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INNATE LYMPHOID CELLS AND CHOLESTEROL METABOLISM IN INTESTINAL BARRIER FUNCTION

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Innate lymphoid cells and cholesterol metabolism in intestinal barrier function

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Al mio papà e alla mia mamma

ABSTRACT

The intestinal mucosa represents one of the largest barrier sites of our body, having to withstand a constant exposure to a plethora of environmental insults (including dietary compounds, xenobiotics, metabolites and microorganisms). While our body has evolved tolerance/ignorance towards some of these factors deemed beneficial for the host, it requires constant maintenance of epithelial barrier integrity and ability to mount pro-inflammatory responses to protect against potentially harmful environmental insults. The intestinal epithelium and innate lymphoid cells (ILCs) are two fundamental players in safeguarding intestinal homeostasis. The goal of this thesis was to study how ILC development/functions and the regenerative capacity of the intestinal epithelium are shaped by the intestinal inflammatory and metabolic milieu.

In **study I**, we investigated whether the pool of adult ILC progenitors in the bone marrow was able to sense and respond to peripheral inflammation. We found that increase in systemic levels of the cytokine Flt3L resulted in expansion of ILC precursors committed to helper ILCs. Although ILCs expand in inflammatory bowel disease patients, this axis does not take place in response to intestinal inflammation. However, in the context of malaria, increased levels of systemic Flt3L correlated with expansion of bone marrow ILC precursors, thus suggesting a potential role for inflammatory ILC lymphopoiesis during malaria.

In **study II**, we explored how alteration in cholesterol metabolism affected the function of intestinal ILCs. We showed that ILC3s, through the receptor EBI2, sensed cholesterol metabolites (oxysterols) produced by colonic stromal cells. Activation of this pathway led to ILC3 migration and thus formation of colonic lymphoid tissues (cryptopatches and isolated lymphoid follicles). Migration of ILC3s to cryptopatches resulted in their acquired ability to produce interleukin (IL)-22, a key intestinal homeostatic cytokine. However, in the context of colitis, augmented oxysterol production promoted EBI2-mediated inflammation and tissue remodeling.

In **study III**, we further investigated the contribution of cholesterol metabolism in intestinal physiology and found that a distinct oxysterol receptor (LXR) controlled the regenerative response of the intestinal epithelium. In the context of intestinal damage, oxysterol production and LXR activation was enhanced. Boosting activation of this pathway in intestinal epithelial cells enhanced regeneration in response to injury by promoting the activity of intestinal stem cells. Remarkably, in the context of tumor, LXR activation limited neoplastic progression, thus representing a novel promising therapeutic target to uncouple regeneration and tumorigenesis.

Taken together, this thesis contributes to our understanding on how ILC and cholesterol metabolism modulate intestinal barrier function and integrity.

LIST OF SCIENTIFIC PAPERS

I. **Parigi SM**, Czarnewski P, Das S, Steeg C, Brockmann L, Fernandez-Gaitero S, Yman V, Forkel M, Höög C, Mjösberg J, Westerberg L, Färnert A, Huber S, Jacobs T, Villablanca EJ.

Flt3 ligand expands bona fide innate lymphoid cell precursors in vivo

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II. Emgård J, Kammoun H*, García-Cassani B*, Chesné J, **Parigi SM**, Jacob JM, Cheng HW, Evren E, Das S, Czarnewski P, Sleiers N, Melo-Gonzalez F, Kvedaraite E, Svensson M, Scandella E, Hepworth MR, Huber S, Ludewig B, Peduto L, Villablanca EJ, Veiga-Fernandes H, Pereira JP, Flavell RA, Willinger T.

Oxysterol Sensing through the Receptor GPR183 Promotes the Lymphoid-Tissue-Inducing Function of Innate Lymphoid Cells and Colonic Inflammation

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III. Das S*, **Parigi SM***, Schewe M, Scharaw S, Webb A, Sorini C, Diaz O, Pelczar P, Frede A, Carrasco A, Pedrelli M, Andersson SJ, Czarnewski P, Nylen S, Antonson P, Mjösberg J, Gustafsson J-A, Gagliani N, Parini P, Huber S, Katajisto P, Villablanca EJ.

Damage-induced Liver X Receptor activation promotes intestinal epithelial barrier regeneration

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II. Seitz C, Liu S, Klocke K, Joly AL, Czarnewski PV, Tibbitt CA, **Parigi SM**, Westerberg LS, Coquet JM, Villablanca EJ, Wing K, Andersson J. "Multi-faceted inhibition of dendritic cell function by CD4+Foxp3+ regulatory T cells." *Journal of Autoimmunity* 2019 Jan pii: S0896-8411(18)30438-4. doi: 10.1016/j.jaut.2018.12.002

III. Brockmann L, Soukou S, Steglich B, Czarnewski P, Zhao L, Wende S, Bedke T, Ergen C, Manthey C, Agalioti T, Geffken M, Seiz O, **Parigi SM**, Sorini C, Geginat J, Fujio K, Jacobs T, Roesch T, Izbicki JR, Lohse AW, Flavell RA, Krebs C, Gustafsson JA, Antonson P, Roncarolo MG, Villablanca EJ, Gagliani N, Huber S. "Molecular and functional heterogeneity of IL-10-producing CD4+ T cells." *Nature Communications*, 2018 Dec 21;9(1):5457. doi: 10.1038/s41467-018-07581-4

IV. Czarnewski P, Das S, **Parigi SM**, Villablanca EJ. "Retinoic Acid and its Role in Modulating Intestinal Innate Immunity". *Nutrients*, 2017 Jan 13;9(1). pii: E68. doi: 10.3390/nu9010068

V. **Parigi SM**, Eldh M, Larssen P, Gabrielsson S, Villablanca EJ. "Breast milk and Solid Food shaping Intestinal Immunity". *Frontiers in Immunology*, 2015 Aug 19;6:415. doi: 10.3389/fimmu.2015.00415. eCollection 2015.

CONTENTS

LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AMP	Anti-microbial peptide
Apc	Adenomatous polyposis coli
Areg	Amphiregulin
BM	Bone marrow
BMP	Bone morphogenetic protein
CBC	Crypt base columnar
CD	Cluster of Differentiation
CHILP	Common helper innate lymphoid cell progenitor
CLP	Common lymphoid progenitor
Cyp27a1	Sterol-27-hydroxylase
DC	Dendritic cell
Dll	Delta-like
EAE	Experimental autoimmune encephalomyelitis
EBI2	Epstein-Barr virus-induced gene 2
EEC	Enteroendocrine cell
EGF	Epidermal growth factor
EILP	Early innate lymphoid progenitor
Eomes	Eomesodermin
ER	Estrogen Receptor
Flt3	FMS-like tyrosine kinase 3 ligand
GALT	Gut-associated Lymphoid Tissue
GPCR	G protein-coupled receptor
HC	Hydroxycholesterol
IBD	Inflammatory bowel disease
Id	DNA-binding protein inhibitor
IEC	Intestinal epithelial cell
IEL	Intra-epithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
ILC	Innate lymphoid cell
ILCP	Innate lymphoid cell progenitor
ILF	Isolated Lymphoid Follicle
ISC	Intestinal stem cell
Lgr5	Leucin-rich repeat containing G-protein coupled receptor 5
LN	Lymph node
LT	Lymphotoxin
LTi	Lymphoid tissue inducer cell
LTiP	Lymphoid tissue inducer cells progenitor
LXR	Liver X receptor
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MΦ	Macrophage
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKP	Natural killer cell progenitor
PC	Paneth cells
RA	Retinoic acid
ROR	RAR-related orphan receptor
RXR	Retinoid X receptor
SILT	Solitary isolated lymphoid tissue
SLO	Secondary lymphoid organ
SREBP	Sterol regulatory element-binding protein
TA	Transit amplifying
TCF	T cell factor
Th	T helper
TNF	Tumor necrosis factor
Treg	Regulatory T cell

1 INTRODUCTION

The intestinal tract represents a highly vulnerable barrier site due to its broad exposure to a massive amount of foreign antigens present in the lumen [1]. Different cell types are involved in preserving the physiological function and integrity of the organ, including immune and non-immune cells from the host (such as intestinal epithelial and stromal cells) interacting with environmental components. Maintenance of intestinal homeostasis requires tightly coordinated pro-inflammatory and tolerogenic responses [1]. While pro-inflammatory intestinal immune responses ensure protection against clinically relevant pathogens, the establishment of immunological tolerance avoids immune reactions against innocuous antigens [2]. Disruption of this delicate balance is a hallmark of intestinal pathologies, such as food allergies or inflammatory bowel disease (IBD) [3], characterized by chronic uncontrolled inflammation and impaired tissue repair.

The intestine as an "immunological" organ: overview. The intestine is characterized by a single layer of intestinal epithelial cells (IECs) at the interface between the luminal environment and the mammalian host. The epithelium is composed of stem cells, absorptive enterocytes and specialized secretory IECs that embody the physical and biochemical barrier protecting the underlying tissue [3]. One of the main functions of secretory IEC is to produce mucus [4] and antimicrobial peptides (AMPs) [5], generating a barrier to keep bacteria at bay. Together with epithelial cells, the immune system contributes to generate an efficient barrier and protection against pathogens. Interspersed between epithelial cells, intraepithelial lymphocytes (IELs), composed mainly by T cells and type 1 innate lymphoid cells (ILC1), help maintaining barrier integrity and protection against pathogens [6,7]. Underlying the epithelium basement membrane, the lamina propria is a loose connective tissue where the majority of the intestinal immune cells are located, embedded in a stromal architecture. Strategically located adjacent to the epithelium, intestinal mononuclear phagocytes, comprising macrophages (MΦ) and dendritic cells (DC), actively sample luminal antigens and coordinate the immune response locally or upon migration to draining lymphoid tissues [8]. Together with abovementioned immune cells, other myeloid (including neutrophils, monocytes, mast cells, eosinophils) and lymphoid cells are found in the intestinal lamina propria.

Adaptive immune responses (including B and T cell responses) are originated in lymphoid structures, such as the mesenteric lymph node (MLN) and the gut-associated lymphoid tissue (GALT), including Peyer's patches and isolated lymphoid follicles (ILF). Upon activation in lymphoid organs, T and B cells migrate and localize in the intestinal tissue where they exert non-redundant effector functions aimed at maintaining intestinal homeostasis [9].

Another more recently identified class of innate lymphocytes, named innate lymphoid cells (ILCs), is highly enriched in the gastrointestinal mucosa. ILCs are mucosal gatekeepers playing a pivotal role in ensuring barrier homeostasis [10]. In the sections to be followed, the development, function and adaptation to the environment of ILCs is discussed.

2 INNATE LYMPHOID CELLS

2.1 ILC classification and function

ILCs are a class of lymphocytes, often defined as the innate counterpart of T cells, with pleiotropic functions at the barrier sites of our body [11]. Despite the high degree of overlap with T cells, ILCs do not react in antigen-specific manner [12], do not rely on priming in secondary lymphoid organs (SLO) and their innate nature enables a kinetically faster response in tissues driven by sensing of stimuli like cytokines, alarmins, stress signals or hormones [13]. ILCs are relatively more represented in mucosal and barrier tissues, likely due to their ability to translate environmental and inflammatory cues into an effector program preventing pathogen-mediated damage, favoring tissue repair and contributing to tissue homeostasis.

Although natural killer (NK) and lymphoid tissue-inducer (LTi) cells have been identified many years ago [14,15], the discovery of novel ILC lineages and a comprehensive and unified classification of ILCs have drastically advanced only in the last decade [11]. ILCs are subdivided into two main lineages: cytotoxic ILCs (comprising NK cells) and helper ILCs (composed of three main subsets, ILC1, ILC2 and ILC3). Mirroring the well-established T cells classification system, NK cells are proposed to be innate counterpart of $CD8^+$ T cells and ILC1, ILC2 and ILC3 of T helper (Th) 1, Th2 and Th17 cells respectively [16]. In line with this approach, master transcription factors and cytokine production defining each subtype (as outlined below) display a high degree of overlap between T cells and ILCs. However, as in the field of T cells, technological advancement allowing transcriptional analysis at the single cell level and lineage tracing tools unearthed a high degree of heterogeneity and plasticity converting this well-defined genealogic tree into a more dynamic continuum [17,18]. Remarkably, an innate counterpart of regulatory T cells (Treg) has long been missing. Recently, a population of regulatory ILCs (named ILCreg) expanding in the intestine in response to inflammation has been identified. ILCregs are characterized by interleukin (IL-) 10 expression, by which they inhibit inflammatory cytokines production by ILC1 and ILC3 [19]. Whether these cells represent an independent subset or rather a transient functional state of other ILC subsets remains unresolved. Nevertheless, other helper ILCs (such as ILC2 and ILC3) can play immunoregulatory functions, thus representing an unconventional innate counterpart of Treg cells. In the following paragraphs a brief description of the different ILCs lineages is outlined (**Figure 1**).

2.1.1 NK cells

The discovery of NK cells dates back to the mid-1970s, when Kiessling, Klein and Wigzell at Karolinska Institutet described for the first time a class of naturally occurring cytotoxic lymphocytes specific for leukemia cells [20]. NK cells are generally characterized by the expression of the transcription factor T-bet and Eomesodermin (i.e. Eomes) driving interferon (IFN)-γ and perforin and granzyme B expression respectively [21]. Similar to ILC1s, NK cells participate in the immune response against intracellular pathogens (such as viruses) and tumor immunosurveillance and are geographically highly enriched in the liver tissue [22]. They

are equipped with activating (e.g. CD16) and inhibitory receptors (e.g. Ly49 and KIRs) modulating their cytotoxicity [23]. Activating receptors can recognize opsonized or stress-induced ligands on target cells, while inhibitory receptors detect MHC-I molecules. According to the "missing-self" hypothesis [24], lack of MHC-I expression on the surface, caused for instance by viral infection or cancerous transformation, fails to engage inhibitory receptors thus sensitizing the cell to NK cell-mediated cytotoxic attack. In this fashion, NK cells are essential to patrol tissues and limit viral shedding or tumor growth when these dangerous insults have hijacked the T cell-mediated recognition machinery [25].

Unlike NK cells, helper ILCs do not retain cytotoxic potential and, with some exceptions, their effector functions mainly rely on the production of soluble mediators, including cytokines, growth factors and metabolic mediators [26]. In the following paragraphs a short outline of the main characteristics of each helper ILC subsets is described.

2.1.2 ILC1

Class I ILCs are mainly found in intestine and liver and, similar to NK cells, express the transcription factor T-bet, but lack Eomes [27]. Mainly upon sensing of IL-12, IL-15 and IL-18 produced by myeloid and non-hematopoietic cells, ILC1s respond by producing IFN-γ and tumor necrosis factor (TNF) [28]. Owing to these features, ILC1s have been shown to be critical in the response against intracellular pathogens (such as *Toxoplasma gondii*) [29,30], viral infections [31], tumor immunosurveillance [32] and induction of classical macrophage activation [30]. While their pro-inflammatory function aids in protecting the host, the flip side is the pathogenic involvement of ILC1 in chronic inflammatory disorders, such as Crohn's disease, where high frequencies of ILC1 are found in the intra-epithelial compartment of the gut mucosa [7,33]. In humans, a high degree of heterogeneity in ILC1s has been recently detailed via single cell transcriptomic analysis and mass spectrometry in different anatomical sites, likely reflecting the tissue adaptation of ILC1 functions [17,34].

2.1.3 ILC2

Group 2 ILCs are essential mediators of type2 immunity thus conferring resistance to helminth infections (such as *Nippostrongilus brasiliensis* in lungs and gut) [35,36] and promoting tissue repair. ILC2s are identified by high expression of the transcription factor GATA-3, essential to regulate their function and development [37]. Found predominantly in lungs, intestine, adipose tissue and skin, ILC2s respond to the epithelium-derived cytokines IL-33, IL-25, TSLP (thymic stroma lymhpopoietin) and to IL-4 and arachidonic acid metabolites [38]. Their effector programs rely on the production of the cytokines IL-9, IL-5 and IL-13 and of the epithelial growth factor amphiregulin (Areg) [39]. By producing these mediators, ILC2s can establish a crosstalk with immune and non-hematopoietic cells, thus driving tissue homeostasis and adaptation to external insults. For instance, in the context of inflammation/infection, epithelial cell death leads to release of the alarmin IL-33 sensed by IL-33 receptors (also known as ST2) on ILC2s resulting in their production of Areg [40]. In turn Areg signals on intestinal epithelial cells driving their proliferation and mucus-producing

goblet cells differentiation [40]. This pathway has been studied in the context of protection against intestinal inflammation [40], skin wound healing [41] and promotion of tissue repair and airway epithelial integrity following H1N1 influenza virus infection in the lungs [42]. Another example of immune-epithelial cells crosstalk mediated by ILC2s is the production of IL-13 in response to intestinal epithelial cells sensing of helminth infections. Tuft cells, chemosensory brush cells present in intestinal epithelial villi, are exclusive producers of the cytokine IL-25 upon parasitic infection [43]. Via the expression of IL17Rb (also known as IL-25 receptor), ILC2s respond to IL-25 by producing IL-13, which in turn skews intestinal stem cells differentiation towards tuft and goblet cells [44,45]. This positive feedback loop orchestrated by ILC2s enables a reprogramming of epithelial cells composition leading to a more competent barrier for the expulsion of large pathogens. Indeed, IL-13 driven mucus production by goblet cells and smooth muscle cells contraction are essential to remodel the tissue and expel worms from the gastrointestinal tract [46,47]. Therefore, by establishing a dynamic crosstalk with non-hematopoietic cells, ILC2s drive homeostasis in tissue- and context-specific manner. In line with this, ILC2s display a high degree of functional adaptation dependent on their geographical location. Single cell RNA sequencing of ILC2s from different organs revealed a high degree of heterogeneity in the expression of cytokines and alarmin receptors. For instance, while *Gata-3* expression is a common feature of all ILC2s regardless of the tissue, IL-33 receptor (*Il1rl1*) expression defines fat and lung ILC2s, IL-25 receptor (*Il17rb*) is mainly expressed by gut ILC2s and skin ILC2s are marked by the expression of IL-18 receptor (*Il18r1*) [48]. These findings suggest that the tissue environment functionally shape ILC2 identity and in turn tissue adaption of ILC2s is required to maintain homeostasis in different anatomical location.

While the aforementioned effector program of ILC2s is pivotal to re-establish homeostasis, exacerbation of ILC2s activation can backfire and drive the pathogenesis of several inflammatory disorders affecting the lungs (such as asthma, chronic sinusitis with nasal polyps, lung fibrosis) [49,50,51,52], the gastrointestinal tract (e.g. eosinophilic esophagitis) [53] and the skin (as in atopic dermatitis) [54]. In these disease settings uncontrolled cytokine production by ILC2s leads, for instance, to unrestrained goblet cells hyperplasia and consequent mucus production with deleterious consequences for normal airway functions [39]. In addition, ILC2s can orchestrate the response of other immune cells ultimately feeding the inflammatory process [55,56].

2.1.4 ILC3

Class 3 ILCs are a heterogeneous group of ILCs mainly enriched in the intestine, skin and tonsils. The common denominator of all ILC3s is the expression of the transcription factor RAR-related orphan receptor gamma t (RORγt), essential for their ontogenesis and function [57]. Belonging to this class are LTi, critical mediators of fetal lymphoid tissue organogenesis, and two other subsets of ILC3s identified based on the expression of natural cytotoxicity receptors (NCR), CCR6 and T-bet [58]. In mice, adult LTi-like CCR6$^+$ T-bet$^-$ ILC3s do not express NCRs and are mainly located in intestinal cryptopatches and ILFs where they can

produce the cytokines IL-22, IL17A and IL17F. CCR6$^-$ T-bet$^+$ ILC3s instead, express the NCR NKp46 and CD49 and produce mainly IL-22. In humans, NCR$^-$ and NCR$^+$ ILC3s are distinguished based on the expression of NKp44. In the following paragraphs a brief outline of the function of LTi, adult LTi-like, NKp46$^+$ ILC3s and of common ILC3 functions is provided.

LTi and adult LTi-like cells in lymphoid tissue organogenesis. LTi and LTi-like cells are essential mediators of lymphoid organ formation both during fetal development and in post-natal life. LTi cells are originated in the fetal liver and are found in lymph nodes (LN) anlagen, where they coordinate lymphoid organogenesis, during embryonic development (day E13.5) [15,59]. How LTi's recruitment and positioning at the site of LN formation is regulated is still poorly understood. The recruitment of LTi to LN anlagen seems to be mediated by the chemokine receptor CXCR5 sensing the chemokine CXCL13 produced by stromal organizer cells, a stromal population driving LN organogenesis [60,61]. Interestingly, production of retinoic acid (RA), by nerve endings might be needed to guide the production of stromal CXCL13 and thus the recruitment of LTi [62]. This finding suggests that neuron-derived signals and sites of RA release are pivotal in determining the location of LN formation at the embryonic stage. Together with CXCL13, CCL21 expression by the lymphatic endothelium aids in attracting LTi cells through their expression of the cognate receptor CCR7 [63]. Once in the LN anlagen, owing to their expression of TNF-related activation-induced cytokine (TRANCE) and TRANCE receptor (TRANCER), LTi cells cluster and signal *in trans* leading to the induction of lymphotoxin-$\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) expression [64]. Interaction between $LT\alpha_1\beta_2$ and its receptor lymphotoxin-β-receptor (LTβR) on stromal cells promotes the differentiation of the latter into stromal organizer cells, mesenchymal cells capable of giving rise to the different mature stromal cell subsets found in mature LN [65]. Signaling through LTβR results in the production of the adhesion molecules vascular cell adhesion molecule 1 (VCAM1), intercellular adhesion molecule 1 (ICAM1) and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1) as well as the chemokines CCL19, CCL21 and CXCL13 [66,67], all necessary for the recruitment and retention of other hematopoietic cells constituting mature lymphoid organs.

Mucosa-associated lymphoid tissues, with the exception of Peyer's patches [68] and unlike other lymph nodes, develop after birth [69]. Cryptopatches, formed around 2 weeks after birth in mice, are small clusters of adult LTi-like cells, dendritic cells and stromal cells dispersed in the intestinal lamina propria in close proximity with the intestinal epithelium [70,71]. Upon CCR6-mediated B cell recruitment, cryptopatches enlarge giving rise to ILF, also known as large solitary isolated lymphoid tissues (SILTs) [72]. Similar to other LN, cryptopatches development relies on lymphotoxin signaling but, in addition, owing to their mucosal location, signals from the intestinal flora seem to play a role in the genesis of large SILT. In particular, commensal bacteria-derived signals were described to induce CCL20 production by ileal intestinal epithelium, conceivably promoting CCR6-mediated B cells attraction [73]. However, which signals control specific LTi-like cells recruitment in cryptopatches (especially in the colon) as opposed to other mature ILC3s, mainly dispersed in the lamina propria, remain largely unexplored. In the second manuscript included in this thesis, we have

described a novel role for the G protein-coupled receptor (GPR) 183 (also known as Epstein-Barr virus-induced gene 2, EBI2) in driving attraction and retention of $CCR6^+$ ILC3s in cryptopatches and ILF (see "Results").

Adult LTi-like ILC3s, by accumulating in the interfollicular regions of lymphoid clusters [74,75], are geographically favored to interact with T and B cells and thus modulate adaptive immune responses. Indeed, via the expression of MHC-II and the lack (or low expression) of co-stimulatory molecules CD80 and CD86, adult LTi-like ILC3s induce immune tolerance by promoting cell death of commensal-specific $CD4^+$ T cells [76,77]. On the other hand, owing to the expression of the costimulatory molecules OX40L and CD30L, they can aid the survival of memory $CD4^+$ T cells and favor T cell-dependent antibody production by B cells [78]. In the intestine, adult LTi-like ILC3s have been shown to be critical to drive IgA response by B cells in a T cell-dependent and independent manner [71]. Mechanistically, by expressing $LT\alpha_1\beta_2$ and secreting $LT\alpha_3$, BAFF and APRIL, ILC3s fuel B cell activation and production of IgA, which is critical to control commensals and pathogens containment in the lumen [79,80]. Hence, by orchestrating the intestinal adaptive immunity and commensal-specific T cell and IgA response, LTi-like ILC3s are important gatekeepers of mucosal homeostasis [81]. Moreover, their strategic positioning in cryptopatches at the bottom of intestinal epithelial crypts and their ability to produce the IL-22 renders them key mediators of stem cells pool maintenance and tissue repair in response to damage (see below for the role of IL-22 in intestinal homeostasis) [82].

$NKp46^+$ ILC3s in the intestine are mainly found interspersed in the lamina propria, owing to their expression of the chemokine receptor CXCR6 interacting with CXCL16 produced by a subset of IL-23-producing DCs. This CXCR6-CXCL16 axis mediates the functional topography of $IL\text{-}22^+$ ILC3s in the gut which in turn is required to achieve successful protection against pathogens, such as *Citrobacter rodentium* [83]. While sharing with the LTi-like ILC3s subset the expression of RORγt and IL-23 receptor, NCR^+ ILC3s rely on *Il12rb2*, *Tbx21* (encoding for T-bet) and *Notch1* for their development [84,85,58] and express the cytotoxicity receptor NKp46 (in mouse) and NKp44 (in human), which are suggested to function as pattern recognition receptors to mount inflammatory responses [86] and protect against pathogens [87]. Given their expression of T-bet and IL-12 receptor, a high degree of plasticity towards class 1 ILCs has been attributed to this subset of ILC3s. Fate-mapping experiments uncovered a population of $T\text{-}bet^+$ $NKp46^+$ cells that had lost RORγt expression and acquired a functional phenotype resembling ILC1 [84]. Further suggesting plasticity, Crohn's disease patients display higher proportion of $CD14^+$ DCs that boost the conversion of ILC3 to inflammatory ILC1 *in vitro* via the production of IL-12. Conversely, culturing ILC1 in the presence of IL-23, IL-1β and RA favors their conversion to ILC3s [88].

Behind this dichotomous distinction of peripheral adult ILC3 subsets, many other populations of ILC3s have been identified through the use of deep-sequencing approaches with single-cell resolution both in mice and humans [18,17]. Many of these newly identified subsets might likely represent distinct functional or developmental states of the same class of cells rather than an ontogenically and functionally distinct subset. In human tonsils, for instance, a subset of ILC3s expressing CD62L and poorly responding to re-stimulation has been identified [89].

Given their naïve-like phenotype, this subset possibly represents a developmental ancestor of mature and activated ILC3s [90].

ILC3s function can be highly influenced and shaped by the environment, particularly by nutrient-derived signals [91]. For example, RA controls the transcription of *Il22* in ILC3s [92], while a vitamin A depleted diet causes drastic reduction of ILC3 numbers and, as a consequence, impaired protection against *C. rodentium* infection [36]. Moreover, maternal retinoid intake shapes the development of fetal LTi cells *in utero*, thus controlling the size of lymphoid organs and the ability to mount protective immunity in the adult offspring [93]. Another receptor expressed by all ILC3s, the aryl hydrocarbon receptor (AhR), can sense diet derived AhR ligands present in cruciferous vegetables [94]. Mice lacking AhR or adult offspring of pregnant mice fed with AhR ligand-depleted diet display a significant reduction in $CCR6^-$ ILC3s coupled with reduced expression of IL-22, thus leading to enhanced susceptibility to *C. rodentium* infection [95,96]. Overall, these data indicate that innate immune protection mediated by ILCs can be pre-programmed as early as at the embryonic stage and, in parallel, mature ILCs can adapt to changes in the microenvironment and nutritional uptake. Cholesterol, a key component of cell membranes and metabolic precursor, can be synthetized by the liver or absorbed through the diet. In particular, "Western diet", epidemiologically associated to many inflammatory diseases, is highly enriched in cholesterol. Whether cholesterol can shape ILCs functions remains elusive. In the second manuscript included in this thesis, we have unraveled a novel role for cholesterol metabolites in the regulation of ILC3 migration and function in colonic cryptopatches.

A shared effector function among virtually all ILC3 subset is the production of the cytokine IL-22. IL-22 is a member of the IL-10 superfamily [97] and, unlike most other cytokines, targets nonhematopoietic cells. IL-22 receptor (composed of IL-10R2 and IL-22R1 subunits) is expressed on intestinal epithelial cells; hence this cytokine represents a strategic mediator of immune-epithelial crosstalk to control mucosal homeostasis. As hinted above, promotion of IL-22 production is driven by a plethora of heterogeneous stimuli encompassing diet (e.g. RA, AhR ligands) [92,98], cytokines (IL-23 and IL-1β) [99,100], microbial flora [101,102] and transcription factor requirements (RORγt in mice) [101]. Early studies proved that IL-22 plays a central role in the protection against intestinal bacterial and viral infection. Molecularly, IL-22 synergizes with IFN-λ signaling to curtail rotavirus infection [283] or promotes the production of AMP by intestinal epithelial cells, specifically lectins of the Reg3 family, necessary to restrain attaching and effacing bacterial pathogens (such as *C. rodentium*) [103]. Adding to this body of evidence, in the second study included in this thesis, we have shown that cholesterol metabolites sensing through EBI2 enables ILC3 migration to colonic cryptopatches and, as a consequence, impact their ability to produce IL-22. In line with our findings, another group has shown that mice lacking GPR183 display reduced protection against *C. rodentium* infection as a result of reduced numbers of $IL\text{-}22^+$ ILC3s [104]. Together with its anti-microbial role, IL-22 is considered as one of the main pro-regenerative cytokines by favoring tissue repair. Using different intestinal epithelial cell damage models, including chemotherapy [105], graft-versus-host disease [106,107] or chemically induced colitis [108,109], several studies demonstrated the pro-regenerative role of IL-22. Mechanistically, sensing of IL-22 by

intestinal stem cells drives signal transducer and activator of transcription 3 (STAT3) phosphorylation and regulation of anti-apoptotic and pro-proliferative pathways [82]. Altogether, these studies propose a model where, by ensuring protection against pathogen invasion and gatekeeping an intact lining of the epithelial barrier, IL-22 acts as a central node in maintenance of intestinal homeostasis. Nonetheless, a tight control of IL-22 level is a prerequisite to prevent uncontrolled growth of intestinal stem cells. Indeed, incautiously boosting pro-regenerative pathways comes at the risk of fueling unrestrained proliferation of stem cells and enhancing the possibility of accumulating malignant transformations. In line with this, IL-22 has been shown to drive tumor progression in the small intestine in the genetically-driven *Apc*$^{Min/+}$ mouse tumor model [110]. To overcome this menace, our body has evolved a system to maintain IL-22 levels in check. Production of IL-22 binding protein (BP), a soluble receptor that neutralizes IL-22 activity, towards the end of the regenerative process has been proven critical to restrain tumor development in the colon [110]. Nevertheless, a more complex picture of IL-22 contribution to colonic tumorigenesis is now emerging. A recent study elegantly showed that IL-22 sensing by intestinal stem cells promotes the DNA damage response, a machinery that controls genome integrity and dampens malignant transformation [111]. Reconciling these data, a dual role of IL-22 as anti- and pro-tumorigenic might rely on the kinetics of expression in specific contexts and/or the organ system and tumor model used.

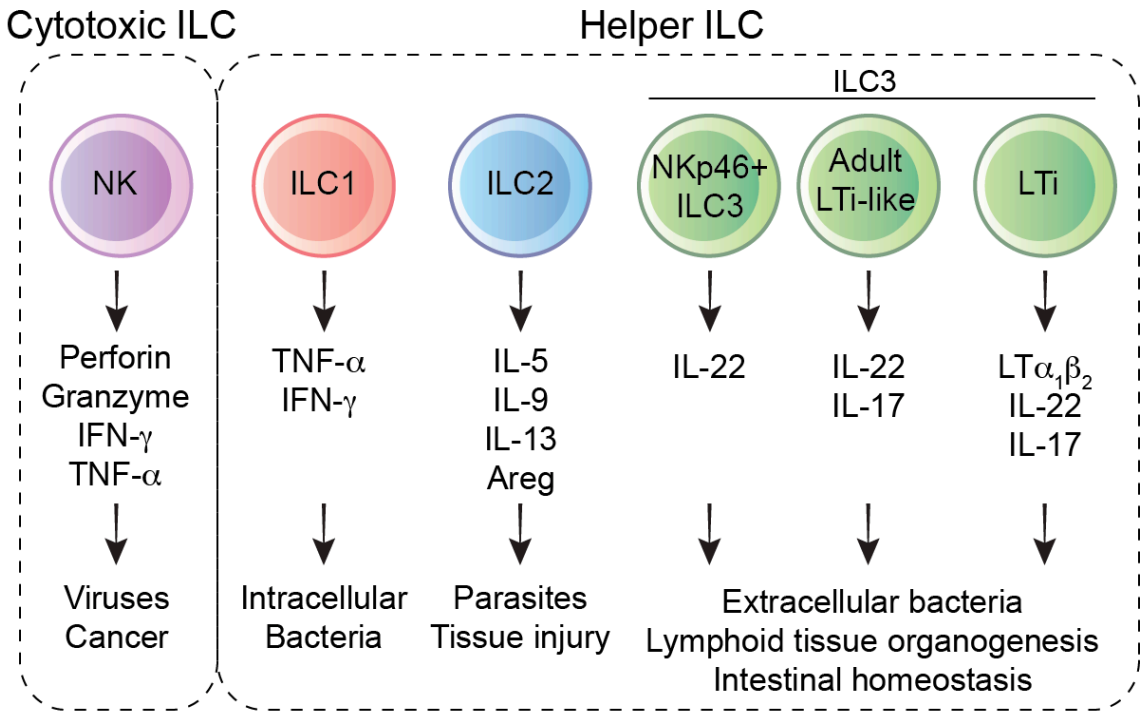

Figure 1. *Schematic representation of ILC subsets and function.*

2.2 ILC development

Several groups over the last decade have focused on unearthing when, where and how ILCs development takes place. Of late, different ILC precursor populations have been identified in mouse and human, both during the fetal stage and in adulthood. The current model of ILC-poiesis propose a step-wise specification where progenitors harboring a broader differentiation potential sequentially lose multipotency, ultimately leading to committed precursors and differentiated mature ILCs [112] (**Figure 2**).

Fetal ILC development. Development of ILCs begins during the fetal period in the fetal liver and intestine [113]. An ILC precursor expressing Arginase 1 (Arg1), CD127 (the receptor for IL-7) and the integrin α4β7 present in the fetal intestine has been proven capable of generating all three types of ILC lineages *in vitro* in the presence of OP9 feeder cells [114]. Similarly, a progenitor population named α-LP, as an acronym of $\alpha4\beta7^+$ Lymphoid Precursor, expressing CD127 and DNA-binding protein inhibitor 2 (Id2), was identified by single cell analysis in the fetal liver as the most primitive ancestor capable to generate all helper ILC subsets and LTi cells [115]. Despite the presence of these multipotent ILC progenitors, the fetal period appears to be the main stage for ILC3, and more specifically LTi development. Two groups have identified a population of $\alpha4\beta7^+$ $ROR\gamma t^+$ $CXCR6^+$ cells in the fetal liver endowed with LTi differentiation potential in a Notch-independent manner [116,117]. Furthermore, single cell analysis of fetal liver progenitors identified a population marked by the expression of CXCR5 and the lack of PLZF, coinciding with LTi progenitors (LTiP) [115]. Less is known regarding the fetal development of other ILC subsets, such as ILC1 or ILC2. Recently, a fate-mapping study of mature ILC2s in different peripheral tissues revealed that the majority of ILC2 pools are generated *de novo* during the post-natal window, and only a minor fraction of peripheral mature ILC2s are derived during the late gestation period [118]. The diverse kinetics of ILC subset development might reflect their differential contribution to physiology. In line with this hypothesis, lineages involved in organogenesis (such as LTi driving lymphoid organogenesis) require pre-natal development while subsets involved in organ homeostasis display a delayed differentiation (post-natal period).

Adult ILC development. In adulthood the bone marrow (BM) is considered as the cradle for ILC development. Starting from common lymphoid progenitors (CLPs), different precursors that had lost B and T cells potential and are uniquely committed to ILCs have been recently described. Specific surface markers, transcription factor requirements and differentiation potential mark these diverse populations of ILC progenitors. Interestingly, a common denominator of all ILC precursors is the expression of the integrin α4β7, which binds to MAdCAM1 and is widely considered as a gut-homing receptor. While α4β7 was shown to be important for migration of ILC2 upon development from the BM to the intestine [119], its role on the other multipotent ILC precursors remains unclear.

A direct ancestor-progeny relationship among the different ILC precursors identified thus far is still missing, as their discovery arose from different studies. In the next paragraphs an attempt to describe in genealogic order (and not the chronological order of discovery) the different ILC progenitors is presented.

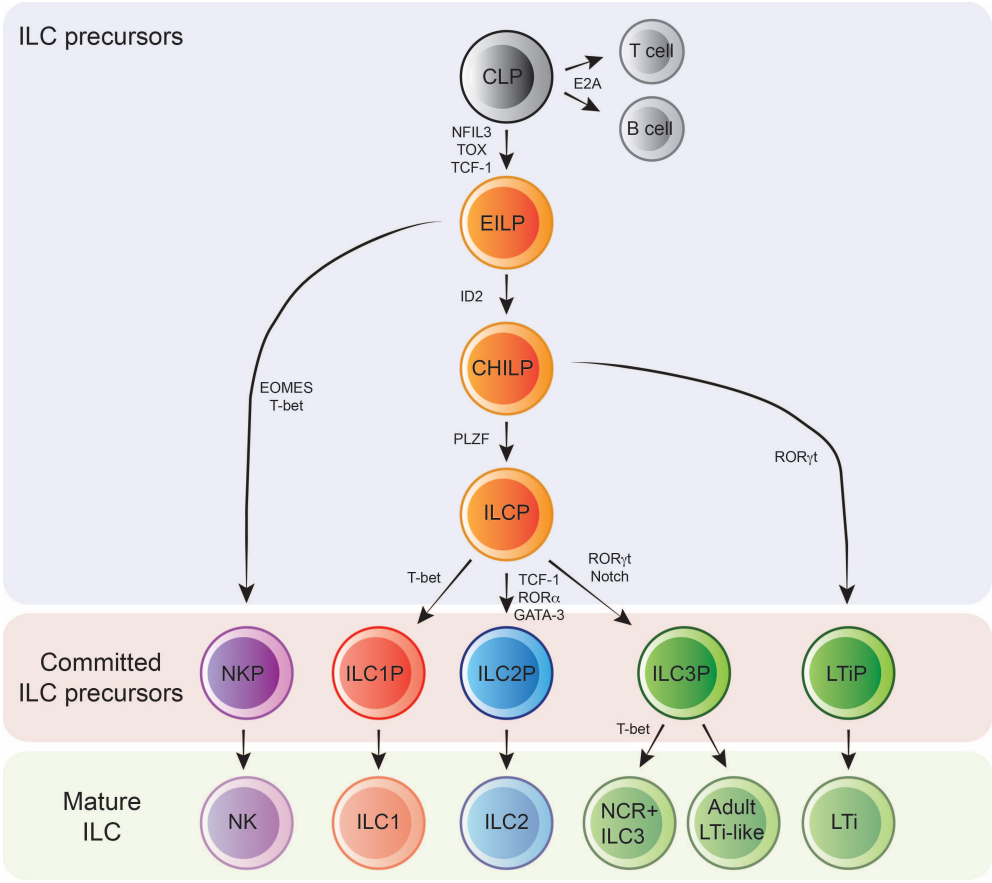

Figure 2. *Schematic representation of ILC development*

2.2.1 EILP

The most primitive ILC ancestor, retaining the potential to differentiate to all ILC lineages (helper and cytotoxic) was named early innate lymphoid progenitor (EILPs). EILPs are marked by the expression of α4β7 and the transcription factor T cell factor-1 (TCF-1, encoded by the gene *Tcf7*). However, unlike other ILC precursors, EILPs lack the expression of CD127, CD90 and of markers specific for committed progenitor populations (such as CD122 for NK progenitors or CXCR6 for helper ILC precursors). Mice deficient in *Tcf7* display a cell-intrinsic severe defect in the generation of all mature ILCs and of committed ILC progenitors, thus locating EILP at the top of the ILCs genealogic tree. Vice versa, isolated EILPs were able to generate all known ILC subsets both *in vivo* and *in vitro* at a clonal level [120]. Together with *Tcf7*, other molecular players are important to regulate ILC development. Nuclear factor interleukin 3-regulated (NFIL3), a transcriptional regulator involved in multiple hematopoietic lineages and in circadian rhythm, appears to be important at the early ILC precursor, as *Nfil3*$^{-/-}$ mice display severe impairment in the generation of all ILC subsets (including cytotoxic and helper ILCs) [121]. Downstream NFIL3, the transcription factor thymocyte selection-associated high mobility group box protein (TOX) has also been proven necessary for early ILC lineage specification [122].

Downstream EILP, the bifurcation into NK-committed progenitors (NKP, expressing CD122 [123] Eomes and T-bet [124]) and helper ILC precursors seems to take place.

2.2.2 CHILP

Common helper ILC progenitors (CHILPs) were identified based on the expression of α4β7, CD127, c-Kit, the transcription factor Id2 and the absence of lineage markers (specific for other mature immune cells population), CD25 (the receptor for IL-2, marking committed ILC2 precursors) and the receptor FMS-like tyrosine kinase 3 (Flt3, also known as CD135, which marks CLPs). This pool of ILC precursors retains the ability to generate all helper ILCs (including LTi) both *in viv*o and *in vitro* and lack NK differentiation potential. Remarkably, CHILPs appear to be a heterogeneous population, comprising multiple progenitors, as seen by their ability to generate single and mixed colonies of different ILC lineages upon single-cell culture *in vitro* [30]. While the first identification of CHILPs was achieved through the use of Id2-GFP reporter mice, a recent study utilizing Id2-RFP mice challenged the current view of CHILP as progenitors committed to helper ILCs and lacking NK potential. This novel reporter mouse showed a more robust fluorescence staining (compared to GFP) and was generated retaining the endogenous expression of Id2, thus allowing a more sensitive analysis. Using Id2-RFP mice, the authors showed that Id2-expressing ILC precursors still retain the ability to generate NK cells both *in vitro* and *in vivo* [125]. Id2, belonging to the ID family of transcriptional repressors, heterodimerizes and thus inhibit the function of E2A, a transcriptional activator required for adaptive lineage progression [126]. In this fashion, Id2 upregulation sets the stage for commitment to ILCs and repression of B and T cell potential.

2.2.3 ILCP

After CHILPs, the bifurcation into LTiP and ILC precursors (ILCP) committed to all the other helper ILC subsets is suggested to take place. ILCPs are identified based on the expression of promyelocytic leukemia zinc finger (PLZF, encoded by the gene *Zbtb16*), a transcription factor originally studied in NKT cells development. Fate mapping experiments showed that ILC1, 2 and 3 but not LTi or NK cells had a history of PLZF expression, thus allowing the identification of ILCP in the BM based on the co-expression of PLZF, α4β7, CD127 and c-Kit. In line with the lineage-tracing results, this progenitor population was shown to give rise to all helper ILCs (but LTi) both *in vitro* and *in vivo* [127].

2.2.4 Committed ILC precursors

Downstream ILCP, unipotent ILC precursors committed to the different lineages have been described. How and where this fate decision takes place remains still largely unresolved. Single cell analysis of fetal ILC progenitors and hierarchical clustering proposed a model whereby, instead of a direct commitment to a specific lineage, ILCPs undergo a stage of simultaneous effector programs expression followed by progressive shut down of programs for alternative fates [115]. A committed precursor to ILC1 (ILC1P) has been identified in the murine BM based on the expression of CD127, NKp46, NK1.1 and the lack of Eomes and RORγt expression. This population represents a *bona fide* immature ILC1 population as shown by their ability to uniquely give rise to ILC1 both *in vitro* and upon adoptive transfer

in lymphopenic mice *in vivo* [30]. Unlike other subsets, ILC2 committed precursors (ILC2P) in the BM are largely represented. ILC2P are characterized by the expression of α4β7, CD127, CD25 and are reliant on the transcription factors GATA-3, TCF1 and RORα for their development [35]. While GATA-3 appears to be required for all helper ILCs development, a marked upregulation of its expression marks the commitment of ILCP to the ILC2 lineage [128] [37]. Despite the high degree of phenotypic overlap with mature ILC2s, ILC2P in the BM are considered as progenitors based on their higher proliferative capacity and limited cytokine production potential in the absence of differentiating stimuli [37]. Interestingly, ILC2P in the BM express the chemokine receptor CCR9, which in conjunction with α4β7 expression drives ILC2P relocation and differentiation in the intestinal lamina propria, thus suggesting an organotropic specific imprinting already during ontogenesis [37]. Remarkably, after initial colonization of peripheral organs, occurring mainly in the post-natal/adult period, ILC2s seem to expand *in situ* following infection rather than depending on *de novo* BM ILC-poiesis [118]. These findings raise questions on the purpose of maintaining a relatively large population of ILC2P in the BM throughout life. A possible explanation is that ILC2P in the BM are rather a functionally mature ILC2 population playing a specific function in the BM niche rather than a committed immature progenitor.

While committed precursors to LTi cells in the fetal liver were the first ones to be identified, much more challenging was the identification of other ILC3 precursors (ILC3P). Indeed, unlike ILC2P and ILC1P, ILC3P are highly infrequent in the adult BM. Nevertheless, a recent study making use of polychromic multiple reporter (Id2, RORα, Bcl11b, Gata-3 and RORγt) mice allowed the identification of an extremely rare population of ILC3P based on the expression of RORγt, Id2, Bcl11b and the low expression of GATA-3 [129]. The paucity of ILC3P in the adult BM has been justified by the hypothesis that early immature ILCP on their way to become ILC3 leave prematurely the BM and complete their maturation in peripheral organs [112]. An alternative explanation might be that we haven't yet found BM ILC3P and we should approach this question in an unbiased fashion rather than exploiting known markers of ILC precursors for their identification.

2.2.5 Human ILC development

In humans attempts to draw an analogous map of ILC development has been performed over the last decade. A common human ILC precursor has been identified in secondary lymphoid organs (tonsil and spleen), although absent in cord blood, peripheral blood, thymus or BM. These progenitors are marked by the expression of CD34, CD45RA, CD117, IL-1R1, integrin β7 and RORγt and display the potential to differentiate into NK and all helper ILC subsets *in vitro* [130]. Downstream this multipotent population, committed progenitors to the NK lineage and to ILC3s have also been characterized in humans. NKPs were identified in the fetal liver, fetal BM, cord blood and adult tonsil by the expression of CD34, CD45RA, CD10, CD7 and the lack of CD127 [131]. ILC3-committed precursors, instead, were discovered in tonsils and in the intestinal lamina propria based on the expression of CD34, CD45RA, CD117, α4β7, RORγt, ID2, KIT, NCR1 and the lack of CD7 and CD127 [132]. While

remarkably similar to common ILC precursors identified in SLOs, ILC3Ps are distinguished based on the lack of IL-1R1.

2.2.6 Tissue residency of mature ILCs

Despite the existence of ILC precursors in the adult BM, the current consensus proposes that the pool of peripheral mature ILCs is maintained independently from BM lymphopoiesis. Indeed, by using parabiotic mice, Rudensky's lab showed that mature ILCs are tissue resident cells, lingering in peripheral tissues for long period of time (up to 130 days) under homeostatic conditions [133]. This finding is hard to reconcile with the evolutionarily conserved energetic expenditure to maintain pool of ILC progenitors in the adult BM. However, in the same study the authors showed that in the context of chronic infections, cells of hematogenous source were partially replenishing the tissue at a late stage [133]. On a similar line, another report from Germain's lab demonstrated that ILC2s from the intestinal lamina propria are endowed with the ability to relocate to the lungs under inflammatory conditions [134]. In parallel, human common ILC progenitors, characterized by IL-1R1 expression, have been shown to respond to IL-1β with enhanced proliferation and differentiation [135]. These results led to the postulation of a model whereby, while mature ILCs largely depend on *in situ* self-renewal under homeostatic condition, an "on demand" ILC-poiesis or tissue relocation takes place upon inflammation (such as infection in mice or IL-1β production in humans) [136]. In the first manuscript included in this thesis, we have expanded on this model and showed that ILC precursors in the adult BM can respond to increased systemic levels of the inflammatory cytokine Flt3L. This phenomenon might represent the lymphoid counterpart of the previously described "emergency myelopoiesis" [137].

3 INTESTINAL EPITHELIAL CELLS

The intestinal epithelial barrier is organized as a single layer of epithelial cells constituting the very first cellular shield facing the external environment and physically separating the luminal content from the host underneath. The main functions of intestinal epithelial cells are absorption of nutrients while ensuring protection against biotic and abiotic environmental stressors. To fulfill these two opposing tasks, the intestinal epithelium has evolved a system to maximize its surface of interaction with the environment (amplifying absorption) and, at the same time, shelter this vast barrier from external insults. The architectural organization in crypt-villus structures and the continuous regenerative behavior of the intestinal epithelium are the recipes to embody this dual role.

Crypt-villus architecture and continuous regeneration. The small intestinal epithelium is composed of crypt-villus units, where the first one represents an invagination, while the latter is a finger-like protrusion of the intestinal wall. The colon, instead, completely lacks villi and is composed of elongated crypts only. The villus is primarily composed of absorptive enterocytes devoted to absorb nutrients. However, by projecting into the lumen, they are constantly exposed to mechanical and environmental stressors. To warrant protection, enterocytes are post-mitotic cells with a remarkably short lifespan (3-5 days). This way, our body continuously gets rid of potentially damaged and infected cells and avoids inheritance of stress signals or mutations to daughter cells. To ensure constant replenishment of enterocytes, the crypts (also called crypts of Lieberkühn) host the factory continuously generating all the differentiated epithelial cells. In this fashion, actively proliferating cells are sheltered from the external environment and hidden in the crypts, while only short-lived cells face directly the luminal content. Intestinal stem cells (ISCs), residing at the bottom of these invaginations, constantly divide giving rise to highly proliferative progenitors (called transit amplifying cells, TA), which then generate all the different mature epithelial cell lineages found in the villi. The current consensus proposes a model of passive mitotic pressure along the crypt-villus axis to explain the continuous upward movement of epithelial cells. This model, also referred as intestinal "conveyor belt", suggests that when a crypt cell divides, pushes the neighboring cells upward thus initiating the journey of enterocytes towards the tip of the villus, where they die of apoptosis and are shed into the lumen [138]. However, this concept was recently challenged by a study proposing that epithelial cells instead migrate actively directed by actin-rich basal protrusions [139].

3.1 The intestinal epithelium: cell type and function

Originating from ISCs, the intestinal epithelium is composed of six differentiated cell types belonging to two lineages: secretory (Paneth, goblet, enteroendocrine and tuft cells) and absorptive cells (enterocytes and microfold or M cells). In the following paragraphs, a brief outline of the main characteristics of each cell type and of the signals governing maintenance and differentiation of the stem cell pool (stem cell niche) is presented. Finally, an overview of how epithelial cells adapt and respond to damage is drawn.

3.1.1 Paneth cells

Paneth cells constitute the primary epithelial niche for stem cells proliferation and maintenance. Located at the base of the crypt, they are interspersed in between ISCs so that each stem cell is in contact with one Paneth cell in a 1:1 ratio. This organization is necessary to promote Paneth cell-dependent stem cell activity, reliant on paracrine Wnt production and contact-dependent Notch signaling (see below "Intestinal stem cell niche") [140]. Despite being a differentiated epithelial lineage, Paneth cells behave quite differently compared to other intestinal epithelial cells. Indeed, their lifespan is fairly prolonged (up to 60 days) and they are the only ones moving downward rather than towards the tip of the villi upon differentiation. This anomalous migration pattern ensures their positioning at the base of the crypt and is mediated by EphB2 and EphB3 signaling [141]. Besides providing niche factors to nurture stem cells, Paneth cells are gatekeepers safeguarding ISCs from external insults. Indeed, by producing anti-microbial products, such as α-defensins, lysozyme and phospholipase A2, Paneth cells provide a shield protecting ISCs [142]. Paneth cells are described only in the small intestine and the colon lacks this population. However, a related population, named deep crypt secretory cells identified by the expression of regenerating family member 4 (Reg4) provides an equivalent function in the large intestine [143].

3.1.2 Goblet cells

Goblet cells are secretory epithelial cells devoted to the generation of the mucus layer protecting the intestinal barrier. By producing transmembrane mucins (forming the glycocalyx) and secreting gel-forming mucins, goblet cells prevent pathogens translocation by trapping bacteria [4]. Regulating their ontogenesis, while Notch signaling prevents their differentiation [144], immune cells-derived interleukins, such as IL-4 and IL-13, boost goblet cells hyperplasia [46]. Recently, Hansson's group described a specialized subset of goblet cells, called sentinel goblet cells, located at the entrance of the colonic crypt sensing the environment through toll like receptors. Upon detection of microbial invasion, sentinel goblet cells start secreting mucin 2 (MUC2) and trigger a similar response from all neighboring goblet cells [145].

3.1.3 Enteroendocrine cells

Enteroendocrine cells (EEC) are specialized hormone producing cells. Among the different hormones produced by EEC are gastric inhibitory peptides, somatostatin, cholecystokinin, glucagon-like peptides, serotonin and ghrelin. While initially thought to be important for nutrients detection and secretion of hormones to stimulate digestion, the role of EEC has now been extended to sensors of microbial metabolites and orchestrators of intestinal immunity [146].

3.1.4 Tuft cells

Tuft cells are the taste buds of the intestinal epithelium, for their ability to chemosense environmental signals, such as microbial metabolites [147]. As described above (see ILC2s),

these cells are important mediators of anti-helminth response and orchestration of type 2 immune responses. By producing IL-25 upon worm infection, tuft cells stimulate IL-13 production by ILC2s and ultimately skew stem cells differentiation towards tuft and goblet cells [43]. Recently, intestinal tuft cells have been described to express the succinate receptor 1 (SUCNR1), necessary to detect succinate metabolites produced by some helminths (such as tritrichomonad protists) and thus activate tuft cells cytokine production [148].

3.1.5 Enterocytes and M cells

Absorptive enterocytes constitute the majority of the differentiated epithelial cells. They populate the villi where they mediate nutrient (lipid, sugar, water, peptide and ion) absorption. They are derived from absorptive progenitors in a Notch signaling-dependent manner [149]. M cells, instead, are a specialized epithelial lineage found specifically above Peyer's patches. These cells are responsible for uptake and transfer of luminal antigens to the immune cells underneath [150]. Their differentiation is dependent on receptor activator of nuclear factor kappa-B ligand (RANKL) produced by subepithelial stromal cells overlying the Peyer's patches [151].

3.1.6 Intestinal stem cells

Giving rise to all the differentiated epithelial cells described above, ISCs are multipotent cells residing in intestinal crypts. Two main populations of stem cells have been thus far identified in the intestinal epithelium, differing based on their location, proliferative potential and physiological function.

CBC stem cells. Crypt base columnar (CBC) stem cells have been identified in the early seventies as constantly dividing cells at the bottom of the crypt by Cheng and Leblond [152]. However, only with the advent of more sophisticated techniques, such as lineage tracing tools and specific markers, a deeper functional validation of their stem cell potential was made possible. Leucin-rich repeat containing G-protein coupled receptor 5 (Lgr5) was found to unequivocally mark CBCs and generation of mouse models to track their progeny allowed to prove that these cells are capable of generating all differentiated intestinal cell types for long periods of time [153]. In parallel, under specific culture conditions, isolated $Lgr5^+$ cells were shown to be able to self-renew and generate mini-guts in a Petri dish (called organoids, i.e. organotypic cultures composed by crypt-villus structures with all intestinal cell lineages) [154]. Therefore, owing to their multipotency and ability to self-renew, CBC can be defined as *bona fide* stem cells. CBC cells divide asymmetrically once a day in mice (with an average cell cycle time of 21.5 hours) [155] and their location at the bottom of the crypt, immersed in an environment of pro-survival niche signals, enables their maintenance of stemness. Nevertheless, given the limited space at the base of the crypt, upon division half of the progeny is randomly pushed out in a process referred as "neutral competition" [156]. Based on this model, cells falling out of the niche will start their differentiation path, while CBCs at a central and bottom position in the crypts have higher chances to persist longer and maintain stemness [157]. Over the last decades, expression analysis of sorted $Lgr5^+$ cells enabled the identification of a specific CBC gene signature, which includes Achaete-scute complex

homolog 2 (*Ascl2*) [158], tumor necrosis factor receptor superfamily member 19 (TNFRSF19 or *Troy*) [159] and Olfactomedin 4 (*Olfm4*) [160].

+4 reserve stem cells. Potten and colleagues described a more quiescent population of label-retaining cells located above the last Paneth cell in the crypt, at the interphase between the crypt base (stem cell zone) and the progenitor zone (where TA cells reside) [161]. Owing to their positioning four cells above the crypt base, these cells were named +4 cells. In addition, due to their enhanced activity in response to damage and limited contribution during homeostasis, these cells are also referred as "reserve stem cells", meaning a pool of stem cells that repopulates the stem cell zone when CBCs are ablated upon injury or genetic ablation [162,163]. This concept is consistent with their low cycling nature, which renders them less susceptible to damage (such as irradiation). Several markers have been proposed to identify this population, including polycomb protein B lymphoma Mo-MLV insert region 1 homolog (*Bmi1*) [164], telomerase reverse transcriptase (*Tert*) [165], homeobox-only protein (*Hopx*) [162] and leucine-rich repeats and immunoglobulin-like domains 1 (*Lrig1*) [166]. However, the jury is still out on whether these can be defined as *bona fide* +4 cells markers, as they can be found expressed by CBCs as well, at least at the transcript level [167,168].

3.2 Intestinal stem cell niche

In order to continuously divide and generate a brand new epithelial layer every 3-5 days, ISCs rely on signals coming from the surrounding microenvironment, called the intestinal stem cell niche. The function of the stem cell niche is to provide signaling molecules, which on one hand nurture and foster self-renewal of ISCs and on the other hand promote differentiation and positioning of stem cell's progeny. To be able to serve this scope, niche cells are mostly located in direct contact or in close proximity to ISCs at the base of the crypt [169,170]. Belonging to the niche are cells of different origin, encompassing epithelial, mesenchymal (fibroblasts, smooth muscle cells, myofibroblasts), enteric neurons and immune cells. In the following sections, the main niche signals involved in intestinal stem cell activity is briefly discussed (**Figure 3)**.

3.2.1 Wnt

Wnt is considered the most important niche factor controlling stem cells maintenance, proliferation and, when uncontrolled, tumor formation. Upon secretion, Wnt binds to the Frizzled-LRP5-LRP6 receptor complex on ISCs leading to sequestration and inhibition of the continuous destruction of β-catenin by the cytoplasmic tumor suppressor adenomatous polyposis coli (Apc). As a consequence, the pool of cytoplasmic β-catenin increases and translocates to the nucleus where it binds the co-factor T-cell factor (Tcf) and drives the transcription of target genes [171]. Among the direct target genes of Wnt pathway are *c-Myc* (driving proliferation of undifferentiated cells) [172], *Ascl2* (considered as a master regulator of stemness) [173], *Troy* [159], ring finger protein (*Rnf*) *43* [174] and zinc and ring finger (*Znrf*) *3* [175] (all considered as negative regulator of Wnt signaling, see below).

Proofs of Wnt signaling as fundamental to promote stemness come from studies where systemic or conditional knock out of *Tcf4* (the main effector of Wnt pathway) result in complete loss of stem cells both during neonatal development [176] and in adulthood [177]. Similarly, mice with overexpression of the secreted Wnt inhibitor Dickkopf1 (Dkk1) in the intestinal epithelium phenocopy mice lacking *Tcf4* [178]. Recently, another secreted Wnt inhibitor, called Notum, has been identified. Interestingly, Notum levels increase with age and inversely correlate with the regenerative capacity of the intestinal epithelium. Thus, reduced Wnt signaling explains the progressive loss of regenerative potential observed upon aging [179]. While inhibition of Wnt signaling strongly affects stemness, on the contrary boosting this pathway comes at the risk of promoting uncontrolled proliferation and hence fueling tumor formation. Indeed, mice with a heterozygous nonsense mutation in the *Apc* locus ($Apc^{Min/+}$ mice) develop spontaneous adenomas resembling the human familial adenomatous polyposis [180].

The main sources of Wnt are Paneth cells (producer of Wnt3) in the small intestine and the mesenchyme underlying the crypts. Therefore, one may suggest that, Wnt being a vital niche factor, having multiple heterogeneous cellular sources ensures functional redundancy that prevents loss of this pathway *in vivo*. Indeed, while Paneth cells-derived Wnt is sufficient *in vitro* to drive organoids growth [140], conditional deletion of epithelial-derived Wnt *in vivo* does not result in any overt phenotype [181]. Similarly, deletion of Wnt secretion by myofibroblast does not alter the normal intestinal epithelial cells physiology [182]. These results indicate that there are redundant sources of Wnt *in vivo* and even if one cell type fails, the system is built to preserve this pathway without compromising intestinal homeostasis. Arguing against this concept, a recent study identified a peri-cryptal Wnt-producing $Foxl1^{+}$ stromal population, called telocytes, as non-redundant niche cells. Ablation of Wnt secretion by these cells causes severe loss of crypt architecture and impaired epithelial renewal [183].

Wnt bioavailability is tightly controlled and Wnt pathway activation is restricted to the lower crypt and decreases while moving upward towards the villus. The way this gradient is maintained is via the binding of Wnt to its receptor. Since Wnt is poorly soluble and does not freely diffuse, upon secretion by niche cells in the surroundings, it binds immediately to Frizzled receptors on ISCs. When ISCs proliferate and differentiate moving upward, they bring membrane-bound Wnt with them. However, with every cell division, the amount of Wnt on ISC surface is halved, thus establishing a decreasing Wnt gradient when moving along the crypt-villus axis [184]. Despite being highly concentrated at the bottom of the crypt, to ensure a fully functional activation of the Wnt pathway, R-spondins are required. R-spondins are soluble proteins that bind to Lgr4 and Lgr5, components of the Wnt receptor on stem cells. Upon binding, R-spondin amplifies Wnt signaling and mediates the sequestration and inactivation of Rnf43 and Znrf3, ubiquitin-ligases normally mediating Frizzled receptors destruction and thus inhibiting Wnt signaling [185,186].

3.2.2 Notch

Notch represents a key signaling factor to control cell fate specification within the intestinal crypt. Efficient Notch pathway activation requires direct contact between cells expressing

Notch receptor on their membranes (Notch 1-4) and cells expressing the transmembrane Notch ligands (DSL ligands, Jagged (Jag) 1 and 2, Delta-like (Dll) 1 and 4). Upon ligand binding, the Notch receptor intracellular domain (NCID) is released through the proteolytic activity of γ-secretases and translocates to the nucleus. In the nucleus, NCID binds to the transcription factor CSL and drives the transcription of target genes [187]. The main target gene of Notch signaling is *Hes1* (hairy and enhancer of split 1), which in turns acts as a repressor of *Atoh1* (atonal homolog 1, also known as Math1) [188], a crucial regulator of secretory lineage differentiation. As a consequence, blocking Notch signaling (for example through γ-secretase chemical inhibition) [144] or overexpressing Atoh1 [189] results in a preponderant fate specification of proliferative precursors into secretory cells. Therefore, Notch signaling represents a central switch to regulate secretory vs. absorptive lineage commitment decision. Moreover, repression of Atoh1 in cells with active Notch signaling leads to downregulation of Dll1 expression, thus preventing the cell to produce its own Notch ligand. This phenomenon is called "lateral inhibition" and functions so that a central cell expressing Notch ligands activates Notch receptors on surrounding cells, which are in turn inhibited to produce their own ligand and propagate the signal [187]. On the other hand, secretory progenitors express Dll1 and as a consequence suppress the secretory fate in surrounding cells, which upon Notch activation commit to the absorptive lineage [190]. Hence, Notch signaling represents a binary switch (stochastically turned "on" and "off" based on lateral inhibition) driving absorptive vs. secretory fate specification and ensuring a constant ratio between different lineages.

In the stem cell niche, Notch ligands are produce by Paneth cells (Dll1 and Dll4), while in the transit amplifying zone are expressed by secretory progenitors (Dll1). Paneth cells-derived Notch ligands signal on Notch-expressing CBCs driving the expression of *Olfm4* and their proliferation and survival [191].

3.2.3 EGF

EGF is a growth factor important to promote survival and proliferation of intestinal stem cells and transit amplifying progenitors. Upon binding of EGF, the EGF receptor tyrosine kinase EGFR (also known as HER1 or ErbB1) homodimerize and initiate a pro-proliferative signaling cascade involving mitogen-activated protein kinase (MAPK), phosphatidyl-inositol 3-kinase (PI3K), c-Jun N-terminal kinases (JNK), phospholipase C (PLC) and Jak/STAT pathways [192]. Owing to its role as an important pro-proliferative signal, EGF levels require a tight control as overactivation of this pathway has been linked to neoplastic progression. To this aim, ISCs express the EGFR inhibitor Lrig1 mediating ubiquitination and degradation of EGFR. Consistent with its role as a negative feedback, genetic deletion of Lrig1 results in abnormal crypt expansion and enlarged intestine in mice [193].

Ligands of EGFR (such as EGF, TGFα, Areg or Epiregulin) are produced by Paneth cells [140], by the mesenchyme underlying the crypt [194] and by immune cells [40]. As a key niche growth factor, EGF is one of the crucial components used in organoids cultures [154]. However, unlike Wnt and Notch, EGF signaling is not required to maintain stem cells identity but rather to

promote their activity. Indeed, removal of EGF does not cause stem cell loss but only induces a quiescent state in Lgr5^{+} cells, which is reversible upon EGF restoration [195].

3.2.4 BMP

Bone morphogenetic proteins, members of the TGF-β superfamily, are essential mediators of intestinal epithelial cells differentiation. Binding of BMPs (BMP2 and 4 in the intestine) to their receptors (Bmpr1 and 2) mediate the phosphorylation of SMAD1, 5 and 8, which then bind to SMAD4 and translocate to the nucleus to mediate target gene expression. Among the direct target genes of BMP signaling are DNA-binding protein inhibitor (Id) 1, 2 and 3 [196]. The main function of the BMP pathway is to counteract pro-proliferative signals in the stem cell niche and instead provide a pro-differentiation signal. As a consequence, deletion of *Bmpr1* in mice results in expansion of ISCs and TA cells and formation of benign polyps [197], recapitulating the phenotype of patient with juvenile polyposis carrying inactivating mutations in the BMP pathway [198]. BMPs are produced by inter-crypts and inter-villus mesenchymal cells [199]. However, while in the villus region BMP signaling is essential to boost differentiation of maturing enterocytes, in the crypt region this pathway needs to be tightly controlled to ensure equilibrium with pro-stemness signals. To this aim, mesenchymal cells producing BMPs in the crypt proximity also produce BMP inhibitors, including Noggin, Chordin-like 1 and Gremlin1 and 2 [200]. This way, an increasing gradient of BMP availability is established moving upward along the crypt-villus axis.

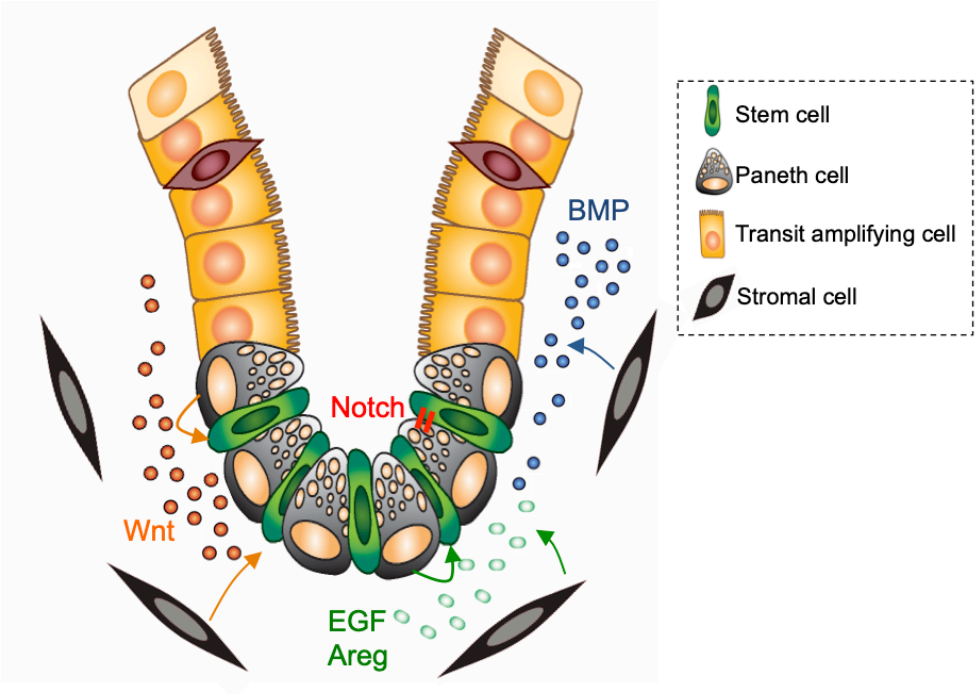

Figure 3. *Schematic representation of a small intestinal crypt and niche signals.*

3.3 Epithelial response to damage

The intestinal epithelium is under the constant risk of extrinsic and intrinsic insults that hamper the homeostatic self-renewal and can result in overt damage. In such scenario, the epithelium needs to boost and exploit a regenerative program that enables repair of the injured barrier. Proving the regenerative capacity of the intestinal epithelium, irradiation, acute inflammation or surgical resection are examples of insults damaging barrier integrity that are followed by an hyper-proliferative response of the crypt and crypt fission (a process whereby two independent crypts are generated from one) [201,202]. The regenerative response to damage is achieved through different strategies: (a) the "two-stem cell model" where rare and quiescent stem cell populations poorly contributing to homeostatic regeneration, are essential in response to damage; (b) niche signals (described above in the "Intestinal stem cell niche") are enhanced over the homeostatic range, thus boosting the proliferative capacity of stem cells; (c) specific inflammatory cues generated in response to injury signal to ISCs and enhance their activity; (d) specific dietary regimens and nutritional intake modulate stem cells activity; (e) when ISCs are ablated by damage, committed progenitors display a plastic behavior and regain stem cell potential ("reverse" stem cell potential) [203]. In the following paragraphs these strategies are briefly presented.

3.3.1 (a) "Two-stem cell model"

As described above, +4 reserve stem cells, owing to their quiescent nature, are less radiosensitive and spared from damage allowing repopulation of the stem cell pool [165,204]. However, another study combining CBC ablation and intestinal damage reported that +4 reserve stem cells are dispensable for tissue repair, while CBCs are essential [205]. Recently, by single cell transcriptomic analysis of intestinal crypts undergoing irradiation-induced damage, a new population called "revival stem cells" has been identified. These cells, poorly represented during steady state, rapidly expand in response to damage and drive barrier regeneration [206].

3.3.2 (b) Niche signals

Most of the niche signals (see above), normally promoting homeostatic stem cell activity, are essential to mediate the regenerative response when damage occurs. For instance, deletion of *c-Myc*, a Wnt target gene, results in severe crypt loss following irradiation [207]. On the other hand, administration of R-spondin 1 favors repair following chemotherapy [208].

The Hippo pathway has recently emerged as key contributor to intestinal regeneration, while poorly contributing to homeostasis. Upon Hippo pathway activation (encompassing a multitude of inputs including mechanotransduction, GPCR signaling, metabolite sensing), the kinase MST (1 and 2) phosphorylates LAT1/2 kinases, which in turn phosphorylate and inactivate Yes-associated protein (YAP) and Tafazzin (TAZ) by sequestering them in the cytoplasm [209]. In the context of chemical or radiation injury, YAP and TAZ negatively regulate Wnt, thus inhibiting uncontrolled stem and Paneth cells expansion upon regeneration and limiting the risk of microadenomas formation [210,194]. Therefore, Hippo signaling ensures that Wnt levels are in the optimal range to allow effective regeneration while avoiding crypt

exhaustion. On the other hand, YAP fuels the EGF pathway by boosting Egfr signaling [194]. Altogether, these studies suggest a role of YAP in driving regeneration by enhancing stem cells growth factor (i.e. EGF signaling) and by reprograming ISCs through Wnt inhibition.

3.3.3 (c) Pro-regenerative inflammation

Barrier damage in the intestine results in exposure to a plethora of exogenous antigens and pro-inflammatory mediators leading to activation of the immune system and efficient mounting of an inflammatory response. While on one hand the immune system ensures elimination of sources of extrinsic and intrinsic damage, on the other hand is actively involved in promoting tissue remodeling and re-establishment of homeostasis. Different secreted interleukins have been shown to play a pivotal role in driving intestinal stem cell activity. As an example, IL-22 produced by ILC3s in response to radiation-induced injury drives stem cells proliferation and barrier repair (see above in "ILC3" section). Similarly, type 2 mediators produced by ILC2s (IL-13 and Areg) boost epithelial cell proliferation and skewing towards secretory cells necessary to fight helminth infection (see above in "ILC2s" section). Recently, a novel role has been assigned to T cell responses in driving intestinal stem cell fate specification and self-renewal. Intestinal T cells have been postulated to interact with ISCs via the expression of MHC-II molecules on the latter. Sensing of pro-inflammatory Th cytokines (such as IL-17 or IL-13) drives ISCs differentiation and fate specification. On the contrary, presence of regulatory cytokines (such as IL-10 normally produced by Treg cells) favors ISCs self-renewal [45]. Therefore, these studies display a new scenario where the stem cell pool is rewired by the inflammatory milieu in the surrounding microenvironment.

3.3.4 (d) Nutrition

Different dietary regimens can highly alter the proliferative response of intestinal stem cells. Starvation (acute food deprivation) and caloric restriction (long-term reduction in nutrient intake) are associated with decreased epithelial surface (less enterocytes and shorter villi), reduced cycling of TA progenitors and in parallel a direct expansion and activation of Paneth cells (through mTORC1) leading to expansion of the CBC pool [211]. This way, in a scenario of limited access to nutrients our body minimizes energy expenditure (by reducing the epithelial surface) and builds up a stock of stem cells poised to re-establish a normal architecture as soon as re-feeding takes place. As a consequence of this quiescent status induced by caloric restriction, mice undergoing fasting are more protected against lethal DNA damage and display stem cells survival and barrier integrity [212].

In contrast to starvation, exposure of mice to a high fat diet results in Paneth cell reduction. However, unexpectedly, this phenomenon is paralleled by expansion of the stem and progenitor cells pool. Mechanistically, PPARδ activation (induced by fatty acids enriched in high fat diet) drives expression of Jag1 and 2 and higher sensitivity to Wnt in CBCs. As a consequence, stem cells become independent of Paneth-derived Notch signaling (by producing their own ligands) and acquire the ability to survive and proliferate in the absence of the epithelial niche [213]. Escaping from the homeostatic control of the stem cell niche, CBCs are exposed to the risk of uncontrolled proliferation and malignant transformation. Indeed,

PPARδ treated TA cells display higher tumorigenic potential, possibly explaining the link between obesity and colorectal cancer [214].

Besides fatty acids (signaling through PPARδ), another link explaining risk of tumor formation in the context of obesity is cholesterol (also enriched in high fat diet). Feeding mice with high cholesterol diet enhances stem cells proliferation and boosting cholesterol biosynthesis results in increase tumor formation in the *Apc*$^{Min/+}$ mouse model [215]. However, our body has a system in place to avoid toxic overload of cellular cholesterol, which relies on cholesterol metabolism into oxidized derivatives called oxysterols (see below in "cholesterol metabolism"). The role of cholesterol metabolism in the context of intestinal regeneration and tumorigenesis remains poorly addressed. In the last manuscript included in this thesis, we have shown how cholesterol metabolism is an adapted tissue response to damage which leads to enhanced intestinal regeneration while limiting the risk of tumor development.

3.3.5 (e) Plasticity of committed progenitors

Many of the damage models used to study regeneration are characterized by almost complete ablation of the stem cell pool at the crypt bottom (as seen in irradiation, chemotherapy or diphtheria toxin mediated stem cells elimination). However, the epithelium recovers in few days and new functional crypts with new CBCs are formed. This finding implies that in a process independent of self-renewal, CBCs can be generated from other cells surviving the damage. Besides the +4 reserve stem cells, several other committed progenitors (including secretory precursors [190,216], Paneth cells [217] and enterocytes precursors [218]) have been shown to de-differentiate, migrate to the crypt bottom and repopulate the stem cell pool, giving rise to all the differentiated enterocytes.

4 CHOLESTEROL METABOLISM

Cholesterol is a crucial building block of the cell membrane and it plays an integral part in allowing transmembrane receptor signaling via the formation of lipid rafts. Moreover, cholesterol serves as the substrate for the generation of several metabolites, such as steroid hormones, bile acid and oxysterols. Despite these homeostatic roles, a tight control of its levels is required as excessive cholesterol is toxic and associated to development of several chronic metabolic disorders, such as diabetes and atherosclerosis [219]. One way to keep cholesterol levels in check is an intrinsic negative feedback mechanism. High cellular cholesterol levels result in the direct inhibition of sterol regulatory element-binding protein (SREBP) transcription factors, which promote cholesterol biosynthesis [220]. On the other hand, metabolic generation of oxysterols represents a second strategy to avoid cholesterol overload. According to the "oxysterol hypothesis", some oxysterols have been shown to directly inhibit SREBP activity and to promote reverse cholesterol transport by binding to their receptor Liver X Receptor (LXR) [221,222]. Reverse cholesterol transport is a process that mediates removal of cholesterol from peripheral tissues, transport to the liver and excretion in the form of bile acid [223,224]. Besides this purely metabolic activity, oxysterols have been shown to be critical in controlling several biological processes including cell proliferation, migration and immune cells function both via regulation of cellular cholesterol load and through other pathways [225]. In the following paragraphs, an overview of the most characterized oxysterols and the function of the main oxysterol receptors is presented.

4.1 Oxysterols

Oxysterols are hydroxylated derivatives of cholesterol generated via either enzymatic or non-enzymatic conversion [226]. Non-enzymatic oxysterols can originate from the diet or they are produced endogenously by free radical attack of cholesterol. Enzymatically produced oxysterols instead are generated via the activity of specific enzymes (mainly belonging to the cytochrome P450 family). This class of oxysterols comprises intermediates of the bile acid synthesis and steroid hormones as well as oxysterols selectively generated in specific cell types [226]. Below a brief description of the biosynthesis and function of the main oxysterols is provided.

4.1.1 27-hydroxycholesterol

27-hydroxycholesterol (27HC) is generated via the enzymatic conversion of cholesterol mediated by the sterol 27-hydroxylase (Cyp27a1), a mitochondrial cytochrome P450 oxidase. Under homeostatic condition, 27HC is the most abundant oxysterol found in circulation [227]. 27HC is a substrate for bile acid generation and Cyp27a1 is involved both in the classic and in the alternative pathway of bile acid production [227]. As a result, mice deficient in *Cyp27a1* display severe defects in bile acid synthesis [228]. Moreover, in line with the "oxysterol hypothesis", 27HC has been shown to inhibit the activity of SREBP and thus limit cholesterol biosynthesis. Besides these metabolic roles, 27HC can bind to two nuclear receptors: the estrogen receptor (ER) and LXR. Binding of 27HC to ER has been linked to a pro-

inflammatory response in MΦ in the context of atherosclerosis [229] and to a pro-metastatic role in breast cancer [230]. Contrary to these pro-inflammatory functions when binding to ER, binding of 27HC to LXR seems to be mainly associated to an anti-inflammatory effect (see below in "Liver X Receptor") [225]. Whether inflammation can induce Cyp27a1 expression and generation of 27HC as a negative feedback mechanism remains unknown. So far a role in inducing Cyp27a1 expression has been attributed to RA [231], the steroid- and bile acid-activated pregnane X receptor (PXR) [232] and LXR [233]. In our study, we have shown that intestinal barrier damage induces upregulation of Cyp27a1 and that this process contributes to intestinal regeneration.

4.1.2 25-hydroxychlesterol

25-hydroxycholesterol (25HC) is generated via the activity of the cholesterol 25-hydroxylase (Ch25h). 25HC can inhibit SREBP activity, bind to LXR (although with a lesser affinity compared to 27HC) [234] and function as an inverse agonist of RORα (26812621), a nuclear receptor involved in circadian rhythm regulation and ILC2 development. Induction of Ch25h is observed in the context of viral infection and appears to be mediated by type I interferon (IFN-I), especially in myeloid cells [235]. In line with this, 25HC has been attributed an antiviral effect by inhibiting viral particle fusion to the cell membrane [236] and by inhibiting cholesterol biosynthesis [237]. In parallel to its anti-viral function, 25HC play an important anti-inflammatory role. Cyster's lab elegantly demonstrated that Ch25h expression dampens production of pro-inflammatory cytokine such as IL-17 from αβ and γδ T cells [238] and IL-1β from MΦs by inhibiting inflammasome activation in response to LPS stimulation [239]. Interestingly, together with IFN-I, endotoxin administration stimulates *Ch25h* expression and 25HC production [240]. Therefore, production of this oxysterol might represent an adapted response to inflammation directed at dampening it.

25HC can be further processed to generate 7α,25-dihydroxycholesterol (7α,25-HC) via the activity of the Cytochrome P450 Family 7 Subfamily B Member 1 (Cyp7b1) enzyme. Expression of this oxysterol is predominant in immune cells where it plays key biological functions by acting on the receptor EBI2 (see below in "EBI2 receptor").

4.1.3 24-hydroxycholesterol

24-hydroxycholesterol (24HC) is produced via the activity of the cytochrome P450 enzyme cholesterol 24-hydroxylase (Cyp46a1). 24HC is considered a brain specific oxysterol (known as cerebrosterol), with a pattern of expression almost exclusively restricted to neurons [241]. By acting on the receptor LXR, 24HC mediates elimination of excess brain cholesterol [242]. Unlike 24HC, which is produced *in loco*, 27HC is also found in the brain tissue, but in this case coming from the circulation and crossing the blood brain barrier. Remarkably, altered levels of 24HC and 27HC have been reported in several neurodegenerative disorders including multiple sclerosis [243], Alzheimer disease [244] and Parkinson's disease [245]. In particular, patients with long history of multiple sclerosis (relapsing-remitting and primary progressive patients) have lower plasma concentration of 24HC compared to early-diagnosed

patients [243]. These results imply a role for oxysterols in the modulation of neuroinflammation and point towards the anti-inflammatory role of LXR (see below in "Liver X Receptor").

4.2 EBI2 receptor

EBI2 is a G protein-coupled receptor that binds to 7α,25-HC. It is expressed on different immune cells (including B cells, T cells, DCs and ILCs) where it plays a critical role in controlling cell migration.

B cells display the highest EBI2 expression among all immune cells and B cell receptor (BCR) stimulation further upregulates it. Activation of EBI2 on B cells drives their migration towards appropriate intra- and extra-follicular regions following antigen recognition, which enables a functional antibody response [246,247]. B cells deficient for EBI2 or disruption of the enzymatic machinery required for the production of EBI2 ligands results in a microanatomic disorganization of B cells coupled with a reduced T cell-dependent antibody response [248,249]. EBI2 ligands are mainly produced by lymphoid tissue stromal cells enriched in the outer follicle and in interfollicular regions [250]. This expression pattern, defined based on the spatial analysis of *Ch25h* expressing stromal cells, creates a micro-gradient of EBI2 ligands that control B cell migration in lymphoid organs. How *Ch25h* induction is governed among different stromal subsets in different area of lymphoid tissue remains unexplored.

Similar to B cells, EBI2 expression on DCs drives their chemotaxis. Splenic $CD4^+$ DCs, via EBI2 activation, expand and localize in the marginal zone bridging channels where they can pick up blood-borne antigens. This process subsequently favors antigen presentation to T cells and generation of a T cell-dependent antibody response [251].

Finally, follicular T cells are guided towards the interface of the B cell follicle and the T cells zone via sensing of 7α,25-HC by EBI2 receptor [252].

Recently, we and others have shown that EBI2 expression on ILC3s is required for their positioning in cryptopatches, thus driving formation of isolated lymphoid follicles [253] and immunity to enteric bacterial infection [104].

4.3 Liver X Receptor

LXR is a sterol activated transcription factor belonging to the class of nuclear receptors. Two isoforms of LXR are present, LXRα (*Nr1h3*) and LXRβ (*Nr1h2*), which bind to the same ligands and regulate the same target genes expression but differ in tissue distribution. While LXRα is mainly expressed in metabolically active tissues and cells types (such as liver, intestine, adipose tissue and macrophages), LXRβ is ubiquitously expressed [219]. As described above, the main ligands activating LXR are oxysterols (predominantly 27HC and 24HC), but also intermediates of the cholesterol biosynthetic pathway, such as desmosterol [254]. To function as a transcription factor, LXR heterodimerizes with retinoid X receptors (RXRs) [255]. When activating ligands are absent, LXR-RXR heterodimer is bound to its response element on the DNA, in complex with co-repressor and thus inhibiting the transcription of target

genes [256]. Upon ligand binding, co-repressors are released and co-activators recruited leading to target gene activation. The main target genes of LXR encode for proteins involved in reverse cholesterol transport, such as the membrane transporters *Abca1* and *Abcg1* mediating cellular cholesterol efflux [257]. Besides directly regulating the transcription of target genes, LXR can negatively regulate gene expression by inhibiting the activity of other transcription factors, such as NF-κB or AP-1, two important pro-inflammatory genes [223,258].

Together with regulating cholesterol homeostasis, LXR plays an important immunological role by suppressing inflammatory responses. In line with this hypothesis, the use of LXR synthetic ligands has been proposed to dampen the progression of various inflammatory diseases in mice, including atherosclerosis, Parkinson's diseases or experimental autoimmune encephalomyelitis (EAE, a mouse model for multiple sclerosis)[219,225]. Interestingly, loss of LXR in mice results in increased susceptibility to chemically induced colitis[259], but whether regulation of oxysterol levels in the intestine has been adapted as a measure to control inflammation remains unaddressed.

The immune-regulatory role of LXR has been extensively characterized in MΦ. The use of LXR agonist *in vitro* on LPS-stimulated MΦs inhibits the expression of pro-inflammatory mediators, including cyclooxygenase 2, inducible nitric oxide synthase, IL-6 and IL-1β[223,260]. *In vivo*, phagocytosis of apoptotic neutrophils raises cholesterol levels in MΦs and, through LXR activation, inhibits the production of IL-23, IL-17 and G-CSF [261]. In addition, LXR activity has been associated with MΦ survival and antimicrobial activity in the context of bacterial infections, such as *Listeria monocytogenes*[262], *Escherichia coli* and *Salmonella typhimurium*[263].

Besides regulating myeloid cells, recent studies propose a role for LXR in regulating lymphocyte biology. Upon T cell activation, induction of sulfotransferase family 2B member 1 (SULT2B1, an enzyme promoting oxysterol sulfation and inactivation) results in decreased LXR activity with consequent cholesterol accumulation required for new membrane formation and proliferation[264]. In line with these results, mice lacking LXR undergo lymphoid hyperplasia and develop autoimmune glomerulonephritis and lupus-like disorders with age[264,265]. In addition, LXR activation participates in T cell differentiation by inhibiting AhR-mediated Th17 induction. As a consequence, loss of LXR is associated with greater severity of EAE, possibly by enhancing the pathogenic effect of Th17[266]. Recently, an indirect control of adaptive immunity has been attributed to LXR, involving regulation of DCs functioning as an orchestrator of T and B cell response. In detail, by modulating cellular cholesterol load in DCs, LXR enhances T cell priming and the production of B cell trophic factors, thus coupling cholesterol metabolism to DC-driven T and B cell proliferation[267]. Furthermore, LXR functions as a regulator of DC biology by inhibiting their migration to lymph nodes and, as a consequence, antigen-presentation. Upon cancer development, tumor cells produce oxysterols, which cause LXR-mediated down-regulation of CCR7 on DCs and tumor immune escape[268]. Overall, oxysterol sensing and LXR activity are important

contributors to immune regulation and control of inflammation. However, their role in intestinal homeostasis remains poorly understood.

Besides modulating immune cell functions, LXR plays crucial anti-proliferative roles in epithelial cells. By overexpressing LXRα in intestinal epithelial cells, Lo Sasso and colleagues showed that genetic (*Apc*$^{Min/+}$) and chemically induced (AOM-DSS) intestinal tumor formation was inhibited [284]. This study suggests that by decreasing the cellular cholesterol pool size, LXRα restrains the proliferative potential of intestinal tumor cells and induces caspase-dependent apoptosis. In the last manuscript included in this thesis we have added on these data and showed that while LXR activation restrains intestinal tumorigenesis, at the same time it promotes epithelial cell regeneration in the context of acute intestinal injury.

5 AIMS OF THE THESIS

The overall aim of this thesis was to investigate how ILC and epithelial cell biology was modulated in the context of intestinal homeostasis, inflammation and tissue response to damage. In particular, the three specific aims were as follows:

Study I: To investigate the ability of ILC progenitors in the bone marrow to respond to peripheral inflammation.

Study II: To functionally characterize how cholesterol metabolism modulates intestinal ILC functions.

Study III: To explore the physiological tissue response to damage and how cholesterol metabolism controls the regenerative potential of the intestinal epithelium.

6 MATERIALS AND METHODS

This chapter briefly summarizes the main materials and methods utilized in the three manuscripts included in this thesis. For a more detailed description, please refer to the "materials and methods" section of each manuscript.

6.1 **Mouse studies** (Study I-II-III)

All experiments were approved by the Stockholm Regional Ethics Committee and the responsible federal health authorities of the State of Hamburg. Mice were maintained under specific pathogen free conditions and both male and female mice were used between 6-20 weeks of age. All experiments were performed in accordance with national and institutional guidelines and regulations.

6.2 **Processing of tissues** (Study I-II-III)

Single cell suspension from spleen, lymph nodes and liver was obtained through smashing on 70μm cell strainers. Liver leukocytes were then enriched through Percoll gradient (Sigma Aldrich). Lung tissue was minced with scissors and digested with 0.15 mg/ml Liberase TL and 0.1 mg/ml DNase I (Roche) followed by Percoll gradient. Bones were crushed with a pestle and filtered through 70μm cell strainers. Intestinal tissue (both small intestine and colon) were first incubated with HBSS containing 10% FCS, 5 mM EDTA, 1 mM DTT and 15 mM HEPES to remove epithelial cells, followed by digestion with serum-free HBSS containing liberase TL (0.15 mg/ml) and 0.1 mg/ml DNase I and Percoll gradient. For intestinal crypts isolation, small intestine was incubated in 10mM EDTA on ice followed by filtration through 70μm cell strainer.

6.3 **Flow cytometry and fluorescent activated cell sorting** (Study I-II-III)

Samples were first stained with live/dead fixable viability dyes (eBioscience) and blocked with Fc-blocking (CD16/32) antibody (eBioscience). Samples were then stained at 4°C with panels of fluorescently labeled antibodies for surface antigens. For intracellular staining, cells were fixed and permeabilized using the fixation and permeabilization buffers (from the Foxp3 staining kit, eBioscience) followed by staining at RT with fluorescently labeled antibodies specific for intracellular proteins. For flow cytometry analysis, samples were acquired using FACS Canto II or FACS LSR Fortessa flow cytometers (BD Biosciences) and analyzed with FlowJo software (TreeStar). For fluorescent activated cell sorting, samples were sorted using a BD FACSAriaI or AriaIII (BD Biosciences). In case of sorting of rare populations (such as CHILPs in Paper I), a pre-enrichment with depletion of lineage$^+$ cells through magnetic beads (Miltenyi Biotec) was performed.

6.4 **Intestinal injury models** (Study I-II-III)

Several intestinal injury models were used in the three manuscripts included in this thesis. Dextran sulfate sodium (DSS) induced colitis (**I-III**) was induced by feeding mice with 2-3% (w/v) DSS (MP Biomedicals or TdB Consultancy) dissolved in drinking water ad libitum for

5-7 days, followed by regular water administration to allow tissue repair. Alternatively (**II**), colitis was induced by injecting 100μg of CD40 (FGK45) antibody intraperitoneally in *Rag1*-deficient mice.

Small and large intestinal damage (**III**) was induced by exposing mice to a dose of 10Gy from an X Radiation Unit.

Chemically induced colon tumor (**III**) was induced by i.p. injection of azoxymethane (AOM, Sigma Aldrich) followed by three cycles of DSS administration in drinking water, alternated with two weeks of regular water.

6.5 **Organoid culture** (Study III)

Following isolation, small intestinal crypts were resuspended in 30%-70% ratio of DMEM/F12 and Matrigel (Corning) and plated in pre-warmed 48 well plates. Complete ENR medium including 500μg/ml of EGF (R&D), 250-500ng/ml of recombinant murine R-Spondin (R&D) and 100ng/ml of Noggin (Peprotech) was overlaid. Complete fresh medium was changed every other day and crypt domain quantification was usually performed at day 3 of culture, followed by harvesting for RNA extraction at day 4-7 of culture.

6.6 **Quantitative PCR analysis** (Study I-II-III)

Tissue collected for RNA extraction was stored in RNALater (Invitrogen) followed by bead-beating lysis (Precellys) in RLT buffer (Qiagen) + 2% β-mercaptoethanol. Cells collected for RNA extraction were resuspended in RLT buffer (Qiagen) + 2% β-mercaptoethanol. RNA extraction was performed using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Alternatively, cells were resuspended in TRIzol reagent (Invitrogen) and RNA was extracted by chloroform phase separation. Reverse transcription to cDNA was performed using iScript RT Supermix (Biorad) (**I-II-III**), SuperScript First-Strand Synthesis System (Invitrogen) (**II**) or High-capacity cDNA Reverse Transcriptase Kit (applied Biosystems) (**II**). Gene expression analysis was performed by quantitative real time PCR using either iTaq Universal SYBR Green Supermix (Biorad) or Taqman probes (Thermo Fisher Scientific).

6.7 **Statistical analysis** (Study I-II-III)

Unpaired two-tailed Student's *t*-test with 95% confidence interval was used when comparing two independent groups. When the same biological sample was tested with different biological stimuli, paired *t*-test was used. When comparing more than two groups (unless specifically indicated), data were analyzed using one-way ANOVA with Bonferroni's multiple comparisons test. Data were analyzed using GraphPad Prism Software 6.0e.

7 RESULTS AND DISCUSSION

This section provides a brief summary of the main findings of each study and discusses their relevance. More details can be found in the manuscripts attached to this thesis.

7.1 Flt3 ligand promotes expansion of ILC precursors (Study I)

Development of ILCs mainly takes place during the fetal and post-natal window. Subsequently, peripheral ILCs display a tissue resident behavior and do not seem to rely on circulating progenitors for their renewal under homeostatic conditions [133]. Nevertheless, the adult murine bone marrow (BM) harbors a population of ILC progenitors endowed with the ability to generate all different mature ILC subsets. A plausible explanation to reconcile this paradox is that BM ILC precursors represent a backup poised to give rise to new ILCs in order to replenish the exhausted mature peripheral pool when inflammation occurs. In line with this hypothesis, at a later time point after infection, peripheral ILCs partially lose their ability to expand locally and start to depend on replenishment from hematogenous sources [133]. We, therefore, tested whether ILC precursors in the BM are capable of sensing peripheral inflammation and react with a demanded ILC-poiesis, similar to what has been shown for myeloid progenitors in the context of "emergency myelopoiesis" in response to systemic infections.

Flt3L is a hematopoietic cytokine that promotes proliferation of myeloid and lymphoid progenitors under homeostatic conditions (including DCs, NK and common lymphoid progenitor cells) [269,270]. In the context of inflammation (such as malaria, cytomegalovirus infection or rheumatoid arthritis) [271,272], Flt3L levels increase and influence hematopoiesis, hence representing a good candidate to test our hypothesis.

We first tested whether increased levels of Flt3L were affecting the pool of ILC precursors in the BM. To this aim, we injected mice with a B16 melanoma cell line constitutively secreting Flt3L (B16-Flt3L), a tool that allowed us to achieve continuous systemic production of this cytokine within two weeks. By comparing the BM of mice B16-Flt3L-injected with mice injected with B16 only, we observed a drastic expansion of CHILPs (common helper ILC precursors) [30] and of ILCP (ILC precursors that lost the ability to generate LTi) without affecting the pool of committed ILC2 progenitors (ILC2P) (**Figure 4**).

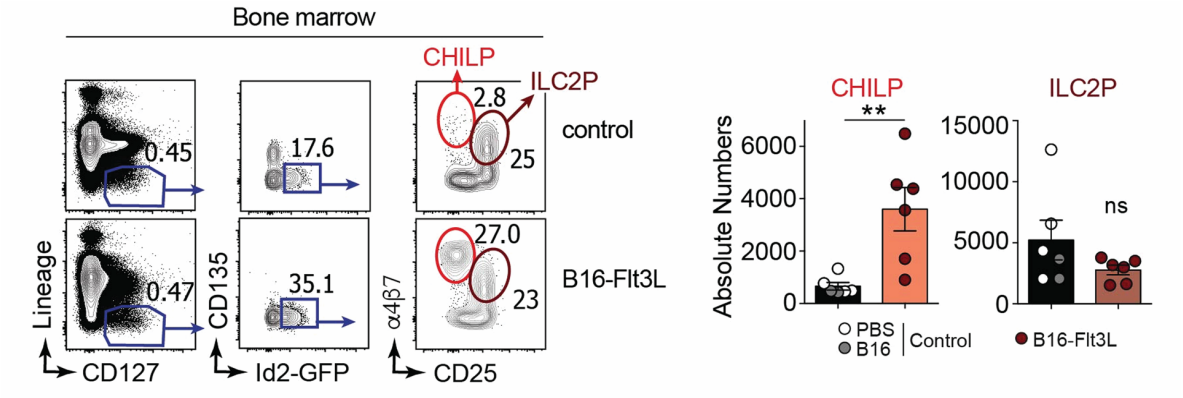

Figure 4. *Expansion of CHILPs in response to B16-Flt3L.*

Interestingly, CHILPs are defined, among other markers, by the absence of Flt3 receptor expression. On the contrary, upstream of CHILPs, CLPs express Flt3 receptor. Whether Flt3L signaling in CLP skews their differentiation towards the ILC lineage is an intriguing hypothesis that remains to be tested.

Given the observed expansion of ILC precursors in the BM, we tested whether increased Flt3L levels affected the peripheral pool of mature ILCs. To our surprise, we did not detect any difference in frequencies and numbers of peripheral ILC1, ILC2 and ILC3s in small intestine, colon, lungs and liver upon B16-Flt3L injection. This finding might be explained by the short-term exposure to high Flt3L levels (two weeks), which might not be enough to affect the pool of CHILPs, generate new ILCs and expand the pool of mature ILCs in the tissues. In line with this hypothesis, a long-term modification of Flt3L levels or Flt3L sensing (by using mice deficient for Flt3 receptor or transgenic for Flt3L) results in expansion of peripheral ILCs [273]. Alternatively, in our system, expansion of mature ILCs does not occur due to lack of peripheral inflammation, which might create an empty and more accessible niche in the tissue for BM-generated ILC replenishment.

To confirm that the expanded pool of CHILPs in the BM were selectively committed to ILCs and to test whether their differentiation potential was altered by Flt3L exposure, we exploited an *in vivo* adoptive transfer approach. Sorted CHILPs from control and B16-Flt3L injected mice were injected into lymphopenic *Rag*$^{-/-}$*Il2rg*$^{-/-}$ mice. Analysis of recipient mice confirmed that CHILPs were selectively giving rise to all helper ILCs *in vivo* and that Flt3L-mediated expansion was not affecting their ability to differentiate uniquely into ILCs (**Figure5**).

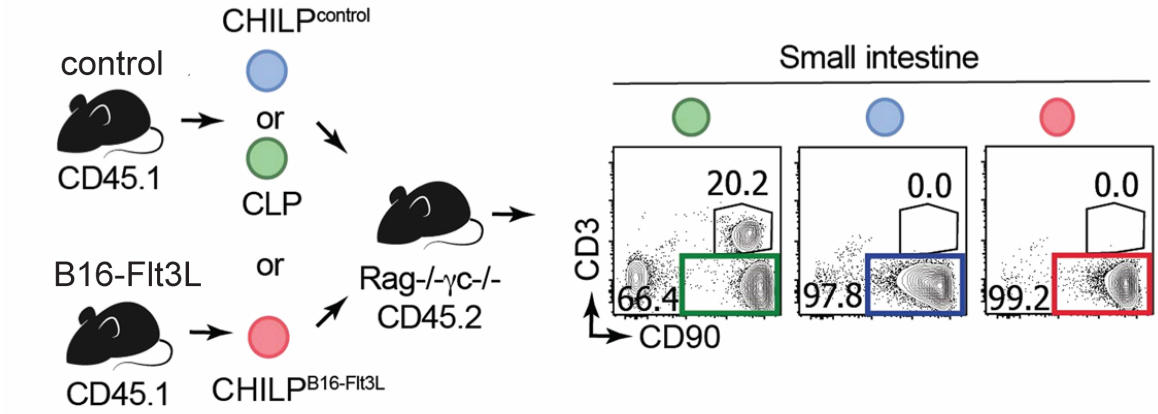

Figure 5. *Flt3L-expanded CHILPs give rise selectively to ILCs.*

Our data alluded to a role of Flt3L in regulating the size of ILC precursors in the BM. With our initial hypothesis in mind, we tested whether this axis occurs upon inflammatory insults, where Flt3L is not artificially modulated (as in our initial model with B16-Flt3L injection). We first tested if Flt3L systemic levels were modulated upon intestinal inflammation and whether this phenomenon was accompanied by expansion of ILC precursors in the BM. We reasoned that ILC precursors might expand in a colitis model since expansion of mature ILCs has been observed in the inflamed colon of inflammatory bowel disease (IBD) patients [33,274] and in mouse models of chronic intestinal inflammation [275]. However, both in the serum of mice exposed to chemically induced colitis and in the plasma of IBD patients, we did not observe

alteration in the Flt3L levels when compared to their respective controls. Similarly, CHILPs numbers were unaffected in the BM of mice undergoing colitis.
On the other hand, in line with previous reports, we observed altered Flt3L levels in circulation when analyzing another inflammatory setting, namely malaria. Both in plasmodium infected mice and in human malaria patients we detected increased Flt3L in serum and plasma respectively. Interestingly, increased systemic levels of Flt3L were kinetically preceding expansion of CHILPs in the BM of infected mice.
Altogether, these data propose a new role for Flt3L in controlling the size of ILC progenitors in the BM. Moreover, we propose a potential involvement of the Flt3L-CHILP axis in the context of specific inflammatory settings (for example malaria). The functional consequences of inflammation-mediated ILC precursors expansion still remain to be addressed. Analysis of ILCs in inflamed tissues, especially in the context of chronic inflammation with enhanced Flt3L levels, or pharmacologic/genetic blocking of this axis during inflammation might represent a future outlook to reveal a novel biological function of "inflammatory ILC-poiesis".

7.2 Oxysterol sensing through EBI2 controls ILC3 function (Study II)

In the second manuscript included in this thesis, we investigated the function and migratory potential of ILCs in the periphery. As immune sentinels and gatekeepers of tissue homeostasis, ILCs are endowed with the ability to sense and respond to cues and various metabolites from the local environment [36,276]. However, whether ILC function is modulated by cholesterol metabolism remains unaddressed. In this study, in collaboration with Tim Willinger's group, we described a novel role for the GPCR Epstein Barr virus-induced gene 2 (EBI2) sensing cholesterol metabolites and driving ILC3 migration, lymphoid tissue formation and inflammation in the colon.

EBI2 (also known as GPR183) is a receptor for hydroxylated derivatives of cholesterol, named oxysterols (in particular 7α,25-dihydroxycholesterol, 7α,25-HC), regulating chemotaxis of B cells, T follicular helper cells and DCs [277]. However, whether EBI2 can control ILC function remains unknown. To address this question we made use of reporter mice for EBI2 (GPR183-GFP mice), *in vitro* migration assay and mice deficient in *Gpr183* or the enzyme necessary to produce EBI2 ligands (i.e. *Ch25h*). We showed that adult LTi-like ILC3s express EBI2 and migrate towards 7α,25-HC *in vitro*. *In vivo*, expression of EBI2 intrinsically on ILC3 was necessary to drive their migration to colonic cryptopatches and thus the formation of isolated lymphoid follicles (ILFs). Moreover, this phenomenon was dependent on the presence of the ligand, as mice lacking *Ch25h* phenocopied mice deficient in *Gpr183* on ILC3s and displayed severe impairment in the formation of colonic cryptopatches. Looking for the source of 7α,25-HC in the tissue, we observed that stromal cells defined by the expression of podoplanin, CD34 and CXCL13 located in cryptopatches and ILFs were the predominant cell types expressing the oxysterol-producing enzymes *Ch25h* and *Cyp7b1* in a microbiota-independent manner.

We then hypothesized that blocking the migration of ILC3s to colonic lymphoid tissues might cause defects in their function. Interestingly, ILC3s from mice lacking EBI2 produced

significantly lower IL-22 compared to EBI2 sufficient mice. This phenomenon was not directly mediated by 7α,25-HC sensing, as purified ILC3s cultured *in vitro* with this oxysterol did not show any impairment in IL-22 production. Conversely, altered ILC3 function seems to be secondary to the migration defect and the reduction in colonic cryptopatches formation.

Expanding these observations beyond physiology, we observed that innate-immune mediated colonic inflammation (i.e. anti CD40) resulted in upregulation of the enzymatic machinery producing 7α,25-HC and concomitant downregulation of oxysterol degrading enzymes (*Hsd3b7*). The increased oxysterol production led to an EBI2-dependent accumulation of ILC3s in inflammatory foci and to an overall increase of colonic inflammation.

Overall this study uncovered a novel mechanism of metabolite sensing by ILCs, which is fundamental towards the ontogenesis of lymphoid tissues in the colon. Moreover, it depicts a non-sessile behavior of ILC3s in the tissue, with oxysterols as cues driving functional positioning within cryptopatches. While EBI2 engagement on ILC3s promotes inflammation in the context of colitis, another oxysterol receptor liver X receptor, known to exert anti-inflammatory functions, is also expressed by ILCs. Hence, an appealing area for future research will be to understand how different oxysterol signals are distributed in the intestine and how ILCs decode them to modulate their functions.

7.3 Damage-induced LXR activation drives intestinal regeneration and limits tumorigenesis (Study III)

In the last manuscript included in this thesis we shifted the focus towards the intestinal epithelium, the first cellular line of defense against environmental stressors. When intestinal damage occurs, a regenerative process emanating from proliferating intestinal stem cells (ISCs) at the bottom of the crypts ensures tissue repair and re-establishment of homeostasis. If regeneration fails to occur, the risk of developing chronic inflammatory disorders (such as IBD or leaky gut syndrome) increases, thus highlighting the importance of finding therapeutic strategies to boost intestinal regeneration [82,278].

With the aim of finding pathways involved in intestinal regeneration in the context of injury, we adopted an unbiased approach analyzing transcriptomic datasets of intestinal tissue undergoing chemically induced colitis (i.e. dextran sodium sulfate-induced colitis, DSS) or irradiation-induced injury. During the process of tissue repair, we observed upregulation of pathways involved in cholesterol metabolism, characterized by the expression of target genes of the transcription factor liver X receptor (LXR). Therefore, we aimed at understanding whether LXR contributes to intestinal regeneration and if it functions as a central hub regulating transcription of genes involved in tissue repair.

To study if LXR favors intestinal regeneration, we fed mice with a modified diet containing the synthetic LXR agonist GW3965 and we exposed them to different intestinal injury models (including DSS colitis and irradiation). Remarkably, we observed that activating LXR *in vivo* in the context of damage promoted tissue repair by boosting crypt cells proliferation and regenerative potential. We therefore decided to investigate the cell type in which LXR activation was necessary to promote intestinal regeneration. While we did not observe any

specific alteration of intestinal immune cell composition when activating LXR *in vivo*, mice specifically lacking LXR in intestinal epithelial cells displayed reduced regeneration upon irradiation. Further proving the role of LXR in the epithelium, intestinal crypts plated *in vitro* in the presence of LXR agonist generated bigger organoids with more newly formed crypt domains and enhanced expression of stem cell markers (**Figure 6**).

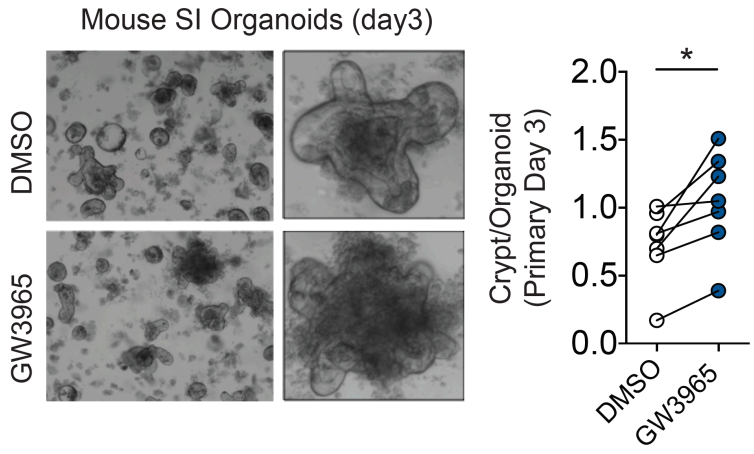

Figure 6. *LXR activation stimulates organoids growth.*

Our data thus far propose a novel role for LXR as necessary and sufficient in epithelial cells to drive intestinal regeneration. The crosstalk between ISCs and epithelial niche cells (Paneth cells, PC) residing in intestinal crypts is essential to promote ISC proliferation and differentiation and thus orchestrate barrier repair. With the aim of unraveling a cellular and molecular mechanism behind this pathway, we first tested in which epithelial cell type LXR activity was required to foster regeneration. By co-culturing wild type ISCs with either wild type or LXR-deficient PC, we observed that LXR expression in PC was indispensable to favor organoids growth *in vitro*. Accordingly, we measured higher expression of stem cell niche signals, such as *Wnt3* and *amphiregulin* (*Areg*), upon GW3965 stimulation, suggesting that LXR activation in the stem cell niche favors intestinal regeneration. Nevertheless, we cannot exclude that LXR activity in stem cells is similarly required to drive their proliferation and differentiation. Future work exploiting mice deficient in LXR selectively in ISCs will thus be required to answer this question.

Since most of the known downstream effects of LXR are regulated by its activity as a transcription factor, we tested which genes were specifically modulated by LXR activation in the intestinal crypt with the aim of finding molecular targets driving regeneration. Towards this, we performed a transcriptomic analysis of small intestinal crypts exposed to GW3965 for 6 hours *in vitro*. Together with the known LXR target genes (such as the cholesterol pumps *Abca1* and *Abcg1*), we found several other genes selectively upregulated in the context of LXR activation. Among these, the fatty acid-regulated transcription factor peroxisome proliferator activated receptor alpha (*Ppara*) and the E3 ubiquitin ligase ring finger protein 186 (*Rnf186*) were validated by qPCR both *in vitro* (organoids culture with GW3965) and *in vivo* (irradiated small intestinal crypts of mice fed with GW3965 or control diet). Interestingly, *Rnf186* gene has been mapped in an ulcerative colitis (UC)-susceptible locus by a genome wide association study [279] and mice deficient in *Rnf186* display enhanced sensitivity to colitis induced by DSS administration [280]. Current investigation in the lab is aiming at understanding whether the

LXR-mediated pro-regenerative effect is molecularly driven by RNF186 activation or via PPARα, another recently described player of intestinal regeneration [179].

Our observations propose that pharmacological LXR activation might represent a therapeutic strategy to boost intestinal regeneration. Accordingly, our initial unbiased characterization of tissue repair suggested that this pathway is physiologically upregulated in the tissue undergoing regeneration. On this basis, we investigated what signal was driving LXR activation in response to intestinal damage. Ligands of LXR are specific oxysterols (mainly 24HC and 27HC) generated by the activity of the enzymes Cyp46a1 and Cyp27a1 respectively. When analyzing the sections of intestinal tissues exposed to DSS- or irradiation-induced damage we observed upregulation of Cyp27a1 coinciding with the regenerative phase. These data suggest that modulation of the cholesterol metabolic machinery generating the LXR ligand 27HC represents an adapted response to intestinal tissue damage. Probing whether generation of this LXR ligand was involved in barrier repair, we tested the regenerative potential of mice lacking *Cyp27a1* enzyme. In line with our hypothesis proposing a pro-regenerative effect of the oxysterol-LXR axis, mice lacking the enzyme generating LXR ligands displayed delayed recovery and impaired epithelial cells proliferation.

Altogether, our results uncovered a novel pathway of intestinal regeneration, which might represent a promising target to improve the outcome of diseases associated with altered barrier integrity (such as IBD or graft versus host diseases). However, boosting regeneration has long been associated with enhanced risk of promoting malignant transformation and cancer progression [281]. This unwanted side effect is molecularly explained by misappropriation by cancer stem cells of homeostatic pro-proliferative and self-renewing signals characteristic of ISCs. Therefore, we wanted to understand if the pro-regenerative effect of LXR activation was at the risk of fueling tumorigenesis. Towards this, we activated LXR pathway *in vivo* (by feeding mice with GW3965 containing diet) and we tested tumor formation using two different models: an inflammation-induced colon tumor model (AOM-DSS) and a genetically driven tumor model affecting the small intestine ($Apc^{Min/+}$). To our surprise, in both models, LXR activation led to a reduction in tumor size and numbers (**Figure 7**).

Figure 7. *LXR activation in vivo dampens tumorigenesis.*

This finding indicates that LXR activity in the epithelium reciprocally regulates regeneration and tumorigenesis. Supporting this notion, tumor biopsies of mice treated with LXR agonist were no longer characterized by upregulation of pro-proliferative signals, such as *Wnt3* and

Areg as observed during regeneration, instead displayed upregulation of pro-differentiating (*Id2*) and pro-apoptotic (*Atm* and *p27*) signatures. To gain mechanistic insights, we observed that expression of the LXR target gene *Ppara* was enhanced by GW3965 administration also in the tumor context. Remarkably, studies in *Ppara* deficient animals showed increased susceptibility to colorectal tumors explained by reduced expression of the tumor suppressor gene *p27* [282]. Thus, PPARα might represent the molecular mediator of LXR-dependent anti-tumor effect and future experiment will be needed to prove this hypothesis.

Overall, we uncovered an unprecedented role of LXR in driving intestinal regeneration, thus revealing a promising therapeutic target to boost tissue repair in the context of chronic inflammatory pathologies marked by aberrant mucosal healing. Further, we showed that, unlike most of the pro-regenerative strategies studied thus far, LXR activation is not associated with enhanced risk of cancer formation. Therefore, understanding how LXR uncouples regeneration and tumorigenesis represents an appealing future outlook for the development of promising and safer therapies.

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