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Adaptation of Intestinal ILC3 Behavior and Function in Response to Environmental Signals

A spatiotemporal regulation of ILC3

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Thèse de doctorat d'Immunologie

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Cover picture shows ILC3 within intestinal villi and cryptopatches in *Rag2^{-/-}Rorc^{GFP}Il22^{TdT}* mice, using immunofluorescence. Intestinal ILC3 stainings: (*Rorc*)-GFP in green and (*Il22*)-TdT in red; nuclear staining: DAPI in blue; actin staining (structures): phalloidin (yellow).

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CHAPTER I

GENERAL INTRODUCTION

I. Intestinal immunosurveillance: importance of IL-22-dependent responses

Mucosal tissues, such as the intestine, represent unique structures exposed to the environment. As such, the mucosal epithelial barrier defines a crucial interface between the body and dietary antigens, the microbial flora as well as potential pathogens. To preserve mucosal homeostasis, it is essential for the epithelium to maintain its integrity while being adaptable, in order to ensure proper mucosal functions (for instance, absorption and transformation of nutrients in the digestive tract) and to respond to specific environmental cues (1). Just below the epithelial barrier, the mucosal tissue is also abundantly populated by a variety of immune cells, critical for both immune tolerance and host protection.

The key role of epithelial and immune homeostasis at mucosal sites is illustrated by the association between dysregulated barrier function and many infectious and inflammatory diseases, including psoriasis, airway inflammation or inflammatory bowel diseases (IBD) (2).

Interactions between the host and the external environment occur to the largest extent in the intestinal mucosae. The intestine constitutes the largest of the body's mucosal surfaces and is continuously exposed to environmental stimuli. The intestinal barrier consists of a single layer of epithelial cells organized into villi and crypts. Villi are finger-like projections of the intestinal epithelium, which extend into the lumen and increase the surface area of absorption, whereas crypts are tubular invaginations of the intestinal epithelium (3).

This surface is continuously renewed by a pool of long-lived, pluripotent intestinal stem cells (ISC) that reside in the base of crypts and give rise to several different types of mature epithelial cells. Although the vast majority of cells bordering the intestinal lumen are absorptive enterocytes, additional specialized cells are also present in the intestinal epithelium. These specialized cells include Paneth cells, goblet cells and neuroendocrine cells that are responsible for antimicrobial peptides (AMP), mucus and hormones production, respectively. Collectively, the diverse functions of intestinal epithelial cells (IEC) result in a dynamic barrier to the external environment (3).

A. IEC: crucial mediators of intestinal homeostasis

The intestinal epithelium represents the barrier between the host and the external environment. As such, IEC are crucial mediators of intestinal homeostasis through engaging in the formation of a physical and biochemical barrier, the sensing of environmental cues as well as the coordination of immune responses (4).

IEC: a physical and biochemical barrier

Segregation between the host and the external environment is achieved by the intestinal epithelium through the formation of a physical and biochemical barrier.

Mucus serves as an important physical barrier, constituting the first line of defense between commensals or potential pathogens and the underlying tissue. Goblet cells secrete highly glycosylated mucins into the intestinal lumen, notably mucin 2 (Muc2), which form intestinal mucous layers (5). The importance of mucin production is emphasized by the development of spontaneous colitis in Muc2-deficient (*Muc2*^{-/-}) mice (6). As small molecules can pass through the mucus gel, the permeability of

the intestinal epithelium requires control mediated by junctional complexes. These junctional complexes are sites where adjacent epithelial membranes are in close apposition and comprise tight junctions, adherens junctions and desmosomes which create together an additional physical barrier (1).

The intestinal barrier is further reinforced by the production of antimicrobial peptides (AMP), establishing a biochemical barrier. Enterocytes are capable of producing some AMP, such as the C-type lectin regenerating islet-derived protein 3-gamma (Reg3 γ). Yet, Paneth cells are the major sources of AMP in the intestine, including defensins (α - and β -defensins), cathelicidins, lysozymes, C-type lectins (Reg3 β and Reg3 γ) and S100 proteins (7). Many AMP exert direct bactericidal activity by impairing several essential features of bacterial biology, such as disrupting surface membranes or entering the bacterium to inhibit intracellular function. Moreover, some AMP also modulate host immunity by recruiting immune cells or by influencing the recognition of microbial products and nucleic acids released upon tissue damage and thus, inflammatory responses (8). For instance, Reg3 γ -deficient mice (Reg3 $\gamma^{-/-}$) mice show increased bacterial colonization of the intestinal epithelium as well as enhanced activation of intestinal adaptive immune responses (9).

Together, the combination of these physical and biochemical barriers limits the quantity of live bacteria – commensal or pathogenic – that can reach the epithelial surface or interact with the underlying mucosal immune system.

IEC: sensors of intestinal homeostasis

In order to maintain barrier function, IEC must be capable of sensing environmental signals and integrating them into an appropriate anti-microbial or immunoregulatory response. IEC express diverse pattern-recognition receptors (PRR), including Toll-like receptors (TLR) (10) and NOD-like receptors (NLR) (11, 12), that provide distinct pathways for the recognition of microbial ligands or endogenous signals associated with pathogenesis (4). Several studies have established a key role for TLR in epithelial homeostasis and repair (13, 14) and in 2001, NOD2 has been identified as the first genetic susceptibility locus for Crohn's disease (15, 16), highlighting the importance of PRR in intestinal barrier function. A specialized regulation of PRR pathways exist in IEC for maintaining hyporesponsive PRR signalling in response to commensals-derived stimuli, such as the expression of negative regulators of PRR-dependent pro-inflammatory signalling (17) or distinct expression of PRR-associated signaling in IEC along the intestinal villus (18).

IEC: regulators of immune responses

As the epithelial surface lies in between the intestinal lumen and the mucosal tissue, IEC must translate external stimuli into suitable signals for tolerizing immune cells, limiting steady-state inflammation and directing correct immune cells responses against pathogens. To communicate with immune cells, IEC release various cytokines and metabolites following PRR signalling triggered by environmental cues. Immunoregulatory functions of IEC are numerous and are reviewed in *reference* (4). One can cite the regulation of intestinal mononuclear phagocytes and subsequent antigen presentation as an example. Imprinted IEC promote the development of interleukin (IL)-10 producing tolerogenic dendritic cells (DC) and macrophages, through IEC-derived thymic stromal lymphopoietin (TSLP), transforming growth-factor- β (TGF β) and retinoic acid (RA) (19–21). Influenced by their

previous interactions with IEC, these tolerogenic intestinal mononuclear phagocytes promote immune tolerance via supporting the differentiation, survival or local expansion of forkhead box P3 (FOXP3⁺) regulatory T cells (T_{reg}).

B. Interleukin (IL)-22: an essential cytokine for the intestinal barrier

IL-22, originally called 'IL-10-related T cell-derived inducible factor', was first identified in 2000 as a gene induced by IL-9 in murine and human T lymphocytes (22–24). IL-22 belongs to the IL-10 family cytokines, comprising IL-10; IL-20 subfamily members including IL-22; and IL-28 subgroup of cytokines, also known as type III interferons (IFN). Studies have shown that IL-22 is expressed at barrier surfaces and that its expression is dysregulated in certain human diseases, such as IBD, which suggests a critical role in the maintenance of barrier function. This is also supported by several mouse studies that have pointed out the role of the IL-22 and IL-22 receptor (IL-22R) axis in promoting tissue homeostasis, inflammation and antimicrobial immunity (2, 25, 26).

In the intestine, IL-22 is produced by both innate and adaptive immune cells, whereas IL-22R expression is restricted to cells of the non-hematopoietic lineage and notably to IEC (27). IL-22 is mainly expressed in response to myeloid-derived pro-inflammatory cytokines, such as IL-1 β , IL-6, TNF α and, chiefly, IL-23, but also in response to dietary metabolites or neuronal-derived factors (25). The IL-22R is a heterodimeric receptor complex, composed of the subunits IL-22RA1 and IL-10R2. Similar to other members of the IL-10 family, downstream signalling is mediated by the activation of the Jak-Stat pathway, inducing phosphorylation of Jak1 and Tyk2 kinases and STAT1, STAT3 and STAT5 transcription factors (2, 26). A soluble form of IL-22R, called IL-22-binding protein (IL-22BP; also known as IL-22RA2) (28) was later found to bind IL-22 and neutralize its activity both *in vitro* and *in vivo* (29–31) (**Figure 1**). The IL-22 IL22-R signalling pathway targets genes encoding molecules involved in barrier function, mucosal homeostasis and tissue repair, immunosurveillance and inflammation (see below).

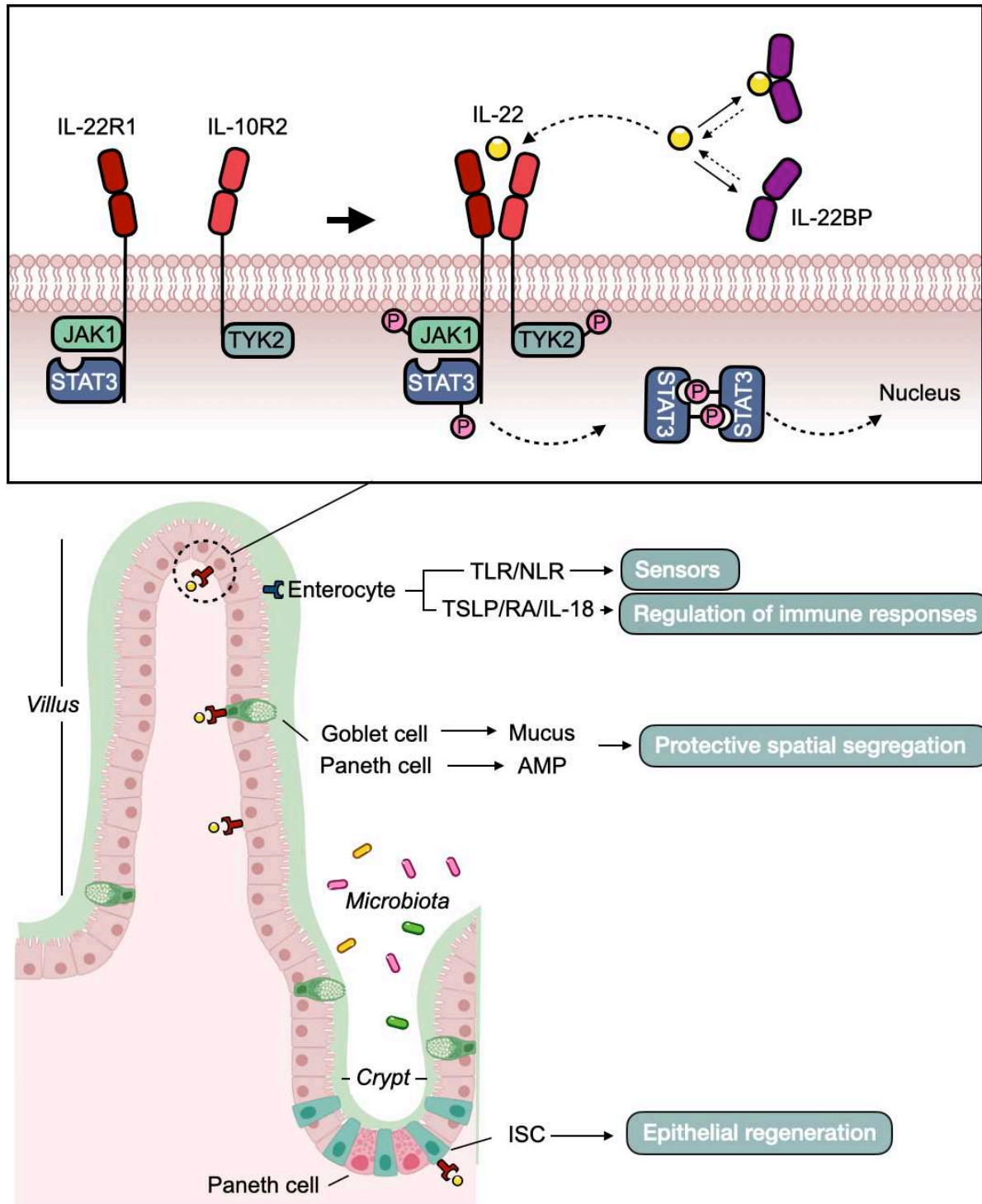


Figure 1 | Role of IL-22 in maintaining the intestinal epithelial barrier

IL-22-dependent epithelial responses support intestinal barrier function and mucosal homeostasis through epithelial regeneration mediated by intestinal stem cell (ISC) and protective spatial segregation provided by mucus-secreting goblet cells and antimicrobial peptides (AMP)-producing Paneth cells. IL-22 mediates its cellular effects via IL-22R1 in a complex with IL-10R2. IL-22BP (IL-22 binding protein) acts as a natural inhibitor on IL-22 activity. STAT3, signal transducer and activator of transcription 3; TLR, Toll-Like Receptor; NLR, NOD-Like Receptor; TSLP, Thymic Stromal Lymphopoietin; RA, Retinoic Acid.

1. IL-22, coordinating immune and epithelial responses

As mentioned above, IL-22R is abundantly expressed across epithelial cells in the intestine. As such, IL-22 is crucial to orchestrate the crosstalk between immune cells and the intestinal epithelium (**Figure 1**).

Role of IL-22 in maintaining barrier function

IL-22 is involved in the maintenance of the intestinal barrier. IL-22 contributes to the formation of intestinal mucous layers. IL-22 directly induces *Muc2* expression in IEC and contributes to facilitate goblet cell restitution in intestinal inflammation. Enhanced mucus production plays a crucial role in IL-22-mediated amelioration of chronic colitis (32). In addition, IL-22 has been shown to promote epithelial glycosylation. IL-22 induces intestinal expression of the fucosyltransferase 2 (*Fut2*) and ablation or deficiency of IL-22 blocks the induction of *Fut2* by IL-22R-positive IEC (33, 34). Besides IL-22 importance for mucus production, it has also been implicated in intestinal permeability via the regulation of tight junctions. As an example, IL-22 production leads to the up-regulation of claudin-2, a key subunit of epithelial tight junctions, and thereby modulates water efflux and pathogen clearance (35, 36). IL-22 is not only associated with the maintenance of the physical barrier but also with the activity of the biochemical barrier, namely the production of AMP. Several studies have shown that both Reg3 β and Reg3 γ production are dependent on IL-22 in the intestine (37, 38). At steady-state, although IL-22 deficiency *per se* does not cause overt pathological consequences, it still results in weakened barrier function and altered microbiota composition. IL-22-dependent induction of AMP promotes the anatomical containment of commensal bacteria, thereby limiting inflammation (39). Altogether, IL-22 has an impact at multiple levels on the intestinal epithelial barrier function and consequently on the segregation between the host and the external environment, which is essential for preserving intestinal homeostasis. As such, regulation of IL-22/IL-22R signalling provides a rapid mean to adapt barrier integrity in case of infections for example.

Role of IL-22 in antimicrobial immunity

Observations that IL-22 induces a marked antimicrobial response raised the idea that IL-22 could exert an important downstream host defensive function during bacterial infection. Several studies have demonstrated the functional importance of early IL-22 production in host protective immunity against enteropathogenic bacteria such as *Citrobacter rodentium*, a model of human enteropathogenic *Escherichia coli* (38, 40–42). An effective IL-22-dependent epithelial response, involving the production of antimicrobial peptides and the maintenance of barrier containment, is critical for pathogen control as illustrated by the susceptibility of IL-22 deficient (*IL22^{-/-}*) mice to infections such as *C. rodentium* or *Klebsiella pneumoniae* (37, 38). Moreover, in recombination-activating gene (Rag) 1 deficient mice (*Rag1^{-/-}*), IL-22 is sufficient to promote protective innate immunity to *C. rodentium* for several weeks post-infection (38, 42), suggesting that the IL-22/IL-22R pathway could be manipulated to enhance mechanisms of innate immunity and protection even in the absence of adaptive immune responses.

Role of IL-22 in tissue repair and tissue inflammation

IL-22RA1 is abundantly expressed across intestinal epithelial lineages and is also present on ISC (43). This suggests an additional role for IL-22 in epithelial regeneration. Upon radiation or methotrexate-induced epithelial damage, STAT3 directly induces prosurvival genes in IEC following activation of IL-22R signalling (43–45). Accordingly, IL-22 has been shown to ameliorate tissue repair in several models, notably in DSS-mediated or T cell-transfer-mediated colitis in the intestine (32, 45, 46). Inhibition or deficiency of IL-22 leads to a reduced frequency of ISC and defective epithelial regeneration (44, 46). In parallel, IL-22 is also required for the activation of the DNA damage response (DDR) and genomic stability. Indeed, ISC deprived of IL-22 signals through selective deletion of IL22-RA1 and exposed to carcinogens escaped DDR-controlled apoptosis, which resulted in an accumulation of mutations and increased malignant tumors development (47). Interestingly, metabolites of glucosinolates, a group of phytochemicals contained in cruciferous vegetables, are parallelly a source of genotoxic stress in IEC and known to activate aryl hydrocarbon receptor (AhR) signalling. AhR signalling is involved in IL-22 production in the intestine, thereby directly regulating components of the DDR in ISC and allowing safe consumption of diet containing DNA-damaging agents (47). Taken together, these studies show that IL-22 is involved in preventing tissue damage and promoting tissue repair both at homeostasis and during acute injury or inflammation.

However, IL-22 also contributes to the expression of genes encoding molecules involved in inflammatory responses, including IL-6, G-CSF, IL-1 α and serum amyloid A (2). In several models of inflammation, IL-22 is necessary to induce tissue inflammation. Muñoz and colleagues demonstrated that, in the intestine, IL-22 is necessary to promote inflammation and notably ileitis following *Toxoplasma gondii* infection (48). Preclinical models have also revealed a potentially more complex role of IL-22 in intestinal inflammation and tissue repair as, in some studies, neutralization of IL-22 improved experimentally-induced colitis (49). In a recent model of murine IBD, macrophage-restricted IL10Ra-deficiency led to severe spontaneous colitis development mediated by IL-22-dependent responses (50).

The opposite findings that IL-22 can both prevent tissue damage and promote pathological inflammation seem paradoxical. However, it is likely that the functional outcome of IL-22 is strongly dependent on context where it is expressed. For example, in the lung, IL-17A is necessary for IL-22-dependent bleomycin-induced airway inflammation (51). In the absence of IL-17A, IL-22 instead promotes repair of the airway epithelium, showing that specific tissue environmental cues might be important in orientating IL-22 effects.

2. Role of IL-22 in human disease

In human, dysregulated IL-22 expression is associated with the development of certain diseases, such as IBD (2). Further understanding IL-22 biology might be relevant for human disease and future therapies. Amongst other things, elevated IL-22 concentrations have been reported in the peripheral blood and intestine of IBD patients and IL-22 expression correlates with pro-inflammatory gene expression, suggesting a key role of IL-22 in the pathogenesis of intestinal diseases (52, 53). Some spontaneous mutations arising in the human population have also hinted towards this. A genome-wide

association study of IL23R, encoding IL-23 which is critical for IL-22 expression, has found this gene to be associated with IBD, consistent with a potential implication of the IL-22/IL-22R pathway in human disease (54).

3. Cellular sources and regulation of IL-22

As discussed above, IL-22 interactions with IL-22R represent a fundamental pathway that integrates signals from the environment and coordinates immune and epithelial responses which regulate intestinal homeostasis and immunosurveillance. IL-22 has multiple and somewhat ambivalent roles, highlighting the importance of its functions as well as the requirement for careful regulation of its expression.

Cellular sources of IL-22 include a variety of innate and adaptive lymphocytes (**Figure 2**). IL-22 was originally found to be expressed in mouse and human T cells (22–24) and was considered to be a T_H1-associated cytokine (55). However, with the advent and identification of T_H17 cells, IL-22 was quickly associated with these cells (56, 57). In humans, T_H22 cells were also described as a source of IL-22 (58, 59) but whether these cells are truly distinct from the T_H17 lineage is still a matter of debate. Additionally, IL-22 can be expressed by unconventional T cells such as oligoclonal $\gamma\delta$ T cells (60, 61) and CD1d-restricted NKT cells (62). IL-22 production by T cell populations requires engagement of their T cell receptor (TCR) with its cognate antigen, so that they are not effectively contributing to steady-state IL-22 levels.

In contrast, innate immune cells might represent a constitutive and rapid source of IL-22. NK1.1⁺ and CD11c⁺ cells were initially thought to be the innate producers of IL-22 (38, 45, 63). Satoh-Takayama and colleagues showed that previously identified IL-22-producing cells might have been contaminating innate lymphocytes instead (41), harboring NK cell and dendritic cell (DC) markers but distinguished from these classical innate populations. Several studies have now shown that the newly identified innate lymphoid cells (ILC), and notably group 3 ILC (ILC3), actually represent the dominant source of IL-22 both in mouse and human (64).

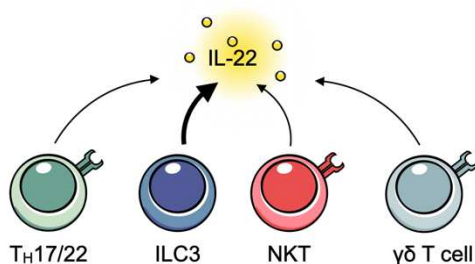


Figure 2 | Cellular sources of IL-22

Cellular sources of IL-22 include a variety of innate and adaptive lymphocytes but ILC3 represent the dominant source of IL-22.

II. Role of ILC3 in intestinal immunosurveillance

While IL-22 can be produced on demand in T cells, it is tonically secreted by ILC3 which are present in large numbers in the intestinal mucosae. Consequently, ILC3 have emerged as essential players in intestinal homeostasis and immunity (65).

ILC3 belong to the recently described ILC family, a novel family of effector lymphocytes (66). Over the past decade, the discovery and investigation ILC has changed our perception of the immune system. The immune system is classically divided into the innate and adaptive arms. On the one hand, innate immune cells express pattern-recognition receptors (PRR), thus supporting rapid immune responses to tissue perturbations. On the other hand, adaptive immunity mediated by lymphocytes relies on precise and diverse antigen-specific receptors, providing delayed but highly specific and long-lasting defense against potential harm. At crossroads between innate and adaptive immunity, ILC represent newly identified members of the lymphoid lineage that display functional characteristics of innate cells, thus challenging this dichotomy.

A. ILC: a new family of innate immune cells

The ILC family

ILC constitute a novel family of innate effector cells with diverse functional properties that play an essential role in early immune responses, especially at mucosal sites. While adaptive lymphocytes are most abundant in lymphoid tissues – from lympho “lymph” and -cyte “cell” – ILC are scarce and disparate in secondary lymphoid organs. Rather, ILC reside mostly in peripheral tissues and are enriched at mucosal sites: in the small intestine lamina propria for instance, ILC represent ~ 15% of leukocytes (CD45⁺ cells; C57BL/6J; (67), in line with our data) at steady-state.

All ILC family members share several features, paradoxically categorizing them as innate cells and lymphocytes. They are derived from common lymphoid progenitors (CLP) but, unlike T and B cells, they develop in the absence of the recombinase machinery and do not express clonotypic antigen receptors. As such, ILC resemble lymphocytes but lack expression of phenotypic markers associated with B-, T- or myeloid lineages. However, in contrast to adaptive lymphocytes, ILC polarization of effector functions is already largely predetermined during development and therefore, ILC are poised for rapid activation. Since ILC are largely devoid of pattern recognition receptors (except for some TLR expressed on human ILC; (68, 69)), they respond to environmental stimuli such as epithelial cells or myeloid cells-derived signals (66). Through their ability to rapidly produce large amounts of cytokines and their strategic location, ILC participate in the initiation and maintenance of immunity, inflammation and tissue homeostasis. Whereas the development of an adaptive immune response requires days – for the activation, expansion, differentiation and migration into tissues of adaptive lymphocytes, ILC can respond within hours to tissue perturbation and therefore represent the first line of defense (65).

Classification of ILC

Despite these features that set them apart, ILC exhibit remarkable developmental and functional similarities with T cells (70). As such, it has been proposed that ILC represent 'innate' counterparts of T cells. ILC have been classified in three distinct groups based on their differential requirements for transcription factors and cytokine outputs, comparable to that of their corresponding adaptive counterparts (70). Group 1 ILC (ILC1) comprise both conventional NK cells and non-cytotoxic ILC1. ILC1 share dependence on the transcription factor T-Box Transcription Factor (T-bet, encoded by *Tbx21*) for their differentiation and production of interferon gamma (IFN γ) and tumor necrosis factor alpha (TNF- α). Group 2 ILC (ILC2) express high levels of the transcription factors Gata binding protein 3 (GATA3, encoded by *Gata3*) and retinoic-acid receptor-related orphan receptor α (ROR α , encoded by *Rora*) to fully differentiate and secrete type 2 cytokines such as IL-5 and IL-13. Group 3 ILC (ILC3) express the transcription factor retinoic-acid receptor-related orphan receptor gamma (ROR γ t, encoded by *Rorc*) and produce IL-17 and IL-22 (**Figure 3**).

For adaptive immunity, a distinction between cytokine-producing 'helper' CD4⁺ T cells and 'killer' CD8⁺ T cells (cytotoxic T lymphocytes) based on their differential developmental pathways. Although all ILC share developmental similarities and thus constitute a related family of innate lymphocytes, the notion of 'helper' versus 'killer' ILC has also emerged based on a distinction between conventional NK cells ('killer' ILC) and other ILC subsets (ILC1, ILC2 and ILC3, 'helper' ILC). Indeed, unlike non-cytotoxic ILC1, conventional NK cells express the transcription factor Eomesodermin (EOMES, encoded by *Eomes*) and exert spontaneous killing activity through the release of cytotoxic granules. Whether these unique functional characteristics and transcription requirements are sufficient to set conventional NK cells apart from the other ILC subsets is still a matter of debate (see Box 1 in (71)). In addition, a group of IL-10-producing ILC has been recently described in the intestine – originally reported as 'ILCreg' – but whether these cells represent a truly distinct subset of ILC (72) or plastic ILC2 (73) remains mysterious.

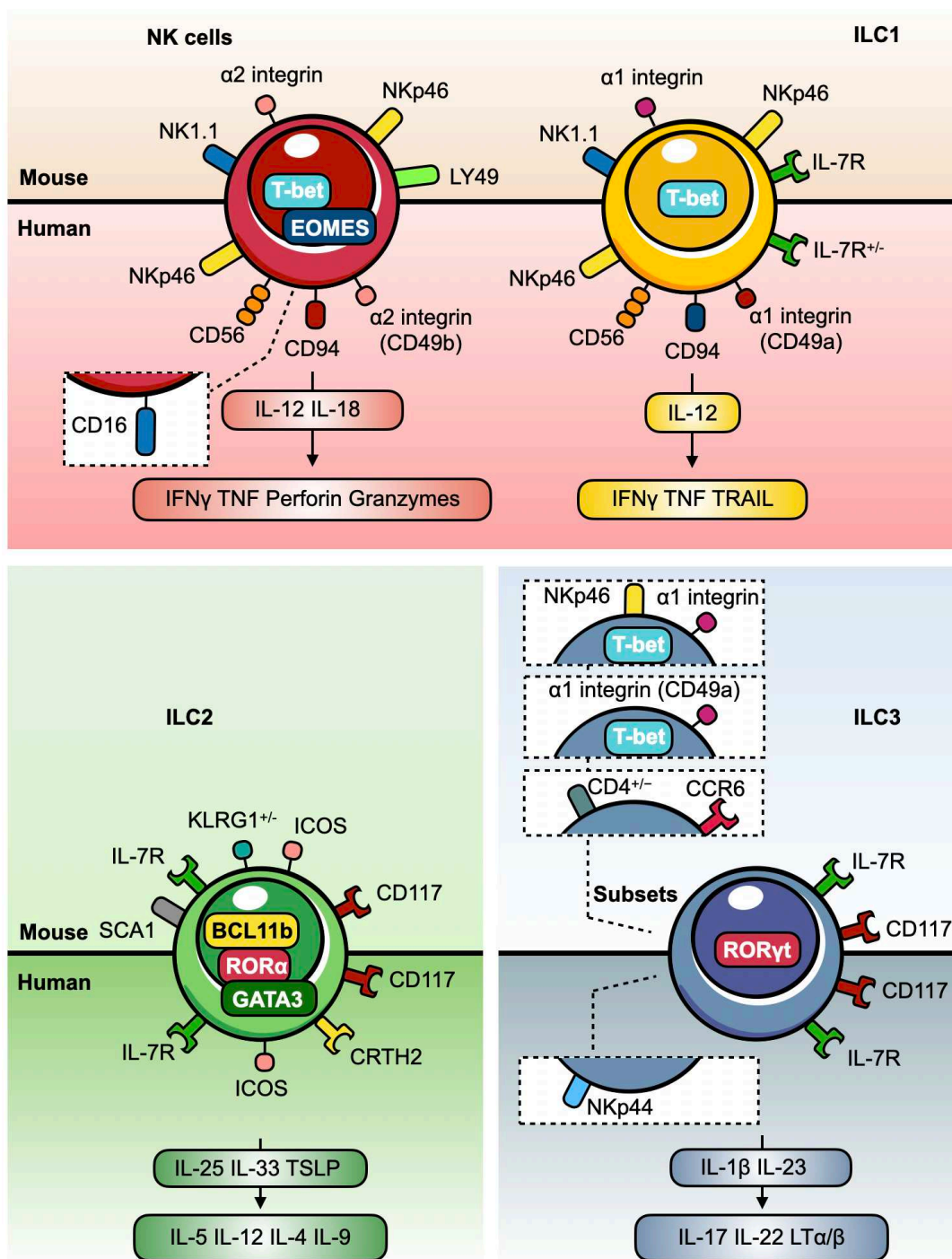


Figure 3 | The innate lymphoid cell family

Characterization of mouse and human innate lymphoid cells (ILC) groups by major surface markers, transcription factors and cytokines. Labels inside the cells indicate the main transcription factors that drive the development of ILC. Most important surface markers to distinguish these subsets are indicated, with subset-specific markers and transcription factors in boxes. Major activating cytokines of ILC are indicated on the arrows with functional outputs pointed out by the arrow. This list is non-exhaustive. T-bet, T-Box transcription factor; EOMES, eomesodermin; BCL11b, B-cell lymphoma/leukemia 11B; ROR α , retinoic-acid receptor-related orphan receptor α ; GATA3, Gata binding protein 3; ROR γ t, retinoic-acid receptor-related orphan receptor gamma; IFN γ , interferon gamma; TNF, tumor necrosis factor; TRAIL, tumor-necrosis-factor related apoptosis inducing ligand; SCA1, stem-cell antigen 1; ICOS, inducible T-cell costimulator; KLRG1, killer cell lectin like receptor G1; CRTH2, chemoattractant receptor-homologous molecule expressed on TH2 cells; TSLP, Thymic Stromal Lymphopoietin; IL-, interleukin.

B. ILC3, new players in intestinal immunity

1. Presentation of ILC3

Group 3 innate lymphoid cells (ILC3) are highly enriched in the intestine and are instrumental in regulating intestinal homeostasis and immunity, in part through their ability to produce large amounts of IL-22. In the intestine, myeloid-derived IL-1 β and IL-23 are crucial for the initiation of ILC3 responses, though many additional signals can activate ILC3. Beyond IL-22, ILC3 can produce a variety of cytokines including IFN γ , IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF) and lymphotoxin (LT). Although all ILC3 share a developmental requirement for ROR γ t, substantial heterogeneity exist within this group. ILC3 can be subdivided in two major subsets, displaying distinct phenotypic and functional features (**Figure 4**):

Mouse ILC3 subsets

CCR6⁺ ILC3, also known as lymphoid-tissue inducer (LTI) cells, were first described in mice as CD3⁻ CD4⁺ α 4 β 7⁺ and lymphotoxin (LT) producing cells (74). They were found colonizing nascent lymph nodes and Peyer's patches (PP) and shown to promote lymphoid tissue organogenesis during fetal life (75). LTI cells can be further subdivided into CD4⁺ (LTI₄) and CD4⁻ (LTI₀) cells: the ratio of these subsets varies depending on the tissue and age of the mice. All LTI cells express the transcription factor ROR γ t, which is essential for their generation (76). Both subsets produce LT α 1 β 2, as well as IL-17 and IL-22 (75, 77–80). The role of CD4 remains unknown, as CD4 deficient mice show normal lymphotoxin and interleukin-dependent functions. Of note, LTI cells also express high levels of major histocompatibility complex (MHC) class II, which is involved in the regulation of autoreactive intestinal T cells (81).

More recently, intestinal CCR6⁻ ILC3 have also been observed and comprise natural cytotoxicity receptor (NCR; NKp46 in mouse and NKp44 in human) ILC3 and heterogenous CCR6^{-/lo} NCR⁻ ILC3. We and others first described intestinal 'NK' cells displaying unique functional properties (41, 82–84). Although these cells express NKp46, they lack typical NK cell effector functions such as cytotoxicity or high IFN γ production. Rather, these cells express the transcription factor ROR γ t and produce large amounts of IL-22 but not IL-17 upon stimulation. Based on these findings, these cells originally termed 'NK22', 'NCR22', 'ILC22' or 'NK-LTI' were included in the ILC3 family and are now referred to as NKp46⁺ ILC3. The functional significance of NKp46 expression on ILC3 remains unclear. In addition, heterogeneous CCR6^{-/lo} NKp46⁻ subsets, denoted double negative (DN) ILC3, have been identified. DN ILC3 are poorly defined but it is likely that these cells represent a heterogenous mix of progenitor and effector cells. These ROR γ t⁺ DN ILC3 can produce IL-17A, IL-22 and IFN- γ , in particular in inflammatory contexts (85–87).

Further studies have shown that a T-bet gradient is required for the development and function of CCR6⁻ ILC3, both NKp46⁺ and NKp46⁻ (86, 88). T-bet is uniformly expressed by all NKp46⁺ and a subset of CCR6⁻ NKp46⁻ ILC3. Interestingly, the integrin α 1 subunit CD49a (encoded by *Itga1*) can also be used to easily identify ILC3 subsets as (i) CD49a and CCR6 expression are mutually exclusive and (ii) CD49a is co-expressed with T-bet (87).

Thus, a remarkable diversity exists within ROR γ t⁺ ILC3 and distinct ROR γ t⁺ ILC3 subsets (**Figure 4**).

Human ILC3 subsets

In humans, heterogeneous populations ROR γ ⁺ ILC3 have also been identified (**Figure 4**). Human fetal LTi cells were first observed in fetal lymph nodes and fetal spleen (89). These cells express *RORC* transcripts as well as lymphotoxins and IL-17 preferentially but lack the CD4 surface marker. Postnatally, ROR γ ⁺ ILC3 are notably present in tonsils, lymphoid tissues and intestinal lamina propria of human subjects (89, 90). They can be subdivided based on their expression of NCR, and notably NKp44 (89–92). Human NKp44⁻ ILC3 resemble fetal LTi cells and are present in peripheral lymph nodes where they produce limited amounts of IL-17, in particular those expressing HLA-DR (human MHC-II) (92, 93). Conversely, human NKp44⁺ ILC3, largely co-expressing NKp46, are found in tonsils and intestine where they exclusively produce IL-22 (90–92). Therefore, in the adult intestine, NKp44⁺ ILC3 are the main IL-22 producing ILC. Contrarily to murine NKp46⁺ ILC3, human ILC3 neither express T-bet nor IFN γ – although expression can be induced after *in vitro* culture (94, 95).

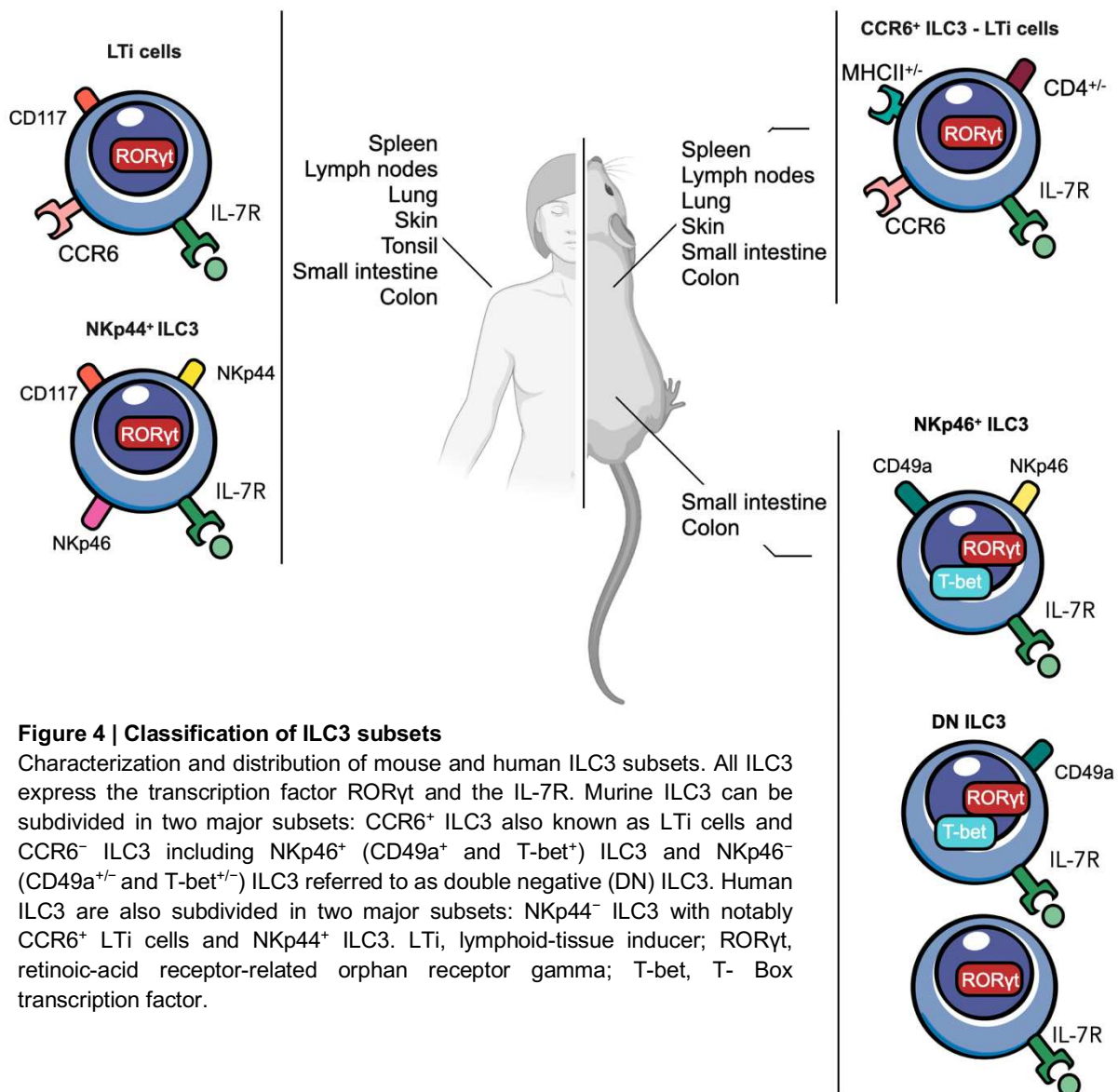


Figure 4 | Classification of ILC3 subsets

Characterization and distribution of mouse and human ILC3 subsets. All ILC3 express the transcription factor ROR γ t and the IL-7R. Murine ILC3 can be subdivided in two major subsets: CCR6⁺ ILC3 also known as LTi cells and CCR6⁻ ILC3 including NKp46⁺ (CD49a⁺ and T-bet⁺) ILC3 and NKp46⁻ (CD49a^{+/-} and T-bet^{+/-}) ILC3 referred to as double negative (DN) ILC3. Human ILC3 are also subdivided in two major subsets: NKp44⁻ ILC3 with notably CCR6⁺ LTi cells and NKp44⁺ ILC3. LTi, lymphoid-tissue inducer; ROR γ t, retinoic-acid receptor-related orphan receptor gamma; T-bet, T-Box transcription factor.

2. Development of mature ILC3

Generation of ILC

Development of ILC initially occurs during fetal life in the liver and continues in the adult in the bone marrow (BM). All ILC subsets depend on the TF Inhibitor of DNA binding 2 (ID2), that acts as a repressor of E proteins transcriptional activators, and on cytokines of the common γ -chain family (γc) to develop from hematopoietic progenitors. Among γc cytokines, IL-7 acts at different stages of differentiation towards ILC3 and is therefore critical for the development of ILC3, as illustrated by impaired ILC3 numbers and function in *Il7^{-/-}* mice (71, 96, 97). Common lymphoid progenitors (CLP) derived from hematopoietic stem cells (HSC) possess precursor potential for T cells, B cells and ILC but not for erythroid and myeloid cells. As E proteins promote B and T lymphopoiesis, *Id2* expression prevents CLP differentiation into T and B cells and promotes instead ILC lineage commitment. These ID2⁺ common ILC precursors (CILCP) can generate all known ILC subsets. It was originally believed that at the stage of the CILCP, two downstream independent pathways co-exist: one that gives rise to cytotoxic ILC, namely conventional NK cells through generation of a NK progenitor (NKP), and another one generating “helper” ILC from a common helper innate lymphoid progenitor (CHILP). However, a recent study from our lab has shown that ID2⁺ CILCP retain potential for all ILC including conventional NK cells (98). ID2⁺ CILCP then give rise to LTi cells as well as to ID2⁺ Promyelocytic Leukemia Zinc Finger (PLZF)⁺ ILC progenitors (ILCP) (99). ILCP generate the different branches of the ILC family in relation with the transcription factors mentioned above in the ILC family classification (**Figure 5**).

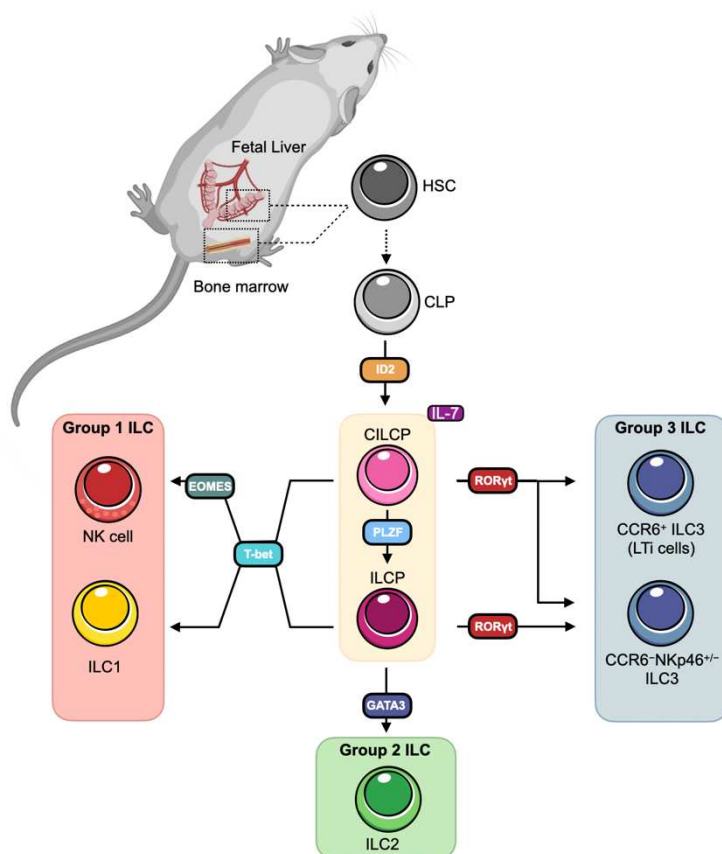


Figure 5 | Simplified model for murine ILC development

All ILC derive from a common lymphoid progenitor (CLP) in the fetal liver or in the adult bone marrow. ID2 expression results in the generation of common ILC progenitor (CILCP) restricted to the ILC lineage. CILCP can further differentiate into a PLZF dependent ILC progenitor (ILCP). Both CILCP and ILCP can give rise to all known ILC subsets, except for LTi cells which are exclusively derived from ID2⁺ PLZF⁻ CILCP. The subsequent development of the three distinct groups of mature ILC requires the engagement of transcription factors as well as extrinsic signals (cytokines, diet-derived metabolites). The common γ -chain cytokine IL-7 acts at different stages of ILC differentiation: at early stages on ILC-restricted progenitors (CILCP and ILC) and at later stages on mature ILC. HSC, haematopoietic stem cell; ID2, inhibitor of DNA binding 2; PLZF, promyelocytic leukemia zinc finger; T-bet, T-Box transcription factor; EOMES, eomesodermin; GATA3, Gata binding protein 3; ROR γ t, retinoic-acid receptor-related orphan receptor gamma; LTi, lymphoid-tissue inducer.

Fetal ILC3 development

ILC3 seed the intestinal tissue during development. All ILC3 require the TF ROR γ t which is essential for their generation, both during fetal and adult life (41, 76, 82–84) (**Figure 6**). Before birth, ILC3 are exclusively LT i cells which can either be LT i_0 or LT i_4 cells. Mature LT i cells are principally found in peripheral lymph nodes and in the gut, starting from embryonic days E12.5 and E13.5, respectively (100–102). Intestinal ILC3 development initially occurs in the fetal liver (FL) where the CLP proliferates and differentiates into CILCP (101). In the FL, CILCP can give rise to ROR γ t⁺ ILC3 progenitors (ILC3P) (103, 104) (**Figure 6**). It is not clear whether progenitors first differentiate in the FL into mature LT i cells and colonize tissues afterwards or if they first migrate into the periphery where they later complete their differentiation (**Figure 7**). A recent study suggests that precursors of CILCP predominantly proliferate in the FL and migrate into nascent lymph nodes where they become mature LT i cells (101), albeit the two options seem possible (104). These results are consistent with previous work showing that fetal CILCP leave the liver and continue their differentiation in intestinal tissues. CILCP identified in the fetal gut are a local source of all ILC populations even though they are largely biased *in vivo* towards LT i cell fate (100).

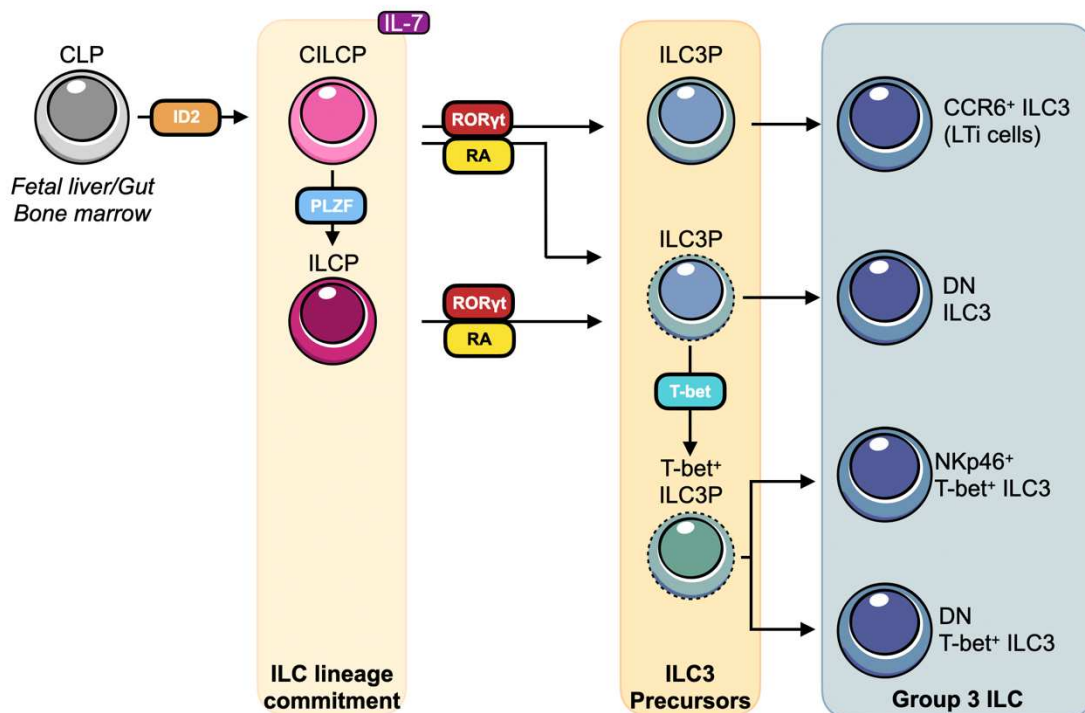


Figure 6 | Simplified model for murine ILC3 development

Murine ILC3 development takes place in the fetal liver, fetal gut as well as in the adult bone marrow. The transcription factor ID2 promotes the commitment of common lymphoid progenitors (CLP) to the ILC lineage. The transcription factor ROR γ t and diet-derived retinoic acid (RA) are essential for the development of mature ILC3 from either ID2⁺PLZF⁻ progenitors (CILCP) or ID2⁺PLZF⁺ progenitors (ILCP). CILCP can further differentiate into an ILC3 progenitor (ILC3P) that has potential for CCR6⁺ and CCR6⁻ ILC3 whereas ILCP-derived ILC3P can only give rise to CCR6⁻ ILC3 including DN ILC3 and NKp46⁺ ILC3. A fraction of CCR6⁻ ILC3 also require the transcription factor T-bet for their development but whether T-bet is upregulated at the precursor (dotted line for putative T-bet⁺ ILC3P) or mature stage is not known. ID2, inhibitor of DNA binding 2; PLZF, promyelocytic leukemia zinc finger; CILCP, common ILC progenitor; ILCP, ILC progenitor; ROR γ t, retinoic-acid receptor-related orphan receptor gamma; T-bet, T-Box transcription factor; DN, double negative; LT i , lymphoid-tissue inducer.

Postnatal ILC3 development

Fetal liver ILC3P not only give rise to LTi cells but also to other ILC3 subsets such as NKp46⁺ ILC3 (Figure 6). While fetal and perinatal ILC3 mostly represent LTi cells, different subsets of ILC3 are present postnatally and progressively increase in complexity (105). After birth, there is a change in ILC3 populations characterized by a drop in LTi cells and an increase in NKp46⁺ ILC3 and other ILC3. This can partly be explained by a higher proliferation rate of these ILC3 subsets compared to LTi cells. As mentioned previously, NKp46⁺ ILC3 and a subset of NKp46⁻ ILC3 require T-bet for their development, in addition to RORγt (86). In the adult, ILC3 mainly localize in the intestinal lamina propria. All intestinal ILC3 subsets are replaced by newly generated cells with a half-life of 3-4 weeks (105), suggesting that an ILC3P replaces fetal-derived ILC3 during adulthood. Despite the fact that ILC are not detected in circulation (67), all ILC3 subsets can be generated in the intestine from adult bone marrow progenitors (105) – either CLP or CILCP (98) – and a recent report has identified extremely rare putative RORγt⁺ ILC3P in adult bone marrow (106). In addition, it has newly been proposed that local precursors could support ILC3 development *in situ* postnatally. ID2⁺ PLZF⁺ ILCP have been found in the intestine of newborn mice where they can give rise to NKp46⁺ ILC3, indicating that ILC differentiation could occur as well outside of the bone marrow (107) (Figure 7).

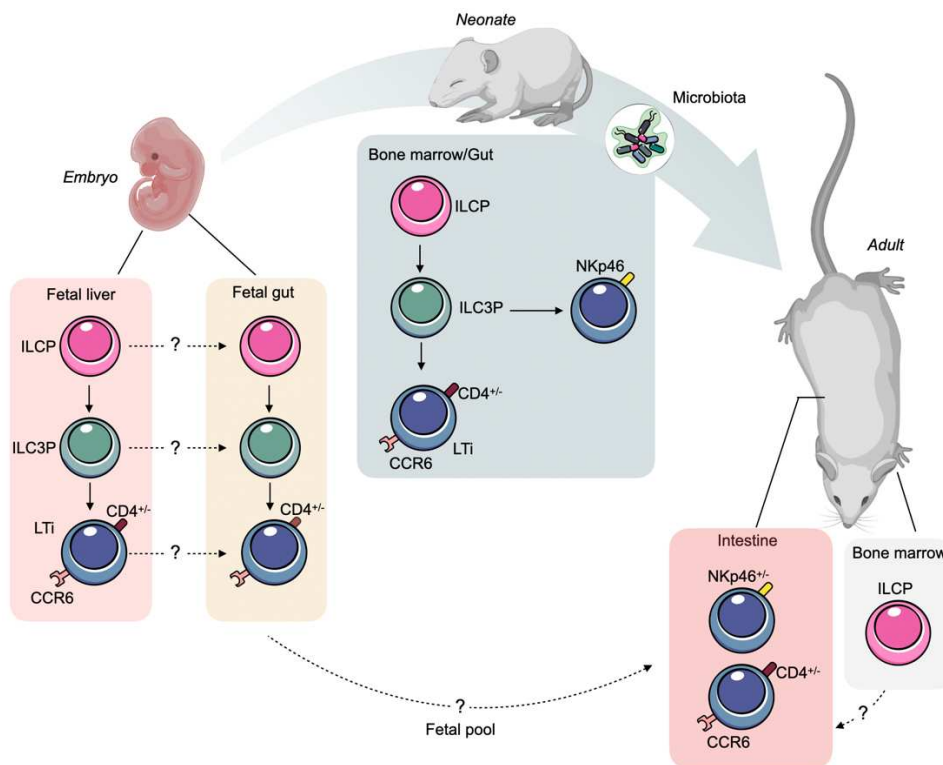


Figure 7 | Fetal versus postnatal murine ILC3 development

A first wave of murine ILC3 development takes place during fetal life and results in ILC3 seeding of the intestine. Mature ILC3 differentiate from ILC3P originally developed from a global ILC-restricted precursor (ILCP). It is not clear whether all ILC3 development occurs in the fetal liver resulting in colonization of the fetal gut by mature ILC3 or if ILC3 precursors (either ILCP or ILC3P) first migrate and complete their differentiation in periphery. While only CCR6⁺ LTi cells develop from ILC3P before birth, ILC3P generate all ILC3 subsets postnatally. Bone marrow progenitors mainly support this second wave of mature intestinal ILC3 development, but local ILC3 differentiation has also been proposed to happen in the intestine of neonates. ILCP, ILC progenitor; ILC3P, ILC3 progenitor; LTi, lymphoid-tissue inducer.

Commitment to ILC3 fate

How ROR γ t is induced in ILC3P remains unclear. *Rorc* can be activated in response to external stimuli such as cytokines, microbial signals or nutritive factors. While STAT3 activation by cytokines (IL-6, IL-23 and TGF β) leads to ROR γ t expression during T_H17 differentiation, these signals are not necessary for ILC3 development (108). The impact of the microbiota on the generation of ILC3 remains controversial. Whereas the generation of LT_i cells is clearly microbiota-independent, some laboratories have reported that certain types of microbiota could contribute to postnatal ILC3 development. It appears that intestinal ILC3 can be recovered in absence of commensal bacteria, although their numbers are impaired compared to control mice (39, 41, 79, 82, 84). At this time, several studies have shown that the microbiota impacts NKp46⁺ ILC3 (41, 82, 84), presumably through stabilization of ROR γ t expression (84). However, whether the microbiota influence ILC3 development or rather ILC3 maintenance is still an open question. Nutrients, and notably retinoic acid (RA), seem to be important to induce ROR γ t in ILC3P *in utero* as well as in adult mice (109, 110) (**Figure 6**). *Rxrg*, a RA receptor, is expressed by fetal CILCP as well as fetal LT_i cells (110). In the periphery, CILCP are exposed to RA which can subsequently induce *Rorc*, which is required for the next step of differentiation (110). In addition, because aryl hydrocarbon receptor (AhR) signalling is required for the generation of ROR γ t⁺ T_H17 cells (111), it was initially proposed to be involved in ILC3 development. Although AhR expression is a hallmark of mature ILC3, both fetal and neonatal ILC3 numbers are normal in *AhR*-deficient which don't show a deficit in the formation of lymph nodes and Peyer's Patches (112, 113). Thus, AhR signalling is likely necessary for the development or maintenance of intestinal ILC3 after but not before birth, in line with the role of dietary-derived AhR ligands in AhR regulation of ILC3 responses (112).

Human ILC3 development

Human ILC development is less well characterized. Recently, our lab has identified a circulating human ILCP that could robustly generate all ILC subsets *in vitro* and *in vivo*, including ROR γ t⁺ ILC3 (114) (**Figure 8**). Human ILCP express high levels of TF such as *ID2* and *PLZF* known to be important for commitment to an ILC fate in mouse. Human ILCP lack active transcripts of genes expressed by mature ILC (*RORC* for instance) but many of these mature ILC identity genes are in an epigenetically poised state. These progenitors are not only found in circulation but also in tissues where they retain ILC multi-potency. Interestingly, human ILCP are present in human fetal liver where they seem biased towards ILC3 fate, similarly to murine fetal CILCP. In addition, committed ILC3P that express high levels of ROR γ t and that give rise mainly to ILC3 have been found in tonsils and intestinal lamina propria (115). Although *RORC* is expressed by committed ILC3P and human ILC3, it does not seem strictly required for ILC3 differentiation in humans. Analysis of *RORC*-deficient patients revealed a lack of IL-17-producing ILC3 but not of IL-22-producing ILC3 or other ILC subsets. This finding suggests that compensary pathways may exist in humans (114).

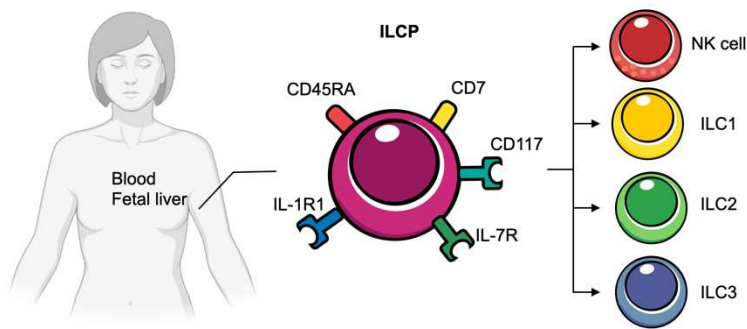


Figure 8 | 'ILC-poiesis': a new model of human ILC differentiation

A human ILC precursor (ILCP) identified in fetal liver and in blood can generate all ILC subsets, including ILC3. Surface markers characterizing human ILCP are indicated.

3. ILC3 functions

Intestinal ILC3 play multiple and diverse roles over an individual's lifetime, ranging from lymphoid tissue organogenesis to orchestration of intestinal immunity. To carry out this multitude of functions, ILC3 harbour several effector molecules. In addition to the expression of surface receptors relevant for their function, ILC3 produce classical T_H cytokines notably IL-17, IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN γ as well as previously unknown ILC factors such as lymphotoxin.

a. Homeostasis

Lymphoid organogenesis

In the fetal period, LT i cells are essential for the formation of lymphoid organs, notably lymph nodes and Peyer's patches (PP). Starting from embryonic day E12.5, retinoic acid (RA) possibly produced by nerve fibers induces expression of the chemokine CXCL13 by mesenchymal stromal cells (MSC) to attract LT i cells. LT i cells colonize nascent lymph nodes (LN) – also known as LN anlagen – where they cluster together with MSC. Subsequently, aggregation of LT i cells facilitates RANKL/RANK interactions on LT i cells which leads to expression of LT α 1 β 2 on LT i cells. From embryonic day E13.5-E14.5, interaction of LT α 1 β 2-expressing LT i cells with lymphotoxin- β receptor (LT β R)-expressing MSC results in differentiation into stromal organizer cells that express chemokines (CXCL13, CCL19 and CCL21) and adhesion molecules (VCAM-1, ICAM-1, Madcam-1). These factors support the attraction and retention of more haematopoietic cells, leading to lymph node growth (116) (**Figure 9**).

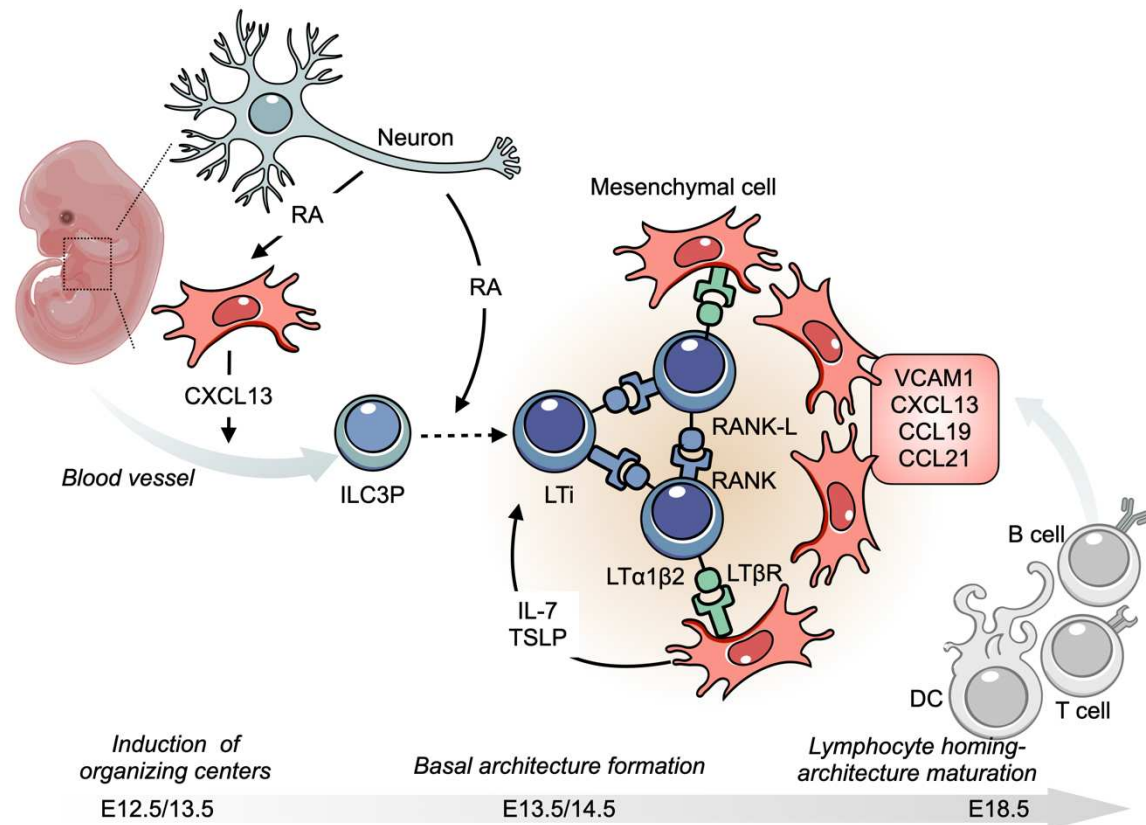


Figure 9 | LTi cells, essential orchestrators of lymphoid organogenesis

Starting as soon as embryonic day 12.5, retinoic acid (RA) induces the expression of CXCL13 by mesenchymal cells which attracts LTi cells from the blood to the lymph node anlagen. Cluster of LTi cells facilitates RANKL/RANK autocrine signalling and upregulation of lymphotoxin- α 1 β 2 (LT α 1 β 2) on LTi cells. Interactions between LT α 1 β 2-expressing LTi cells and lymphotoxin- β receptor (LT β R)-expressing mesenchymal cells results in expression of chemokines, adhesion molecules and cytokines by mesenchymal cells. These factors support the attraction and retention of lymphocytes and therefore, lymph node formation. ILC3P, ILC3 progenitor; LTi, lymphoid-tissue inducer; TSLP, Thymic Stromal Lymphopoietin; RANK, receptor activator of nuclear factor κ B; RANKL, RANK ligand; VCAM1, vascular cell adhesion protein 1; DC, dendritic cell.

Barrier function

As intestinal ILC3 are the dominant source of IL-22 at steady-state, their role is crucial in maintaining barrier function (see Part I *Role of IL-22 in maintaining barrier function*). ILC3 are ideally positioned to ensure segregation between the host and the external environment, particularly commensal microbes. In mice and human intestinal tissues, ILC3-derived IL-22 induces AMP production to promote anatomical containment of bacteria, thus preventing bacterial dissemination and systemic immune activation (39). As mentioned earlier, IL-22 also promotes the physical exclusion of commensal bacteria through induction of mucins and goblet cell hyperplasia, and by regulating the expression of tight-junction components. ILC3 further support microbiota homeostasis through control of epithelial fucosylation. Ablation of IL-22 or LT in ILC3 results in a marked reduction in epithelial fucosylation, demonstrating that both cytokines are critical for this process (33). Since commensal bacteria metabolize fucose from host fucosylated proteins, this process is important to maintain mutualistic bacterial species and disruption of fucosylation leads to shifts in microbial communities. IL-22 production

by ILC3 is also important for the bacterial containment of specific communities such as *Alcaligenes spp.* or segmented filamentous bacteria (SFB) – either directly through the induction of AMP (39, 117) or indirectly by regulating T_H17 cells that limit SFB colonization (118, 119) (**Figure 10**).

While maintaining integrity is essential for the epithelium, remaining adaptable is as important to ensure proper mucosal functions such as nutrient absorption. ILC3-derived IL-22 modulates lipid absorption by IEC. Upon weaning, which represents a period of intense microbial colonization, microbial detection by myeloid cells leads to IL-22 release from ILC3 and consecutive upregulation of AMP like *Reg* proteins that insulate the barrier from bacteria. Increased IEC expression of antimicrobial molecules is concomitant with reduced IEC expression of key lipid transporter genes, which results in decreased lipid absorption (117). In line with this, Talbot and colleagues also observed that reduced IL-22 production by ILC3 enhances the expression of lipid-binding proteins and transporters (120). The authors propose that food consumption rapidly activates a population of enteric neurons that inhibits IL-22 production by ILC3, thereby increasing lipid absorption during meals. Therefore, ILC3 have emerged as orchestrators not only of barrier integrity but also of barrier adaptability. By indirectly sensing environmental cues, either microbes or food, ILC3 tune barrier function to fit its requirements at a given time, either protection or absorption. Interestingly, these studies suggest a trade-off between nutrient absorption and intestinal immunity and a pivotal role for ILC3 in prioritizing conflicting tasks (121).

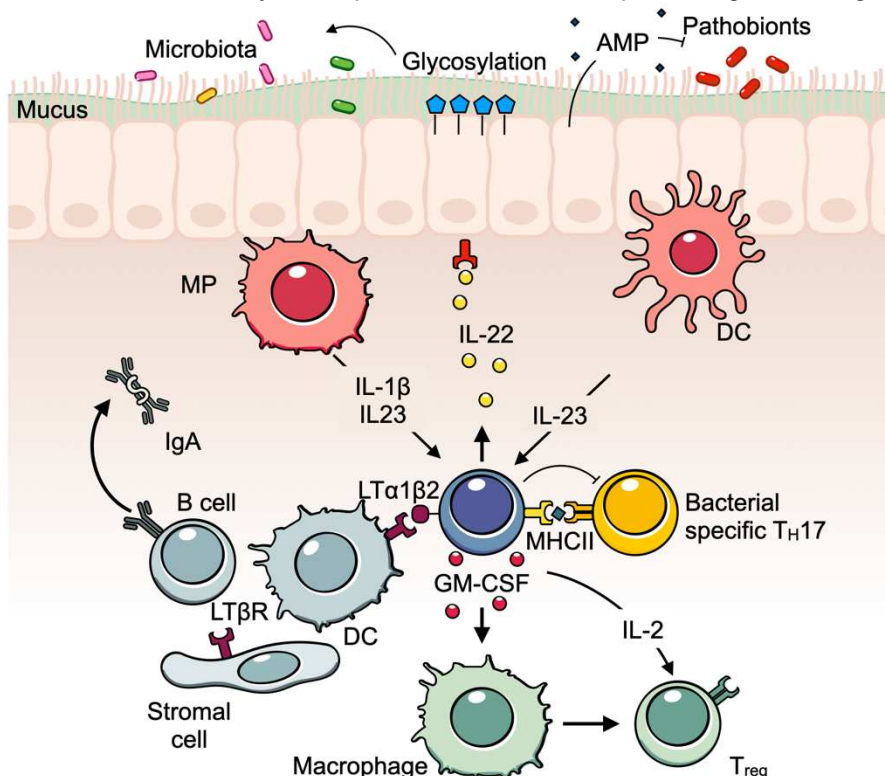


Figure 10 | ILC3 host-protective functions in intestinal homeostasis

Intestinal ILC3 exert multiple functions that are dependent on soluble factors, namely cytokines, and cellular interactions, through membrane-bound receptors, in order to maintain intestinal homeostasis. This is achieved through diverse mechanisms including the promotion of epithelial barrier function (epithelial glycosylation, mucus and AMP production) and immunoregulatory adaptive immune responses (support of T_{reg} function, intestinal IgA production and deletion of commensal-specific autoreactive T cells). AMP, antimicrobial peptides; MP, mononuclear phagocyte; DC, dendritic cell; MHCII, major histocompatibility complex class II; GM-CSF, granulocyte-macrophage colony-stimulating factor; T_{reg} , regulatory T cells; LT α 1 β 2, lymphotoxin- α 1 β 2; LT β R, lymphotoxin- β receptor; IgA, immunoglobulin A.

Tissue repair

Several reports have linked ILC3-associated signals and intestinal stem cell function including protection and regeneration of the epithelium. IL-22 is constitutively produced by small intestinal ILC3 (122) and plays a crucial role in intestinal repair (see Part I *Role of IL-22 in tissue repair and tissue inflammation*). Through IL-22, ILC3 safeguard epithelial stem cells after intestinal damage using methotrexate or in a mouse model of graft-versus-host disease where allogeneic T cells attack ISC (43, 44). The role of ILC3-derived IL-22 in epithelial repair is only partially understood but seems to rely on IL-22 dependent activation of STAT3 in ISC and subsequent expression of pathways associated with wound healing (45). Interestingly, it has been shown that ILC3 also have effects on epithelial regeneration beyond IL-22 production. The crypt regeneration-associated Hippo- yes-associated protein 1 (YAP1) pathway is amplified by the presence of ILC3 independently of IL-22, while ILC3-derived IL-22 appears to be important for stem cell protection and maintenance (123). In addition, ILC3 also adjust the DNA damage response (DDR) in ISC at homeostasis. In the intestine, a group of aryl hydrocarbon receptor (AhR) ligands, glucosinolates, are genotoxic and induce DDR in ISC. Since the aryl hydrocarbon receptor (AhR) is expressed on ILC3 and is a key regulator their IL-22 expression, ILC3 can integrate these diet-derived signals and adapt on-demand ISC responses to genotoxic compounds present in diet (47) (**Figure 11**).

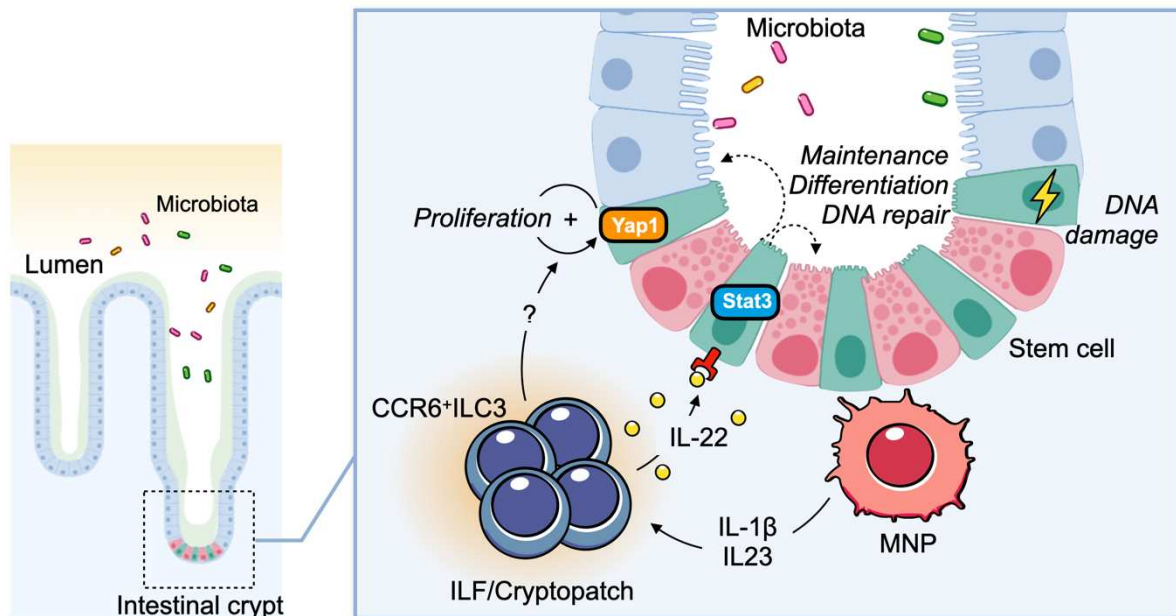


Figure 11 | Intestinal ILC3 orchestrate tissue repair

Interactions between intestinal ILF ILC3 and intestinal stem cells (ISC) within crypts are essential for epithelial regeneration, repair and DNA damage repair (DDR). Both IL-22-dependent and independent mechanisms are involved in the aforementioned functions, whether it be at steady-state or following tissue damage. MNP, mononuclear phagocyte; YAP1, yes-associated protein 1; STAT3, signal transducer and activator of transcription 3; ILF, isolated lymphoid follicle.

Regulation of adaptive immunity

In addition to their role as regulators of epithelial cells, intestinal ILC3 are increasingly appreciated to have broader immunoregulatory functions via interactions with the adaptive immune system.

CCR6⁺ ILC3 are mainly positioned in cryptopatches in the adult intestine. Cryptopatches (CP) are lymphoid clusters at the bottom of intestinal crypts that can be detected starting 2 weeks after birth (124). Initially, only LT_i cells and dendritic cells (DC) cluster in the CP but both are important for the development and maintenance of these structures (125). In response to microbial and dietary signals, CP recruit B cells into the structure and thereby further mature into isolated lymphoid follicles (ILF) (77, 112, 113) that support IgA production in the intestine. LT_i cells are essential for the development of these structures as well as for subsequent IgA responses. ILF provide a privileged site for ILC3-B cell interaction and thus lead to a T-cell-independent stimulation of IgA (126). It was discovered that LT_i cells actually stimulate both T cell-dependent IgA production via soluble LT α 3 involvement in T cell homing to the gut and T cell-independent IgA production via interaction of membrane-bound LT α 1 β 2 with LT β R on myeloid cells (127). Thus, soluble and membrane-bound lymphotoxins produced by ILC3 distinctly organize adaptive immune responses in the gut and control commensal microbiota composition (**Figure 10**).

ILC3 are also crucial to regulate the balance between inflammatory and tolerogenic T cells responses in the intestine – either through soluble mediators or direct cell-to-cell interaction. For instance, intestinal resident macrophages sense the microbiota and produce IL-1 β to promote GM-CSF production by ILC3. Such GM-CSF production improves antigen-presenting cells ability to produce IL-10 that supports survival and expansion of regulatory T cells (T_{reg}), thus promoting oral tolerance (128). Specific ILC3 subsets control this balance through distinctive mechanisms associated to different effector molecules. IL-2 is involved the generation, maintenance and function of T_{reg}. NKp46⁺ ILC3 have recently been described an essential and non-redundant source of IL-2, again in response to macrophage-derived IL1 β production following microbial sensing, that maintains T_{reg} cell homeostasis and promotes oral tolerance uniquely in the small intestine (129). Alternatively, CCR6⁺ ILC3 inhibit pro-inflammatory CD4⁺ T cell responses in the intestine rather than favoring T_{reg} development. At steady-state, MHC-II-expressing CCR6⁺ ILC3 present peptides from the commensal flora to CD4⁺ T cells without providing co-stimulation and directly induce cell death of autoreactive commensal bacteria-specific T cells in a process coined ‘intestinal T cell selection’, thus preventing intestinal inflammation (81, 130). Altogether, these studies advocate for the microbiota-guided role of intestinal ILC3 in preserving a tolerogenic state at steady-state (**Figure 10**). However, ILC3 can also drive pathogenic T cell development. During colitis, tumor necrosis factor-like cytokine 1A (TL1A)-dependent expression of the co-stimulatory molecule OX40 ligand (OX40L) on MHC-II⁺ ILC3 leads to the activation of antigen-specific intestinal T_H1 cells required for chronic T cell colitis (131).

b. Immunity

Immunity to pathogens

Initial studies characterizing the functions of IL-22 showed that it was involved in the induction of AMP in the intestine. Subsequent studies have demonstrated the functional importance of IL-22 in host protective immunity (see Part I *Role of IL-22 in antimicrobial immunity*). Intestinal ILC3 and notably NKp46⁺ ILC3 were discovered in part because of their high IL-22 production and it was immediately hypothesized and observed that these cells mediated protection against enteropathogenic bacteria *C. rodentium* (41). Indeed, previous studies had shown that resistance to intestinal infection with *C. rodentium*, a model organism for attaching-and-effacing *Escherichia coli*, was strictly IL-22 dependent and that mucosal IL-22 was retained in *Rag2*^{-/-} mice lacking B and T cells (38). Since then, it has been found that ILC3 – via IL-22 production but not only – are protective in various infection models, including *C. rodentium*, *Salmonella typhimurium*, *Candida albicans* and rotavirus infection.

The contribution of ILC3-derived IL-22 to host protection has been intensively investigated in the context of *C. rodentium* infection. The secretion of IL-23 by intestinal mononuclear phagocytes is required for proper ILC3 activation. As previously mentioned, intestinal IL-22 stimulate the production of AMP, mucus and epithelial fucosylation. Altogether, these IL-22 dependent responses increase resistance to *C. rodentium* through limitation of pathogen replication and dissemination. In immunocompromised mice (*Rag2*^{-/-}), ILC3-derived IL-22 is required for survival and immunity to *C. rodentium* infection (38, 41, 42, 132), whereas in the presence of adaptive immunity ILC3-derived IL-22 is required to prevent morbidity in the early phase of infection before the generation of a T cell response (40, 42, 132).

IL-22 can also act synergistically or additively with other factors to promote host defense in the intestine. A synergistic requirement between IL-22 ILC3 and IFN λ on epithelial cells has been reported in rotaviral infection. Rotaviral infection triggers the release of IL-1 α from epithelial cells, which stimulates ILC3-derived IL-22 production. Cooperation between these IL-22 and IFN λ is required for optimal activation of STAT1 and expression of interferon-stimulated genes (ISG), thereby supporting resistance to rotaviruses (133). IL-22 can also act with ILC3-derived factors. LT α 1 β 2-LT β R triggers IL-23 release by myeloid cells which is required for IL-22 production by LTi cells and therefore for protection against *C. rodentium* (134). Interestingly, fucosylation of the intestinal epithelium requires IL-22 and LT α – maybe through IL-22 regulation by the lymphotoxin pathway – and protects against *S. Typhimurium* infection (33).

Even though IL-22 is the main effector cytokine secreted by both CCR6⁺ and CCR6⁻ ILC3, ILC3 produce other molecules involved in immunity against infections. In particular, T-bet upregulation in CCR6⁻ ILC3 is associated with IFN γ production. One key cytokine controlling *Salmonella* infection is IFN γ . During *S. Typhimurium* infection, 80% of IFN γ -producing cells early following infection are NKp46⁺ T-bet⁺ ILC3. The production of IFN γ by NKp46⁺ ILC3 is essential for the release of mucus and contributes to innate barrier protection against *S. Typhimurium* (86). Besides ILC3 role in antiviral and antimicrobial responses, ILC3 and notably CCR6⁺ also contribute to antifungal immunity. IL-17 mediated immunity has emerged as a crucial host defense mechanism against fungal infections. In a mouse model

of oropharyngeal infection with *Candida albicans*, Gladiator and colleagues have observed that ILC3 are the main source of IL-17A and IL-17F in the oral mucosae (135). Since *Rag1*^{-/-} mice control *C. albicans*, resistance is not T cell dependent. However, *Rorc*^{-/-} mice fail to control *C. albicans* infection, indicating that ILC3 are essential to promote IL-17-dependent anti-fungal immunity. Patients with *RORC* mutations have selective immunodeficiencies to *C. albicans* and *Mycobacterium tuberculosis* infections (136). Although these mutations result in absence of IL-17-producing T cells, the lack of IL-17-producing ILC3 (114) might also contribute to this phenotype (Figure 12).

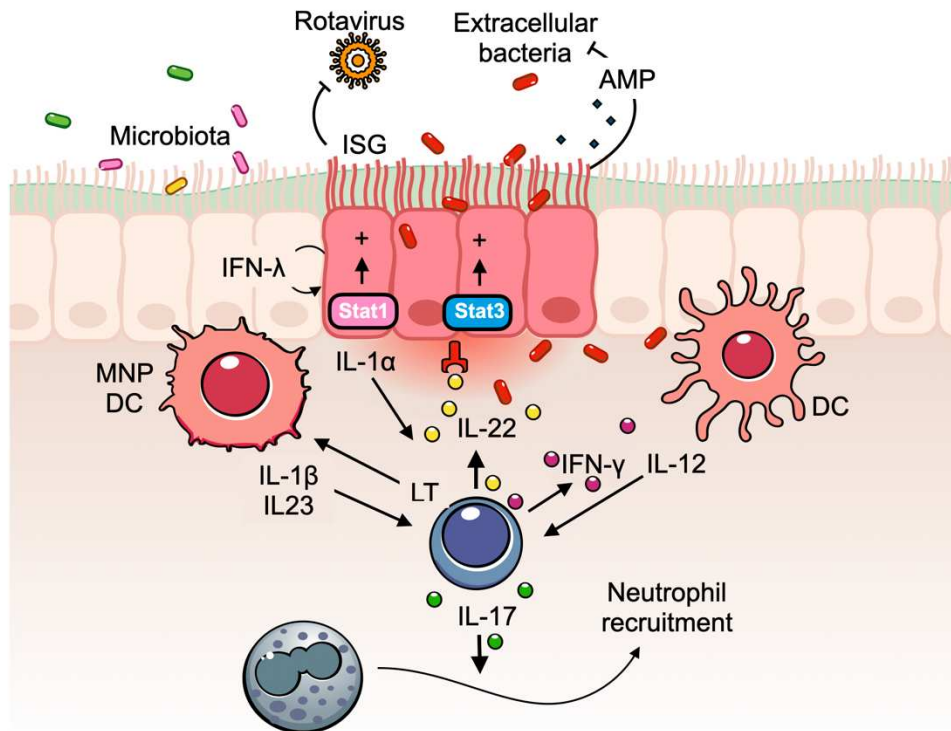


Figure 12 | ILC3 promote protective immunity against intestinal pathogens

Upon pathogen challenge, epithelial and myeloid-derived signals activate ILC3. Intestinal ILC3 produce IL-22, which is involved in the protection against a wide variety of pathogens, but also other cytokines such as IFN γ and IL-17. IL-17 production allows the recruitment of neutrophils in the lamina propria whereas IL-22 secretion in response to IL-1 β and IL-23 drives epithelial responses including the production of antimicrobial peptides (AMP), and therefore promote host defense in the intestine. MNP, mononuclear phagocyte; DC, dendritic cell; LT, lymphotoxin; STAT1/3, signal transducer and activator of transcription 1/3; IFN, interferon; ISG, interferon-stimulated genes.

Inflammation

Detrimental ILC3 responses have been reported in several models of colitis and have been related to the activity of ILC3-derived cytokines.

Accumulation of IFN γ and IL-17-secreting DN ILC3 in the intestine following *Helicobacter hepaticus* is required for the induction of innate bacterial driven colitis in *Rag*^{-/-} mice (85). Similarly, during *Salmonella* infection, the production of IFN γ by NKp46⁺ ILC3 is essential to protect the barrier but is also involved in the development of enterocolitis (86). Additionally, in both mice and humans, ILC3 secrete GM-CSF, which can recruit myeloid cells and further promote intestinal inflammation (137). Furthermore, although IL-22 can be protective in several models of colitis, it can also promote

pathological inflammation (see Part I *Role of IL-22 in tissue repair and tissue inflammation*). For instance, in another model of IL-23 driven colitis, ILC3-derived IL-22 was necessary to promote the development of α CD40-induced intestinal inflammation (85) (**Figure 13**). The association of ILC3 with intestinal inflammation has prompted long-standing interest in the role of ILC3 in inflammatory bowel disease (IBD). A definitive role for ILC3 in IBD hasn't been established but several studies have reported altered ILC3 numbers or functions (138).

In line with a disease-causing role of ILC3, Crohn's disease patients exhibit higher numbers of IL-17-producing ILC3 (139). In a cohort of pediatric patients with Crohn's disease, the expression of MHCII on ILC3 was lower than in control subjects without IBD and correlates with increased numbers of T_H17 cells, in accordance with a role of MHCII-expressing ILC3 in limiting pathogenic T cells (81). Conversion of IL-22-producing ILC3 to IFN γ -producing ILC1 'ex ILC3' (see Part IV *ILC3 plasticity*) was also observed in the intestine of Crohn's disease patients (140) (**Figure 13**), further supporting the hypothesis that alteration of ILC3 phenotypes and functions can promote the development of IBD. Changes in IL-22 producing ILC3 populations in the intestine of IBD patients have also been observed but since IL-22 can be either protective or pathogenic, the role of these IL-22⁺ ILC3 remains unclear.

Cancer

While ILC3 responses appear to be protective through their role in promoting barrier homeostasis and repair, there is also evidence that ILC3-derived cytokines participate to the malignant transformation of epithelial cells and tumorigenesis.

The negative role of ILC3-associated cytokines was first hinted in a mouse model of human colorectal carcinoma in which tumor growth was driven by intestinal IL-23 and IL-17 (141). Colonic inflammation resulted from defects in epithelial barrier integrity and microbial segregation, two functions that can be associated with ILC3 responses. Furthermore, aberrant crypt lesions and transition of *H. hepaticus*-induced colitis to carcinogen-induced colorectal cancer using azoxymethane (AOM) was proven to rely on IL-17 and IL-22 produced by DN ILC3 (142). Therefore, it seems that sustained ILC3 activation can lead to cancer development. Indeed, work focusing on IL-22 and IL-22BP has revealed that uncontrolled IL-22 signals increase epithelial proliferation and tumorigenesis in *Il22bp*^{-/-} mice treated with DSS + AOM (29). Similarly, in the APC^{min/+} mouse model which spontaneously develop multiple intestinal adenomas due to a point mutation in the tumor-suppressing gene adenomatous polyposis coli (APC), *Il22* deficiency decrease tumor numbers whereas *Il22bp* deficiency promotes the development of colorectal tumors (29) (**Figure 13**).

Although IL-22 is protective against genotoxic stress in the intestine through induction of DDR-dependent apoptosis in colonic ISC, thus limiting cancer transformation (47), its function in controlling epithelial proliferation could be harmful too and accelerate tumor progression. The conflicting results obtained in *Il22*^{-/-} mice may be dependent on the context and on the phase of the response. While IL-22 protects the intestinal barrier against tumorigenesis at steady-state, it may enhance tumor proliferation in cells that have already lost cell-cycle control (29, 142, 143). At homeostasis, *Il22*^{-/-} mice exhibit more tumors than controls, in accordance with IL-22 protective role at steady-state and possibly during early phases of pathogenesis (47). However, the fact that *Il22*^{-/-}APC^{min/+} mice develop less

tumors than APC^{min/+} suggests that IL-22 may be detrimental in an already inflammatory protumorigenic environment (29). Accordingly, the pro-inflammatory cytokine IL-18 reduces IL-22BP levels *in vitro*, possibly driving intestinal IL-22 perpetuation (29). Further investigation is required to understand the role of IL-22 and ILC3 in cancer, but the tissue environment is likely to regulate these responses. Understanding the environmental signals involved in ILC3 biology may be key to address ILC3 role in pathogenesis and harness their potential in therapeutics.

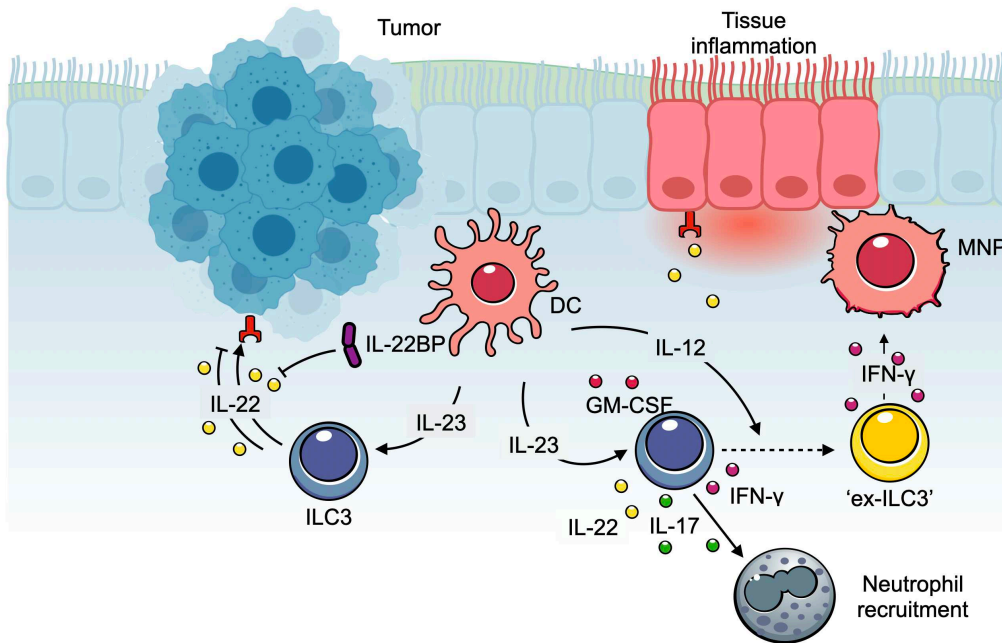


Figure 13 | Pro-inflammatory and tumorigenic functions of intestinal ILC3

ILC3 are amplified in a variety of inflammatory diseases and contribute to intestinal pathology. Although IL-22 mediates host-protective functions, it can also promote pathological inflammation and tumorigenesis depending on the context. IL-22 can be neutralized by IL-22BP-producing DC. Moreover, ILC3 or converted 'ex-ILC3' can secrete a combination of additional cytokines such as IFN γ , IL-17 and GM-CSF that are involved in the development of intestinal inflammation in response to myeloid-derived IL-12 or IL-23 respectively. MNP, mononuclear phagocyte; DC, dendritic cell; IFN γ , interferon gamma; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-22BP, IL-22 binding protein.

III. Localization and migration of intestinal ILC3

A. Tissue distribution of ILC3

ILC are widely distributed across the body and are present both in lymphoid and non-lymphoid tissues. They are highly enriched at mucosal sites, in relation to their function in initiating and maintaining tissue homeostasis and immunity. ILC are differentially distributed across the organism and the relative abundance of ILC groups differs between tissues both in mice and human (144–146). For instance, ILC2 are present in the lung, colon, skin and adipose tissues but not in the liver whereas conventional NK cells and ILC1 can be easily found in the liver but are in minority in the aforementioned organs (70, 145). The intestine contains all ILC family members, with ILC3 and ILC2 forming the predominant intestinal ILC subsets in the murine small and large intestine, respectively.

ILC3 are mostly found in the gastrointestinal tract where they represent up to 9% of leukocytes (CD45⁺ cells; C57BL/6J; (67), in line with our data) and can be recovered in large numbers (**Figure 14**). Furthermore, ILC3 and particularly CCR6⁺ ILC3 can be encountered in lymphoid tissues such as secondary lymphoid organs where they reside in interfollicular regions or in other peripheral tissues like the lung or the skin to some extent. Within the intestine, regional differences in ILC3 distribution can be observed. ILC3 numbers seem to increase from the proximal to the distal small intestine (3) and ILC3 subsets are differentially distributed. NKp46⁺ ILC3 are almost exclusively present in the small intestine where CCR6⁻ ILC3 (including NKp46⁺ ILC3) are the most abundant ILC3 population, whereas CCR6⁺ ILC3 constitute the majority of colonic ILC3 (84) (**Figure 14**). Although age influences the distribution of ILC3 subsets (105), as well as the bacterial density or composition along this axis probably, the main determinants of this specific spatial distribution remain unknown.

The differential tissue distribution of ILC and the mechanisms driving this compartmentalization are not fully understood. One reason for this could be the temporal seeding of tissues. Indeed, an ILC pool residing in a given tissue can be derived from different waves of development from fetal to adult life. For instance, intestinal colonization by ILC progenitors begins in fetal life, a time where CILCP appear to preferentially differentiate into ILC3. Moreover, ILC3 development proceeds in a least two waves: a first wave where fetal ILC3P give rise to LT_i cells followed by a second postnatal wave where adult ILC3 including NKp46⁺ ILC3 develop (105). This layered ontogeny could explain regional differences observed for intestinal ILC3 subsets. Whether ontogeny of ILC differs between tissues remains unclear but progress has recently been made on this long-standing question. Schneider and colleagues have shown that adult ILC2 pools are composed of cells of fetal, postnatal and adult-derived origin using fate-mapping strategies (147). Interestingly, the relative contribution of these three distinct waves to the adult ILC2 pool and the kinetics of dilution of the ILC2 pool by *de novo* generated ILC2 vary between tissues. Another possible explanation for differential ILC repartition could be the organ-specific expression of homing receptors on mature ILC, as this has been hypothesized for T cells. It was postulated early that unique chemokine and integrin combinations drive T cell homing in a multi-step model and that organ-specific 'area codes' may exist wherein each tissue display a unique molecular signature for entry (148). Supporting this hypothesis, distinct homing molecules have been identified for T cell homing to various

peripheral tissues (149). As an example, T cells that undergo activation in gut-associated mucosal tissues upregulate the integrin $\alpha 4\beta 7$ and the chemokine receptor CCR9 that both play important roles in homing to the small intestine mucosa (150). Similar mechanisms could be involved in ILC homing to unique tissue sites, with $\alpha 4\beta 7$ and CCR9 acting as possible intestinal homing receptors for ILC too. Finally, ILC distribution could depend on the presence of maintenance signals including cytokines, microbial signals or nutrients (**Figure 14**). Aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that is essential for the maintenance and function of ILC3 (151). AhR ligands include physiologic ligands generated by microbiota, diet, and host metabolism present in the gastrointestinal tract, which could explain the preferential distribution of ILC3 there. Differences in microbial or nutrient composition along the intestinal tract could lead to opposite proportions of CCR6⁺ and CCR6⁻ ILC3 between small intestine and colon. In the colon, the main metabolites of undigested carbohydrates by bacterial fermentation are short-chain fatty acids (SCFA) (152). SCFA do not serve only as an energy source but also play an important role in the modulation of immune responses and their receptors such as FFAR2, FFAR3 and GPR109A are expressed by many immune cells (152). FFAR2 expression is enriched on colonic ILC3 and interestingly, ILC3-specific deletion of FFAR2 impairs the proliferation and IL-22 secretion of CCR6⁺ ILC3 specifically (153). Thus, the high concentration of SCFA might contribute to the maintenance and abundance of CCR6⁺ ILC3 in the colon compared to the small intestine.

B. Tissue residency versus tissue trafficking of ILC3

ILC are present in almost every tissue examined so far. In a pivotal study, Gasteiger and colleagues have investigated how ILC maintain their presence in lymphoid and peripheral tissues using parabiotic mice (67). Unlike most lymphocytes, mature ILC (besides conventional NK cells) are largely tissue-resident cells under physiologic or inflammatory conditions, both in lymphoid and non-lymphoid organs. This suggests that ILC are maintained by local-self renewal, although rare seeding from the bone marrow could occur. However, recent studies have challenged this concept and hypothesized that ILC, including ILC3, could retain some migratory capacities.

Dutton and colleagues have used Kaede photoconvertible mice to label all cells within a single peripheral lymph node (154). This approach allows to monitor migratory and resident populations as well as to track labeled cells. In peripheral lymph nodes, both migratory and resident ILC population could be identified. Similarly to T cells, lymph node entry and egress by migratory ILC rely on CCR7 and S1PR, respectively. Most ILC3 were tissue-resident but a small fraction also had the ability to migrate. Previous work from this team had already showed that CCR6⁺ ILC3 have the capacity to constitutively migrate from the intestine to the mesenteric lymph node in a CCR7-dependent fashion (155) (**Figure 14**). The functional significance of these ILC migratory populations remains unclear. CCR6⁺ ILC3 constitutive trafficking could be critical for 'intestinal T cell selection' occurring in the mesenteric lymph node (81). MHC-II-expressing CCR6⁺ ILC3 could acquire peptides from the commensal flora in the intestine and subsequently migrate to interfollicular regions of the mesenteric lymph node for presentation and deletion of autoreactive T cells. Additionally, it has been proposed that ILC migration could be augmented upon inflammation or infection. Circulation of inflammatory ILC2 has been detected

upon inflammation by three complementary techniques and supposedly contributes to anti-helminth defense and tissue repair (156). Using the Kaede photoconvertible mice approach, one could address whether ILC3 trafficking from or to the intestine occurs in these conditions, compare functional capacities of resident versus migratory ILC3 and understand the biological relevance of these ILC3 with migratory capacities.

While murine ILC appear mostly tissue-resident with little capacity for migration, ILC can be readily detected in the circulation of human individuals. Circulating ILC comprise both ILC2 and ILCP (114). It has been proposed that mature ILC in tissues, including ILC3, are maintained by migration and differentiation of blood ILCP into tissues under the influence of local environmental factors. Lim and colleagues have later suggested that IL-1 β following disruption of tissue homeostasis could promote migration of blood ILCP into tissues (157). However, IL-1 β lacks direct chemotactic activity and effective chemotactic cues that could be involved in this process haven't been investigated. Further studies are required to clarify tissue residency versus tissue trafficking of ILC, and to establish to what extent mature ILC in tissues are replenished by hematogenous progenitors.

C. Homing of ILC3

Mature ILC3 are largely tissue-resident cells but the mechanisms regulating ILC3 homing to tissues are still only partially understood. As for T cells, ILC3 homing involves integrins and chemokine receptors (**Figure 14**).

Seeding of peripheral tissues by ILC3 progenitors of mature ILC3 initially occurs during embryogenesis (100, 101, 110). ILC3 are among the first lymphocytes to colonize fetal tissues including secondary lymphoid organs and intestine (74, 76, 102). Both fetal and adult ILC3 progenitors and mature ILC3 express the integrin $\alpha 4\beta 7$ and the chemokine receptor CXCR6. The interaction of mucosal addressin cell adhesion molecule-1 (MadCam-1) on high endothelial venules (HEV) with $\alpha 4\beta 7$ integrin expressed on LTi cells allows their recruitment to developing lymph nodes and Peyer's patches (74, 158). $\alpha 4\beta 7$ integrin is a homing receptor for intestinal T cells and could act accordingly for intestinal seeding by rare ILC3 progenitors in adult life, as MadCam-1 is then mainly expressed by endothelial cells in the intestine and gut-associated mucosal tissues (159). The chemokine receptor CXCR6 is also expressed on fetal and adult ILC3P (104, 160), as well as on all adult intestinal ILC3 subsets (132). While the absence of CXCR6 does not affect the development of ROR γ t⁺ cells in the fetus (104), it has been suggested to impair ROR γ t⁺ cells migration to the periphery and to be compensated by higher ILC3 proliferation (160). Moreover, this receptor has a critical role for NKp46⁺ ILC3 homeostasis as CXCR6-deficient mice have a marked reduction in intestinal NKp46⁺ ILC3 numbers (132). Interestingly, CCR6⁺ ILC3 do express CXCR6 but are not affected by its absence. This divergent effect of CXCR6 on NKp46⁺ and CCR6⁺ ILC3 could be explained by chemokine receptor redundancy. CCR6⁺ ILC3, also known as LTi cells, express several chemokine receptors such as CXCR5, CCR7 and of course CCR6.

CXCR5 has been shown to drive LTi cells clustering at lymph node anlagen and consequently, lymphoid organogenesis. Retinoic acid, possibly produced by nerve fibers, induces CXCL13 production by mesenchymal stromal cells to attract CXCR5-expressing LTi cells, thereby fixing sites of future

development of lymph nodes and Peyer's patches (78, 161–163). The function of CXCR5 in lymphoid tissue organogenesis is complemented by CCR7 through CCL19/CCL21 attraction of CCR7-expressing LT_i cells to nascent lymph nodes (164, 165). Despite CCR6 expression by all LT_i cells, absence of this receptor does not seem to affect lymph node or Peyer's patch formation (166) and LT_i cells are still found in intestine (105) but their spatial positioning is disorganized (77). Together, CXCR5, CCR7 and CCR6 might support homing of CCR6⁺ ILC3 during fetal life, whereas CXCR6 might have a role in the development of ILC3 subsets that appear after birth, such as NKp46⁺ ILC3.

Recently, a role for CCR9 in intestinal homing of ILC3 has been uncovered (167), as for T cells. In the small intestine, dietary vitamin A metabolites are converted to retinoic acid (RA) by CD103⁺ DC and delivered to T cells in gut-associated lymphoid tissues where they promote α 4 β 7 and CCR9 expression (150). Its ligand, CCL25 (thymus-expressed chemokine), is constitutively expressed in the small intestine but not in the colon by intestinal epithelial cells and drives intestinal T cell homing. Kim and colleagues observed that CCR9 was expressed on intestinal ILC3 and that ablation of CCR9 resulted in a reduction of intestinal ILC3 in the small intestine but not in the colon (167). Prolonged *in vitro* exposure to RA induced CCR9 expression on ILC3 and vitamin-A deficient diet of mice resulted in decreased CCR9 expression on intestinal ILC3. Thus, the authors have proposed that RA signals are involved in ILC3 homing to the gut by activation of CCR9.

In addition to homing receptors that are necessary to recruit ILC3, other receptors should retain ILC3 within tissues. All intestinal ILC3 constitutively express CD69 (83, 160), a lectin involved in lymphocyte retention (168, 169). In T cells, CD69 is expressed upon activation and antagonizes the sphingosine-1-phosphate receptors (S1PR) that promote lymphocyte egress (170, 171). Moreover, some CCR6⁻ ILC3 express the integrin α -subunit CD49a, a collagen-binding protein that has been shown to promote T cell retention in tissues (172).

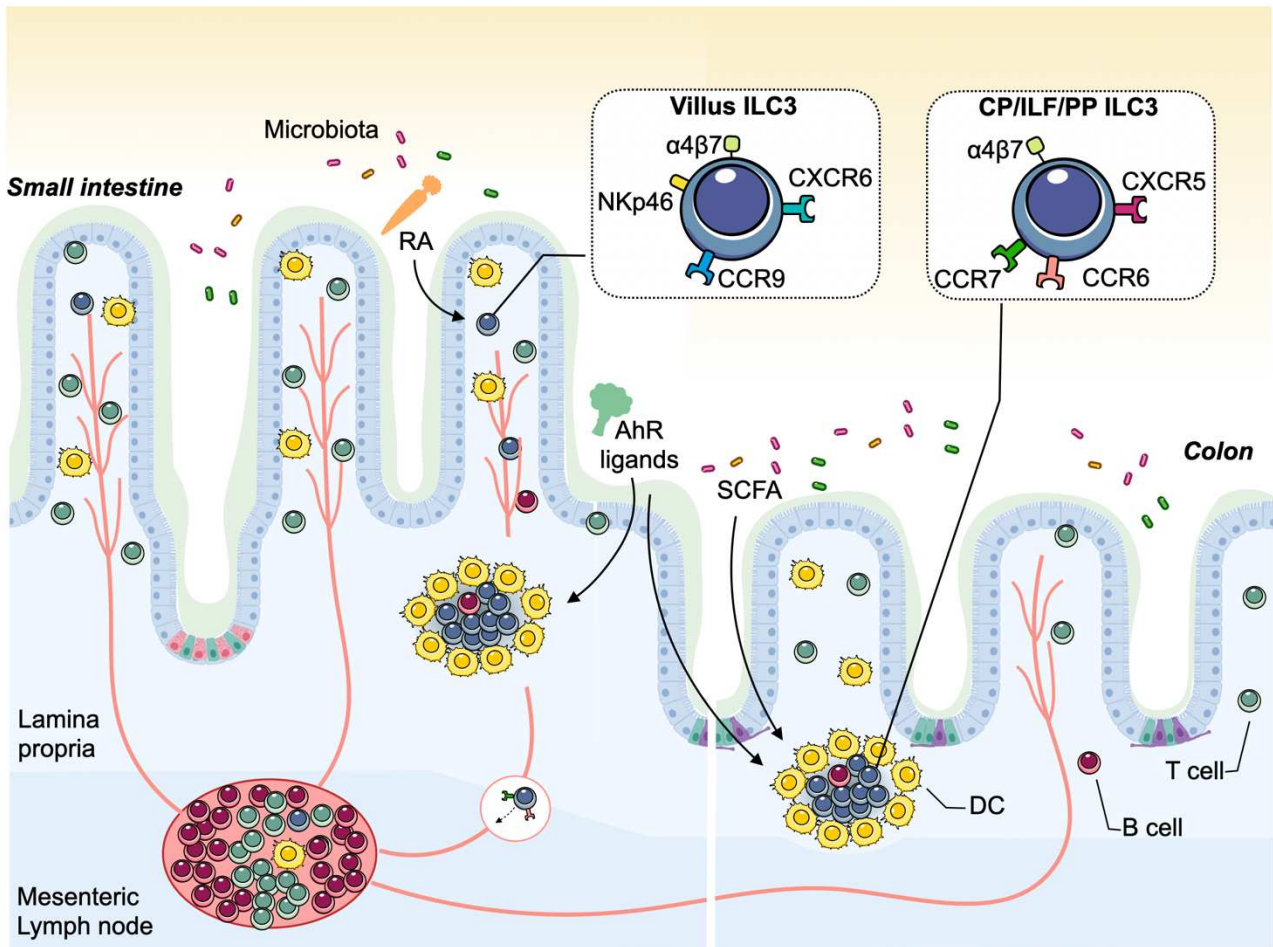


Figure 14 | Localization and migration of intestinal ILC3

ILC3 are predominantly distributed in the gastrointestinal tract: while CCR6⁺ ILC3 are found both in the small intestine and colon, NKp46⁺ ILC3 presence is restricted to the small intestine. NKp46⁺ ILC3 are positioned within intestinal villi whereas CCR6⁺ ILC3 cluster into cryptopatches (CP) and isolated lymphoid follicles (ILF) in the basal lamina propria. Chemokines receptors and integrins play an important in the homing and spatial positioning of intestinal ILC3 subsets, as well as extrinsic factors derived from the microbiota and the diet. Although a small proportion of CCR6⁺ ILC3 can migrate from the intestine to the mesenteric lymph node, most intestinal ILC3 are tissue-resident. RA, retinoic acid; SCFA, short-chain fatty-acids; DC, dendritic cell; PP, Peyer's patches.

D. Spatial positioning and cellular behavior of intestinal ILC3

Intestinal ILC3 play multiple and diverse roles, yet it is poorly understood how these tissue-resident cells are spatially positioned within the intestine to fulfill these roles. Localization of ILC3 within the intestinal tissue may affect their ability to sense signals from specialized niches and to quickly respond to environmental cues under physiologic or inflammatory conditions. Understanding ILC3 spatial positioning and migration may help to explain how these cells exert such powerful effects on intestinal immunity.

Intestinal ILC3 topography: description and regulation

In the intestine, NKp46⁺ ILC3 are highly enriched and dispersed in the intestinal lamina propria whereas CCR6⁺ ILC3 mainly reside in the basal intestinal mucosa clustered in isolated lymphoid follicles (ILF) (173). The spatial distribution of ILC3 within the intestine is guided by chemokine receptors. We have recently shown that the CXCL16/CXCR6 chemokine-chemokine receptor axis regulates ILC3 diversity, localization and function (132). Indeed, ablation of CXCR6 generated a selective loss of the NKp46⁺ ILC3 subset and impaired their localization into the intestinal villi. Interestingly, CCR6⁺ ILC3 do express CXCR6 but were not affected by its absence, remaining clustered in ILF. More recent work has established that metabolic signals also ensure that ILC3 are properly positioned in the intestine. As an example, Emgård and colleagues have found that oxysterol-sensing through the G protein-coupled receptor GPR183 (also known as EBI2) guide ILC3 positioning within ILF, as colonic ILC3 lacking GPR183 exhibit aberrant localization and failed to form ILF (174) (**Figure 14**).

Link between ILC3 topography and function

Interestingly, both CXCR6 and CCR6 chemokine receptors control ILC3 topography while having an impact on ILC3 IL-22 production too. IL-22 production has been correlated with *Cxcr6* expression in ILC3 and CXCR6 is essential in locating IL-22-producing NKp46⁺ ILC3 into the villus (132). Ablation of CXCR6 leads parallelly to the loss of IL-22-producing NKp46⁺ ILC3 and to a strong reduction of IL-22 production from remaining NKp46⁺ ILC3 within the gut, thus resulting in the inability of the host to control *C. rodentium* infection (132). In the intestine, CD11b⁺ (CD103⁻ CX3CR1⁺) macrophages are the major producers of CXCL16 at steady-state and are responsible for CXCL16 increase after *C. rodentium* infection (132). Simultaneously, intestinal CD11b⁺ macrophages produce IL-23 (132, 175, 176). Together, this would allow CXCR6⁺ ILC3 to be attracted to CD11b⁺ DC during infection and to receive signals promoting IL-22 production. In contrast, although ILC3 subsets are not altered in CCR6-deficient mice, their expression of IL-22 is significantly increased and, as a consequence, the production of antibacterial peptides by epithelial cells is augmented (105). Collectively, these data indicate that chemokine receptors, notably CXCR6 and CCR6, regulate the topography and function of ILC3 and suggest that ILC3 localization within the intestinal tissue is critical for their function.

Lymphocyte migration in intestinal immunosurveillance

Functional analyses of ILC3 have mainly focused on cytokine production, regardless of their tissue localization. Furthermore, little is known about ILC3 movement within tissues, although this could be a mechanism through which ILC3 modulate tissue responses. In T cell biology, a lot of effort has been made to understand lymphocyte migration, initially in an attempt to explain how a single T cell could make contact with rare antigen-presenting cells exhibiting their specific epitope (149). It has now become clear that T cell migratory behavior is closely linked to their function. High-resolution microscopy techniques such as two-photon microscopy have allowed dynamic imaging of T cells within tissues, including the intestine, and to understand the function of their migratory behavior. For

instance, intestinal CD4⁺ intraepithelial lymphocytes (IEL) exhibit unique movement patterns at steady-state and scan the epithelial layer in a microbiota-dependent fashion (177). Upon enteric infection, this behavioral pattern changes and CD4⁺ IEL ‘squeeze’ between IEC. This unique movement – termed ‘flossing’ – is involved in protecting the host against enteric infections, as illustrated by the increase incidence of *Salmonella* invasion when microbial-sensing by IEC is abolished and intestinal CD4⁺ IEL ‘flossing’ is disrupted. This highlights how lymphocyte migratory behavior can impact their role in intestinal immunosurveillance. Applying these approaches to the study of intestinal ILC3 could be useful to understand ILC3 cellular behavior and its impact on ILC3 function, notably in barrier protection.

IV. Influence of tissue environment on ILC3 biology

A. Environmental signals in ILC3 maintenance

Intestinal ILC3 are ideally positioned to sense a wide range of external and host-derived signals. ILC3 have the ability to integrate these multiple inputs and adapt their effector responses in order to maintain tissue homeostasis or to respond to tissue perturbation. Both 'endogenous' factors comprising stromal cells, innate and adaptive immune cells, neurons and 'exogenous' factors including microbes and metabolites are involved in tuning ILC3 function. Indeed, several studies have shown that transcriptional programs and cytokine secretion of ILC3 can be modulated by extrinsic factors (**Figure 15**).

1. Endogenous signals

Maintenance and activation of ILC3 relies on a cross-talk with stromal cells as well as immune cells. Stromal cells form specialized tissue niches that support ILC3 survival in secondary lymphoid tissues and gut-associated mucosal tissues. In addition to IL-7 role in ILC3 development (see Part II *Generation of ILC*), IL-7/IL-7R α signalling appears to be involved in ILC3 homeostasis as exogenous delivery of IL-7 can expand ILC3 *in vivo* in the adult (96, 97). Besides IL-7, TSLP can also signal through a heterodimer of IL-7R α and TSLPR and reinforce ILC3 survival (178). Studies have identified diverse stromal cells as the sources of IL-7 and TSLP (179), highlighting the role of stromal-cell derived factors in ILC3 maintenance. In the intestine, oxysterols (in particular 7 α ,25-hydroxycholesterol; 7 α ,25-OHC) are required for GPR183-mediated recruitment of ILC3 to colonic patches. ILF-resident CD34⁻ podoplanin⁺ stromal cells locally synthesize 7 α ,25-OHC and CD34⁺ podoplanin⁺ stromal cells located outside of ILF express GPR183-ligand-degrading enzymes, thus creating a gradient of oxysterols between ILF and the intestinal lamina propria necessary for proper ILC3 positioning and function (174). This indicates that stromal cells can regulate ILC3 homeostasis beyond survival, in a cytokine-independent fashion.

As ILC lack pattern-recognition receptors, ILC3 activation is mainly regulated by soluble factors, such as cytokines, inflammatory mediators or neuronal factors. Among these multiple factors, myeloid-derived IL-1 β and IL-23 are crucial for ILC3 activation. The important role of IL-23 was established in the context of *C. rodentium* infection. It had been previously observed that mice deficient in either the *p19* (encoded by *Il23a*) or *p40* (encoded by *Il12b*) subunits of IL-23 lose their ability to control *C. rodentium* infection. In a key study, Zheng and colleagues demonstrated that IL-22 was essential for early host defense against *C. rodentium* and that IL-23 was required for induction of IL-22 (38). Subsequent studies drawing attention to the role of ILC3-derived IL-22 in host protection against enteropathogens have also emphasized the requirement of IL-23 for ILC3 activation (41, 42), which is released mainly by CX3CR1⁺ mononuclear phagocytes but also by CD103⁺ dendritic cells depending on TLR stimulation (117, 175, 176, 180–182). In absence of IL-23, CX3CR1⁺ macrophages or TLR signalling via MYD88, *C. rodentium* infection cannot be controlled by the host unless IL-22 is administered, demonstrating that CX3CR1⁺ macrophages support innate IL-22 production via IL-23

(175, 176, 183). While IL-23⁺ cells are suggested to be distributed across the intestine during *C. rodentium* infection (175), only a rare population of macrophages constitutively produces IL-23 in the intestine. These IL-12/23 p40⁺ macrophages preferentially reside in CP/ILF where they activate neonatal ILC3 in response to TLR stimulation by the microbiota, thus restricting ILC3 activation and IL-22 distribution at steady-state (122). In addition, IL-23 can also trigger IFN γ and IL-17 production by ILC3, together with IL-22, and drive innate intestinal pathology in response to *H. hepaticus* infection (85). Myeloid-derived IL-1 β is another critical activator of ILC3, that potentiates IL-23-induced IL-22 production and can induce ILC3 production of other cytokines too. IL-1 β production by intestinal macrophages is augmented during *C. rodentium* infection, whose specific virulence factors regulate the release of IL-1 β via caspase-11 inflammasome and ensuing IL-22 production (183). Multiple signals, either endogenous or exogenous, modulate IL-23 production or IL-23 function and impact indirectly ILC3 activation. For instance, TL1A synergizes with IL-23 and IL-1 β , presumably via mitogen-activated protein kinase (MAPK) downstream of DR3 (TL1A receptor), to induce protective IL-22 in intestinal ILC3 during acute colitis (131, 175). In contrast, other signals can suppress the activation of ILC3. IL-10 signalling in CX3CR1⁺ macrophages contributes to the control of ILC3 activation, since IL-10R-deletion in CX3CR1⁺ cells induces uncontrolled IL-22 production in ILC3 and the development of spontaneous colitis as a result of elevated IL-23 production by IL-10R-deficient macrophages (50). Interestingly, TL1A expression is dependent of the microbiota and IL-23 production by IL-10R-deficient macrophages too (50, 131), suggesting that exogenous signals from the microbiome may have an important role in indirectly regulating ILC3 activation (see below). Intriguingly, a positive feedback loop driven by ILC3 and depending on IL-23 controls their IL-22 production in the gut. LT β -expressing ILC3 activation of LT β R signalling in CD11c⁺ cells is essential for IL-23 release and controls IL-23-dependent IL-22 responses of ILC3 in the ILF (182). Furthermore, additional myeloid-derived signals have a direct effect on ILC3 responses. Upon *S. typhimurium* infection, CCR6⁻T-bet⁺ ILC3 produce IFN γ in response to IL-12 and participate in protection against *Salmonella* but also in *Salmonella*-driven enterocolitis (86). Together, this highlights the integral role of myeloid cells in modulating intestinal ILC3 effector responses through a multitude of myeloid-derived signals, at steady-state as well as upon tissue perturbation.

Interestingly, recent studies have identified a reciprocal interaction between intestinal T cells and ILC3 (184). On top of ILC3 regulation of T cell responses, it appears that T cells influence ILC3 functions. While competition between ILC3 and T cells for survival factors has been proposed in lymphoid tissues (81, 185), there is no evidence that T cells exert a direct impact on ILC3 activation in the intestine. Yet, it has been observed by many that IL-22⁺ ILC3 numbers and IL-22 production are increased in *Rag2*^{-/-} mice in intestine and in lymphoid tissues (79, 117, 155, 186). This could be the result, amongst other things, of absence of direct regulation by adaptive immune cells or indirectly of altered microbiota composition and enhanced environmental signals such as myeloid-derived factors as a consequence of expansion and activation of the myeloid compartment. Work from Korn et colleagues has shown that restoration of adaptive immunity through adoptive transfer could reduce IL-22 production and IL-22-dependent responses (186). In particular, adoptive transfer of conventional CD4⁺ T cells was sufficient to regulate IL-22 innate responses. How conventional CD4⁺ T cells regulate ILC3 activation remains to be determined. In a model of α -CD40-induced colitis in *Rag2*^{-/-} mice, T_{reg} can prevent IL-23 and IL-1 β

secretion by CX3CR1⁺ macrophages through engagement of latent activation gene-3 (LAG3) with MHC-II on macrophages, thus limiting excessive IL-22 production by ILC3 and suppressing ILC3-driven colitis (187). However, T_{reg} transfer was not sufficient to reduce IL-22-dependent innate responses (186). A recent study has shown that both T_{reg} and T_H17 cells could limit ILC3 activation through independent mechanisms (117). While T_{reg} seem to limit ILC3 activation through control of IL-23, perhaps via LAG3 or IL-10 dependent mechanisms (50, 187), T_H17 cells potentially restrain ILC3 activation by limiting the expansion of specific microbial species (117), such as segmented filamentous bacteria (SFB; see below). Further studies should help define how T cells regulate directly and indirectly intestinal ILC3 activation, but this likely involves multiple and complex pathways.

A previously unappreciated role for nervous system-derived molecules in modulating ILC3 action has been recently uncovered, including neurotransmitters and neurotrophic factors. The enteric nervous system is the largest accumulation of neurons outside the central nervous system (188). Intestinal ILC3 are closely entangled with projections from enteric neurons and enteric glial cells and express several receptors for molecules secreted by the nervous system. A role for glial cells in the intestinal immune system has been suggested early on. Indeed, the receptor tyrosine kinase RET, which is essential for enteric nervous system formation, has been shown to be required for the proper migratory behavior of haematopoietic cells during embryogenesis and formation of Peyer's patches which represent gut-associated lymphoid follicles (102). In the adult, intestinal ILC3 located in lymphocytes aggregates (CP/ILF) express RET and glial cells project into these lymphoid clusters. Ablation of RET in ILC3 doesn't affect the structure of these clusters but impairs IL-22 and AMP production. Enteric glial cells sense microenvironmental cues via MYD88 and release neurotrophic factors that control innate IL-22 dependent responses and gut defense (189). In addition to glial cells, enteric neurons can also modulate ILC3 function. In particular, intestinal CCR6⁺ ILC3 express the Vasoactive Intestinal Peptide Receptor 2 (VIPR2) for the VIP neuropeptide. CCR6⁺ ILC3 in ILF reside in close proximity to enteric neurons, and notably VIPergic neurons (120, 190). Feeding induces the production of VIP by enteric neurons, however the role of VIP on ILC3 and IL-22 is currently debated. A first study has reported that ILC3 stimulation in presence of VIP decreases IL-23-induced IL-22 production. Accordingly, conditional depletion of VIPR2 in ILC3 increases the numbers of IL22⁺ ILC3 and AMP expression. These results were confirmed using Designer Receptor Exclusively Activated by Designer Drugs (DREADD) to stimulate or inhibit firing of VIPergic neurons (120). Feeding decreases innate IL-22, which is thought to promote lipid absorption by intestinal epithelial cells during meals (117, 120). In contrast, another study reported that VIP enhances ILC3 production of IL-22 after IL-7/IL-23 stimulation. Accordingly, the percentage of IL-22⁺ ILC3 is higher following exogenous VIP administration and fed mice exhibit increased ILC3-derived IL-22, which is abrogated in VIPR2-deficient mice (190). Currently, the discrepancies observed between these two studies remain unexplained and more work is required to address this question. Nevertheless, these studies argue for a role of nervous system-derived molecules in regulating ILC3, either by activating or suppressing IL-22 production.

Emerging evidence suggest a circadian regulation of ILC3, in favor of the implication of the brain in harmonizing ILC3 responses. Intestinal ILC3 express high amounts of circadian clock genes, including *Clock*, *Arntl*, *Nr1d1* and *Nfil3* (191–193). Circadian clock gene expression in ILC3 is associated with

rhythmic expression of ILC3-associated transcription factors (*Rorc*, *Ahr*), cytokines (*Il17a*, *Il22*) and diurnal oscillations in cytokine production (190–193). Deletion of *Arntl* – also known as BMAL1 –, which binds DNA rhythmically and activates expression of core clock genes (194), from ROR γ t-expressing cells results in a decrease of ILC3 numbers and frequencies (191, 192). Abolition of the molecular clock in ILC3 induces a downregulation of ROR γ t expression, a downregulation of surface expression of gut-homing receptors and affects IL-17 and IL-22 production (190–193). Microbiota, feeding regimens and light and dark cycles have an impact on circadian patterns in ILC3 (190, 191). Therefore, neuronal regulation of ILC3 appears to harmonize ILC3 functions in the intestine to environmental signals.

2. Exogenous signals

The activity of ILC3 is not only shaped by host-derived environmental signals. ILC3 are particularly enriched at intestinal sites, next to commensal communities and maintain close relations with the microbiota (195, 196). The microbiota is known to regulate ILC3 responses, mostly indirectly by modulating myeloid cell responses since ILC3 lack pattern-recognition receptors but also directly through microbial-derived metabolites. Alterations in intestinal microbial communities have been shown to affect ILC3 phenotype and functionality (197). Contradictory results have been obtained in germ-free (GF) mice regarding ILC3 function. Several studies have identified impaired NKp46⁺ and CCR6⁺ ILC3 functions in GF mice, as reflected by a lack of IL-22 expression and compromised maturation of intestinal CP into ILF, respectively (82, 84, 117, 132), whereas others reported unchanged (39) or elevated (79) IL-22 production by ILC3. On the one hand, commensal microbes modulate ILC3 activity to induce regulatory responses. The microbiota is sensed by intestinal resident macrophages which release IL-1 β and elicit GM-CSF production by ILC3. GM-CSF is involved in antigen-presenting cells ability to produce IL-10 and promote T_{reg} conversion and expansion (128). Moreover, induction of IL-1 β following microbial-sensing by macrophages is also necessary for ILC3-derived IL-2 production, which plays a role in T_{reg} maintenance (129). Together, these complementary pathways promote immune tolerance in the intestine by shaping ILC3 activity. On the other hand, microbial communities can also influence ILC3 activity to induce pro-inflammatory responses. Segmented filamentous bacteria (SFB) preferentially attaches in the ileum and induces T_H17 responses via myeloid-derived IL-23 (198, 199). Recent studies have shown that SFB colonization additionally activates ileal ILC3 and IL-22 secretion by stimulating IL-23 production. Subsequent IL-22R signaling in IEC results in liberation of serum amyloid A (SAA) in the terminal ileum which indirectly enhances IL-17 production by T_H17 cells and limits SFB colonization (118, 119). Interestingly, ILC3 hyperactivation in *Rag2*^{-/-} mice requires SFB colonization. While in wild-type mice T_H17 cells control SFB colonization and restrain ILC3 activation, SFB abundance is increased in *Rag2*^{-/-} mice and results in exaggerated IL-22 responses by ILC3 (117). In parallel to indirect regulation of ILC3 responses, the microbiota has been proposed to directly stimulate ILC3 responses, in humans especially. A fraction of human CD127⁺ RORC⁺ LTi cells freshly isolated from tonsils express TLR2 and *in vitro* stimulation of expanded human CD127⁺ LTi cells with the bacterial lipopeptide PAM3CSK4 (a TLR2 ligand) induces CD127⁺ LTi cells proliferation and IL-22 production (68). Thus, microbial sensing by TLR can influence human ILC3 function. Direct recognition of microbes by ILC3

could also be mediated by natural cytotoxicity receptors (NCR), as NCR are involved in recognition of microbial-derived ligands (200). Engagement of NKp44 using an anti-NKp44 antibody results in TNF- α production by human tonsil-derived NKp44⁺ RORC⁺ ILC3 whereas cytokines (IL-1 β , IL-23 and IL-7) preferentially induce IL-22 production. NKp44 engagement together with cytokine stimulation acts synergistically to induce multi-functional NKp44⁺ RORC⁺ ILC3, producing both TNF- α and IL-22 (91). These results are in favor of a direct action of microbes on ILC3 responses. However, NKp44 is not expressed in mouse and NKp46 seems dispensable for IL-22 production by murine intestinal ILC3 (91, 201). Further investigation is required to determine possible pathways of direct microbial sensing by ILC3.

Dietary metabolites can also have a direct impact on ILC3 activity. Part of diet-derived metabolites are produced by the microbiota. As mentioned previously, short-chain fatty acids (SCFA) are produced in the colon by bacterial fermentation (152). SCFA activation of FFAR2 on CCR6⁺ ILC3 supports CCR6⁺ ILC3 maintenance and functions, such as IL-22 production and colonic patches formation. Therefore, SCFA impact multiple aspects of colonic ILC3 function in intestinal homeostasis and immunity, as illustrated by the susceptibility of mice carrying ILC3-specific genetic deletion of FFAR2 to DSS-induced inflammation and to *C. rodentium* infection (153). ILC3 are also influenced by specific SCFA including butyrate, a fermentation product of dietary fibers by the gut microbiota. Although *Rag1*^{-/-} *Gpr109a*^{-/-} mice develop spontaneous colitis associated with elevated colonic ILC3 numbers and IL-17 production, the receptor of butyrate Gpr109 is not expressed on ILC3. This indicates that microbial-derived metabolites can also exert an effect on ILC3 indirectly. Gpr109 suppression of ILC3 responses and colonic inflammation is dependent on the inhibition of IL-23 production by colonic myeloid cells by Gpr109 (202). Furthermore, dietary metabolites that do not necessitate transformation by the microbiota shape ILC3 responses. Sensing of oxysterols through GPR183 not only guide ILC3 positioning but also boost IL-22 production by colonic ILC3 (174). Further highlighting the role of dietary ligands in ILC3 biology, all ILC3 subsets express the aryl hydrocarbon receptor (AhR), a transcription factor whose ligands include dietary ligands, such as phytochemicals (e.g., flavonoids and glucosinolates) contained in cruciferous vegetables (112). AhR responsive elements are clustered with ROR-responsive elements in intestinal ILC3, including the *Il22* promoter, suggesting that *Il22* is a direct target of AhR and ROR γ t in ILC3 and that innate IL-22 is directly regulated by diet-derived factors (113). Accordingly, AhR-deficient mice display reduced numbers of IL-22-producing ILC3 and are more susceptible to *C. rodentium* infection (112, 113, 203). AhR is also involved in the maintenance of all ILC3 subsets, as shown by the decrease of ILC3 numbers (both NKp46⁺ and CCR6⁺) after birth but not during fetal life and the maintenance of secondary lymphoid structures but not of tertiary lymphoid organs (112, 113, 203). It had been proposed that microbial-derived AhR ligands are unlikely to be involved in AhR regulation of ILC3 responses since germ-free mice had normal numbers of ILC3 and development of cryptopatches wasn't affected. Rather, a diet devoid of phytochemicals recapitulated the phenotype observed in AhR-deficient mice, in favor of a crucial role of dietary-derived AhR ligands for ILC3 maintenance and function (112). AhR seems to be involved in ILC3 homeostasis by promoting ILC3 proliferation post-birth and expression of pro-survival genes such as *Bcl2* (112, 113). In humans, it has also been reported that NKp44⁺, NKp44⁻ ILC3 and ILC1 have differential expression of AhR and that conversion of NKp44⁺ ILC3 towards pro-inflammatory

ILC1 (see after) in the inflamed ileum of Crohn's disease patients is accompanied by a downregulation of AhR, suggesting that AhR also maintains proper human ILC3 function (204, 205). Another crucial dietary ligand that can directly modulate ILC3 responses is retinoic acid (RA), a vitamin A-derived metabolite that can be synthesized from β -carotene in carrots for example. RA binds to heterodimers formed by RA receptors (RAR) and retinoid X receptors (RXR), which bind DNA RA response elements including the *Rorc* locus. Thus, RA stimulation results in ROR γ t upregulation and ROR γ t-associated effector functions. Besides RA crucial role during development, RA is also crucial for ILC3 homeostasis and IL-22 production. Vitamin-A deficiency results in reduced ILC3 numbers, IL-22 secretion and AMP production, which can all be restored by RA administration (109, 206). Moreover, RA controls the number of ILF and subsequent IgA production by maintaining intestinal ILC3 (206). RA could maintain intestinal ILC3 through *Rorc*-dependent transcriptional programs but also through induction of the integrin α 4 β 7 and chemokine receptor CCR9, two gut homing receptors (167).

Overall, this non-comprehensive review of the impact of 'endogenous' and 'exogenous' signals in ILC3 maintenance and function highlights the integral role of tissue environment in ILC3 biology. This crucial role of local environment in shaping ILC3 responses is also reflected by tissue-specific signatures of ILC3 and subsequent tissue-specific immune responses (207).

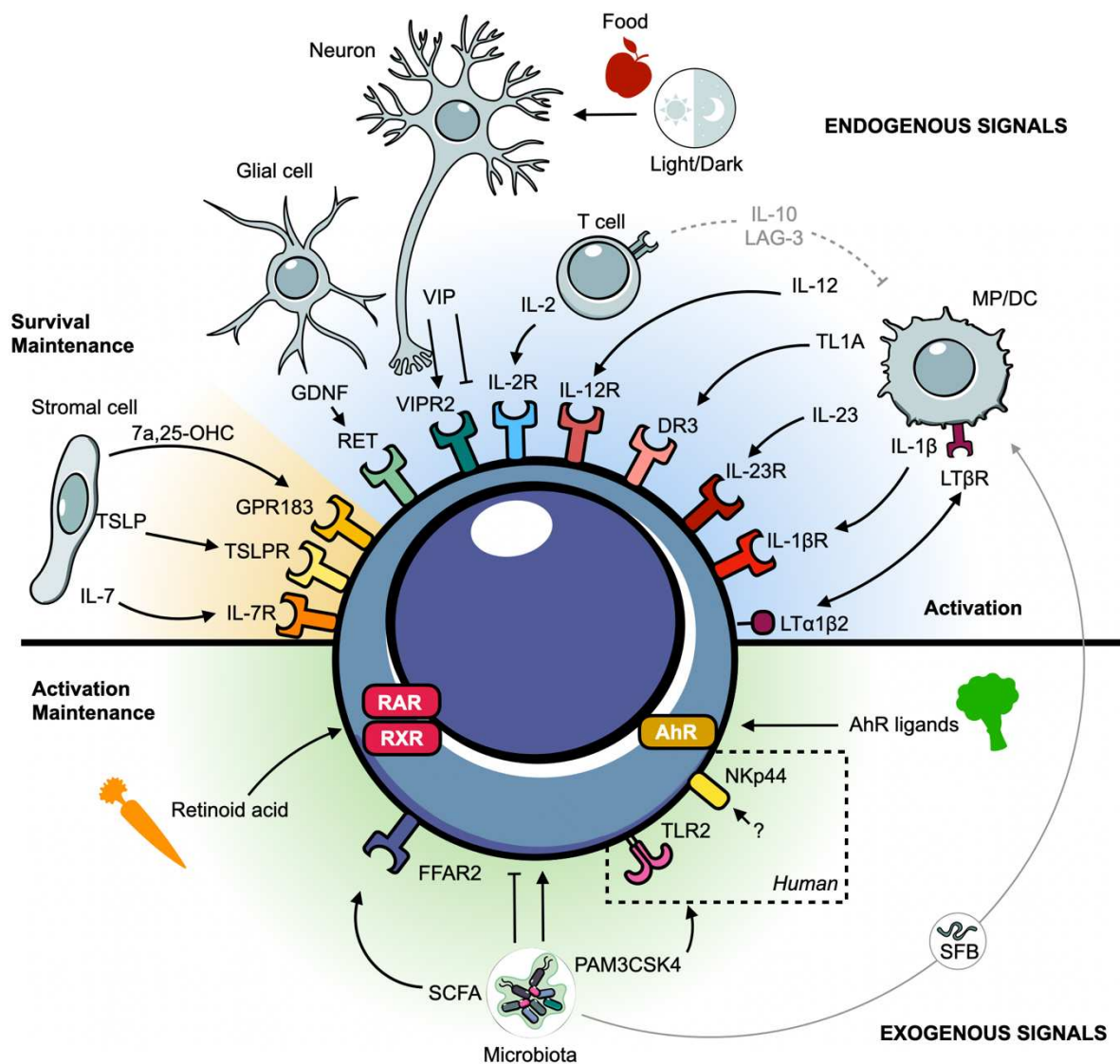


Figure 15 | Impact of tissue environment on ILC3 biology

Environmental signals are essential for many aspects of ILC3 biology. Intestinal ILC3 sense and integrate a broad range of endogenous (top) and exogenous (bottom) signals which influence their survival and maintenance (yellow), activation (blue) or both (green). Consequently, local tissue environment has an important impact on the nature and magnitude of intestinal ILC3 responses. Although depicted as one cell, it should be noted that some environmental cues can modulate CCR6⁻ and CCR6⁺ ILC3 distinctly. TSLP, Thymic Stromal Lymphopietin; 7α,25-OHC, 7α,25-dihydroxycholesterol; GDNF, glial-derived neurotrophic factor; VIP, vasoactive intestinal peptide; LAG-3, lymphocyte-activation gene 3; TL1A, tumor necrosis factor-like cytokine 1A; DR3, death-receptor 3; MP, mononuclear phagocyte; DC, dendritic cell; LTα1β2, lymphotoxin-α1β2; LTβR, lymphotoxin-β receptor; AhR, aryl hydrocarbon receptor; TLR, Toll-like receptor; PAM3CSK4, Pam3CysSerLys4; SFB, segmented filamentous bacteria; SCFA, short-chain fatty-acids; FFAR2; free fatty acid receptor 2; RAR, retinoic acid receptor; RXR, retinoid X receptor; IL-, interleukin.

B. Impact of tissue environment: ILC3 plasticity

As previously mentioned, many external and internal factors influence ILC3 biology. Consequently, intestinal ILC3 are not 'fixed' but exhibit 'plastic' properties in response to environmental signals. Several studies have shown that transcriptional programs and cytokine secretion of ILC3 could be modulated by extrinsic factors (**Figure 16**).

For instance, murine intestinal NKp46⁺ ILC3 can down-regulate *Rorc* expression and up-regulate T-bet in response to IL-12 and IL-18 (84). These plastic ILC3, denoted 'ex-ILC3' ILC1 owing to their lost *Rorc* expression, acquire ILC1 functions and the ability to robustly produce IFN γ (84, 208). Although these ex-ILC3 haven't been extensively studied, it has been proposed that they are required for protection against some enteropathogens such as *S. Typhimurium* (86). ILC3 to ILC1 plasticity could similarly be observed in humans where myeloid-derived IL-12 could promote IFN γ secretion by human ILC3 (209). In humans, ILC1 and ILC3 can reversibly differentiate, as human ILC1 can turn into RORC⁺ IL-22-producing ILC3 when stimulated with IL-1 β and IL-23 \pm retinoic acid (209).

Intestinal ILC3 also display significant plasticity among ILC3 subsets, with CCR6⁻ ILC3 plasticity being particularly versatile and reversible. Unexpectedly, multiple studies have reported phenotypic plasticity of NKp46 expression, although its functional significance on ILC3 remains unclear. CCR6⁻ ILC3 can upregulate NKp46 expression after T-bet activation (88). Upregulation of T-bet and NKp46 is promoted by cytokines such as IL-12 and IL-23 as well as by microbial signals (86, 88). Conversely, fate-mapping studies have identified NKp46⁻ ILC3 with a previous history of NKp46 expression and established that a balance between Notch and TGF- β signaling is involved in NKp46 modulation (87, 88, 210).

Beyond phenotypic plasticity, CCR6⁻ ILC3 can also acquire functional plasticity as evoked in aforementioned studies (87). While ILC3 plasticity can be beneficial for protection against enteropathogenic bacteria, it can also lead to intestinal pathology. In the context of *Salmonella* infection, ex-ILC3-derived IFN γ contributes to *Salmonella*-mediated enterocolitis (86). In the context of *Helicobacter hepaticus* infection, IL-23-stimulated CCR6⁻ ILC3 become polyfunctional IFN γ -, IL-17- and IL-22-producing cells and drive *H. hepaticus*-induced colitis (85).

Collectively, these reports highlight the fact that environmental signals not only tune ILC3 function but also have the capacity to change their identity. The remarkable plasticity of ILC3 might reflect the requirement for these cells to adjust to the broad range of stimuli encountered in the intestine.

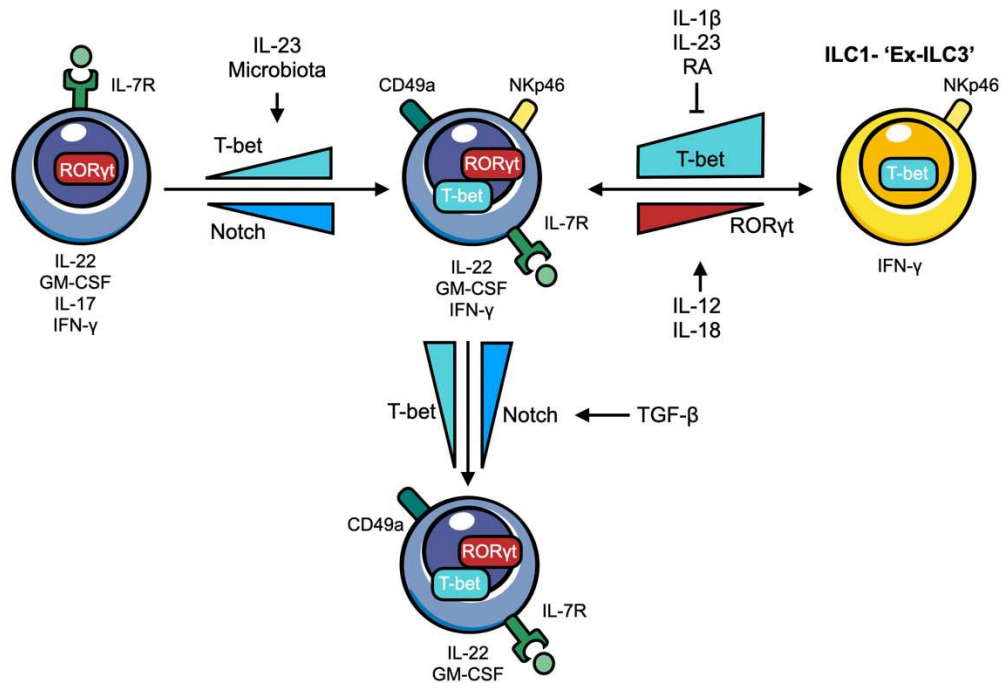


Figure 16 | Plasticity of mature ILC3

Although ILC3 subsets are 'fixed' in principle, with defined phenotypes and functional profiles, they have the ability to adapt to their local environment and to become 'plastic'. Plasticity of mature ILC3 takes place between ILC3 subsets which adjust their transcription factor and surface marker expression, but also between ILC3 and ILC1 which is a reversible conversion. Transcriptional programs and cytokine secretion of ILC3 can be modulated by extrinsic factors, such as cytokines, microbial-derived and diet-derived signals. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN γ , interferon gamma; RA, retinoic acid; TGF- β , transforming growth factor beta; ROR γ t, retinoic-acid receptor-related orphan receptor gamma; T-bet, T-Box transcription factor.

C. Impact of tissue environment: ILC3 long-term fate?

Accumulating evidence indicates that intestinal ILC3 are shaped by the microenvironment and exhibit considerable adaptive capacities, as illustrated by their 'plastic' properties. As ILC3 are tissue-resident cells (67), they can come into contact with multiple stimuli in the intestine. It is therefore tempting to ask how these cells might adapt over the lifespan of an individual and to question whether some of these environmental cues could have a long-lasting impact on ILC3. Interestingly, ILC3 are believed to persist long-term in tissues (67) where they rapidly produce cytokines in response to local signals (65), reminiscent of tissue-resident memory T (T_{RM}) cells. This raises the question as to whether ILC3 show properties consistent with immunological memory.

For the most part, innate immunity is associated with rapid and pleiotropic responses whereas adaptive immunity is recognized for its specificity and its ability to retain long-term immune memory of past infections. However, this classical view of immunological memory has recently been challenged by studies suggesting that innate immunity can be influenced by previous encounters and provide protection against subsequent infections. This form of immunological memory has been termed 'trained immunity' or 'innate immune memory' (211). The first line of evidence for innate immune memory comes from the observation of acquired resistance to infection in plants, denoted 'systemic acquired resistance' (SAR). After an initial localized injury, plants exhibit enhanced immunity throughout all tissues that

confers long-term protection against subsequent infections. Additional evidence obtained in invertebrates suggests that innate immune cells display memory traits. Although they lack functional equivalents of T and B cells, insects can develop heightened resistance to secondary infection after an initial encounter with a pathogen and demonstrate cross-protection between infections with pathogens. This contradicts the paradigm that innate immune responses cannot adapt and suggests that innate immune cells could build immunological memory in vertebrates too.

Indication of innate immune memory in humans has been provided by the field of vaccination. In fact, non-specific effects of vaccines are supported by a number of epidemiological studies in humans. Immunization with live attenuated vaccines, such as bacillus Calmette-Guérin (BCG), reduces mortality from non-target diseases (212). Cross-protection observed in human epidemiological studies can be attributed either to classical protection by cross-reactive T cells or to unspecific protection of innate immunological memory. Experimental models of infection or vaccination in mice have supported the latter hypothesis (211).

In recent years, emerging evidence has shown that after infection or vaccination, innate immune cells display long-term changes through epigenetic, transcriptional and functional reprogramming. This process has been extensively studied in humans during monocyte-to-macrophage differentiation. It was reported that stimulation of macrophages by β -glucan induces epigenetic reprogramming, accompanied by changes in positive histone regulatory marks. This process is in turn responsible for the increased responses of the 'trained' cell for genes encoding host defense molecules upon re-stimulation with a second stimulus. Alternatively, epigenetic rewiring following monocyte priming by LPS can lead to decreased responses of 'tolerized' macrophages upon re-stimulation, which might be useful to promote tolerance at barrier sites (213).

Within the ILC family, NK cells have been proposed to possess 'adaptive' features and to develop into long-lived 'memory' NK cells (214). A role for NK memory responses was first observed in mice during hapten-induced contact hypersensitivity (CHS). Hapten-induced CHS responses could occur in ears of *Rag2*^{-/-} mice to the same extent as in wild-type animals. Depletion of NK cells abolished CHS responses upon re-challenge, raising the idea that NK cells could mediate antigen-specific responses (215). Antigen-specific memory NK cells have been extensively studied in viral infections notably. In a model of murine cytomegalovirus (MCMV) infection, a MCMV-specific activating NK cell receptor Ly49H and its cognate antigen m157 have been identified and their interaction confers protection of C57BL/6 mice. Taking advantage of this MCMV-specific receptor on NK cells, Sun et al. tracked NK cells. They reported that after MCMV infection, NK cells expand in an antigen-specific manner similar to T cells and later differentiate into long-lived 'memory' NK cells in the absence of the antigen, with enhanced functional responses to MCMV upon reinfection (216). These properties of memory NK cells were also observed in humans (214). In humans, similar expansion of a NK cell population carrying the activating receptor NKG2C has been appreciated in human cytomegalovirus (HCMV)-seropositive donors (214). Upon HCMV infection or reactivation in immunosuppressed individuals, these NKG2C⁺ NK cells can expand and show a unique transcriptional profile as well as an enhanced ability to produce cytokines and to degranulate in response to HCMV-infected cells (217, 218). A new study demonstrates that the NKG2C receptor can recognize with fine specificity peptides derived from the HCMV protein UL40 in

the context of the non-classical major histocompatibility complex (MHC) class I molecule HLA-E2. Together with co-stimulation and cytokine signalling, this drives the expansion of a population of adaptive NKG2C⁺ NK cells (219). Similarly to primed macrophages populations, the NKG2C⁺ adaptive NK cell population exhibits epigenetic remodeling, including hypo-methylation of regulatory regions of the IFNG locus (220).

In addition to signals mediated by activating NK receptors, pro-inflammatory cytokines are essential for the generation of memory NK cells both in mouse and humans. Of note, cytokine-induced adaptive NK cells can develop in an antigen-independent fashion. *In vitro*, a brief exposure of murine NK cells to IL-12, IL-15 and IL-18 results in 'memory-like' NK cells with enhanced ability to secrete high amounts of IFN γ and to express perforin and granzymes. Adoptive transfer of 'cytokine-induced' memory NK cells reveals that they persist long-term and maintain these cell-intrinsic superior functional capacities (221). More recently, lung ILC2 have been shown to acquire memory-like properties in the context of allergic inflammation (222). Unlike NK cells, ILC2 do not recognize antigens and are instead primarily activated by cytokines. Previously activated ILC2, also referred to as 'experienced' ILC2, persist after resolution of lung inflammation. Experienced ILC2 show enhanced recall responses to a secondary challenge by ILC2-activating cytokine IL-33 or an allergen in a cell-autonomous fashion, which could be partly attributed to increased IL-25 responsiveness.

On the whole, a growing body of evidence supports the existence of innate immune memory and incites to broaden our view of immunological memory. In light of this, it is reasonable to wonder whether intestinal ILC3 can generate a similar degree of adaptation and whether experienced ILC3 show evidence of immunological memory at the functional level.

GLOBAL CONTEXT AND OBJECTIVES

The intestinal barrier comprises a layered defense system involving epithelial and immunological strategies which are critical for both tolerance and protection against potential pathogens. In contrast to the clearly established role for the adaptive immune system in maintaining this balance, knowledge on how the innate immune system generates regulatory and protective immune responses within the intestine is less defined. Emerging evidence highlights an essential role for tissue-resident ILC3 in intestinal homeostasis and immunity. Through their ability to rapidly produce large amounts of cytokines and their strategic location, ILC3 promote rapid and essential innate immune responses. Yet, the spatio-temporal regulation of intestinal ILC3 responses remains poorly understood.

On the one hand, distinct environmental signals from intestinal spatial niches determine lymphocyte activation and the nature of their responses. In particular, migration within tissues plays an essential role in adaptive lymphocyte function in intestinal immunosurveillance. Still, whether ILC3 migrate and adapt their cellular behavior in response to tissue-specific cues, such as environmental insults, to support intestinal barrier function is largely unknown.

On the other hand, continuous exposure to local environmental stimuli within the intestine shapes lymphocyte function and subsequent immune responses. Specifically, pathogen encounter results in the development of immunological memory for adaptive lymphocytes, thus sustaining long-term intestinal immunosurveillance and recall responses to pathogens. Similarly, whether ILC3 activation has any long-term impact on ILC3 responses and how this helps to contribute more efficiently to intestinal immune defense remains to be determined.

In my thesis, we aim to answer these questions and to understand tissue adaptation of intestinal ILC3 behavior and function in response to environmental stimuli. In [Chapter II](#), we first develop a new approach combining ILC3 reporter and intravital imaging to gain insight into ILC3 cellular behavior. We explore the dynamics of intestinal ILC3 in steady-state and inflammatory conditions and their regulation. In [Chapter III](#), we next investigate the impact of multiple pathogen encounter on ILC3 responses and its functional consequence for long-term mucosal defense. In [Chapter IV](#), I will ultimately discuss our findings on these two novel and complementary aspects of ILC3 biology and their relevance in intestinal immunosurveillance.

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CHAPTER II

INFLAMMATION TRIGGERS ILC3 PATROLLING OF THE INTESTINAL BARRIER

Type 3 innate lymphoid cells (ILC3) include tissue-resident lymphocytes that critically regulate intestinal homeostasis and immunity. While it is known that ILC3 subsets occupy spatially distinct compartments within the intestine, cellular dynamics within and between these sites are unknown. Whether ILC3 change their cellular behavior to adapt to environmental signals, including tissue perturbation, may have important consequences for barrier defense. Here, we use intravital imaging to reveal distinct compartmentalized ILC3 behaviors in the mouse small intestine. Largely immotile under steady-state conditions, villus ILC3 acquire novel migratory attributes and enhanced cytokine expression in response to inflammation. Local endogenous and exogenous signals, T cells and bacterial ligands respectively, regulate intestinal ILC3 behavior. In particular, T/ILC3 competition for the chemokine CCL25 is a major determinant of ILC3 'patrolling'. Our results highlight significant differences in ILC3 and T cell behavior with prominent adaptation of intestinal ILC3 that promote patrolling of mucosal perturbations.

This chapter is part of a manuscript in preparation and the collaborative work of: Angélique Jarade, Zacarias Garcia, Solenne Marie, Abdi Demera, Immo Prinz, Philippe Bousso, James P. Di Santo and Nicolas Serafini

MAIN TEXT

Mucosal tissues, such as the intestine, represent unique structures exposed to the environment. As such, the intestinal epithelial barrier defines a crucial interface between the host and the external environment (1). Maintenance of barrier function is essential to preserve homeostasis of the entire body and prevent systemic dissemination of commensal and pathogenic microbes. To support mucosal homeostasis and defense, the intestinal tissue is abundantly populated by a variety of immune cells that surveil and modulate barrier function in response to specific environmental signals (2). Upon enteric infection, both the innate and adaptive immune system initiate an essential type 3 response resulting in the release of cytokines, such as interleukin (IL)-22 which orchestrates immune-epithelial cell crosstalk (3). An effective IL-22-dependent epithelial response, involving the production of antimicrobial peptides and the maintenance of barrier containment, is critical for pathogen control as illustrated by the susceptibility of IL-22 deficient (*Il22^{-/-}*) mice to infections such as *C. rodentium* or *Klebsiella pneumoniae* (4, 5). Several studies have shown that type 3 innate lymphoid cells (ILC3) represent the dominant source of intestinal IL-22 (3, 6) and, consequently, critical actors of early immune responses against enteropathogenic bacteria (7–9).

Although it is presumed that ILC3 localization allows them to quickly respond to environmental and pathogenic stimuli, little is understood about the impact of tissue localization on ILC3 responses. In the intestine, NKp46⁺ ILC3 are enriched and dispersed in the intestinal lamina propria villi whereas CCR6⁺ ILC3 mainly reside in the basal intestinal mucosa clustered in isolated lymphoid follicles (ILF) (10). We have recently described the importance of the CXCL16/CXCR6 chemokine-chemokine receptor axis in regulating ILC3 diversity, localization and function (11). Indeed, ablation of CXCR6 generated a selective loss of the NKp46⁺ ILC3 subset and impaired their localization into the intestinal villi. Loss of IL-22-producing NKp46⁺ ILC3 within the gut resulted in the inability of the host to control bacterial infection, suggesting that ILC3 localization within the intestinal tissue is critical for their function (11).

Intra-tissue distribution of ILC3 may affect their ability to sense signals, to make cellular interactions and to modulate tissue responses but remains poorly characterized. In particular, migration within tissues is a key determinant of adaptive lymphocyte function in intestinal immunosurveillance (12–14), but whether tissue-resident intestinal ILC3 migrate and adapt their behavior in response to environmental cues is largely unknown.

We applied intravital multi-photon imaging to assess intestinal ILC3 behavior under steady-state and inflammatory conditions. As ILC3 express the transcription factor ROR γ t (*Rorc*) and produce IL-22 (*Il22*), we imaged *Rorc*^{GFP} and *Il22*^{TdT} reporter mice to identify ILC3 and IL-22 expressing ILC3 *in vivo* (**Fig. S1A**). Still, as *Rorc*^{GFP+} T_H17 cells are also labeled using this approach (**Fig. S1A**), we further generated *Rag2^{-/-} Rorc^{GFP} Il22^{TdT}* mice lacking adaptive lymphocytes that allowed us to selectively visualize and track *Il22⁺*-expressing ILC3 (**Fig. S1B**). In order to study responses in a lymphocyte-replete setting, we created mixed bone marrow (BM) chimeras by transfer of BM from *Rag2^{-/-} Rorc^{GFP} Il22^{TdT}* mice and wild-type mice that express cyan fluorescent protein under the control of the Actin promoter (*Actb*^{ECFP};

CFP) into lethally irradiated CD45.1 congenic C57BL/6J mice. Seven weeks post-transfer, we verified that chimeras were correctly reconstituted with lymphocyte populations in the small intestine from CD45.2⁺ donor cells, including B cells (CD19⁺) and T cells (CD3⁺) (**Fig. 1A**). As expected, there were no GFP⁺ T cells in the intestinal lamina propria of BM chimeric mice (**Fig. 1A**). However, we could detect a small population of GFP⁺ ILC3 (CD19⁻CD3⁻Rorc^{GFP+}CD127⁺) representing 1.8% of CD45.2⁺ cells, of which about half expressed *I22* transcripts (**Fig. S1C**).

We next used intravital imaging to study ILC3 populations in the small intestine from BM chimeras (**Fig. 1A**). Since intestinal ILC3 can be histologically detected in lamina propria villi and crypts (10), we imaged both these sites in chimeric mice. In agreement with the FACS analysis, we observed a subset of intestinal GFP⁺ ILC3 and a larger population of CFP⁺ cells within the intestinal villi (**Fig. 1B**). CFP⁺ cells exhibited diverse *in vivo* dynamics, ranging from immobility to rapid migration (**Fig. 1B and 1D**), which likely reflects the high heterogeneity among the CFP⁺ population, containing *inter alia* B cells, T cells, myeloid cells. Interestingly, contrary to intestinal T cells (14), we found that ILC3 observed in the intestinal villi were mostly non-motile cells that lacked *I22* expression (**Fig. 1B**). Tracking of ILC3 showed that they displayed low speed mean (<2 $\mu\text{m}/\text{min}$), were largely arrested (arrest coefficient $\sim 90\%$; defined by the percentage of time where instantaneous velocity <3 $\mu\text{m}/\text{min}$) and confined (straightness ratio ~ 0.18 ; defined as the ratio track displacement/track path length which reflects directionality), confirming that intestinal ILC3 in villi are poorly motile (**Fig. 1C and 1D**). In contrast, GFP⁺ ILC3 in intestinal crypts were clustered in ILF and abundantly expressed *I22* transcripts, in line with a previous study (6) (**Fig. 1B**). ILC3 residing in ILF exhibited even more restricted motility, with speed mean, motion and straightness ratio reduced and arrest coefficient increased, compared to villus ILC3 (**Fig. 1C and 1D**). Altogether, these identify specific features of compartmentalized intestinal ILC3 with overall limited motility, and IL-22 expression exclusively observed in crypts at steady-state.

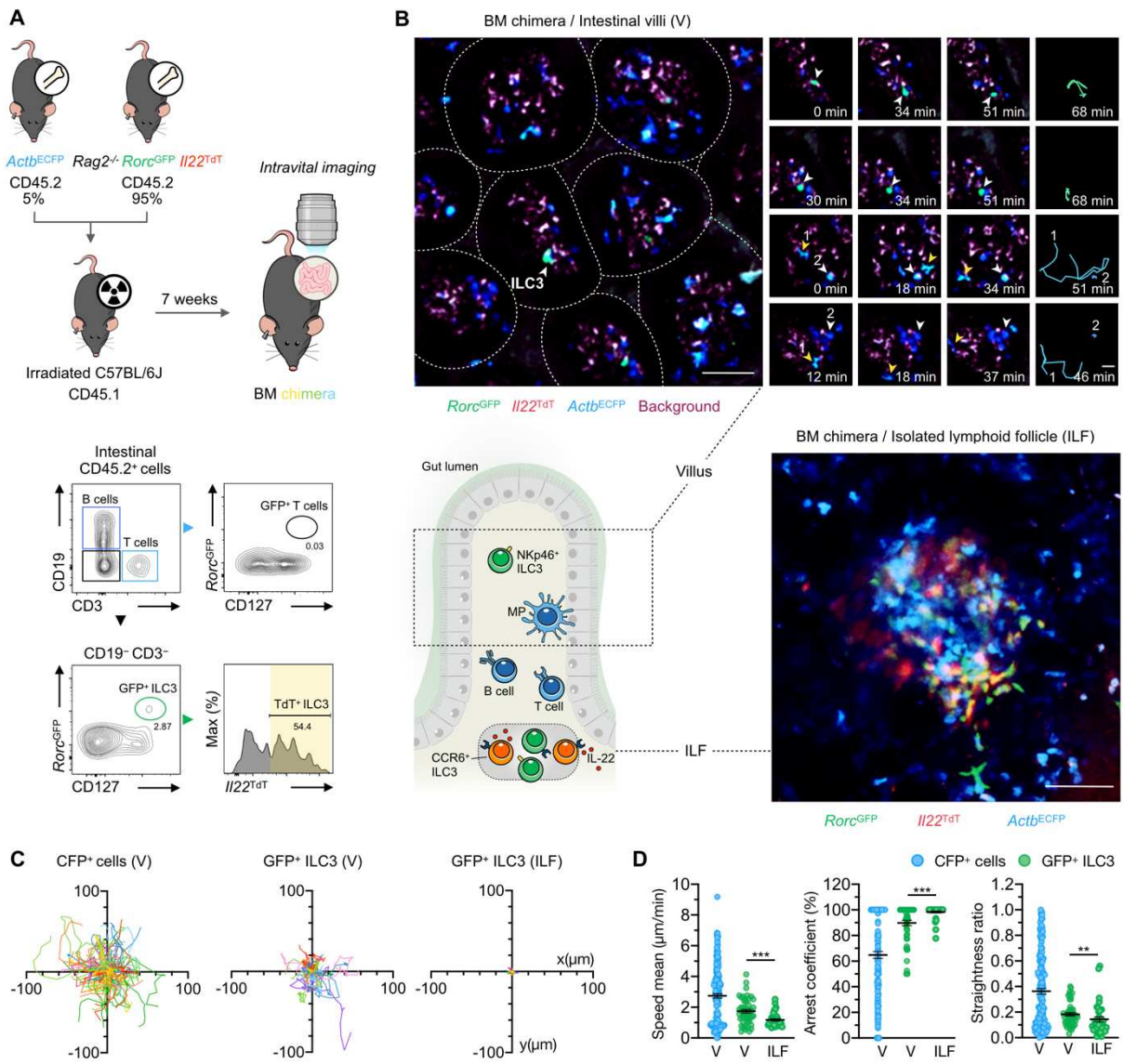


Figure 1 | Intestinal ILC3 are compartmentalized and poorly motile at steady-state

(A) Top: Bone marrow (BM) chimeric mice were created between a cyan fluorescent protein (CFP) and a *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} (GFP staining ILC3 only and Tomato staining *Il22* transcripts). Mixed bone marrow was injected into lethally irradiated congenic C57BL/6J mice and later analyzed for reconstitution. Seven weeks post-transfer, intestine from BM chimera was imaged by multi-photon microscopy. Bottom: Flow cytometry analysis of reconstitution by donor cells (CD45.2⁺) in the intestinal lamina propria (left). Scheme of intestinal longitudinal cross-section (right), depicting ILC3 localization within intestinal lamina propria in villi (V) and isolated lymphoid follicles (ILF). (B) Representative image (left; scale bar 50 μm) and time-lapse images (right; scale bar 15 μm) of ILC3 and CFP⁺ cells in intestinal villi (V; top) of BM chimera or in intestinal isolated lymphoid follicles (ILF; bottom) of BM chimera. (D) Individual tracks of intestinal CFP⁺ cells (left) and GFP⁺ ILC3 (middle) in intestinal villi or intestinal lymphoid follicles (right). (E) Graphs show mean speed, arrest coefficient (percentage of time where instantaneous velocity < 3 μm/min) and straightness ratio (track displacement/track path length; reflecting directionality) of indicated populations in the intestine. Results in (C-D) are from two (CFP⁺ cells and GFP⁺ ILC3 in ILF) or nine movies (GFP⁺ ILC3 in V) obtained in two independent experiments (n=170 CFP⁺ cells; n=49 GFP⁺ ILC3 (V); n=38 GFP⁺ ILC3 (ILF)). Each line corresponds to the mean ± SEM of the values obtained – only ILC3 tested (***P<0.001; **P<0.002; two-tailed Mann-Whitney test).

Our previous results suggested that the NKp46⁺ ILC3 subset selectively localizes to the intestinal villus lamina propria (11). To understand cellular dynamics of this ILC3 subset, we imaged *Ncr1*^{GFP} *Il22*^{TdT} mice (encoding NKp46 and IL-22, respectively) under steady-state conditions (**Fig. 2A-B**). Because *Ncr1*^{GFP} is expressed by both ILC1 and NKp46⁺ ILC3, we focused our analysis on double positive *Ncr1*^{GFP+} *Il22*^{TdT+} cells which represent a rare and pure population of *Il22*-expressing NKp46⁺ ILC3 (**Fig. 2A and S2A**). We found that migration of NKp46⁺ *Il22*⁺ ILC3 was limited (average speed of ~ 3.4 μ m/min) and the majority of cells remained arrested over the track duration (>60%) (**Fig. 2B**). We next assessed whether intestinal NKp46⁺ ILC3 can adapt their behavior in response to environmental stimuli. We injected bacterial flagellin, that can mimic bacterial infection (15, 16) and characterized its impact on ILC3 homeostasis, function and behavior (**Fig. 2C-H**). We found that absolute numbers of NKp46⁺ *Il22*⁺ were clearly increased upon flagellin stimulation (**Fig. 2C and S2B**). Analysis of ILC3 subsets showed that frequencies of *Il22*-expressing cells were rapidly augmented among both ILC3 subsets five hours post-flagellin (**Fig S2C**). Accordingly, imaging of *Ncr1*^{GFP} *Il22*^{TdT} mice revealed a marked increase of double positive cells in intestinal villi that rapidly changed their motility in response to flagellin challenge (**Fig. 2D, S2D and S2E**). In this context, activated NKp46⁺ ILC3 migrated within the intestinal villi with enhanced velocity compared to steady-state ILC3 (**Fig. 2D, 2E and 2F**). NKp46⁺ ILC3 exhibited unique movements in the intestinal lamina propria after challenge, shifting from 'spot' migration patterns corresponding to restricted motility to 'wavy' migration patterns typical of patrolling cells (17) (**Fig. 2G and S2F**), resulting in increased tissue scanning by *Il22* expressing-NKp46⁺ ILC3 (**Fig. 2H**). Together, these data indicate that environmental signals, such as acute inflammation caused by bacterial challenge, impact ILC3 behavior and induce a patrolling function that is associated with enhanced IL-22 expression.

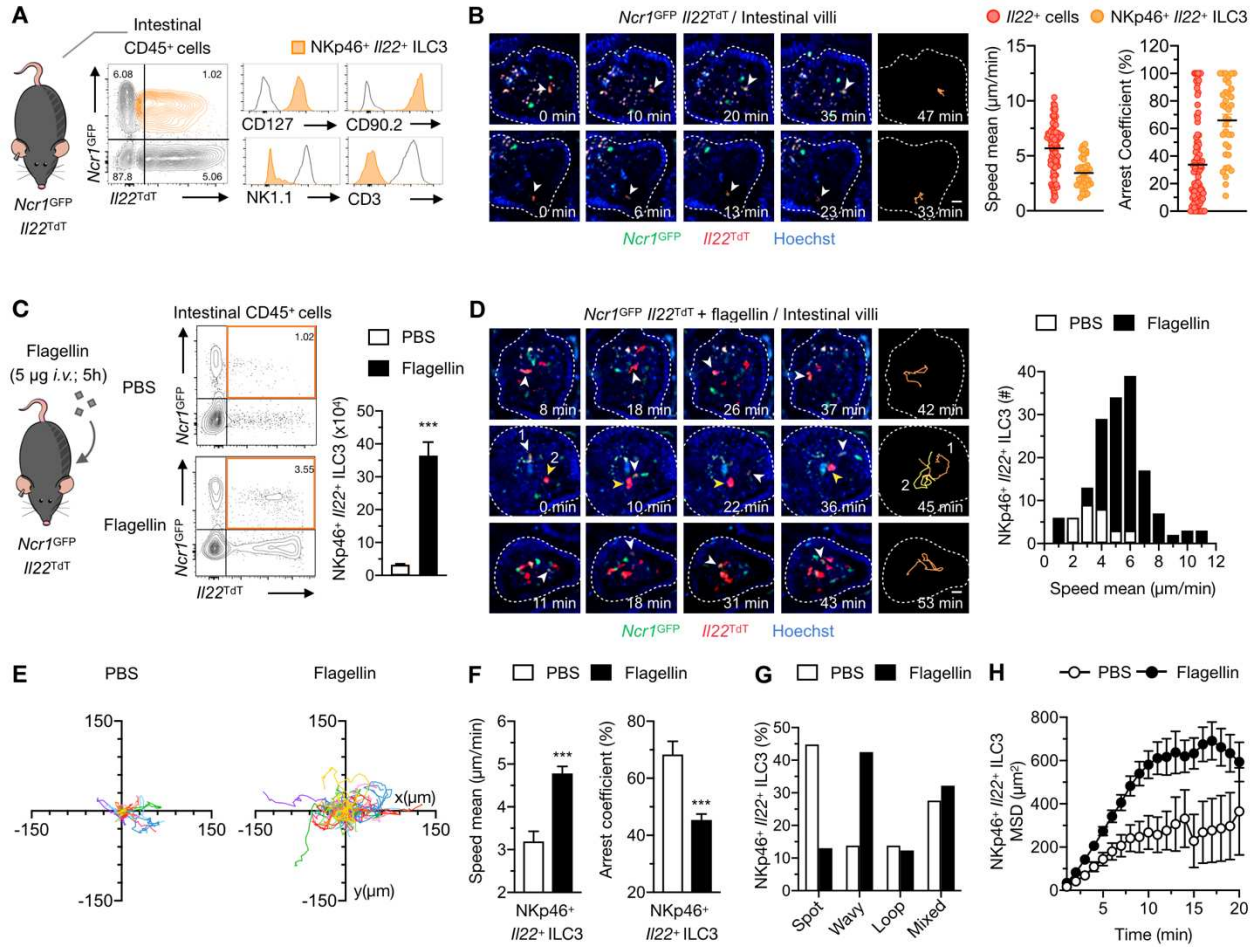


Figure 2 | Monitoring of intestinal tissue during inflammation by patrolling ILC3

(A) Flow cytometry analysis of intestinal CD45⁺ cells from *Ncr1*^{GFP} *I122*^{TdT} reporter mice and focus on *Ncr1*^{GFP+} *I122*^{TdT+} cells. Analysis of CD127, CD90.2, NK1.1 and CD3 expressions in CD45⁺ *Ncr1*^{GFP+} *I122*^{TdT+} cells to confirm NKp46⁺ *I122*⁺ ILC3. (B) Time-lapse images (scale bar 15 µm) of NKp46⁺ *I122*⁺ in intestinal villi. Graphs show mean speed and arrest coefficient of NKp46⁺ *I122*⁺, compared to *I122*⁻ (NKp46⁻) cells. (C) *Ncr1*^{GFP} *I122*^{TdT} mice were treated with flagellin (5 µg, *i.v.*) five hours prior imaging. Flow cytometry analysis of intestinal NKp46⁺ *I122*⁺ ILC3, pregated on CD45⁺ cells, in *Ncr1*^{GFP} *I122*^{TdT} mice and graph showing absolute numbers of NKp46⁺ *I122*⁺ ILC3, without or with flagellin stimulation. (D) Time-lapse images (scale bar 15 µm) of NKp46⁺ *I122*⁺ in intestinal villi five hours post-flagellin stimulation. Graph shows mean speed distribution of NKp46⁺ *I122*⁺, without (white) or with (black) flagellin. (E) Individual tracks of intestinal NKp46⁺ *I122*⁺, without (left) or with (right) flagellin. (F-H) Graphs show mean speed, arrest coefficient (F), distribution of ILC3 patrolling behaviors (G) and mean square displacement (MSD, H) of NKp46⁺ *I122*⁺, without (white) or with (black) flagellin stimulation. Results in (B, D-H) are from at least nine movies per condition obtained in three independent experiments (n=95 *I122*⁺ cells; n=29 and n=156 NKp46⁺ *I122*⁺ ILC3, without or with flagellin respectively). Each bar corresponds to the mean ± SEM of the values obtained – only NKp46⁺ *I122*⁺ ILC3 tested (****P*<0.001; ***P*<0.002; two-tailed Mann-Whitney test).

Our results are consistent with a crucial role of the local environment in shaping ILC3 responses. Intestinal ILC3 are ideally positioned to sense a wide range of external and host-derived signals and have the ability to integrate these multiple inputs and adapt their effector responses (18). Consequently, after demonstrating the role of external stimuli on ILC3 responses, we further asked whether intercellular interactions could also affect ILC3 behavior. Previous reports have documented increased intestinal ILC3 numbers and IL-22 production in *Rag2*^{-/-} mice (19–21). We next interrogated whether an absence of adaptive immunity affects ILC3 behavior through intravital imaging of *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} mice (**Fig. 3A and S1B**). We discovered that large numbers of patrolling ILC3 were present in the intestinal villi at steady-state (**Fig. 3A and 3D**). There, ILC3 constantly scanned the tissue without limitations, defined by low confinement (straightness ratio ~ 0.5) and little stop (arrest coefficient ~ 35.9-41.8%), and at high velocity (speed mean ~ 4.6-4.9 $\mu\text{m}/\text{min}$), independently of their *Il22* expression (**Fig. 3A, 3D and 3E**). Indeed, consistent with previous findings (19–21), ILC3 largely expressed *Il22*. These results indicate that behavior of villus ILC3 is modulated by adaptive immune cells.

Several recent studies have identified a reciprocal interaction between intestinal T cells and ILC3 (22). In particular, adoptive transfer of conventional T cells was sufficient to down-regulate heightened IL-22 responses in ILC3 in *Rag2*-deficient mice (19, 20). To test whether T cells could regulate ILC3 dynamics, we adoptively transferred CFP⁺ T cells into *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} mice, allowed them to reconstitute for 2 weeks and then imaged intestinal ILC3 (**Fig. 3B**). In contrast with ILC3 in *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} mice, ILC3 in T cell reconstituted *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} mice hardly explored the intestinal tissue (straightness ratio ~ 0.2-0.3) with a strongly reduced velocity (<2.2 $\mu\text{m}/\text{min}$) and were mainly arrested (arrest coefficient ~ 83.2-91.1%), regardless of *Il22* expression (**Fig. 3B, 3D and 3E**). Thus, T cells appear to suppress ILC3 patrolling behavior under steady-state conditions. To assess the balance between T-cell mediated suppression and inflammation-mediated activation of ILC3 patrolling, we challenged T cell-reconstituted *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} mice with flagellin (**Fig. 3C**). In this setting, despite the presence of T cells, both *Il22*⁻ and *Il22*⁺ intestinal ILC3 exhibited enhanced patrolling behavior with augmented velocity, reduced arrest and confinement compared to unchallenged intestinal ILC3 – although not to the same extent as in *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} where T cells are missing (**Fig. 3C, 3D and 3E**). Taken together with the observation that T cells patrol the intestine under physiologic and inflammatory conditions (**Fig.S3**), these data support the idea that T cells and ILC3 responses are coordinated within the gut: under non-infectious conditions, T cell control suppresses ILC3 patrolling but inflammation associated with bacterial challenge can override this T cell control allowing inducible ILC3 patrolling of the intestinal barrier.

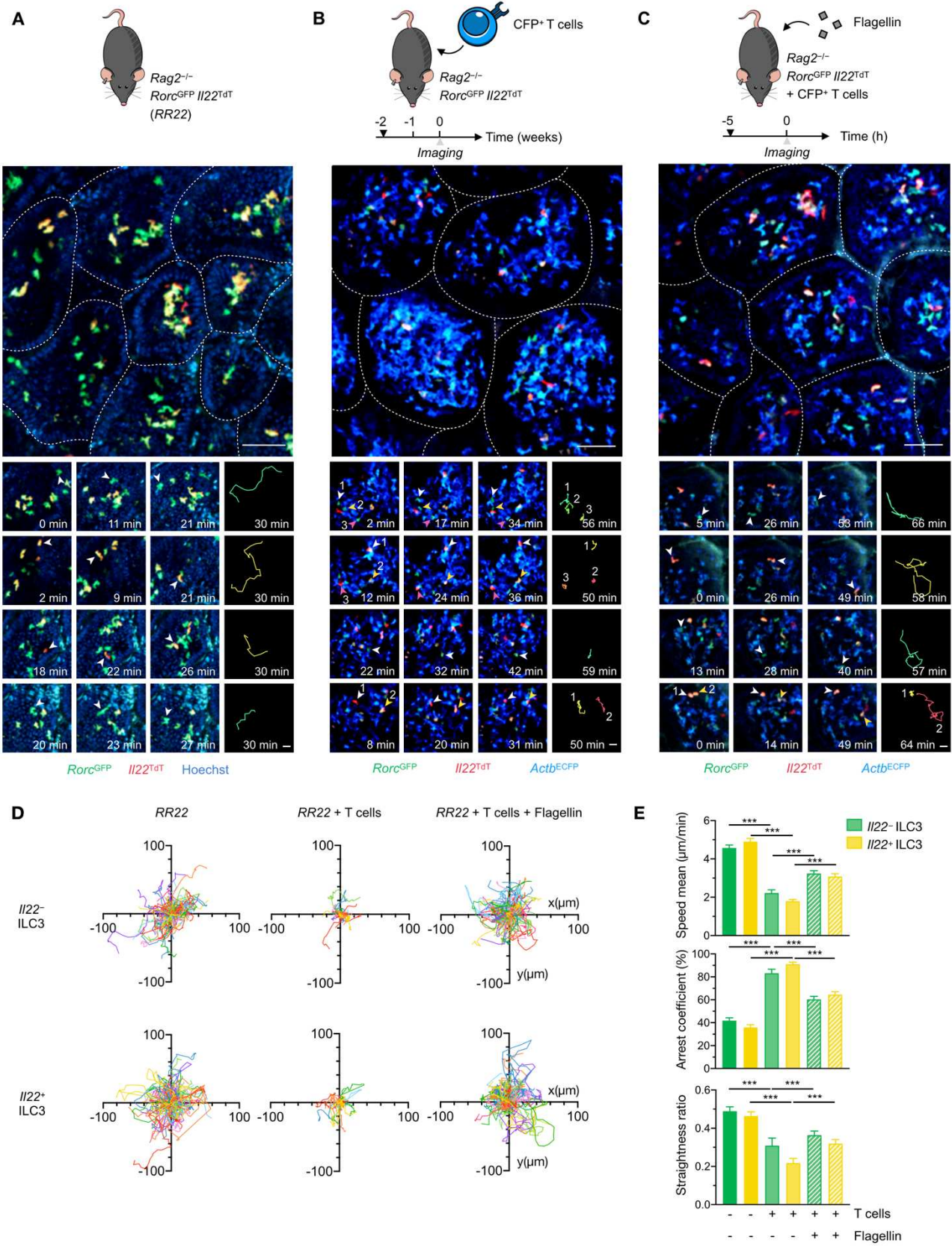


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Figure 3 | Local environmental stimuli regulate ILC3 patrolling

(A) Representative image (top; scale bar 50 μm) and time-lapse images (bottom; scale bar 15 μm) of ILC3 within intestinal villi of *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} (RR22) mice. (B) T cells were isolated from spleen and lymph nodes of a *Actb*^{ECFP} reporter mice were adoptively transferred into *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} (RR22; GFP staining ILC3 only and Tomato staining *Il22* transcripts) transgenic mice. Two weeks later, intestine of recipient mice (RR22 + CFP⁺ T cells) was imaged by multi-photon microscopy. Representative image (top; scale bar 50 μm) and time-lapse images (bottom; scale bar 15 μm) of ILC3 and CFP⁺ T cells in intestinal villi of RR22 mice adoptively transferred with CFP⁺ T cells. (C) Flagellin (5 μg) was *i.v.* injected into adoptively transferred RR22 (RR22 + CFP⁺ T cells) mice five hours prior to intravital imaging. Representative image (top; scale bar 50 μm) and time-lapse images (bottom; scale bar 15 μm) of ILC3 and CFP⁺ T cells in intestinal villi of RR22 + CFP⁺ T cells stimulated with flagellin. (D) Individual tracks of *Il22*⁻ (top) or *Il22*⁺ (bottom) ILC3 in RR22 (left), RR22 + CFP⁺ T cells (middle) and RR22 + CFP⁺ T cells + flagellin (right) mice. (E) Graphs show mean speed, arrest coefficient and straightness ratio of indicated populations in RR22, RR22 + CFP⁺ T cells and RR22 + CFP⁺ T cells + flagellin mice. Results in (D-E) are from one to three movies per condition from one of three independent experiments (n=109 and n=120 for *Il22*⁻ and *Il22*⁺ ILC3 in RR22 mice, respectively, and n=39 and 120 *Il22*⁻ ILC3; n=45 and 109 *Il22*⁺ ILC3 for RR22 + CFP⁺ T cells mice without or with flagellin, respectively). Each bar corresponds to the mean \pm SEM of the values obtained (***)*P*<0.001; one-way ANOVA).

We next aimed to define the molecular mechanisms driving intestinal ILC3 migration patterns that we have observed. As chemokines play a fundamental role in lymphocyte migration and function (23) and have been shown to be involved in ILC3 homing to the intestine and trafficking from the intestine to lymph nodes (11, 24, 25), we hypothesized that chemokine/chemokine receptors might regulate intestinal ILC3 patrolling. We first assessed chemokine receptors expression on sorted intestinal NKp46⁺ and CCR6⁺ ILC3 (Fig. 4A) and confirmed previously identified receptors including *Ccr6*, *Ccr9* and *Cxcr6* (11, 25, 26). We then examined by flow cytometry the expression at the protein level of newly and previously identified chemokine receptors on ILC3 subsets (Fig. 4B). We only detected common expression of CCR7, CXCR4 and CXCR6 on both ILC3 subsets – albeit at different levels – and differential expression of CCR9 on the NKp46⁺ ILC3 subset (Fig. 4B and 4C). We next assessed expression of relevant chemokines (*Ccl19* and *Ccl21* for CCR7, *Cxcl12* for CXCR4, *Cxcl16* for CXCR6, *Ccl25* for CCR9 and *Ccl20* for CCR6); Fig. 4C) from whole ileal tissue extracts of wild-type, *Rag2*-deficient and T cell-reconstituted *Rag2*-deficient mice (Fig. 4D). Interestingly, *Ccl25*, *Cxcl12* and *Cxcl16* were upregulated in *Rag2*^{-/-} mice suggesting a role in ILC3 patrolling.

To assess the role for these chemokines on ILC3 patrolling *in vivo*, we neutralized their activity *in vivo* using a combination of blocking monoclonal antibodies against CXCL12, CXCL16, CCL25 and CCL21 (Fig. 4E and S4A). We imaged intestinal ILC3 prior and after injection to visualize the effect of chemokines blockade on ILC3 patrolling in real-time (Fig. 4E). We found that the speed of intestinal ILC3 strongly decreased 20 minutes following chemokine neutralization, an effect that was not observed when an isotype combination was injected instead (Fig. 4F, 4G and S4B-D). Chemokine blockade impaired ILC3 patrolling and tissue scanning, which was reflected by the reduction of ILC3 trajectories, speed means and straightness ratios (Fig. 4F, 4H and 4I). Patrolling ILC3 became predominantly arrested, with a high arrest coefficient (over 70%) (Fig. 4F and 4I). All the effects of chemokine blockade were rapidly observed within the first hour post-injection but were even more pronounced after two hours of blockade (Fig. 4I). Overall, these results unraveled the essential role of intestinal chemokines in regulating intestinal ILC3 dynamics and behavior.

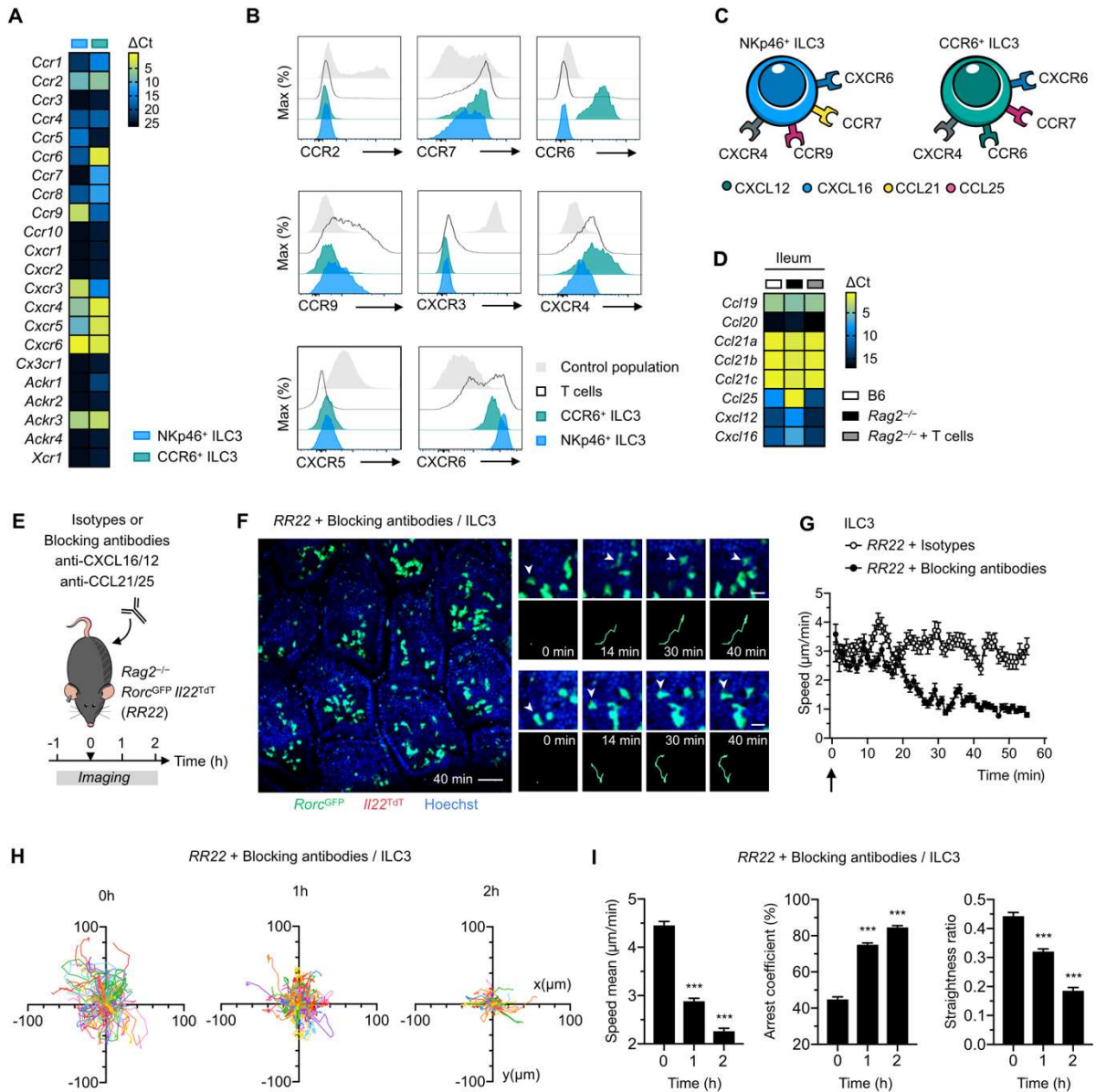


Figure 4 | Chemokines are involved in the control of ILC3 migration

(A) Heatmap depicting differential chemokine receptors expression in sorted NKp46⁺ ILC3 and CCR6⁺ ILC3, as detected by bulk multiplex Biomark. (B) Flow cytometry analysis of chemokine receptors profiles of intestinal NKp46⁺ ILC3, CCR6⁺ ILC3 and T cells. A negative or positive population is shown as a control for each chemokine receptor. (C) Scheme of chemokine receptor expression profiles of intestinal ILC3. (D) Heatmap depicting differential chemokines expression in whole ileum of B6 (white), *Rag2*^{-/-} (black) and *Rag2*^{-/-} adoptively transferred with T cells (black), as detected by bulk multiplex Biomark. (E-I) Intestinal ILC3 were imaged in *Rag2*^{-/-} *Rorc*^{GFP} *I122*^{Tdt} mice using intravital imaging for one hour. Then, mice were injected *i.v.* with a combination of either isotype controls (50 μg mouse IgG1, 100 μg rat IgG2a and 50 μg rat IgG2b) or blocking monoclonal antibodies (anti-CXCL12, anti-CXCL16, anti-CCL21 and anti-CCL25; 50 μg each) and subsequently imaged for 2 hours. (F) Representative image (left; scale bar 50 μm) and time-lapse images (right; scale bar 15 μm) of ILC3 in intestinal villi after blocking antibodies injection (at 0 min). (G) Speed over time of intestinal ILC3, following either isotypes (white) or blocking antibodies (black) injection at time 0 (arrow). (H) Individual tracks of intestinal ILC3 before (-60-0 min; left), 1h (0-60 min; middle) and 2h (60-120min; right) after blocking antibodies injection. (I) Graphs show mean speed, arrest coefficient and straightness ratio of intestinal ILC3 at indicated timepoints after blocking antibodies injection. Results in (G-H) are from three movies per condition obtained in three independent experiments (n=364 ILC3 tracks for 0h; n=617 ILC3 tracks for 1h; n=310 tracks for 2h). Each bar corresponds to the mean ± SEM of the values obtained (****P*<0.001; one-way ANOVA).

In particular, our multiplex chemokine analysis indicated that *Ccl25* was the most upregulated and abundant chemokine in *Rag2*^{-/-} mice (**Fig. 4D**). To analyze the specific role of CCL25 on patrolling ILC3, we injected *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} mice with monoclonal antibodies against CCL25 alone (**Fig. 5A and S4E**). We observed that patrolling was strongly affected following mCCL25 neutralization specifically (**Fig. 5B and S4F-H**), with a progressive decrease in ILC3 speed (**Fig. 5C**). CCL25 blockade limited ILC3 migration, with reduced trajectories, speed mean (2 μ m/min), augmented arrest coefficient (~ 85%) and confinement (~ 0.3) two hours post-injection (**Fig. 5D and 5E**). These results identify the CCR9/CCL25 axis as a critical regulator of ILC3 patrolling within the intestine.

Interestingly, some intestinal T cells share CCR9 expression with ILC3 (27) (**Fig. 4C**), which suggests that T cells may regulate ILC3 patrolling through indirectly modulating factors driving ILC3 migration, such as CCL25, but also through directly competing for them. While competition between ILC3 and T cells for survival factors has been proposed in lymphoid tissues (28, 29), evidence that T cells and ILC3 compete for chemokines has not been reported. To further address T/ILC3 chemokine competition for CCL25, we adoptively transferred 5×10^6 total T cells from *Ccr9*^{-/-} mice into *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} recipients (**Fig. 5F and S5A-B**). In this setting, we found that villus ILC3 could patrol despite the presence of T cells (**Fig. 5G and S5C**). In contrast, ILC3 patrolling was ablated as expected when control *Ccr9*-expressing T cells were transferred (**Fig. 5H**), suggesting that T cell regulation of ILC3 patrolling occurs, at least in part, through regulation of CCL25 accessibility by CCR9⁺ T cells. To assess whether available CCL25 could be involved in T cell regulation of ILC3 patrolling, we treated adoptively transferred *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} + *Ccr9*^{-/-} T cells with blocking CCL25 antibodies (**Fig. 5I**). CCL25 neutralization resulted in reduction of ILC3 patrolling, with ILC3 arresting and displaying reduced speed mean as well as straightness ratio in presence of CCL25 antibodies (**Fig. 5J and 5K**). These findings indicate that regulation of CCL25 availability by CCR9⁺ T cells partly accounts for T cell influence on ILC3 patrolling.

Figure 5 | The CCL25/CCR9 axis is involved in ILC3 patrolling and in T cell regulation of ILC3 patrolling within intestinal villi

(**A-E**) Intestinal ILC3 were imaged in *Rag2*^{-/-} *Rorc*^{GFP} *Il22*^{TdT} (*RR22*) mice using intravital imaging for one hour prior injection. Then, mice were injected *i.v.* with either isotype control (50 μ g rat IgG2b) or CCL25 blocking antibody (50 μ g) and subsequently imaged for 2 hours. (**B**) Representative image (left; scale bar 50 μ m) and time-lapse images (right; scale bar 15 μ m) of ILC3 in intestinal villi after anti-CCL25 injection (at 0 min). (**C**) Speed over time of intestinal ILC3, following either isotype (white) or anti-CCL25 (black) injection at time 0 (arrow). (**D**) Individual tracks of intestinal ILC3 before (-60-0 min; left), 1h (0-60 min; middle) and 2h (60-120min; right) after anti-CCL25 injection. (**E**) Graphs show mean speed, arrest coefficient and straightness ratio of intestinal ILC3 at indicated timepoints after anti-CCL25 injection. (**F-K**) *RR22* mice were adoptively transferred with *Ccr9*^{-/-} T cells. Two-weeks later, intestinal ILC3 were imaged using intravital imaging. In parallel, adoptively transferred *RR22* mice with *Ccr9*^{-/-} T cells were imaged for one hour prior injection. Then, mice were injected *i.v.* with anti-CCL25 as before and subsequently imaged for 1h. (**G**) Representative image (left; scale bar 50 μ m) and time-lapse images (right; scale bar 15 μ m) of ILC3 in intestinal villi of *RR22* adoptively transferred with *Ccr9*^{-/-} T cells. (**H**) Graphs show mean speed, arrest coefficient and straightness ratio of intestinal ILC3 in presence of either control (*Ccr9*^{+/+}; white) or *Ccr9*^{-/-} (gray) T cells. (**J**) Representative image (left; scale bar 50 μ m) and time-lapse images (right; scale bar 15 μ m) of ILC3 in intestinal villi of *RR22* + *Ccr9*^{-/-} T cells mice after anti-CCL25 injection (at 0 min). (**K**) Graphs show mean speed, arrest coefficient and straightness ratio of intestinal ILC3 in presence of *Ccr9*^{-/-} T cells at indicated timepoints after anti-CCL25 injection. Results in (D-E, H and K) are from at least two movies per condition obtained two independent experiments (D-E ILC3 tracks: n=131 for 0h; n=516 for 1h; n=179 for 2h / H ILC3 tracks: n= 84 for *Ccr9*^{+/+}; n= 338 for *Ccr9*^{-/-} / K ILC3 tracks: n=152 without; n=516 with @CCL25). Each bar corresponds to the mean \pm SEM of the values obtained (***P*<0.001; ***P*<0.002; one-way ANOVA test in (E) and Welch's t-tests in (H) and (K)).

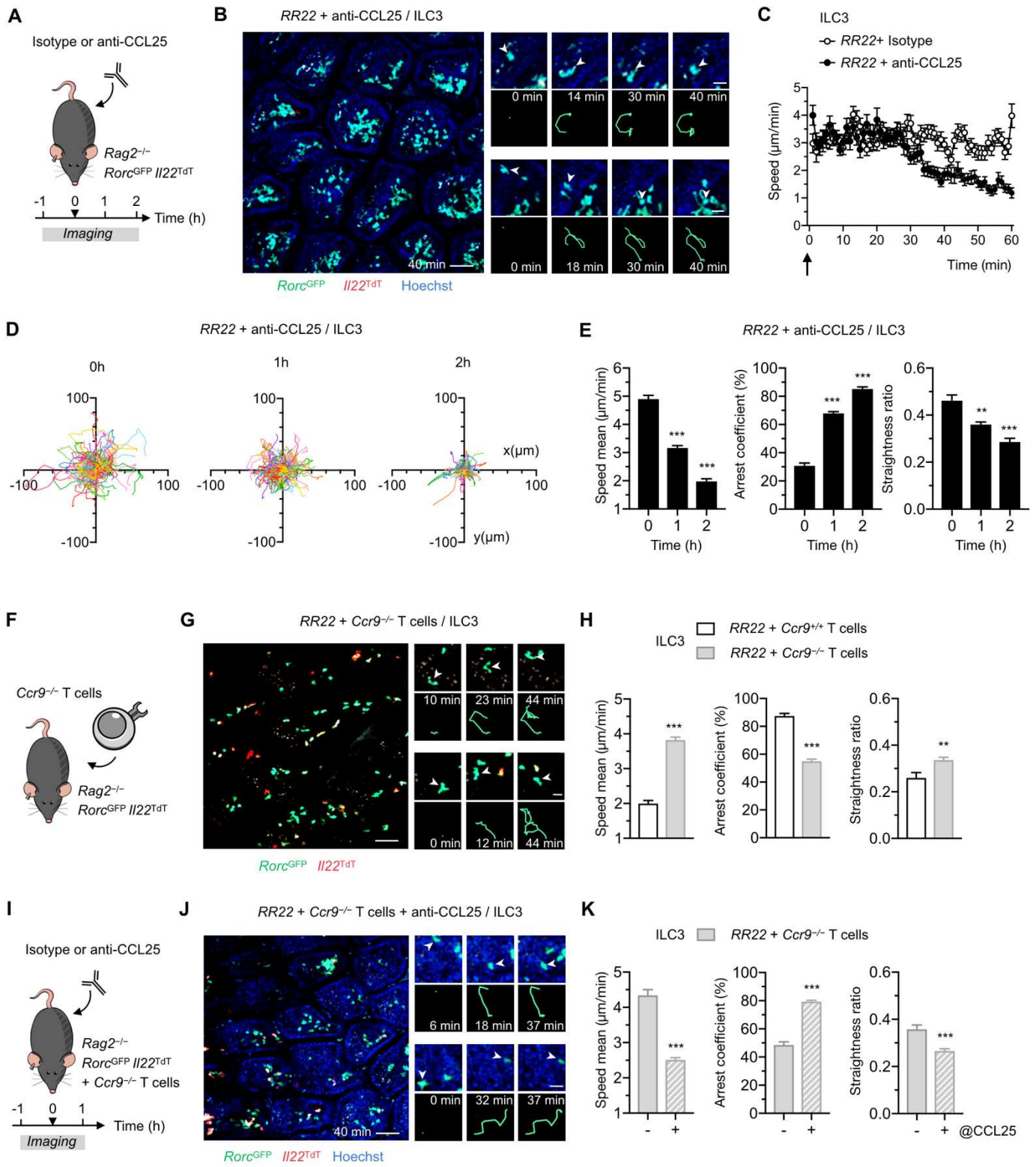


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Collectively, our results uncover a previously unrecognized coordinated intestinal ILC3/T cell response to inflammation that promotes monitoring of the intestine by patrolling ILC3, presumably supporting proper epithelial barrier function. While ILC3 are critical for maintaining intestinal homeostasis and immunity (30), unbalanced ILC3 responses can lead to intestinal pathology. Indeed, dysregulated ILC3 numbers and functions are associated with the development of intestinal diseases, such as inflammatory bowel disease (IBD) (31). We provide evidence here that intestinal ILC3 cellular behavior and function are tightly regulated but also readily adaptable. We observed that naïve ILC3 are compartmentalized within the intestine, along with *IL22* distribution which is restricted to intestinal crypts, and poorly motile independently of ILC3 priming reflected by *IL22* expression. Upon inflammation, either induced by the bacterial ligand flagellin or spontaneously observed in *Rag2^{-/-}* mice, *IL22* repartition was modified within the intestine with large numbers of *IL22*-expressing ILC3 present within intestinal villi, possibly allowing a dynamic regulation of the intestinal barrier. Unexpectedly, we found that these quick ILC3 responses were also accompanied by a shift in ILC3 cellular behavior. Local environmental signals provided tissue-resident ILC3 with novel migratory attributes: ILC3 rapidly increased their motility to become patrolling cells, resulting in enhanced tissue scanning. Thus, the intestinal mucosa appears continuously exposed to environmental stimuli that can induce immune responses through the recruitment of sentinel cells including patrolling ILC3. We identified the CCL25/CCR9 axis as one of the signals controlling intratissular migration of intestinal ILC3, in addition of its role in ILC3 and T cell homing to the gut (25, 27), as well as a critical pathway for T cell regulation of ILC3 patrolling. Adoptive transfers of T cells showed that they prevented ILC3 migratory behavior at steady-state but not upon inflammation, in part through directly regulating CCL25 availability within the intestine, providing the first evidence that ILC3 and T cells could compete within intestinal niches for soluble factors. In light of these and previous findings, we propose that signals from the microenvironment promote tissue adaptation of intestinal ILC3 responses, including patrolling behavior and IL-22 production, which contributes to maintaining the balance between regulatory and immune protective ILC3 functions. Further investigations are required to define the biological role of patrolling ILC3 in intestinal immunosurveillance, as it has been suggested for intestinal T cells migration (13). In the long-term, this work could lead to novel therapeutic approaches for intestinal diseases, based on manipulating the signals controlling patrolling ILC3.

MATERIALS AND METHODS

Mice

All mice were bred in dedicated animal facilities of the Institut Pasteur. *Actb^{ECFP}* (CFP, (32)), *Rorc^{GFP}* (33), *Il22^{TdT}* (34), *Ncr1^{GFP}* (35) and *Rag2^{-/-}* (36) have been previously described and used on a C57BL/6 background. C57BL/6J and C57BL/6 Ly5.1 mice were purchased from Charles River laboratories and kept in dedicated animal facilities of the Institut Pasteur. All experiments involving mice were performed according to guidelines issued by the Institut Pasteur Ethics Committee and were approved by the French Ministry of Research (projects dha170001, CETEA 2013-033 and CETEA 17500).

Adoptive transfer of T cells

Cells from the spleen and the lymph nodes of CFP⁺ or *Ccr9^{-/-}* mice were isolated by being passed through a steel wire mesh, filtered on a 40- μ m cell strainer and after red blood cells lysis using ACK buffer. To enrich for T cells, single-cell suspensions were first incubated with biotin-conjugated anti-B220 (RA3-6B2, eBioscience), anti-CD19 (1D3, eBioscience), anti-CD11b (M1/70, BD Biosciences), anti-CD11c (N418, eBioscience), anti-Gr1 (RB6-8C5, eBioscience), anti-NK1.1 (PK136, BD Biosciences) and anti-Ter119 (TER-119, BD Biosciences) antibodies followed by anti-biotin microbeads (Miltenyi) and negatively selected by magnetic cell separation with MACS technology (Miltenyi). 5×10^6 enriched T cells were intravenously transferred to *Rag2^{-/-}Rorc^{GFP}Il22^{TdT}* (RR22) mice and recipient mice were analyzed at day 14 post-transfer.

Generation of BM chimeric mice

Cells from the bone marrow of CFP⁺ and RR22 mice were isolated by crushing the bones with a mortar and pestle, being filtered on a 40- μ m cell strainer and after red blood cells lysis using ACK buffer. Bone marrow cells were mixed at a 1:19 ratio – 0.5×10^6 and 10×10^6 cells from CFP⁺ and RR22 mice, respectively. Mixed bone marrow cells were intravenously transferred into lethally irradiated (9 Gy X-ray irradiation one day prior injection) congenic C57BL/6J mice. BM chimeric mice were analyzed seven weeks later for reconstitution (donor cells in the small intestine lamina propria) and imaged by multi-photon microscopy.

Isolation of intestinal cells

Small intestine was collected from euthanized mice and placed into cold complete medium: RPMI 1640 GlutaMAX (Gibco) supplemented with 5% fetal calf serum (Eurobio) and 10mM HEPES (Sigma-Aldrich). The mesenteric adipose tissue and Peyer's patches were first pulled out before cutting the small intestine longitudinally and removing feces. Intestinal tissue was washed in PBS (Gibco) to eliminate mucus, cut into 1-2 cm pieces and intestinal epithelial cells were eliminated by shaking incubation in complete medium containing 5 mM EDTA (Invitrogen) for 20 min at 37°C. Subsequently, the intestinal tissue was minced and incubated twice in a digestion solution containing 0.5 mg Collagenase VII (Sigma-Aldrich) for 15 min at 37°C in a shaking incubator to isolate the lamina propria lymphocytes

(LPL). LPL were filtered through a 40- μ m cell strainer and kept in complete medium for downstream analysis.

Flow cytometry

Cells were first blocked with FcR Blocking Reagent (Miltenyi) and stained with Flexible Viability Dye (eFluor 506 or 780, eBioscience) for 15 min, followed by 30 min of surface antibodies staining on ice except for chemokine receptors (at 37°C for 30 min then at 4°C for 15 min). Cells were generally fixed in 4% PFA (Sigma-Aldrich). Only for experiments involving intranuclear transcription factors (TF) staining, cells were fixed, permeabilized and stained using Foxp3/TF Staining Buffer Kit (eBioscience). Intranuclear and cell surface staining were performed with the following antibodies (from BD Biosciences, eBioscience and Biolegend): anti-CCR2 (SA203G11), anti-CCR6 (140706), anti-CCR7 (4B12), anti-CCR9 (CW-1.2), anti-CD11b (M1/70), anti-CD19 (1D3), anti-CD127 (A7R34), anti-CD3 (145-2C11), anti-CD3 (500A2 or 17A2), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD5 (53-7.3), anti-CD8a (53-6.7), anti-CD4 (GK1.5), anti-CD90.2 (30-H12), anti-CD90.2 (53-2.1), anti-CXCR3 (CXCR3-173), anti-CXCR4 (QA16A08), anti-CXCR5 (L138D7), anti-CXCR6 (SA051D1), anti-KLRG1 (2F1), anti-NK1.1 (PK136), anti-NKp46 (29A1.4), anti-ROR γ t (Q31-378), TCR β (H57-597). All the samples were acquired on a custom-configured LSR Fortessa or sorted using a FACSAria III (BD Biosciences). For the sorting of ILC3 subsets, we applied the following gating strategies: Live/CD45.2⁺/CD3⁻/NK1.1⁻/KLRG1⁻/CD90.2⁺CD127⁺ and sub-gated on NKp46⁺ or CCR6⁺ cells. Data were analyzed with FlowJo software (TreeStar).

Intravital two-photon imaging

Mice were first anaesthetized with a mixture of ketamine (Imalgene; 62.5 mg/kg), xylazine (Rompun; 12.5 mg/kg) and acepromazine (Calmivet; 3.1 mg/kg), and the abdomen hair was shaved. The abdominal skin and wall musculature were incised along the *linea alba* to expose the intestine. A loop of the terminal ileum (2-cm upstream the caecum) was exposed and cut open on the opposite side of the mesentery using an electrical cautery. Feces were gently removed using PBS and mice were placed on a heated (37°C) steel plate. Mice were immobilized by placing vinyl polysiloxane-based paste (3M) on both sides of the abdomen and a Superfrost Plus slide (Menzel-Gläser, VWR) was placed across the paste with a PBS-soaked tissue paper (Kimtech, Kimberly-Clark) on top. The intestinal tissue was positioned on the slide, immobilized with a coverslip (Menzel-Gläser, VWR) on top held by paperclips and covered with Supragel ultrasound gel (LCH). During imaging, mice were supplied with oxygen and their temperature was controlled and maintained at 37°C using a heating pad, heating cover and objective heater for the tissue. When indicated, intestine and blood vessels labeling was performed by intravenous (*i.v.*) injection of Hoechst 33342 (Invitrogen; 40 μ l of 10 mM solution, prepared in PBS) and EvansBlue (Sigma-Aldrich; 25 μ l of 2 μ g/ μ l solution, prepared in PBS), respectively. Two-photon imaging was performed with an upright microscope FVMPE-RS (Olympus) and a \times 25/1.05 numerical aperture, water-dipping objective (Olympus). Excitation was provided by an Insight DeepSee dual laser (Spectra-Physics) tuned at 920. The following filters were used for fluorescence detection: CFP or Hoechst

(483/32), GFP (520/35), mTom (593/40) and background (624/40). To create time-lapse sequences, we typically scanned a 40- to 60- μm -thick volume of tissue at 7- μm Z-steps and 60-s intervals.

Image analysis

Movies were processed with Imaris (Bitplane) or Fiji (ImageJ2) softwares and analyzed as two-dimensional projections of three-dimensional data to avoid wrong tracking related to motion (intestinal villi or diaphragmatic movement). As such, represented speeds of motile cells are likely to be slightly underestimated. When necessary, drifting correction was also applied using the “Correct Drift” function in Imaris to minimize XY tissue drift. DiPer (37) was used to calculate mean square displacements and tracking behaviors. Movies and figures based on two-photon microscopy are shown as two-dimensional projections of three-dimensional data. For optimal contrast rendering, background was pseudo-colored in magenta in some images.

***In vivo* treatments**

To induce intestinal inflammation, mice were *i.v.* injected with purified flagellin from *Salmonella typhimurium* (FLA-ST Ultrapure, InvivoGen; 5 μg) five hours prior analysis. For chemokine neutralization, mice were *i.v.* injected during imaging with a mixture of Hoechst 33342 (see above) to control *i.v.* injection and the following monoclonal blocking antibodies (50 μg) : anti-CXCL12 (MAB310), anti-CXCL16 (MAB503), anti-CCL21 (MAB4572) and anti-CCL25 (MAB481, all from R&D Systems). Control mice were *i.v.* injected in accordance with the following isotype controls (50 μg): mouse IgG1 (MAB002), rat IgG2a (MAB006) and rat IgG2b (MAB0061, all from R&D Systems).

Immunofluorescence staining and confocal imaging

The ileal portion of the small intestine was cut longitudinally, washed in PBS and prepared using the Swiss-rolling technique. Intestinal rolls were fixed overnight in 4% PFA followed by dehydration in 30% sucrose (Sigma-Aldrich) before embedding in Tissue-Tek OCT compound (Sakura Finetek). Samples were frozen in an isopentane bath cooled with liquid nitrogen and stocked at -80°C . 8- μm sections were cut on a CM3050 S cryostat (Leica) and adhered to Superfrost Plus slides (Menzel-Gläser, VWR). Frozen sections were first hydrated with PBS-TS (PBS supplemented with 0.1% Triton X-100, 1% FCS, 1% bovine serum albumin (BSA; Sigma-Aldrich) and filtered) and blocked for one hour at room temperature (RT) with PBS-TS 10% FCS. Slides were then incubated overnight at 4°C with the following antibodies diluted in PBS-TS: anti-RFP (Rockland), phalloidin (Invitrogen) and anti-CD3 (500A2, BD Biosciences). The next day, slides were washed, incubated for one hour at RT with secondary conjugated antibodies, washed again and incubated for 2 minutes at RT with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich; 1 $\mu\text{g}/\text{ml}$). After staining, slides were mounted with Fluoromount-G (Southern Biotech) and examined on an Axio Imager Z.2 microscope (Zeiss).

Bulk RNA isolation and multiplex qPCR (Biomark)

For ILC3, $3.3 - 7.4 \times 10^3$ and $2.2 - 4.5 \times 10^3$ NKp46⁺ and CCR6⁺ ILC3 bulks were sorted directly into RLT buffer (Qiagen). For whole ileal tissue, dissected ileum was snap-frozen in liquid nitrogen, crushed with a mortar and pestle and homogenized into RLT buffer. Samples were stored at -80°C until mRNA purification using RNeasy Micro and Minikit with a DNase digestion step using RNase-Free DNase set (Qiagen) for ILC3 and ileum, respectively. mRNA quantity, quality and integrity were checked on Bioanalyser system (Agilent) or on a Nanodrop spectrophotometer (Thermofisher) for ILC3 and ileum, respectively. To synthesize cDNA, RT-PCR was performed using Transcriptor High Fidelity cDNA synthesis kit (Roche). From the cDNA, we followed the protocol 'Fast Gene Expression Analysis Using Evagreen® on the Biomark™' from Fluidigm. Briefly, pre-amplified cDNA was obtained after a pre-amplification step with Delta Genes™ Assays (Fluidigm) using Taqman Preamp Master Mix (Applied Biosystems) and a subsequent purification step using exonuclease I (NEB), and was then diluted 1:5 in TE buffer (Invitrogen). Sample mix was prepared as follows: diluted pre-amplified cDNA (3.6 µl), DNA binding dye Sample loading reagent (Fluidigm; 4 µl) and SsoTM Fast Evagreen Supermix with low ROW (Biorad; 0.4 µl). Assay mix was prepared as follows: primers (Fluidigm; 4µl, 10µM) and assay loading reagent (Fluidigm; 4µl). A 96.96 dynamic array integrated fluidic circuit (IFC; Fluidigm) was primed with control line fluid, and the chip was loaded with assays and samples using an HX IFC controller (Fluidigm). The experiments were run on a Biomark HD (Fluidigm) for amplification and detection (70°C-2400s; 60°C-30s; 95°C-60s; (96°C-5s,60°C-20s)X30 cycles; Melting curve: 60°C-3s; 60 to 95°C 1°C/3s). Real-time PCR Analysis software (Fluidigm) was used to view Ct data and amplification curves for the run and export results. The relative abundance of mRNA was normalized to *Ppia*.

Statistical analysis

All statistical tests were performed using Prism v.8 (GraphPad). Points in graphs indicate individual cells (except for speed over time analysis, where points indicate mean of individual cells), lines indicate means and error bars indicate cells SEM. For image analysis, in bar graphs, bars indicate means and error bars indicate individual cells SEM. For flow cytometry analysis, in bar graphs, bars indicate means and error bars indicate sample SEM. The statistical tests employed are detailed in the figure legends. Briefly, for small individual cell numbers or biological samples (< 30), normal distribution was not assumed and non-parametric tests were systematically used. When over 30 individual cells were analyzed, normal distribution was tested and, according to the results, parametric tests or non-parametric tests were performed.

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AUTHOR CONTRIBUTIONS

A.J., J.P.D. and N.S. conceived the study. A.J. and N.S. designed and performed experiments, together with Z.G. for all intravital imaging experiments. S.M. performed Biomark experiments and analyzed data. A.D. and I.P. provided *Ccr9*^{-/-} lymphocytes for adoptive transfer experiments. A.J. analyzed data and performed statistical analysis. J.P.D. and P.B. provided key intellectual expertise, resources and funding acquisition. A.J. wrote the original draft and J.P.D., P.B. and N.S. corrected it. N.S. supervised the study.

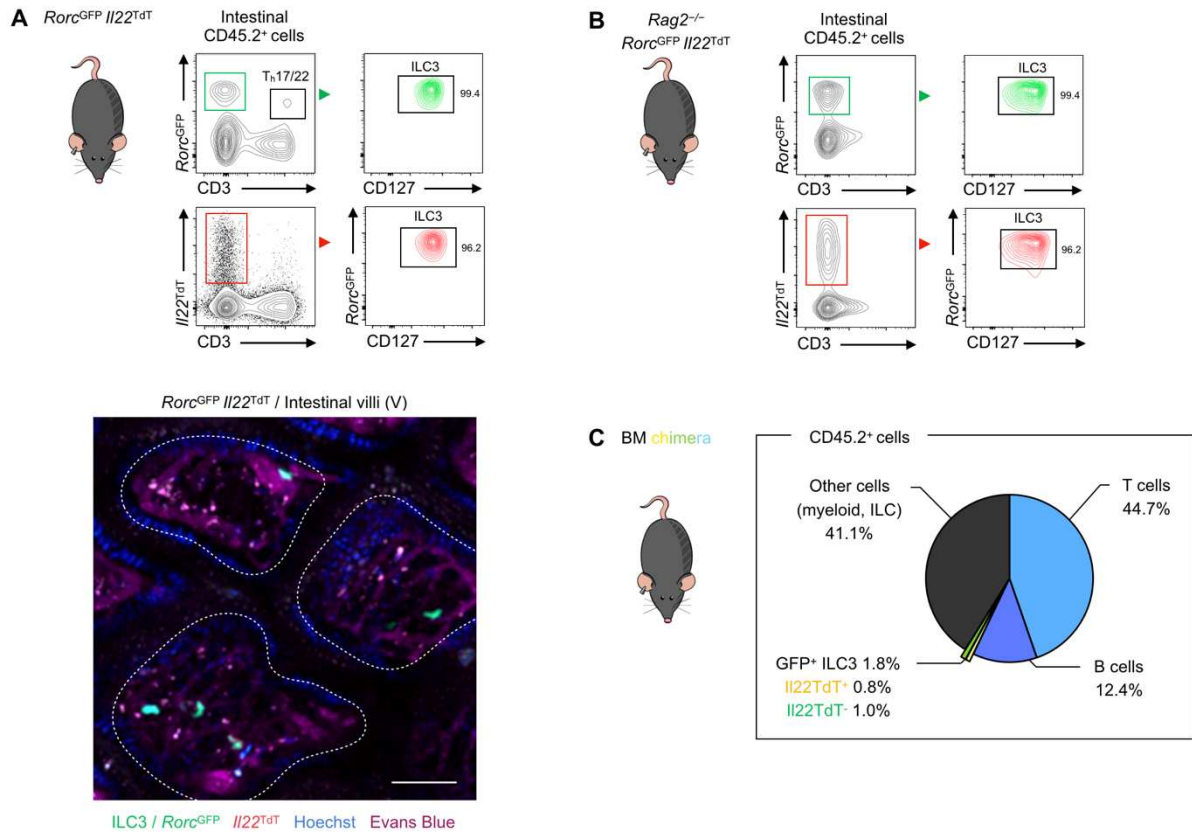
COMPETING INTERESTS

The authors declare no competing financial interests.

SUPPLEMENTARY INFORMATION

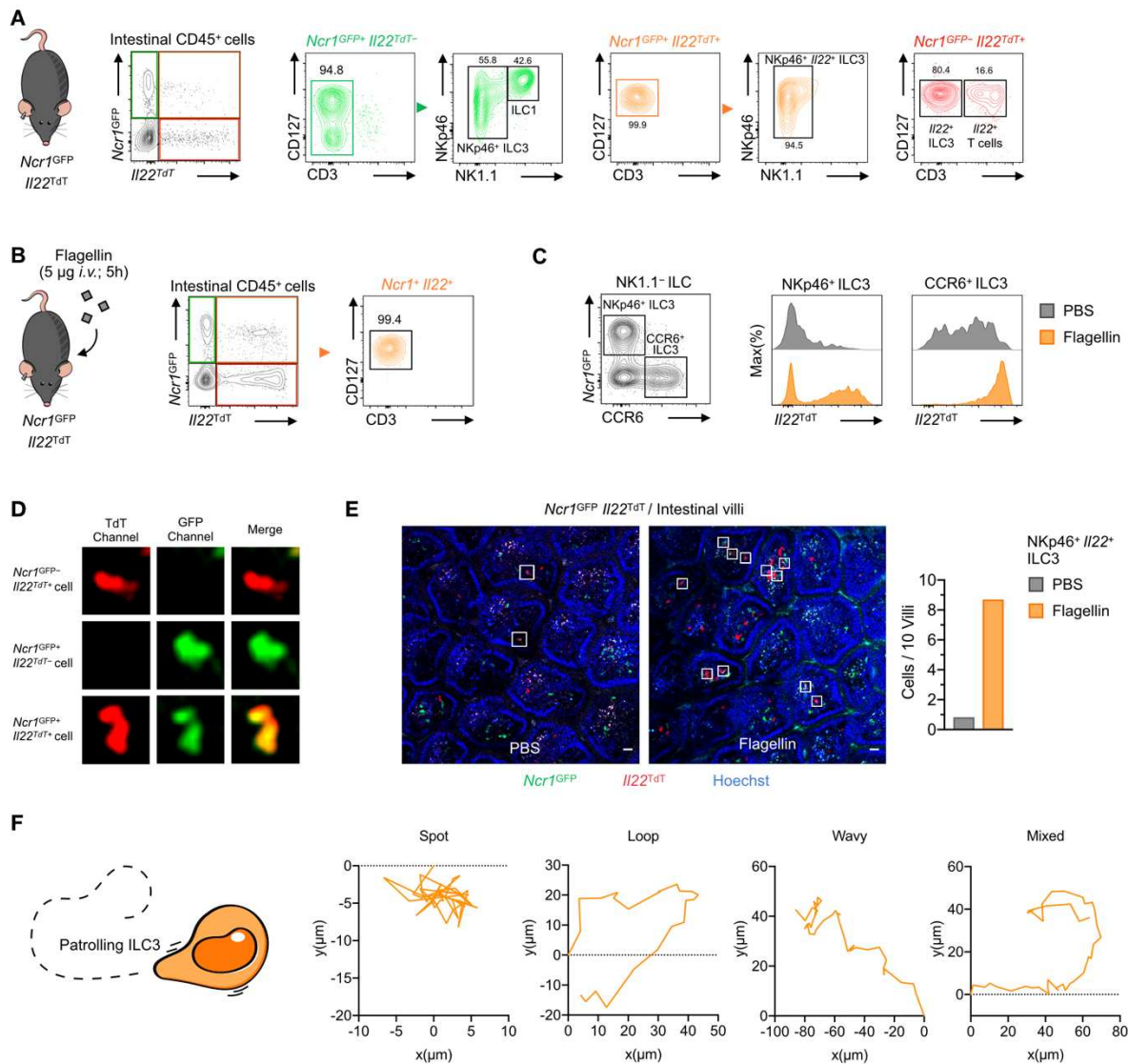
Supplemental Figures 1 - 5

Figure S1

**Figure S1 | BM chimera, a novel model to investigate ILC3 dynamics *in vivo***

(A) Flow cytometry analysis of small intestine from *Rorc^{GFP} Il22^{TdT}*. Representative intravital image of ILC3 (scale bar 50 μ m) in intestinal villi (V) of *Rorc^{GFP} Il22^{TdT}* mice. Mice were injected with Hoechst and Evans blue before imaging to visualize all nuclei (blue) and blood vessels (magenta). (B) Flow cytometry analysis of small intestine from *Rag2^{-/-} Rorc^{GFP} Il22^{TdT}* transgenic mice. (C) Bone marrow (BM) chimeric mice were created by cell transfer. Mixed bone marrow from *Rag2^{-/-} Rorc^{GFP} Il22^{TdT}* and *Actb^{ECFP}* (CFP) mice was injected into lethally irradiated congenic C57BL/6J mice and later analyzed for reconstitution. Pie chart showing the proportions of different subsets amongst donor cells analyzed by flow cytometry.

Figure S2

Figure S2 | *Ncr1*^{GFP} *I22*^{Tdt}, a model to study NKp46⁺ ILC3 responses upon bacterial challenge

(A-B) Flow cytometry analysis of small intestine leukocytes from *Ncr1*^{GFP} *I22*^{Tdt} mice, without (A) or with (B) 5 μ g of flagellin (*i.v.*; 5h). (C) Flow cytometry analysis of *I22* transcripts in NKp46⁺ and CCR6⁺ ILC3 (CD45⁺ CD3⁻ CD5⁻ CD90⁺ CD127⁺ cells) from PBS (gray) or flagellin- (orange) treated *Ncr1*^{GFP} *I22*^{Tdt} mice. (D) Representative images of observable cells in *Ncr1*^{GFP} *I22*^{Tdt} mice by intravital imaging. Single GFP and TdT channels images are shown, as well as merged images. (E) Representative images of NKp46⁺ *I22*⁺ ILC3 in intestinal villi of from PBS (left) or flagellin- (right) treated *Ncr1*^{GFP} *I22*^{Tdt} mice. White squares indicate NKp46⁺ *I22*⁺ ILC3. Graph shows the increase of NKp46⁺ *I22*⁺ ILC3 within intestinal villi after flagellin treatment. Results were calculated on 52-86 villi from three to five movies per condition from one of three independent experiments. (F) Sample tracks showing different NKp46⁺ *I22*⁺ ILC3 patrolling behaviors (spot; loop; wavy; mixed tracks) used in Figure 2.

Figure S3

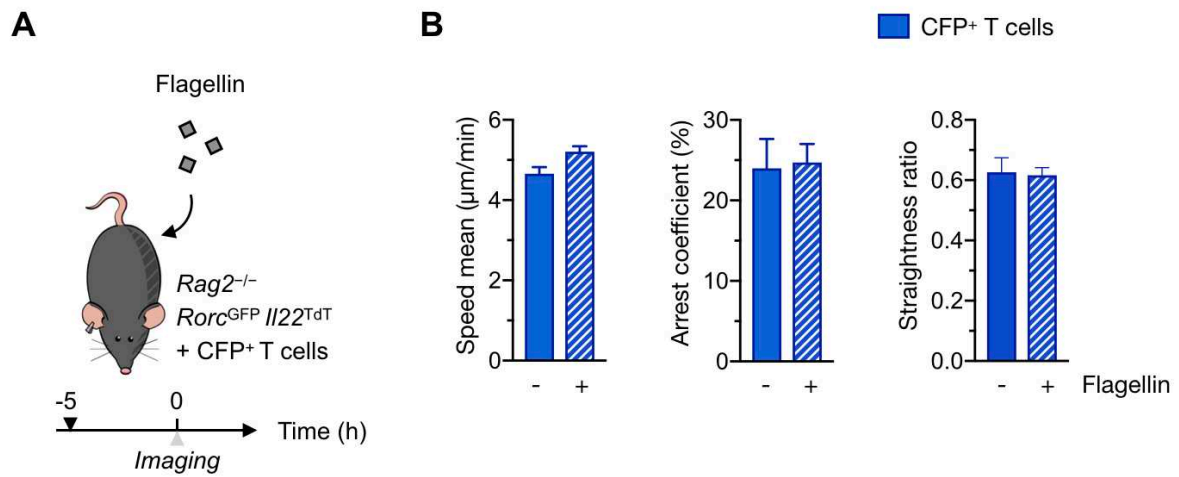


Figure S3 | Analysis of intestinal T cells migration in the absence or presence of flagellin

(A) T cells were isolated from spleen and lymph nodes of a CFP transgenic mice and were adoptively transferred into *Rag2^{-/-} Rorc^{GFP} I122^{TdT}* mice. Two weeks later, intestine of recipient mice (*RR22* + CFP⁺ T cells) was imaged by multi-photon microscopy. (B) Graphs show mean speed, arrest coefficient and straightness ratio of adoptively transferred CFP⁺ T cells in the intestine from PBS (full bar) or flagellin- (dashed bar) treated mice. Results are from at least one movie per condition from one of three independent experiments (n=27 and 80 CFP⁺ T cells for *RR22* + CFP⁺ T cells mice without or with flagellin, respectively). Each bar corresponds to the mean ± SEM of the values obtained (two-tailed Mann-Whitney test).

Figure S4

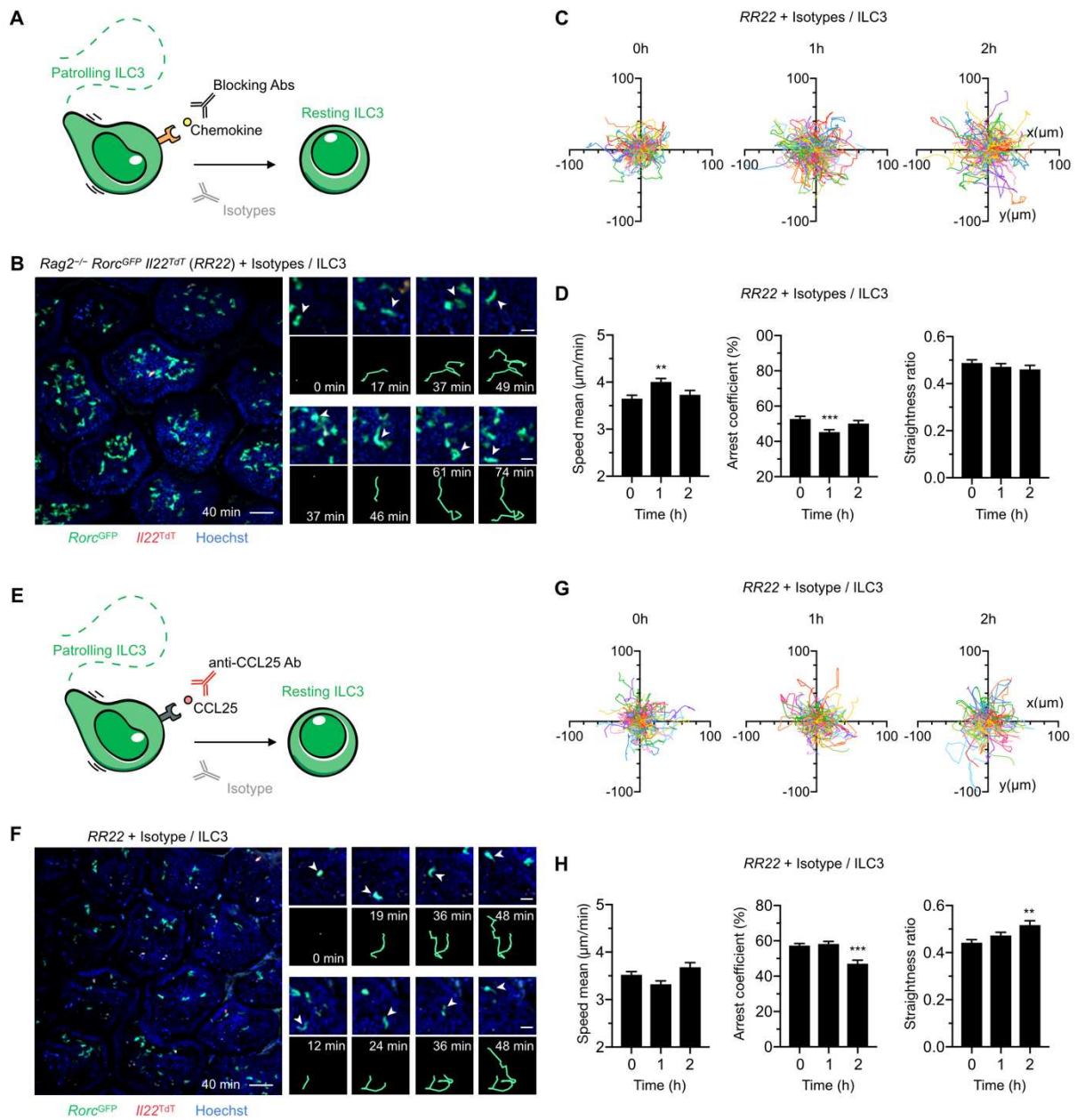


Figure S4 | Isotypes control have little effect on ILC3 migration

(A-H) *Rag2^{-/-} Rorc^{GFP} Il22^{TdT} (RR22)* mice were injected *i.v.* with combination of isotypes in (A-D): 50 μg mouse IgG1, 100 μg rat IgG2a and 50 μg rat IgG2b, or in (E-H) 50 μg rat IgG2b only. (B and F) Representative image (left; scale bar 50 μm) and time-lapse images (right; scale bar 15 μm) of ILC3 in intestinal villi after isotype controls injection at 0 min (combination in B or rat IgG2b alone in F). (C and G) Individual tracks of intestinal ILC3 before (-60-0 min; left), 1h (0-60 min; middle) and 2h (60-120min; right) after isotype controls injection (combination in C or rat IgG2b alone in G) of *RR22* mice. (D and H) Graphs show mean speed, arrest coefficient and straightness ratio of intestinal ILC3 at indicated time points after isotype controls (combination in D or rat IgG2b alone in H) injection. Results in (C-D) are from two to four movies per condition obtained in two independent experiments (n=266 ILC3 tracks for 0h; n=340 ILC3 tracks for 1h; n=259 tracks for 2h). Results in (G-H) are from three to four movies per condition obtained in two independent experiments (n=349 ILC3 tracks for 0h; n=304 ILC3 tracks for 1h; n=178 tracks for 2h). Each bar corresponds to the mean ± SEM of the values obtained (***P*<0.002; ****P*<0.0001; one-way ANOVA).

Figure S5

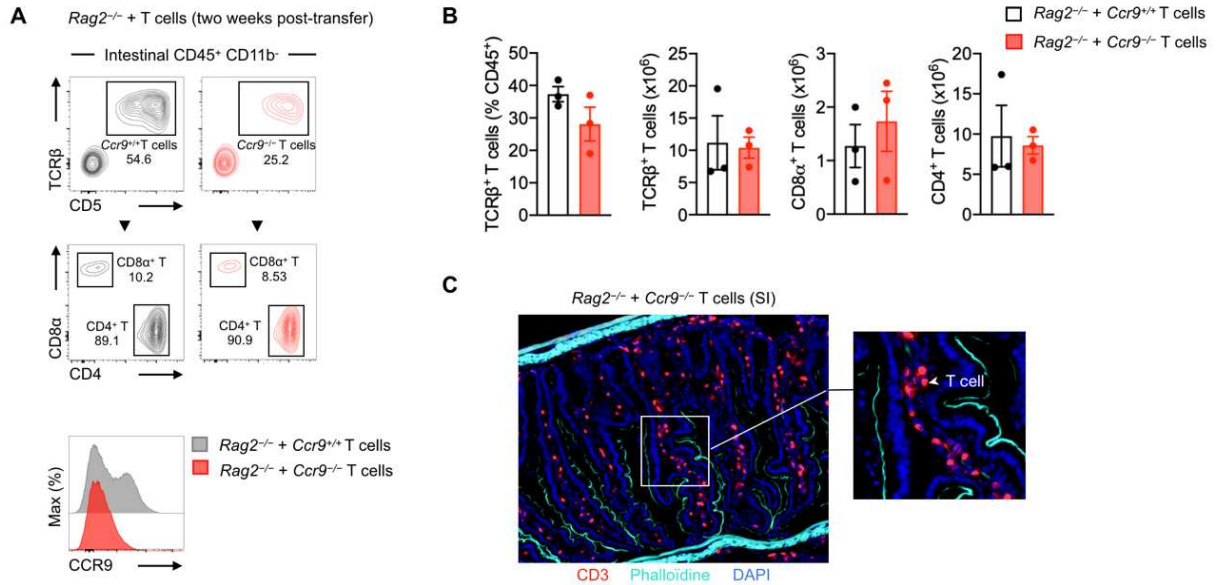


Figure S5 | *Ccr9*^{-/-} T cells home into the intestinal lamina propria after adoptive transfer

(A) Flow cytometry analysis of intestinal T cells in *Rag2*^{-/-} mice two weeks post-adoptive transfer of either control (*Ccr9*^{+/+}; gray) or *Ccr9*^{-/-} (red) T cells. (B) Graphs show absolute numbers of total TCR β ⁺ T cells, as well as CD8 α ⁺ and CD4⁺ T cells subsets, in the small intestine of *Rag2*^{-/-} mice two weeks post-adoptive transfer of either control (*Ccr9*^{+/+}; gray) or *Ccr9*^{-/-} (red) T cells. (C) Immunofluorescence of ileum from *Rag2*^{-/-} mice adoptively transferred with *Ccr9*^{-/-} T cells (scale bar 50 μ m). Results in (B) were obtained in three independent experiments (n=3 mice per group). Each bar corresponds to the mean \pm SEM of the values obtained (***P*<0.001; ***P*<0.002; two-tailed Mann-Whitney test).

CHAPTER III

TRAINED ILC3 RESPONSES PROMOTE INTESTINAL DEFENSE

Group 3 innate lymphoid cells (ILC3) are innate immune effectors that contribute to host defense, inflammation and tissue remodeling. Whether ILC3 functions are stably modified following pathogen encounter is unknown. Here we assess the impact of a time-restricted enterobacterial challenge to long-term ILC3 activation. We found that intestinal ILC3 persist for months in an activated state following exposure to *Citrobacter rodentium*. Upon re-challenge, these “trained” ILC3 proliferate, display enhanced interleukin (IL)-22 responses and have a superior cell-intrinsic capacity to control infection compared to naïve ILC3. Metabolic changes in trained ILC3 support IL-22 production via heightened cell fitness. Accordingly, a limited encounter with a pathogen can promote durable phenotypic and functional changes in intestinal ILC3 that contribute to long-term mucosal defense.

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MAIN TEXT

The intestinal tract is richly populated by a diverse set of specialized immune cells that promote barrier function and maintain microbial tolerance (1). In this context, effector T cells have a fundamental role in immune protection and generate memory T cells that support the long-term immune-surveillance and recall responses to pathogens encountered. Recently, additional novel cytokine-producing effector lymphocyte populations, denoted innate lymphoid cells (ILC), have been identified that promote immune defense and tissue homeostasis (2, 3). In particular, group 3 ILC (ILC3) are highly enriched in intestinal sites where they are essential orchestrators of lymphoid tissue development, barrier function and mucosal homeostasis (4, 5). ILC are largely devoid of pattern recognition receptors (6) and are indirectly activated during immune responses by soluble factors (cytokines, chemokines) derived from epithelial and hematopoietic sentinel cells (4). For example, barrier disruption that accompanies bacterial invasion initiates epithelial and myeloid-derived IL-1 β and IL-23 production that activates ILC3; these in turn secrete interleukin (IL)-17 and IL-22 that limits infection and restores tissue homeostasis (7–10). Still, how the functional competence of ILC3 is established during the lifespan of an individual is poorly understood. ILC3 are active during the fetal period to promote lymphoid tissue organogenesis (11, 12) and exposure to microbial flora in early post-natal life is associated with changes in ILC3 subset distribution and cytokine production (7, 13). Whether these different periods of ILC3 activation have any long-term impact on ILC3 effector functions is unclear. ILC may be considered as innate versions of T helper (T_H) cells due to their dependency on specific transcription factors for differentiation and their similar cytokine outputs (4, 14, 15). Still, whether ILC3 subsets exhibit any degree of adaptation to commensal or pathogen encounter that results in heightened immunological function (a hallmark of adaptive T cell responses) remains to be demonstrated.

To study whether persistent functional changes occur in intestinal ILC3 following microbial encounter, we utilized the mouse pathogen *Citrobacter rodentium* (*C. rodentium*) that provokes an enterocolitis in C57BL/6 mice sharing several disease phenotypes with human enteropathogenic *Escherichia coli* infection (16). In immunocompetent mice, *C. rodentium* attaches and replicates in the distal small intestine and colon, provoking both innate and adaptive immune responses, including dendritic cell (DC)-induced ILC3 activation as well as generation of antigen-specific T and B cells, respectively (7, 10). Moreover, *C. rodentium* provokes transient microbial dysbiosis via disruption of commensal communities (17). In order to characterize specific roles for intestinal ILC3 responses to *C. rodentium* that are independent of adaptive immune priming and infection-associated dysbiosis, we limited the duration of the pathogen encounter using a highly efficient antibiotic treatment (Abx, ciprofloxacin; (18)) administered after infection. As a proof of concept, we showed that ciprofloxacin treatment was able to rescue susceptible *Rag2^{-/-}Il2rg^{-/-}* mice after *C. rodentium* infection (**Fig. S1A-E**; (7)) demonstrating its efficacy in rapidly clearing this pathogen. We next applied this experimental protocol to immunocompetent mice reasoning that a time-restricted exposure to *C. rodentium* would create a context in which tissue-resident ILC3 would be normally activated but adaptive immune responses and intestinal microbial changes would be limited. This was the case as this short course of antibiotic treatment was sufficient to restrict the T_H22 response while ILC3-dependent IL-22 responses

were activated normally (**Fig. S1F-H**). The long-term impact of ciprofloxacin on commensal bacterial communities in wild-type mice before (**Fig. S2**) or after infection (**Fig. S3**) was negligible.

We characterized innate ILC3 and adaptive T_H22 immune responses in this modified *C. rodentium* infection model using *Rorc*^{GFP} and *IL22*^{TdT} reporter mice (**Fig. 1A, 1B and S4**). The responses following initial limited *C. rodentium* infection (infection followed by ciprofloxacin treatment) and re-infection (30 days later) were compared. While overall T cell and ILC3 populations were relatively stable during the initial infection, reinfection of antibiotic treated mice resulted in a rapid increase in the absolute numbers of ILC3s in the intestinal lamina propria with little effect on T cells (**Fig. 1B**). Analysis of ILC3 subsets (defined as NKp46⁺ and CCR6⁺ ILC3) showed that numbers of both ILC3 subsets increased after re-infection (**Fig. 1B and 1C**) with enhanced Ki67 expression (**Fig. 1D**). We found that absolute numbers and frequencies of IL-22-expressing ILC3 (using *IL22*^{TdT} reporter and intracellular protein detection; **Fig. 1E and S5**) were clearly increased during initial infection but showed an enhanced response during re-infection, while IL-22-expressing T cells were largely unchanged (**Fig. 1E, 1F and S5**). We further performed *C. rodentium* challenge/re-challenge experiments in mice not treated with ciprofloxacin (**Fig. S6A**). We detected elevated IL-22⁺ ILC3 responses in this *C. rodentium* re-challenge model indicating that enhanced ILC3 responses can develop independently of the antibiotic treatment (**Fig. S6B**). Moreover, CD4⁺ T cell numbers and frequencies of IL-17A-, IL-22- and IFN- γ -producing T cells were not significantly different in our re-challenge model compared to the classical reinfection model, suggesting that T cell responses are largely unaffected by the antibiotic treatment (**Fig. S6B-D**). Together, these results suggest that homeostasis and function of intestinal IL-22-producing ILC3 can be modified following a subclinical *C. rodentium* infection.

As IL-22 production by T cells and ILC3 activates epithelial responses during pathogen infection at mucosal barriers (19) and is essential for resistance to *C. rodentium* (20), we assessed the impact of an initial limited infection on bacterial growth during re-infection. We followed infection by live imaging using a bioluminescent *C. rodentium* strain 3 days after primo infection (denoted “CR”) or 3 days after reinfection of antibiotic-treated *C. rodentium* mice (denoted CR-Antibiotic-CR or “CRACR”) (**Fig. 1H**). While *C. rodentium* infection was clearly established in CR mice, we failed to image bacteria in CRACR mice consistent with resistance to infection in this context (**Fig. 1H**). We confirmed that bacterial growth in CRACR mice was strongly suppressed (<10⁴ CFU/g feces; **Fig. 1I**), suggesting that enhanced ILC3 function might play a role in the observed mucosal defense against bacterial re-challenge. Whereas a peak in DC activation was noted during re-challenge, other innate immune effector cells (myeloid cells, macrophages) were largely unchanged consistent with the absence of pathogenic bacteria in the tissue of CRACR mice (**Fig. S7**). Taken together, these results suggest that a limited initial exposure of intestinal ILC3s to pathogenic bacteria can generate a highly functional and persistent ILC3 subset – that we will refer to as “trained-ILC3” (ILC3^{TR}) – that responds more potently to stimulation compared with “naïve” ILC3.

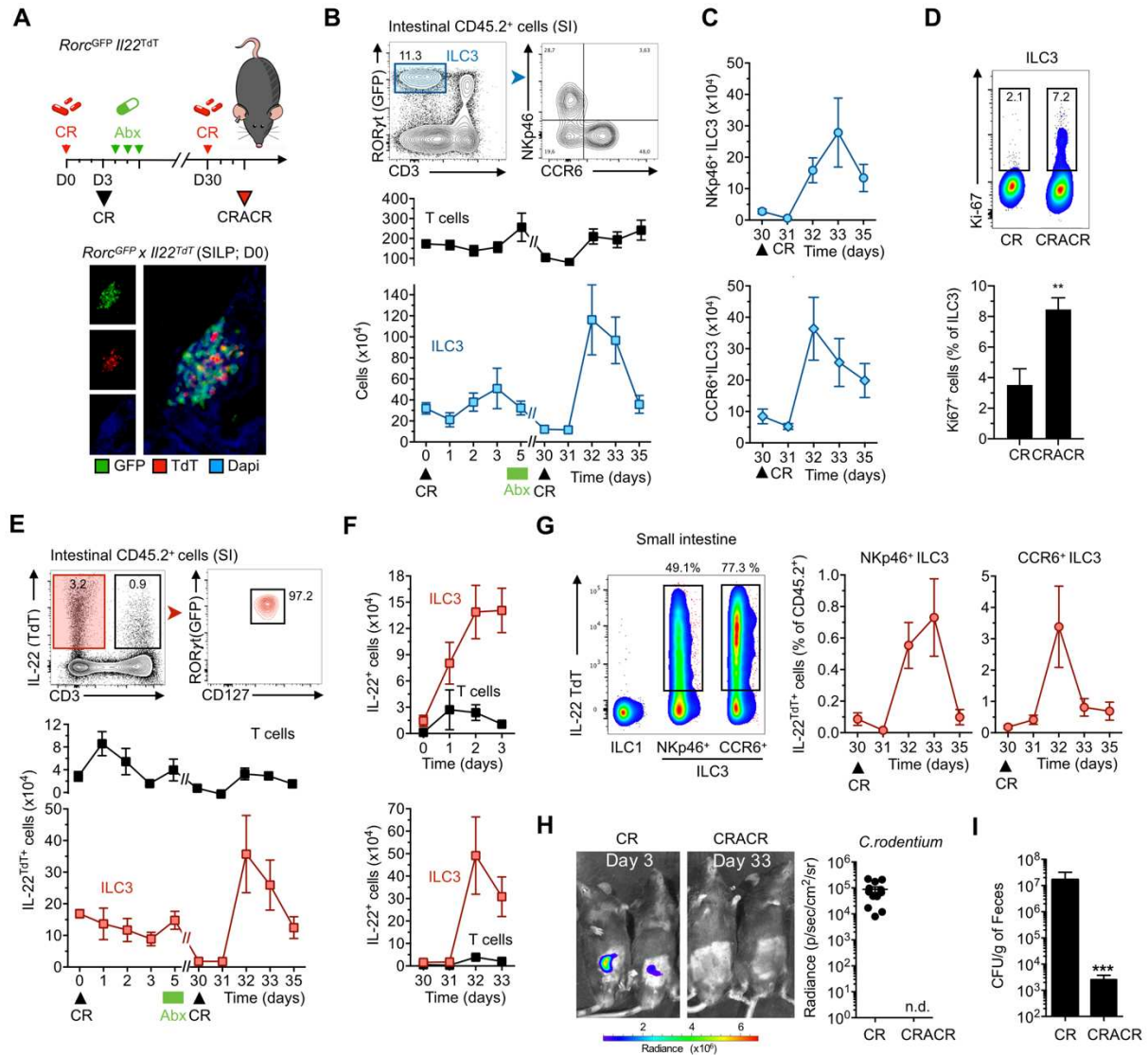


Figure 1 | Trained ILC3 efficiently control infection after re-challenge with pathogenic bacteria

(A-I) *C. rodentium* (CR) infected *Rorc^{GFP} Il22^{TdT}* reporter mice received antibiotic treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) 4 days after infection. One month later, the mice were re-infected with CR (CRACR). (A) Immunofluorescence staining of small intestine of RORγt⁺ and IL-22⁺ cells in *Rorc^{GFP} il22^{TdT}* reporter mice. (B) Intestinal NKp46⁺ and CCR6⁺ ILC3 (RORγt^{GFP} CD3⁻ CD45⁺ cells) were analyzed by flow cytometry. Absolute numbers of ILC3 and T cells in the small intestine lamina propria at indicated timepoints after bacterial infection, antibiotic (Abx)-treatment and re-infection (n=4-12 for each time point). (C) Absolute numbers of intestinal ILC3 subsets (NKp46⁺ and CCR6⁺ ILC3) were determined by flow cytometry. Representative data of 3 independent analysis (n=3-7 for each time point). (D) Frequency of Ki67⁺ ILC3 was analyzed by flow cytometry (n=6). (E) Representative flow cytometry plots of small intestinal IL-22^{TdT} ILC3 (*Rorc^{GFP} CD3⁻ CD45⁺* cells) and T cells (CD3⁺ CD45⁺ cells) frequencies at day 0, pre-gated on CD45.2⁺ cells. Absolute numbers of IL-22^{TdT} cells were analyzed by flow cytometry. Representative data of 6 independent analysis (n=4-12 for each time point). (F) Absolute numbers of IL-22⁺ (protein) cells were analyzed after *ex-vivo* stimulation with IL-23 and IL-1β (50 ng/ml each; n=4-9 for each time point) in CR and CRACR conditions. (G) Flow cytometry analysis of *Il22^{TdT}* expression in ILC1 (NKp46⁺ NK1.1⁺ CD3⁻ cells), NKp46⁺ ILC3 and CCR6⁺ ILC3. Clustering of smooth density plot is shown (SSC^A vs IL-22^{TdT}). Representative data of 3 independent analysis. Frequency of IL-22^{TdT} NKp46⁺ and NKp46⁻ ILC3 subsets in small intestine lamina propria after re-infection (CRACR; n=3-7 for each time point). (H-I) After infection, bacterial growth was monitored by IVIS imaging (H) and fecal bacterial culture (I). Representative pseudo color images are shown (H; scale bar, photon/s/cm²/sr) and average radiance analysis was performed. Data are representative of 3 independent experiments containing 12 mice per group. Mean ± SEM; *** p<0.001, Mann-Whitney test.

We next assessed the durability of ILC3^{TR} responses on *C. rodentium* re-infection 4 months after limited primo infection (**Fig. 2A**). We found that day 3 fecal bacterial loads were significantly reduced ($<10^4$ CFU/g) in re-infected mice compared with control mice following primary infection (**Fig. 2B**). Moreover, absolute numbers of total ILC3 (**Fig. 2C**) and IL-22⁺ ILC3 (**Fig. 2D and 2E**) were significantly increased in CRACR mice. Concerning ILC3 subset distribution at 4 months following initial limited *C. rodentium* infection, we found that CCR6⁺ ILC3 and “DN” ILC3 (9) persisted in contrast with NKp46⁺ ILC3 (**Fig. 2F**). Accordingly, CCR6⁺ and “DN” ILC3 were dominant IL-22 producers in this context (**Fig. 2G**). Variability within the NKp46⁺ ILC3 subset may be explained by the phenotypic instability of NKp46 expression that can be modulated by environmental signals (21, 22). Supporting this idea, we observed predominant CD49a⁺ expression (a marker of NKp46⁺ ILC3; (21)) on “DN” ILC3 in the absence of CD304 expression (Nrp1; marker of CCR6⁺ ILC3; **Fig. S8**).

To address whether ILC3^{TR} persist following a limited bacterial exposure, we utilized a fate-mapping strategy to track intestinal ILC3. As ID2 is an essential transcription regulator required for ILC development and remains highly expressed in differentiated ILCs (23, 24), we generated *Id2^{CreER2T/+} Rosa26^{RFP}* to allow tamoxifen-induced temporal expression of red fluorescent protein (RFP) in ID2-expressing cells. To follow ILC3 fate in CR mice, we administrated tamoxifen before *C. rodentium* infection and analyzed RFP⁺ cells 3 days later. In CRACR mice, we administrated tamoxifen after limited *C. rodentium* infection and we analyzed RFP⁺ cells 3 days after re-infection (**Fig. 2H**). All ILC were labeled in naïve mice, with a higher percentage of RFP⁺ ILC3 after *C. rodentium* infection (**Fig. 2I and 2J**). In contrast, most RFP⁺ ILC1 and ILC2 were lost in CRACR mice, whereas labeled RFP⁺ ILC3 subsets were maintained (**Fig. 2I and 2J**). Taken together, these results indicate that diverse ILC3^{TR} subsets can persist and provide long term protection after a limited exposure to pathogenic bacteria.

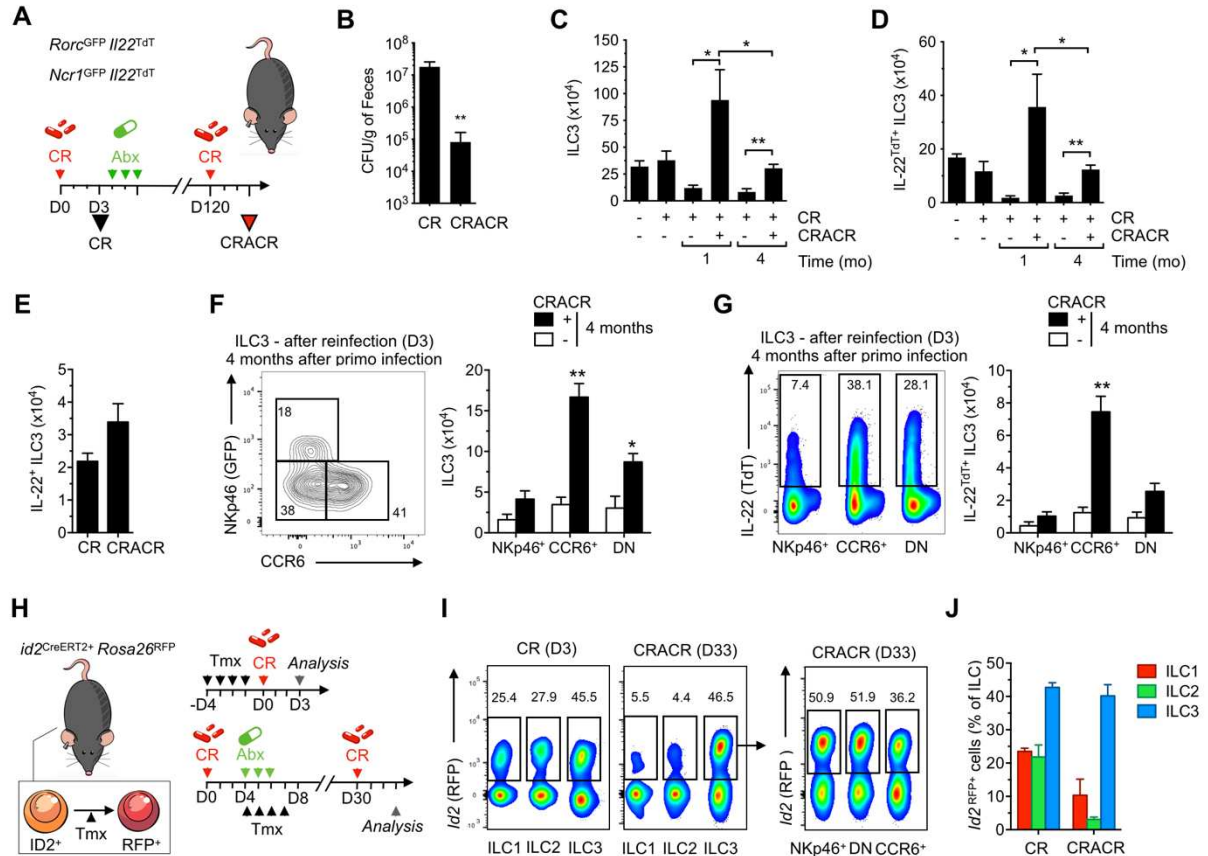


Figure 2 | Long-term persistence of trained ILC3

(A) Infected *Rorc*^{GFP} *x* *IL22*^{TdT} and *Ncr1*^{GFP} *x* *IL22*^{TdT} reporter mice received antibiotic (Abx)-treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) 3 days after *C. rodentium* infection (CR). Four months later, the mice were re-infected with CR (CRACR). (B) Bacterial growth (CR, n=8 and CRACR, n=12) was monitored by fecal bacterial culture. (C-D) Absolute numbers of total (C) and IL-22^{TdT}⁺ (D) ILC3 in the small intestine lamina propria 1 and 4 months after primo infection (n=9), Abx-treatment and 3 days after re-infection (CRACR; n=5). (E) Absolute numbers of intestinal IL-22⁺ ILC3 (protein; n=5) were analyzed after *ex-vivo* stimulation with IL-23 and IL-1 β (50ng/ml) 3 days after infection or re-infection. (F-G) ILC3 (F) and IL-22^{TdT}⁺ ILC3 (G) subsets in CRACR mice were analyzed by flow cytometry and the absolute numbers were calculated based on the FACS analysis. Clustering of smooth density plot is shown (SSC^A vs IL-22^{TdT}). (H) *Id2*^{CreERT2} *T*⁺ *x* *Rosa*^{RFP} mice received antibiotic treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) 3 days after CR infection. Before or after CR infection, mice received Tamoxifen (Tmx) by *i.p.* injection. In indicated case, infected mice were re-infected with CR 1 month later. (I-J) Flow cytometry analysis of *Id2*^{RFP}⁺ cells in ILC1 (NKp46⁺ NK1.1⁺ CD3⁻ cells), ILC2 (KLRG1⁺ CD127⁺ NK1.1⁻ CD3⁻ cells) and ILC3 subsets. Clustering of smooth density plot is shown (SSC^A vs *Id2*^{TdT}). Percentage of *Id2*^{RFP}⁺ cells in ILC in CR and CRACR mice were analyzed by flow cytometry (n=3). Mean \pm SEM; (C-D) *p<0.05, **p<0.01, Kruskal-Wallis test. (F-G) *p<0.05, **p<0.01, Mann-Whitney test.

To further determine if IL-22 produced by ILC3^{TR} is required for immune defense in absence of T cells, we studied CRACR *Rag2*^{-/-} mice (**Fig. 3A**). Absolute numbers of total ILC3 and IL-22⁺ ILC3 were significantly increased in CRACR *Rag2*^{-/-} mice (**Fig. 3B**) and these mice harbored significantly reduced bacterial loads compared to the CR infected *Rag2*^{-/-} mice (**Fig. 3C**). To experimentally address whether IL-22 production from 'trained' ILC3 can provide protective immunity to *C. rodentium* reinfection in absence of T cells, we reinfected *Rag2*^{-/-} and *Rag2*^{-/-}*Il22*^{-/-} mice (**Fig. 3A**). As expected, *Rag2*^{-/-} and *Rag2*^{-/-}*Il22*^{-/-} mice resisted a limited *C. rodentium* infection after antibiotic treatment. In contrast, *Rag2*^{-/-}*Il22*^{-/-} mice were highly susceptible and succumbed during re-infection (**Fig. 3D and 3E**). We next investigated whether ILC3^{TR} are sufficient to provide protection against *C. rodentium* in a naïve lymphocyte replete host. We adoptively transferred ILC3 isolated from CR or CRACR *Il22*^{+/+} mice to *Il22*^{-/-} mice that had been previously infected with *C. rodentium* (**Fig. 3F**). All CR infected *Il22*^{-/-} mice as well as *Il22*^{-/-} mice receiving naïve ILC3 exhibited body weight loss and succumbed about 2 weeks after infection (**Fig. 3G**). In contrast, *Il22*^{-/-} mice receiving ILC3^{TR} recovered from infection and showed significantly enhanced survival (**Fig. 3G**). These results demonstrate that the protective capacity of trained ILC3 operates in a cell-intrinsic and T-cell-independent fashion during pathogen re-challenge.

Whereas antigen specificity has long been considered as a feature of adaptive T and B cell memory (25), innate cells such myeloid cells (26, 27), NK cells (28–30) or ILC2 (31) have been shown to have 'memory-like' properties. To investigate whether ILC3^{TR} exhibit pathogen-specific properties, we analyzed whether *C. rodentium* induced ILC3^{TR} could protect against an unrelated enteropathogen (*Listeria monocytogenes*; LM). We have previously reported that LM infection elicits IL-23 release that can trigger ILC3 activation and IL-22 production, which is involved in the intestinal epithelial response (32). We first infected mice with *C. rodentium* (CR), then following ciprofloxacin treatment re-challenged the mice with LM (**Fig. S9A**). We compared the responses to mice that had not received the initial CR exposure. As shown below, we found elevated ILC3 responses are also observed following re-challenge with *L. monocytogenes* (CRALM compared to LM; **Fig. S9B**). Together, these results indicate that ILC3^{TR} responses are not restricted to CR, and suggest that intestinal ILC3 'training' can potentially extend protection to unrelated pathogens. As IL-23 and IL-1 β strongly activate intestinal ILC3, we assessed whether ILC3^{TR} can be generated by these cytokines in the absence of infection. While repeated injection IL-23 and IL-1 β *in vivo* (**Fig. S9C**) triggered intestinal ILC3 for higher IL-22 production (**Fig. S9D**), we failed to detect increased ILC3 numbers or enhanced Ki67 expression in this non-infectious context (**Fig. S9E**). Together, our results demonstrate that trained ILC3 lack antigen-specificity and require tissue-dependent signals beyond IL-1 β and IL-23 for their generation.

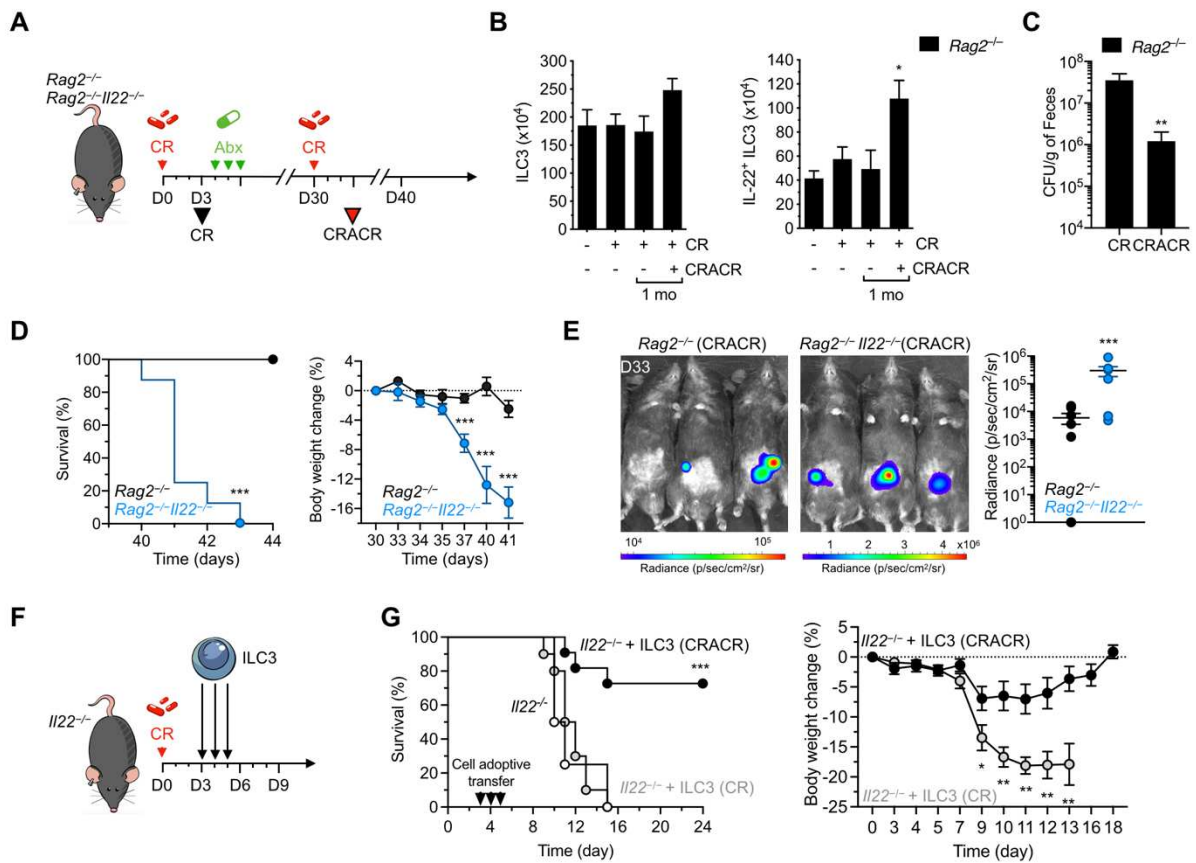


Figure 3 | IL-22 mediated protection is an intrinsic feature of trained ILC3

(A-C) *C. rodentium* (CR) infected *Rag2*^{-/-} and *Rag2*^{-/-}*IL22*^{-/-} mice received antibiotic treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) 4 days after infection. One month later, the mice were re-infected with CR (CRACR). (B) Flow cytometry analysis of ILC3 and IL-22⁺ ILC3 in *Rag2*^{-/-} mice (n=3). (C) Bacterial growth was monitored by fecal bacterial culture (n=5). Mean ± SEM; *p<0.05, **p<0.01, Mann-Whitney test. (D) Survival and body weight were assessed in re-infected *Rag2*^{-/-} and *Rag2*^{-/-}*IL22*^{-/-} mice (n=7). (E) *In vivo* CR growth dynamics were assessed 3 days after re-infection. Pseudo color representative images were obtained using IVIS imaging (scale bar, photon/s/cm²/sr). Relative CR growth was determined in *Rag2*^{-/-} and *Rag2*^{-/-}*IL22*^{-/-} mice (n = 7 for each time point). Mean ± SEM; *** p<0.001, Mann-Whitney test. (F) ILC3 were purified from infected (CR) or re-infected (CRACR) mice 3 days after infection and transferred into CR-infected *IL22*^{-/-} mice. (G) Survival and body weight were assessed at the indicated times after infection (n=4 for *IL22*^{-/-}, n=10 for *IL22*^{-/-} + ILC3 (CR), n=11 for *IL22*^{-/-} + ILC3 (CRACR), pool of two independent experiments).

To understand mechanisms governing ILC3^{TR} differentiation, we performed RNA-seq analysis on intestinal CD49a⁺ and CCR6⁺ ILC3 sorted from naïve (control, C), *C. rodentium* infected (CR), *C. rodentium* infected and antibiotic-treated (CRA) and CRA *C. rodentium* re-infected mice (CRACR; **Fig. 4A and 4B**). We validated previously reported differences between intestinal CD49a⁺ (that include NKp46⁺ ILC3) and CCR6⁺ ILC3 (that include LTi-like CD4⁺ ILC3) (7, 9, 21). Upon *C. rodentium* infection, we found that > 1000 genes in CCR6⁺ ILC3 and > 500 genes in CD49a⁺ ILC3 were modified with a > 2-fold-change (**Fig. 4B**) demonstrating that ILC3 are transcriptionally responsive during infection by pathogenic bacteria (**Fig. S10A**). In contrast, when we compared gene expression profiles between CR and CRACR conditions, only 152 genes in CCR6⁺ ILC3 and 138 genes in CD49a⁺ ILC3 showed a ≥ 2-fold-change, suggesting that ILC3 maintain their transcriptomic profiles after *C. rodentium* infection (**Fig. S10A**). Moreover, in all conditions, the differences observed between CCR6⁺ and CD49a⁺ ILC3 were maintained (**Fig. S10B**). Pathway analysis revealed modification of cell cycle and effector functionalities in ILC3 subsets in CRACR, CRA and CR mice compared to control naïve mice (**Figure 4C**). Activated and memory NKp46⁺ ILC3s highly expressed genes involved in the lymphoid function, including *Il22*, *Gzmb*, *Gzmc* (**Fig. S10C**). In contrast, few lymphoid lineage genes were modified in CCR6⁺ ILC3, although *Il22* and *Il17f* expression was increased, while expression of multiple transcription factors, including *Zbtb16* (encoding PLZF), *Ikzf2* (encoding Helios), *Ikzf3* (encoding Aiolos) and *Zeb1* (encoding Tcf-8) decreased (**Fig. 10C**).

Recent studies have demonstrated that rewiring of cellular metabolism induced by external signals is essential for the generation of both classical and innate immunological memory (33, 34). Multiple metabolic pathways (including nutrient absorption, glycolysis, TCA and urea cycle, lipid oxidation, OXPHOS and ATP generation) converge to govern cell survival, proliferation, differentiation and long-term persistence of these cells (37, 38). Our analysis of metabolic pathways showed a metabolic shift from glycolysis and glutaminolysis to enhanced tricarboxylic acid cycle, oxidative phosphorylation (OXPHOS), fatty acid synthesis and oxidation-associated gene expression in CCR6⁺ and CD49a⁺ ILC3 after *C. rodentium* infection. This modification is preserved under CRA and CRACR conditions (**Fig. 4D and S10C**).

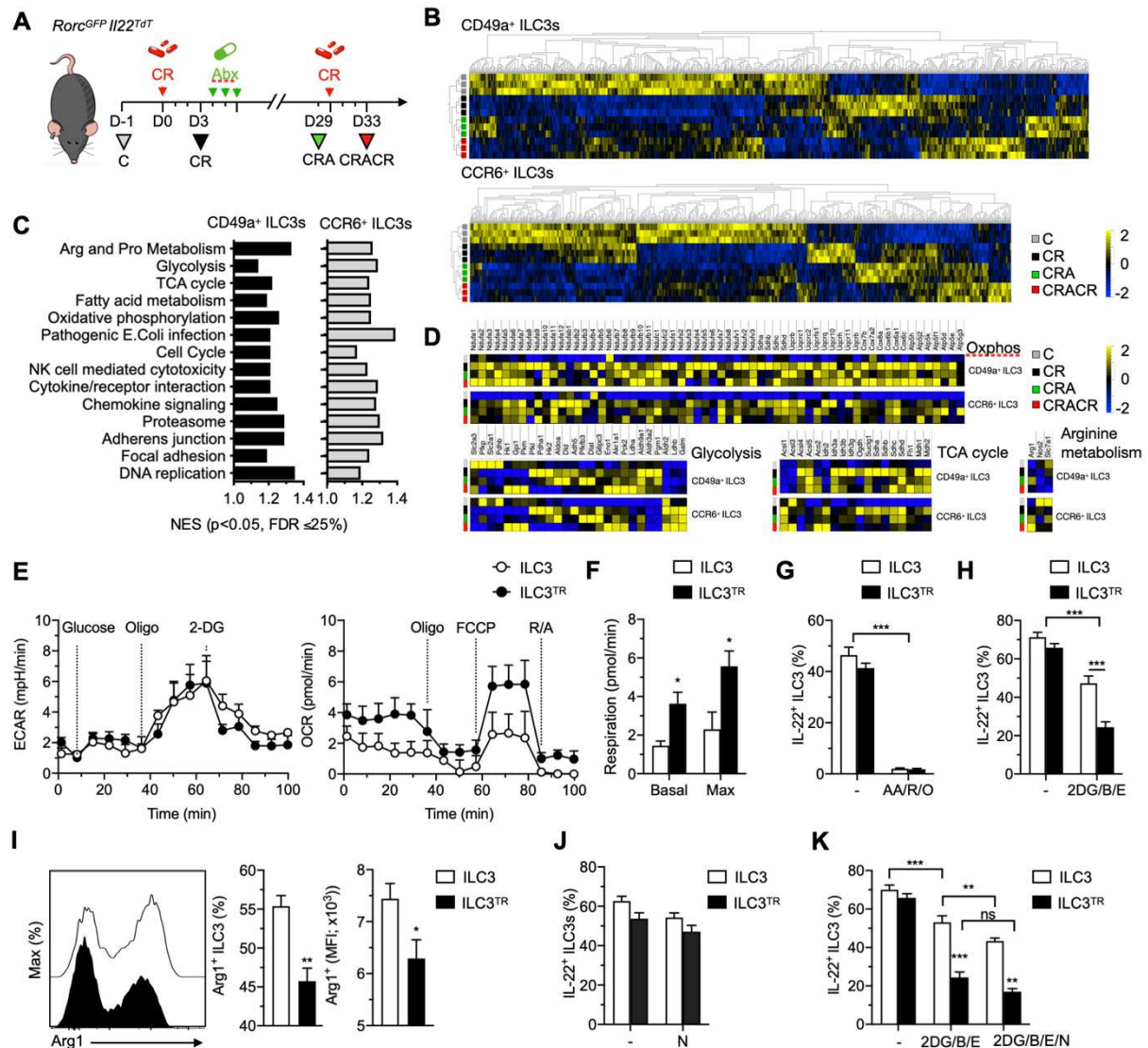


Figure 4 | Gene expression and metabolic profiles reveal a specific signature of trained ILC3

(A) Mice were orally infected with *Citrobacter rodentium* (CR) and treated with antibiotic (CRA). 1 month later treated and infected mice were re-infected (CRACR). CCR6⁺ and CD49a⁺ ILC3 from naïve (C) and infected mice (CR, CRA and CRACR) were sorted for gene expression analysis by RNA-seq. (B-C) Heatmap showing the relative expression levels of differentially expressed genes in intestinal ILC3 subsets. (B). Pathway analysis was performed and gene pathways were organized into clusters to compare CR, CRA, CRACR vs C conditions (n=3; C). (D) Heatmap with clustering of differentially expressed metabolism associated genes between the indicated populations and conditions. (E-F) OCR and ECAR metabolic profiles of freshly sorted ILC3 (ILC3) and trained ILC3 (ILC3^{TR}) were determined by Seahorse in response to the mitochondrial uncoupler (FCCP), Oligomycin (oligo), Rotenone/antimycin (R/A) and 2-deoxyglucose (2-DG). Basal and maximal respiration were determined (F). Data are representative of 3 independent experiments. (G) ILC3 were cultured with IL-7, Flt3-L, SCF (20ng/ml), IL-23 and IL-1β (25ng/ml) in DMSO or 5μM Antimycin, 5μM Rotenone and 2nM Oligomycin (AA/R/O) for 18h and IL-22 production was analyzed by flow cytometry. (H) ILC3 were cultured with 2-DG, BPTES (B) and Etomoxir (E). 2h later cells were stimulated for 2h with IL-23 and IL-1β and IL-22 expression was analyzed by flow cytometry. (I) Flow cytometry analysis of Arg-1 expression in naïve ILC3 and ILC3^{TR}. (J) ILC3 were stimulated with IL-23 and IL-1β (50 ng/ml) in the presence of the Arg1 inhibitor, Nor-NOHA (N), in culture for 4h. DMSO was used as a control. (K) ILC3 were cultured with 2-DG, B, E and N. 2h later cells are stimulated for 2h with IL-23 and IL-1β and IL-22 protein was analyzed by flow cytometry. Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, Mann-Whitney test.

To further characterize metabolic changes associated with ILC3^{TR}, we studied bioenergetic profiles of naïve and trained ILC3. We measured the extracellular acidification rate (ECAR) and cellular oxygen-consumption rate (OCR) that define mitochondrial respiration and aerobic glycolytic activity, respectively. ILC3^{TR} showed high OCR indicating elevated mitochondrial fitness (**Fig. 4E and 4F**). As ILC3^{TR} show enhanced IL-22 production (**Fig. 1F and Fig. 4C**), we investigated whether changes in OXPHOS activity in ILC3 could influence their IL-22 production. Using mitochondrial electron transport inhibitors (Antimycin A, Rotenone and Oligomycin), we found that IL-22 production from ILC3 and ILC3^{TR} required OXPHOS (**Fig. 4G**). Diverse nutrients fuel immune cell metabolism, with glucose, glutamine and fatty acids representing the main nutrient support mitochondrial bioenergetic pathways (35). To assess nutrient requirements for IL-22 production, ILC3 were incubated with inhibitors targeting glycolysis (2-DG), glutamine conversion (BPTES) and fatty acid β -oxidation (etomoxir). Treatment with these inhibitors had a more modest effect on naïve ILC3s but significantly decreased IL-22 production from ILC3^{TR} (**Fig. 4H**). ILC3 subsets showed strong expression of urea cycle-associated genes, including *Arg1* (**Fig. 4C**) previously shown to drive ILC2 and T cell proliferation as well as pro-inflammatory functions (36, 37). Arginase-1 protein was clearly expressed by naïve ILC3 with somewhat reduced levels in ILC3^{TR} (**Fig. 4I**). We next assessed whether modifications in arginine metabolism could affect IL-22 production by ILC3s. An Arg-1 inhibitor (N-Hydroxy-nor-L-arginine; Nor-NOHA) did not affect IL-22 production from *ex-vivo* naïve or 'trained' ILC3 stimulated with IL-23 and IL-1 β (**Fig. 4J**). However, Arg1 inhibition synergized with 2-DG, BPTES and etomoxir to significantly reduce IL-22 production from naïve ILC3 (**Fig. 4K**) but not from ILC3^{TR}. These results suggest that L-arginine acts an additional metabolic source in naïve ILC3 further distinguishing their metabolic needs from trained ILC3.

Infection or tissue inflammation is classically associated with the development of adaptive immunological memory, conferring resistance upon secondary encounter. Recently, several studies showed that components of the innate immune system can adapt to environment inflammatory signals generating cells with novel long-lived phenotypes (38, 39). Here, we describe memory-like attributes for intestinal 'trained' ILC3 that emerge and persist following initial pathogen encounter. Upon reinfection, ILC3s proliferate and robustly produce IL-22, thus promoting mucosal defense. Although not studied here, 'trained' ILC3 may be generated at other tissue sites during the course of their activation. As observed for memory T cells (40, 41), altered metabolic activity is closely associated to ILC3 fate. As such, trained ILC3 may provide a novel avenue for immune manipulation for prevention or treatment of disease caused by pathogens that invade barrier surfaces or inflammatory processes that affect other organs.

MATERIALS AND METHODS

Mice

Gender- and age-matched mice between 6–12 weeks old were used. *Rorc*^{gfp/+} (42), *Il22*^{Tdrt/+} (43), *Id2*^{CreERT2} (44), *Il22*^{-/-} (45) have been described. *Rag2*^{-/-}*IL2rg*^{-/-} mice were maintained at CDTA (TAAM laboratory, Orléans, France), while all other strains were housed at the Institut Pasteur. *Rorc*^{gfp/+} and *Rosa*^{RFP} mice were provided by Gérard Eberl. All mice were on the C57BL/6 background and were kept under specific pathogen-free conditions, provided with food and water ad libitum. To generate *Id2*^{RFP} mice, *Id2*^{CreERT2} mice were crossed with *Rosa*^{RFP} mice. Where noted, tamoxifen (10 mg/ml in 10 % ethanol in Corn oil; 100 mg/kg) was administered by daily intraperitoneal injection during 4 consecutive days. Mice were transferred and maintained in the A3 isolators prior to infection. For antibiotic treatment, mice were treated 3 days with Ciprofloxacin (Sigma Aldrich; 200 µl in H₂O; 100 mg/kg/day) 4 days after *C. rodentium* infection.

Experimental animal protocols were performed in accordance with the guidelines of the Animal Care Use Committee at the Institut Pasteur and were approved by the French Research Ministry (project #2013-0033).

C. rodentium infection and imaging

The bioluminescent *Citrobacter rodentium* strain IC180 was used in this study (46). Log phase cultures of bacteria were grown from overnight culture, washed in PBS and mice were orally infected with 10⁹ CFU as previously described (16). Animals were monitored for survival, suffering and were weighted. Feces were collected from live mice and homogenized in sterile PBS, following infection and/or antibiotic treatment. The bacterial load was determined by plating serial dilutions of the homogenates.

For *in vivo* tracking of *C. rodentium*, mice were anesthetized and the abdominal region was shaved. Mice were placed in a confinement box with oxygen (TEM SEGA) and imaged using an IVIS 100 system (Caliper Life Sciences). Image acquisition and analysis were performed with the Living Image 3.2 software (Caliper Life Sciences) as described (9).

L. monocytogenes infection

LM were grown from overnight culture. Bacteria were diluted in brain-heart infusion medium to reach mid-log growth phase as previously described (32). Bacteria in PBS were washed in PBS and mice were orally infected with 10⁹ CFU as previously described (16). Animals were monitored for survival, suffering and were weighted.

Isolation of cells from intestinal tissue and *ex vivo* stimulation

Small intestines were collected from euthanized mice. Peyer's patches were removed and intestinal epithelial cells were eliminated by shaking incubation in 0.5 mM EDTA and 1mM HEPES (Sigma Aldrich) for 20 minutes at 37°C. Subsequently, the intestinal tissue was minced and incubated 2 times in a digestion solution containing 0.5 mg/mL collagenase VII (Sigma-aldrich) for 20 min at 37°C in a shaking

incubator to isolate the lamina propria lymphocytes (LPL). LPLs were further purified using a 40% Percoll, filtered through a 70 μm nylon mesh and kept in 10% FCS RPMI. For cytokine *ex-vivo* stimulation, $1-5 \times 10^6$ cells were incubated at 37°C with IL-1 β (Peprotech), IL-23 (R&D Systems) at several concentrations (see details in figure legends) and BD GolgiPlug (BD) in 10% FCS DMEM for 3h (37°C; 5% CO₂). To assess metabolic pathways, inhibitors were added during the stimulation. 2.5 mM of [³H]2-deoxy-d-glucose (2-DG), 10 mM of bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES), 10mM of etomoxir (E), 5 μM of rotenone (R), 5 μM of antimycin A (AA), 2 nM of oligomycin A (O; all from Sigma) and 0.5 mM of N^ω-hydroxy-L-arginine (Nor-NOHA; N; Cayman chemical).

Flow Cytometry

For FACS analysis, single-cell suspensions were stained with Flexible Viability Dye eFluor 506 (eBioscience) and blocked with FcR Blocking Reagent (Miltenyi) for 15 minutes, followed by 30 minutes of surface antigens staining with a combination of fluorescently conjugated monoclonal antibodies (from BD Biosciences, eBioscience and Biolegend) on ice. For experiments involving intracellular and intranuclear transcription factor staining, cells were fixed, permeabilized, and stained using BD Transcription Factor Staining kit and Foxp3/Transcription Factor Staining Buffer Kit respectively, according to the manufacturer's instructions (BD Biosciences and eBioscience). All the samples were acquired on a custom-configured LSR Fortessa (BD Biosciences) and the data were analyzed on FlowJo10 software version 10 (TreeStar).

Immunofluorescence staining and confocal imaging

The small intestine was excised, washed and fixed in paraformaldehyde (Sigma-Aldrich) 4% (wt/vol) overnight followed by dehydration in 30% sucrose before embedding in Tissue-Tek (Sakura Finetek). Samples were frozen in a bath of isopentane cooled with liquid nitrogen and were stocked at -80°C. 8- μm sections were cut and adhered to Superfrost Plus slides (VWR). Frozen sections were first hydrated for 5 min in PBS containing 0.1% (vol/vol) Triton X-100 and 1% (vol/vol) normal goat serum and blocked for 1 h at 20 °C with 10% (vol/vol) bovine serum in PBS 0.3% Triton X-100. Slides were then incubated overnight at 4 °C with primary polyclonal antibody in, were washed three times, were incubated for 1 h at 20 °C with secondary conjugated antibody, then were washed once, incubated for 5 min at 20 °C with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich), washed three times and mounted with Fluoromount-G (SouthernBiotech). The following antibodies were used for staining: anti-GFP (Abcam), anti-RFP (Rockland) and anti-CD3 (clone 500A2; BD). Slides were examined with an AxioImager Apotome microscope (Carl Zeiss).

Fecal microbiota analysis

Feces were collected from mice and 16S rRNA sequencing was performed. DNA was first extracted from the feces using the NucleoSpin Soil kit (Machery-Nagel). Since V3 region of the 16S rRNA were found to be most suitable for distinguishing bacterial species (47), the 16S rDNA was amplified with V3F (CCTACGGRAGGCAGCAG) and V4R (GGACTACHVGGGTWTCTAAT) barcoded primers. PCR products were cleaned, quantified and pooled, followed by sequencing using a Illumina v3 300-bp paired end reads MiSeqKit at Biomix NGS platform of the Institut Pasteur.

After removing reads containing incorrect primer or barcode sequences and sequences with more than one ambiguous base, we recovered from 30 samples a total of 4.466.810 reads (83,6% mapped, 102.129 mapped reads on average). The bioinformatics analysis was performed as previously described (48). Briefly, amplicons were clustered into Operational Taxonomic Units (OTU) with VSEARCH (v1.4) and aligned against the SILVA reference database. The clustering was performed at 98% sequence identity threshold, producing 383 OTUs. The input amplicons were then mapped against the OTU set to get an OTU-abundance table containing the number of reads associated with each OTU. The normalization, statistical analyses and multiple visualization were performed with SHAMAN (SHiny application for Metagenomic Analysis (shaman.c3bi.pasteur.fr) based on R software. Principal coordinates analysis (PCOA) based on Bray-Curtis distance matrix was computed at OTU level to describe the similarity between microbiota.

Bulk RNA isolation and RNA-seq analysis

$2-12 \times 10^4$ cells from each population were FACS Aria (BD Bioscience) sorted directly in RNA lysis buffer (Qiagen). mRNA was purified using RNeasy microkit (Qiagen) and quality and integrity were checked on Bioanalyser system (Agilent) according to manufacturer's instructions. The samples with RNA integrity number (RIN) above 8 were used to generate cDNA. rRNA sequences were eliminated by enzymatic treatment (Zap R, Clontech). The RNAseq libraries were prepared with the Smarter Stranded Total RNA-seq Kit – Pico input mammalian (Clontech) and checked by Bioanalyzer (Agilent). Clusters were generated for the resulting libraries, with Illumina HiSeq SR Cluster Kit v4 reagents. Sequencing was performed with the Illumina HiSeq 2500 system and HiSeq SBS kit v4 reagents. Runs were carried out over 65 cycles, including seven indexing cycles, to obtain 65-bp single-end reads. Sequencing data were processed with Illumina Pipeline software (Casava version 1.9). Data were normalized and analyzed in R software with the Bioconductor packages.

ECAR and OCR measurements

Oxygen consumption rates (OCRs) and extracellular acidification rates (ECARs) were measured for freshly sorted ILC3 (10^4 cells) from 3 mice for each condition. XF media (containing 10 mM of glucose, 2 mM of L-glutamine, and 1 mM of sodium pyruvate) was used under basal conditions. Addition of 1 μ M of oligomycin, 1.5 μ M of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and 1 μ M of RO + 1 μ M of AA was performed using portal injection in an extracellular flux analyzer (Seahorse Bioscience) as previously described (49).

Cell transfer

CR and CRACR infected mice were sacrificed on day 2, 3, 4 post infection (or reinfection) and for each condition 1.5×10^3 intestinal ILC3 were sorted. 3 days before the cell transfer, the recipient mice were infected with CR. Immediately after purification, cells were transferred by *i.v.* injection into CR infected *I122^{-/-}* mice.

Statistical analysis

Experimental results are reported as mean \pm SEM. Statistical differences between groups were determined with the Mann-Whitney test. p values < 0.05 were considered significant. Significance for survival rates of Kaplan-Meier curves was calculated with the log rank test. All data were analyzed with Prism 6-7 software (GraphPad).

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AUTHOR CONTRIBUTIONS

N.S and J.P.D. designed the study and wrote the manuscript. N.S. and A.J. designed, performed and analyzed the experiments. O.D. assisted with the LM experiments. P.G analyzed the intestinal microbiota. N.S., O.S., H.V.; R.L., and J.Y.C. performed RNA-seq analysis. L.S. provided metabolism expertise. G.F. provided microbiology expertise. J.P.D. directed the study.

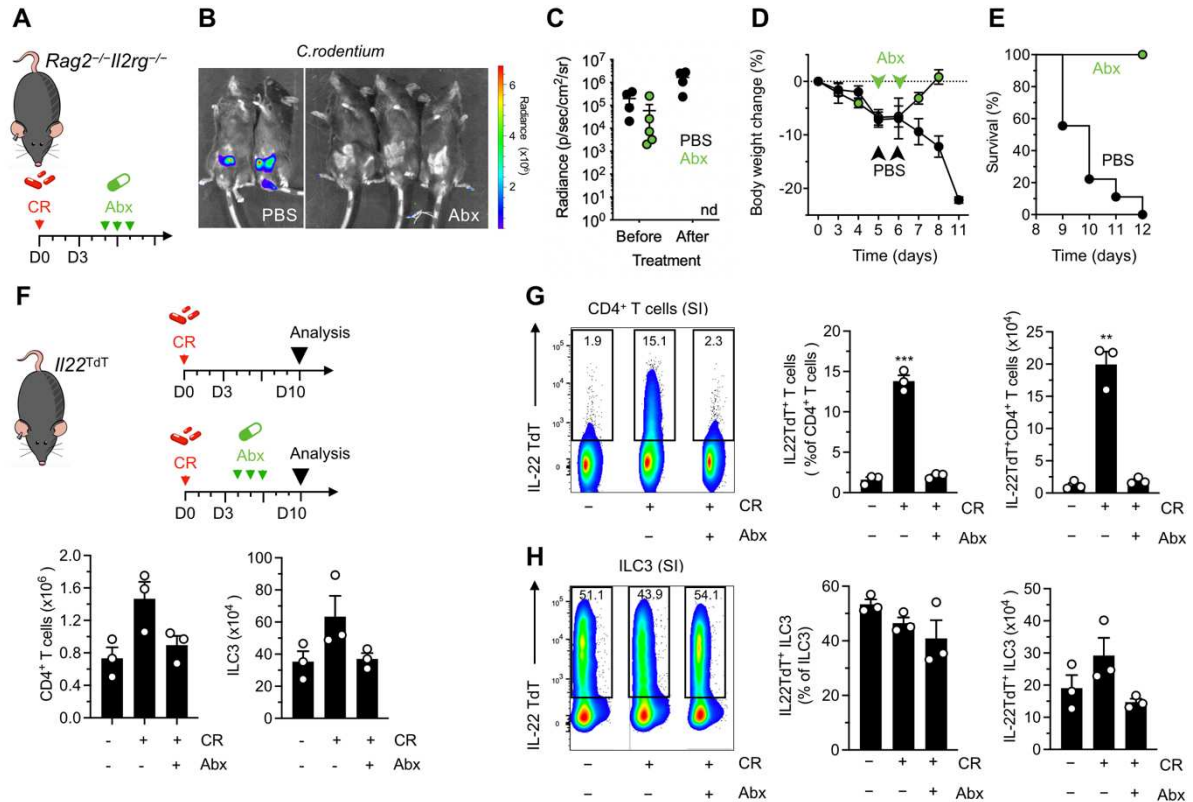
COMPETING INTERESTS

The authors declare no competing financial interests.

SUPPLEMENTARY INFORMATION

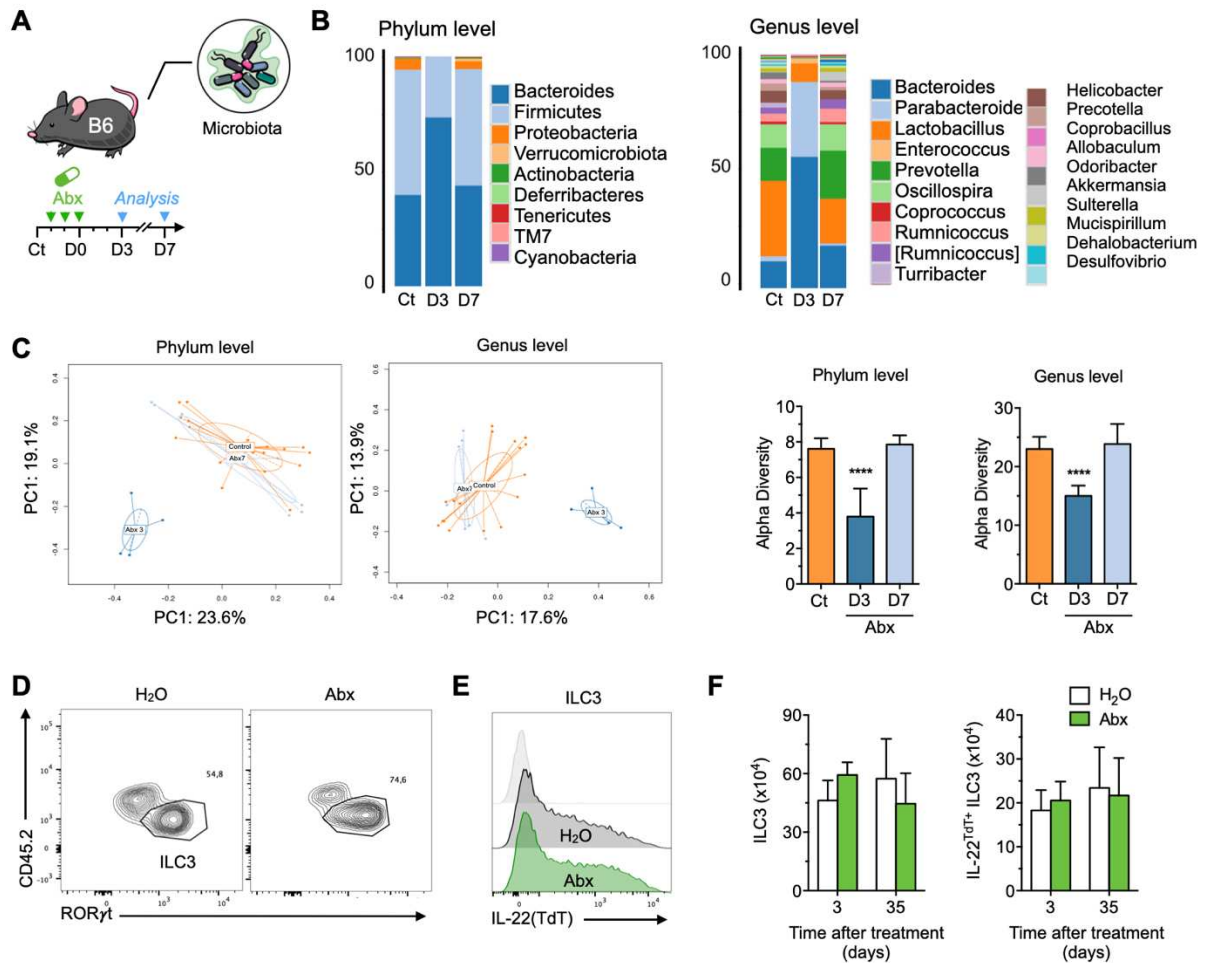
Supplemental Figures 1 - 10

Figure S1

**Figure S1 | ILC3 and T cell responses in CR and CRA models**

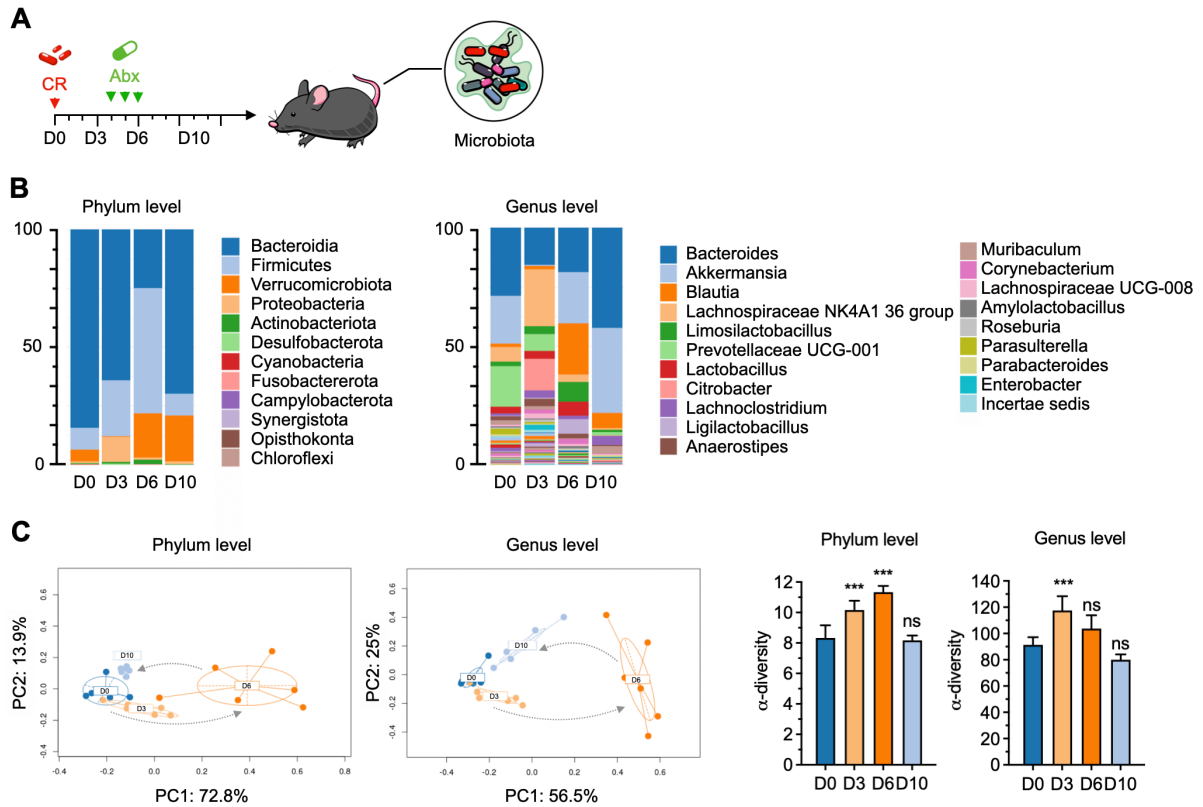
(A-E). Infected Rag2^{-/-}Il2rg^{-/-} mice received antibiotic treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) or PBS, 5 days after *C. rodentium* (CR) infection. The antibiotic was administered by oral gavage. (B-C) After infection, mice were examined for CR growth dynamics using noninvasive Xenogen technology. Representative pseudo color images are shown (day 8; B) and average radiance analysis was calculated (C). Data are representative of 2 independent experiments (scale bar, photon/s/cm²/sr; n=4-5) (D-E) Body weight (D) and survival (E) were monitored in groups containing 5-8 mice per group. (F) Infected Il22^{TdT} reporter mice received antibiotic treatment by oral gavage (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) or PBS, 4 days after CR infection. Mice were sacrificed at day 10 after infection and absolute numbers of CD4⁺ T cells and ILC3 were determined (n=3). (G-H) Representative flow cytometry analysis and quantification of frequencies and absolute numbers of IL22^{TdT} CD4⁺ CD5⁺ CD4⁺ T cells (G) and Il22^{TdT} ILC3 (H; n=3). Mean ± SEM; **p<0.01, ***p<0.001, Mann-Whitney test.

Figure S2

**Figure S2 | Effect of antibiotic treatment on intestinal microbiota and ILC3 phenotype**

(A-C) C57BL/6 mice received antibiotic treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) (A). The antibiotic was administered by oral gavage. (B) Relative abundance of major bacterial phylum and genus in the fecal microbiota from control or Abx treated mice (3 and 7 days after administration) were quantified by 16S rRNA gene sequencing. (C) 16S rRNA Operational taxonomic units (OTU) are clustered according to principal-coordinate (PC) analysis of untreated and treated (3 days and 7 days after Abx treatment). Percentage of variation, using Bray-Curtis distance, explained by plotted PC1 and PC2 are indicated on the x and y axes respectively. The alpha diversity, shown as total observed OUT and Shannon index, in untreated and treated (3 days and 7 days after Abx treatment). Mean and standard error of mean (SEM) values are indicated, and subgroups are compared with a Kruskal-Wallis test. **** P < 0.0001. (D-G) Intestinal ILC3 subsets were analyzed in control and antibiotic (Abx) treated mice (D). Expression of *IL22* (TdT) in ILC3 from control (black line) and Abx treated mice (green line; E). Absolute numbers of ILC3s and *IL22*^{TdT+} ILC3 in small intestine lamina propria (F). Data are representative of 3 independent experiments containing 6-18 mice per group. Mean and standard error of mean (SEM) values are indicated, and subgroups are compared with a Mann-Whitney test.

Figure S3

**Figure S3 | Impact of CR infection and antibiotic treatment on intestinal microbiota**

(A-C) *C. rodentium* (CR) infected mice received antibiotic treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) by oral gavage and feces were collected before infection (day 0; D0) and at indicated time points after infection and antibiotic treatment (A). (B) Relative abundance of major bacterial phylum and genus in the fecal microbiota were quantified by 16S rRNA gene sequencing. (C) 16S rRNA Operational taxonomic units (OTU) are clustered according to principal-coordinate (PC) analysis of untreated and treated mice. Percentage of variation, using Bray-Curtis distance, explained by plotted PC1 and PC2 are indicated on the x and y axes respectively. The alpha diversity, shown as total observed OUT, in untreated and treated mice. Mean and standard error of mean (SEM) values indicated, and subgroups are compared with a Kruskal-Wallis test. Data are representative of 1 experiment containing 6 mice. Results are shown as the mean \pm SEM. ** $P < 0.01$; *** $P < 0.001$.

Figure S4

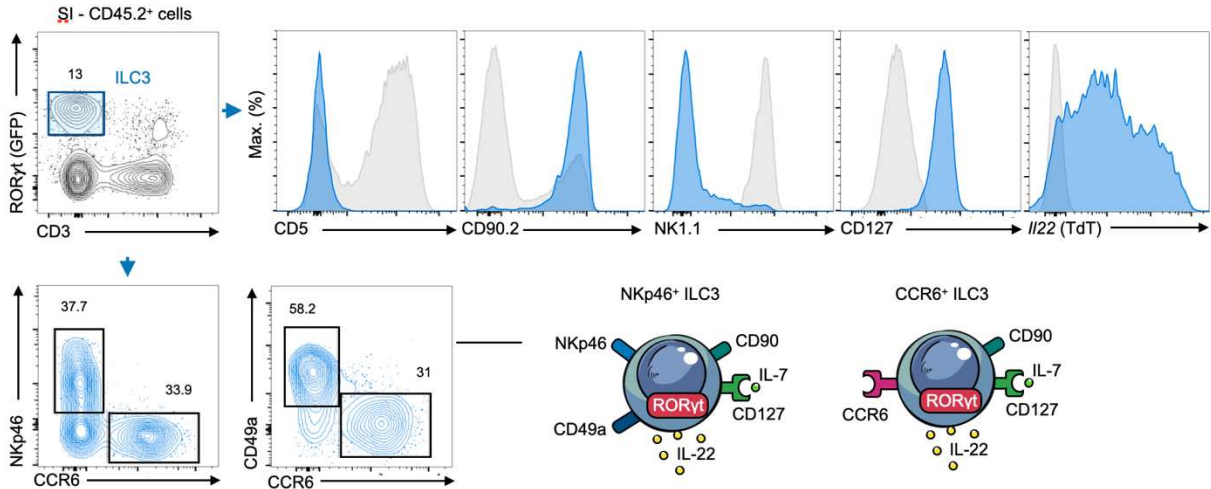
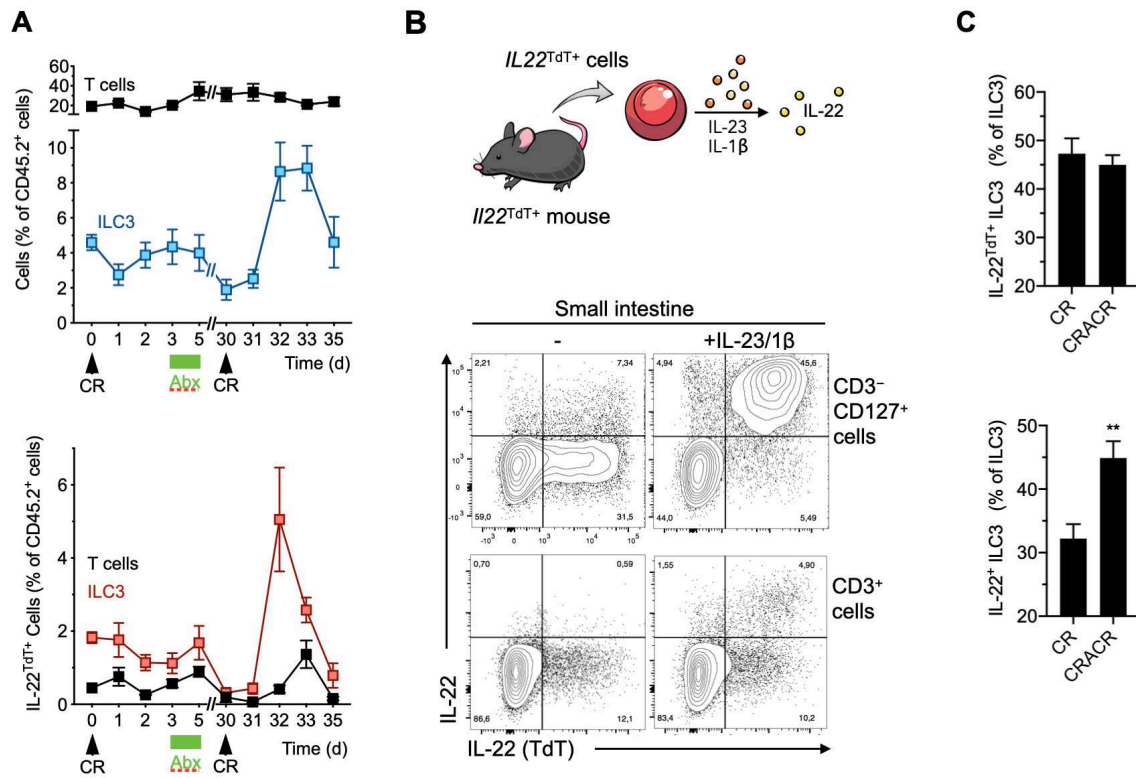


Figure S4 | Identification of small intestine ILC3 by flow cytometry

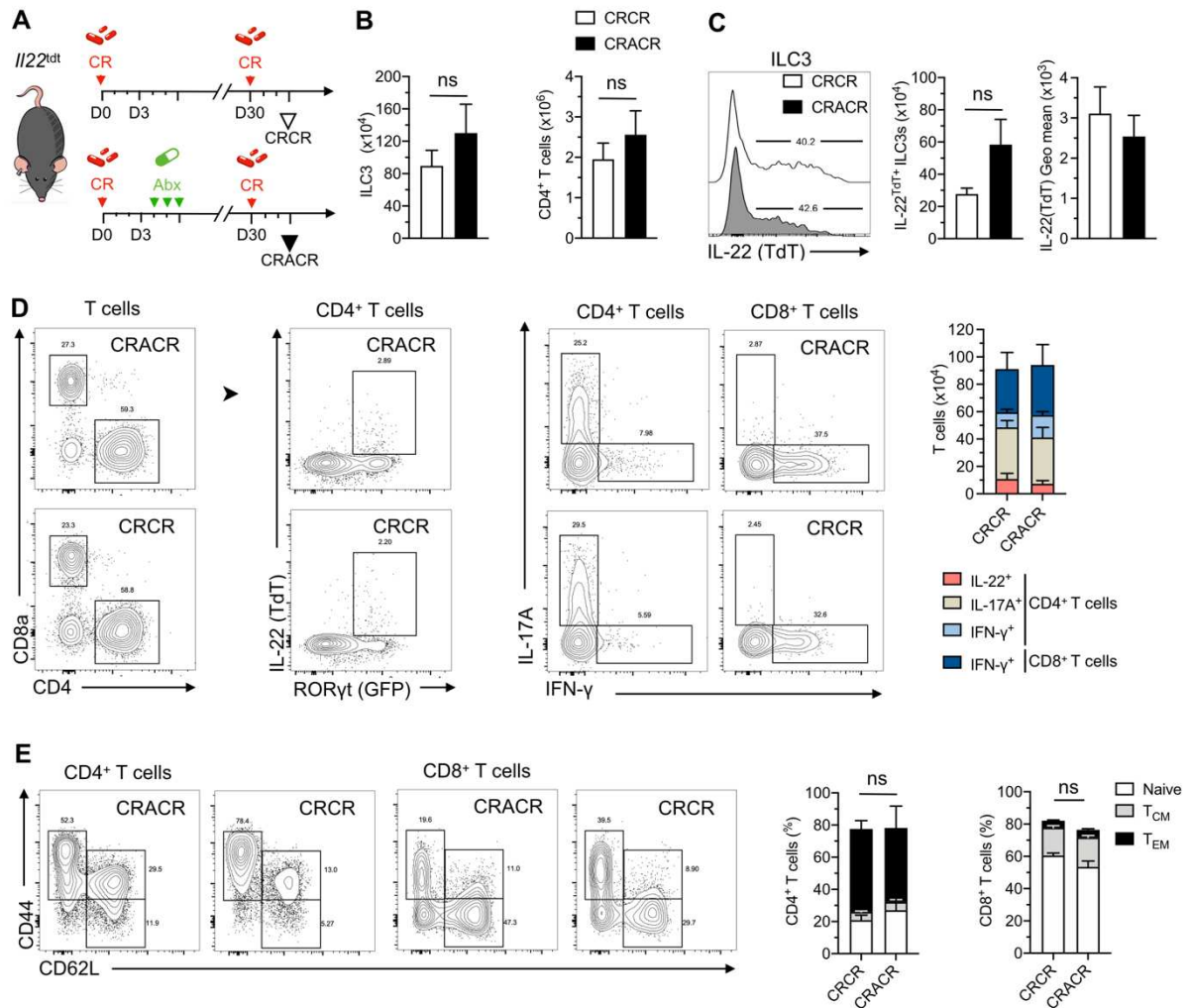
Small intestine ILC3 were analyzed by flow cytometry and gated on CD45.2⁺ CD3⁻ RORγt^{GFP+} cells. ILC3 were analyzed for CD5, CD90.2, NK1.1, CCR6, NKp46, CD49a, CD127 and I22^{TdT} expression.

Figure S5

**Figure S5 | Expression of *IL22^{TdT}* and IL-22 in intestinal ILC3 and T cells**

(A) *C. Rodentium* (CR) infected *Rorc^{GFP}IL22^{TdT}* reporter mice received antibiotic treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) 4 days after CR infection. One month later, the mice were re-infected with CR (CRACR). Frequency of total and IL-22^{TdT+} T cells and ILC3 were analyzed by flow cytometry at indicated time points. Representative data of 6 independent analysis (n=4-12 for each time point). (B) Representative flow cytometry analysis of IL-22 (TdT) and IL-22 expression in CD127⁺ CD3⁻ cells and T cells (CD3⁺ cells) from *il22^{TdT}* reporter mice after ex-vivo stimulation with IL-23 and IL-1 β . (C) Frequency of IL22⁺ and IL22^{TdT+} ILC3 in CR and CRACR mice (n=6). Data are representative of 3 independent experiments. Mean \pm SEM; **p<0.01, Mann-Whitney test.

Figure S6

**Figure S6 | ILC3 and T cell responses in CRCR and CRACR models**

(A-E) *C. rodentium* (CR) infected *I122^{TdT}* reporter mice received water (CRCR, n=7) or antibiotic treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days; CRACR, n=6) 4 days after CR infection. One month later, the mice were re-infected with CR. (B-E) Absolute numbers of ILC3 and CD4⁺ T cells (B), cytokine-producing ILC3/T cells (C) and tissue resident subsets of T cells (D; T_{CM}, central memory CD44⁺ CD62L⁺; T_{EM} effector memory CD44⁺ CD62L⁻; CD44⁻ CD62L⁺ naive T cell) were analyzed by flow cytometry in small intestine lamina propria in CRCR and CRACR conditions. Mean ± SEM. Mann-Whitney test.

Figure S7

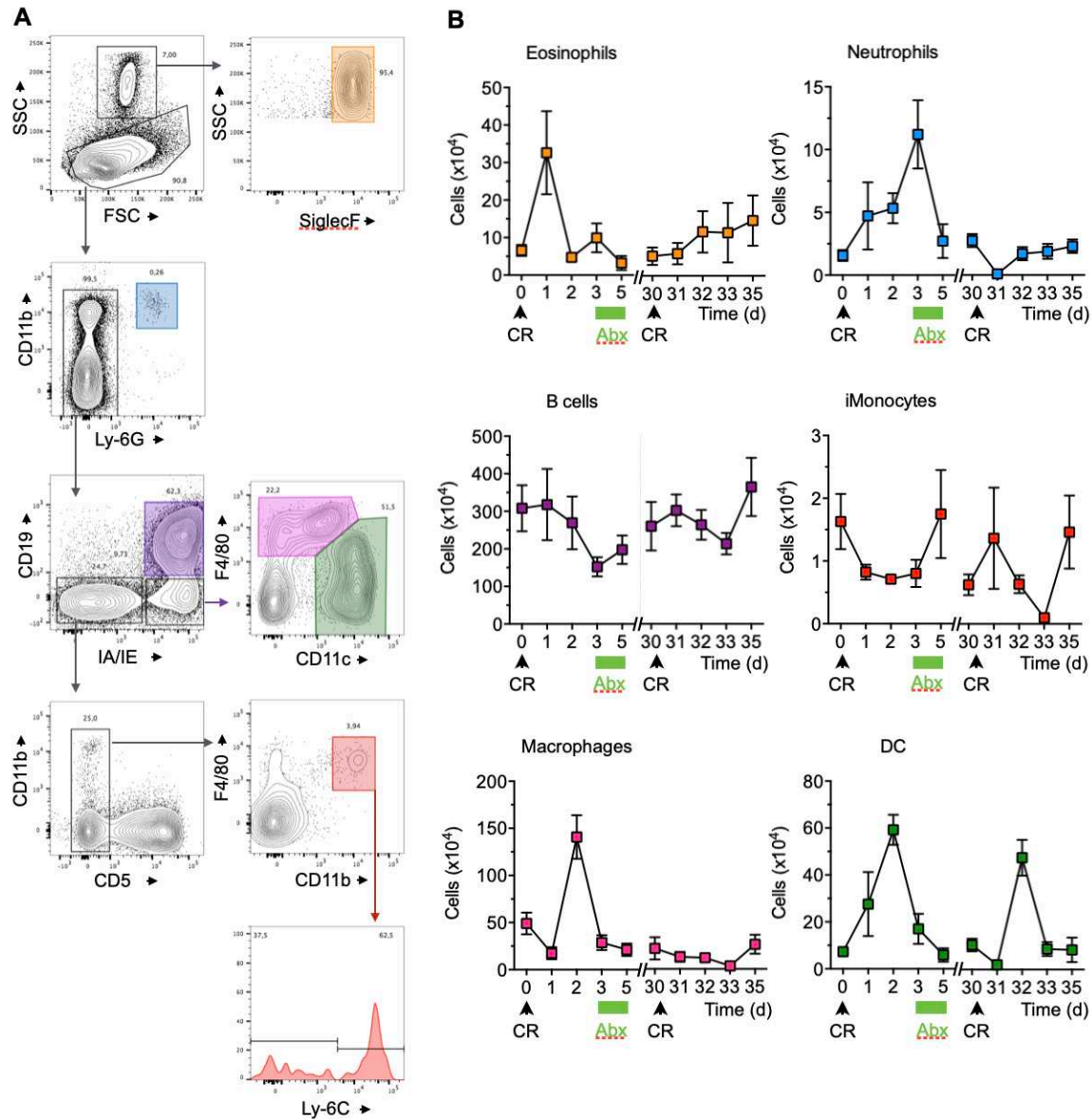


Figure S8

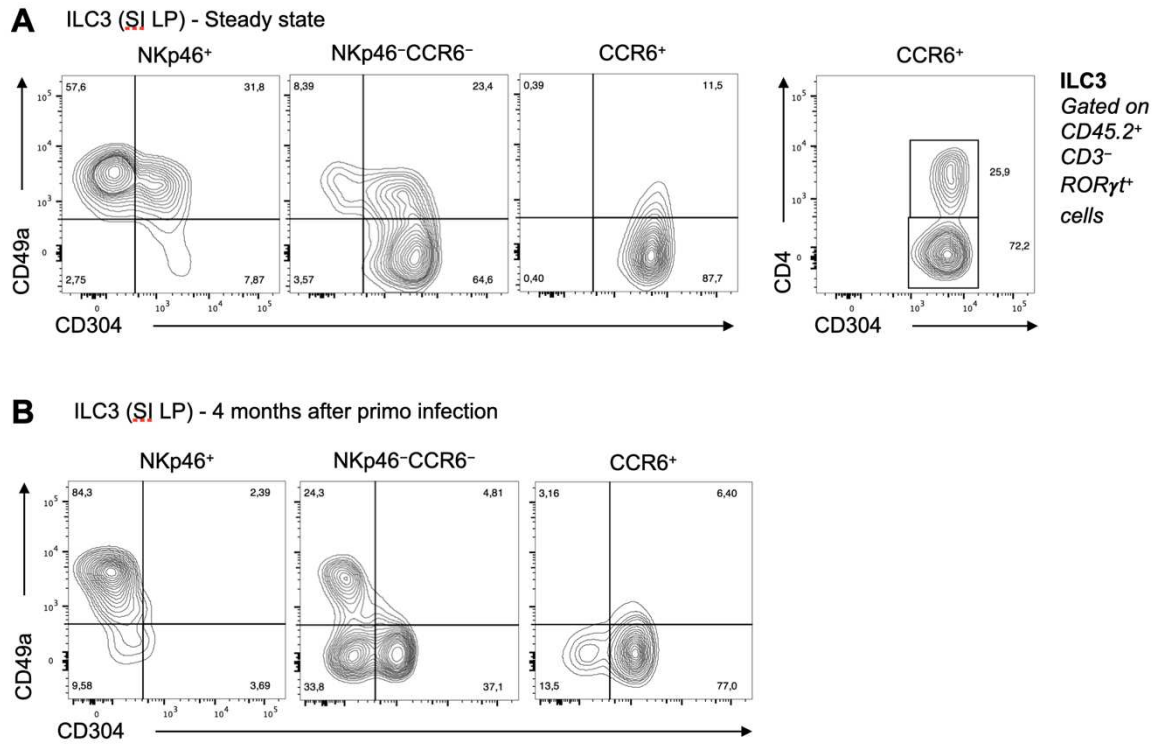
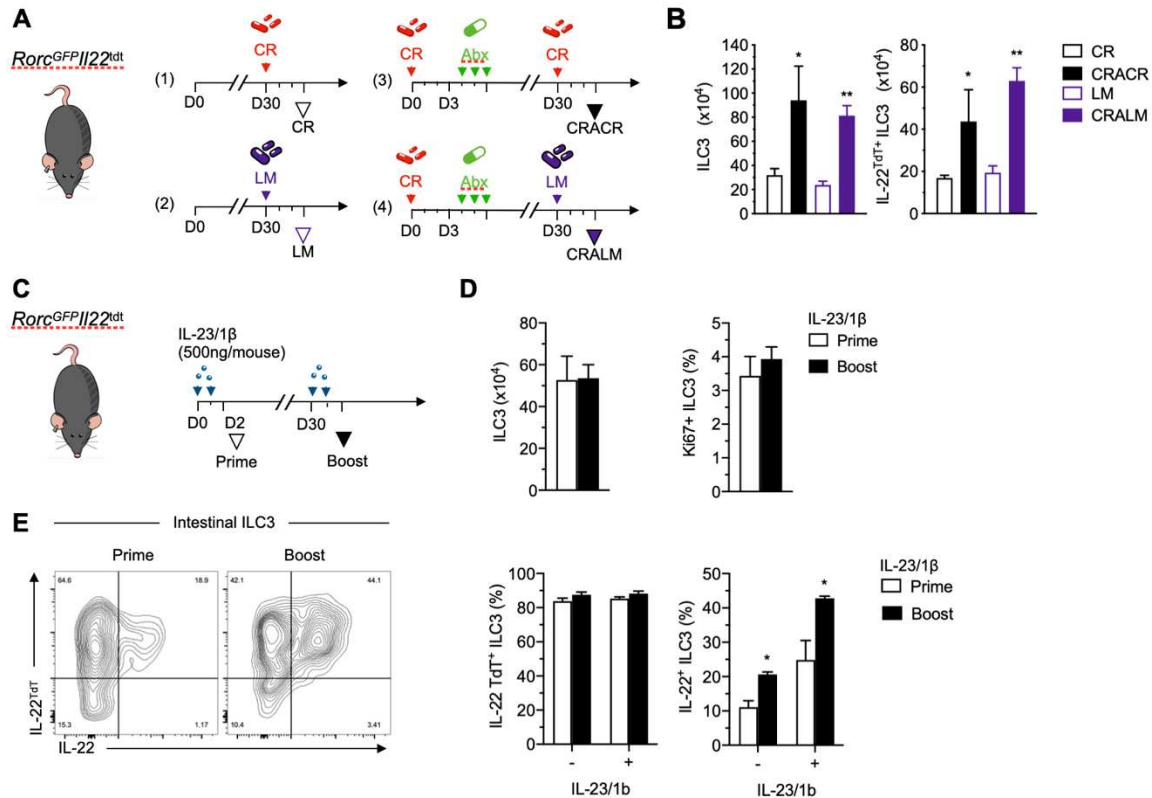


Figure S8 | Expression of CD49a, CD304 and CD4 on cell surface of gut ILC3 subsets

(A-B) Expression of CD49a, CD304 (or Nrp1) and CD4 on ILC3 (CD45⁺ Rorc^{GFP+} CD3⁻ cells) from small intestine Rorc^{GFP} Il22^{TdT} reporter mice before *C. rodentium* infection (A) or 4 months after infection and 3 days after *C. rodentium* reinfection. Data are representative of 3 independent experiments.

Figure S9

**Figure S9 | ILC3 response to unrelated pathogen and cytokine challenge.**

(A) *C. Rodentium* (CR) infected *Rorc^{GFP} Il22^{Tdt}* reporter mice received antibiotic treatment (ciprofloxacin, Abx; 100 mg/kg/day for 3 days) 4 days after infection. One month later, the mice were re-infected with CR (3; CRACR) or *L. monocytogenes* (4; CRALM). To control, one group was infected with CR (1) or *L. monocytogenes* (2; LM). (B) Intestinal ILC3 and IL-22^{Tdt}+ ILC3 were analyzed by flow cytometry (n=4). (C) *Rorc^{GFP} Il22^{Tdt}* mice received two *i.p.* injections of IL-23 and IL-1β. One month later, they received two *i.p.* injections of IL-23 and IL-1β. (D-E) Numbers of ILC3, percentages of Ki67+ and IL-22+ primed and boosted ILC3 were determined by flow cytometry after 3h of restimulation with IL-23/1β (50ng/ml). Data are representative of two independent experiments (n=6). Mean ± SEM; *p<0.05; **p<0.01, Mann-Whitney test.

Figure S10

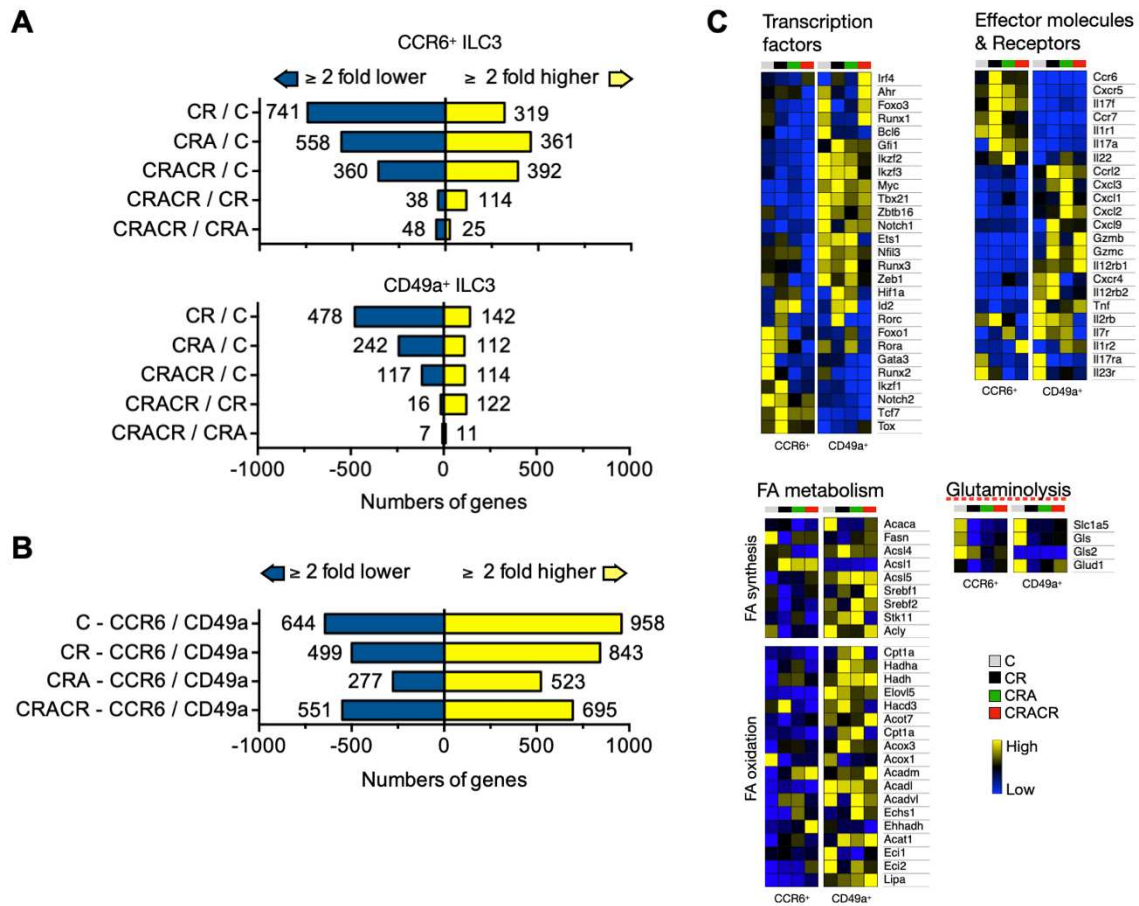


Figure S10 | Gene expression analysis of ILC3 subsets by RNA-seq

(A-B) Numbers of genes with a ≥ 2 -fold decrease (blue) and increase (yellow bar) in expression levels between the indicated populations and conditions. (C) Heatmap showing the relative expression levels of differentially expressed genes in ILC3 subsets in the small intestine of C, CR, CRA and CRACR mice.

CHAPTER IV

GENERAL DISCUSSION

I. Compartmentalization of intestinal ILC3

During my thesis, we first observed compartment-specific features of ILC3 in the mouse small intestine using intravital imaging. In line with existing studies, we found that ILC3 subsets occupy spatially distinct compartments within the intestine (1–3). In addition, we highlighted compartmentalization of multiple ILC3 functions, including IL-22 expression as well as previously unappreciated ILC3 migratory behaviors. These observations raise several questions: what are the consequences of intra-tissue distribution on ILC3 behavior and function? What is the biological relevance of ILC3 compartmentalization?

A. Consequences of topographic compartmentalization on ILC3 responses

We described compartment-specific features of intestinal ILC3, presumably associated to their distinct topography. Intestinal ILC3 were found both in lamina propria villi and crypts, recapitulating preceding histological reports demonstrating enrichment of NKp46⁺ ILC3 and CCR6⁺ ILC3 within intestinal lamina propria villi and basal intestinal mucosa, respectively (1–3). However, we detected IL-22 expression exclusively in ILC3 clustered in ILF within intestinal crypts at steady-state. In contrast, ILC3 acquired migratory capacities only within intestinal villi in response to inflammation. The impact of differential ILC3 localization on ILC3 function remains unclear but hypotheses can be drawn.

Impact of tissue niches on functional programs

As a result of compartmentalization, ILC3 reside in distinct tissue niches. Within tissues, immune cells rely on neighboring cells and local environmental stimuli to initiate functional programs. As the presence of various tissue-resident cells and environmental factors is dependent on spatial location, niches provide a specialized environment for immune responses, including ILC responses. In particular, a conserved niche for ILC2 has been described. ILC2 localization was determined using an *Il5*^{RFP} reporter in multiple organs and were generally found in perivascular spaces. In the lung, ILC2 predominantly localize close to IL-33⁺ PDGFR α ^{low} adventitial stromal cells (ASC), which produce TSLP to support ILC2 proliferation and activation, and therefore ILC2-driven type 2 inflammation in the lung (4). In turn, ILC2-derived IL-13 promotes ASC expansion and IL-33 expression, confirming that tissue niches reinforce cellular crosstalk. Similarly, white adipose tissue-resident ILC2 are enriched with IL-33⁺ mesenchymal stromal cells (MSC) lying around vessels. Co-culture of ILC2 with MSC results in increased ILC2 proliferation and cytokine production and requires cell-contact, as separation of MSC and ILC2 in transwells ablates these effects. Indeed, ligation of lymphocyte function-associated antigen 1 (LFA-1) on ILC2 by ICAM-1 on MSC is responsible for ILC2 proliferation and IL-5 production (5). This further emphasizes the importance of vicinity of cellular partners within tissue niches for the initiation of ILC responses.

Interestingly, it has already been shown that the intestinal environment segregates different T cells populations. While CD4⁺ IEL reside in the epithelium, FoxP3⁺ T_{reg} are located mainly in the lamina propria. FoxP3⁺ Treg can migrate to the epithelium where they adapt to the local environment and convert to CD4⁺ IEL in a microbiota-dependent fashion, a process involving dynamic regulation of

transcription factors. The authors provide evidence that both CD4⁺ IEL and FoxP3⁺ T_{reg} perform complementary roles in limiting tissue inflammation (6). This highlights how discrete niches in the intestine shape immune cell identity and function, leading to an intra-tissue specialization of these cells. In our study, we also found that the intestinal environment segregates different ILC3 populations with compartment-specific features.

Intestinal tissue niches for IL-22 production of ILC3

Distinct signals and intercellular interactions from discrete intestinal 'niches' most certainly determine intra-tissue ILC3 adaptation. Differential spatial expression of myeloid-derived IL-1 β and IL-23 has been previously identified and proposed to account for compartment-specific IL-22 expression within the intestine. Indeed, intestinal cryptopatches represent specialized anatomic and functional sites, where primed macrophages that are constitutively positive for IL-12/23 p40 preferentially reside clustered with activated IL-22⁺ LTi cells (7). These macrophages have been proposed to induce IL-22 expression in neonatal LTi upon weaning in response to microbial signals, although how sensing of the microbiota might occur within cryptopatches is not clear as crypts in the small intestine seems devoid of bacteria (8). Moreover, ILC3-glia interactions within intestinal cryptopatches could contribute to compartment-specific IL-22 distribution. As mentioned in the introduction, ILC3 are adjacent to neurotrophic-factor-expressing glial cells that project into ILC3 clusters and influence IL-22 production through the neuroregulatory receptor RET expressed on ILC3 (9). Interestingly, cryptopatches are also surrounded by a unique subset of cDC termed CIA-DC that produce IL-22BP and regulate intestinal IL-22 levels (10), in favor of cryptopatches acting as specialized niches for regulation of intestinal IL-22 expression at steady-state. In contrast, we have observed that flagellin-induced intestinal inflammation abolished compartment-specific expression of IL-22. Following bacterial challenge, IL-22 expression was dispersed throughout the intestine with large numbers of NKp46⁺ IL-22⁺ ILC3 present within lamina propria villi, in addition to IL-22 expression in ILF-associated CCR6⁺ ILC3. In the context of *C. rodentium* infection, IL-23⁺ mononuclear phagocytes are suggested to be distributed across the intestine and to coordinate the intestinal topography of IL-22 (3, 11). Whether other factors may contribute to differential IL-22 expression post-infection remains unknown.

Intestinal tissue niches for ILC3 migration

Conversely, we have observed patrolling ILC3 only within intestinal villi in response to inflammation. Differential patterns of migration associated with localization have been previously observed for cutaneous T cells (12). Upon herpes simplex virus (HSV) infection, epidermal T cells are less motile than dermal T cells, independently of their phenotype (CD8⁺ versus CD4⁺ T cells). These findings suggest that the relative motility of cells in the skin is mostly determined by their intra-tissue distribution, in the epidermis or in the dermis. Moreover, CD8⁺ T cell motility has been shown to differ between the four layers of the small intestine, indicating that intestinal tissue architecture dictates lymphocyte migratory behaviors as well (13). Similarly, ILC3 migration could be segregated by the intestinal environment. As we have just now described this novel aspect of ILC3 biology, the molecular mechanisms that control it remain to be discovered and how discrete niches in the intestine shape ILC3 cellular behavior is completely unknown. We demonstrated that the chemokine CCL25 (Thymus-

Expressed Chemokine; TECK) is a critical regulator of ILC3 migratory function. Several studies have shown that CCL25 expression is restricted to epithelial cells distributed from the one-third lower villi to the crypt in the small intestine (14–16). Therefore, it is probable that CCR9 expression rather than CCL25 repartition drives villi-specific ILC3 patrolling. Indeed, we have detected CCR9 expression only in intestinal NKp46⁺ ILC3 which are dispersed in the villus, but not on intestinal CCR6⁺ ILC3 which are located in crypts. Still, other signals controlling ILC3 migration may exist and be provided by specialized intestinal niches, thus not ruling out the importance of topographic compartmentalization on ILC3 migratory behavior.

ILC3 and T cells, two partners for a single niche?

In light of the above considerations, one can reasonably assume that ILC3 homeostasis and function can be controlled by distinct signals provided by different gut environments. It would be of interest to study whether ILC3 and T cells reside in distinct or overlapping niches in the intestine. Indeed, intestinal ILC3 are increased in *Rag1*^{-/-} mice and exhibit enhanced IL-22 production but how T cells control these functions remains obscure (17, 18). Given that ILC3 and T cells largely depend on the same factors for their survival and activation, they could likely be competing with each other. This notion is supported by two studies suggesting that ILC3 from lymphoid tissues can compete with T cells for IL-2 and IL-7 consumption (19, 20). Furthermore, we have uncovered T/ILC3 competition for the chemokine CCL25 as a major determinant of ILC3 patrolling. The presence of both CCR9⁺ T cells and CCR9⁺ NKp46⁺ ILC3 at common intestinal sites could determine this competition and subsequent ILC3 responses.

Overall, compartmentalization of ILC3 within the intestine results in occupation of specialized tissue niches that may impact ILC3 biology. Additional work is required to bridge localization and function of these cells. To better understand the consequences of discrete microenvironments in the gut on ILC3 responses, approaches combining spatial information and single-cell RNA sequencing will be useful in the future (21).

B. Biological relevance of ILC3 compartmentalization?

The observation that different mechanisms govern ILC3 distribution within the intestine (see general introduction), thereby resulting in compartment-specific features (see above), opens the possibility for different roles of ILC3 present in villi or in crypts.

Importance of IL-22 within intestinal crypts

Although all ILC3 possess the ability to produce IL-22, intestinal IL-22 production from villus or ILF ILC3 may have distinct functional consequences. While IL-22 is secreted by NKp46⁺ ILC3 mainly when IL-23 is released in response to pathogens, it is constitutively produced at steady-state by CCR6⁺ ILC3 residing in ILF and can be increased after infection (3, 7, 22, 23). Disturbing the topography of IL-22 cells in the villus has as a consequence a marked reduction in overall IL-22 production and a corresponding decrease of antimicrobial peptides by intestinal epithelial cells. Therefore, localized IL-22 production in the villus plays an important role in maintaining epithelial defense and mucosal barrier function in response to pathogens. CXCR6 expression appears to be a key mechanism required for

regulating this function. In crypts, IL-22 might serve a similar function in host protection and additional functions as well. It appears that IL-22 blockade disrupts the organization of ILF and subsequent antibody production during *C. rodentium* infection (23), suggesting a unique role of IL-22 present in crypts in protection against enteropathogens (**Figure 17**). Besides its role in anti-microbial immunity, IL-22 is also involved in other homeostatic processes in crypts. IL-22⁺ CCR6⁺ ILC3 are ideally positioned, close to intestinal stem cells (ISC), to protect and regenerate the epithelium in response to intestinal damage or genotoxic stress (24–27). In addition to CCR6⁺ ILC3, Paneth cells producing signals for stem cell maintenance (epidermal growth-factor, EGF; Wnt3) are found at the base of each crypt as well as pericryptal CD34⁺ gp38⁺ mesenchymal stromal cells which also produce niche factors (Wnt2b) and constitute together an essential niche for ISC (28, 29). This underlines the requirement of crypt-associated IL-22 specifically for optimal function, as all the members of the ISC niche are found there (**Figure 17**). Curiously, human β -defensins that are produced by Paneth cells can interact with CCR6 (30) and could attract and retain CCR6⁺ ILC3 to further ensure an operative ISC niche. Therefore, control of intestinal IL-22 production occurs through several mechanisms, independent (CXCR6-mediated) or dependent (CCR6-mediated) of ILF, that may have distinct functional consequences.

Restriction and dynamic regulation of IL-22 within intestinal crypts

Yet, the rationale for restriction of IL-22 expression to ILF at steady-state remains unclear. As mentioned in the general introduction, IL-22 has two facets: protective or detrimental, most likely dependent on the context. Therefore, restricting IL-22 to ILF for tissue homeostasis and adapting IL-22 distribution upon tissue perturbation might represent a way to tightly regulate the levels of this major cytokine within the intestine and to maintain the balance towards protective and not pathogenic functions. In the intestine, tissue damage may occur frequently as the intestinal epithelial barrier is continuously exposed to environmental stimuli including bacteria and noxious compounds. As such, the intestinal epithelium needs to be continually renewed, a process that requires IL-22 (see general introduction). Therefore, restriction of IL-22 to crypts allows to support this vital function while maintaining low IL-22 expression elsewhere to minimize potential harm. To avoid uncontrolled IL-22 activation in crypts, which could have irreversible negative consequences since stem cells reside there, ILC3 themselves program a subset of IL-22BP-producing dendritic cells (DC) that neutralize bioactive IL-22 (10). In accordance with expression of IL-22 exclusively in crypts, these crypt-associated DC reproduce the major source of IL-22BP in the intestine at steady-state. In addition to preventing ISC damage and malignancy, tight control of crypt-associated IL-22 has also been proposed to support lipid absorption, another essential homeostatic function for the host (10, 18, 31). As low levels of IL-22 are favorable to lipid transporters expression, the IL-22/IL-22BP module promotes absorption (10). Intriguingly, although absorptive enterocytes specialized with metabolic and digestive functions are positioned all along the intestinal villi, it is IL-22 expression from crypts and not villi that are believed to control this process. This may be because reducing IL-22 levels for nutrient assimilation comes at a cost for barrier protection against bacteria and requires rapid adaptation aligned on feeding rhythms. Since VIPergic neurons project into ILF, dynamic modulation of IL-22 levels upon food intake is possible in crypts, as opposed to intestinal villi (31). An interrogation remains though regarding the physiological relevance of crypt-associated IL-22 in lipid absorption. As a matter of fact, digestion and absorption of

lipids occurs to the greatest extent in the proximal small intestine, as illustrated by the contribution of malabsorption to weight loss following gastric bypass surgery where a stomach pouch is directly connected to the jejunum (32). Yet, in the three aforementioned studies, crypt-associated IL-22 levels have been associated with lipid metabolism in the ileum but have little or no effect in the duodenum and jejunum, suggesting that this circuit may fine-tune ileal nutrient absorption but likely has a minimal impact on global lipid digestion. Therefore, it appears that restriction of IL-22 to crypts at homeostasis mostly serves stem cell maintenance and protection, while avoiding unbalanced IL-22 and IL-22-dependent harmful effects (**Figure 17**). Still, there is potential for dynamic regulation of IL-22 upon tissue perturbation. Upon enteric infection, IL-22 expression is upregulated both in villi and in crypts in response to newly activated IL-23⁺ mononuclear phagocytes dispersed throughout the intestinal lamina propria (3, 11, 33, 34). This helps maintaining the intestinal barrier and limiting pathogen invasion, thus promoting host protection.

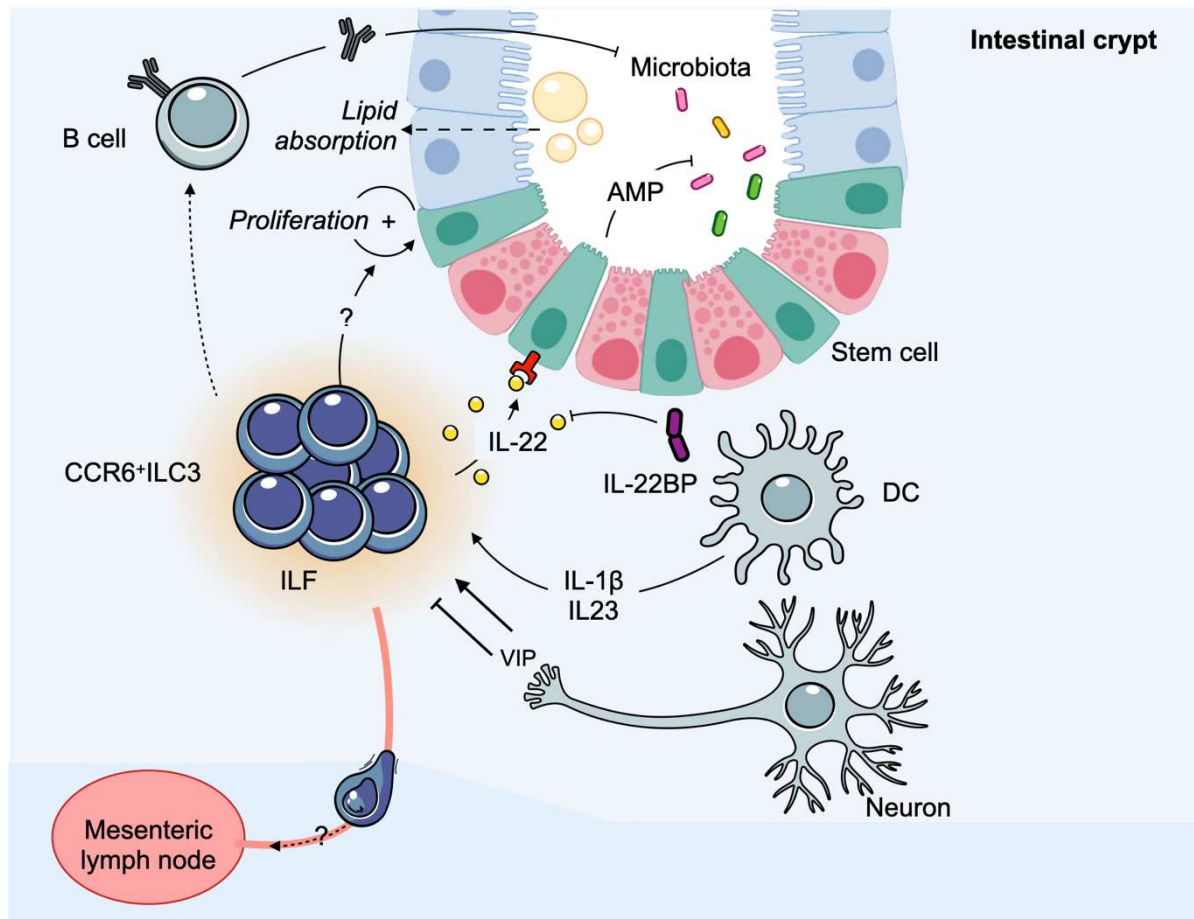


Figure 17 | Compartmentalization of ILC3 function within intestinal crypts

Intestinal ILC3 within isolated lymphoid follicles (ILF) exhibit limited motility at steady-state, which is likely associated with CCR6⁺ ILC3 essential functions in intestinal crypts. CCR6⁺ ILC3 within crypts exert both IL-22 independent functions such as support of intestinal IgA production and IL-22 dependent functions such as antimicrobial peptide (AMP) production, stem cell maintenance and lipid absorption. At steady-state, IL-22 expression is restricted to crypts where IL-22 activity can be dynamically regulated. IL-22BP, IL-22 binding protein; VIP, vasoactive intestinal peptide; DC, dendritic cell.

Relevance of compartmentalization for IL-22-independent ILC3 functions

Furthermore, ILC3 exhibit other compartment-specific features than IL-22 expression associated with their different roles in villi or in crypts. As previously mentioned, CCR6⁺ ILC3 within crypts express LT α 1 β 2, which is not the case of other ILC3 subsets within intestinal villi. Compartmentalization of CCR6⁺ ILC3 within ILF provides a privileged site for ILC3-DC-B cells interactions and subsequent antibody production. Indeed, membrane-bound LT α 1 β 2 interacts with LT β R on myeloid cells and promotes T cell-independent class switching and differentiation of B cells to IgA plasma cells (35, 36). Therefore, compartmentalization of ILC3 within ILF is also necessary for CCR6⁺ ILC3 IL-22-independent functions, including the maintenance, maturation and function of these essential structures (**Figure 17**). Indeed, ILF have been proposed to act as 'local' lymph nodes in the gut that, on the one hand, maintain immunoregulatory responses at homeostasis in order to control commensal microbiota composition and, on the other hand, induce local protective adaptive immune responses upon infection (1, 23). Interestingly, it has been demonstrated that gut lymph nodes are unique depending on the segment drained and this compartmentalized gut lymph node drainage of the intestine dictates site-specific adaptive immune responses (37). It would be relevant to test whether CCR6⁺ ILC3 also exhibit regional specialization and whether ILF along the intestinal tract also participate to segment-specific local intestinal immunity. In contrast, NKp46⁺ ILC3 could exert immunoregulatory functions within intestinal villi. In support of this idea, T_{reg} have been described in intestinal lamina propria villi (6) and NKp46⁺ ILC3 have been recently identified as the main source of IL-2 within the intestine, which is essential for T_{reg} maintenance and immunoregulatory function (38). Thus, additional compartment-specific features of ILC3 drive distinct yet complementary roles in intestinal homeostasis.

Relevance of compartment-specific migratory behaviors

Lastly, our study has revealed site-specific ILC3 migratory behaviors in response to bacterial challenge. Inflammation triggered ILC3 patrolling of the intestinal barrier in lamina propria villi by NKp46⁺ ILC3 but did not induce patrolling of CCR6⁺ ILC3 from ILF (data not shown). One could hypothesize that CCR6⁺ ILC3 must remain in ILF close to crypts to carry their fundamental role in niche protection. In the intestine, pathogens such as *Shigella* (39) directly target crypts for early entry. Robust production of IL-22 within crypts may be important to induce antimicrobial peptides (AMP) production by local Paneth cells and to protect stem cell from damage. Moreover, epithelial cell renewal plays an important role in the control of bacterial colonization by several enteropathogens including *C. rodentium* (40) and is dependent on crypt-associated IL-22 production. While epithelial cell death allows the elimination of damaged cells and limits persistent bacterial colonization, epithelial regeneration maintains the integrity of the intestinal barrier. Taken together with the requirement of CCR6⁺ ILC3 within ILF to amplify antibody production, these reasons might explain why ILC3 migratory behavior is solely observed in lamina propria villi and why CCR6⁺ ILC3 remain still in ILF upon flagellin-induced intestinal inflammation.

We will focus our discussion on ILC3 motility within intestinal villi, although we are not closed to the idea that CCR6⁺ ILC3 could move. At homeostasis, constitutive trafficking of CCR6⁺ ILC3 from the intestine to the mesenteric lymph node has been described (41). One day after photo-conversion of 2-3 cm of intestinal tissue, ~ 30% of mesenteric CCR6⁺ ILC3 had migrated from the intestine which

represents roughly 750 cells. Although this could be critical for 'intestinal T cell selection', this process is rare and is not necessarily synchronized: we might not detect it as we image surfaces of 0.26 mm², which underlines the importance of using different approaches to study ILC3 migration. Moreover, it would be of interest to image CCR6⁺ ILC3 in different inflammatory contexts. Pearson and colleagues have shown using intravital imaging of *Rag1*^{-/-}*Il23*^{gfp/+} mice that ILC3 within ILF exhibit little motility (42), consistent with our observations in *Rag2*^{-/-}*Rorc*^{GFP}*Il22*^{TdT} (data not shown) despite intestinal inflammation and intense ILC3 patrolling within lamina propria. However, they found that ILC3 could enter and exit the cluster after anti-CD40 treatment, suggesting that there could be conditions where CCR6⁺ ILC3 mobilization occurs. It is not clear whether these images were acquired in the small intestine or colon of *Rag1*^{-/-}*Il23*^{gfp/+} mice but it is tempting to speculate that colonic CCR6⁺ ILC3 is different motility than that of ileal CCR6⁺ ILC3. In fact, NKp46⁺ ILC3 are absent from the colon and CCR6⁺ ILC3 motility might be required for protection in this context. Alternatively, since the mucosal architecture is very different between small intestine and colon (43), with no villi in the latter, ILC3 patrolling might not be required for monitoring of the intestinal tissue. Although technically challenging because of anatomical constraints (the mesocolon being short), performing intravital imaging of colonic ILC3 would be helpful to answer these questions.

Overall, several exciting questions remain regarding the relevance of topographic and functional compartmentalization of intestinal ILC3. Among them, we would like to address whether environmental signals, including tissue perturbation, might affect ILC3 compartmentalization. We would also like to understand how this compartmentalization may change over an individual's lifetime, especially with regards to the weaning to adult transition period where tissue remodeling takes place.

II. Intestinal ILC3 migration

As the intestine is continuously exposed to a multitude of environmental stimuli, including invading pathogens, immunosurveillance of the intestinal barrier is of great interest. In the last decade, a few studies have assessed migration of T cells within the intestine and shown that this contributes to intestinal physiology and immunity against enteropathogens (6, 13, 19, 44–48). Although ILC3 motility within tissues could be a mechanism through which ILC3 rapidly modulate tissue responses, their migratory behavior has never been evaluated in the intestine. During my thesis, we decided to study this novel aspect of ILC3 biology and assess intestinal ILC3 dynamics. We interrogated whether tissue-resident ILC3 adapt their cellular behavior in response to environmental signals, including intestinal inflammation, and how this process may be regulated.

A. ILC3 migration in intestinal homeostasis

To visualize ILC3 behavior within the intestine, we have combined recently developed ILC3 reporter mouse models with intravital imaging. We have observed distinct compartmentalized ILC3 migratory behaviors in the mouse small intestine, which has also been documented for intestinal CD8 $\alpha\beta$ T cells (13) – here, we will focus on villus ILC3 since we have previously discussed ILC3 migration within ILF. Surprisingly, using two complementary approaches, we have found that ILC3 exhibit limited motility within intestinal villi at steady-state. Imaging of villus ILC3 in reconstituted bone marrow (BM) chimera revealed that these cells were poorly motile despite residing within the gut, which is permanently exposed to environmental stimuli. In theory, the gut environment is favorable to cellular activation, as illustrated by the activated phenotype (CD44⁺ CD69⁺) of ‘resident’ T cells such as natural intraepithelial lymphocytes (49). In line with this, we distinguished remarkably dynamic migration patterns among CFP⁺ cells in the intestine of BM chimeric mice. Thus, our observations on villus ILC3 contrasted the general view that ILC3 are in a ‘ready-to-go’ state and presumably monitor the intestinal tissue in the same way as T cells. However, ILC3 limited motility is in accordance with previous studies based on parabiosis or direct illumination reporting that ILC3 are mostly tissue-resident cells (41, 50, 51).

To confirm these results, we decided to use a complementary approach to avoid common limitations of BM chimeric mice, such as the fact that this is a somewhat artificial model with limited numbers of ILC3 derived from an adult progenitor which might not recapitulate the full spectrum of endogenous ILC3 and with possibly toxic effects linked to sub-lethal irradiation. Using double transgenic *Ncr1*^{GFP} *Il22*^{TdT} mice to discriminate endogenous ILC1 and NKp46⁺ ILC3, we found that double positive NKp46⁺ *Il22*⁺ ILC3 also exhibited limited motility in lamina propria villi at steady-state, confirming our previous results. We noted that ILC3 motility was increased in villus NKp46⁺ *Il22*⁺ ILC3 of *Ncr1*^{GFP} *Il22*^{TdT} mice compared to villus ILC3 of BM chimeric mice: whether this is due to not strictly comparable ILC3 populations, distinct environments or to the fact that NKp46⁺ *Il22*⁺ are possibly ‘primed’ since they express *Il22* is not clear. Still, NKp46⁺ *Il22*⁺ ILC3 exhibit little motility overall when compared with other intestinal *Il22*⁺ cells which include a mixture of NKp46⁻ ILC3 and T cells. Unfortunately, we currently lack optimal models to image ILC3 *in vivo* (detailed discussion in **Box 1 ‘Current challenges for the study of intestinal ILC3 migration’**). It would be of interest to develop additional reporters to assess the migratory behavior of

'unprimed' ILC3 only, especially because these NKp46⁺IL22⁺ ILC3 represent a low percentage of total ILC3 at steady-state.

At the moment, we ignore how and why the motility villus ILC3 is restricted at steady-state. Could these cells stick to neighboring structures? For instance, we know that CCR6⁺ ILC3 strongly interact with stromal cells through integrins and adhesion molecules for lymphoid organogenesis during fetal life (52). Could villus ILC3 express integrins, such as the α 1 sub-unit (CD49a) or α 4 β 7, that allows them to connect to intestinal elements? Alternatively, villus ILC3 could be devoid of factors required for leukocyte migration, such as leukocyte function-associated antigen-1 (LFA-1) which is broadly required for patrolling (53). For instance, active LFA-1 drives T-cell attachment and lamellipodial movement at the leading edge whereas it causes T-cell detachment at the trailing edge (53). A deeper characterization of receptors and signaling networks involved in lymphocyte migration on ILC3 may help to explain the curious migratory behavior of ILC3 within intestinal villi. We will discuss later the biological relevance of this unusual behavior with regards to classical lymphocyte migration, notably within the intestine.

Box 1 'Current challenges for the study of intestinal ILC3 migration'

Despite the potential important consequences of intestinal ILC3 migration on their role in tissue homeostasis and immunity, this aspect of ILC3 biology has only recently come into focus – possibly owing to limited tools which constitute an obstacle for its study. ILC3 share many developmental, phenotypical and functional similarities with T cells, making easy distinction between these cells difficult. Until now, studies investigating ILC3 tissue residency and trafficking have exclusively relied on FACS-based approaches and include parabiosis, intravenous antibody-labeling or photoconversion with subsequent flow cytometry analysis. Flow cytometry identification of ILC3 from CD45⁺ cells requires a combination of positive (ROR γ t and CD127 minimum; NKp46 and CCR6 minimum for ILC3 subsets) and exclusion markers (CD3 and CD5 at least; more without ROR γ t). These markers are all shared with other intestinal populations, which is incompatible with simple imaging of intestinal ILC3 within tissues. For intravital imaging of lymphocyte populations within tissues, several strategies exist:

1) Antibody labeling. This approach is not appropriate for ILC3 since there is no phenotypic marker specific for ILC3. Still, we tested it in *Ncr1*^{GFP} *Il22*^{TdT} mice with an A594-conjugated NK1.1 antibody: despite its low molecular weight and efficient labeling in vessels, this antibody was not able to cross the vascular endothelium and enter the intestinal tissue (data not shown). Isolation and injection of cells.

2) Isolation and injection of cells. This strategy has been extensively used in the T cell field to label T cells *ex vivo* or study antigen-specific T cells, but has some drawbacks for ILC3. Smaller number of ILC3 can be recovered and these cells can't be expanded as there are currently no optimal conditions for ILC3 culture *in vitro*. Transfer of mature naïve ILC3 is challenging: as evidenced by our *in vivo* results, intestinal ILC3 are not prone to migration. Progenitor cells can be transferred alternatively but are rare and require a lymphocyte-deplete environment. Moreover, the digestion process required to extract lamina propria lymphocytes (LPL) may affect surface expression of receptors involved in migration on ILC3. This constitutes a major drawback to evaluate ILC3 migration *in vitro* using transwell, Matrigel or poly-L-lysine-based assays: we couldn't assess ILC3 migration *in vitro*, even though cells were purified from *Rag2*^{-/-}*Rorc*^{GFP}*Il22*^{TdT} or re-stimulated *ex vivo* with cytokines (data not shown). While T cells isolated from lymph nodes were able to migrate in our *in vitro* assays, it was not the case of T cells isolated from gut (data not shown), indicating that either intestinal cells have a 'resident' phenotype or that isolation of LPL impacts lymphocyte migratory abilities. To get around these obstacles, one could consider using ILC3 from lymph nodes or spleen to study ILC3 migration *in vitro*.

3) Mouse fluorescent reporters. Possible reporters for ILC3 include the *Id2*^{RFP} and *Rorc*^{GFP} mice but intestinal T cells share expression of these two transcription factors with ILC3. To avoid this issue, we and others have used these reporters on a *Rag2*^{-/-} background, a straightforward approach with limitations though. Since the major issue is to distinguish ILC3 from T cells, one could associate aforementioned reporters with T cell-specific transgenic mice. We have obtained *Lck*^{Cre}*Rosa26*^{RFP}*Rorc*^{GFP} animals where RFP expression is restricted to T cells. However, considering the high but incomplete Cre recombinase efficiency (~80%), we couldn't exclude that RFP⁻GFP⁺ cells contained T cells (data not shown). Another option is to combine several reporters, as we did in the *Ncr1*^{GFP} *Il22*^{TdT} model but only partial information can be collected – in this case, exclusively on NKp46⁺IL22⁺ ILC3. Overall, much remains to be learned about ILC3 migration and the pathways that regulate it but the field awaits for the development of new strategies to explore these questions in depth. Understanding ILC3 development, maintenance and function will be useful to generate new tools and investigate ILC3 migration in the future.

B. ILC3 patrolling in intestinal inflammation

In spite of the surprising observation that intestinal ILC3 exhibit limited motility at steady-state, we were curious to see whether ILC3 could adapt their cellular behavior in response to environmental stimuli – particularly in the context of gut inflammation. To this aim, we used the TLR5-ligand flagellin since this is the main TLR expressed on intestinal epithelial cells (IEC) in the small intestine (54) and flagellin was previously shown to activate ILC3 to produce IL-22 (55). We found that flagellin-induced inflammation promotes ILC3 patrolling of the intestinal barrier by NKp46⁺IL22⁺ ILC3, which is associated with enhanced IL-22 expression. This raised two questions: what is the origin of these patrolling NKp46⁺IL22⁺ ILC3 within villi? What signals, either environmental or cell-intrinsic, could trigger this cellular behavior? (Figure 18)

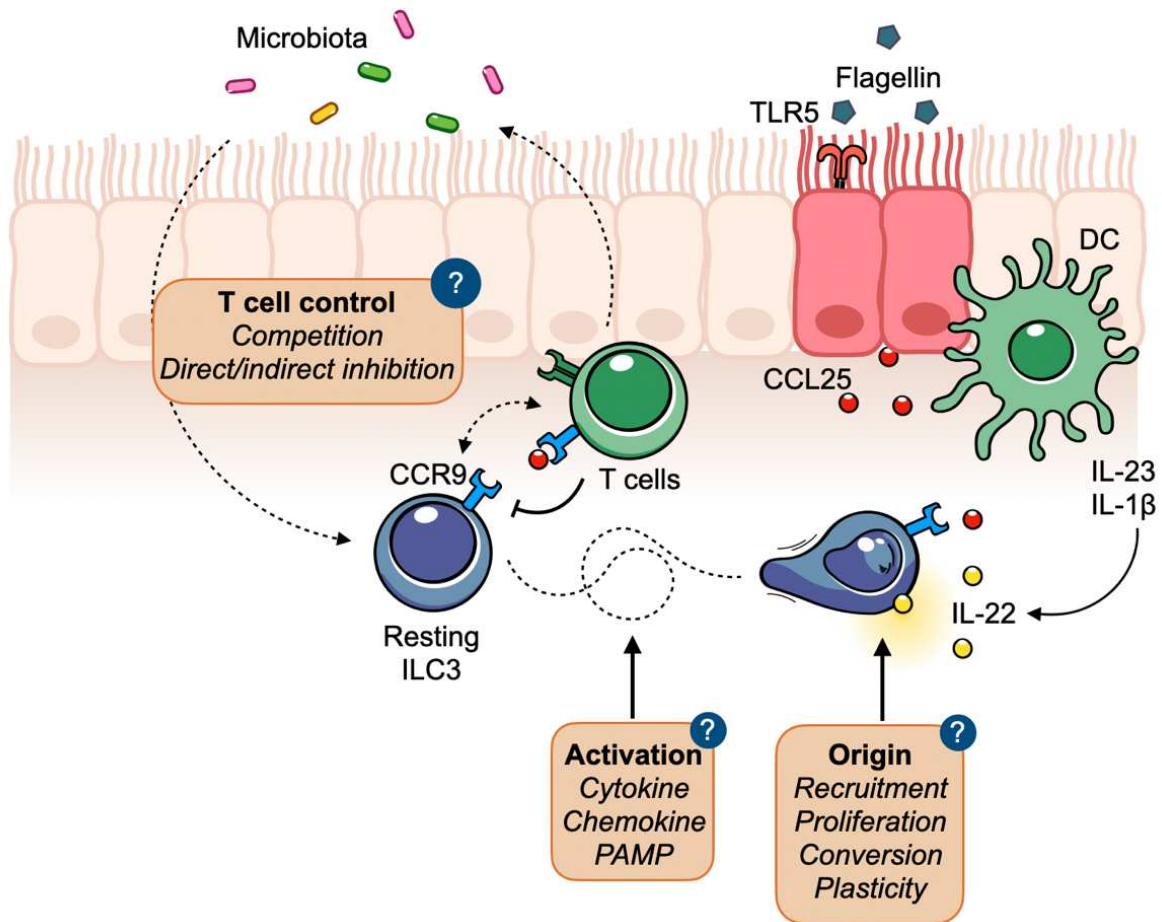


Figure 18 | Environmental regulation of ILC3 patrolling

The intestinal mucosa is continuously exposed to environmental stimuli that can induce immune responses through adaptation of cells including patrolling ILC3. Local endogenous and exogenous signals regulate ILC3 migratory behavior. T cells control ILC3 patrolling through competition for the chemokine CCL25 and possibly also through other mechanisms. Intestinal inflammation activates ILC3 and triggers CCL25-mediated patrolling of the intestinal barrier but other signals responsible for this cellular behavior, such as microbial or bacterial-derived signals, are not excluded. TLR5, Toll-like receptor 5; PAMP, pathogen-associated molecular patterns; DC, dendritic cell.

1. Origin of patrolling ILC3 within intestinal villi

Although this hasn't been formally tested in inflammatory conditions prone to ILC3 migration such as *C. rodentium* infection, ILC3 have been shown to be preferentially tissue-resident cells under various physiological and inflammatory conditions (50, 51). Therefore, it is unlikely that patrolling ILC3 are recruited from the blood following flagellin challenge. Rather, these cells are probably already within the tissue and acquire this novel migratory behavior in response to local inflammation. Both absolute numbers of NKp46⁺ //22⁺ and frequency of //22-expressing cells among NKp46⁺ cells were rapidly augmented five hours post-flagellin. This suggests that it is improbable that these large numbers of NKp46⁺ //22⁺ ILC3 derive from local proliferation of pre-existing NKp46⁺ //22⁺ ILC3. Alternatively, tissue-resident NKp46⁺ ILC3 could convert to patrolling NKp46⁺ //22⁺ in response to local environmental stimuli including IL-1 β and IL-23. These cells may already occupy lamina propria villi, a privileged hypothesis considering previous histological analyses of intestinal NKp46⁺ ILC3 (56) and presence of large numbers of villus *Ncr1*^{GFP+} cells at steady-state in our model, or be recruited from somewhere else in the tissue. Otherwise, patrolling NKp46⁺ //22⁺ following flagellin challenge could also arise from plastic ILC3 from villi or ILF – notably CD49a⁺ or DN ILC3 – that upregulate NKp46 and IL-22. This could happen in response to IL-23 or microbial-derived signals (57) (**Figure 18**). Indeed, we have proposed that ILC3 plasticity might reflect the requirement for these cells to adapt to environmental stimuli encountered in the intestine, as suggested by the high-degree of heterogeneity among these cells. A single-cell RNA-sequencing (RNAseq) study of intestinal ILC has highlighted five distinct transcriptional, and possibly functional, states for ILC3 (58). Comparison of this data with the bulk sequencing data of NKp46⁺ and CCR6⁺ ILC3 has provided evidence that the gene expression profiles of these two ILC3 subsets span several transcriptional states identified for ILC3, thus in favour of a dynamic interchange between transcriptional and functional states in response to environmental cues. In the gut, this process may represent a good way to mount rapid and appropriate immune responses for tissue-resident ILC3, which poorly get recruited or renewed. Finding a model to settle the matter is challenging (see **Box 1**). Still, to gain insight into the modification of NKp46⁺ ILC3 migratory behavior upon intestinal inflammation, we could combine the *Ncr1*^{GFP} reporter with the newly developed *Rorc*^{Kat} model (59) to address global NKp46⁺ ILC3 migration within villi, independently of their //22 expression.

2. Signals driving ILC3 patrolling within intestinal villi

Could environmental signals be responsible for ILC3 patrolling?

We demonstrated that flagellin-induced inflammation could trigger ILC3 patrolling of the intestinal barrier. Intestinal ILC3 responses are shaped by the local environment, thus environmental cues could drive ILC3 patrolling behavior. Moreover, we know that ILC3 are poised for rapid activation in response to epithelial- and myeloid-derived signals (60) and both IEC and intestinal mononuclear phagocytes express TLR5 (54, 61). Therefore, could sensing of flagellin results in epithelial and myeloid activation, resulting in the release of ILC3-modulating factors?

Previous studies have shown that TLR5⁺ dendritic cells are required for flagellin-mediated induction of IL-22, through IL-23 production (55, 62). Could cytokines, especially IL-1 β and IL-23, be involved in ILC3 patrolling as well? In our study, we have observed that there is no strict correlation between *Il22* expression – a possible indicator of cytokine priming – and ILC3 migratory behavior: *Il22*⁺ cells in ILF remain still, NKp46⁺ *Il22*⁺ ILC3 exhibit little motility at steady-state and most importantly, ILC3 patrol independently of *Il22* expression in *Rag2*^{-/-}*Rorc*^{GFP}*Il22*^{TdT} mice. Still, this doesn't dismiss a role for IL-1 β and IL-23 in ILC3 migration. We tested this hypothesis in our *Rag2*^{-/-}*Rorc*^{GFP}*Il22*^{TdT} + CFP⁺ T cells model: while flagellin was able to induce ILC3 patrolling and *Il22* expression, simple injection of IL-1 β and IL-23 augmented *Il22* expression but did not trigger this cellular behavior (data not shown). This indicates that other signals beyond IL-1 β and IL-23 are required for the adaptation of ILC3 cellular behavior, likewise for the adaptation of their trained ILC3 function.

TLR are recognized for their ability to induce different transcriptional and functional programs compared to cytokines (54). TLR5 stimulation induces epithelial proinflammatory and chemokine gene expression (63). Chemokines are good candidates to keep in mind when thinking about possible signals driving ILC3 patrolling. Chemokines organize recruitment of many leukocytes to achieve the right magnitude of responses and are modulated during inflammation (64). In neutralization experiments, we have demonstrated an important role for CCL25 in ILC3 migratory behavior. Yet, it remains unclear whether flagellin results in CCL25 upregulation and whether this chemokine alone drives ILC3 migration. We could examine the effect of injection of recombinant mouse CCL25, although we wouldn't be able to control *in vivo* the bioactivity of CCL25 as we have for IL-1 β and IL-23 looking at *Il22* expression. Besides, CCL25 is constitutively produced within the intestine (14–16) and tissue-resident already express CCR9: unless a 'lock' signal exists within the intestine, it is unclear why ILC3 wouldn't monitor the intestinal tissue at steady-state if patrolling is exclusively dependent on CCL25.

Additional unknown environmental factors might be responsible for ILC3 patrolling. One could imagine performing mass-spectrometry analysis of ileal tissue to screen for such signals. Alternatively, cell-intrinsic activation of ILC3 following flagellin may be required for migration (**Figure 18**).

Could cell-intrinsic activation be responsible for ILC3 patrolling?

Although we often think of ILC3 as primed 'ready-to go' cells, this might not be the case: one could propose that intestinal ILC3 receive a signal that would change their activation upon flagellin-stimulation, resulting in increased motility. As a matter of fact, cell-intrinsic activation is required for migration of myeloid and lymphoid populations. As an example, in response to TLR-mediated recognition of PAMP, DC undergo an activation process which leads to an increase in motility corresponding to upregulation of CCR7 (65). Subsequently, T cells make prolonged contacts with antigen-bearing DC which initially results in T cell arrest and later in T cell migration. In both cases, cell-intrinsic activation and ensuing dynamics occur in response to antigenic signals. Could bacterial ligands trigger an ILC3-intrinsic program required for intestinal patrolling in a similar fashion? Although ILC3 are largely devoid of common PRR, they may express atypical receptors (**Figure 18**). To test this hypothesis directly, we could isolate and stimulate intestinal ILC3 with flagellin in an *in vitro* migration assay but this requires prior optimization (see **Box 1**). One possible receptor that we haven't investigated in our study is the NCR NKp46. Multiple ligands, either endogenous or exogenous, have been described for NKp46

including bacterial ligands (66). Moreover, cross-linking of NKp46 with antibodies results in calcium release in NK cells and we know that calcium signalling is involved in lymphocyte migration, notably in T cells (67). Therefore, we could imagine that NKp46 could be involved in ILC3 patrolling. Instead of using heterozygous *Ncr1*^{GFP/+} mice as we did in our study, we could challenge NKp46-deficient *Ncr1*^{GFP/GFP} mice. For the moment, we haven't identified direct signals that could adjust ILC3 activation and migration and this still needs to be investigated.

Could it be a combination of environmental and cell-intrinsic activation?

Tissue environment plays an important role in ILC3 responses and may contribute to ILC3 patrolling upon intestinal inflammation. In parallel, short flagellin stimulation did not apparently modified the motility of other intestinal populations such as T cells, indicating that adaptation of ILC3 migratory behavior may not be purely environmental and require additional cell-intrinsic activation. We could evaluate this by sorting intestinal NKp46⁺ ILC3 prior or post-flagellin stimulation and performing scRNAseq to understand the cellular pathways induced upon bacterial challenge which could be associated with increased cell motility. For instance, in contrast to intestinal dendritic cells which upregulate CCR7 to migrate to the mesenteric lymph node, we haven't observed an upregulation of our 'relevant' chemokine receptors following flagellin treatment (data not shown) but novel receptors may be expressed specifically in inflammatory settings.

Interestingly, we noted that ILC3 IL-22 expression and migratory behavior are not coupled, suggesting that IL-22 doesn't represent the one and only read-out of ILC3 responses. Patrolling behavior of ILC3 might reflect a different form of activation and raises the question of its function in ILC3 biology (see later). Adaptation of ILC3 cellular behavior was observed following bacterial challenge but also at steady-state in *Rag2*^{-/-}*Rorc*^{GFP}*Il22*^{TdT} mice. We showed the presence of intestinal inflammation at steady-state in this model, with augmented pro-inflammatory cytokines as well as chemokines (data not shown), which could contribute to ILC3 hyperactivation and patrolling phenotype. Also, *Rag2*^{-/-} exhibit alterations in their microbiota (18, 68) and these microbial signals may directly alter ILC3 programs. As for flagellin-induced inflammation, permanent activating environmental signals combined with a distinct activation state for ILC3 could explain their patrolling behavior at steady-state in the *Rag2*^{-/-} model. Furthermore, we have proposed that this phenotype results from the absence an aforementioned 'lock' signal in *Rag2*^{-/-} mice which would prevent ILC3 patrolling. We have demonstrated that T cells are involved in the regulation of ILC3 patrolling and will now discuss this aspect.

C. T cell influence on ILC3 patrolling

We demonstrated that adoptive transfer of conventional T cells was sufficient to limit migratory behavior of intestinal ILC3 in *Rag2*-deficient mice, thus indicating that T cells suppress ILC3 patrolling under steady-state conditions. Regulation of ILC3 cellular behavior by T cells could be achieved either directly or indirectly, through control of signals involved in ILC3 motility (**Figure 18**).

1. T/ILC3 competition

In our paper, we demonstrated that T cells prevent ILC3 migratory behavior at steady-state, in part through regulating CCL25 availability, within the intestine. We proposed that T/ILC3 competition for the chemokine CCL25 is a major determinant of ILC3 'patrolling'. In support of T/ILC3 competition, we found that they both express CCR9 as previously described (69–71). In transwell assays, CCR9⁺ T cells and splenic CCR9⁺ ILC3 migrate to CCL25 – a process that is dependent on their CCR9 expression (69, 70, 72). Since we couldn't assess intestinal ILC3 migratory capacities to CCL25 *in vitro* (see **Box 1**), we directly tested it *in vivo* and found that CCL25 neutralization prevented ILC3 patrolling of the intestine. This effect was observed rapidly, usually in less than one hour, in favor of a direct effect on CCL25 on ILC3 migratory behavior rather than of an indirect effect of another CCR9⁺ population that would secondarily affect ILC3 motility. To further prove that ILC3 migrate *in vivo* in response to CCL25, we could cross our *Rag2*^{-/-}*Rorc*^{GFP}*Il22*^{TdT} reporter on a *Ccr9*^{-/-} background and evaluate whether intestinal ILC3 are still able to patrol at steady-state. Although CCR9 is involved ILC3 homing, small numbers of intestinal ILC3 could be imaged but compensation mechanisms may emerge in absence of CCR9. Finally, we verified that CCL25 was consumed and measured CCL25 levels in ileal explants using enzyme-linked immunosorbent assay (ELISA). We didn't detect changes in ileal CCL25 concentrations in wild-type, *Rag2*^{-/-} or *Rag2*^{-/-} transferred T cells (data not shown), suggesting that CCL25 is consumed in all these conditions. Based on our adoptive transfer experiments using *Ccr9*^{-/-} T cells, we concluded that T/ILC3 competition for the chemokine CCL25 is a major determinant of ILC3 'patrolling'. Interestingly, although ILC3 were able to migrate in presence of *Ccr9*^{-/-} T cells, their migratory behavior was not as pronounced as in *Rag2*^{-/-} mice, suggesting that T cells may control ILC3 migration through additional mechanisms.

2. Direct regulation through cell-contact

In lymph nodes, ILC3 have been suggested to perform MHC-II-mediated antigen-presentation to T cells, thus directly regulating T cells responses through cell-contact. Conversely, we and others demonstrated that T cells could in turn modulate ILC3 responses in the intestine (17, 18, 73, 74). From our experiments, we can't report a role for direct cell-contact between ILC3 and T cells in intestinal ILC3 regulation. We observed neither short nor long contacts between GFP⁺ ILC3 and CFP⁺ cells in lamina propria villi of BM chimeric mice, in which all T cells are CFP-labeled since they only derive from CFP⁺ BM. Similarly, we performed preliminary imaging experiments in the small intestine of *Lck*^{Cre}*Rosa26*^{RFP}*Rorc*^{GFP} but didn't detect such interactions at steady-state (data not shown). We would be intrigued to examine this in other organs such as the mesenteric lymph node or the spleen, where CCR6⁺ ILC3 expressing MHC-II reside in the interfollicular region (75) and possibly interact with T cells for antigen presentation. Accordingly, intestinal and splenic CCR6⁺ ILC3 exhibit different expression of MHC-II associated pathways, differ in their capacity to stimulate T cells *in vitro* and this phenotypical and functional heterogeneity appears to be related to their tissue localization (76).

3. Indirect regulation: which option to control signals driving ILC3 patrolling?

Because we didn't noted interactions between T cells and ILC3 *in vivo*, we hypothesized that T cells could indirectly control ILC3.

Niche occupancy?

Besides intense ILC3 patrolling, *Rag2*^{-/-} mice also exhibit increased ILC3 numbers and IL-22 production compared to lymphocyte-replete animals (17, 18). In the general introduction, we proposed that this could be the result of enhanced environmental signals. Yet, biased differentiation from the common lymphoid progenitor (CLP) and an empty niche could also contribute to ILC3 expansion. Niche occupancy by T cells following adoptive transfer could indirectly regulate ILC3 behavior. Our transfer of *Ccr9*^{-/-} T cells argues against this idea. Indeed, ILC3 still patrol in spite of T cell presence within intestinal villi.

Regulation of intermediary signals?

Alternatively, T cells could control potential signals involved in ILC3 migration not by consumption but by regulating pathways involved in their production. We will mainly focus on CCL25, a chemokine produced by IEC at steady-state (14–16), which we know is involved in ILC3 patrolling. Analysis of chemokine expression in whole ileal extracts revealed that T cells down-regulate expression of *Ccl25* transcripts. Since we observed that flagellin overrides T cell control of ILC3 patrolling, it would be of interest to interrogate ileal CCL25 expression in this context. Given the short stimulation, it is unlikely that flagellin regulates *Ccl25* expression at the RNA level but it could trigger the release of CCL25 the protein level, as IEC express TLR5 and can produce CCL25 (54, 61). We could also address whether T cells regulate the protein levels of CCL25 in the intestine. As mentioned earlier, we already did it using ELISA on explants but because CCL25 appears to be consumed, we sought to measure CCL25 directly in producers via flow cytometry (intracellular staining) or cell lysis followed by protein extraction. We definitely believe that T cells modulate many aspects of the intestinal environment, including DC and IEC, that are known to regulate ILC3 responses and also ILC3 patrolling possibly. Our preliminary data have shown that T cells regulate many epithelial and myeloid-derived cytokines and chemokines (data not shown). Additionally, many studies have shown that T cells regulate epithelial or myeloid activation and in particular, functions that could have an impact on ILC3 responses (17, 18, 73, 74).

Furthermore, T cells can shape the activity of ILC3 through modulation of the microbiota. This thought deserves some attention as it has been previously observed that the microbiota is involved in IEC activation and control of lymphocyte migratory behavior in the intestine. A first paper has shown that TLR-sensing of microbial signals in IEC results in 'flossing' of $\gamma\delta$ intraepithelial lymphocytes (IEL) (46). A second paper also described that carbohydrates, which are degraded and fermented by commensal bacteria (77), induce a transcriptional program in IEC which is associated with specific localization and motility of $\gamma\delta$ IEL (78). Therefore, we suppose that microbes could affect the production of signals involved in ILC3 motility – through IEC but also through DC. Indeed, *Rag2*^{-/-} exhibit alterations of microbial communities (18, 68), which could result in ILC3 patrolling. To investigate the effect of commensals on ILC3 patrolling, we imaged antibiotic-treated *Rag2*^{-/-}*Rorc*^{GFP}*Il22*^{TdT} mice and observed

a significant reduction of ILC3 motility (data not shown), indicating a possible role of microbes in this process. We also performed analysis of gene expression in whole ileal extracts in this antibiotic-treated *Rag2^{-/-}* model and discovered that expression of many environmental factors was modified, suggesting that the microbiota could modulate ILC3 patrolling in part via activation of epithelial and myeloid cells as proposed in other studies. Since T cells are involved in control of microbial communities (79), one could hypothesize that this is an additional pathway through which T cells regulate ILC3 patrolling. Interestingly, ILC3 motility as well as the expression of several genes were more affected in presence of T cells compared to antibiotic treatment alone, arguing that T cell suppression of ILC3 patrolling is not purely mediated through microbiota control.

T cell influence on ILC3 patrolling: mechanism or mechanisms?

All in all, we understand that there may be many co-existing networks for T cell regulation of ILC3 responses and patrolling notably. Here, we have only focused on the CCR9/CCL25 axis but ILC3 migratory behavior is likely modulated by several other CCL25-independent mechanisms which provide additional ways for T cells to keep ILC3 'in check' and promote coordinated intestinal responses. In our demonstration, we have performed adoptive transfer of total T cells but we haven't invested time in understanding whether specific T cell subsets suppress ILC3 motility. An alternative strategy to dissect the pathways regulating ILC3 patrolling could be to transfer distinct T cell subsets to identify precise subsets influencing ILC3 cellular behavior and to subsequently test the impact of these T cell-associated functions on patrolling ILC3 (**Figure 18**).

III. Functional relevance of patrolling ILC3

The studies presented here uncover a previously unappreciated aspect of intestinal immune responses to inflammation. We show that signals from the microenvironment promote tissue adaptation of intestinal ILC3 responses, with an emphasis on ILC3 patrolling of the intestinal barrier upon gut inflammation. In this part of the discussion, we interrogate the functional relevance of this ILC3 movement in intestinal immunosurveillance, with respect to previous knowledge of lymphocyte migration and of the role of intestinal ILC3.

A. ILC3 migration program: sentinels or explorers?

Patrolling ILC3: explorers?

Previous dynamic imaging studies have revealed that lymphocytes (NK cells, T cells and B cells) exhibit robust cellular migration under basal conditions and respond with altered motility when they get activated. As such, these crawling cells which sample their environment can be seen as ‘explorers’ (80). Because ILC and T cells share many developmental and functional similarities (81, 82), this overlap may extend to common residency and migration programs. In order to make contact rare antigen-presenting dendritic cells, naïve T cells actively scan within the lymph node with mean speed of 11-14 $\mu\text{m}/\text{min}$ and velocities reaching up to 25 $\mu\text{m}/\text{min}$ (83). In presence of antigen, T cells interact with DC and eventually arrest to establish stable long-lasting contacts, resulting in T cell activation (84). By 48 hours, many T cells regain motility and disseminate in the periphery. Although intestinal ILC3 are tissue-resident, they also rely on myeloid-derived signals, namely cytokines, for their activation (60). How ILC3 gain access to their activating signals remains unclear: in light of the above considerations, we wondered whether ILC3 could be ‘explorers’ that would sample the intestinal tissue to find their activating mononuclear phagocyte.

Strikingly, the migratory behavior of ILC3 stood out among the ‘explorer’ behavior shared by their lymphocyte relatives. Instead of scanning the intestinal tissue, as it has been observed for tissue-resident T cells (13, 46, 85), ILC3 were largely immotile at steady-state. Intestinal ILC3 only patrolled after activation in response to environmental signals. On the basis of a parallel with the migration of their T cell analogues, one possibility is that these ‘naïve’ ILC3 instead represent ‘primed’ cells, which have received or are receiving a signal that would promote their arrest and subsequent activation. In support of this idea, microbiota-sensing by intestinal macrophages continuously sustain *IL22* expression in a subset of ILC3 (7). This priming could represent an intermediary step in ILC3 activation and be required for future rapid responses and IL-22 production, although this remains to be proven for ILC3. Since tissue environment plays an important role on ILC3 biology, it is possible that environmental priming may be required for their optimal function, such as patrolling of the barrier in response to bacterial challenge. In the gut, microbial-derived signals have been shown to be involved IEL positioning and in subsequent dynamic responses to intestinal microbes (46). If ILC3 are truly similar to T cells and other lymphocytes, they could exhibit a similar pattern of migration coupled to their activation, although over different time periods. Naïve ILC3 would be motile and may correspond to ILC3 in a newborn mouse.

These cells would get primed in response to microbial signals and consequently arrest. Following tissue perturbation, primed ILC3 could become rapidly activated and patrol the intestinal barrier. This temporal classification of ILC3 activation would fit with the idea of sequential ILC3 responses throughout life recently proposed based on intestinal ILC3 p-STAT3 and *I122* expression over time (18) (**Figure 19**).

However, this model is purely speculative and previous studies on cellular dynamics have already pointed out limitations of parallels between T cells and ILC. In particular, comparison of NK cells and CD8⁺ T cells within lymph nodes revealed notable differences, such as maintenance of a motile behavior for NK cells during their activation and short contacts with DC (86). Similarly, within the tumor environment, NK cells and CD8⁺ T cells exert cytotoxic activity with distinct dynamics (87). While T cells need to form stable interactions with DC or antigen-expressing tumors for their activation and effector functions, NK cells actively patrol their environment which allows them to make multiple short-contacts and either rapidly eliminate tumor cells or rapidly enhance their effector functions thanks to their proximity with multiple DC. Therefore, it appears that members of the ILC family exhibit distinct migration patterns compared to T cells. This may be because they are poised for rapid responses to environmental signals and do not require a prolonged phase of activation by mononuclear phagocytes to turn on an effector program, in contrast to T cells.

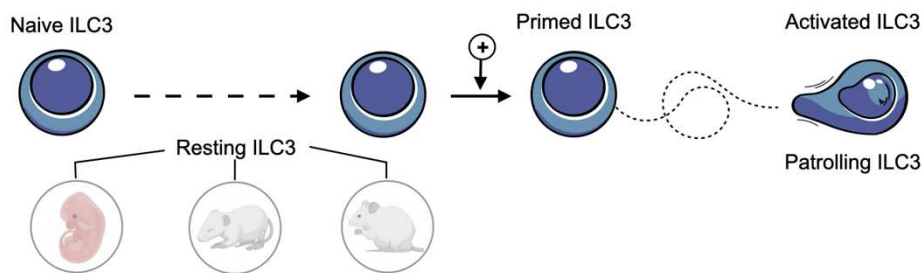
Patrolling ILC3: sentinels?

Although the majority of immune cells behave as 'explorers', including lymphocytes but also monocytes or neutrophils, there is one notable exception to this dynamic behavior. Antigen-presenting cells are generally sessile in tissue, unless induced to migrate in response to microbial or inflammatory stimuli, rather corresponding to 'sentinels' (80). Our observations revealed that intestinal ILC3 were mostly fixed at steady-state but adapted their migratory behavior to patrol the surrounding tissue upon gut inflammation. As such, tissue-resident ILC3 may be 'local sentinels' or 'gatekeepers' that would get rapidly activated but how this happens remains unclear (**Figure 19**). Mononuclear phagocytes express a variety of PRR that allows them to rapidly sense environmental cues, resulting in an activation program which allows them to upregulate CCR7 and subsequently migrate (65). Moreover, mononuclear phagocytes are disseminated throughout the intestinal tissue (88) and make extensions in the lumen (89, 90) which allows them to perceive stimuli. In contrast, murine ILC3 lack common PRR and require myeloid-derived signals for their activation. The dense network of mononuclear phagocytes within the intestine may explain how these cells quickly receive activating signals and patrol. In our system of intravenous administration of flagellin, we may generate a coordinated activation of mononuclear phagocytes since the signal is disseminated throughout the entire intestinal villi, resulting in synchronized ILC3 patrolling. It would be of interest to test whether oral administration of flagellin, instead of intravenous, could stimulate myeloid cells locally and lead to compartmentalized ILC3 patrolling within villi specifically at the site of antigen sampling. Taken together with the fact that ILC3 already express CCR9 under basal conditions (69) and remain in tissues following activation, we propose that ILC3 patrolling behavior may be in fact specific to the ILC family.

Obviously, since NK cells circulate, they are *de facto* apart from the other members of the ILC family in terms of migration. Still, viral infection stimulates NK cell patrolling within the bone marrow to a similar extent as flagellin triggers ILC3 patrolling within the intestine (91). In the skin, an innovative experiment

using two-photon live imaging described that dermal ILC2 patrol their local environment, consistent with a potential role in cutaneous immunosurveillance (92). Again, these cells showed a distinct migratory phenotype compared to cutaneous T cells. The movement of dermal ILC2 was characterized by brief migratory periods followed by extended pauses, resulting in an average speed similar to that of migratory dermal DC rather than to that of T cells. Another recent study using imaging of live viable precision-cut lung slices and lung intravital imaging revealed that pulmonary IL-13⁺ ILC2 accumulate in the perivascular and peribronchial regions after intranasal allergen or IL-33 challenge, thus indicating that ILC2 distribution can be modified in response to local environmental cues (93). Accumulation of ILC2 after IL-33 challenge was associated with increased ILC2 motility around blood vessels and airways. Most ILC2 exhibited ‘amoeboid-like’ exploratory movement and enhanced motility compared to pulmonary T cells. Chemokines and extracellular matrix (ECM) proteins were involved in the regulation of ILC2 dynamics, although the chemotactic pathways in question diverge from those classically implicated in T cells dynamics.

Model #1: ‘Sentinel’ ILC3



Model #2: ‘Explorer’ ILC3

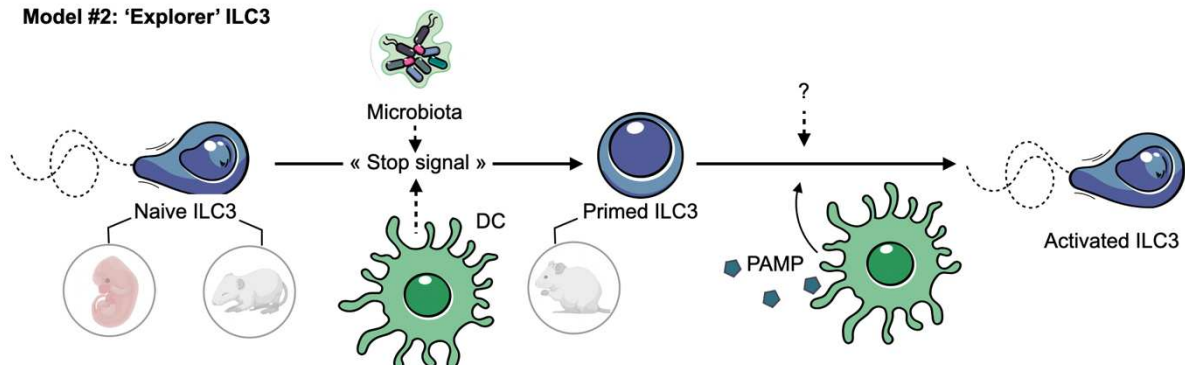


Figure 19 | A model for ILC3 patrolling: sentinels or explorers?

Migration of immune cells can be connected to two reductionist models: 1) A model of ‘sentinels’, based on antigen-presenting cell migration, in which cells are sessile in tissue and only migrate in response to microbial or inflammatory stimuli. 2) A model of ‘explorers’, based on lymphocyte migration, in which cells are motile under basal conditions to sample their environment and later respond with altered motility once they get activated. Intestinal ILC3 are sessile at steady-state but actively patrol in response to inflammatory signals, fitting to a ‘local sentinel’ model. Alternatively, intestinal ILC3 imaged in adults could actually represent previously patrolling cells undergoing priming to later migrate and respond to inflammatory signals, corresponding to the ‘explorer’ model. PAMP, pathogen-associated molecular patterns; DC, dendritic cell.

Therefore, members of the ILC family appear to share common migratory patterns and in particular, increased motility in response to tissue perturbation. Tissue-residency and environmentally-induced patrolling may define a novel migration program of 'gatekeeper' cells. ILC3 are pre-positioned in the intestine, which is a site of continuous exposure to environmental stimuli, and remain there to develop essential early immune responses which possibly include patrolling. Whether this cellular behavior is truly shared by other ILC remains to be determined. Indeed, dermal ILC2 locally patrol at steady-state (92) and this hasn't been evaluated for lung ILC2 (93). Future studies will contribute to our understanding of ILC migration and define whether a common ILC-intrinsic migration program exists or whether ILC migration is predominantly defined by their tissue environment.

B. Role of ILC3 patrolling in intestinal immunosurveillance

Tissue perturbations such as infection or inflammation promote migration of multiple immune cells, thereby being essential to the development of tissue-specific immune responses. For instance, CX3CR1⁺ monocytes crawl inside blood vessels and these patrolling monocytes rapidly invade tissues following tissue damage and infection (94). Tissue invasion is concomitant with the initiation of innate immune responses supported by a monocyte-to-macrophage program involving upregulation of pro-inflammatory mediators and wound-repair metalloproteinases. There is no reason to think that this general principle doesn't apply to patrolling ILC3. Adaptation of intestinal ILC3 migratory behavior in response to intestinal inflammation may have important functional consequences and we will now speculate on what these could be.

Patrolling ILC3: critical role for the barrier?

One key objective in intestinal immunosurveillance is to maintain epithelial barrier function. In the intestine, migration of $\gamma\delta$ T cells towards epithelial cells in response to enteric infection is essential to host defense, presumably through the maintenance of epithelial tight junctions (45, 46, 95). Similarly, ILC3 patrolling within the intestinal tissue may reinforces barrier function during the early phase of enteric infection, possibly through the diffusion of IL-22. Indeed, during infection, an effective IL-22-dependent epithelial response has critical consequences on mucosal barrier containment through the production of mucus and antimicrobial peptides (AMP) (33, 34). Consequently, ILC3 tissue scanning within intestinal villi could promote the 'delivery' of IL-22 to intestinal epithelial cells, thus promoting protection against enteropathogenic bacteria (**Figure 20**).

Recent studies address quantitative aspects of cytokine propagation to explain how cytokine cell-to-cell communication shapes and fine-tunes immune responses (96). Cytokine concentrations are heterogenous within tissues and determined by adaptable diffusion/consumption mechanisms (97). Briefly, cytokines are freely diffusing molecules and the size of the niche around producers depends mostly on the density and consumption rate of cytokine-consuming cells. Because cytokine-consuming cell close to the source have a greater probability of capturing the cytokine and to deplete it from the total pool, a cytokine gradient is established with the cytokine concentration decreasing when the distance from the producers increases. Typically, the aforementioned parameters vary over the course of an immune response, with variations of cytokine-producing and -consuming cells numbers as well as

affinity for cytokines. Interestingly, the IL-22R is found exclusively on epithelial cells within the intestine and it has been reported that IL-22R expression can be enhanced *in vitro* by IFN γ (98), so possibly under inflammatory conditions. Augmented IL-22R likely results in increased IL-22 consumption rates, with robust depletion of IL-22 from the tissue, leading to a diminution of the cytokine niche size. As such, IL-22 producing cells may need to head closer to their targets to diffuse properly the cytokine, which could explain the patrolling behavior of ILC3 within intestinal villi.

Most importantly, cytokine gradients have been shown to be coupled to functional gradients, thus contributing to pathogen clearance at barrier sites. Müller and colleagues have demonstrated in a pioneer study that CD4⁺ T cells rely on such cytokine gradients to control pathogens beyond sites where the initial antigen presentation occurs (99). Although CD4⁺ T cells contact a minority of infected cells, they promote inducible nitric oxide synthase (iNOS) expression in bystander cells within an 80 μ m radius of a CD4⁺ T cell thanks to by-stander activity of IFN γ , thus supporting clearance of the infection. Intracellular defense mechanisms induced by IFN γ were strongest in the vicinity of sites of antigen presentation and slowly declined with distance to these spots, showing that proximity with producers confers functional and defensive advantages. Therefore, tissue scanning by IL-22-producing ILC3 could allow them to provide sufficient IL-22 concentrations to ensure optimal IL-22R signalling and epithelial activation, further promoting barrier defense (**Figure 20**). To test this hypothesis, we could limit ILC3 patrolling via neutralization of CCL25 and assess barrier function in these conditions. Following treatment, we could look at impaired barrier permeability or we could seek for a reduction of IL-22 dependent signatures in IEC gene expression. Direct examination of cytokine activity *in vivo* is challenging but the development of new probes could allow to us to interrogate this aspect of cellular communication. For instance, probes have been generated to assess IFN γ activity such as a fluorescent reporter combining a STAT1-GFP fusion protein and a nuclear mCherry protein (100); however, this probe was introduced *in vitro* in tumor cells before injection and subsequent imaging. Since IL-22R is expressed by non-hematopoietic cells, different approaches need to be developed to examine IL-22 activity and understand the impact of ILC3 patrolling on the epithelial barrier.

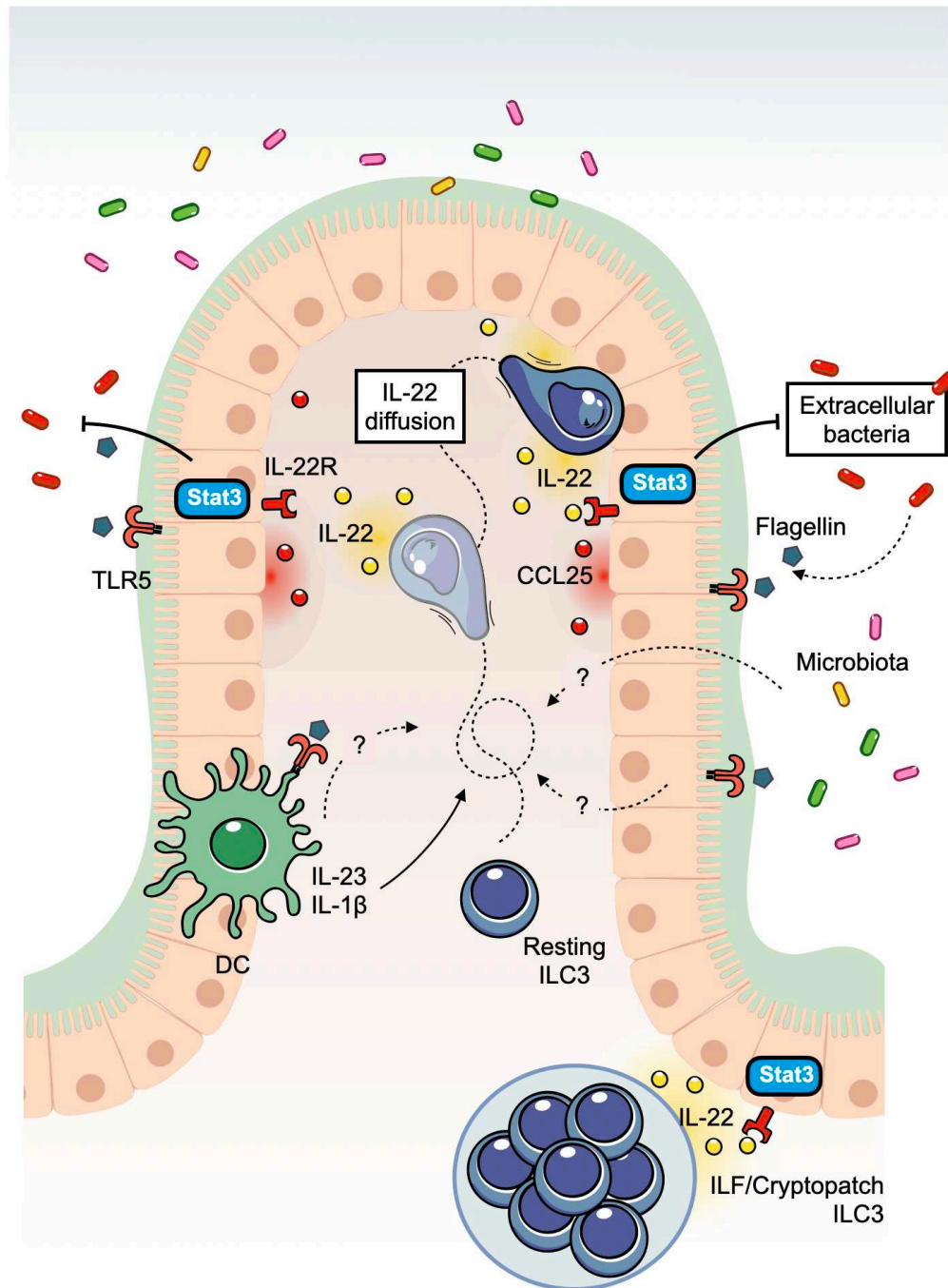


Figure 20 | Functional relevance of ILC3 patrolling of the intestinal barrier

During enteric infection, an effective IL-22-dependent epithelial response is critical to maintain the integrity of the mucosal barrier and consequently to promote host defense. Tissue scanning of ILC3 within intestinal villi following pathogen challenge could be important to support elevated IL-22 concentrations close to intestinal epithelial cells and the optimal induction of their functional programs. TLR5, Toll-like receptor 5; STAT3, signal transducer and activator of transcription 3; ILF, isolated lymphoid follicle; DC, dendritic cell.

Adaptation of ILC3 behavior: a must-have for coordinated intestinal responses?

In light of the above considerations, we propose that intestinal ILC3 patrolling is fully integrated with their IL-22 function and that monitoring of the intestine promotes proper epithelial barrier function. Thus, our study provides support for a coordinated tissue-immune cell response to environmental signals that requires adaptation of intestinal ILC3 responses.

Indeed, ILC3 are engaged in a fundamental epithelial-immune circuit which receives environmental inputs and constantly adapts to sustain intestinal homeostasis and immunity. The cornerstone of this circuit is the intestinal epithelial barrier, which lines a crucial interface to segregate the host and the external environment. Since the IL-22/IL-22R pathway regulates barrier function, ILC3 are ineluctably at the center of this circuit and orchestrate the crosstalk between immune cells and the intestinal epithelium. Surely, mononuclear phagocytes also play an important role in this circuit as myeloid-derived IL-23 is essential for IL-22 production.

Interestingly, the distinct constituents of this circuit cross-regulate each other, in order to achieve coordinated intestinal responses. Epithelial cells have been shown to control ILC3 activation and subsequent IL-22 production. As an example, rotavirus infection induces IEC-derived IL-1 α that directly enhances IL-22 production by ILC3, which is required for subsequent control of rotavirus infection (101). As such, the crosstalk between IEC and ILC3 appears bidirectional and required for optimal barrier defense responses and host protection. From our results demonstrating that CCL25 controls ILC3 patrolling, we speculate that epithelial cells may also determine ILC3 migratory behavior within the intestine through their CCL25 production, but this remains to be shown. A feedback loop may exist where CCL25 attracts IL-22⁺ ILC3 close to the epithelium to promote optimal IL-22 responses, including CCL25 production which in turn reinforces IL-22⁺ ILC3 proximity and barrier function. Such an IEC-ILC3 module, based in part on IEC activation, would allow to maintain the balance between regulatory and immune protective ILC3 functions. Moreover, inputs from the symbiotic microbiota can also modulate ILC3 activation and intestinal responses. At weaning, specific microbial species such as segmented filamentous bacteria (SFB) stimulate IL-23 production which activates intestinal ILC3 and promote IL-22 dependent responses (18, 102, 103). These transient IL-22 responses are essential to cope with the massive microbial colonization occurring at weaning through proper segregation, in order to maintain intestinal homeostasis. In contrast, in adults the microbiota sustains epithelial-derived IL-25 production which indirectly reduces innate IL-22, thereby favoring intestinal homeostasis (104). Whether coordination of intestinal ILC3 responses by the microbiota also applies to ILC3 patrolling behavior is unknown. In a set of preliminary experiments, we have shown that antibiotic treatment of adult *Rag2*^{-/-}*Rorc*^{GFP}*IL22*^{TdT} mice lessens ILC3 migratory behavior, indicating that the microbiota harmonizes intestinal ILC3 responses at several levels – including at least IL-22 production and patrolling.

Altogether, our work adds to the already existing body of evidence stating that a complex circuit stands in the intestine to achieve coordinated immune responses. Specifically, we underline a novel aspect of this circuit which is the adaptation of ILC3 migratory behavior. We have outlined above how different members of this circuit (IEC, DC, microbes) intermingle and could all be able to promote tissue adaptation of ILC3. Yet, in our study, one more member of this circuit has come into focus: T cells. We have found that T cells could regulate ILC3 patrolling, through direct competition for CCL25, but we

have also suggested that T cells could exert this function indirectly through modulating other actors of the intestinal circuit. Indeed, others have already observed that T cells adjust ILC3 activation (see general introduction). Briefly, T cells control the abundance and composition of the microbiota through *inter alia* T-cell dependent IgA production (35, 79). Moreover, T cells control ILC3-derived IL-22 through the aforementioned regulation of commensal bacteria but also through control of mononuclear phagocytes activity (18, 73, 74). Considering their role in modulating ILC3-derived IL-22, T cells are therefore also required for the induction or suppression of transcriptional programs in epithelial cells (18, 78). As such, T cells have the potential to promote tissue-adaptation of ILC3 and subsequent intestinal responses through multi-layered modulation of this complex circuit.

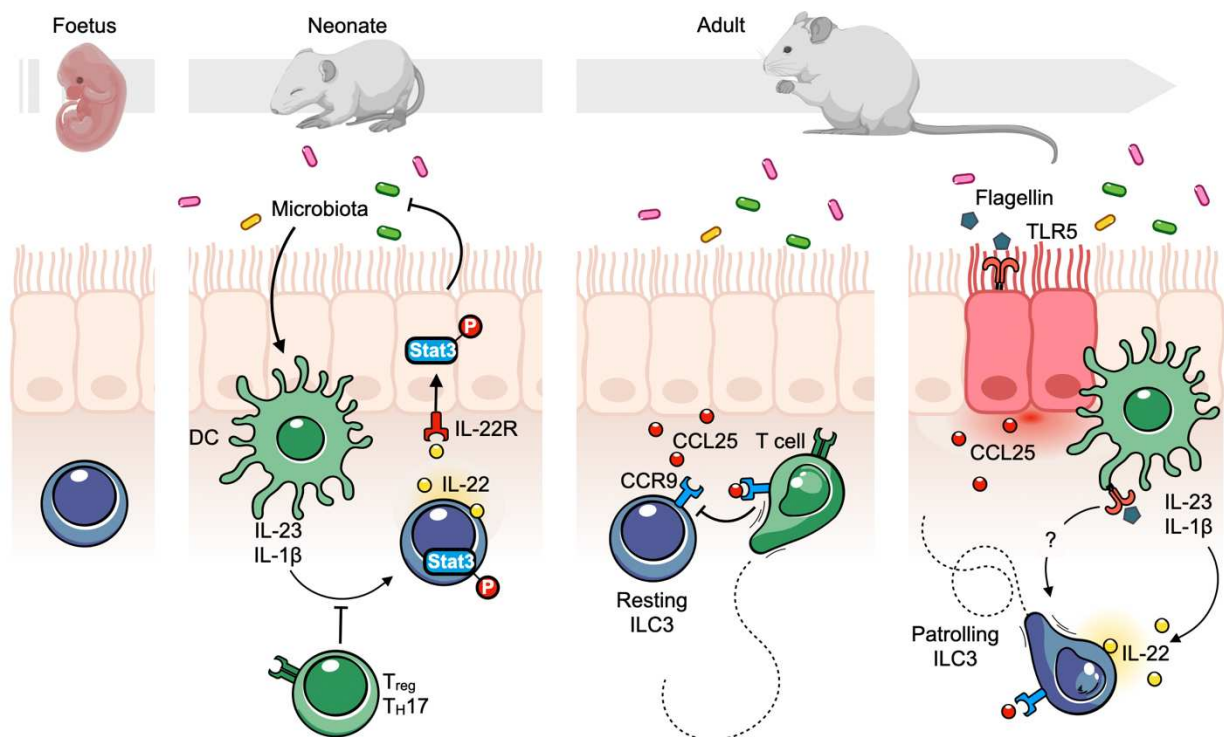


Figure 21 | Adaptation of ILC3 responses for coordinated intestinal responses across the life span

Tissular environmental signals shape intestinal ILC3 responses throughout life to promote appropriate immune responses and cope with change. During the weaning to adult transition period, microbial-derived signals indirectly stimulate IL-22 production in ILC3 to reinforce epithelial barrier function and spatial segregation. This process is then regulated by intestinal T cells which limit both ILC3 IL-22 production and ILC3 migratory behavior to support mucosal homeostasis, although through distinct mechanisms. Disruption of intestinal homeostasis, notably during pathogen challenge, leads to the activation of immune cells, including ILC3 which upregulate IL-22 production and patrol the intestinal barrier in a concerted effort to provide protective immunity against pathogens. DC, dendritic cell; STAT3, signal transducer and activator of transcription 3; T_{reg}, regulatory T cell; TLR5, Toll-like receptor 5.

We believe that this intestinal circuit could be important at multiple moments throughout life and notably at weaning to cope with the microbiota, at homeostasis to maintain regulatory responses and upon enteric infection to promote immunity. Mao and colleagues have previously proposed that innate and adaptive lymphocytes sequentially shape the microbiota and lipid absorption through control of IL-22 production by ILC3 (18). They have demonstrated that microbial colonization at weaning results in

robust ILC3 activation and IL-22 production. Innate IL-22 responses are then regulated by CD4⁺ T cells to maintain a balance and limit prolonged activation of IL-22 dependent programs in epithelial cells, which is mainly important to allow lipid metabolism according to the authors. Of note, despite the presence of T cells, ILC3 can still be activated and produce IL-22 upon enteric infection (3, 33). In a similar fashion, we propose that the microbiota promotes ILC3 patrolling during the weaning period in absence of T cells, which may prime these cells and allow them to complete their functional program. Cellular behavior of ILC3 is then regulated by T cells that surveil the intestinal tissue in order to maintain intestinal homeostasis, succeeding to patrolling ILC3 poised for rapid activation. Upon intestinal inflammation, T cell control over ILC3 is exceeded to allow essential immune responses through the recruitment of sentinels including patrolling ILC3. Indeed, the pathogen can disrupt the homeostatic intestinal circuit at several levels and lead to a reactive intestinal circuit. Bacterial infection simultaneously disturbs microbial communities, stimulates TLR-mediated activation IEC and DC – including IL-1 β /IL-23 but maybe also CCL25 – and subsequently ILC and T cell responses (62, 63, 105). Altogether, we see that this circuit comprises multiple layers of regulation that are capable of integrating environmental stimuli and adapting throughout life to achieve coordinated intestinal immune responses, including to tissue perturbation (**Figure 21**). Our results highlight prominent adaptation of intestinal ILC3 that promote patrolling of mucosal perturbations and raises several questions: does intestinal inflammation trigger tissue-adaptation of all the intestinal circuit members, and to which extent? Could we interrogate this circuit during the weaning to adult transition period, as proposed above? If tissue perturbation shapes ILC3 responses, what is the long-lasting impact on subsequent ILC3 responses?

Long-term fate of patrolling ILC3?

We have described that bacterial challenge provides naïve ILC3 with novel migratory attributes in the third chapter. In parallel, we have assessed the long-term fate of activated ILC3 in a *C. rodentium* infection model. In line with a long-lasting adaptation of ILC3 upon mucosal perturbation, we have shown that ‘trained’ ILC3 have a superior capacity to control infection compared to naïve cells and that this ameliorated protection is linked to their memory-like attributes including rapid proliferation and enhanced cytokine production that emerge and persist following initial pathogen encounter. Thus, we propose that changes in ILC3 localization and/or migratory behavior following primary challenge could persist and contribute to improved protection against reinfection (**Figure 22**).

While it was initially believed that secondary T cells responses were just faster and more effective but occurred similarly as primary responses – that is initiation in lymph nodes followed by migration into tissues, it has now become clear that memory T cells need to be pre-positioned in tissues to immediately intercept pathogens and ensure protective immune responses (106, 107). Specifically, effector T cells can differentiate *in situ* into memory T cells and remain in tissues without recirculating following infection, where they are referred to as resident memory T cells (T_{RM}) (106). Interestingly, re-localization of CD8⁺ and CD4⁺ T cells was observed in the skin following herpes simplex virus (HSV) infection. While CD8⁺ and CD4⁺ T cells were intermixed and dispersed throughout the skin during the initial phase of the response, segregation of CD4⁺ and CD8⁺ T cells to the dermis and epidermis respectively was noted during the memory phase (12). Resident memory CD8⁺ T cells (T_{RM}) within the epidermis provide enhanced local protection against subsequent HSV infection whereas circulating effector memory CD4⁺ T cells in the dermis are involved in global skin immunity, independently of the primary inoculation site (12, 108). Interestingly, these two populations also exhibit distinct migration patterns within the skin which may support their different functions in cutaneous immunosurveillance: intravital imaging revealed slow epidermal killer T cells (local responders) above rapidly trafficking dermal helper T cells (global responders) (12). As such, it appears that T cell localization and migratory behavior within tissues determines their role in immunosurveillance and is essential for optimal protection against reinfection through accelerated pathogen clearance. Specifically, in the small intestine, whether memory T cells localization and migration impacts their ability to mediate enhanced secondary responses against enteropathogenic bacteria is unknown. T_{RM} are present in large numbers in the small intestine, where they localize mainly within the epithelial layer and retain the ability to migrate within the intestine (106), but the functional significance of these observations remains unclear.

Still, we believe that intratissular distribution and migration represent important features of intestinal memory cells responses, including trained ILC3 responses. Taking advantage of existing our *Rorc*^{GFP} *Il22*^{TdT} reporters or combining this reporter with a fate-mapping strategy (e.g., *Id2Cre*^{E^{ERT2}} *Rosa26*^{EYFP}) to label persisting ILC3 specifically, we could assess the localization of trained ILC3 within the intestine by immunofluorescence and understand whether a possible different repartition of trained cells could confer a protective advantage against reinfection. In addition, either in our controlled *C. rodentium* infection model or in a newly developed model based on multiple flagellin injections, we could perform intravital imaging of trained ILC3 before and after rechallenge to evaluate whether trained ILC3 display unique migratory attributes that form an integral part of their durable functional changes and heightened

responses. In support of these hypotheses, pathway analysis of our transcriptomic data revealed modification of chemokine signaling in intestinal ILC3 after primary *C. rodentium* infection in accordance with adaptation of patrolling ILC3, but also after secondary infection (data not shown) suggestive of a distinct migratory behavior for trained ILC3.

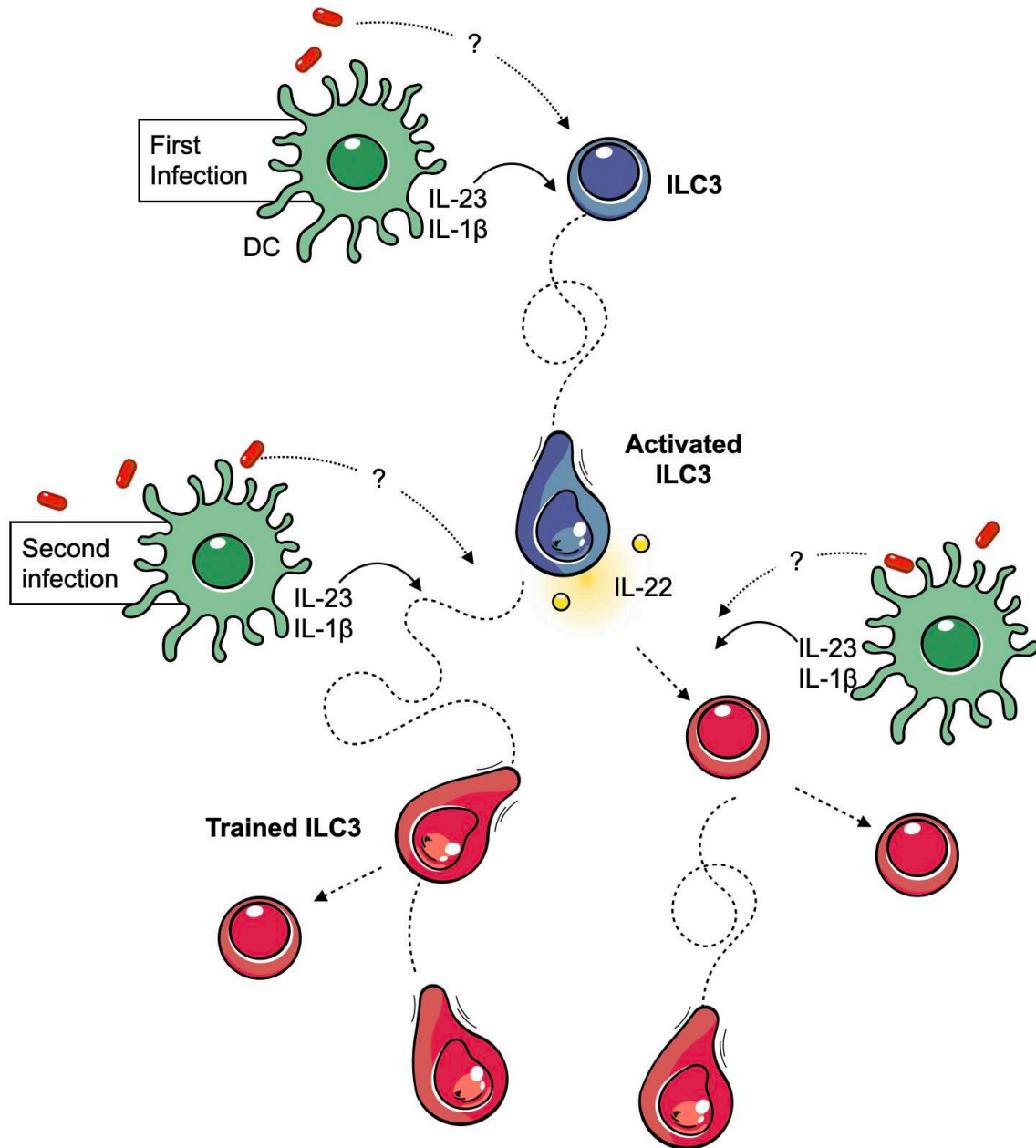


Figure 22 | Trained ILC3 patrolling: an advantage for rapid secondary responses?

Bacterial challenge triggers ILC3 patrolling of the intestinal barrier, which may have important functional consequences on barrier function, as well as generation of trained ILC3. Trained ILC3 persist long-term and exhibit enhanced IL-22 production upon reinfection. Persistent or enhanced ILC3 migratory behavior could contribute to improved protection against reinfection but their existence remains unexplored. DC, dendritic cell.

IV. ILC3 and immunological memory

In the second chapter of this thesis, we highlighted remarkable adaptation of intestinal ILC3 functions in response to tissue perturbation that promotes patrolling of the intestinal barrier. In the course of mucosal response to infection, effector T cells also play a fundamental role in immune protection and generate memory T cells that support the long-term immune-surveillance and recall responses to pathogens encountered. In the third chapter of this thesis, we assessed whether adaptation of intestinal ILC3 could persist long-term and result in heightened immunological function.

In this collaborative work, we demonstrated that limited pathogen exposure can promote durable functional changes in intestinal ILC3, which contributes to long-term mucosal defense (**Figure 23**). Here, we will further discuss ‘trained’ ILC3 properties and examine whether these are consistent with the development of immunological memory. Later, we will consider the role of ‘trained’ ILC3 in lymphocyte immune response to infection and the potential impact of our study.

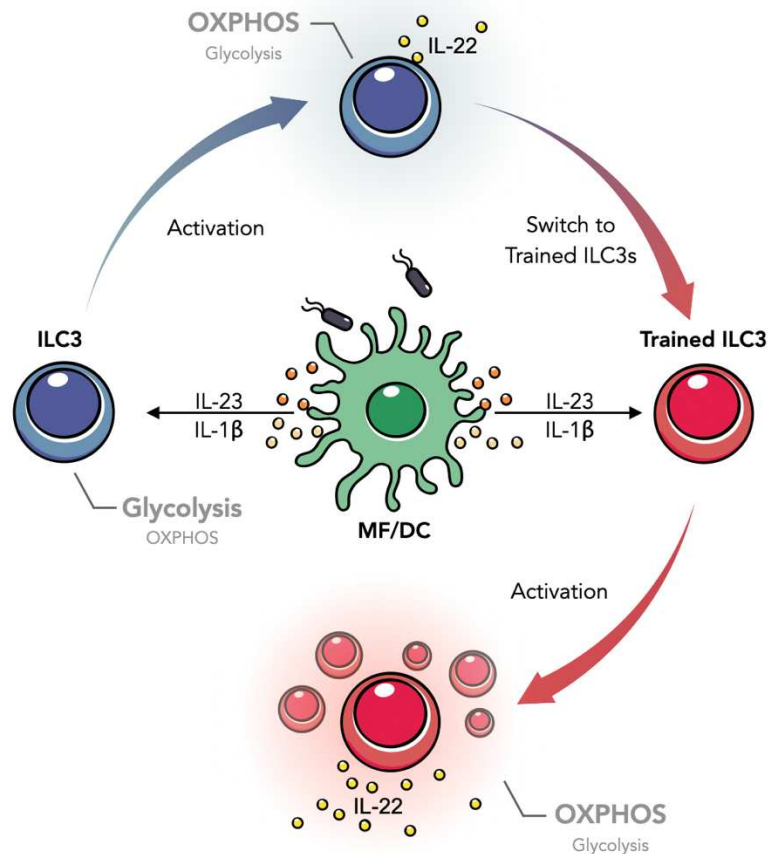


Figure 23 | Trained ILC3 responses promote intestinal defense

Intestinal ILC3 persist in an activated state following exposure to enteropathogenic bacteria. Upon re-challenge, these trained ILC3 rapidly proliferate and display enhanced IL-22 responses, resulting in a superior cell-intrinsic capacity to control infection compared to naïve ILC3. Generation of trained ILC3 is associated with a rewiring of their cellular metabolism. MF, macrophage; DC, dendritic cell; OXPHOS, oxidative phosphorylation.

A. Properties of 'trained' ILC3

Generation of trained ILC3

In our study, we evaluated the long-lasting impact of enteric infection on ILC3 activation and demonstrated that intestinal ILC3 establish 'memory-like' states that confer protection against reinfection. Yet, the precise signals driving ILC3 training remain undefined.

A hallmark of classical immunological memory is specificity (109). Given that ILC do not express clonotypic antigen receptors but rather rely on myeloid cues for their activation (60), they don't meet the criteria for specificity. Yet, antigen-specific NK cells have been shown to develop during cytomegalovirus (CMV) infection both in mice and humans (110), raising questions as to whether specificity may exist in our system. We investigated whether trained ILC3 responses were restricted to *C. rodentium* infection but re-challenge with the unrelated pathogen *L. monocytogenes* uncovered that there was no specificity of ILC3 recall responses. Accordingly, unlike memory NK cells that harbor receptors binding viral antigens (such as Ly49H in mice and NKG2C in humans) (111, 112), a specific receptor that drives ILC3 training and would preferentially be associated with 'trained' ILC3 compared to naïve ILC3 could not be identified from our RNAseq analysis. Of note, our bulk sequencing approach may not allow sufficient resolution regarding the existence of different IL-22 producing ILC3 populations that mediate protection against reinfection, such as 'trained' ILC3 but also newly activated ILC3. Therefore, a single cell approach could be helpful to prevent the mix of heterogeneous ILC3 populations and to rule out the existence of a specific receptor on trained ILC3. The lack of specificity of ILC3 recall responses supports the idea that ILC3 immunological memory is distinct from adaptive immune memory and more likely resembles innate immune memory or 'trained' immunity. Trained immunity can be generated in response to endogenous and exogenous stimuli, such as infection or inflammation (113). Contrary to other innate immune cells, all members of the ILC family lack common pattern recognition receptors (PRR) in mice (60). Therefore, while trained macrophages can be induced following recognition of pathogen-associated molecular patterns (PAMP) including β -glucan and LPS, training of ILC3 must take place in a different way (114). Memory-like NK cells and ILC2 have been shown to develop in responses to cytokines, in an antigen-independent fashion (115) (**Figure 24**). We tested the possibility that 'trained' ILC3 could be generated by cytokines alone, namely IL-1 β and IL-23, which could explain unspecific protection against diverse enteropathogenic bacteria. Indeed, both restricted to *C. rodentium* and *L. monocytogenes* infection trigger the release of IL-23 by intestinal mononuclear phagocytes, which is responsible for ILC3 activation and IL-22 production (3, 34, 116). Although IL-1 β and IL-23 stimulation effectively enhanced IL-22 production in activated ILC3, it failed to generate 'trained' ILC3 harboring all the memory-like features of these cells such as increased proliferation upon re-challenge.

Altogether, our results show that 'trained' ILC3 lack antigen-specificity and require signals beyond IL-1 β and IL-23 to be generated, presumably bacterial-associated signals (**Figure 24**). As murine ILC3 lack common PRR, direct bacterial recognition is unlikely but could occur through unidentified receptors. Alternatively, pathogenic bacteria can modulate multiple members of the 'intestinal circuit', as discussed before, and ILC3 indirectly. One way to understand the nature of these signals would be to evaluate the transcriptional and functional signatures of intestinal ILC3 to anticipate their responsiveness and, in

parallel, to perform tissue profiling by mass spectrometry to identify relevant candidates. For the moment, further investigations are required to define the signals essential to the generation of ‘trained’ ILC3.

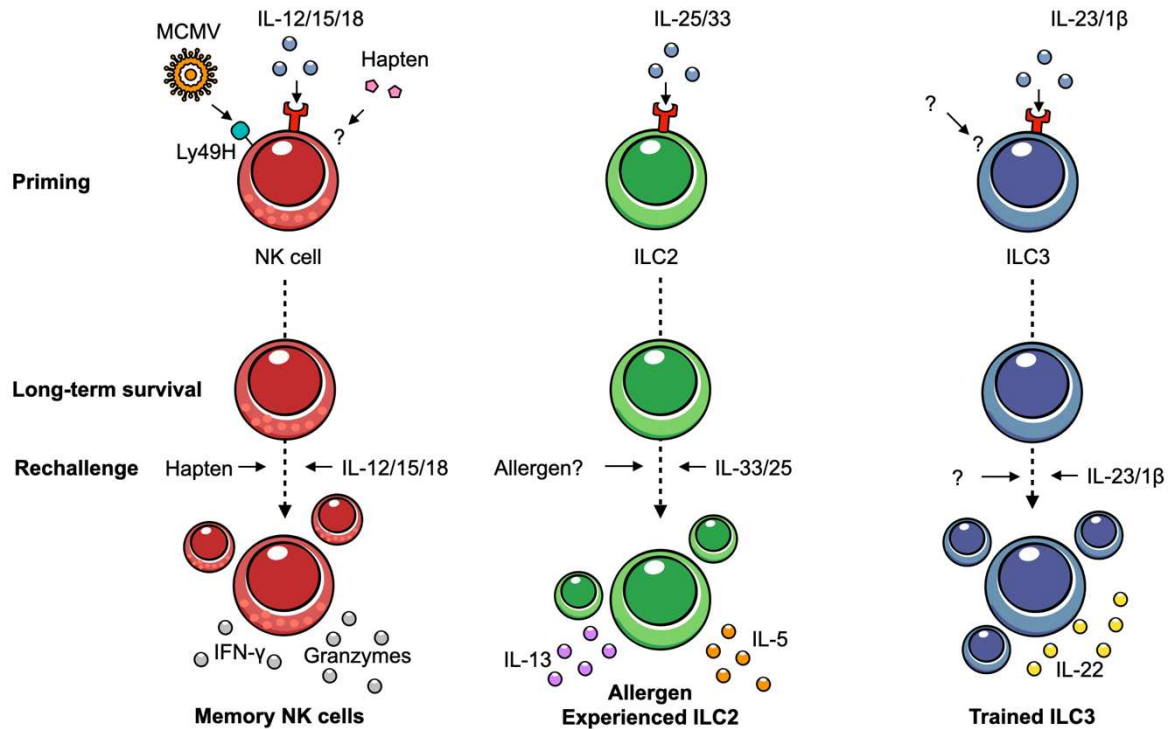


Figure 24 | ILC and immunological ‘innate’ memory

Diverse members of the ILC family show evidence of immunological memory at the functional level. Adaptive features were initially described for NK cells which can develop into both antigen-dependent and -independent long-lived memory NK cells. In contrast, ILC2 and ILC3 develop unspecific ‘memory’. While lung ‘experienced’ ILC2 display enhanced recall responses to allergens/cytokines following priming with cytokines, cytokines are not sufficient for the generation of intestinal ‘trained’ ILC3. Following initial pathogen encounter, long-lived ‘trained’ ILC3 can promote IL-22 mediated long-term intestinal defense against the same or an unrelated enteropathogen. MCMV, murine cytomegalovirus; IFN γ , interferon gamma.

Durability of trained ILC3 responses

A prerequisite for the generation of memory responses is that cells should be long-lived and maintained in absence of stimulation or antigen persistence (117). Although ILC are believed to persist long-term in tissues, we addressed whether this applies to our system and how ‘trained’ ILC3 are maintained.

We assessed the long-term maintenance of ILC3 in a cell fate-mapping approach relying on *Id2*^{CreERT2+} *Rosa26*^{RFP} mice to label ILC after infection. We showed that RFP⁺ ILC3 were maintained upon reinfection, indicating that ‘trained’ ILC3 can persist long-term. In support of long-term stable maintenance of ILC3, we observed a 2.4-fold increase in proliferation in ‘trained’ ILC3 compared to naïve ILC3 corresponding to an identical 2.4-fold increase in total ILC3 numbers, suggesting that ILC3 recall responses are supported by long-lived ILC3 rather than newly generated ILC3. To test the durability of ILC3 responses, we re-challenged mice 4 months after primo-infection – instead of one in all other experiments – and observed significant increase in ILC3 and IL-22⁺ ILC3 numbers together

with reduced bacterial loads, indicating that trained ILC3 responses are maintained for months which is compatible with the generation of innate immune memory. Interestingly, the impact of primary infection on ILC3 recall responses and subsequent protection was more pronounced at one month than four, in agreement with the transient nature of trained immunity. Of note, trained ILC3 were maintained in absence of antigen persistence as we used highly efficient antibiotic-treatment to rapidly clear the pathogen.

Trained immunity can be maintained in mononuclear phagocytes for a few weeks up to several months despite the fact that monocytes are short-lived cells (average half-life 5-7 days) (118). Recently, it has been shown that induction of trained immunity could take place in bone marrow progenitor cells as well as in blood monocytes and tissue macrophages, thereby making a distinction between central and peripheral trained immunity (119). Regarding tissue-resident ILC3, central trained immunity is unlikely because ILC are not maintained through replenishment from hematogenous sources, except for rare seeding of bone marrow ILCP perhaps but this hasn't been formally demonstrated. Rather, ILC are mainly self-renewing cells that are maintained and expanded locally (51), a property more consistent with peripheral immunity. However, this observation is not definitive: during infection, hematogenous cells would help to replenish the pool of resident ILC. This has been tested in the context of parasitic but not bacterial infection (51). In our experiments, we observed that RFP⁺ ILC1 and ILC2 were not maintained upon reinfection, which is not surprising for ILC1 as they contain circulating NK cells but is unexpected for ILC2. Decrease in RFP⁺ ILC2 following enteric infection could reflect circulation of ILC2, which has already been proposed in helminth infection (120), apoptosis and/or renewal from hematopoietic progenitors. In a series of unpublished experiments, we took advantage of the *Id2*^{CreERT2+} *Rorc*^{fl/fl} mouse model to interrogate maintenance of tissue-resident ILC3 upon bacterial infection. A previous study demonstrated that despite deletion of ROR γ t in mature ILC3, these cells persist in tissues (121) so we used this property to track ILC3 fate. Similarly to our fate-mapping experiment, we labeled ILC3 Δ ^{Rorc} after infection but with a 90% efficiency here. We found that these ILC3 Δ ^{Rorc} were maintained up to 4 months after infection and didn't detect newly generated ILC3^{Rorc}, indicating that ILC3 are essentially self-maintained even after bacterial infection. As such, the reduction of trained ILC3 responses observed at four months compared to one month is probably not the result of renewal of the ILC3 pool with 'untrained'/naïve cells. Instead, this phenomenon may reflect a progressive loss of ILC3 'trained' signatures imprinted by previous insults.

Signatures of trained ILC3

Memory or trained immune cell should be long changed following pathogen encounter, leading to a functionally enhanced response towards a second challenge (117). Epigenetic reprogramming of transcriptional pathways, including intracellular immune signalling but also cellular metabolism, mediates trained immunity in innate immune cells (118). Consequently, signatures indicative of trained cells functional reprogramming can be assessed at three levels minimum: epigenetic, transcriptional and metabolic.

In our study, we have not initially performed analyses of epigenetic modifications in 'trained ILC3' due to their limited number during the 'contraction' phase (<10⁵ cells). Instead, we have directly

performed transcriptional analyses of 'trained' ILC3 which provides information on pathways that might be targeted by epigenetic modifications. We could discern many persisting changes in gene signatures, including variations in cell cycle and cytokine/receptor interaction pathways consistent with our functional data. Most differentially expressed genes belonged to multiple metabolic pathways, in accordance with recent studies showing that metabolic rewiring in response to external stimuli is essential for the generation of trained immunity. Strikingly, while increased aerobic glycolysis is a hallmark of trained monocytes and is required for their enhanced responsiveness (118, 122) we didn't observe reprogramming of cellular metabolism towards this energetic pathway and this was confirmed in our extracellular flux analysis of glycolysis. The fact that trained ILC3 don't upregulate glycolysis, in contrast to trained monocytes but also effector T cells, and that they already express many glycolysis-associated genes could be the result of the tissue-residency of these long-lived cells within an activated environment suggesting that naïve ILC3 are already 'primed' cells in a way. Our transcriptomic analysis of metabolic networks revealed a metabolic shift towards enhanced tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), fatty acid synthesis and oxidation-associated gene expression (**Figure 23**). Accordingly, our extracellular flux analysis of mitochondrial respiration validated these observations and revealed that OXPHOS was essential for IL-22 production in both naïve and trained ILC3, suggesting that augmented OXPHOS is required for heightened ILC3 functions. A growing body of evidence suggests that sustained-dependence on OXPHOS is a specific feature of 'type 3' immune responses, as this has also been reported for $\alpha\beta$ T_H17 cells and IL-17⁺ $\gamma\delta$ T cells (123, 124). We also reported that 'trained ILC3' upregulate nutrient uptake to support IL-22 production, most likely through mitochondrial bioenergetic pathways. Interestingly, naïve ILC3 can exploit additional metabolic sources for their function whereas trained ILC3 seem less versatile and restricted to the use of specific energetic pathways. This could reflect that the cellular metabolism of trained ILC3 is 'epigenetically' constrained compared to that of naïve ILC3. Altogether, we found that the metabolic rewiring of trained ILC3 appear to support their IL-22 production via heightened cell fitness. Interestingly, this metabolic shift was observed after primary infection and maintained in trained ILC3. This suggests that trained ILC3 acquire stable modifications of their transcriptional and metabolic pathways upon primary infection and remain continually altered, reminiscent of stable epigenetic reprogramming in trained innate immune cells.

Epigenetic reprogramming refers to sustained changes in gene expression or activity and cell physiology which do not involve permanent genetic alterations (125). This process involves several mechanisms (histone modifications, chromatin modification, DNA methylation, etc.) and is affected by the environment (125). Therefore, epigenetic remodeling provides a good mean to promote training immunity in response to infection but is only transient, resulting in short-lived innate immune memory. Many key effector genes are thought to be already accessible at the chromatin level in ILC because these cells are rapid responders and exhibit remarkable plasticity (126–128). Nonetheless, altered epigenetic landscape could support transcriptional and functional reprogramming of ILC3, including of metabolic genes, and account for their novel 'memory-like' properties. Performing an analysis of epigenetic remodeling at genetic loci will be useful in the future to further confirm the distinct memory phenotype of trained ILC3 and to further understand the mechanisms underlying enhanced ILC3 recall responses. Moreover, since primary infection itself can induce epigenetic and consequently

transcriptional responses (129), an in-depth analysis of our transcriptomic data comparing trained ILC3 to activated ILC3 will also improve our understanding of ILC3 training. Of note, all these reprogramming events (epigenetic, metabolic, transcriptomic, functional) are closely intricately. Indeed, previous studies have revealed that the active interplay between cellular metabolism and epigenetics is necessary for the induction of trained immunity (118). Metabolites derived from bioenergetic pathways modulate the activity of chromatin-modifying enzymes involved in epigenetic reprogramming and promote enhanced gene expression, thus facilitating recall responses (118, 129). In trained monocytes, initiation of these mechanisms relies on PRR signalling; hence how this could be initiated in ILC3 remains to be determined.

Contribution of trained ILC3 responses to tissue protection

In a broad sense, immunological memory refers to the ability of the immune system to store and recall information of previous insults, leading to a modified secondary response. While immunological memory was originally considered to be an exclusive feature of adaptive immune cells, it has now become clear that multiple additional lineages show evidence of immunological memory at the functional level. Global memory responses are observed at the tissue level, with several layers of protection (Figure 25). Three of them are particularly relevant for protection against *C. rodentium* infection:

- 1) Memory T cells, as T_H17 are an important source of IL-22 which is essential for host protection against *C. rodentium* (130)
- 2) Trained myeloid cells that could theoretically provide essential signals for the activation of IL-22 producing ILC3 and T cells
- 3) Trained epithelial stem cells which have recently been shown develop memory in the skin and the gut, resulting in accelerated responses (131, 132) and possibly in a stronger intestinal barrier to subsequent challenge.

In addition, we have described 'trained' properties for ILC3 which are particularly relevant in the context of long-term protection against enteropathogenic bacteria. Our initial observation was the association between reduced bacterial loads upon reinfection and enhanced IL-22⁺ ILC3 responses. In light of the above considerations, protection against reinfection could be the result of a trained barrier simply independently of immune responses, of trained macrophages with a superior capacity to secrete IL-23 and therefore to induce IL-22 responses, of antigen-specific memory T cells or of trained ILC3 with a cell-intrinsic superior capacity to produce IL-22⁺. In a series of experiments on *Rag2*^{-/-} mice, we showed that the generation of trained ILC3 responses and reduction of bacterial loads can happen in absence of T cells and depended on IL-22 as expected. Because IL-22 mediated protection could not solely be attributed to ILC3 but also to enhanced myeloid-derived signals or enhanced IL-22 dependent epithelial responses, we performed adoptive transfers and demonstrated that trained ILC3 had a superior cell-intrinsic capacity to protect the host compared to naïve ILC3 when in a similar environment regarding myeloid and epithelial activation. As such, long-term adaptation of ILC3 represents a complementary layer to global memory tissue responses and confers a survival advantage to the host.

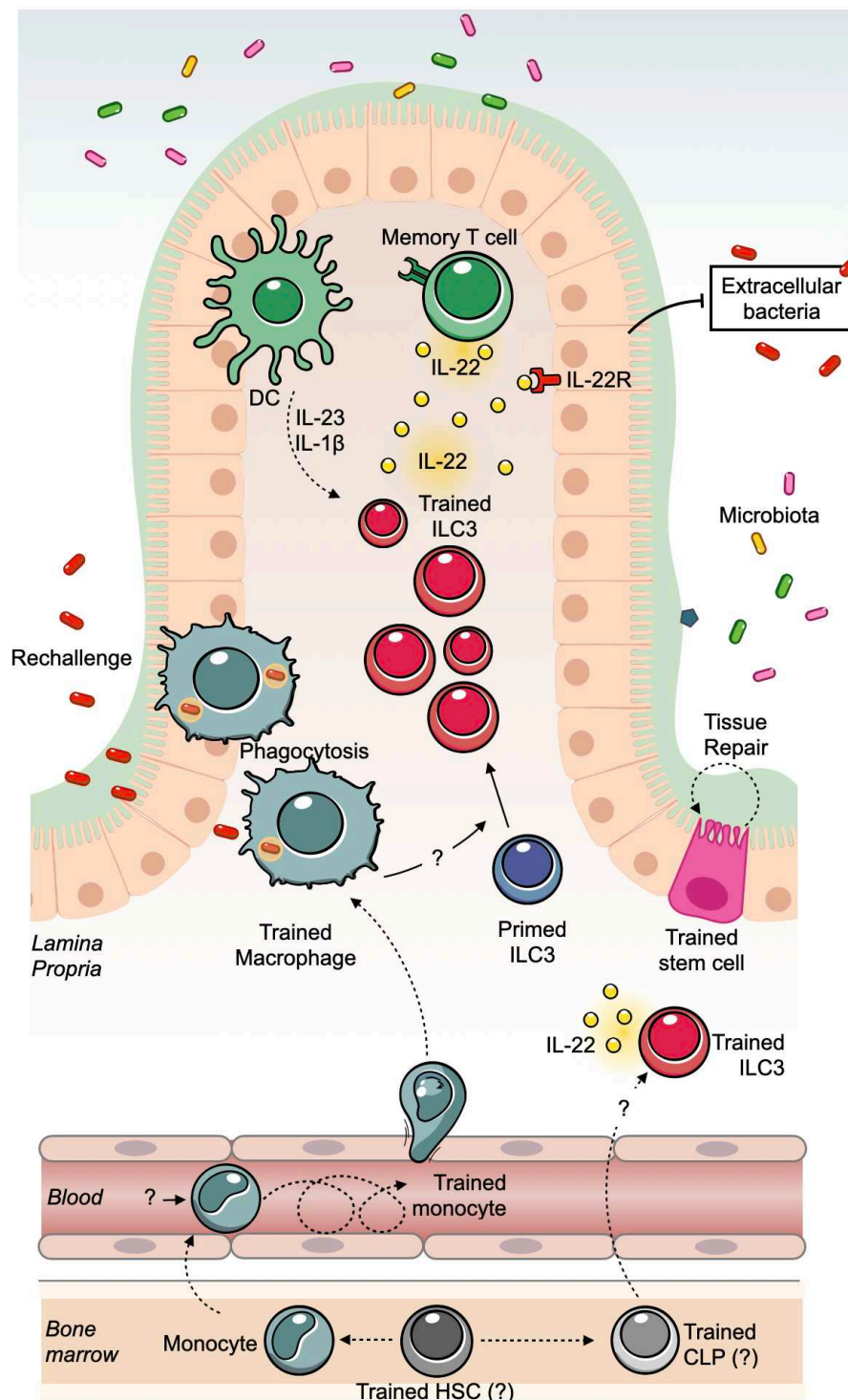


Figure 25 | Global tissue-memory

In contrast to the classical view of immunological memory, multiple lineages appear to develop immunological memory of previous insults and to contribute together to global memory responses at the tissue level. In addition to memory T cells, innate immune cells such as monocytes or ILC3 can be trained within tissues. Moreover, training of cells from the non-hematopoietic system have appears to take place, at least in epithelial stem cells. Altogether, memory and trained cells provide several layers of protection against subsequent insults, notably in the context of immunity against extracellular bacteria. DC, dendritic cell; HSC, hematopoietic stem cell; CLP, common lymphoid progenitor.

B. Role of ‘trained’ ILC3 in intestinal responses

Reactivation of ILC3: a form of immunological memory?

During an immune response, several critical objectives must be attained: to specifically recognize invading micro-organism, to react quickly to limit tissue damage, to efficiently eliminate the disease-causing pathogen and to establish long-lasting immunological memory in the case of pathogen re-encounter. An effective immune response that achieves all these goals requires a concerted effort between different cell types that sense pathogens and then alert effector cells to complete the process.

During enteric bacterial infection, both ILC3 and T cells are critical players in the early and late stages of the intestinal immune response, respectively. While the role of memory T cells is well recognized in recall immunity (109), ILC3 were not previously thought to develop into long-lived memory cells that can participate in recall immunity. Here, we have shown that ILC3 in the intestinal mucosa, when activated during enteric infection, generate cells that have ‘memory-like’ properties compared to their naïve counterparts that we have denominated ‘trained ILC3’. Similarly to memory T cells, these long-lived ILC3 exhibit durable phenotypic and functional changes and participate in recall responses to promote long-lasting intestinal defense. Although trained ILC3 can display individual features of memory T cells, there are some notable differences that set these two subsets apart. First, while activation of effector T cells is antigen-driven and generate memory T cells with antigen specificity (109), ILC3 do not respond to antigen but to cytokines (and apparently additional signals) and show no specificity of their recall responses. Second, in contrast to T cells which undergo selection for clonal expansion and memory, there appears to be no selection for the generation of ‘trained’ ILC3. This implies that a smaller number of memory T cells clones may be maintained compared to ‘trained’ ILC3 numbers (133). Third, T cell memory extends over years (134) whereas trained ILC3 responses lasts months post-exposure but seems transient, thereby being shorter-lived than classical immunological memory. With these differences in mind, we speculate that innate ILC3 memory and adaptive T cell memory can have distinct functional consequences that we discuss below.

Functional relevance of trained ILC3 responses

In our study, we have identified an essential role for intestinal ILC3 trained responses in protection against both *C. rodentium* and *L. monocytogenes*. Contrary to adaptive recall responses which provide protection against a specific pathogen, our results suggest that trained ILC3 potentially provide protection against a wide variety of pathogens. Because the intestine is continuously exposed to external stimuli, the development of cross-protective responses is particularly suited for intestinal immunosurveillance. Furthermore, the vast majority of enteropathogens induce essential IL-22 responses for the maintenance of the intestinal barrier, even though host survival does not strictly depend on IL-22 for all enteropathogens (101, 116, 135–140). Therefore, IL-22 mediated protection of trained ILC3 appears fundamental in the intestine. Our experiments using *C. rodentium* and *L. monocytogenes* illustrate well this point: despite the fact that only protection against *C. rodentium* is strictly IL-22 dependent, both pathogens induce IL-22 responses and trained ILC3 responses consequently improve pathogen clearance in both models (data not shown). It would be of interest to

identify enteropathogens with IL-22-unrelated immune responses and to see whether trained ILC3 can be generated and protect the host in this context. A possible candidate could be enteropathogenic *Yersinia pseudotuberculosis*: a recent report has shown that *Il22*^{-/-} mice are resistant to *Y. pseudotuberculosis* infection and that infection does not induce intestinal IL-22 expression, indicating that *Y. pseudotuberculosis* protection does not require the involvement of IL-22 – even though this is an extracellular pathogen (141). Of note, broad protection against pathogens is not an exclusive feature of innate immune cells. Cross-reactive memory T cells may confer heterologous immunity (142) and most importantly, pathogen-independent memory T cells can also provide nonspecific host resistance against infection such as *Toxoplasma gondii* infection in absence of pathogen recognition, notably through IL-12-dependent IFN γ production and promotion of CD4⁺ T cell responses (143). This suggests that both ILC and T cell-derived pathogen-independent cytokine production could contribute to broad protection against enteropathogenic bacteria, further blurring the traditional boundaries between innate and adaptive immunity.

Additional hypotheses for the biological relevance of trained ILC3 responses include the possibility to mount higher global secondary intestinal responses. After primary infection, memory T cells can either become circulating and re-circulate through secondary lymphoid organs (central memory T cells; T_{CM}) as well as tissues (effector memory T cells; T_{EM}) or become tissue-resident (resident memory T cells; T_{RM}) (107). Because T_{RM} cells are strategically pre-positioned within tissues, they are crucial players of early recall responses but represent only a fraction of the memory T cell pool (106, 107). As such, trained ILC3 which are rapidly poised for reactivation could contribute to intestinal responses to prevent a gap in early immunological protection. Moreover, the existence of short-lived trained ILC3 responses bear supplemental advantages. Because epigenetic remodeling presumably drives ILC3 trained immunity, trained ILC3 responses could adapt to the multiple challenges encountered throughout life and are *de facto* more flexible than adaptive immune cells whose responses are restricted to a single antigen. Consequently, for each insult, different memory T cell clones must develop whereas the same trained ILC3 could respond which leads to the question of space. Limitations in the size of the niche and access to survival factors probably restrain the number of memory T cell clones that can be maintained, thus emphasizing the requirement of trained ILC3 responses on the side. Along these lines, if trained cells are more flexible and can rely on a variety of signals, maintaining large numbers of these cells may be achieved easier for the host. Last of all, trained ILC3 responses might be important for the protection from autoimmunity. In our experimental approach, we used antibiotic-treatment to limit the extent of T cell memory responses. In these settings, we were able to generate trained ILC3 which promote pathogen control. Early control of infections mediated by trained ILC3 could limit subsequent activation of T cells, and notably repeated activation, and the numbers of differentiated T cells. Differentiated T cells pose a constant threat of autoimmunity to the host due to their ability to readily elicit effector function (lower TCR-activation threshold) and to enter peripheral tissues. As such, trained ILC3 responses may inhibit the onset of autoimmune responses. This last argument is purely speculative and should be taken cautiously as excessive trained immunity has rather been associated with autoimmune diseases notably autoimmune disorders (118).

Trained ILC3 responses and therapy?

Our observations on trained ILC3 in the context of subclinical *C. rodentium* infection could be relevant to human health. Indeed, our approach to identify trained ILC3 using ciprofloxacin does seem pertinent considering the fact that ciprofloxacin is currently used for treating *Escherichia coli*-induced mild/severe diarrheas (144, 145). In light of these considerations, it is probable that trained ILC3 responses also develop in humans in response to enteropathogenic infections. Trained ILC3 may provide a novel avenue for immune manipulation for prevention or treatment of disease caused by pathogens that invade barrier surfaces or inflammatory processes.

In humans, the first evidence for trained immunity has been partially provided by the field of vaccination. In fact, non-specific effects of vaccines are supported by a number of epidemiological studies. Immunization with live attenuated vaccines, such as bacillus Calmette-Guérin (BCG), reduces mortality from non-target diseases (146). Subsequent trials with the BCG vaccine have shown that this vaccine induces non-specific protection from reinfection, notably through activation of innate immune cells like monocytes but also NK cells (147, 148). Whether vaccines generate trained ILC3 responses responsible for non-specific effects of vaccination is completely unknown but should be taken in consideration when evaluating the effects of licensed human mucosal vaccines such as oral polio vaccines. While central trained immunity has been described for monocytes and could provide a mean to robustly prime adaptive immune responses against a variety of pathogens, the impact of apparently shorter-lived trained ILC3 responses on vaccination-mediated long-term protection is less clear. Longitudinal studies in mice will help us to define the lifespan of trained ILC3 responses and a deeper understanding of how these responses are generated could be useful to improve the durability of these responses and translate our research to humans.

Alternatively, trained ILC3 responses may be detrimental in the context of intestinal inflammatory disorders such as inflammatory bowel disease (IBD). Detrimental ILC3 responses have reported in several mouse model of colitis including pathogen-induced innate colitis (57, 149). Therefore, we could investigate whether trained ILC3 responses in response to pathogens – *C. rodentium* or a different enteropathogenic bacteria more prone to induction of polyfunctional ILC3 responses – promote the development of colitis. Several studies have shown that ILC3 numbers and functions were dysregulated in IBD patients compared to healthy individuals, emphasizing the importance of ILC3 (150). If excessive trained ILC3 responses are observed in colitis, temporarily suppressing them might help to break the cycle of inflammation and could ameliorate patient outcomes. Moreover, it is possible that trained ILC3 responses promote epithelial stem cells homeostasis and tissue repair, although we have not addressed this question in our study. The short-lived nature of trained immunity might represent an advantage in this context: temporarily limiting trained immunity pharmacologically would limit inflammation and restoration of trained immunity through treatment termination would then promote repair of previously damaged tissue.

Interestingly, epigenetic rewiring following monocyte priming by LPS can lead to the generation of 'tolerized' macrophages (113, 114). It would be of interest to see whether specific signals may induce tolerance programs in ILC3 to be able to exploit this function in therapy. An ideal tolerance program for ILC3 would allow to maintain reasonable levels of IL-22 for intestinal homeostasis while avoiding

uncontrolled IL-22 activation or decreasing pathogenic functions previously associated to IBD such as IFN γ and IL-17 production (151, 152). As such, inducing tolerance in ILC3 could help to treat intestinal inflammatory disorders. In contrast, defective trained immunity resulting from a tumor microenvironment could prevent effective immune responses, as it has been shown for myeloid cells (153). Enhancing trained ILC3 responses may help to fight cancer, but ILC3 responses in the tumor environment should first be carefully examined. Indeed, inflammation has also been associated with tumors and the role of IL-22 and ILC3 in cancer remains poorly understood, but the tissue environment is likely to regulate these responses. Therefore, it will be important to assess ILC3 responses in this context and their influence on tumorigenesis to determine whether trained ILC3 responses should be promoted or inhibited.

Overall, there is still a long road ahead for immune manipulation of trained ILC3. In order to harness their full potential, we must comprehend several aspects of trained ILC3 including the mechanisms that drive the generation of these cells, the durability of their responses, the nature of their responses in different contexts which implies the mechanisms that regulate them and their role in human health. Hopefully, this work and future collective work will increase our understanding of intestinal ILC3 responses and lead to novel therapeutic approaches for intestinal diseases that are relevant for patients.

CONCLUDING REMARKS

In summary, my thesis provides new elements to better understand how ILC3 function is regulated in the intestine in response to pathogens from a 'spatio-temporal' standpoint. We first uncovered a previously unrecognized coordinated intestinal ILC3/T cell response to inflammation that promotes monitoring of the intestine by patrolling ILC3, presumably supporting proper epithelial barrier function. We then described memory attributes for intestinal 'trained' ILC3 that emerge and persist following initial pathogen encounter, with an essential role in long-term mucosal defense.

Throughout our work, we have highlighted prominent tissue-adaptation of intestinal ILC3 to environmental signals promoting protective immune responses within the intestine. Our findings raise new questions regarding the compartment-specific features of intestinal ILC3, the biological relevance of ILC3 migration as well as the long-term fate of ILC3 in diverse contexts. Our research also presents original approaches in complement to existing functional analyses for the investigation of ILC3 behavior and of their role in intestinal immunosurveillance.

In the long term, identification of novel functions and regulatory pathways influencing ILC3 responses may be relevant to human mucosal immunity. Indeed, several studies have reported altered ILC3 numbers and functions in intestinal disease such as Inflammatory Bowel Disease (IBD). As such, harnessing ILC3 potential may help to develop novel therapeutic approaches for intestinal diseases in the future.

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APPENDIX

SUMMARY

The intestinal barrier comprises a layered defense system involving epithelial and immunological strategies which are critical for both tolerance and protection against potential pathogens. In contrast to the clearly established role for the adaptive immune system in maintaining this balance, knowledge on how the innate immune system generates regulatory and protective immune responses within the intestine is less defined. In particular, type 3 innate lymphoid cells (ILC3) include tissue-resident lymphocytes that critically regulate intestinal homeostasis and immunity. Through their ability to rapidly produce large amounts of cytokines and their strategic location, ILC3 promote rapid and essential innate immune responses. In my thesis, I have addressed the spatio-temporal regulation of intestinal ILC3 to uncover essential regulatory mechanisms controlling ILC3 responses and understand how ILC3 balance immune regulation with protective responses against pathogens within the gut.

First, we described a novel experimental approach combining ILC3 reporter mouse models with intravital imaging to gain new insight into ILC3 cellular behavior under basal or inflammatory conditions. We revealed compartment-specific features of ILC3 within the intestine. While ILC3 are largely immotile under steady-state conditions, villus ILC3 acquire novel migratory attributes and enhanced cytokine expression in response to inflammation. Diverse environmental cues, such as local endogenous and exogenous signals, modulate intestinal ILC3 behavior. Specifically, we provided evidence that ILC3 and T cells compete for local signals, notably for the chemokine CCL25, which ultimately defines ILC3 'patrolling' and represents a key regulatory mechanism of ILC3 biology *in vivo*. Our results highlight significant differences in ILC3 and T cell behavior with prominent adaptation of intestinal ILC3 that promote patrolling of mucosal perturbations.

Second, we assessed the long-lasting impact of pathogen exposure in the intestine on ILC3 responses using the mouse enteropathogen *Citrobacter rodentium*. We found that intestinal ILC3 persist for months in an activated state after pathogen clearance. Limited encounter with *C. rodentium* promotes durable phenotypic and functional changes in intestinal ILC3, including metabolic reprogramming. Upon re-challenge, previously exposed or 'trained' ILC3 proliferate, display enhanced interleukin (IL)-22 responses and have a superior cell-intrinsic capacity to control infection compared to naïve ILC3. Our studies underline 'memory-like' properties of intestinal ILC3 that emerge after bacterial infection and that allow them to contribute more efficiently to intestinal immune defense. Of note, we observed elevated ILC3 recall responses against multiple intestinal pathogens, suggesting the importance of 'trained' ILC3 in long-term protection against a wide variety of pathogens.

Collectively, our work defines the spatio-temporal regulation of ILC3 in the intestine. We uncover prominent tissue-adaptation of intestinal ILC3 to environmental signals promoting protective immune responses within the intestine. A better understanding of the regulatory pathways influencing ILC3 biology may lead to future therapeutic approaches for intestinal diseases.

Keywords: Type 3 Innate Lymphoid Cells (ILC3), Mucosal Immunity, Intestinal Immunosurveillance, Lymphocyte Migration, Intravital Imaging, Trained Immunity, Bacterial Infection

RÉSUMÉ

La muqueuse intestinale comporte plusieurs lignes de défense, dont la barrière épithéliale ainsi que le système immunitaire, afin de garantir la protection contre de potentiels agents pathogènes tout en assurant l'homéostasie par le maintien de la tolérance. Contrairement au rôle clairement défini du système immunitaire adaptatif dans le maintien de cet équilibre, celui de l'immunité innée dans la mise en place de réponses tolérogènes ou défensives au sein de l'intestin demeure peu compris. Au sein de ce système, on trouve notamment les cellules lymphoïdes innées de type 3 (ILC3), des lymphocytes résidant dans les tissus et impliqués dans la régulation de l'homéostasie et de l'immunité intestinales. Grâce à leur production rapide de grandes quantités de cytokines ainsi qu'à leur emplacement stratégique, les ILC3 jouent un rôle essentiel dans la réponse innée mucoale. Au cours de ma thèse, j'ai étudié la régulation spatio-temporelle des ILC3 intestinales afin de découvrir des mécanismes régulateurs des réponses ILC3 et de comprendre comment celles-ci soutiennent l'équilibre entre l'activation et la régulation de la réponse immunitaire dans l'intestin.

Dans un premier temps, nous avons établi une nouvelle approche expérimentale combinant l'utilisation de souris rapportrices des ILC3 et l'imagerie intravivale, permettant d'étudier le comportement des ILC3 intestinales dans des conditions basales ou inflammatoires. Nous avons observé que les fonctions des ILC3 sont compartimentalisées au sein de l'intestin. Alors que les ILC3 sont constitutivement immobiles, elles migrent dans les villosités intestinales et augmentent leur expression de cytokines lors d'une inflammation. Ce comportement des ILC3 intestinales est modulé par divers signaux environnementaux, tels que des signaux locaux endogènes et exogènes. Plus précisément, nous avons montré qu'il existe une compétition entre les ILC3 et les lymphocytes T pour des signaux locaux, notamment pour la chimiokine CCL25, qui détermine *in fine* la migration intratissulaire des ILC3 et constitue ainsi un mécanisme de régulation clé des ILC3 *in vivo*. Nos résultats mettent en évidence des différences biologiques majeures entre les ILC3 et les lymphocytes T, et notamment les capacités d'adaptation considérables des ILC3 intestinales qui scannent le tissu lors de perturbations de la barrière intestinale telles des cellules sentinelles ou « en patrouille ».

Dans un second temps, nous avons évalué l'impact de l'exposition aux pathogènes au sein de l'intestin sur les futures réponses ILC3 en utilisant le pathogène intestinal murin *Citrobacter rodentium*. Nous avons montré que les ILC3 persistent dans l'intestin dans un état activé pendant des mois suivant une infection et l'élimination du pathogène. Une exposition limitée à *C. rodentium* induit des modifications durables du phénotype et de la fonction des ILC3 intestinales, dont une reprogrammation de leur métabolisme. Lors d'une réinfection, les ILC3 « mémoires » ou « expérimentées » prolifèrent rapidement, produisent davantage d'interleukine (IL)-22 et sont intrinsèquement capables de mieux contrôler l'infection par rapport à des ILC3 naïves. Nos travaux soulignent que les ILC3 intestinales acquièrent des propriétés de « mémoire » suite à une infection bactérienne initiale, leur permettant de mieux participer à l'immunité intestinale et de renforcer les défenses de la muqueuse intestinale. Nous avons remarqué des réponses ILC3 mémoires contre différents pathogènes intestinaux, suggérant l'importance des ILC3 « mémoire » pour la protection à long-terme contre de multiples pathogènes.

En conclusion, nos résultats permettent dans l'ensemble de définir la régulation spatio-temporelle des ILC3 dans l'intestin. Nos travaux mettent en évidence les capacités d'adaptation intra-tissulaire remarquables des ILC3 intestinales en réponse aux signaux environnementaux, favorisant la mise en place de réponses immunitaires protectrices au sein de l'intestin. Une meilleure compréhension des voies régulant la biologie des ILC3 permettrait de développer de futures approches thérapeutiques contre les maladies intestinales.

Keywords: Cellules Lymphoïdes Innées de Type 3 (ILC3), Immunité Mucosale, Immunosurveillance intestinale, Migration Lymphocytaire, Imagerie Intravitale, Mémoire Innée, Infection Bactérienne

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Lescale C., Lenden Hasse H., Blackford AN., Balmus G., Bianchi J.J., Yu W., Bacoccina L., Jarade A., Clouin C., Sivapalan R., Reina-San-Martin B., Jackson S.P., Deriano L. *Cell Rep.* (2016)

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Trained ILC3 Responses Promote Intestinal Defense.

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Inflammation Triggers ILC3 Patrolling Of The Intestinal Barrier.

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« L'espoir c'est un truc qui est toujours le plus fort. »¹

¹ Cette photo a été prise par Nico lors d'une de nos expériences d'imagerie avec Sacha à l'Institut Pasteur en 2019. Elle me rappelle nos nombreux rendez-vous de « l'équipe de l'intestin », des instants à part, dans notre bulle, où nous avons partagé de très bons moments ensemble. Cette citation est tirée de mon roman préféré « La Vie devant soi » écrit par Romain Gary en 1975. Elle est à l'image de cette thèse, vallonnée, mais où nous avons toujours continué à y croire.