes wound healing* and protects intestinal epithelial cells from genotoxic stress-induced DNA damage	- Overproduction of IL-17 promote inflammation and angiogenesis, and disrupt the intestinal epithelial barrier - Overproduction of IL-22 dysregulates epithelial proliferation	(96, 102, 103, 106, 108, 109, 112, 113)

*Indirect evidence of involvement in CRC development.

in tumor development and progression. Mechanistically, IL-33 was found in adenomatous polyposis coli (*APC*)^{min/+} mice to promote intestinal polyp formation by activating stromal cells and modulating angiogenesis (85, 87), and by promoting cell growth and proliferation of primary CRC cells (88). Serum soluble ST2 levels were inversely correlated with the tumor stage despite being elevated in CRC patients. Indeed, soluble ST2 was shown to act as a decoy receptor suppressing IL-33-driven angiogenesis (89) and inhibiting CRC growth, while loss of ST2 expression conferred protection in AOM/DSS-induced CRC (90). Conversely, reduced levels of serum IL-33 is observed in CRC patients compared with healthy individuals and decreased expression of ST2L, the transmembrane isoform of the ST2 receptor, in CRC tumors is associated with higher tumor grade and worse prognosis (91). These conflicting results indicate that the IL-33/ST2 axis may playing a dual role in CRC pathogenesis and is capable of both promoting or protecting against CRC tumor development and progression depending on the environmental context. IL-33 has multiple downstream targets as ST2 is not only expressed on ILC2 but is also found

on other intestinal cell types such as epithelial cells or regulatory CD4⁺ T cells (86). Therefore, the role of the IL-33/ST2/ILC2 pathway in CRC warrants further investigation using conditional mouse models, which would allow cell-specific responses to be dissected.

Type 3 Innate Lymphoid Cells

Inflammation is a key driver of cancer progression and the link between IBD and the development of CRC is well-established (92). This inflammation can be exacerbated by the loss of barrier protection occurring early during CRC development (93), allowing for the infiltration of microbes and their associated products into the tumor microenvironment. This activates dendritic cells and macrophages to produce IL-23 (94), a key cytokine regulating ILC3 function and plasticity (53, 95). Additional stimuli such as IL-12, IL-18, TGF- β , or Notch ligand can profoundly influence ILC3 plasticity (50–52). In the presence of IL-12 or TGF- β , IL-22-expressing ILC3 can acquire ILC1 features and transdifferentiate into IFN- γ producing cells (52). At the molecular level, the transcription factors T-BET, AIOLOS, and Signal Transducer and Activator of Transcription (STAT) 4 induce an ILC1 transcriptional program, repressing in parallel the ILC3 lineage genes (52, 53). Transitional ILC3/ILC1 cells were found in human intestinal epithelium (52) and accumulate in inflamed lesions of patients suffering from Crohn's disease (51) suggesting that such plasticity may also occur in inflamed CRC lesions.

IL-23 has been found to be overexpressed in both mouse and human CRC tumors (93, 96, 97) and IL-23 deficiency has been shown to protect against tumor development in a number of mouse models of cancer (97). Conversely, chronic IL-23 injection induces ILC3-dependent duodenal adenomas development in mice (98). Furthermore, local IL-23 expression drives ILC3 accumulation and activation within the colonic lamina propria (99, 100). These intestinal ILC3 express higher levels of the activating receptors ICOS and CD69 (59, 60), regulators of tumor responses CD244 and CD39 (59), and exhibit enhanced production of the downstream signaling cytokines IL-17 and IL-22 that contribute to the pathogenesis of CRC (96) (Table 3 and Figure 1C).

Several studies have reported high IL-17 expression in both mouse and human CRC compared with normal adjacent tissues (96, 101–103). ILC3s produce high levels of IL-17 but other immune and non-immune intestinal cell types also express this cytokine. These include Th17 cells, $\gamma\delta$ T cells, and Paneth cells (104). Importantly, IL-17 transcripts are increased during progression of tumors from the adenoma to carcinoma stages in CRC patients (105). Furthermore, IL-17 can alleviate cell cycle inhibition, thereby promoting epithelial proliferation and intestinal inflammation (106) and increase angiogenesis by inducing VEGF expression (103). Administration of anti-IL-17 antibodies have been shown to ameliorate inflammation severity (96) while genetic ablation of IL-17 was able to reduce tumor burden (93, 100, 106, 107). Although, these studies show that IL-17 is a pro-inflammatory cytokine involved in CRC pathogenesis, IL-17 may also play a protective role. Lin and colleagues (102) reported better patient overall survival associated with high

IL-17 levels (102) and neutralizing IL-17 exacerbated Crohn's disease and DSS-mediated colitis by increasing gut permeability (108, 109) (Table 1).

In a similar pattern to IL-17, the ILC3-driven IL-22 pathway plays both protective and pathogenic roles during colitis and tumorigenesis (Table 3). Under normal conditions, IL-22 is required for homeostatic epithelial cell proliferation through STAT3 activation (110, 111). Furthermore, IL-22 production by ILC3 and $\gamma\delta$ T cells protects the intestine from genotoxic stress-induced DNA damage through effective initiation of the DNA damage response program (112). Loss of IL-22 or IL-22 receptor on epithelial cells results in delayed tissue repair, exacerbated inflammation (113) and tumor development when intestinal cells are exposed to carcinogens (112). Conversely, sustained IL-22-dependent epithelial proliferation leads to tumor progression (Figure 1C) and neutralizing IL-22 reduced CRC development (96). In addition to this, loss of IL-22 binding protein, a soluble decoy receptor neutralizing IL-22-driven cell activation, has been shown to increase epithelial cell proliferation and was associated with a higher tumor burden (114).

These studies unanimously suggest that IL-23 signaling in ILC3 is central in driving CRC development. Indeed, the absence of ILC3 despite chronic IL-23 injections protects mice from tumor development (98). However, the downstream cytokine IL-17 and IL-22 signaling pathways seem to play a dual role in CRC. These findings suggest that initial activation of the IL-23/ILC3 pathway is sufficient to trigger tumor development but additional mechanisms influence CRC progression and outcomes. Collectively, further investigations are warranted to shed light on the role of these cytokines in CRC pathogenesis.

CONCLUDING REMARKS

Despite increasing evidence for the crucial role of the immune system in dictating patient's prognosis, the evaluation of the tumor immune cell infiltration in primary CRC is not yet fully integrated into assessments in routine clinical practice (115).

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It is, however, clear that adaptive immune cells, particularly CD8⁺ memory T cells, play an important role to limit tumor recurrence and prolong patient's survival (30). How adaptive immune cells are influenced by resident innate immune cell populations, the first line of immune defense, is still poorly understood (116). Direct and indirect evidence tend to suggest that ILCs could play a dual role in colorectal cancer. However, most of the aforementioned findings resulted from studying knockout mice or mice lacking adaptive immune cells. Thus, further studies are warranted to ascertain the role of ILCs in immunocompetent animals using genetically-modified conditionally-deficient mouse models. Collectively, given their function and strategic location, harnessing ILC responses would open up new possibilities through the development of combination therapies to further constrain CRC progression.

AUTHOR CONTRIBUTIONS

QH and NJ wrote the initial draft. WC designed the figure. All authors provided critical insight, edited, and approved the final version.

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The reviewer DW declared a past co-authorship with one of the authors GB to the handling editor.

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ARTICLE

TCF-1 limits the formation of Tc17 cells via repression of the MAF–ROR γ t axis

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Interleukin (IL)-17-producing CD8⁺ T (Tc17) cells have emerged as key players in host-microbiota interactions, infection, and cancer. The factors that drive their development, in contrast to interferon (IFN)- γ -producing effector CD8⁺ T cells, are not clear. Here we demonstrate that the transcription factor TCF-1 (*Tcf7*) regulates CD8⁺ T cell fate decisions in double-positive (DP) thymocytes through the sequential suppression of MAF and ROR γ t, in parallel with TCF-1-driven modulation of chromatin state. Ablation of TCF-1 resulted in enhanced Tc17 cell development and exposed a gene set signature to drive tissue repair and lipid metabolism, which was distinct from other CD8⁺ T cell subsets. IL-17-producing CD8⁺ T cells isolated from healthy humans were also distinct from CD8⁺IL-17[−] T cells and enriched in pathways driven by MAF and ROR γ t. Overall, our study reveals how TCF-1 exerts central control of T cell differentiation in the thymus by normally repressing Tc17 differentiation and promoting an effector fate outcome.

Introduction

CD8⁺ T cell differentiation traditionally results in the emergence of two subsets: effector cells that produce IFN- γ and memory cells that mediate immune protection against pathogen infections. More recently, it has emerged that other specialized CD8⁺ T cell populations develop in immune responses and are critical to orchestrate complete immune protection. These subsets include tissue-resident memory T cells, follicular cytotoxic T cells, and two CD8⁺ T cell subsets that produce IL-17, namely, mucosal-associated invariant T (MAIT) cells and IL-17-producing CD8⁺ T (Tc17) cells.

Tc17 cells are present in multiple disease settings such as gastrointestinal cancers (Zhuang et al., 2012; Wu et al., 2014) and graft versus host disease (Gartlan et al., 2015). They have been shown to protect against exposure to lethal fungal infection and to respond to microbial colonization in the skin (Naik et al., 2015; Linehan et al., 2018; Harrison et al., 2019) and influenza virus infection (Hamada et al., 2009). Despite these cardinal immunomodulatory roles, the transcriptional circuit that

controls Tc17 cell differentiation is not well understood. The acquisition of the ability of a CD8⁺ T cell to produce IL-17 appears to occur when the T-box factors, T-box transcription factor (T-BET) and EOMES (Intlekof et al., 2008), or T-BET and Blimp (Xin et al., 2016), are down-regulated. RAR-related orphan receptor γ (ROR γ t) also promotes IL-17 production in CD8⁺ T cells by binding the IL-17 conserved noncoding sequence-2 enhancer region (Ysebrant de Lendonck et al., 2013). However, forced expression of ROR γ t only results in a small increase in Tc17 cells (Ysebrant de Lendonck et al., 2013), suggesting that while ROR γ t is necessary, it is not sufficient for IL-17 expression, and other, as yet undefined factors must be required to cooperate with ROR γ t to drive specification of the Tc17 program.

T cell factor 1 (TCF-1) is a transcription factor previously shown to be critical for differentiation of early thymic progenitor cells (Germar et al., 2011; Weber et al., 2011). More recently it has been shown that TCF-1 also plays a role in thymic T cell development to establish CD4⁺ and CD8⁺ T cell fate (Steinke

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et al., 2014; Xing et al., 2016). TCF-1 is critical for CD8⁺ memory T cell responses during infection (Jeannet et al., 2010; Zhou et al., 2010; Xing et al., 2016) and more broadly directs the divergence of CD4⁺ T cell lineages into T helper type 2 cells (Yu et al., 2009) and follicular cytotoxic and T follicular helper subsets (Choi et al., 2015; Wu et al., 2015; Xu et al., 2015; Leong et al., 2016), but suppresses the differentiation of T helper type 17 (Th17) cells and promotes their stem cell-like properties (Ma et al., 2011; Yu et al., 2011; Zhang et al., 2018; Karmaus et al., 2019). In situations of chronic stimulation such as cancer or chronic infection, TCF-1 is critical for sustaining CD8⁺ T cell responses (Nanjappa et al., 2012, 2017; Im et al., 2016; Utzschneider et al., 2016; Wu et al., 2016; Kurtulus et al., 2019). Despite the significant impact of TCF-1 at various stages of T cell development, whether it plays a role in the generation of Tc17 cells has not yet been established.

In this study, we demonstrate that the loss of TCF-1 results in the emergence of conventional IL-17-producing CD8⁺ T cells, which we have defined as Tc17 cells. Ablation of TCF-1 during T cell development revealed that divergence to a Tc17 cell subset was constitutive when TCF-1-mediated repression was removed in double-positive (DP) thymocytes. We identify MAF BZIP transcription factor (MAF) and RORγt as critical for the formation of Tc17 cells and as direct targets of TCF-1. Loss of TCF-1 was associated with global changes in the chromatin architecture in CD8⁺ T cells, leading to increased accessibility of *Rorc* and Tc17 effector genes *IL-17a* and *IL-17f* in Tc17 cells. In contrast, we observed reduced accessibility of *Tbx21*, *Eomes*, and *Irf4* loci in Tc17 cells, genes normally required for the induction of effector and central memory CD8⁺ T cells. Finally, we demonstrate that IL-17-producing CD8⁺ T cells are present in healthy humans and express MAF and RORγt. Collectively, our study reveals the regulatory role of TCF-1 as instrumental in suppressing the MAF-RORγt axis in developing T cells to limit Tc17 formation.

Results

TCF-1 restricts Tc17 cell development

Regulation of RORγt expression is dramatically down-regulated as DP thymocytes transition to single-positive (SP) CD4⁺ or CD8⁺ T cells (Fig. 1 A, upper panel; and Fig. S1 A). In contrast, TCF-1 expression is maintained throughout positive selection in the thymus and is not down-regulated until SP naive T cells migrate to the periphery (Fig. 1 A, lower panel). We wanted to determine whether sustained TCF-1 was critical for the dynamic regulation of RORγt expression that is essential for T cell maturation (He et al., 2000). To investigate this, we assessed splenic TCRβ⁺ T cells from 6-wk-old *Tcf7*^{+/+} and *Tcf7*^{-/-} mice by unbiased clustering t-Distribution Stochastic Neighbor Embedding (tSNE) multiparameter analysis of CD4, CD8, CD44, CD62L, and RORγt staining. While the frequency of a number of populations was altered in the absence of TCF-1, one of the most striking differences was associated with a significant enrichment of RORγt⁺CD8⁺ T cells (Fig. 1 B). These cells were expanded in the thymus, secondary lymphoid tissues, and nonlymphoid tissues such as the small intestine, up to 25-fold across all tissues examined (Fig. 1 C). By contrast, in naive WT mice, very few of

these cells were normally detected. RORγt⁺CD8⁺ T cells developing in the absence of TCF-1 had an activated phenotype marked by high CD44 and low CD62L expression, but in addition, they also expressed CD25 (Fig. 1 D and Fig. S1 B), a marker that is not highly expressed in WT mice at steady state. In the absence of TCF-1, these TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺ T cells readily expressed RORγt (Fig. 1 D) and produced IL-17 in response to stimulation ex vivo with PMA and ionomycin (Fig. 1 D). Further characterization did not show any aberrant production of inflammatory cytokines such as IFN-γ, IL-6, IL-22, TNF-α, or IL-2 (Fig. S1 C).

Given that MAIT cells share some features similar to RORγt⁺IL-17⁺CD8⁺ T cells that develop in the absence of TCF-1, including their expression of CD8α, RORγt and IL-17 production (Rahimpour et al., 2015), we examined TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺RORγt⁺ T cells from the spleen of WT and *Tcf7*^{-/-} mice for the transcription factor promyelocytic leukemia zinc finger protein (PLZF), a factor essential for MAIT cell development (Koay et al., 2016). In contrast to cells from *Tcf7*^{+/+} mice, cells from *Tcf7*^{-/-} mice did not express PLZF (Fig. S1 D). Furthermore, in contrast to the RORγt⁺CD8⁺ T cells found in *Tcf7*^{-/-} mice, we found that MAIT cells required TCF-1 for their development (Fig. 1 E). Thus, TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺RORγt⁺ T cells found in the spleen of WT mice are MAIT cells and are distinct from those found in *Tcf7*^{-/-} mice. This suggests that the RORγt⁺CD8⁺ T cells present in TCF-1-deficient mice are Tc17 cells consistent with recent work showing that Tc17 cells are not present at steady-state in wild-type mice bred in specific pathogen-free facilities (Linehan et al., 2018). Furthermore, Tc17 cells from *Tcf7*^{-/-} mice did not show a highly restricted semiinvariant TCR-α chain as featured by MAIT cells (TRAV1-TRAJ33, also designated Vα19-J33; Fig. 1 F). Instead, *Tcf7*^{-/-} Tc17 cells expressed a very diverse range of both Vα and Vβ chains (Fig. 1 F). Thus, TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺RORγt⁺ T cells in mice at steady state are predominantly composed of MAIT cells, while TCF-1 deficiency results in the emergence of a distinct subset of Tc17 cells that exhibit a diverse TCR repertoire. To determine if the Tc17 cells we observed were induced by commensal microbes, we treated pregnant mothers before the birth of pups and continued treatment of pups with a cocktail of antibiotics to eliminate bacterial stimulation. This revealed that similar to untreated *Tcf7*^{-/-} mice, Tc17 cells develop despite the ablation of bacterial communities (Fig. S1 E). To further examine if the Tc17 cells that develop in the absence of TCF-1 could emerge in response to microbe-derived peptides recognized by the non-classical MHC class Ib molecules, H2-M3 as seen in previous work (Linehan et al., 2018), we probed Tc17 cells for their ability to bind H2-M3 tetrameric complexes loaded with the *Staphylococcus epidermidis*-derived N-formyl methionine peptide, MIIINA. CD8⁺ T cells isolated from the spleen and ears of naive *Tcf7*^{+/+} and *Tcf7*^{-/-} mice together with WT mice that had been infected with *S. epidermidis* for 7 d were examined (Fig. S1 F). Although f-MIIINA:H2-M3 tetramer-positive cells were enriched in the spleen and ears of *S. epidermidis*-associated mice, *S. epidermidis*-specific cells were not expanded in these tissues in *Tcf7*^{-/-} mice (Fig. S1 F). Collectively, these data

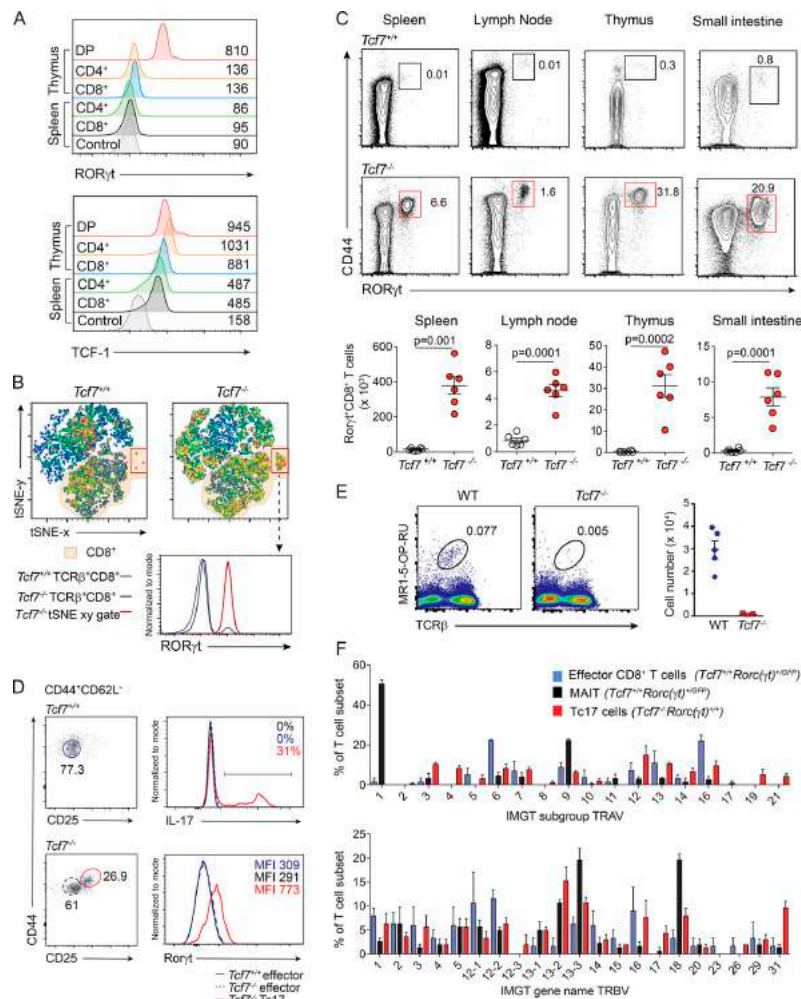


Figure 1. Loss of TCF-1 results in the emergence of IL-17-producing CD8⁺ T (Tc17) cells. (A) Expression of RORγt (upper panel) and TCF-1 (lower panel) in various T cell populations from thymus and spleen of WT mice. Populations include DP (TCRβ⁺CD4⁺CD8⁻) thymocytes and CD4⁺ (TCRβ⁺CD4⁺CD8⁻) or CD8⁺ (TCRβ⁺CD4⁻CD8⁺) T cells isolated from spleen or thymus. Control for RORγt expression represents fluorescence minus one stain of CD8⁺ T cells from WT mice and for TCF-1 expression shows spleen CD8⁺ T cells from *Tcf7*^{-/-} mice. Data show representative plots of one of two independent experiments (*n* = 4 mice/experiment). (B) tSNE analysis of TCRβ⁺ cells analyzed by flow cytometry for CD4, CD8, CD62L, CD44, and RORγt from spleen of *Tcf7*^{+/+} and *Tcf7*^{-/-} mice. Dot plots are displayed as pseudocolor plots denoting areas of high and low population density. Orange shading indicates CD8⁺ T cells. Histogram shows RORγt expression. Representative of three independent experiments (*n* = 6 mice). (C) Contour plots gated on TCRβ⁺CD8⁺CD4⁻ T cells show the frequency (upper panels) and number (lower panel) of RORγt⁺CD8⁺ T cells in various tissues in *Tcf7*^{+/+} and *Tcf7*^{-/-} mice. Data show representative plots (upper panels) and the mean ± SEM of individuals pooled from three independent experiments (lower panels; *n* = 6, *P* values calculated using Student's *t* test). (D) IL-17 production and RORγt expression by *Tcf7*^{-/-} Tc17 cells (red, TCRβ⁺CD8⁺CD4⁻CD62L⁻CD25⁻) and *Tcf7*^{+/+} or *Tcf7*^{-/-} effector cells (blue and black, TCRβ⁺CD8⁺CD4⁻CD62L⁻CD25⁻) FACS-purified from the spleen and lymph nodes of *Tcf7*^{+/+} and *Tcf7*^{-/-} mice, followed by stimulation with PMA and ionomycin for 4 h in vitro. Histograms are representative of one of two independent experiments (*n* = 3 mice/genotype/experiment). (E) Flow-cytometric analyses of MAIT cells in spleen of WT and *Tcf7*^{-/-} mice. Data show representative staining from an individual mouse for each genotype and data from one of two similar experiments (*n* = 3–5 mice/genotype). (F) Single-cell PCR analysis of TCR Vα (upper panel) and TCR Vβ (lower panel) usage of effector CD8⁺ T cells (TCRβ⁺CD4⁻CD8⁺CD44⁻CD62L⁻*Rorc(gt)*^{-/-}) and MAIT cells from *Tcf7*^{+/+}*Rorc(gt)*^{-/-}*GFP* mice (TCRβ⁺CD4⁻CD8⁺CD44⁻CD62L⁻*Rorc(gt)*^{-/-}*GFP*) and Tc17 cells from *Tcf7*^{-/-} mice (TCRβ⁺CD4⁻CD8⁺CD44⁻CD62L⁻CD25⁻). Data show the mean expression ± SEM of 50 individual cells analyzed for TCR Vα and TCR Vβ usage from each of three mice.

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demonstrate that the Tc17 cells that emerge in the absence of TCF-1 are not driven by the normal intestinal microbiota, and TCF-1 plays a critical role in suppressing development of these Tc17 cells.

TCF-1 suppresses the transcriptional profile associated with IL-17-producing cells

To identify the molecular mechanisms underlying the emergence of Tc17 cells, we compared their gene expression profile to effector and other CD8⁺ T cell subsets. We took advantage of our finding that Tc17 cells express CD25 and performed RNA-sequencing (RNA-seq) on FACS sorted naive (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁻), effector (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺), and memory CD8⁺ T cells (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺) isolated from *Tcf7*^{+/+} and *Tcf7*^{-/-} mice and Tc17 cells (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺) from *Tcf7*^{-/-} mice (Fig. 2). Multidimensional scaling analysis (Fig. S2 A) and heat map (Fig. 2 A) analysis revealed that Tc17 cells had a distinct transcriptional profile compared with naive, effector, and memory CD8⁺ T cells from both *Tcf7*^{+/+} mice and *Tcf7*^{-/-} mice (Fig. 2 A). Given that Tc17 cells had an activated phenotype (CD44⁺CD62L⁺) but are not present in WT mice at steady state (Fig. 1 and Linehan et al., 2018), we compared them directly to the expression profile of WT effector cells. We identified 671 genes up-regulated in Tc17 cells compared with WT effector cells and 644 down-regulated genes (Fig. 2 B). Genes were called differentially expressed (DE) if they achieved an FDR of ≤0.05. Among the up-regulated genes was the transcription factor *Rorc*, a critical factor required for all IL-17-producing cell types (Santori et al., 2015; Fig. 2, A and B). Also included in this list of up-regulated genes were genes that have previously been shown to a play role in regulating IL-17 production in CD4⁺ T cells or γδ T cells such as *Maf*, *Sox13*, and *Blk* (Bauquet et al., 2009; Laird et al., 2010; Gray et al., 2013; Tanaka et al., 2014), the expression of which in Tc17 has not previously been described (Fig. 2, A and B). In addition, a number of cytokine and chemokine receptors such as *Il7r*, *Il27ra*, *Il23r*, *Il1r1*, *Ccr4*, *Ccr6*, and *Ccr9* were up-regulated by Tc17 cells compared with naive, effector, and memory CD8⁺ T cells (Fig. 2 A). Further investigation of transcription factors known to control effector cell differentiation revealed that *Tbx21*, *Id3*, *Irf4*, and *Eomes* were down-regulated in Tc17 cells compared with WT effector cells (Fig. 2, A and B). These observations were corroborated by flow cytometry for protein expression of T-BET, EOMES, MAF, and IRF4 (Fig. 2 C). Our analyses of *Tbx21/Eomes* double knockout mice did not show increased constitutive Tc17 cells at steady state (data not shown). This is consistent with previous reports showing that T-BET and EOMES suppress the differentiation of IL-17-producing CD8⁺ T cells following lymphocytic choriomeningitis infection but exhibited normal T cell composition at steady state (Intlekofer et al., 2005, 2008). This analysis identifies TCF-1 as a critical transcriptional repressor of Tc17 cell differentiation and normally preserves the expression of genes associated with conventional effector CD8⁺ T cell differentiation.

Tcf7-deficient Tc17 cells and pathogen-induced Tc17 cells exhibit a conserved transcriptional profile that is distinct to Th17 cells

Our data and others show that Tc17 cells are not present in WT mice housed in SPF facilities at steady state (Linehan et al.,

2018). Although we show that *Tcf7*^{-/-} Tc17 cells do not recognize the f-MIIINA:H2-M3, we investigated whether transcriptionally these cells were similar to or different from the Tc17 cells shown to develop in the skin following imiquimod treatment or in association with the commensal bacteria *S. epidermidis* (Linehan et al., 2018). Gene set enrichment analysis between our RNA-seq data and the published dataset (Linehan et al., 2018) showed a high correlation between genes DE in *Tcf7*^{-/-} Tc17 cells and both imiquimod (Fig. S2 B) and *S. epidermidis*-induced (Fig. S2 C) Tc17 cells (*P* < 0.0001 in all comparisons). Venn diagram analysis comparing all three Tc17 cell types showed that 190 genes highly expressed in *Tcf7*^{-/-} Tc17 cells were shared among all three Tc17 cell types, including the transcription factors *Rorc* and *Maf* (Fig. 2 D). In addition, 240 shared genes were down-regulated in all three Tc17 cell types including *Tbx21* and *Eomes*, transcription factors previously shown to suppress Tc17 development following lymphocytic choriomeningitis virus infection (Intlekofer et al., 2008). To understand how similar or distinct *Tcf7*^{-/-} Tc17 cells are from Th17 cells, we compared RNA-seq data of murine T helper type 0 and Th17 cells from a published dataset (Ciofani et al., 2012) to identify the Th17 specific gene set. This was then compared with the DE gene list from our own Tc17 analyses (Fig. 2 E). This revealed that while Th17 and Tc17 cells share a common gene set as would be expected of IL-17-producing cells (166 genes), a large proportion (87%) of genes were distinct from those expressed by Th17 cells (1,149 genes). Thus, Tc17 cells express a unique transcriptional program that establishes their regulation as distinct from Th17 cells.

Tc17 cells exhibit enhanced capacity for lipid metabolism and tissue repair

To understand how Tc17 cells are distinct from other T cell subsets, we used gene network analyses to uncover novel features of these cells. Metascape analyses (<http://metascape.org>; Tripathi et al., 2015) of genes down-regulated in Tc17 cells revealed a dampening of pathways associated with T cell activation, immune effector function, negative regulation of the immune system, and cell adhesion (Fig. S2 D). Interrogation of up-regulated genes revealed gene clusters associated with lipid metabolism, inflammation, and immune cell development (Fig. S2 E). Lipid metabolism plays an integral role in the survival, proliferation, and differentiation of pathogenic Th17 cells (Berod et al., 2014) but has not previously been described for Tc17 cells. Comparison of the Tc17 profile with other CD8⁺ T cell subsets revealed that genes involved in phospholipid and glycerophospholipid metabolism were specifically enriched in Tc17 cells (Fig. 2 F). This included target genes of the sterol response element binding proteins (*Srebp*) and master regulators of fatty acid and cholesterol synthesis, including *Gpd2*, *Ldlr*, *Soat1*, *Sqle*, *Srebp2*, and *Mvd* (Walls et al., 2016). Intermediates of this pathway have recently been shown to act as agonists for RORγt (Hu et al., 2015; Santori et al., 2015). Further gene ontology analyses revealed an enrichment in genes involved in wound healing (Log10 (*P*) = -6.6), particularly genes involved in chemotaxis (*Ccr2*, *Csf2*, *Tgfb1*, *Tgfb3*, and *Furin*), angiogenesis (*Vegfb* and *Csf2*), and tissue remodeling (*Mmp25*), was increased in Tc17 cells

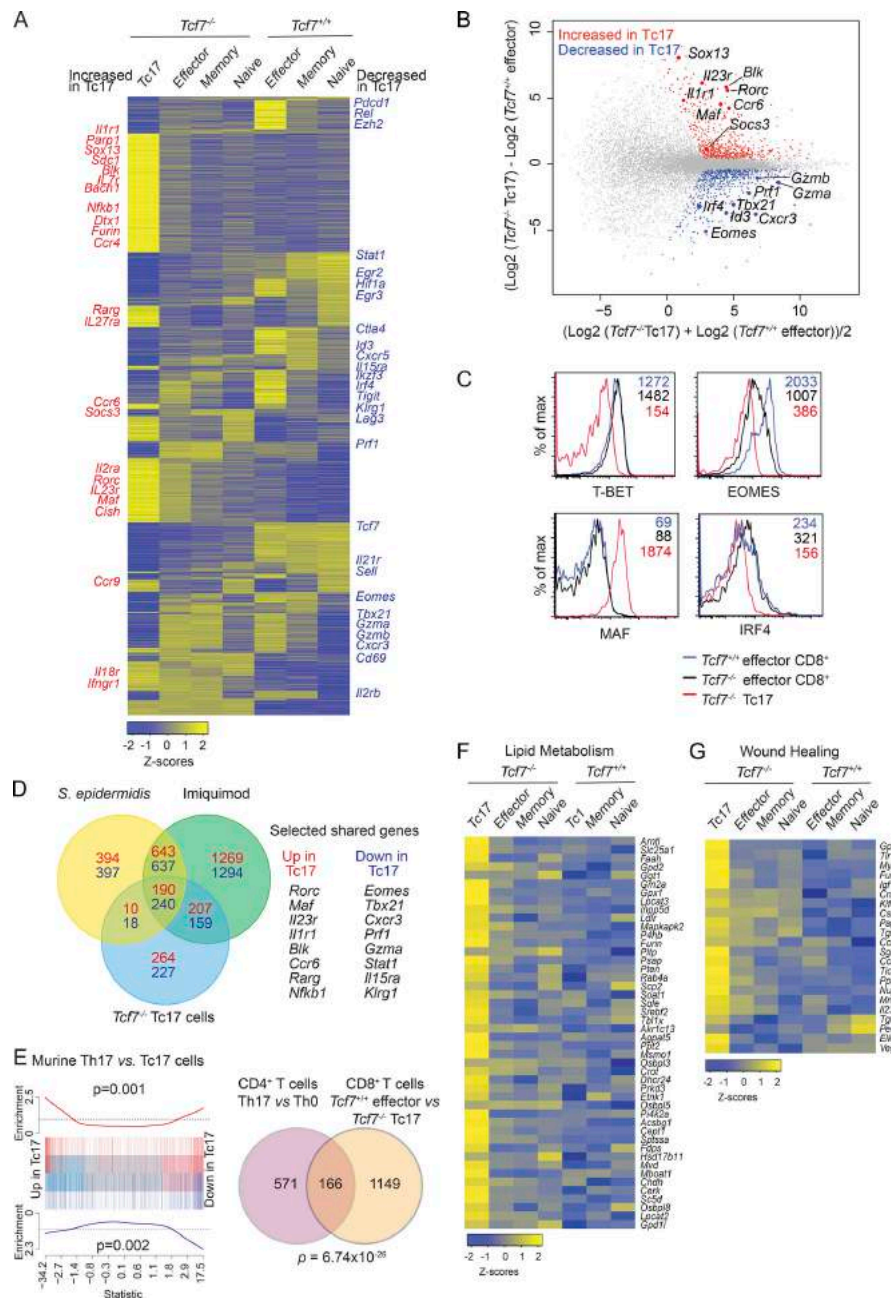


Figure 2. TCF-1-deficient Tc17 cells are transcriptionally distinct from other CD8⁺ T cell subsets. (A) Heat map showing DE genes by Tc17 cells (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺) compared with naive (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺), effector (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺), and memory (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺) CD8⁺ T cell populations from *Tcf7*^{+/+} and *Tcf7*^{-/-} mice. Relative expression (Z-scores) of genes are shown; rows are scaled to have a mean of 0 and an SD of 1. For each T cell population, two independent biological replicates were analyzed. Data show the mean expression values for each group. (B) Mean-difference plot comparing *Tcf7*^{-/-} Tc17 cells with *Tcf7*^{+/+} effector cells showing up-regulated genes in red and down-regulated genes in blue. Data show log2-fold change compared with mean expression. (C) Transcriptional profile of Tc17 cells. Intracellular analyses of the expression of T-BET, EOMES, MAF, and IRF4 in WT effector cells, *Tcf7*^{-/-} effector cells, and Tc17 cells. Effector CD8⁺ T cells were identified as TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺RORγt⁺ (effector WT, blue; *Tcf7*^{-/-}, black), while Tc17 cells were TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺RORγt⁺ (*Tcf7*^{-/-}, red). Data show the MFI of expression for each CD8⁺ T cell population and show representative profiles from one of two similar experiments (*n* = 4 mice/genotype). (D) Venn diagram analysis of shared genes among Tc17 cell types. Comparison of *Tcf7*^{-/-} Tc17 versus WT effector CD8⁺ T cells DE genes with DE genes in imiquimod-induced skin Tc17 cells and DE genes in topical *S. epidermidis*-induced Tc17 cells. Number of genes up-regulated in Tc17 cells compared with effector cells for each comparison is shown in red, and the number of down-regulated genes is shown in blue. (E) Comparison between RNA-seq analyses of murine T helper type 0 versus Th17 cells from a published dataset (GSE40918, eight libraries; Ciofani et al., 2012) with *Tcf7*^{+/+} effector versus *Tcf7*^{-/-} Tc17 cells. (F and G) Heat map analysis showing up-regulated genes in Tc17 cells enriched for lipid metabolism (F) or wound healing (G) in Tc17 cells compared with naive, effector, and memory CD8⁺ T cell populations from *Tcf7*^{+/+} and *Tcf7*^{-/-} mice. Relative expression (Z-scores) of genes are shown, color coded according to the legend. Rows are scaled to have a mean of 0 and an SD of 1.

compared with all other CD8⁺ T cell subsets (Fig. 2 G). This is consistent with a recent study in which tissue repair pathways have been shown to be increased in Tc17 cells (Linehan et al., 2018; Harrison et al., 2019).

TCF-1 regulates chromatin architecture to suppress Tc17 cell potential

In addition to TCF-1 directly binding DNA, this factor has recently been shown to have intrinsic histone deacetylase activity, capable of coordinating the opening of chromatin in early T cell development, and to repress CD4⁺ T cell fate in CD8⁺ T cells (Xing et al., 2016; Johnson et al., 2018). This provides a second process by which TCF-1 is able to regulate gene expression. We therefore hypothesized that TCF-1 regulates chromatin accessibility as a mechanism to suppress Tc17 cell potential. To this end, we performed Assay for Transposase-Accessible Chromatin (ATAC) sequencing (ATAC-seq) on *Tcf7*^{-/-} Tc17 cells, effector and memory CD8⁺ T cells purified by flow-cytometric sorting from *Tcf7*^{+/+} and *Tcf7*^{-/-} mice. Our unsupervised clustering analysis revealed that 19.7% of regulatory elements were more accessible and unique to Tc17 cells compared with only 8.2% of *Tcf7*^{-/-} Tc1, 5% of *Tcf7*^{-/-} memory, 6.8% of *Tcf7*^{+/+} effector, and 4.6% of *Tcf7*^{+/+} memory T cells (Fig. 3 A), thus suggesting that Tc17 constitute a distinct CD8⁺ T cell subset. These accessible regions were assigned to a gene if they were located within 20 kilobase pairs upstream of the transcription start site or 5 kbp downstream of the 3' end of gene loci. To determine the strength of the relationship between TCF-1-regulated genes identified by our RNA-seq, we correlated gene expression with ATAC-seq peak scores for each DE gene (Fig. 3 B). A significant positive correlation (regression slope, 0.48; P value, 1.94×10^{-99}) emerged between accessible chromatin and DE genes in *Tcf7*^{-/-} Tc17 cells and *Tcf7*^{+/+} effector cells (Fig. 3 B). Analysis of the chromatin accessibility of loci encoding key transcription factors associated with T cell differentiation showed that *Rorc* was more accessible in Tc17 cells (Fig. 3 B and Fig. S3 A), while transcription factors with decreased gene expression also have decreased chromatin accessibility (e.g., *Eomes*, *Tbx21*, *Id3*, and *Irfa*; Fig. 3 B and Fig. S3 A). Thus, TCF-1 appears to be a major molecular switch controlling chromatin accessibility of key genes associated with the divergence between effector cells and Tc17 cells.

Tc17 cells are primed for IL-17 production

Our ATAC-seq analysis revealed that 1,726 regions of chromatin (1,621 genes) were more accessible in *Tcf7*^{-/-} Tc17 cells compared with *Tcf7*^{+/+} effector CD8⁺ T cells. Most of these genes (1,471) were not associated with genes actively expressed in Tc17 cells at steady state (Fig. 3 C). These included accessible chromatin regions associated with Tc17 effector genes such as *Il17a* and *Il17f* (Fig. 3 C). We found that many more genes (5,580) were less accessible in Tc17 cells and open in effector cells. Of these, 5,312 were not associated with actively expressed genes in effector cells, including *Ifng*, *Tnf*, and *Il2* (Fig. 3 C and Fig. S3 B). A close examination of the *Il17a* locus revealed three regions with increased accessibility in Tc17 cells compared with effector CD8⁺ T cells (Fig. 3 D). Using the ECR Browser (<http://ecrbrowser.dcode.org>), we found the stretch of DNA upstream of the *Il17a* loci contained MAF binding motifs and ROR response elements (RORE), predicted to bind RORγt (Fig. 3 D, upper panel). Two regions were also identified in the distal region of *Il17a* that were more accessible in Tc17 cells compared with effector cells (Fig. 3 D) containing numerous TCF-1 and LEF-1 binding motifs (Fig. 3 D). The promoter region of *Il17f* also displays increased accessibility in *Tcf7*^{-/-} Tc17 cells (Fig. 3 D, lower panel), containing RORE binding motifs. These results show that the *Il17* loci are poised for gene expression in Tc17 cells developing in the absence of TCF-1, but remained closed in effector cells from both WT and TCF-1-deficient mice. Finally, a global analysis of transcription factor binding motifs revealed that RORE and MAF binding motifs were among the most enriched in accessible chromatin regions specific to Tc17 cells (Fig. 3 E and Table S1). Given that RORγt and MAF were among the most highly expressed transcription factors in Tc17 cells (Fig. 2 A), we hypothesized that lack of TCF-1 releases suppression of RORγt and MAF to orchestrate Tc17 differentiation.

Early deletion of TCF-1 drives constitutive formation of Tc17 cells

TCF-1 is essential for establishing T cell development in both the thymus and periphery (Yu et al., 2009, 2011; Ma et al., 2011; Xu et al., 2015; Leong et al., 2016; Xing et al., 2016; Johnson et al., 2018). Nevertheless, the mechanisms underpinning Tc17 cell development in the absence of TCF-1 remain unclear. To

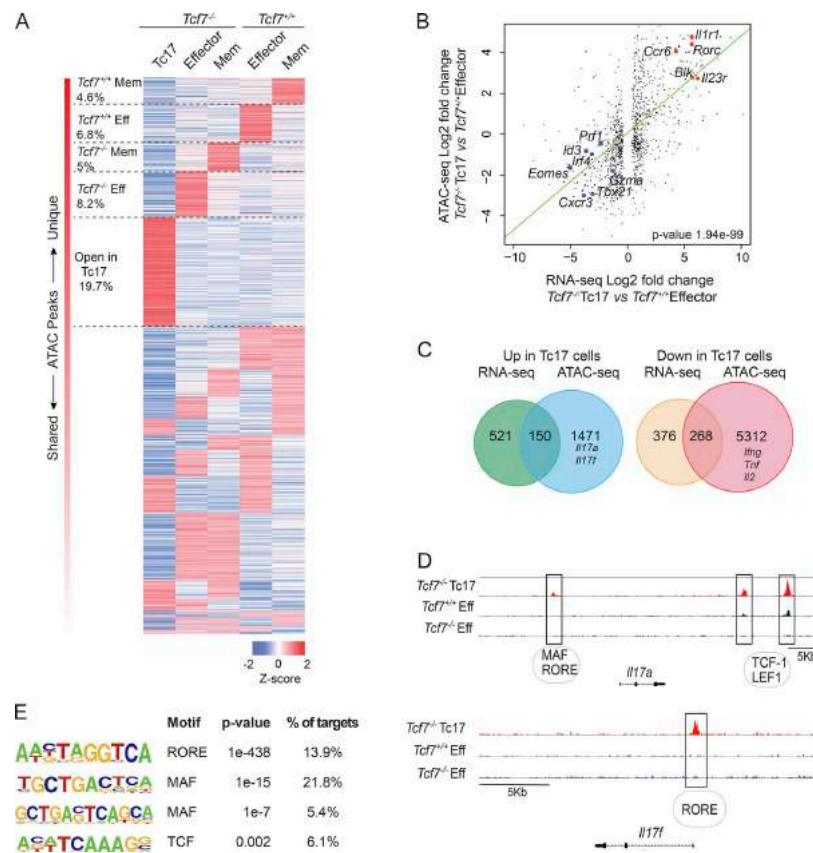


Figure 3. TCF-1 maintains the chromatin landscape of CD8⁺ T cells to suppress Tc17 potential. (A) Heat map of regions of chromatin accessibility across the genomes of *Tcf7*^{-/-} Tc17 cells (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺), effector (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺) and memory (TCRβ⁺CD4⁺CD8⁺CD44⁺CD62L⁺CD25⁺) CD8⁺ T cells from *Tcf7*^{+/+} and *Tcf7*^{-/-} mice. Average relative peak abundance (Z-score) calculated from two independent biological replicates from log2-CPM value of peak regions. (B) Correlation analysis of DE genes from RNA-seq analysis of *Tcf7*^{-/-} effector CD8⁺ T cells and *Tcf7*^{-/-} Tc17 cells (x axis) with gene accessibility (y axis). The green line is the regression line with a slope of 0.48. P value of the linear regression analysis is shown in the figure. (C) Overlap of DE genes from RNA-seq analysis and genes associated with differentially accessible chromatin regions in *Tcf7*^{-/-} Tc17 cells and *Tcf7*^{+/+} effector CD8⁺ T cells. (D) Genome browser of normalized ATAC-seq reads across the *Il17a* and *Il17f* loci from *Tcf7*^{-/-} Tc17 and effector CD8⁺ T cells from *Tcf7*^{+/+} and *Tcf7*^{-/-} mice. Predicted transcription factor binding sites are listed below as assessed by using the evolutionary rate covariance browser. (E) De novo motif analysis using HOMER in regions of chromatin with increased accessibility in Tc17 cells. Eff, effector CD8⁺ T cells; Mem, memory CD8⁺ T cells.

determine where loss of TCF-1 influenced the emergence of Tc17 cells, we generated two different mouse strains, allowing us to address the temporal consequences of TCF-1 loss at different stages of T cell development. To understand the impact of TCF-1 loss at the early stages, *Tcf7*^{fl/fl} mice were crossed with *Cd4Cre*^{+/T} mice (Sawada et al., 1994; *Tcf7*^{fl/fl}*Cd4Cre*^{+/T}), resulting in the loss of TCF-1 at the DP stage in the thymus (Fig. S4 A). For the second strain, *Tcf7*^{fl/fl} were crossed to *dLck*^{Cre} mice (*Tcf7*^{fl/fl}*dLck*^{Cre}), whereby Cre-recombinase gene expression is driven by the distal promoter of the lymphocyte protein tyrosine kinase (*dLck*; Wang et al., 2001) and induces ablation of TCF-1 after thymic

positive selection, resulting in a peripheral CD8⁺ T cell population lacking *Tcf7* (Fig. S4 B).

Analysis of *Tcf7*^{fl/fl}*Cd4Cre*^{+/T} mice revealed the constitutive presence of Tc17 cells in the periphery (Fig. 4 A, upper panel). These results confirm that TCF-1 suppression of Tc17 cells is intrinsic to the T cells and is not driven by the lymphopenic environment of germline TCF-1-deficient mice. In sharp contrast, late deletion of TCF-1 did not impair CD8⁺ T cell differentiation as virtually no Tc17 cells were detected in *dLck*-deleted mice at steady state (Fig. 4 A, lower panel). Our analysis of Th17 cells is consistent with a previous report of Zhang et al. (2018)

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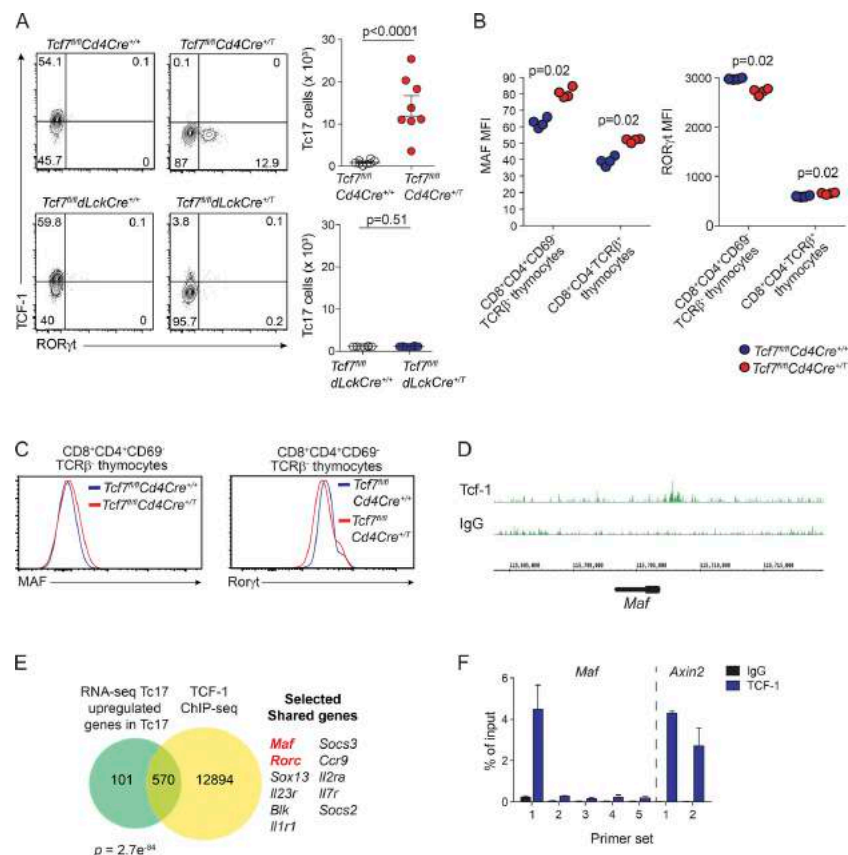


Figure 4. TCF-1 controls Tc17 generation and MAF expression in developing thymocytes. (A) Flow-cytometric analyses and enumeration of RORγt and TCF-1 expression in TCRβ⁺CD8⁺CD62L⁺CD44⁺ splenocytes isolated from *Tcf7^{fl/fl}CD4Cre^{+/+}* and *Tcf7^{fl/fl}CD4Cre^{-/-}* mice (upper panels) and *Tcf7^{fl/fl}dLckCre^{+/+}* and *Tcf7^{fl/fl}dLckCre^{-/-}* mice (lower panels). Data are pooled from two independent experiments and show the mean ± SEM (*n* = 4 or 5 mice/genotype/experiment). Exact *P* values were calculated using Student's *t* test. (B) Geometric MFI of MAF and RORγt expression in DP thymocytes (CD4⁺CD8⁺TCRβ⁺CD69⁻) and CD8 SP thymocytes (CD4⁻CD8⁺TCRβ⁺). Data are representative of two independent experiments (*n* = 4 mice/group/experiment) and show the mean MFI from one experiment. Exact *P* values were calculated using the Mann-Whitney *U* test. (C) Representative histograms of MAF and RORγt expression in DP thymocytes (CD4⁺CD8⁺TCRβ⁺CD69⁻) from B. (D) Genome browser of normalized TCF-1 ChIP-seq reads across the *Maf* locus is shown in green identified by ChIP-seq dataset from Dose et al. (2014) from WT mouse thymocytes. (E) Venn diagram analyses of up-regulated genes as determined by RNA-seq of *Tcf7^{-/-}* Tc17 cells versus *Tcf7^{+/+}* effector cells, compared with TCF-1 target genes identified by ChIP-seq dataset from Dose et al. (2014). FDR of 10⁻⁵ was applied when calling ChIP-seq peaks. Selected shared TCF-1 target genes DE by Tc17 cells are shown with key transcription factors highlighted in red. (F) ChIP analysis of TCF-1 binding in *Maf* loci of mouse thymocytes. TCF-1 binding in the *Axin2* loci is shown as a positive control. Data show the mean ± SD pooled from two independent experiments.

showing that TCF-1 also acts to suppress Th17 cells in *Tcf7^{-/-}* mice (Fig. S4 C). However, in contrast to Tc17 cells, in Th17 cells, this occurs before expression of the CD4 coreceptor, as we did not observe an increase in Th17 cells in *Tcf7^{fl/fl}CD4Cre^{+/+}* mice compared with *Tcf7^{fl/fl}CD4Cre^{-/-}* control mice (Fig. S4 D). Collectively, these findings highlight the different role TCF-1 plays in suppressing Th17 versus Tc17 differentiation. Thus, continuous expression of *Tcf7* in the thymus is critical to repress the

constitutive emergence of Tc17 cells before thymic positive selection.

TCF-1 directly suppresses *Maf* expression in developing thymocytes

We show that TCF-1 plays a critical role in suppressing Tc17 cell development following deletion in DP thymocytes. Both MAF and RORγt were highly expressed in Tc17 cells, and MAF and

ROR γ t binding motifs were significantly enriched in accessible chromatin regions of Tc17 cells. Therefore, we determined whether TCF-1 controls expression of ROR γ t and MAF at the DP stage of T cell development in the thymus by analyses of Tcf $^{fl/fl}$ Cd4Cre $^{+/+}$ and Tcf $^{fl/fl}$ Cd4Cre $^{+/T}$ mice. We observed a slight reduction in the number of DP (CD8 $^{+}$ CD4 $^{+}$ CD69 $^{-}$ TCR β $^{-}$) thymocytes in Tcf $^{fl/fl}$ Cd4Cre $^{+/T}$ mice but no reduction in SP CD8 $^{+}$ T cells (TCR β $^{+}$ CD8 $^{+}$ CD4 $^{-}$; Fig. S4 E). Interestingly, when analyzing these same populations for MAF expression, we observed a significant increase in MAF expression in DP thymocytes of Tcf $^{fl/fl}$ Cd4Cre $^{+/T}$ mice (Fig. 4 B, left panel; and Fig. 4 C). Increased expression was maintained as they matured to SP CD8 $^{+}$ T cells (Fig. 4 B, left panel). Consistent with previous reports (Xing et al., 2016; Johnson et al., 2018), in the absence of TCF-1, ROR γ t expression was reduced in DP thymocytes compared with control mice (Fig. 4 B, right panel; and Fig. 4 C, right panel), and expression was increased in SP CD8 $^{+}$ T cells (Fig. 4 B, right panel). These results show that TCF-1 suppresses MAF expression in developing thymocytes and places MAF upstream of ROR γ t in development of Tc17 cells in TCF-1-deficient mice.

Previous reports investigating the development of Tc17 cells have not shown whether MAF is required for development of these cells, nor whether TCF-1 could be a direct regulator of MAF expression. Therefore, we wanted to determine if TCF-1 could directly bind the promoter region of MAF as a potential mechanism to suppress its expression. Analysis of TCF-1 chromatin immunoprecipitation (ChIP)-sequencing (ChIP-seq) of thymocytes (Dose et al., 2014) revealed that TCF-1 normally binds the promoter DNA regions of *Maf* (Fig. 4 D). Comparison of genes up-regulated in Tc17 cells and direct TCF-1 target genes in thymocytes revealed that 570 genes were bound by TCF-1, including *Maf* and *Rorc* (Fig. 4 E). Analysis of TCF-1-bound genes that were down-regulated in Tc17 cells identified 512 genes including *Tbx21*, *Irf4*, *Id3*, *Prfl*, *Klrg1*, and *Gzma* (Fig. S4 F). We confirmed direct binding of TCF-1 to *Maf* loci at the same site identified in the ChIP-seq analysis by performing additional ChIP coupled with quantitative PCR (ChIP-qPCR) analyses for TCF-1 in mouse thymocytes (Fig. 4 F). Collectively, these results demonstrate that TCF-1 binds directly to the gene loci of *Maf* to limit its expression in developing T cells.

Tc17 cells are present in healthy humans

Our results and other reports show that Tc17 cells are very rare in mice at steady state (Hamada et al., 2009; Naik et al., 2015), but they appear to occur at higher frequency in mice from the wild, in humans, and under inflammatory conditions (Hamada et al., 2009; Zhuang et al., 2012; Wu et al., 2014; Gartlan et al., 2015; Naik et al., 2015; Amicarella et al., 2017; Linehan et al., 2018). To further investigate Tc17 cells in humans, we analyzed IL-17 and IFN- γ production in T cells from blood and spleen of healthy donors after in vitro stimulation with PMA and ionomycin followed by intracellular staining. MAIT cells and $\gamma\delta$ T cells were excluded using antibodies for Va7.2 and the $\gamma\delta$ T cell receptor, respectively (Fig. S5 A). While CD4 $^{+}$ T cells (CD3 $^{+}$ CD4 $^{+}$ CD8 $^{-}$ CD45RO $^{+}$ Va7.2 $^{-}$ TCR $\gamma\delta$ $^{-}$) were the main producers of IL-17 (Fig. 5 A, upper panel), a small but consistent number of IL-17 $^{+}$ CD8 $^{+}$ cells were detected (CD3 $^{+}$ CD4 $^{-}$ CD8 $^{+}$ CD45RO $^{+}$ Va7.2 $^{-}$ TCR $\gamma\delta$ $^{-}$ IL-17 $^{+}$;

Fig. 5 A, lower panel). Comparison of IL-17 $^{+}$ CD8 $^{+}$ cells in human blood and spleen demonstrated a slightly higher frequency of these cells in human spleen (Fig. 5 B).

Human Tc17 cells express MAF and RORC

Next we analyzed whether human IL-17 $^{+}$ CD8 $^{+}$ T cells have a distinct gene expression profile compared with IL-17 $^{+}$ CD8 $^{+}$ T cells. We used flow cytometry to purify human Tc17 cells from healthy spleen using a human IL-17 cytokine capture kit following a brief (3 h) restimulation with PMA and ionomycin. IL-17 $^{+}$ CD8 $^{+}$ T cells (CD3 $^{+}$ CD4 $^{-}$ CD8 $^{+}$ Va7.2 $^{-}$ TCR $\gamma\delta$ $^{-}$ IL-17 $^{+}$), IL-17 $^{+}$ CD8 $^{+}$ T cells (CD3 $^{+}$ CD4 $^{-}$ CD8 $^{+}$ Va7.2 $^{-}$ TCR $\gamma\delta$ $^{-}$ IL-17 $^{+}$) along with the analogous CD4 $^{+}$ T cell populations, IL-17 $^{+}$ CD4 $^{+}$ T cells (CD3 $^{+}$ CD4 $^{+}$ CD8 $^{-}$ Va7.2 $^{-}$ TCR $\gamma\delta$ $^{-}$ IL-17 $^{+}$) and IL-17 $^{+}$ CD4 $^{+}$ T cells (CD3 $^{+}$ CD4 $^{+}$ CD8 $^{-}$ Va7.2 $^{-}$ TCR $\gamma\delta$ $^{-}$ IL-17 $^{+}$), were isolated for genomic analyses by RNA sequencing. Our analysis of DE genes (FDR <0.1) revealed 296 genes down-regulated in IL-17 $^{+}$ CD8 $^{+}$ T cells and 457 up-regulated genes compared with IL-17 $^{+}$ CD8 $^{+}$ T cells. Heat map analysis highlighted the unique gene expression profile of IL-17 $^{+}$ CD8 $^{+}$ T cells compared with IL-17 $^{+}$ CD8 $^{+}$ T cells, IL-17 $^{+}$ CD4 $^{+}$ T cells, and IL-17 $^{+}$ CD4 $^{+}$ T cells (Fig. 5 C). *IL17A* and *IL17F* were the two most DE genes when human IL-17 $^{+}$ CD8 $^{+}$ T cells or IL-17 $^{+}$ CD4 $^{+}$ T cells were compared with the respective control IL-17 $^{+}$ CD8 $^{+}$ T cells or IL-17 $^{+}$ CD4 $^{+}$ T cells (Fig. 5 D and Fig. S5 B), indicating that the cytokine capture approach effectively isolated IL-17-producing cells. Importantly, signaling molecules *IL23R* and *SOCS3*, which were significantly up-regulated in murine IL-17 $^{+}$ CD8 $^{+}$ T cells, were also expressed by human IL-17 $^{+}$ CD8 $^{+}$ T cells (Fig. 5 D). In addition, we observed an increase in secreted cytokines *IL22*, the chemokine *CCL20*, and cell surface receptor *CD7* (Fig. 5 D). Analysis of the transcription factors *RORC* and *MAF*, which we identified as highly expressed in mouse Tc17 cells and regulated by TCF-1, showed that they were also expressed by human IL-17 $^{+}$ CD8 $^{+}$ T cells (Fig. 5, D and E). Collectively, these results show that IL-17 $^{+}$ CD8 $^{+}$ T cells, which exist in healthy humans, are Tc17-like and share a similar gene profile to murine Tc17 cells but are distinct from human IL-17 $^{+}$ CD8 $^{+}$ cells, which lack ROR γ t and MAF expression.

Human Tc17 cells are distinctly regulated compared with Th17 cells

As both Tc17 cells and Th17 cells express similar molecular regulators, we investigated whether Tc17 cells exhibited a distinctly separate transcriptional program or were regulated via a highly conserved program between the two T cell subsets. Therefore, we compared our IL-17-producing human CD4 $^{+}$ and CD8 $^{+}$ T cell subsets analyzed by RNA-seq in this study. We observed a significant overlap ($P = 1.12 \times 10^{-8}$) with genes DE between IL-17 $^{+}$ CD4 $^{+}$ and IL-17 $^{+}$ CD4 $^{+}$ cells, and genes DE between IL-17 $^{+}$ CD8 $^{+}$ and IL-17 $^{+}$ CD8 $^{+}$ cells. Despite this, an overwhelming 96% of genes DE were specific to IL-17 $^{+}$ CD8 $^{+}$ cells (Fig. 5 F). Thus, collectively, these analyses strongly support that while CD4 $^{+}$ and CD8 $^{+}$ IL-17-producing cells share a core set of genes, in humans, Tc17-like cells exist, and these IL-17 $^{+}$ CD8 $^{+}$ cells express a unique transcriptional program that establishes their regulation as distinct from Th17-like IL-17 $^{+}$ CD4 $^{+}$ cells.

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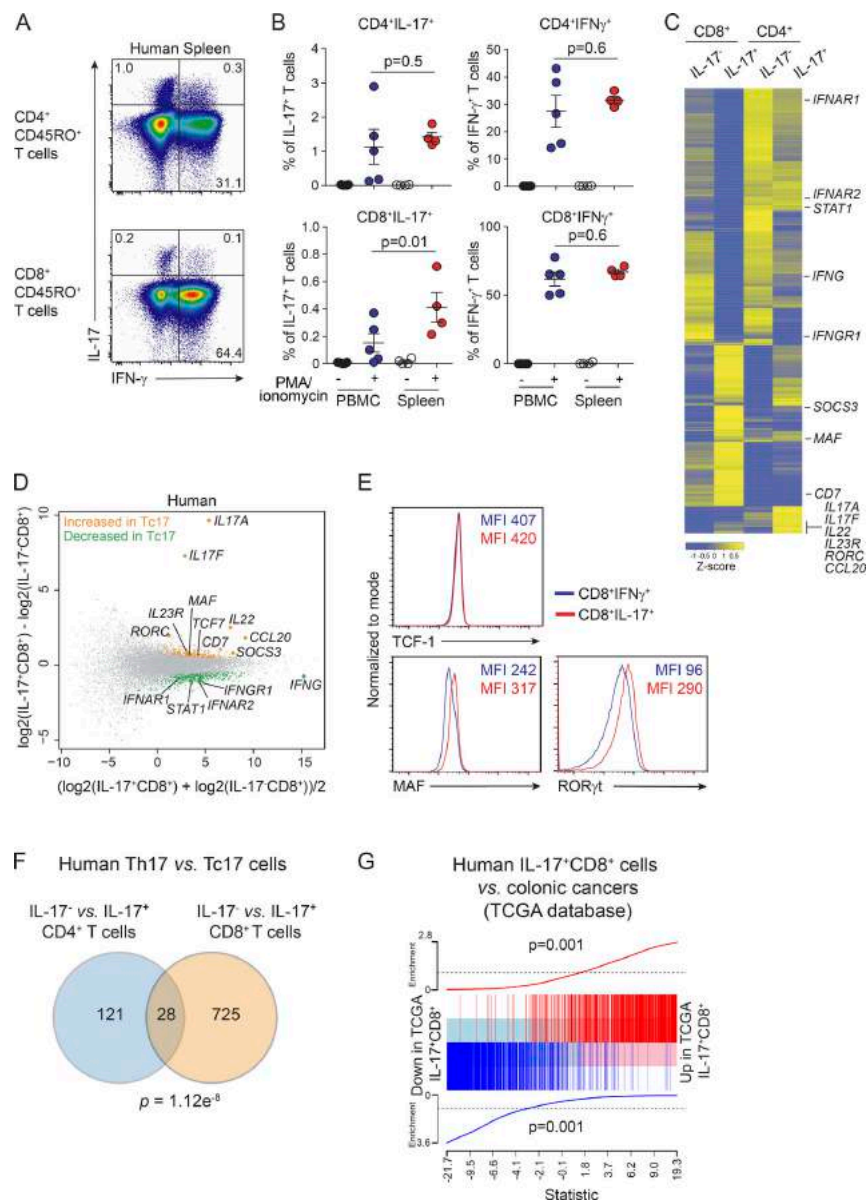


Figure 5. Tc17 cells occur in healthy humans and express MAF and RORC. (A) Representative dot plot analysis from four individual donors of IL-17 and IFN- γ production by T cells from human spleen. Cells are gated on CD45RO⁺CD4⁺ T cells (CD3⁺CD4⁺CD8⁺Va7.2-TCR γ δ -CD45RO⁺) or CD45RO⁺CD8⁺ T cells (CD3⁺CD4⁺CD8⁺Va7.2-TCR γ δ -CD45RO⁺). (B) Total cell numbers of IL-17 or IFN- γ -producing CD45RO⁺CD4⁺ T cells (upper panel) or CD45RO⁺CD8⁺ T cells (lower panel) from peripheral blood mononuclear cells ($n = 5$ donors) and spleen cells ($n = 4$ donors) from healthy donors determined by intracellular flow cytometry staining following stimulation with PMA and ionomycin for 4 h. Cells are gated as in A. Results are pooled from four independent experiments. Individual responses are shown together with the mean $\pm$ SEM; exact P values were calculated using Student's t test. (C) Heat map displaying DE genes.

Yellow color represents high expression, and blue represents low expression in effector IL-17-CD8⁺ versus IL-17-CD8⁺ T cells and IL-17-CD4⁺ versus IL-17-CD4⁺ T cells. Cells were sorted from spleens of three healthy donors for RNA-seq using a human IL-17 cytokine capture kit following restimulation with PMA and ionomycin. IL-17-CD8⁺ T cells (CD3⁺CD4⁺CD8⁺Vα7.2-TCRγδ⁺IL-17⁺), IL-17-CD8⁺ effector T cells (CD3⁺CD4⁺CD8⁺Vα7.2-TCRγδ⁺IL-17⁺), IL-17-CD4⁺ (CD3⁺CD4⁺CD8⁺Vα7.2-TCRγδ⁺IL-17⁺), and IL-17-CD4⁺ cells (CD3⁺CD4⁺CD8⁺Vα7.2-TCRγδ⁺IL-17⁺). (D) Mean difference plot highlighting genes significantly increased in human spleen IL-17-CD8⁺ T cells (yellow) versus genes increased in human spleen IL-17-CD8⁺ T cells (green). Data show log2-fold change compared with mean expression. (E) Protein expression of TCF-1, MAF and RORγt as assessed by flow cytometry from spleen cells of healthy donors. Cells are gated on CD3⁺CD4⁺CD8⁺Vα7.2-TCRγδ⁺IFN-γ⁺ (blue) or CD3⁺CD4⁺CD8⁺Vα7.2-TCRγδ⁺IL-17⁺ (red). Data show representative histograms from two independent experiments and MFI. (F) Comparison of RNA-seq analyses of human IL-17-CD4⁺ versus IL-17-CD4⁺ with IL-17-CD8⁺ versus IL-17-CD8⁺ T cells. (G) Enrichment of up-regulated (Up signature) and down-regulated (Down signature) genes in human splenic IL-17-CD8⁺ cells in the colonic cancers identified from the TCGA database (Up signature P value = 0.001; Down signature P value = 0.001).

IL-17 has been shown to promote progression of colorectal cancer (Grivennikov et al., 2012; Hyun et al., 2012; Wang et al., 2014). Therefore, to determine whether we could identify colorectal cancer samples likely to have infiltrating Tc17 cells, we analyzed 593 patient samples from The Cancer Genome Atlas (TCGA; <https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) database. Using Nearest Shrunken Centroid (Tibshirani et al., 2002) and Random Forest (Liaw and Weiner, 2002) classification algorithms, we developed gene expression-based classifiers for the CD8⁺IL-17⁺ Tc17-like cells from our human RNA-seq data. We then used these classifiers to query the TCGA Colon and Rectal Cancer samples to identify those samples with evidence in their transcriptome of a Tc17 expression profile. The samples from TCGA that were predicted by both independent classification methods to resemble Tc17 cells represent the set most likely to contain Tc17 infiltrating cells. To validate the likely Tc17 infiltration in these TCGA samples, we next tested whether these samples are enriched for genes DE in Tc17 splenic cells in our RNA-seq data. We separated putative Tc17 infiltrated samples from other tumor samples, and performed a differential expression analysis between these human tumor sets, then used gene set enrichment analysis to probe the gene expression data for the Tc17 gene expression signature. A significant enrichment of Tc17 genes (1,621 total shared genes between Tc17 cells and colorectal cancers, $P = 0.001$) was detected amongst these samples (Fig. 5 G). This gene set was distinct from those detected for IL-17-CD8⁺, IL-17-CD4⁺, and IL-17-CD4⁺ T cells, indicating that Tc17 cells exhibit a expression pattern and transcriptional profile separate from other CD8⁺ and CD4⁺ T cell subsets, including the related Th17 cells. Furthermore, this signature is identifiable in a small subset of colonic cancer samples.

MAF and RORγt drive Tc17 differentiation

Here we identified increased expression of MAF in TCF-1-deficient DP thymocytes and showed that TCF-1 can bind the promoter region of *Maf*. To investigate the role of *Maf* in Tc17 cell development and to determine whether interaction of TCF-1 with other genes identified in our RNA-seq experiments, namely *Rorc*, also play a role in the development of Tc17 cells, we crossed the *Tcf7*^{-/-} mouse strain to the *Maf*^{+/-} heterozygous and *Rorc*(γt)^{9f} reporter lines. The mice were analyzed for the constitutive formation of Tc17 cells (Fig. 6, A and B). In MAF-deficient mice, loss of a single copy of *Maf* resulted in a nearly fourfold reduction in the generation of Tc17 cells (Fig. 6 A). Strikingly, the Tc17 population was almost completely ablated

when a single copy of *Rorc*(γt) was deleted (Fig. 6 B). Thus, *Maf* and *Rorc*(γt) both play important roles in the development of Tc17 cells at steady state in the absence of TCF-1. Collectively, these data demonstrate that TCF-1 suppresses Tc17 production through the sequential regulation of the transcription factors MAF and RORγt.

Discussion

The transcriptional network required for the differentiation of Tc17 cells is largely unknown. Deciphering these networks may have important clinical implications to enable manipulation of CD8⁺ T cell differentiation to promote wound healing, resolve infection, and direct T cell responses in cancer (Hamada et al., 2009; Zhuang et al., 2012; Wu et al., 2014; Naik et al., 2015; Nanjappa et al., 2015; Linehan et al., 2018). We show that TCF-1 bound directly to DNA regulatory regions of MAF and RORγt and can limit the expression of MAF and RORγt to suppress the emergence of Tc17 cells. This uncovered a mechanism by which the sequential suppression of MAF and RORγt by TCF-1 centrally in the thymus determines the CD8⁺ T cell fate outcome of maturing thymocytes. Indeed, sustained expression of TCF-1 was observed to be necessary for expression of genes important for effector and memory CD8⁺ T cells, while TCF-1 loss enabled divergence of DP thymocytes into Tc17 cells.

TCF-1 plays critical roles in the development of T cells both in the thymus (Okamura et al., 1998; Germar et al., 2011; Weber et al., 2011; Yu et al., 2012) and in peripheral cells (Zhou et al., 2010; Lin et al., 2016). Here we focus on the role of TCF-1 in the thymus, where it is critical to suppress Tc17 cell differentiation, while deletion in mature T cells did not affect Tc17 cell number or frequency. Despite this, how TCF-1 expression shapes the heterogeneity of fate outcomes in the thymus has not been fully elucidated. Our results show that loss of TCF-1 activates the MAF/RORγt circuit during thymic differentiation to guide CD8⁺ T cell heterogeneity.

MAF has been shown to be important in CD4⁺ T cell subsets, particularly in the generation of regulatory T cells (Xu et al., 2018) and follicular helper T cells (Bauquet et al., 2009; Kroenke et al., 2012), and to promote Th17 differentiation (Tanaka et al., 2014). In γδ T cells, MAF antagonizes TCF-1 and LEF-1 to promote the formation and maintenance of RORγt⁺ IL-17-producing γδ T cells (Zuberbuehler et al., 2019). However, the role of MAF in CD8⁺ T cells is not well characterized and to our knowledge has not previously been described in Tc17 cells. We show for the first time that MAF is critical for Tc17 cells, and

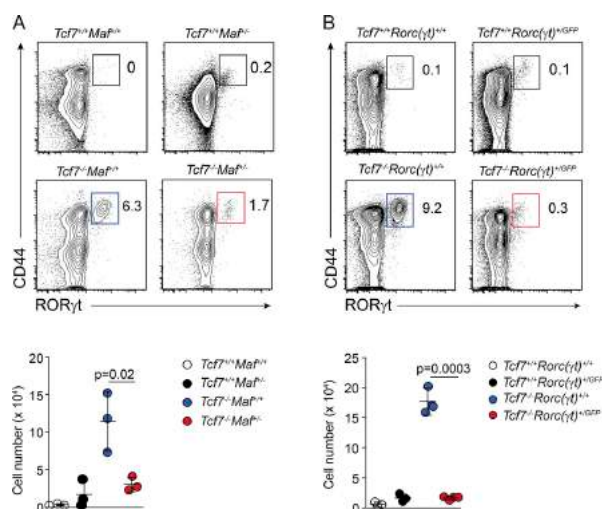


Figure 6. *Maf* and *Rorc* are required for Tc17 differentiation. (A) Enumeration of Tc17 cells in WT or *Tcf7*^{-/-}; *c-Maf*^{-/-} mice. Contour plots show the frequency of RORyt-expressing cells within live TCRβ⁺CD4⁺CD8⁺ T cells (upper panel). Scatter plots show the total number of TCRβ⁺CD4⁺CD8⁺RORyt⁺ Tc17 cells per spleen for each genotype (lower panel). (B) Frequency and total number of Tc17 cells in WT and *Tcf7*^{-/-}; *Rorc(yt)*^{+GFP} mouse strains. Contour plots show the frequency of RORyt-expressing cells within live TCRβ⁺CD4⁺CD8⁺ T cells (upper panel). Scatter plots show the total number of TCRβ⁺CD4⁺CD8⁺RORyt⁺ Tc17 cells per spleen for each genotype (lower panel). (A and B) Lower panels data show individual mice (mean ± SD) pooled from three independent experiments (*n* = 1 or 2 mice/group/experiment). Exact P values were calculated using Student's t test.

TCF-1 acts to suppress *Maf* expression in the thymus. Recent evidence shows that TGF-β can drive MAF expression to promote exhaustion of conventional αβ CD8⁺ T cells in melanoma, resulting in defective anti-tumor responses (Giordano et al., 2015). TGF-β has been implicated in Tc17 differentiation in vitro (Hamada et al., 2009; Huber et al., 2013), but it remains to be determined whether TGF-β links the TCF-1-MAF-RORyt axis in Tc17 cells. In T cells, we identified potential TCF-1 binding sites and increased chromatin accessibility in the distal region of *Il17a*. This is consistent with previous reports in CD4⁺ T cells, where TCF-1 has been shown to directly bind *Il17a* regulatory regions and suppress IL-17 production (Yu et al., 2011; Zhang et al., 2018). Our data build on this, showing that TCF-1 also limits expression of the transcription factor RORyt in TCRβ⁺CD8⁺ T cells. Our data further extend this by uncovering a pathway placing MAF upstream of RORyt, normally repressed by TCF-1 to limit the emergence of Tc17 cells.

Transcription factor activity and intrinsic histone deacetylase activity of TCF-1 appear to be essential for coordinating the opening of chromatin early in T cell development, and to establish the dichotomy between CD4⁺ and CD8⁺ T cells (Xing et al., 2016; Johnson et al., 2018). Here, we extend these findings and show that in addition to regulating central T cell lineage choices, TCF-1 suppresses Tc17 differentiation. We found that TCF-1 has a fundamental role in establishing chromatin architecture of CD8⁺ T cells and suppressed the opening of 1,726 chromatin regions in Tc17 cells. We predict some of these will be directly involved in the differentiation of Tc17 cells. TCF-1 was also instrumental in establishing the chromatin landscape of effector cells by cementing three times as many open chromatin regions in effector CD8⁺ T cells. Thus, these results revealed that TCF-1 plays a global role in defining the chromatin landscape of CD8⁺ T cells.

We found that Tc17 cells were significantly enriched for pathways that regulate phospholipid and glycerophospholipid metabolism, in striking contrast to both WT and *Tcf7*^{-/-} naive, effector, and memory CD8⁺ T cell subsets. Lipid metabolism is indispensable for generating the main components of cell membranes, provides energy, and is necessary for cell signaling (Howie et al., 2018). These same pathways are exploited by Th17 cells and tumor cells to enhance their metabolic rate and survival (Berod et al., 2014). The gene expression profile of metabolic genes exhibited by Tc17 cells suggests that these cells have requirements distinct from canonical CD8⁺ T cells that fuel subset differentiation. Interestingly, Th17 cells also exhibit a metabolic profile skewed toward phospholipid metabolism (Berod et al., 2014), and these pathways have recently been shown to produce ligands for RORyt (Hu et al., 2015; Santori et al., 2015), further highlighting the potential importance of lipid metabolism for Tc17 cells.

In humans, IL-17 plays a key role in defense against fungal infections (Puel et al., 2011), while several reports have correlated IL-17-producing cells with the development of cancer, including colorectal and gastric cancer (Zhuang et al., 2012; Wu et al., 2014). While this is the case, these studies have generally not clearly distinguished between Tc17, Th17, MAIT, or γδ T cells in humans or assessed their phenotype in detail. We start to delineate a potential role for Tc17 cells in human colorectal cancers by generating a gene expression signature of human Tc17 cells to test in TCGA datasets. We were able to identify a Tc17 signature in a small subset of colorectal cancer samples, indicating that this T cell subset plays a possible role in this disease. We show that Tc17 cells are present in healthy human blood and spleen and exhibit a transcriptional profile that is distinct from IL-17⁺CD8⁺ T cells. The expression profile of human Tc17 cells aligned with our analyses of Tc17 cells in mouse and Tc17 cells that develop in response to imiquimod and

commensal bacteria *S. epidermidis* (Naik et al., 2015; Linehan et al., 2018). Thus, the development of Tc17 cells appears to be conserved between man and mouse.

Tc17 cells are emerging as critical players in host-commensal microbiota interactions, infection, and cancer. Despite this, the molecular program that underpins their generation in favor of effector cells has not been previously defined. Together, our work identifies TCF-1 as a core transcriptional switch that in combination with MAF and ROR γ t defines the Tc17 fate outcome in developing thymocytes. This is mediated through the genome-wide programming of chromatin architecture and suppression of Tc17 identity genes. Thus, these findings are important in identifying the target pathways regulating Tc17 cells that may have important clinical implications for promoting tissue repair, homeostatic preservation of barrier function, and the elimination of intestinal tumors.

Materials and methods

Mice

Tcf7^{-/-} (Verbeek et al., 1995), *Id2*^{flp} (Jackson et al., 2011), *Rorc*(γ t)^{flp} (Eberl et al., 2004), *c-maf*^{-/-} (Kawauchi et al., 1999), *Tcf7*^{flp/fl} (Steinke et al., 2014), *Cd4*Cre (Sawada et al., 1994), and *dLck*Cre (Zhang and Bevan, 2012) have been previously described and were all maintained on a C57BL/6 (Ly5.2) background (originally derived from the Jackson Laboratory). The *Tcf7*^{-/-} line was crossed to the *maf*^{-/-} and *Rorc*(γ t)^{flp/+} strains to generate the *Tcf7*^{-/-}*maf*^{-/-} and *Tcf7*^{-/-}*Rorc*(γ t)^{flp/+} lines, respectively. *Tcf7*^{-/-}*maf*^{-/-} mice were maintained as a heterozygous line on the C57BL/6 background as mice lacking both alleles exhibit early postnatal mortality (Kawauchi et al., 1999). Mice were bred and maintained in-house. All mice were used at 6–8 wk old, and procedures were performed in accordance with approvals for the Animal Ethics Committee of the Walter and Eliza Hall Institute of Medical Research.

Human samples

Peripheral blood mononuclear cells were isolated from five healthy control donors (Volunteer Blood Donor Registry). Healthy human spleens from four donors were obtained from biopsy and cadaveric organ donors (Victorian Cancer Biobank and New South Wales Organ Transplant Registry). This study was performed according to the principles of the Declaration of Helsinki and was approved by local human research ethics committees (Melbourne Health, 2009.162; Walter and Eliza Hall Institute, 10/02).

Isolation of lymphocytes and flow cytometry

Spleen, thymus, and lymph node cells were isolated by mashing of organs over a 70- μ m filter, and cells were resuspended in PBS. Intestinal lymphoid cells were isolated from the intestine by incubation for 45 min at 37°C in Ca²⁺- and Mg²⁺-free HBSS plus 1 mM EDTA, 15 mM Hepes, and 10% FCS, with gentle shaking for removal of intestinal epithelial cells. Supernatants were discarded, and tissues were then incubated with gentle shaking for 45 min in 1 mg/ml (wt/vol) collagenase type III (Worthington), 200 μ g/ml DNase I (Roche), and 0.4 U/ml Dispase (Gibco) in

RPMI-1640 medium plus 2% (vol/vol) FCS. Preparations were filtered, and mononuclear cells were isolated on a 40–80% Percoll gradient. Lymphocytes were recovered from the interface.

Single-cell suspensions were stained with the following antibodies to mouse cell surface receptors: CD3 (145-2C11), CD4 (GK1.4), TCR β (H57-597), TCR $\gamma\delta$ (GL3), CD8 α (53-6.7), CD45.2 (104), CD44 (IM7), and CD62L (MEL-14). For human cell surface receptors, surface staining was performed with antibodies to CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8; eBioscience), TCRV α 7.2 (3C10; BioLegend), $\gamma\delta$ TCR (11F2; BD Biosciences), CD45RA (HI100), and CD45RO (UCHL1; eBioscience). Intracellular cytokine staining was performed following stimulation for 4 h with PMA (50 ng/ml) and ionomycin (100 ng/ml) in the presence of Brefeldin A (1 μ g). Intracellular staining was performed using the Transcription Factor Staining Buffer Set (eBioscience) with antibodies to murine ROR γ t (AFKJS-9 or Q31-378), TCF-1 (C63D9), T-BET (4B10), EOMES (Dan11Mag), IRF4 (3E4), MAF (Sym0F1), PLZF (Mags.21F7), IL-17 (Ebio17B7), IFN- γ (XMGL2), IL-2 (JES6-5H4), IL-6 (MP5-20F3), IL-22 (IL22)OP, and TNF- α (MP6-XT22); or antibodies to human IL-17 (eBio64-DEC17) and IFN- γ (45.B3). Single-cell suspensions of mouse thymus, spleen, and lymph node were prepared and stained with PE-mouse MRI-5-OP-RU tetramers (Corbett et al., 2014; Koay et al., 2016). *S. epidermidis*-specific CD8⁺ T cells were tracked via the use of a peptide-MHC tetramer, f-MILINA:H2-M3 (Linehan et al., 2018). Cells were analyzed using a FACS Canto II or Fortessa X20 (BD Biosciences), and FlowJo software was used for analysis. Flow-cytometric sorting was performed with a FACSAria (BD Biosciences).

Murine T cell isolation

Murine CD8⁺ T cells were enriched from lymph nodes and/or spleens by negative selection of single-cell suspensions incubated with antibodies against Mac-1 (M1/70), Ter-119, GR1 (RB6-8C5), MHC-II (M5/114), and CD4 (GK1.5) for 20 min at 4°C. Antibody-bound cells were removed by anti-rat IgG Ab-conjugated magnetic beads (Dynabeads; DYNAL). T cell subsets were purified by flow-cytometric sorting (FACS Aria flow cytometer; BD Biosciences).

Topical association of mice with *S. epidermidis* and lymphocyte isolation from ear skin

S. epidermidis strain (NIHLM087) association and skin digests were performed as previously described (Linehan et al., 2018; Park et al., 2019). Briefly, *S. epidermidis* was cultured for 18 h in tryptic soy broth at 37°C. For topical association of bacteria, each mouse was associated by placing 2.5 ml of bacterial suspension (~2.5 $\times$ 10⁹ total bacteria) across the flank and ear skin surface using a sterile cotton swab. Application of bacterial suspension was repeated every second day for 7 d. On day 7, ears were harvested, and skin was incubated in Liberase TL/DNase solution (0.25 mg/ml Liberase; Sigma-Aldrich; and 0.1 μ g/ml DNase; Sigma-Aldrich) at 37°C for 20 min and the epidermis separated from the dermis, then chopped and incubated for a further 60 min before passing through a 70- μ m filter before staining for flow cytometry.

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Oral antibiotic treatment

Mice were provided autoclaved drinking water supplemented with ampicillin (0.5 mg/ml; Sigma-Aldrich), gentamicin (0.5 mg/ml; Sigma-Aldrich), metronidazole (0.5 mg/ml; Sigma-Aldrich), neomycin (0.5 mg/ml; Sigma-Aldrich), vancomycin (0.25 mg/ml; Sigma-Aldrich), and sucralose (4 mg/ml; Splenda; McNeil Nutritionals, LLC; [Abt et al., 2012](#)); or ampicillin (1 mg/ml), streptomycin (5 mg/ml), colistin (1 mg/ml; Sigma-Aldrich) and vancomycin (0.25 mg/ml) added to the sterile drinking water of mice ([Routy et al., 2018](#)). Splenda was added to make the antibiotic cocktail more palatable. Mice were placed on antibiotic treatment for up to 1 wk before birth and continued for the duration of the experiment. Mice were analyzed at 4 wk of age. Antibiotic activity was confirmed by collecting feces at weekly intervals and bacterial colonization quantified using 16S ribosomal RNA gene copy number determined by real-time PCR ([Denman and McSweeney, 2006](#)).

Ex vivo single-cell multiplex RT-PCR for paired CDR3- β and CDR3- α analysis

Single effector (TCR β ⁺CD8⁺CD44⁺CD62L⁺ROR γ ⁺) cells from Tc7^{-/-}Rorc(yt)⁺GFP mice, MAIT cells (TCR β ⁺CD8⁺CD44⁺CD62L⁺ROR γ ⁺) from Tc7^{-/-}Rorc(yt)⁺GFP mice, and Tc17 (TCR β ⁺CD8⁺CD44⁺CD62L⁺CD25⁺) cells from Tc7^{-/-} mice were isolated by sorting single cells with a FACS Aria into 80 wells of a 96-well twin-tec plate (Eppendorf). The CDR3 α and CDR3 β regions were determined by a single-cell multiplex RT-PCR ([Dash et al., 2011](#)). mRNA transcripts were reverse-transcribed to cDNA using a VILO kit (Invitrogen) according to the manufacturer's instructions. The CDR3 α and CDR3 β regions were amplified by nested PCR using 23 TRAV (T cell receptor alpha variable gene) and 19 TRBV (T cell receptor beta variable gene) primers for external and internal rounds of PCR, together with constant region TRAC and T cell receptor beta constant primers ([Dash et al., 2011](#)). For the internal round of PCR, 2.5 μ l of the external product was used as template, with either a set of TRAV or TRBV internal primers. Positive PCR products were purified with Exo-SAP-IT at 37°C for 15 min and at 80°C for 15 min. Cleaned PCR products were sequenced using TRAC or T cell receptor beta constant internal primers. Sequences were analyzed using FinchTV, and V and J region usage identified using the ImMunoGeneTics information system (http://www.imgt.org/IMGT_vquest). Where dual TCR α or TCR β transcripts were detected for a single cell, sequencing with the relevant TRAV or TRBV internal primers was performed to separately identify each transcript.

Chromatin immunoprecipitation

Primers for ChIP-PCR were designed around peaks identified in published TCF-1 ChIP-seq data on thymocytes and published primer sequences for control gene Axin 2 ([Zhou et al., 2010](#)). Primer sequences are outlined in Table S2. ChIP analyses were performed on WT thymocytes that were cross-linked with 1% paraformaldehyde and lysed in lysis buffer (1% SDS, 1 mM EDTA, and protease inhibitors). Cross-linked DNA was sonicated with a Bioruptor (Diagenode). Protein G Dynabeads (Invitrogen) were incubated with 20 μ g anti-TCF-1 (H-11; Santa Cruz

Biotechnology) or 20 μ g of purified rabbit IgG. Coupled antibody was added to 200 μ g chromatin, followed by incubation at 4°C overnight. Unbound chromatin was removed using a series of four washes. After elution, bound chromatin was reverse-cross-linked and subjected to phenol-chloroform immunoprecipitation. Enrichment of recovered DNA was measured by real-time PCR.

RNA sequencing and analysis

Murine CD8⁺ T cells

CD8⁺ T cell subsets were purified by flow-cytometric sorting (FACS Aria flow cytometer; BD Biosciences) from Tc7^{-/-}IL17⁺CD8⁺ Tc17 cells (TCR β ⁺, CD8⁺, CD44⁺, CD62L⁺, and CD25⁺) and naive (TCR β ⁺, CD8⁺, CD44⁺, CD62L⁺, and CD25⁻), effector (TCR β ⁺, CD8⁺, CD44⁺, CD62L⁺, and CD25⁺), and memory (TCR β ⁺, CD8⁺, CD44⁺, CD62L⁺, and CD25⁻) CD8⁺ T cells from Tc7^{-/-} and Tc7^{-/-} mice. Total RNA was prepared from each subset using an RNeasy mini kit (Qiagen). 2 $\times$ 10⁵ T cells and two biological replicates were generated and subjected to 100-bp single-end sequencing on an Illumina HiSeq2000 at the Australian Genome Research Facility (Melbourne, Australia). Around 20 million reads were generated for each replicate and aligned to the GRCm38/mm10 build of the *Mus musculus* genome using the Subread aligner ([Liao et al., 2013](#)). Genewise counts were obtained using featureCounts ([Liao et al., 2014](#)). Reads overlapping exons in annotation build 38.1 of the National Center for Biotechnology Information (NCBI) RefSeq database were included. Genes were filtered from downstream analysis if they failed to achieve a counts per million (CPM) mapped reads value of ≥ 0.5 in at least two libraries. Counts were converted to log2-CPM, quantile-normalized, and precision-weighted with the voom function of the limma package ([Law et al., 2014](#); [Ritchie et al., 2015](#)). The log2-CPM values were then converted to log2-reads per kilo exonic bases per million mapped reads values. A linear model was fitted to each gene, and empirical Bayes' moderated *t*-statistics were used to assess differences in expression ([Smyth, 2004](#)). Genes were called DE if they achieved a false discovery rate (FDR of ≤ 0.05 and also had ≥ 8 reads per kilo exonic bases per million mapped reads in one or both of the two cell types being compared. Functional gene annotation was performed using Metascape analyses ([Tripathi et al., 2015](#)).

Human T cells

Total RNA was prepared from three biological replicates each using 2 $\times$ 10⁴ purified human splenic IL-17⁺CD8⁺ T cells (CD3⁺, CD4⁺, CD8⁺, Va7.2⁺, TCR δ ⁺, and IL-17⁺), IL-17⁺CD8⁺ effector T cells (CD3⁺, CD4⁺, CD8⁺, Va7.2⁺, TCR δ ⁺, and IL-17⁺), IL-17⁺CD4⁺ (Th17: CD3⁺, CD4⁺, CD8⁺, Va7.2⁺, TCR δ ⁺, and IL-17⁺), and IL-17⁺CD4⁺ (T helper type 1: CD3⁺, CD4⁺, CD8⁺, Va7.2⁺, TCR δ ⁺, and IL-17⁺) using an RNeasy micro kit (Qiagen). Libraries were prepared using the SMART-Seq v4 Ultra Low Input RNA kit (Clontech Laboratories). Samples were sequenced on an Illumina NextSeq 500 generating 75 bp paired-end reads. Human RNA-seq data were analyzed in a similar way as the analysis of mouse RNA-seq data. Genes were called DE if they achieved an FDR of ≤ 0.01 .

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ATAC sequencing and analysis

CD8⁺ T cell subsets were purified by flow-cytometric sorting (FACS Aria flow cytometer, BD Biosciences) from naive WT and *Tcf7*^{-/-} mice. For each biological replicate ($n = 2$), 5×10^4 cells of each subset were prepared for sequencing as previously described (Buenrostro et al., 2013, 2015). Samples were sequenced on an Illumina NextSeq 500 generating 75 bp paired-end reads. Reads were aligned to the GRCm38/mm10 build of the *M. musculus* genome using the Subread aligner. Only uniquely mapped reads were retained. ATAC-seq peaks were called using Macs2 with an FDR cutoff of 0.01. Overlapping peaks called from different samples were merged into a single peak region. Mapped reads in each sample were then assigned to these regions using featureCounts. Region counts were converted to log2-CPM and quantile-normalized with the voom method in limma. Regions were assigned to genes based on the overlap between genomic span of a region and gene body that is extended 20 kb upstream and 5 kb downstream. To determine enriched transcription factor binding motifs, de novo motif analysis using Homer was applied to accessible chromatin regions unique to Tc17 cells.

Analysis of chromatin immunoprecipitation sequencing

ChIP-seq data for TCF-1 has been published previously (Dose et al., 2014) and was downloaded from the GEO database (accession no. GSE46662). Sequence reads were aligned to the GRCm38/mm10 build of the *M. musculus* genome using the Subread aligner, and only uniquely mapped reads were retained. Binding peaks were called using the Homer program (Heinz et al., 2010). Chromatin input control was used to test the significance of binding enrichment. An FDR cutoff 1×10^{-5} was applied. Called peaks were assigned to a gene if they overlapped with the body of the gene plus 20 kb upstream and 5 kb downstream regions. The NCBI RefSeq mouse gene annotation (build 38.1) was used in the assignment of peaks to genes.

Visualization using tSNE plots

Unbiased clustering tSNE (van der Maaten and Hinton, 2008) were generated using FlowJo software to map multiparameter expression of CD4, CD8, CD62L, CD44, and ROR γ t in splenic TCR β ⁺ cells from the spleen of WT (*Tcf7*^{+/+}) and *Tcf7*^{-/-} mice and analyzed by flow cytometry. Data are displayed as a pseudo dot plot where color intensity indicates increasing cell frequency.

Classification of human tumor samples from TCGA

To obtain classification signatures for Tc1, Tc17, T helper type 1, and Th17 cell line models, two gene expression-based classifiers were developed using Random Forest (Liaw and Weiner, 2002) and Nearest Shrunken Centroid (Tibshirani et al., 2002) algorithms. While the Random Forest classifier is capable of capturing nonlinear relationships between the genes that characterize different subpopulations of T cells, the shrunken centroid classifier captures linear associations. The shrunken centroid classifier implemented in the pamr CRAN package (Hastie et al., 2014) was trained on human splenic T cell RNA-seq data using a threefold cross-validation training procedure with the default parameters. The Random Forest classifier implemented in the randomForest CRAN package was trained

using 500 trees, with three genes chosen at random at each split. The default parameters were used for the remaining parameters of the algorithm. Each classifier was then applied to a total number of 593 cancer samples in the TCGA Colon and Rectal Cancer datasets to predict if a sample in the Colorectal Cancer cohort is likely to have an expression profile resembling any of the four T cell sub-types. The samples predicted independently by both classifiers to resemble Tc17 expression profiles were considered to be the ones most likely to contain Tc17 infiltrating cells. To validate the likely Tc17 infiltration in these TCGA samples, we next tested whether these samples are enriched for genes DE in Tc17 splenic cells in our RNA-seq data. We separated putative Tc17 infiltrated samples from other tumor samples, discarding any samples that had a Tc17 prediction by only a single classifier, and performed a differential expression analysis between these human tumor sets using the voom function in limma (Law et al., 2014; Ritchie et al., 2015). The enrichment of the splenic Tc17 gene expression in TCGA gene expression data was then assessed using the mroast gene set enrichment test in limma using genes DE between splenic Tc17 cells and combined other T cell types as the gene set.

Quantification and statistical analyses

Statistical parameters including the exact value of n , the definition of center, dispersion, and precision measures (mean $\pm$ SEM), and statistical significance are reported in the figures and figure legends. Analyses were performed using GraphPad Prism software (version 7.0). Data were considered statistically significant when $P < 0.05$ by Student's t test, Mann Whitney U test, or ANOVA with Kruskal-Wallis test.

Data and materials availability

Raw data files for the RNA sequencing and ATAC sequencing have been deposited in the NCBI Gene Expression Omnibus under accession nos. GSE83841, GSE96741, and GSE119839.

Online supplemental material

Fig. S1 shows the transcriptional and functional phenotype of Tc17 cells at steady state and following treatment with antibiotics. Fig. S2 shows the gene network analysis of *Tcf7*^{-/-} Tc17 cells. Fig. S3 shows TCF-1 changes in chromatin accessibility of genes required for function of effector CD8⁺ T cells. Fig. S4 shows the deletion efficiency and T cell development in *Tcf7*^{fl/fl}CD4Cre^{+/+} mice. Fig. S5 shows the differential gene expression in human IL-17⁺CD4⁺ versus IL-17⁺CD4⁺ cells. Table S1 shows de novo motif enrichment using Homer from ATAC-seq peaks enriched in Tc17 cells, and Table S2 shows primer sequences for quantitative ChIP-PCR analysis.

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Parallel worlds of the adaptive and innate immune cell networks

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Adaptive and innate immune cells have typically been functionally and temporally segregated even though they share a number of salient features. Over the past decade, significant advances have been made in understanding the composition and diversity of both innate and adaptive cell populations. This has shed light on how cells from two distinct pathways are so highly complementary. Innate lymphoid cells (ILCs) are pivotally positioned in tissues to form a stable population akin to tissue-resident T cells that protects the body. Nevertheless, the pathway by which different lymphocytes enter tissues, terminally differentiate and are replenished to maintain populations remains incompletely understood. Recent evidence challenges our assumptions about the sedentary lifestyles of so called 'tissue-resident cells' and pushes us to consider their roles in orchestrating protection of the immune system beyond the classical models.

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Introduction

Immune cells are largely concentrated in primary and secondary lymphoid tissues, but it is increasingly clear that a vast number of lymphocytes can accumulate in non-lymphoid tissues even at steady-state. These cells affect critical functions in maintaining immune homeostasis together with mediation of responses to infectious or inflammatory stimuli.

'Tissue residency' refers to immune cells that establish in the tissue parenchyma or stroma where they can reside for an extended period of time without moving from tissue to

tissue. Demarcating immune cells into 'tissue-resident' versus 'circulating' cells provides a framework for defining two patterns of immune cell behavior matching the specialized functions of these cells. Circulating cells move between the blood and secondary lymphoid tissues, while tissue-resident immune cells move between the blood and nonlymphoid tissues thereby establishing long-term within the peripheral compartments. Tissue-residency implies that cells are relatively fixed or static in tissues providing a mechanism by which immune surveillance can be effectively executed through sampling of constituents of the blood or lymph that filter through organs. This makes them potent controllers of infection at the point of entry of infectious organisms to the body and provides a time and energy efficient method of eliminating pathogens.

The classic paradigm in immunology involves innate immune cells residing at barriers and in tissues mediating front-line defense, while adaptive cells undergo a series of triggers to arm them with effector molecules and migratory potential to continuously patrol for pathogen invaders and recruit the larger secondary army. This was challenged when it was discovered that some T cells remained in non-lymphoid tissues for much longer than anticipated [1,2] and this retention was governed by specific interactions between lymphocyte receptors and the extracellular matrix [3]. These initial studies ushered a number of elegant studies [4–7] that demonstrated that T_{RM} are both located, and functionally different from, effector and memory cells allowing CD8⁺ T cells to establish their weaponry alongside innate cells at the frontier of tissue invasion.

The concept of tissue-residency initially emerged from studies of a distinct subset of CD8⁺ memory T cells which develop within the affected tissues following a primary infection or insult and do not spread beyond this point or contribute to the circulating pool [4]. Now, it has become increasingly clear that other immune cell types exhibit tissue-residency and establish relatively stable populations within non-lymphoid tissues. For example, tissue-resident regulatory T cells (Tregs) have been defined and these cells exhibit a different transcriptome from other non-lymphoid tissue Treg populations and accumulate as animals age [8]. Deeper investigation revealed that this population of Tregs undergo an unexpected multi-step maturation program that relies on priming in the spleen before exiting the lymphoid tissue and establishing in non-lymphoid tissues accompanied by a final

diversification in the visceral adipose tissue [9*] (Figure 2). Collectively, these studies highlight the complexity of the lifecycle of lymphocyte lineages as they establish tissue residency.

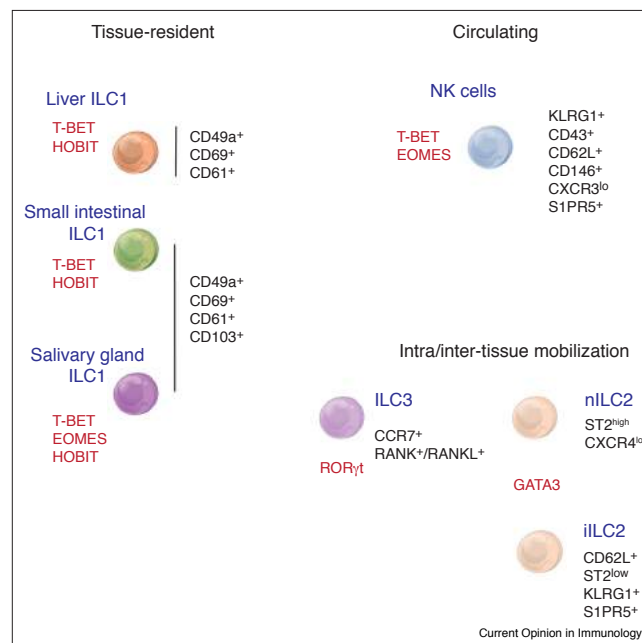
In the innate system, ILCs have been seen as long-term inhabitants statically embedded in peripheral tissues and largely lacking a pathway for circulation around the body. The level of tissue residency has mainly been determined using parabiosis. In this approach, the vasculature of two mice is conjoined allowing circulating lymphocytes to exchange between the two hosts. Strikingly, T_{RM} and ILC fail to exchange at a very low frequency within the reference timeframe of the experiment leading to the notion that this is not their typical pathway [10–12,13*]. After initial seeding, expansion and maturation of ILCs, little redistribution of these cells was found to occur at steady-state. Under inflammatory conditions ILCs greatly expanded, but their relocalization to different tissues remained modest [13*,14]. While the data strongly support the static nature of these populations, it does not discount the possibility that the maintenance of tissue-resident cells may depend on circulating progenitors which are not generated *in situ*, nor

that circulating cells adopt a phenotype distinct from the mature tissue-resident cells obscuring their detection, like ILC progenitors in humans [15*]. Detailed genomic profiling has revealed that tissue-resident cells differ both transcriptionally and metabolically from their circulating counterparts [16,17*,18] and these alterations are likely to markedly skew our modeling of the circulatory and migratory pathways undertaken by ILCs.

Factors required to establish residency of T_{RM}

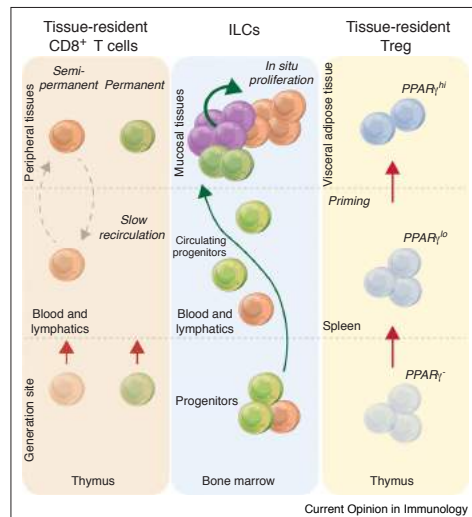
The hallmarks of tissue residency are defined by (i) core surface receptors which include CD69, CD103, CD49a and CD44, (ii) homing molecules such as S1PR and (iii) metabolic activity (Figure 1). Critically, CD103 and CD49a (VLA-1, very late antigen-1) not only define key markers of T_{RM} but form the binding partners for the ligands E-cadherin and collagen, respectively, that tether cells within the tissues [3]. CD44, like CD103 and CD49a, is a receptor for hyaluronic acid, a component of the extracellular matrix [19,20]. Classically, CD44 is a marker of T cell activation on newly generated effector cells but functionally it is also likely to be important in T_{RM} [21,22].

Figure 1



Surface receptors (black) and transcription factors (red) that promote resident or migratory behavior of different ILC subsets in mice between or within tissues.

Figure 2



Tissue-residency defines the final dominant destination of an immune cell. The pathway to tissue-residency is typified by three different scenarios. Firstly, following activation, tissue-resident CD8⁺ T cells localize to peripheral tissues and largely remain at that site while a smaller fraction of cells are retained for long periods but can slowly recirculate. In the second setting, ILCs localize in mucosal tissues and do not undergo recirculation but largely replenish through slow division at the tissue site. However, progenitors that exhibit a phenotype distinct from mature cells, are likely to travel in the circulation for deposition in non-lymphoid tissues, particularly mucosal sites. The third pathway highlights the importance of intermediary tissues such as the spleen where Tregs destined to become visceral adipose tissue-resident cells undergo a priming phase which is an essential part of their developmental pathway. Expression of PPAR γ is also upregulated to facilitate the cellular metabolism requirements in adipose tissue.

Transcriptional regulators of T_{RM}

Governance of the T_{RM} phenotype is orchestrated via a constellation of transcription factors. These include HOBIT (homolog of Blimp1 in T cells or ZNF683), BLIMP1, KLF2, RUNX3, T-BET and EOMES. In mice, HOBIT and BLIMP1 cooperate synergistically to regulate the expression of genes involved in retention of cells in tissues, and their egress [18]. This is largely mediated by their regulation of two T-box transcription factors T-BET and EOMES which when downregulated induce responsiveness to TGF- β and upregulation of CD103 [23,24]. Low levels of T-BET are, however, essential to maintain responsiveness to IL-15 enabling the formation of T_{RM} and their function [25]. The HOBIT-BLIMP1 transcriptional module is also required to regulate other tissue-resident lymphocytes such as

natural killer T (NKT) cells and liver-resident ILC1 [18] (Figure 1). Interestingly, the role of HOBIT in ILC1 is not consistent between ILC1s from different organs. HOBIT is expressed by ILC1s across organs such as the salivary gland and small intestine and yet only liver ILC1s are affected by the loss of HOBIT [26]. Many factors may be involved in regulating the egress of cells from a tissue and it cannot be assumed that transcription factors associated with tissue residency will have conserved functions in all organs. In human tissues, HOBIT is more broadly expressed than in mouse tissues while T_{RM} also lack expression of T-BET and EOMES. Thus, how HOBIT fits into the picture as a key regulator not completely clear. HOBIT expression in man occurs at lower levels on both circulating and tissue resident CD8⁺ T cells but is strongly expressed in natural killer (NK) cells suggesting that its role in defining T_{RM} is not universal across species [27]. T_{RM} cells in humans appear to depend more closely on factors such as NOTCH-1 and HIF-1 α , or organ-specific factors such as RUNX3, BATF (Basic Leucine Zipper *ATF*-Like Transcription Factor), AHR (aryl hydrocarbon receptor) and AP-1 as seen in the lung [28]. RUNX3 has been shown to regulate circulating effector T cells but also directs the differentiation and maintenance of CD103⁺ T_{RM} cells [29]. Both NOTCH-1 and AHR are necessary for the maintenance of T_{RM} cells [17,28,30]. Collectively, multiple transcription factors have a significant impact on the development of T_{RM}, and true master regulators have not yet been fully established. Nevertheless, a distinct set of genes, overlaid by local tissue influences, may universally define these cells (Figure 1).

T_{RM} also appears to have important roles in tumors, particularly those lodged in peripheral tissues. This is well illustrated by several recent studies that demonstrate a positive association between T_{RM} cells and an enhanced clinical outcome for melanoma, breast tumors, lung adenocarcinoma, ovarian carcinoma and the efficacy of cancer vaccination [7,31–36]. Tumor-infiltrating T cells are a heterogeneous population that include effector, effector memory and T_{RM}. Single cell RNA sequencing of infiltrating CD8⁺ T cell within tumors identified T_{RM} gene signatures that could be clustered based on their level of expression of previously characterized immune checkpoints genes including PD-1, TIM-3, CTLA-4, LAG3 and TIGIT, or mitotic genes [34,35]. In breast cancer, the T_{RM} gene signature was associated with a significantly better prognosis in early stage triple-negative breast cancer, a subtype of breast cancer with an aggressive clinical behavior [35]. The efficacy of anti-PD-1 treatments observed in patients harboring triple-negative tumors [37] suggest that they may rely on PD-1^{high}-expressing T_{RM} cells much more than CD8⁺ non-T_{RM} (CD8⁺CD103⁻) cells as the clinical prognosis was aligned to a significantly enriched baseline T_{RM} gene signature in responders to anti-PD-1 therapy [35]. In lung

adenocarcinoma, three clusters T_{RM} were identified [34]. Strikingly, one cluster expressed many more immune check-point genes than the other two clusters. Interestingly, patients that showed poorer enrichment for T_{RM} genes that included immune check point genes had improved survival outcomes [34]. These studies illustrate the complexity and heterogeneity of tumor-infiltrating T cells even within the T_{RM} subset which has thought to be a relatively homogeneous population. Collectively, these results highlight the central importance of having high quality, rather than a large number of, infiltrating T cells to influence patient survival.

Tethering and letting go

While transcription factors are essential in dictating the developmental pathway of T_{RM} , retention in tissues depends on mechanisms that tightly regulate lymphocyte tethering and egress to ensure stable positioning within tissues and at barrier surfaces for front-line immune protection. The most well studied receptor ligand pairs that establish tethering are CD103 and E-cadherin, and CD49a and collagen [3,16,17*]. Loss of either of these receptor/ligand partners via blocking or genetic deletion results in continuous circulation of cells in the blood without establishment in the tissues. Transforming growth factor (TGF)- β induces CD103 expression and promotes retention of both CD8⁺ memory T cells and salivary gland ILC1, but not liver or gut, ILC1 [17*,23,38,39]. The capacity to 'leave' tissues is dictated by receptors such those in the S1PR family. S1PR1 mediates chemotaxis of T cells toward sphingosine-1-phosphate found in lymphatic endothelial cells and efferent lymph [40]. S1PR1 is antagonized by CD69 which assists in maintaining T_{RM} in the skin epidermis and lungs [17*,41]. It is regulated by multiple transcription factors including HOBIT/BLIMP1, KLF2 and RUNX3 [18,28,29*,42,43].

Similar to T_{RM} , ILC appear to be almost exclusively tissue-resident at steady-state. Under parabiotic conditions exchange between conjoined mice is negligible even two months after surgery. This is in stark contrast to T cells which show ~50% interchange between the respective hosts [13*,44*]. Despite this, ILC exhibit an array of chemokine and cytokine receptors that suggest they have the capacity to become mobile and may not be permanently resident. NK cells express S1PR5, which is regulated by T-BET, and is critical for the egress of NK cells from the bone marrow and lymph nodes [45–47]. L-selectin, or CD62 ligand (CD62L), is expressed by most lymphocytes including naïve and memory CD8⁺ T cells but on activation is cleaved as on effector T cells which allows them to enter the circulation and migrate to non-lymphoid tissues and sites of inflammation. Similarly, the ability of NK cells to return to lymphoid tissues is dependent on CD62L expression. A subset of activated or inflammatory KLRG1⁺ST2 (IL-33 receptor)^{low} ILC2,

are highly responsive to stimulation with IL-25 or helminth-induced activation and can develop into circulating cells [44*]. These cells express the receptor S1PR5 similar to NK cells and exhibit SIP-mediated chemotaxis enabling mobilization into the circulation and deposition in distant tissue sites [45]. The failure to detect migration in ILC2 previously might be attributable to the use of antibiotics that potentially downregulate the ability of microbes to induce homing receptors [13*]. ILC2 can also be mobilized from bone marrow in response to a fungal aeroallergen challenge with *Alternaria* extract through ST2-dependent downmodulation of CXCR4 [48] while chronic allergen exposure promotes an increase in blood and lung ILC2 through a β 2 integrin-dependent manner [49]. Using the elegant Kaede transgenic mouse in which photoconversion of fluorescent dyes can be induced to tag individual ILC, ROR γ t⁺ ILC3 were shown to migrate from mucosal and peripheral sites to local secondary tissues. For LT α i-like ILC3 at least, this depended on CCR7 expression [50] while positioning of ILC3 in cryptopatches relies on RANK/RANKL-mediated signals [51]. Thus, stability of ILC in tissues is likely to be important to their protective functions at steady-state while tissue-specific and systemic inflammatory cues drive multiple signals to mobilize ILC populations to effector sites paralleling the induction of adaptive immune cells (Figure 2).

Functional changes in tissue residency

The metabolic state of immune cells is essential for their survival and to repopulate distinct tissue sites. These metabolic constraints on glucose and oxygen availability are reflected in the adaptations of lymphocytes in different tissue microenvironments. Effector and effector memory T cells rely on aerobic glycolysis to catabolize glucose but T_{RM} are positioned within the parenchyma of tissues such as the lung, epidermis or solid tumors that are typically low in glucose and require different energy programs. P2RX7, a receptor for adenosine triphosphate (ATP), is vital for the generation and maintenance of memory cells, specifically memory precursor and central memory T cells which exhibit higher energy requirements compared with effector and effector memory T cells. Long-lasting memory cells express higher levels of P2RX7 to sense extracellular ATP, a signal released by damaged cells. In preparation for the increase in energy requirement, metabolic reprogramming occurs, whereby the mitochondria is modified for more efficient oxidative phosphorylation. T_{RM} also have high expression and dependency on the P2RX7 receptor for their development and maintenance [52*]. It is not known whether P2RX7 regulates their metabolism, but it is likely that maintenance of long-lived memory and T_{RM} will have similar metabolic requirements and regulatory mechanisms.

In the skin, T_{RM} preferentially use the free fatty acid transporters FABP4 and FABP5 to metabolize fatty acids. These transporters are also required for the survival of

T_{RM} in both normal and psoriatic skin and lung [53]. Fatty acids are a substrate found in the skin, lung, the gut through the action of bacteria [54] and are abundant in melanomas. They are important contributors to energy generation but also enhance molecules such as acetyl-CoA which drive the production of cytokines such as IFN- γ [55]. However, conditions in different tissues vary both anatomically and temporally as in the case of increasingly hypoxic conditions that might emerge in the setting of tumors as they grow in size. This alters the oxygen concentration which is essential for fatty acid catabolism. Thus, it is likely that tissue-resident immune cells have specific but multiple strategies for coping with the dynamic environment in which they reside (Figure 2). Peroxisome proliferator-activated receptor γ (PPAR γ), also known as NR1C3 (nuclear receptor subfamily 1, group C, member 3) regulates glucose metabolism and is expressed by a variety of immune cells including ILC2, NKT cells and Treg cells. Treg cell expansion in adipose tissue is directly induced by IL-33 [56] and ILC2 express both PPAR γ and ST2 [57,58] but whether this is a sensing pathway for ILC2 in adipose tissue or other sites is yet unknown. It seems likely, however, that the ability of ILCs to finely sense metabolites would be critical to maintain tissue homeostasis.

Conclusions

Here we have outlined the parallels that exist between the innate and adaptive immune cells in their distribution and behavior. Collectively, they form a complex but highly synergistic network although key pieces of the puzzle that coordinate their regulation and interactions remain unknown. Progress on these elements will be essential to our understanding. Firstly, T_{RM} exhibit a distinct transcriptional profile but it is not yet known whether they arise from a distinct progenitor separate from effector and memory T cell lineages. Secondly, are tissue-resident lymphocytes replenished from local proliferation *in situ* or do they rely on circulating progenitors that can re-seed the interstitial compartments? In ILCs, proliferation has been thought to occur at a low rate within the tissues themselves leading to the idea that the numerical maintenance of the population occurs in isolation from circulating cells. This model, however, has been fortified by the paucity of evidence supporting circulating ILCs which has in part been blindsided by the lack of recognition that circulating cells may 'look' completely different from the mature populations. Thirdly, we need to think broadly about the identity of circulating precursors as they may not mimic the resident populations. Thus, a complete rethink of how we tackle resolving the elements of the tissue-tissue and tissue-vascular migratory pathways is warranted. Fourth, the factors that establish life-long maintenance in tissues, as opposed to long-term positioning followed by mobilization are poorly resolved. Whether these are similar or different for ILCs and T cells is not yet known, nor how collaboration is

mediated between two distinctly different resident immune cell subsets. Fifth, while some key transcriptional regulators have been elucidated, the complete blueprint remains to be dissected. Transcription factor such as HOBIT, which is a key coordinator in the murine lineages, are differently expressed in human tissues suggesting it is not a master regulator. In addition, how the overlay of different tissue microenvironments modulates the transcriptional architecture remains to be uncovered. Addressing these and related questions are likely to shed new light on our understanding of the immune cell networks that protect our borders.

Conflict of interest statement

Nothing declared.

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Bach2: An Instrument of Heterogeneity for Long-Term Protection

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Generation of functionally diverse effector and memory killer T cells is essential for immediate and long-term protective immunity. Herndler-Brandstetter et al. (2018) report that *Bach2* promotes functional plasticity of effector T cells and the transition into the long-term memory compartment by regulating the expression of the inhibitory receptor KLRG1.

A hallmark of CD4⁺ T cells is their high degree of developmental plasticity and functionally heterogeneous subsets. In contrast, CD8⁺ T cells have long been thought to differ from their CD4⁺ counterparts, having carved out a very unidirectional path to form either effector or memory cells with a highly restricted ability to adopt new fates. However, it has begun to emerge that CD8⁺ T cells can also exhibit significant heterogeneity although the mechanisms regulating these fate transitions are only just starting to be teased apart.

Effector cells appear early in infection. They upregulate the expression of the inhibitory receptor killer cell lectin-like receptor G1 (KLRG1) and concomitantly downregulate interleukin-7 receptor (IL-7R). These cells are highly reactive and produce abundant cytolytic molecules

such as granzymes and perforin to directly attack pathogen-infected cell. Their capacity to self-renew is relatively limited but a proportion of these cells go on to form effector memory (Tem) cells and subsequently express variable levels of KLRG1. Concurrently, CD8⁺ T cells that express the surface molecules CD62L and IL-7R form central memory (Tcm). These are long-lived and functionally quiescent but molecularly poised to rapidly respond to re-exposure to pathogens to generate effector cells. The receptor KLRG1 has conventionally been used to define terminally differentiated CD8⁺ T cell subsets as they arise in an infection. Nevertheless, how its expression relates to, or regulates, T cell fate outcome has not been fully defined. In this issue of *Immunity*, Herndler-Brandstetter et al. identify that *Bach2* is a key regulator of

the differential expression of KLRG1 on the surface of CD8⁺ T cells and dictates their entry to the effector or memory pool. This plasticity facilitates effective protection against pathogens depending on the environmental stimuli received.

During an infection, antigen-specific CD8⁺ T cells undergo profound clonal expansion to form a heterogeneous population (Figure 1). After infection, 90%–95% of these cells die by apoptosis leaving a minor population of cells that persist and generate long-lived memory T cells. A long-standing debate in the field has centered on how these different fates emerge. For example, do memory cells derive solely from naive T cells that give rise to a discrete group of memory precursor cells early in infection? Alternately, do effector cells arise directly from naive T cells immediately after activation and



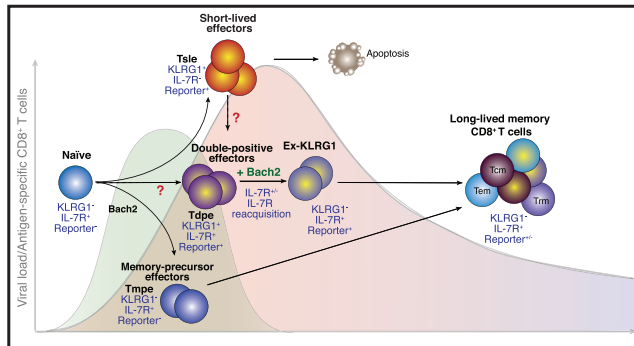


Figure 1. KLRG1 Triggering Shapes CD8⁺ T Cell Differentiation

Pathogen infection drives the rapid proliferation of naive CD8⁺ T cells toward the generation of short-lived effector (Tse) cells that can kill infected cells. The inflammatory stimuli result in differential expression of KLRG1 demarcating KLRG1⁺IL-7R⁺ double-positive effector (Tdpe) and memory-precursor effector (Tmpe, KLRG1⁺IL-7R⁺) cells. Both these cell types are detectable around 3–5 days after infection although it is not clear whether the Tdpe cells originate from naive T cells or another intermediate subset. All CD8⁺ effector cells report KLRG1 activity when tracked by lineage tracing. As the pathogen is eliminated, inflammatory signals are downregulated, leading to contraction of T cell populations and the loss of Tse via apoptosis. Tdpe and Tmpe remain long after the virus is cleared. Tmpe retain the multipotent capacity to differentiate into multiple types of long-lived memory cells including central memory, effector memory, and resident memory CD8⁺ T cells. Tdpe appear to be the major source of ex-KLRG1 cells and exhibit significant developmental plasticity orchestrated by Bach2-mediated downregulation of KLRG1. Ex-KLRG1 cells appear to have similar potential to develop into the various memory populations as Tmpe.

then “dedifferentiate” to populate the memory compartment after pathogen clearance? A recent breakthrough has shed light on some of these questions regulating the CD8⁺ T cell differentiation pathway (Youngblood et al., 2017). Using DNA methylation analyses, Youngblood et al. (2017) showed that a subset of virus-specific effector cells could be epigenetically remodeled by the methyltransferase Dnmt3a. This allowed memory T cells to develop from effector T cells demonstrating the crucial interconnection between these two populations and the capacity for the effector subset to potentially continuously seed the memory compartment. Epigenetic remodeling by a number of epigenetic writer proteins is instrumental for remodeling the ability of a T cell to acquire or adopt different fates. How this type of transition is mediated through surface receptors in cells during a response has not been elucidated.

Previously, KLRG1, which recognizes the classical cadherin family, was thought to be largely dispensable for both virus-induced NK cell responses and CD8⁺ T cell differentiation (Gründemann et al., 2010). It was speculated that this receptor only exhibited weak inhibitory function

that might easily be overcome by the strong stimulus of a virus infection such as lymphocytic choriomeningitis virus (LCMV). Indeed, strong T cell receptor and inflammatory stimuli induce high expression of KLRG1 (Joshi et al., 2007; Xin et al., 2016). Not content with this explanation, Herndler-Brandstetter et al. (2018) generated a novel *Klrp1^{Cre}* reporter system for KLRG1 (Herndler-Brandstetter et al., 2018). This allowed the simultaneous tracking of a fluorescent reporter protein (eYFP or tdTomato) tag driven by *Klrp1* together with KLRG1 surface expression via antibody staining. Tracking the CD8⁺ T cell response to the bacterial *Listeria monocytogenes* infection revealed a surprising discordance between these two parameters. Initially, Reporter⁺ cells were all KLRG1⁺ as was expected but as the infection progressed (day 6) cells began to emerge that had lost KLRG1 expression but remained Reporter⁺ (Figure 1). These were termed “exKLRG1” cells, reflecting the historical imprint of KLRG1 expression as they progressed along the differentiation pathway. Strikingly, these cells were identified in the blood and comprised 20%–35% of the pathogen-specific memory pool.

When tested, they efficiently developed into long-lived memory cells and did not appear to differ in their memory potential from the classical KLRG1⁺Reporter⁺ CD8⁺ T cells, similar to stem memory (Tscm) cells (Restifo and Gattinoni, 2013), that were normally thought to form memory. Fate mapped exKLRG1 cells could give rise to all CD8⁺ T cell memory subsets indicating that they could readily replenish the Tcm, Tem, and tissue-resident memory (Trm) lineages. In the case of Trm, this also implies that the population is indeed heterogeneous and arises from multiple sources.

Molecular analyses demonstrated that exKLRG1 effector cells displayed open chromatin at the effector-related genes *Klrp1* and *Cx3cr1* and memory-related genes *Irf7* and *Bach2*. This correlated with lower expression of CX₃CR1 in exKLRG1 cells but showed that only KLRG1⁺ and exKLRG1 cells could maintain CX₃CR1 expression during early memory T cell formation. Despite the loss of surface KLRG1, exKLRG1 cells retained some features of their effector origin including enhanced cytotoxic and interferon-γ potential together with higher responsiveness to IL-12. Subsequently, these exKLRG1 cells have the capacity to mediate immune protection, similar to KLRG1⁺ effector cells. Collectively, this imparts exKLRG1 cells with all the memory capability normally associated with conventional KLRG1⁺Reporter⁺ CD8⁺ T cells (Figure 1). Furthermore, the open chromatin state in multiple effector gene regions of the exKLRG1 cells may provide an early response advantage and flexibility in responding to a variety of inflammatory stimuli at the body's surfaces. What is not clear in this system is whether tagging via the eGFP-Cre fusion protein depends on a T cell achieving a cumulatively high level of stimulus before fluorescence is achieved. Indeed, only KLRG1^{hi} expressing cells efficiently expressed the Cre protein and cells with intermediate KLRG1 expression had heterogeneous Cre expression. Thus, the number of fate mapped cells may be an underestimate of the true contribution of exKLRG1 cells to memory populations and their functions.

The transcription factor *Bach2* is a highly conserved repressor that controls the terminal differentiation and maturation

of both T and B lymphocytes (Kometani et al., 2013; Roychoudhuri et al., 2016). Loss or mutation of *Bach2* is associated with severe autoimmune disease (Afzali et al., 2017; Roychoudhuri et al., 2013) but transient downregulation in effector cells is essential for them to fully execute their differentiation program. In contrast, memory populations retain high expression. The AKT-mTOR-Foxo1 pathway regulated this differentiation step and was necessary to promote the generation of exKLRG1 memory cells, a process that could be inhibited using Rapamycin. *Bach2* has been previously thought to mediate its main action through stimulation of the T cell receptor. Now, these new findings highlight that *Bach2* plays multiple roles in dictating the fate outcome of CD8⁺ T cells through mechanisms not directly linked to T cell receptors. More broadly, this suggests that a number of previously unappreciated receptors and signaling molecules may play key roles in fine tuning the dose of transcription factors such as *Bach2* and thus the fate outcomes of T cells. One such receptor may be IL-7R as exKLRG1 cell development occurs concurrently with the reacquisition of IL-7R expression. Furthermore, exKLRG1 cells appear to only develop from KLRG1⁺IL-7R⁺ double-positive effector (Tdpe) T cells and not KLRG1⁺IL-7R⁻ short-lived effector (Tsle) cells (Figure 1). Yet during the early stages of exKLRG1 development, not all exKLRG1 cells express IL-7R. Further work will be required to understand the

temporal regulation of these receptors, whether the upregulation of IL-7R is a prerequisite for the downregulation of KLRG1, and how this influences CD8⁺ T cell fate choices. How this plays out in a chronic infection or inflammation was not addressed in this study. It will be important to elucidate the factors responsible for the induction of KLRG1 as a key determinant of fate outcome. Previously, high expression of KLRG1 has been strongly associated with senescence and exhaustion but a role in the dynamic regulation in T cell fate outcomes has not been appreciated.

Collectively, these findings demonstrate that CD8⁺ T cells exhibit significant phenotypic and functional diversity in generating memory populations. Moreover, it highlights the multiple pathways that have evolved to sustain a long-term memory pool over the course of a lifetime and through the assaults of multiple infections.

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Shaping Innate Lymphoid Cell Diversity

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Innate lymphoid cells (ILCs) are a key cell type that are enriched at mucosal surfaces and within tissues. Our understanding of these cells is growing rapidly. Paradoxically, these cells play a role in maintaining tissue integrity but they also function as key drivers of allergy and inflammation. We present here the most recent understanding of how genomics has provided significant insight into how ILCs are generated and the enormous heterogeneity present within the canonical subsets. This has allowed the generation of a detailed blueprint for ILCs to become highly sensitive and adaptive sensors of environmental changes and therefore exquisitely equipped to protect immune surfaces.

Keywords: innate immunity, differentiation, gene expression, immune protection, innate lymphoid cell

INTRODUCTION

The importance of innate immunity has been recognized for many years. These cells play a key role in rapid responses to pathogens to protect the mucosal and external surfaces of the body. Natural killer (NK) cells and lymphoid tissue-inducer (LTi) cells are the founding members of the innate lymphoid family with the former identified more than 40 years ago. Over the past 10 years, a number of new family members have been discovered revealing an entire network of innate cells that complement the adaptive immune network. These cells went largely unrecognized for several decades which begs the question as to how they were overlooked. In this review, we summarize the current knowledge on innate lymphoid cell (ILC) differentiation and critically discuss the key challenges in the field in understanding ILC homeostatic regulation.

CANONICAL ILC SUBSETS

The ILC family is divided into three major groups: group 1 ILCs (ILC1s) which includes NK cells and ILC1s that produce interferon- γ (IFN- γ) and depend on the transcription factors Eomesodermin (Eomes) and T-bet; group 2 ILCs (ILC2s) that secrete IL-5 and IL-13 and are characterized by Gata3 expression; and group 3 ILCs (ILC3s) that express the RAR-related orphan receptor, Ror γ t, and includes LTi cells and multiple subsets of ILC3s capable of producing IL-17 and/or IL-22 (Figure 1A). The ILCs are distinguished from adaptive immune cells by their lack of germline recombined antigen-specific receptors and generalized lack of lineage-specific markers normally used to distinguish B and T cells. ILCs are not thought to traffic through tissues and are often referred to as “tissue-resident” (1) but their precursors can be isolated in humans from blood (2) indicating that these cells are not completely sessile throughout their life cycle. At the very least, they transit around the body to achieve their strategic positioning close to barrier surfaces to allow them to respond rapidly to local environmental changes.

Innate lymphoid cell subsets were initially categorized based on their phenotype, function, and the key transcriptional regulators that drive their development. In many aspects, these subsets mirror CD4⁺ T cell subsets although some populations such as ILC1s have been quite difficult to position due to their lack of specific distinguishing markers (3, 4). The current classification model has served as an important framework to focus our thinking around canonical subset classifications. However, recent analyses of elegant reporters and genomic probing of individual cells has revealed that ILCs

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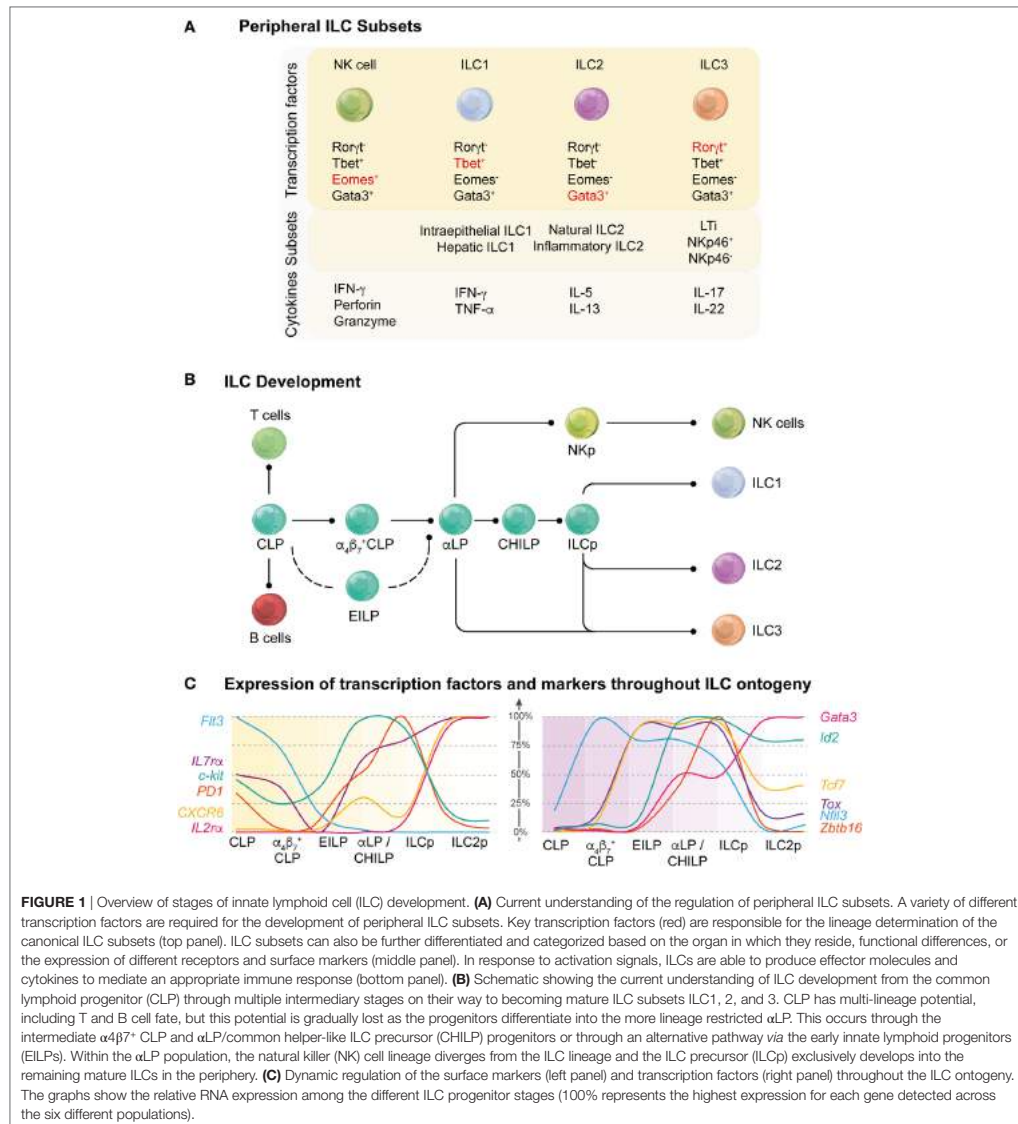


FIGURE 1 | Overview of stages of innate lymphoid cell (ILC) development. **(A)** Current understanding of the regulation of peripheral ILC subsets. A variety of different transcription factors are required for the development of peripheral ILC subsets. Key transcription factors (red) are responsible for the lineage determination of the canonical ILC subsets (top panel). ILC subsets can also be further differentiated and categorized based on the organ in which they reside, functional differences, or the expression of different receptors and surface markers (middle panel). In response to activation signals, ILCs are able to produce effector molecules and cytokines to mediate an appropriate immune response (bottom panel). **(B)** Schematic showing the current understanding of ILC development from the common lymphoid progenitor (CLP) through multiple intermediary stages on their way to becoming mature ILC subsets ILC1, 2, and 3. CLP has multi-lineage potential, including T and B cell fate, but this potential is gradually lost as the progenitors differentiate into the more lineage restricted α LP. This occurs through the intermediate $\alpha\beta\gamma^+$ CLP and α LP/common helper-like ILC precursor (CHILP) progenitors or through an alternative pathway via the early innate lymphoid progenitors (EILPs). Within the α LP population, the natural killer (NK) cell lineage diverges from the ILC lineage and the ILC precursor (ILCp) exclusively develops into the remaining mature ILCs in the periphery. **(C)** Dynamic regulation of the surface markers (left panel) and transcription factors (right panel) throughout the ILC ontogeny. The graphs show the relative RNA expression among the different ILC progenitor stages (100% represents the highest expression for each gene detected across the six different populations).

are dynamically tuned resulting in enormous heterogeneity (5–8). Potentially, this property would enable ILCs to respond to diverse stimuli in “real time.” It is widely accepted that CD4⁺ T cell subsets display extraordinary plasticity allowing them adapt to a broad spectrum of inflammatory signals, but such a program among ILCs has not been appreciated until recently. Indeed, the capacity for ILCs to exhibit a highly flexible program may be an essential

element for tuning ILCs to ensure responsiveness to continuous changes in signals encountered at mucosal barriers.

Recent findings in the field have identified conceptually new ideas about how the immune system is regulated and how the innate arm might contribute to this process. For example, the ILC network forms an extensive interface between the external environment and the adaptive immune system. Their regulation

is highly dynamic and relies on a highly integrated molecular signaling network, resulting in heterogeneity and plasticity. Finally, it appears to be highly complementary to the adaptive immune system providing a fail-safe mechanism for ensuring immune protection and repair processes. Excitingly, we are only just beginning to understand how this network of cells might work.

CORE TRANSCRIPTION FACTORS ESTABLISH THE ILC DIFFERENTIATION FRAMEWORK

Innate lymphoid cells arise from the common lymphoid progenitor (CLP) through multiple intermediary stages with changes in surface expression of key surface molecules and the temporal regulation of transcriptional regulators to become mature ILC1, 2, and 3 subsets (Figure 1B). Induction of the downstream molecular program involves the induction of $\alpha_4\beta_7$, which identify the $\alpha_4\beta_7^+$ CLP (9) [also called α LP1 (10)] follow by the downmodulation of Flt3 expression leading to the emergence of the α lymphoid progenitor (α LP, also called α LP2) (10, 11). While the α LP can

generate all ILC subset, a subpopulation seems to have lost the ability to generate NK cells and named common helper-like ILC precursor (CHILP) (12). The distinction between the α LP and CHILP is not clear as they appear to be very highly similar in their surface marker or transcription factor expression. Finally, the induction of promyelocytic leukemia zinc finger (PLZF) in the ILC precursor (ILCp) mark the bifurcation between LTi and NK cells with the other ILC1, 2, and 3 subsets (13). Their fate is guided by lineage-determining transcription factors that are also involved in specifying different subsets of T cells. Transcription factors control multiple aspects of the development of immune cell lineages including proliferation, migration, metabolism, and effector function (Table 1). Some transcription factors play unique roles in defining the fate of early progenitors, but increasingly it is emerging that overlapping and synergistic contributions by transcription factors may be critical in setting the threshold for fate decisions and the function of an individual cell. A major challenge for the field now is to understand the combinatorial interactions between transcription factors and how they define ILC developmental choices.

The emergence of the innate cells from the CLP and the divergence of this pathway away from the adaptive lineages is

TABLE 1 | Requirement for different transcription factors during innate lymphoid cell (ILC) development.

Gene	Progenitors	Mature cells				Mouse phenotype
		Natural killer (NK) cells	ILC1	ILC2	ILC3	
Nuclear factor, interleukin 3 (<i>E4bp4</i>)	✓	–	–	–	–	Loss of α LP, small and fewer Peyer's patch; normal lymph nodes, significantly reduced NK cells (14, 15)
Inhibitor of DNA binding 2 (<i>Id2</i>)	–	✓	n.d.	n.d.	n.d.	Complete loss of lymph node and Peyer's patch formation, significantly reduced NK cells in KO and reduced IL-15 responsiveness in cKO (16, 17)
RAR-related orphan receptor gamma, ROR γ t (<i>Rorc</i>)	✓	–	–	–	✓	Complete loss of lymph node and Peyer's patch formation, loss of all ILC3s (18)
B-cell lymphoma/leukemia 11B BCL11B (<i>Bcl11b</i>)	✓	–	–	✓	–	Impaired function of ILC2 via dysregulation of Gfi1 and IL-33 receptor (ST2) (19–21)
Thymocyte selection-associated high mobility group protein (<i>Tox</i>)	✓	n.d.	n.d.	n.d.	n.d.	Normal NKp, loss of lymph node, and Peyer's patch formation, reduced NK cells, loss of mature NK cells (22–24)
ETS proto-oncogene1, ETS1 (<i>Ets1</i>)	✓	✓	n.d.	✓	n.d.	Reduced NK cells, hyporesponsive to IL-15 and impaired killing and degranulation, impaired ILC2 development.
T cell-specific transcriptions factor 1 (<i>Tcf7</i>)	✓	–	n.d.	n.d.	n.d.	Small Peyer's patches, reduced NK cells in bone marrow but normal peripheral compartment (25, 26)
Promyelocytic leukemia zinc finger (<i>Zbtb16</i>)	✓	–	✓	✓	✓ [not lymphoid tissue-inducer (LTI)]	Not required in peripheral NK cells of LTI cells (13)
GATA-binding protein 3, GATA3 (<i>Gata3</i>)	✓	✓/–	✓	✓	–	Loss of GATA3 impairs NK cell maturation (27–29)
Growth factor independent 1 transcriptional repressor, GFI1 (<i>Gfi1</i>)	n.d.	n.d.	n.d.	✓	n.d.	Regulates GATA3 expression together with responsiveness via IL-33 receptor (ST2) (30)
<i>Tbx21</i>	–	✓	✓	n.d.	✓	Reduced NK cells, ILC1 and NCR $^+$ ILC3; reduced mNK cells
<i>Eomesodermin</i>	–	✓	n.d.	n.d.	n.d.	Reduced NK cells and loss of mNK
<i>Pdcd1</i>	–	✓	n.d.	✓	n.d.	Normal secondary lymphoid tissue formation (5, 9)

✓ required for development and/or maintenance; –, not required for development and/or maintenance; mNK cells, mature NK cells; KO, germline deletion; cKO, conditional deletion; n.d., not determined.

an extremely controlled process that involves the coordinated actions of several transcription factors. Detailed analysis of the transcriptional landscape of the ILC development from the earliest precursor to the committed cells has revealed that regulation of the different developmental stages is highly dynamic. The sequential expression of nuclear factor interleukin 3 (NFIL3), inhibitor of DNA binding 2 (ID2), thymocyte selection-associated high mobility group box protein (TOX), and GATA-binding protein 3 (GATA3) establishes the framework for ILC differentiation (7, 9) (Figure 1C). ID2 counterbalances the effects of E proteins to direct cell choices away from T and B cell outcomes (31). Other transcription factors such as EOMES, PLZF, transcription factor 1 (TCF-1), and RUNX influence subset divergence. We now have new insight to the key factors that determine the fate outcome of progenitor cells under steady-state conditions. However, it still remains unclear how higher order genomic architecture establishes and maintains the differentiation program.

THE EARLY REGULATORS: A QUARTET

Two major transcription factors, NFIL3 and TOX, have emerged as critical initiators of development of early α LP. Induction of NFIL3 appears to be the critical initiating step in driving the α LP toward the ILC lineage (7, 9). NFIL3 is induced in α 4 β 7⁺ CLP, but the factors responsible for this induction have yet to be elucidated (Figures 1B,C). Concomitantly, TOX and ID2 are only expressed at low levels in the CLP but TOX expression rapidly increases in the early innate lymphoid progenitors (EILPs) while ID2 levels remains low until the late α LP and common helper ILC (CHILP) stages (12, 32–34). Nevertheless, the expression of ID2 has two patterns in ILCps; the first phase in which ID2 is expressed at low levels (and the E protein E2A is concomitantly high) and does not appear to be required for ILC development, and the second in which ID2 is strongly upregulated with concurrent downregulation of E2A and is essential for ILC lineage progression (9, 31). Indeed, while the deletion of NFIL3 blocks the development of the α 4 β 7⁺CLP, ID2 deletion has does not appear to affect the development of the α LP. However, all cells derived from the ILC progenitor are absent in ID2^{-/-} mice suggesting that ID2 is more important in late differentiation and in maintaining the long-term identity of ILCs (16, 35). Loss of ID2 in ILCs has also been shown to repress genes belonging to the stem cell program such as *Gfi1b*, *Tal1*, *Lmo2*, *Gata2*, and *Hhex* (9).

Precisely how NFIL3 and TOX regulate the development of the ILC progenitors remains unclear. TOX-deficient progenitors appear to lack the expression of key factors thought to be essential for ILC development including *Gata3*, *Rora*, *Rorc*, *Tcf7*, and *Zbtb16* (22). Despite this, *Nfil3* expression, which could be regulated by TOX, was not found to be different from that of wild-type cells. Thus, more work will be necessary to ascertain whether NFIL3 is a direct target of TOX or not. NFIL3 has been shown to directly bind to ID2; however, it is not clear that this binding is actually responsible for the induction of ID2 (36). In ILC progenitors, ID2 expression has been found to be reduced when NFIL3 is deleted in the hematopoietic compartment. However, in mature cells, ablation of NFIL3 did not alter ID2 expression suggesting that the developmental stage of the cell influenced the interactions

(37, 38). Furthermore, overexpression of either ID2 or TOX in NFIL3-deficient CLPs revealed that both transcription factors could at least partially rescue ILC development independent of NFIL3. Therefore, it is likely that the key role of NFIL3 is to promote the emergence of ILCs by induction of the expression of these two key transcription factors.

EXPRESSION PATTERNING OF NFIL3 AND ID2 ESTABLISHES THE LANDSCAPE FOR ILCs

Although several details of the fine tuning of NFIL3 remain unanswered, the timing and action of NFIL3 is very interesting. *Nfil3*^{flax/flax} mice crossed to the *Id2*^{ERT2Cre} strain generated a model in which deletion of NFIL3 could be timed relative to ID2 (9). This approach demonstrated that NFIL3 expression preceded that of ID2 but that surprisingly, deletion of NFIL3 in ID2⁺ cells either *in vivo* or *in vitro* did not affect the subsequent development of any of the ILC subsets. These findings are consistent with earlier work showing that NFIL3 was not required for the maintenance of mature NK cells (14). What was particularly unexpected, however, in the study, was the very transient nature of the NFIL3 expression which was both necessary and sufficient to promote ILC development. Indeed, only a short pulse of NFIL3 expression in the progenitors was required and it subsequently rapidly decreased as ILC progressed through each developmental stage (7, 9) and is repressed in mature cells in the periphery (15).

This pattern of short-lived expression found in NFIL3 may represent a more generalized pattern for orchestrating the complex integration of different transcriptional signals. This expression pattern has also been reported for *Zbtb16* (that encodes PLZF), another transcription factor important for ILC development but which was originally implicated in NKT cell development (39). Mature ILCs do not express PLZF; however, lineage tracing experiments revealed that 60–75% of ILCs exhibited a fluorescent imprint marking their previous expression of PLZF during development (13). In this setting, only ~5% of LTi cells and ~20% of NK cells were labeled indicating that bifurcation of these lineages from other ILC subsets occurred before the induction of PLZF. The role of the transient expression of PLZF in ILCp is not known. Using the PLZF-reporter mice, Constantinides et al. (13) also showed that PLZF is expressed only transiently. This allowed the identification of the ILCp, but PLZF was subsequently downregulated after this stage. PLZF does not appear to be absolutely required for ILCs as deletion of PLZF results in ~4-fold reduction in the number of ILCs that develop in contrast to ablation of NFIL3 which results in more than a 10-fold reduction. PLZF is expressed after NFIL3 coordinately with E2A and ID2 resulting in a stepwise progression through the early progenitor stage to generate E2A^{hi}PLZF^{hi}ID2^{lo}, E2A^{hi}PLZF^{hi}ID2^{lo}, E2A^{lo}PLZF^{hi}ID2^{hi}, and E2A^{lo}PLZF^{hi}ID2^{hi} expressing cells (31). In competitive situation, PLZF-deficiency appears selective, mainly affected ILC2s in the lamina propria and ILC1s in the liver (13). It was notable that ~40% of the ILC2s in this study were not labeled in these tracing experiments, so it

remains possible that they could be derived from an alternative pathway that is independent of PLZF.

LOSS OF IL-7R EXPRESSION: A NEW PROGENITOR OR AN ALTERNATIVE PATHWAY?

The identification of the early ILC progenitor in the bone marrow, or EILP, noted for its high expression of TCF-1, has raised some questions around the linear model of ILC development (25). This EILP lacks B or T cell potential but can generate all ILC subsets including the NK cells similar to the α LP progenitor capacity, but it differs from the other precursors described as it lacks IL7R α expression (25). One possibility is that IL-7R α expression is lost between the α LP and the ILCp stages. Such changes could be regulated *via* posttranslational modifications though the biological relevance for such downregulation is unclear. A second possibility is that the EILP is a precursor for an alternative pathway for ILC development. EILPs express high levels of NFIL3, TOX, and TCF-1, low levels of ID2 and PLZF is almost undetectable. Interestingly, the EILPs are not affected by ID2 deletion, thus the EILPs appear to be very similar to the IL-7R-expressing α LP. A comparison between these two cell types may help to better understand the relationship between the IL7R α^+ and IL7R α^- ILCps and define the factors that regulate IL-7R signaling in ILCs that is essential for their development. To date, TCF-1 and ID2 are known to be upregulated in EILP but additional transcriptional requirements of this progenitor have not been investigated. Therefore, whether EILP represents an intermediate stage of the ILCp, or an alternative precursor that does not fit in the current linear model of ILC development, remains an open question.

HETEROGENEITY AND PLASTICITY OF ILCs ARE KEY TO MAINTAIN HOMEOSTASIS

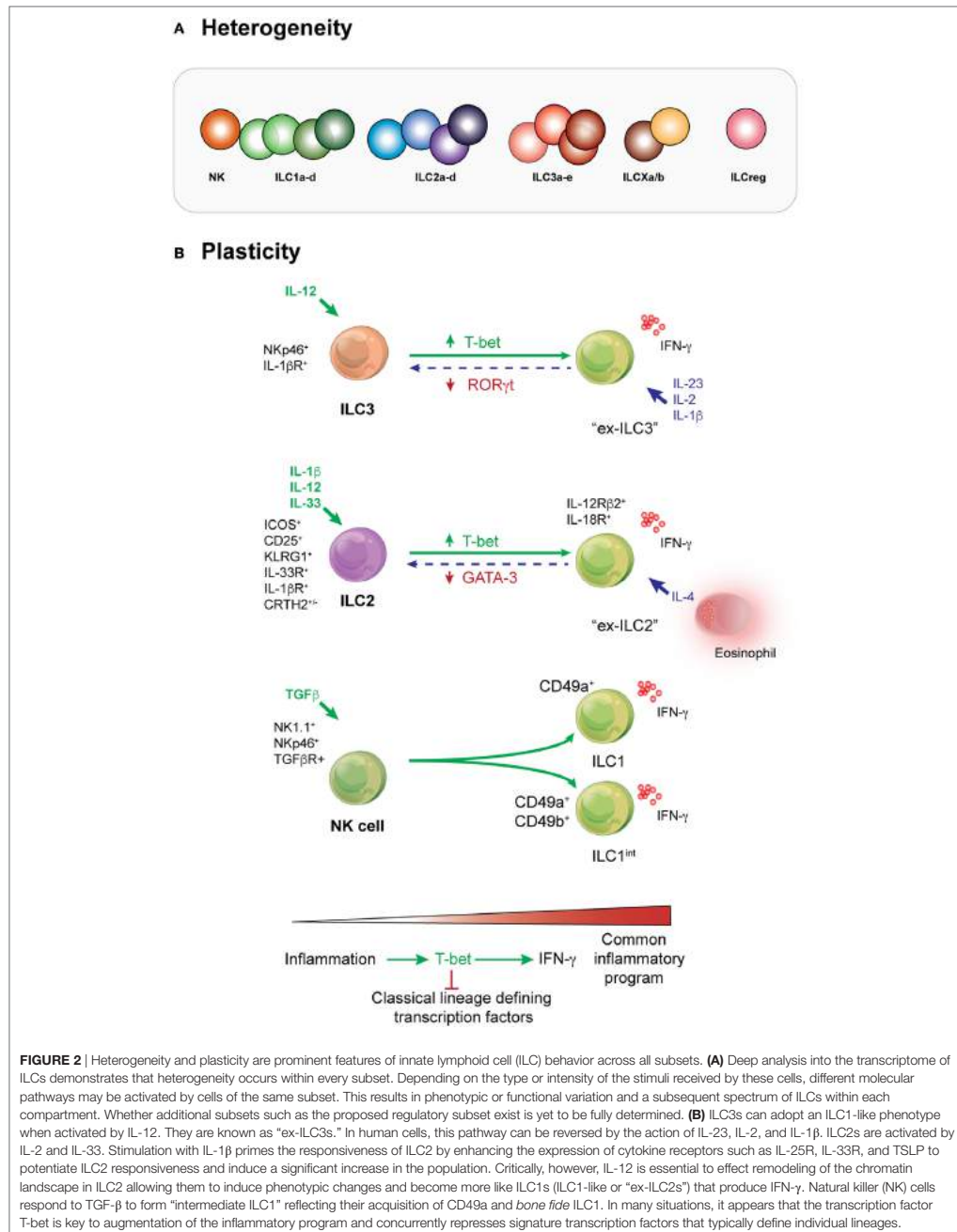
Initial categorization of ILC subsets relied on cytokines and effector molecules they produced combined with signature transcription factors that appeared to be central regulators of the different subsets. The patterns found in ILCs were aligned to the categorization of CD4 $^+$ T cell subsets. Unlike CD4 $^+$ T cells, however, it quickly emerged that several ILC phenotypes did not necessarily neatly fit into the unified nomenclature coined in 2013 with subsets described as being “like” other subsets (40). The full spectrum of ILC heterogeneity has recently been unveiled using comprehensive single-cell sequencing combined with mass cytometry approaches which has allowed the field to bridge between our understanding of ILCs in mouse and man (2, 6, 41, 42) (Figure 2A). This approach revealed that potentially as many as 15 transcriptionally distinct identities could be delineated in the small intestine and that crosstalk orchestrated *via* cytokines such as IL-12 and IL-23 and microbial signals were substantially responsible for mediating this subset plasticity (6). It is undoubtable that these approaches are transforming our understanding of diversity in immune cell subsets but these data also throw up new technical and intellectual challenges in understanding

how diversity arises. Historically, various surface markers have been considered to represent “lineage specificity” but we now recognize that this is seldom the case, challenging that how we interpret complex data and underlying that subsets cannot be defined exclusively by particular markers or transcription factors (43). Nevertheless, it provides a rich landscape for understanding how different stimuli affect the homeostatic balance of ILC subsets. It has been revealed that ILCs exhibit tissue-specific characteristics and this in part reflects alterations in phenotype that can be significantly regulated by responses to inflammation and infection.

The first example of ILC ability to adopt a different phenotype was the identification of ILC3s that downregulated ROR γ t but expressed T-bet, NK1.1, and produced IFN- γ (also known as ex-ILC3s) (12, 44, 45) under chronic stimulation and ROR γ t $^+$ ILC3 that produced IL-17 which were found in the large intestine (46) (Figure 2B). IL-12 was found to be a key driver of this pathway. Similarly, IL-1 β , together with combinations of IL-4, IL-12, and IL-33, can drive ILC2s to adopt an IFN- γ -producing phenotype called ex-ILC2 (47–49). ILC2s can be divided into two subsets: homeostatic or natural ILC2s which typically reside in barrier tissues and respond to IL-33; and inflammatory ILC2s which are generally not found in peripheral tissues at steady-state but respond to IL-25 which induces multipotency (50–53). In this setting, T-bet (encoded by *Tbx21*) was induced, whereas GATA3 was diminished. These studies highlight the variability that can occur in ILCs, particularly *in vitro* as demonstrated for ILC2s (47–49). Indeed, even NK cells can undergo this sort of transformation (54). Signaling through the TGF- β pathway can convert NK cells (CD49a $^+$ CD49b $^+$ Eomes $^+$) into intermediate ILC1 (CD49a $^+$ CD49b $^+$ Eomes $^+$) and *bone fide* ILC1 (CD49a $^+$ CD49b $^+$ Eomes $^{+/-}$) within a tumor microenvironment. Strikingly, these latter ILC1 were disabled in their capacity to control local tumor growth and to prevent metastasis, while NK cells retained their ability to undertake immune surveillance (54). It is striking that once activated through these different pathways, each subset converges on an “IFN- γ -producing ILC1-like” phenotype. This suggests that this fate may represent a common outcome for multiple ILC subsets, even perhaps an essential adaptive program for all ILCs. It remains a challenge, however, to understand how these IFN- γ -producing cells arise and whether it represents a protective response or the first steps to loss of immune control (Figure 2B). Teasing this apart will require much more extensive study particularly in the context of pathogen infection, inflammation, and tumor development.

ARE MORE SUBSETS POSSIBLE?

How ILCs populate tissues after birth or maintain their presence in peripheral tissue is not clear. Our current understanding indicates that ILCs are almost exclusively tissue resident and that they do not routinely circulate throughout the body. This perception is predicated on the findings that during engraftment following irradiation, donor ILCs largely fail to replace ILCs originally found in the host (1). Instead, they depend on local proliferation to expand and replace ILCs, and it is only late in an infection or physiological disturbance that replenishment from blood-borne precursors



restores the integrity of the ILC compartment. But have we got this right? These findings depend on a number of assumptions. For example, it is presumed that the transcriptional regulators, surface molecules, maturity, and frequency of relatively “mature” ILCs are also the most useful for pinpointing circulating ILCs. In addition, the high similarity of markers in murine ILCs that are comparable in man may have obscured our ability to identify circulating progenitors, or more mature cells, that are critical to maintain tissue homeostasis.

Circulating Precursors

The first clue that programming might potentially differ between mouse and man came from Scoville et al. (55) who observed that ROR γ t was expressed in all human ILCs. This was in striking contrast to murine ILCs where ROR γ t expression was highly restricted to the ILC3 subset (18). Interestingly, CD34⁺ progenitors expressing c-kit and ROR γ t could generate all ILC subsets, including NK cells. These progenitors selectively resided in secondary lymphoid tissues. This helped to identify the pathway of ILC development in man; however, the involvement of these precursors remained unclear. Later, an extensive analysis of the c-kit⁺ ILCs from the blood and tissues revealed that circulating ILCp in humans exist and do not typically express many of the markers associated with mature cells but do express low levels of ROR γ t (2). Such cells are maintained in RORC-deficient patients and retain the potential to produce different populations of ILC except ILC3. Thus, it appears that high expression of ROR γ t is necessary to generate an ILC3 in both man and mouse, but these observations raise the question of whether in mice similar progenitors have been simply discounted due to their low expression of this transcription factor. However, while the role of ROR γ t in the development of human ILCps is unclear, transcriptomic and epigenomic analysis of circulating human ILCps revealed the upregulation of many transcription factors known to be critical for ILC development in mice such as *NFIL3*, *ID2*, *TOX*, *TCF7*, *ZBTB16*, and *GATA3* (2). This suggests that transcriptional regulation of ILC development share common factors in human and mouse.

Regulatory ILCs

Innatelymphoid cell subsets appear to largely mimic those defined for CD4⁺ T cells. Recent evidence suggests that this extends to the presence of regulatory ILCs. Among the ILCs, NK cells have been described to produce IL-10 (56, 57) that acts to depress B cell (58) and dendritic cell immune responsiveness (59, 60) and suppress activation. The Ohashi group (61) strengthens the notion that a regulatory subset might exist. During their evaluation of a tumor-infiltrating cell-based adoptive immunotherapy for ovarian cancer, it was noticed that a high frequency of CD56⁺CD3⁻ cells was strongly correlated with suppression of tumor-infiltrating cell outgrowth and proposed that these cells played a regulatory role. While this effect has only been tested *in vitro*, they ascertained that regulatory and conventional CD56⁺CD3⁻ ILCs exhibited high levels of the transcription factors *ID2*, *ZBTB16* (*PLZF*), *RUNX3*, and *TOX*, but similar amounts of *EOMES*, *TBX21*, *GATA3*, *RORA*, and *AHR*, factors also shared with NK cells, ILC2s, and ILC3s. Very recently, however, Wang et al. (62) provide the first description of regulatory ILCs in mice and humans which were shown to

be important in response to gut inflammation such as *Citrobacter rodentium*. This subset arises from an ID2-expressing progenitor and depends on a second inhibitor of DNA-binding protein, ID3 but not PLZF or ROR γ t. Intriguingly, this subset lacks expression of any of the classical transcription factors required for the early steps in development by other ILCs including NFIL3, TOX, TCF-1, GATA3, or PLZF. Thus, it remains unclear whether the early progenitor is the ILCp, or alternatively a distinct progenitor gives rise to this subset. However, like other ILC subsets they lack typical lineage markers but do express CD25 and CD90 while autocrine expression of TGF- β 1 drives expansion of this IL-10⁺ subset during inflammation and results in suppression of activation of ILC1 and ILC3.

Combined, these important studies point toward the existence of a regulatory subset, but important questions still remain. For example, how and when do regulatory ILCs emerge, what other transcription factors drive this process, and do they express receptors that can be targeted to restore immune homeostasis during chronic and autoimmune diseases. If they do, this proves another avenue to unleash the protective power of ILCs either within tumors or during inflammation and in maintaining normal homeostasis to prevent autoimmunity.

CONCLUDING REMARKS

With increasing understanding of the regulation of the ILC, we realize how extensive is their ability to adapt their microenvironment. While ILC subsets are often seen as innate counterpart of T helper cells, it may be interesting to imagine the ILCp as an innate counterpart of naive T cells. ILC subsets also to appear to be an extremely plastic population that can profoundly change their predicted responses in reaction of extracellular mediators. One important challenge will be to identify the large variety of environmental and host-derived signals they can integrate to understand the role of ILCs in the homeostasis of the tissue and during inflammation.

AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript and read, edited, and approved the final manuscript.

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