Type 3 innate lymphoid cells: Guardians of epithelial barriers

Type 3 innate lymfoïde cellen:
Bewakers van epitheliale barrieres

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by

Patricia Aparicio Domingo
born in Madrid, Spain

Erasmus University Rotterdam

Doctoral Committee

Promotor: Prof.dr. J.J. Cornelissen

Other members: Prof.dr. I.P. Touw
Prof.dr. R.W. Hendriks
Dr. M.C. Coles

Co-promotor: Dr. T. Cupedo

A mis padres

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Patricia Aparicio Domingo and Tom Cupedo
Department of Hematology, Erasmus University Medical Center,
Rotterdam, the Netherlands

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CHAPTER 1

General Introduction

GENERAL INTRODUCTION AND SCOPE

Mucosal surfaces segregate the external environment from the host and represent a major entry point for pathogenic microorganisms. Therefore, these are strategic sites where multilevel interactions between epithelial cells and the immune system are necessary to maintain host homeostasis. Epithelial cells integrate signals from the local surroundings and translate them into immune mediators acting on the hematopoietic compartment underneath the epithelium. Epithelial-derived signals can trigger specific types of immune responses directly by influencing effector lymphocytes, or indirectly by activating myeloid cells that ultimately regulate lymphocyte activity. Disruption of the fine-tuned crosstalk that occurs at mucosal surfaces, increases the risk of infection and inflammation, and can lead to chronic diseases and cancer development. Thus, it is essential to understand the mechanisms by which immune cells interact with the local environment, and how these signals regulate tissue-specific responses in health and disease.

In the past few years, an emerging group of innate lymphoid cells (ILCs) has been identified in mucosal tissues[1-4]. ILCs are found close to epithelial barriers, where they constitute a major source of the homeostatic cytokine interleukin 22 (IL-22) (Figure 1). ILC cytokine profiles resemble those produced by helper T cells, yet they lack antigen specificity and provide a quicker immune response. Recent studies brought to light a previously unappreciated role of ILCs in protecting against enteric pathogens and promoting homeostasis in mucosal tissues. However, the role of ILCs in tissue damage in the absence of pathogenic infections remains unknown.

This thesis aims to investigate the contribution of type 3 innate lymphoid cells (ILC3) in intestinal epithelial damage responses. Activation mechanisms and effector functions of ILC3 will be discussed in the context of intestinal damage.

INTESTINAL EPITHELIUM

The intestinal epithelium consists of a single layer of epithelial cells that efficiently combines the uptake of nutrients from the diet, with providing a physicochemical barrier between the microbiota and our body[5].

Differential expression within intestinal epithelial cells of pattern recognition receptors (PRR), including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors, guarantees an effective epithelial response to microbiotic pressure while keeping the tolerogenic state that is necessary along the epithelium of the intestine[6].

The small and large intestines are highly compartmentalized tissues lined by columnar epithelial cells connected by tight junctions. The small intestine is divided, from proximal to distal, into duodenum, jejunum an ileum, and it is characterized by the presence of epithelial projections towards the lumen called villi that efficiently increase the absorption

surface. The ceacum delineates the beginning of the large intestine, composed by the ascending, transverse and descending colon. In contrast to the small intestine, the large intestine lacks villi and contains the highest microbiota load[7].

Invaginations of the epithelium into the underlying lamina propria generate the intestinal crypts or crypts of Lieberkühn, where pluripotent intestinal stem cells (ISCs) reside. In the small intestine, each villus is surrounded by at least six intestinal crypts, where highly proliferative ISCs give rise to all differentiated epithelial cells[8,9]. Specialized epithelial cells migrate upwards from the crypts to the tip of the villi, where they are released from the epithelial layer by anoikis[8-10]. This epithelial cell-renewal and migration of differentiated cells takes place in only 3-5 days, making the intestinal epithelium one of the tissues with the highest proliferative rate in the human body[11].

Epithelial barrier

Intestinal epithelium combines the digestive function with the maintenance and regulation of barrier properties[5]. Based on their function, intestinal epithelial cells can be classified into absorptive and secretory cells. The vast majority of intestinal epithelial cells are absorptive enterocytes that carry out metabolic and digestive functions[7]. A characteristic brush border formed by microvilli covers the surface of the enterocytes, contains digestive enzymes, and greatly increases the absorption surface[12]. Moreover, enterocytes can secrete antimicrobial peptides (AMPs), including the C-type lectin regenerating islet-derived protein III gamma (REG3γ) that contributes to the host-microbial segregation[7]. The secretory lineage includes enteroendocrine cells, goblet cells and Paneth cells. While enteroendocrine cells secrete hormones and peptides that regulate the neuroendocrine system[13], goblet cells and Paneth cells actively participate in the barrier formation by production of mucins and AMPs[14,15] (Figure 1).

Goblet cells

Mucins produced by goblet cells are the major component of the mucus layer, which forms a highly charged gel, called glycocalix, and coats the intestinal epithelium. These gel-forming mucins are highly glycosylated proteins that are toxic to bacteria and prevent them from directly reaching the epithelium[16,17]. The mucus layer represents the first line of defense and as such the number of mucus-producing goblet cells increases from proximal to distal in accordance to the bacteria load[14,16]. In the colon, with the highest microbiota content, the mucus barrier is formed by an inner and an outer layer, whereas the small intestine is coated by a single layer of mucus[17,18]. In the small intestine and in the outer layer of mucus in the colon, the mucine Muc2 is the most abundant structural component of the mucus. Deficiencies in this *Muc2* in mouse models cause severe colitis after exposure to the colitogenic agent dextran sulfate sodium (DSS) and have been associated with colorectal cancer[19,20].

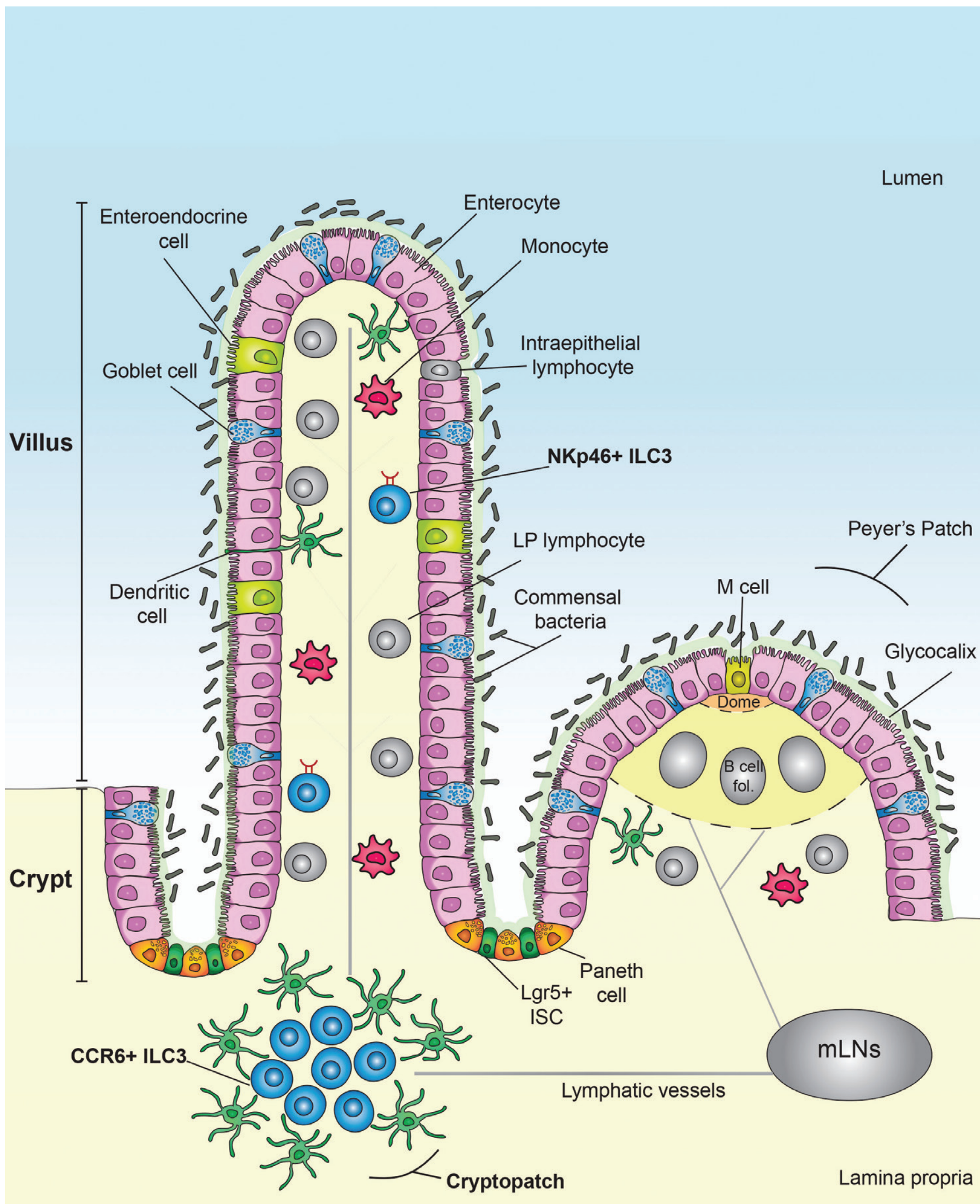

Figure 1. Schematic overview of the small intestinal epithelium and lamina propria. Intestinal epithelium is formed by a single layer of epithelial cells. Luminal projections of differentiated epithelial cells including enterocytes, goblet cells and enteroendocrine cells form the villi. Epithelium invaginations form the intestinal crypts wherein Paneth cells and intestinal stem cells reside. The luminal side of the epithelium is coated with a mucus layer (glycocalix) that protects epithelial cells from direct contact with the microbiota. Peyer's patches containing B cell follicles and interfollicular T cell areas connect with the luminal side via microfold (M) cells located on top of the dome area of the lymphoid aggregate. Cryptopatches are situated deeper in the lamina propria, close to intestinal crypts and consist of clusters of CCR6+ILC3 enclosed by dendritic cells. NKp46+ILC3 are located scattered throughout the villi together with lamina propria lymphocytes and monocytes[5,7].

The production of mucus is influenced by immune mediators including IL-13 and IL-9 produced during type 2 immune responses, for instance during worm expulsion[21,22]. Importantly, a novel function for ILCs in mucus production has been recently identified. In a model of salmonella infection, IFNγ produced by lamina propria NKp46$^+$ILC3 play a significant role in the maintenance of the mucus layer[23]. Moreover, in a Th2–mediated colitis model, IL-22 gene delivery enhanced activation of colonic epithelial cells and induced STAT3-dependent expression of mucus-associated molecules and restitution of mucus-producing goblet cells, which translated into rapid amelioration of local intestinal inflammation[24]. This study is in line with the recent findings that describe ILC3-derived IL-22 as an essential factor for mucus maintenance and epithelium integrity in the context of DSS-induced damage[25].

In addition, goblet cells secrete trefoil-factor 3 (TFF3), a small peptide that contributes to the mucin crosslinking, and gives structural integrity to the mucus layer[26,27]. Moreover, a protective role for TFF3 has been shown, as it contributes to intestinal epithelial wound healing and participates in the active migration of epithelial cells that takes place in both the homeostatic epithelial restitution and the re-epithelialization upon damage[28,29].

Paneth cells

Paneth cells are secretory cells of the small intestine characterized by their numerous cytoplasmatic granules harboring several AMPs, including defensins, lysozyme, cathelicidins, and similar to enterocytes, REG3γ[30,31].

The antimicrobial-rich granules are secreted into the lumen and mucus layer where they kill their target microorganisms by disrupting the membrane integrity or by interfering with the synthesis of the bacterial wall[31,32]. Defensins (cryptdins in mice) are the most abundant antimicrobial peptides in the intestine of mammals with bactericidal activity against Gram-positive and Gram-negative bacteria. Functional activity of α-defensins depends on proteolytic cleavage by the matrix metalloproteinase 7 (MMP7) expressed by Paneth cells, and mice with genetic defects in this gene are more susceptible to enteric pathogens[33]. Other AMPs produced by Paneth cells include lysozymes and phospholipases, that specifically hydrolyses bacterial wall peptidoglycans and phosphorglycerides, respectively[33].

The contribution of Paneth cells to the epithelial barrier has been demonstrated in different mouse models. Depletion or aberration of Paneth cell function leads to increased bacterial translocation and higher susceptibility to infection with Gram-positive and negative bacteria[30]. Furthermore, defects in NOD2, a PRR expressed predominantly by Paneth cells in the intestinal epithelium, correlates with lower expression of α-defensin and abnormal activation of T helper 1 (Th1)-inflammatory responses in mice[34]. In humans, NOD2 mutations have been described in Crohn's diseases patients and several disease susceptibility genes for Crohn's disease are associated with dysfunction of Paneth

cells[35,36]. Paneth cells are therefore key players in shaping the chemical component of the intestinal barrier.

Besides their well-known antimicrobial capacity, Paneth cells secrete signaling molecules that are thought to regulate stem cell survival and function[37]. Strikingly, Paneth cells are the only specialized epithelial in the intestine cell with longer self-renewal cycles (3-6 weeks instead of 3-5 days) that upon terminal differentiation migrates downwards into the crypt to settle in between ISCs[38]. At these positions Paneth cell-derived factors, including Wnt3a, epidermal growth factor (EGF) and Notch ligands, directly influence stem cell behavior and constitute the stem cell niche[37].

Intestinal stem cells

Crypt residing stem cells are responsible for the maintenance and regeneration of intestinal epithelium. Proliferation of ISCs is driven by activation of the canonical Wnt pathway[9] and deficiencies in Wnt-associated proteins such as Tcf4 or β-catenin result in reduced proliferative capacity[39,40]. Similar to the Wnt pathway, the Notch pathway is essential to maintain proliferation in the crypt compartment, but in addition, Notch signaling plays a critical role in controlling lineage fate decisions. This was clearly demonstrated in studies using genetic deletions in the Notch pathway, that resulted in a complete conversion of all epithelial cells into goblet cells[41] and gain of function experiments that revealed the opposite effects, with loss of goblet cells and reduction of neuroendocrine and Paneth cells[42]. Although Paneth cells provide important factors required for stem proliferation and survival, it is likely that in vivo additional cellular sources contribute to this, as Paneth cell ablation or conditional loss of Wnt3a is not sufficient to inhibit stem cell-driven epithelial renewal[43,44].

In the past few years, enormous advances have been made in the field of stem cell research and different techniques have been developed to elucidate the dynamics and plasticity of intestinal stem cells. Fate-mapping technologies as well as three-dimensional ex-vivo cultures of crypts, have allowed the identification and characterization of different populations of epithelial cells with cell-renewal capacity[45,46].

Proliferative crypt base columnar (CBC) cells residing at the bottom of the crypts, express the recently identified marker leu-rich repeat-containing G protein-couple receptor 5 (LGR5)[10]. Lgr5 is a Wnt target gene that acts by amplifying the canonical Wnt pathway by recruiting Wnt agonists, such as roof plate-specific spondin (R-spondin 1-4)[47]. Subsequently, other CBC stem cell markers have been identified, including olfactomedin-4 (OLFM4), Achaete–Scute homologue 2 (ASCL2), Musashi homologue 1 (MSI1) and SPARC-related modular calcium-binding 2 (SMOC2)[48-51].

Lgr5^{+} ISCs are rapidly cycling cells that during homeostasis give rise to a progeny of transient amplifying cells (TA) that eventually differentiate into specialized epithelial cells[10]. Based on multicolor tracing experiments and computer modeling, it is currently

assumed that Lgr5+ ISCs undergo stochastic symmetrical division, in which they generate either two stem cells or two TA cells[52,53]. The current model implies a balanced supply of stem cells that is achieved by competition among the dividing cells for the restricted niche space. Based on the high grade of specialization of the intestinal epithelium, it is likely that external factors, provided by surrounding cells, rather than random events, regulate the fate choices and positioning of CBC stem cells.

In addition to CBC stem cells, label retaining cells with stem cell properties are found at the fourth position of the intestinal crypt (+4 stem cells)[54]. These quiescent stem cells are more resistant to damage and are able to differentiate into TA cell as well as Lgr5+ CBC[55]. Markers for +4 stem cells, including BMI1, homeodomain-only (HOPX), telomerase reverse transcriptase (TERT) and Leu-rich repeats and immunoglobulin-like domains 1 (LRIG1), have been recently identified[45,50,56]. Nevertheless, the expression of these markers can also be found throughout the proliferative crypt compartment, and therefore they are not unique markers for +4 stem cells[46,50].

To maintain tissue homeostasis and prevent microbiota translocation, it is essential that intestinal epithelium regenerates rapidly after damage. Recent studies suggest that during acute epithelial damage, in which Lgr5+ cells are transiently depleted, +4 quiescent stem cells take over and contribute to survival of the epithelium. *In vivo* lineage tracing studies have shown that indeed BMI1+ stem cells repopulate the Lgr5 stem cell pool for subsequent epithelial regeneration[46]. Furthermore, other mechanisms seem to also contribute to the remarkable regenerative ability of the epithelium. For example, a certain grade of plasticity exists within committed cells and stem cells. In a model of irradiation induced-damage, label-retaining cells (LRC) identified as Paneth cells rapidly converted into self-renewing, multipotent stem cells[57,58]. In a different study, an early secretory progenitor expressing the Notch ligand Delta-like 1 (DLL1) , differentiated into Lgr5+ CBC following acute irradiation, contributing to subsequent epithelial regeneration[59].

Therefore, several complementary mechanisms safeguard the maintenance and regeneration of the crypt-villus axis. Although specific factors have been identified that support the *in vitro* culture of intestinal crypts[60], the signals that regulate intestinal regeneration *in vivo* remain largely unknown.

Due to the rapid nature of the epithelial regeneration and the high epithelial plasticity, it is probable that factors produced by neighboring cells regulate epithelial responses. Indeed, it has been proposed that pericryptal stromal cells support ISCs by production of factors including Wnt ligands, the Notch ligand Delta-like 1 (DLL4), EGF and Noggin [60]. Importantly, a role for the immune system in stem cell protection from immune cell-mediated killing has recently been shown. In this study, radio-resistant ILC3 protected ISCs from alloreactive T cells in a mouse model of graft versus host disease (GvHD)[61]. In this model, IL-22 derived from CCR6+ILC3 prevented stem cells from being killed by T cells and promoted survival and regeneration of the intestinal epithelium.

Epithelial-immune interactions

Mucosal surfaces are rich in immune cells and represent one of the most complex immunological sites in the body[5,62]. Epithelial integrity can only be achieved if a proper communication between the immune compartment and epithelial cells exists[63,64]. This communication occurs in a bidirectional manner, where intestinal epithelium integrates microbiota-derived signals into clues that modulate immune cells, and translates factors derived from the immune system into epithelium function[5,62,65]. Several studies illustrate this active crosstalk. For example, it has been shown that commensal bacteria activate IEC via PRR signaling to produce factors including thymic stromal lymphopoietin (TSLP), transforming growth factor beta (TGFβ)[63,64] and retinoid acid (RA)[66] to induce tolerance via antigen presenting cells (APC). Moreover, B cell function can be influenced by epithelial-derived factors such as B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL), which are important for the regulation of B cell class switching and mucosal immunoglobulin A (IgA) responses[67,68]. Important for epithelium homeostasis, IEC express the polymeric immunoglobulin receptor (pIgR) that directly transport secretory IgA from the lamina propria to the lumen[69].

During inflammation or infection, IEC express a broad array of chemokines and cytokines, including CXCL1/2[70], MIP-2[71] and IL-8[72] that recruit neutrophils and macrophages and mediate immune responses. Furthermore, IL-25 derived from epithelial cells has been identified as an important modulating factor for Th2 responses[73] and to dampen ILC3 activation[74]. In contrast to IL-25, IEC-derived IL-23 promotes ILC3 production of IL-22 in a lymphotoxin-β-Receptor-dependent manner[25].

INNATE LYMPHOID CELLS

The family of innate lymphoid cells (ILCs) combines aspects of the innate and adaptive immune response and are part of the first line of defense at barrier surfaces. Similar to adaptive immune cells, ILCs express IL-7 and IL-2 receptors, but are set apart from adaptive lymphocytes by their independence of RAG genes and the resulting absence of specific antigen receptors[75,76] The capacity to produce effector molecules associated with adaptive immunity, but without the antigen restriction, endows ILCs with the ability to respond much quicker than adaptive cells.

The current classification of ILC family members makes a first distinction based on their cytotoxic capacity[77,78]. In this first level of division, NK cells represent the only cell type with cytotoxic and killing activity. On the non-cytotoxic side, ILCs are further classified based on the cell-surface markers, transcription factors and expression of effector cytokines into group 1, 2 or 3 (ILC1, ILC2 and ILC3). A certain grade of plasticity exists between these groups, which reflect the versatility of ILCs in immune responses. As such, important roles in intestinal homeostasis, infection and tissue wound healing have been attributed to ILCs (Figure 2).

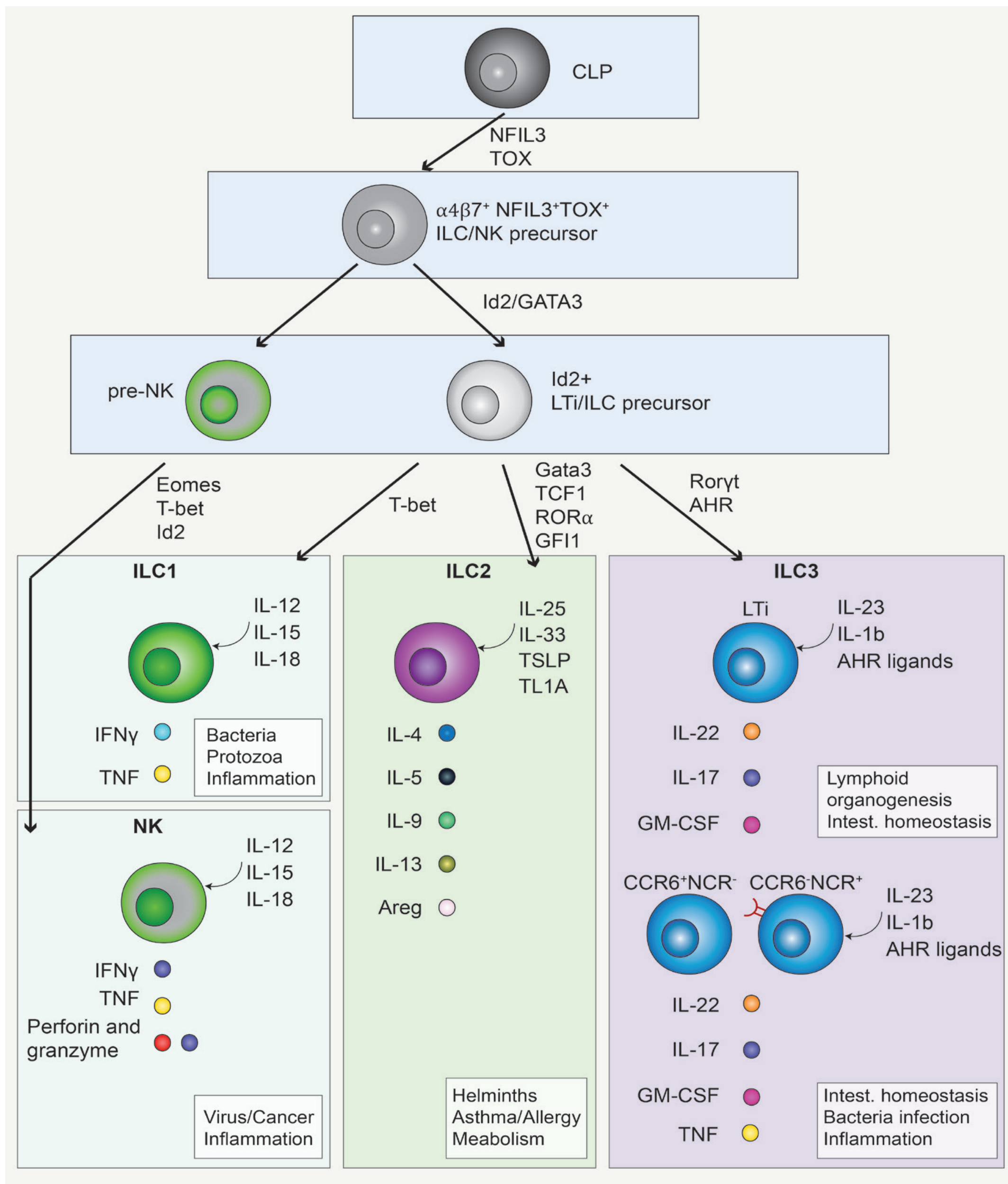

Figure 2. Development and function of the family of innate lymphoid cells. A common lymphoid precursor (CLP) gives rise to a α4β7 expressing ILC/NK cell precursor. Downstream of the common ILC/NK cell precursor is the pre-NK cell and the Id2 expressing LTi/ILC precursor. Pre-NK cells expressing Eomes, T-bet and Id2 develop into NK cells, which respond to IL-12, IL-15 and IL-18 to carry out effector functions by producing IFNγ, TNF and perforin, among others. LTi/ILC precursor can acquire expression of different transcription factors to develop into the different ILC family members. T-bet expression characterizes type 1 innate lymphoid cells (ILC1), which respond to IL-12, IL-15 and IL-18 by secreting IFNγ and TNF. Gata3 expressing ILC2 produce IL-4, IL-5, IL-9, IL-13 and Amphiregulin (Areg) in response to IL-25, IL-33, TSLP and TL1A. Expression of the transcription factor Rorγt and the Aryl Hydrocarbon receptor (AHR) characterize ILC3. Within the ILC3 group, multiple subsets are found. LTi cells, $CCR6^+NCR^-$ ILC3 and $CCR6^-NKp46^+$ ILC3 are activated by IL-23 and IL-1β and AHR ligands to secrete IL-22, IL-17 and GM-CSF. Main functions are indicated in white boxes[75,76].

In the last few years, extensive studies have been carried out to understand the lineage relationships and development of the ILC family members. It is nowadays accepted that NK cells and ILC family members derive from a common lymphoid precursor (CLP) expressing the integrin α4β1[77], which gives rise to the ILC/NK cell precursor expressing the transcription factors NFIL3[79] and TOX[80]. The expression of the transcriptional inhibitor Id2[81,82] and the transcription factor GATA3[78,83] specifically generate the ILC precursor, whereas the NK cells develop independently of these transcription factors. Downstream of the $Id2^+$ precursor, T-bet[84], GATA3[85-87] and Rorγt[88] delineate the ILC1, ILC2 and ILC3 subsets respectively (Figure 2).

ILCs display several features normally associated with activated cells of the adaptive immune system. Similar to T cells, ILC3 can express surface lymphotoxin-α1β2 (LT) and secrete IL-2[89,90]. Importantly, ILCs produce cytokines normally secreted by specific T helper cells and represent the innate version of T helper responses.

NK cells are well known for their tumor killing activity and their role in immune responses against virus[91]. The cytotoxic NK cells respond to IL-12, IL-15 and IL-18 by producing IFNγ, TNFα as well as perforins and granzymes[91]. Similar to NK cells, $T\text{-}bet^+$ ILC1 resemble Th1 cells and respond to IL-12 and IL-18 by producing TNFα and IFNγ[92]. Both NK cells and ILC1 have been implicated in chronic inflammation of the intestine, with a pathogenic role in mouse models of colitis and human inflammatory bowel disease (IBD)[92,93].

The expression of GATA3 identifies ILC2. Similar to Th2 cells, ILC2 produce an array of type 2 cytokines including IL-4, IL-5, IL-9 and IL-13, that can be directly regulated by epithelial-derived IL-25, IL-33 and TSLP[94-96]. ILC2 are involved in immunity against helminthes[94,95] and are associated with allergic and asthmatic diseases[97,98]. In addition, ILC2-derived amphiregulin has been shown to play an essential role in lung tissue remodeling after influenza infection[99]. In the gut, IL-33-stimulated ILC2 induce goblet cell hyperplasia and enhance mucus secretion during helminth infection by producing IL-5 and IL-13[94,95].

Expression of the transcription factors Rorγt (encoded by the Rorc gene) and the transcriptional inhibitor Id2 delineates the heterogeneous group of ILC3[82,88,100,101]. Similar to Th17 cells, some of the ILC3 subsets require the expression the aryl-hydrocarbon receptor (AHR) for development and function[102-104]. Moreover, ILC3 secrete IL-22, GM-CSF and TNFα, in response to IL-23 and IL-1β, reminiscent of Th17 cells[1,2,4,74,89,105,106]. ILC3 display a higher level of complexity compared to other ILC family members, as different subsets can be distinguished in this group. The classical lymphoid tissue inducer cells (LTi), involved in lymphoid organogenesis, were the first subset of ILC3 identified[90]. In the last few years, other subsets have been characterized based on the expression of surface markers CCR6 and the natural cytotoxicity receptors (NCRs)[4,23,106-108].

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Intestinal ILCs

All three types of ILCs, are found in the gut and require CCR9 to migrate from peripheral tissues into the small intestine[109]. ILC2 can be programed in the bone marrow to express gut homing receptors, whereas ILC1 and ILC3 are recruited to the intestine upon homing receptor switch regulated by retinoid acid (RA) in the periphery[109]. Intestinal ILC1 are though to initiate IFN-γ responses against pathogens, but production of this cytokine has also been associated with pathological inflammation, suggesting that ILC1 may be involved in chronic inflammation. In line with this concept, increase in intraepithelial[92] and lamina propria[93] ILC1 has been found in Crohn's disease patients (CD) and appeared to be at the cost of reduction in ILC3 numbers. Indeed, recent studies demonstrated that certain plasticity exists between intestinal ILC1 and ILC3 that is driven by the environmental clues[110]. While the presence of DC-derived IL-12 favors the differentiation of ILC3 into ILC1 accompanied by the downregulation of Rorγt, the opposite effect occurs in the presence of IL-23 and IL-1β, where ILC3 differentiation is promoted [110].

Although the role of ILC2 in intestinal inflammation is less clear, it has been proposed that they play an important role in controlling eosinophil homeostasis through IL-5 secretion[111]. Moreover, intestinal ILC2 are shown to control resistance and susceptibility to parasite infections[95,112].

Intestinal ILC3 are probably the best characterized intestinal ILC subset and their location and function will be described in the following paragraphs of this thesis.

ILC3 IN LYMPHOID ORGANOGENESIS

The lymphoid tissues of the intestines are collectively referred to as the Gut Associated Lymphoid tissues (GALT) and can be divided into programmed tissues that form during development, such as the gut-draining lymph nodes and the Peyer's patches and induced tissues that form after birth driven by external signals[113].

Lymph nodes

In mice, at least 40 lymph nodes are present[114] and humans are estimated to have between 500 and 700 individual lymph nodes[115]. Development of these secondary lymphoid organs is induced by activation of the local mesenchyme by ligation of the LTβR through membrane bound LT expressed on LTi cells[116]. This activation results in secretion

of homeostatic chemokines and expression of adhesion molecules. As a consequence, additional (precursor) LTi cells are recruited to the developing lymph node anlage. Via paracrine interactions LTi cell precursors are allowed to differentiate into mature LTi cells, establishing a positive feedback loop resulting in the maturation of specific stromal cell subsets that in progressive developmental steps will control the influx of rearranging adaptive immune cells[116].

Peyer's Patches

Peyer's patches are located on the anti-mesenteric side of the small intestine, directly underlying the intestinal epithelium. Inbred mouse strains have on average 6-10 Peyer's patches, although the exact number varies between strains. The human small intestine contains approximately 240 Peyer's patches, with an increasing density towards the terminal ileum[117]. Peyer's patches are characterized by the presence of more then one B cell follicle and distinct interfollicular T cell areas with high endothelial venules[118]. The intestinal epithelium overlying the Peyer's patches contain Microfold cells (M cells) that actively transport particles from the intestinal lumen into the Peyer's patch[119] (Figure 1).

In mice, Peyer's patch development starts at embryonic day (E) 15.5, when $CD117^+CD11c^+$ Lymphoid tissue initiator cells (LTin) cluster at distinct sites on the anti-mesenteric wall of the fetal small intestine co-localizing with VCAM-1 expressing stromal cells[120,121]. In additional developmental steps, LTi cells are recruited to the developing Peyer's patches and the VCAM-1^+ stroma will start to organize the anlage into distinct microdomains[120,122]. In the absence of LTi cells, as is the case in mice with a targeted deletion of Rorc or Id2, Peyer's patch development is terminated at E16.5.

Cryptopatches

Since the intestinal immune system has to be able to respond to changes in the intestinal microbiota by mounting immune responses against pathogens while being in a homeostatic equilibrium with commensal bacteria[113] it has evolved a unique set of lymphoid tissues that functions as a dynamic system with the ability to respond swiftly to changes in microbial pressure through the generation of IgA producing plasma cells. The basis for this flexible immune compartment is formed by small lymphocyte clusters termed cryptopatches that contain mainly ILC3 and dendritic cells, but only very few B or T cells[123] (Figure 1). The murine small intestine contains approximately 1500 cryptopatches and the colon about 150[123]. Similar to other programmed lymphoid tissues, development of cryptopatches depends on LTβR signaling and Rorγt expressing ILC3[124,125]. Under the influence of microbial-derived signals, cryptopatches have the capacity to attract B cells and transform into an isolated lymphoid follicle[126,127].

Isolated lymphoid follicles

Isolated lymphoid follicles (ILFs) are histologically characterized by the presence of a single B cell follicle containing follicular dendritic cells and scattered T cells without a well defined T cell area[126]. Similar to their CP ancestors, ILFs remain in direct contact with M cell containing intestinal epithelium. Upon activation, B cells within the ILF can enter germinal center reactions and differentiate into plasma- and memory B cells[125]. Not surprising, the majority of plasma cells generated in ILFs produce IgA, a protective immunoglobulin that can be actively transported across the epithelial barrier upon migration of plasma cells into the lamina propria.

The transition from CPs into ILFs depends on microbial derived signals, and in germ-free mice no ILFs develop. In addition, ILC3-mediated activation of stromal cells in the CP is essential for the regulated transition from CP to ILF, establishing ILC3 as master regulators of development of cryptopatches and differentiation of ILFs, in line with their function during embryonic development of lymphoid tissues[124,125].

As mentioned in the previous paragraph, ILFs contain an ill-defined T cell area. This already suggests that ILFs are lymphoid organs in which T cells are not essential for supporting B cell responses, a notion that is confirmed by the fact that ILFs can generate IgA producing plasma cells in the absence of T cells[125.] As such, a third function of ILC3 in CP-ILF development and function becomes apparent. Through their production of TNFα, ILC3 can activate production of MMPs (matrix metalloproteinases) by stromal cells and macrophages within the ILF. These MMPs can in turn activate latent TGFβ which is an important switch factor for the generation of IgA[125.].

ILC3 IN THE INTESTINE

ILC3 are present in the entire intestinal tract, although they are predominantly found in the distal part of the small intestine[7]. In the fetal murine intestine LTi cells are the predominant ILC population[128,129]After birth, additional ILC3 populations appear in the intestines including cells with low CD117 expression and a population that express the natural cytotoxicity receptor (NCR)[46,129]. In adult humans and mice, a substantial population of mucosal ILC3 is characterized by the expression of NCRs[3,4,106]. Murine intestinal-derived ILC3 express NKp46, while human intestinal-derived ILC3 predominantly express NKp44[1,2,108].

In the mouse intestine, a subset of $CCR6^+$ ILC3 that can express the T cell marker CD4, are found in lymphoid clusters called cryptopatches, close to the intestinal crypts. Outside of the cryptopatches, in the lamina propria of the villi, most CCR6- ILC3 expressing NKp46 are located[1,4] (Figure 1). NCRs are Ig-like transmembrane receptors that belong to a family of activating receptors expressed on NK cells that include NKp30, NKp46 and NKp44[130]. Human NK cells constitutively express the first two while Nkp44 is only expressed upon

stimulation. NKp46 is conserved as a constitutive NK cell marker in mice. In addition to these NCRs, human ILC3 can express the NK marker CD56[1,2] and mouse ILC3 express low levels of NK1.1[3,4,106].

The distribution of ILC3 within the lamina propria seems to be based on chemokine gradients as NKp46$^+$ ILC3 express CXCR6 and accumulate in the villi in response to DC-derived CXCL16[131]. The anatomical segregation of ILC3 within the lamina propria suggests specialized functions for CCR6 and NKp46 expressing ILC3. First clues about the specialization of CCR6$^+$ ILC3 in the cryptopatches arose from the protective role that ILC3-derived IL-22 exhibited in T cell mediated killing of ISCs[61]. Although lamina propria NKp46$^+$ ILC3 are also an important source of IL-22 in the mouse intestine, the specific function of this subset remains unclear. Mouse models with *C. rodentium* infection have shown that NKp46 expression is not required to maintain intestinal integrity[132]. Although these experiments failed to identify a function for NKp46, this infectious colitis model mostly affects the large intestine rather than the small intestine, where most of the ILC3 are found. Furthermore, NCR ligands are presumed to be induced upon damage or stress, and response against infectious pathogens and epithelial damage may be regulated by different mechanisms. During tissue damage, ILC3 activation may be locally regulated at the site of damage and thus local cell surface ligands could drive activation of ILC3. Therefore to understand the role of NCR expression on ILC3, different models besides bacterial infection are needed.

ACTIVATION OF ILC3

ILC3 directly influence intestinal epithelial cells through production of cytokines that affect epithelial fitness and function[4,70,133-135]. In both mice and humans, ILC3 have the capacity to produce the Th17 associated cytokines IL-17a and IL-22[2,4,134-138]. Especially the latter is associated with ILC3 located in mucosal tissues where it has been shown to play an essential role in intestinal homeostasis and host defense[74,132,139]

In murine intestines ILC3 are the main cellular source of IL-22[134], are located directly underneath the epithelium, both in the lamina propria as well as in cryptopatches, ideally positioned for regulating the intestinal epithelium. Ligation of the IL-22R expressed by IEC regulates the production of antimicrobial peptides, which are secreted into the intestinal lumen and are important for maintaining the balance with the microbiota[4,70,133-135].

While *in vitro*, both mouse and human ILC3, respond to IL-23 with induction of IL-22 secretion[4,135-137,140], *in vivo* murine intestinal ILC3 respond to IL-1β and do not depend on IL-23 for their homeostatic production of IL-22[107,134].In cultured human tonsil-derived ILC3, IL-23 alone is not sufficient to induce IL-22 and requires an additional co-stimulatory signal. This second signal can be either a cytokine such as IL-2 or a TLR ligand[89]. Tonsil-derived ILC3 also secrete IL-2, making them a unique non-T cell source of this cytokine[89].

In addition, human ILC3 express TLRs and signaling through TLR2 can function as a co-stimulatory signal in conjunction with IL-23 or IL-15[89]. *In vivo*, the TLR-2 ligand Zymosan is able to induce IL-22 production in murine ILC3 in an indirect manner, dependent on IL-23[105]. A likely intermediate in this ILC3 activation are macrophages/dendritic cells that express TLRs and can produce IL-23.

ILC3 also have the ability to produce the pro-inflammatory Th-17 cytokine, IL-17a. In humans, ILC3 derived from fetal developing lymph nodes contain transcripts for IL17a, while lacking IL-22 transcripts[2]. The function of IL-17 in lymph node development is still unclear, as IL-17 deficient mice do not have obvious defects in lymphoid organogenesis. ILC3 in murine fetal intestines also produce IL-17a, in concert with IL-22[134].

The only reported induction of IL-17a in cultured human ILC3 involved signaling through the IL1R[141]. This suggests that specific inflammation-related signals might control the production of IL-17a in an antigen-independent manner. *In-vitro*, induction of IL-17a occurred in cells that were also exposed to IL-7 and/or SCF and it is conceivable that in parallel to the induction of IL-22, a combination of signals is needed for IL-17a secretion. Since IL-17a is a very potent pro-inflammatory cytokine its expression in T helper cells is under tight control and requires a strong TCR signal in conjunction to the right cytokine environment. Since ILC3 lack specific antigen receptors, control of IL-17a production will probably be regulated through a combination of specific signals to avoid untimely release of this cytokine.

There are contradictory data on the role of the microbiota in controlling activation and IL-22 production by ILC3, especially regarding the NCR+ subset. Initial experiments, had shown that in germ-free mice, LTi cells develop normally, as shown by the presence of lymph nodes in these mice, but the frequency of $NKp46^{+}$ILC3 was significantly reduced[4,136]. In line with the reduction of ILC3, IL-22 production in the intestines was impaired.

In contrast, other reports indicate opposite results, where murine ILC3 from the sterile fetal intestine, express IL-22 and IL-17 transcripts, which levels decrease as microbiota colonization takes place[74].

Moreover, commensal microbiota-induced production of IL-25, was shown to be responsible for the decrease in IL-22 secretion by adult murine intestinal ILC3 and in germ free mice IL-22 production by ILC3 was increased[134].

At present it is very difficult to reconcile these different viewpoints. There is clearly an important role for the microbiota in the regulation basal function of intestinal LTi cells. This mere fact might already reveal part of the underlying problem, as microbial content is likely to differ in the various animal facilities. This would however not explain the opposite findings with germ free mice.

Of note, IL-23 is not essential for the production of IL-22 by ILC3 in the intestines, indicating that additional mechanisms of regulation are at play[134]. The microbiota and

ILC3 thus maintain a fine balance tuned to optimize homeostasis. When this balance is disrupted by experimentally induced epithelial damage, IL-22 production by ILC3 increases and aids in the restoration of the intestinal barrier through direct effects on epithelial cells[142].

Besides the TLR- and cytokine-induced activation of ILC3, additional mechanisms contribute to secretion of cytokines by ILC3. A large number of mucosal ILC3 express NCRs and this phenotype is associated with an activated status. Since NKp46 acts as an activating receptor on NK cells, it is reasonable to consider that expression of NCRs on ILC3 might function as activating receptors as well. Indeed, *in vitro* experiments have shown that NKp44 is functional on human tonsil-derived ILC3 and its triggering promotes the expression of pro-inflammatory cytokines[143]. Although engagement of NKp44 alone is capable of inducing TNFα production, additional cytokine signals are necessary to promote IL-22 and GM-CSF secretion by ILC3. In this model of activation, IL-23, IL-1b and IL-2 act synergistically with NKp44 to induce the homeostatic cytokine production. Since NCR ligands are thought to be damage-induced ligands[144] , these findings suggest a local activation of ILC3 that in contrast to soluble cytokines would drive a specific inflammatory response based on the expression of NCR ligands in the tissue. Certainly, NKp44 ligands expressed by tumor epithelial cell lines trigger ILC3 activation and production of both IL-22 and TNFα[143], demonstrating a functional role of NCR expression on human ILC3.

On the contrary, the functional role of NKp46 expression on murine ILC3 remains elusive as NKp46 cross-linking does not induce production of cytokines[143]. These results together with the observation that NKp46 is dispensable during *C. rodentium* infection[132] suggest that NCR expression on murine ILC3 might be redundant. Nevertheless, murine NKp46^{+} ILC3 represent a consistent and abundant population in the intestinal mucosa, and it is likely that additional signals trigger their activation. Perhaps NKp46-mediated activation of ILC3 requires co-stimulatory signals that are only found *in vivo* under certain physiological conditions and thus limiting system production of inflammatory cytokines.

ILC3 MAINTAINING INTESTINAL HOMEOSTASIS

Formation of inducible lymphoid tissues in the intestines is part of the normal development of the homeostatic equilibrium of intestinal immunity and the commensal microflora. However, there are many examples of lymphoid tissues forming in adulthood in response to chronic immune activation during infections, auto-immune diseases and some forms of cancer[145]. The exact function of these so-called tertiary lymphoid tissues is still unresolved and might be either beneficial or pathological depending on the disease, the tissue or even the stage of disease. Many of the effector molecules expressed by fetal ILC3 that are essential for induction of the programmed lymphoid tissues and even of the CPs are also expressed by activated T cells, B cells or NK cells. This suggests that

during inflammation, when tertiary lymphoid tissues can form, many different immune cells might have lymphoid tissue inducing capacity.

The intestinal immune system keeps equilibrium with the commensal flora by being in a continuous state of activation. However, most of the organogenic events that occur in the intestines, such as the maturation of ILFs, are controlled by ILC3 rather than by activated adaptive immune cells. Nonetheless, during chronic inflammatory diseases like Crohn's disease or persistent infections like *Helicobacter pylori* in the stomach, lymphoid neogenesis occurs which is most likely independent of ILC3[145]. In experimental systems, the lymphoid tissue inducing capacity of activated B cells in the intestines was compellingly shown. Mice with a targeted deletion of Rorc, which lack all ILC3, Peyer's patches and CP, harbor about 3 fold more B cell aggregates in the non-inflamed colon in comparison to wild type mice[142]. These aggregates, which are mainly ILF and colonic patches, develop in mice in the absence of ILC3 and cryptopatches.

ILFs induced in the absence of ILC3 are histologically indistinguishable from those found in wild type mice. However, there is a clear shift in function. In spite of the increase in total numbers of ILF in the absence of Rorγt, the number of IgA producing plasma cells is strongly reduced[125]. Instead, IgG producing cells are now found within the lamina propria[142]. This shift away from protective IgA and towards a more inflammation-related B cell response does not lead to overt intestinal inflammation during normal homeostatic conditions. The immune system is still in equilibrium with the microbiota and bacterial dissemination is effectively prevented[142]. However, this changes at times of intestinal damage. When the intestinal epithelium of these mice is damaged by DSS administration the number of ILF increases in Rorγt$^{-/-}$ mice in a manner that is still comparable to wild type mice**.** However, in the absence of ILC3 this inflammation-induced ILF formation is driven by LT-expressing activated B cells[142]. Upon DSS exposure, Rorγt deficient mice succumb to a severe colitis, which is accompanied by wasting disease and excessive recruitment of neutrophils[142]. The shift in immunoglobulin production seems to be involved in this exacerbated response as disease severity could bee partially reverted by the administration of intra-venous immunoglobulin.

These data suggest that ILC3 control the balance between IgA and IgG that is generated in ILF, ensuring that during homeostasis and especially during responses to epithelial damage, protective IgA is the dominant isotype produced in response to the commensal microbiota. In the absence of ILC3, IgA producing plasma cells are reduced concomitant with an increase in IgG-secreting plasma cells[125,142]. Indeed, recent studies unveiled the mechanisms by which ILC3 balanced IgA responses[146]. While soluble LTα (sLTα3) produced by ILC3 regulated T cell dependent IgA via T cell homing in the lamina propria, membrane-bound LTβ controlled T cell independent IgA via dendritic cells[146].

In addition, the number of ILFs increases in an LTβR-dependent, yet ILC3-independent

manner[146]. One might therefore speculate that intestinal lymphoid tissues that are not under the control of ILC3, including presumably those formed during chronic inflammatory responses, abide to different rules and are subject to alternative forms of regulation. If this regulation changes, and IgG plasma cells are formed, this can aggravate disease.

Another study also underlines the essential crosstalk of commensal bacteria and ILC3 in intestinal epithelial homeostasis. ILC3 regulate intestinal epithelial glycosylation, which is an important mechanism for host-microbiota symbiosis[147]. IL-22 and LT expression by ILC3 contributes to epithelial fucosylation by inducing expression of fucosyltransferase2 (Fut2) in IEC. Epithelial fucosylation not only promotes symbiosis in the intestine but also protects against *Salmonella thyphimurium* infection.

ILC3 and adaptive immunity

Other mechanisms by which ILC3 contribute to intestinal homeostasis is by directly regulating adaptive immune responses. A subset of intestinal CCR6^{+}ILC3 that expresses MHCII controls CD4^{+}T cell responses against commensal bacteria and protects against commensal bacteria-specific inflammatory responses[148]. MHCII^{+}ILC3 can process and present antigens yet they lack co-stimulatory molecules. The regulatory function of ILC3 takes place in a MHCII-dependent manner, independently of IL-22, IL-17 or IL-23. In addition, ILC3 regulate adaptive immune responses in homeostasis by promoting regulatory T cell (Treg) function[149]. Microbiota-stimulated macrophages produce IL-1b that acts on ILC3 to induce GM-CSF secretion[149]. Although GM-CSF is also produced by IEC, ILC3 represent the main source of this cytokine at steady state in the murine intestine. GM-CSF produced by ILC3 mediates the secretion of regulatory cytokines, including IL-10 by macrophages and dendritic cells that in turn induce Treg development and function. This microbiota-macrophage-ILC3 axis highlights the importance of ILC3-derived GM-CSF in Treg function and intestinal homeostasis. Since GM-CSF plays an important role in mucosal wound healing[150], it is reasonable to speculate that GM-CSF produced by ILC3 might also act on epithelial cells after damage when higher levels of this cytokine might be required to restore epithelial integrity.

Interestingly, the crosstalk between ILC3 and T cells seems to occur in a bidirectional manner. Besides ILC3 regulating T cell responses, lamina propria CD4^{+}T cells also influence ILC3 biology. CD4^{+}T cells restrict ILC3 numbers and function in a TCR-dependent manner[151].

Nevertheless, the best-characterized homeostatic function of ILC3 is based on the IL-22 effects promoting AMP production by IEC. Expression of Reg3β and Reg3γ by intestinal epithelial cells is directly linked to IL-22R signaling, and deficiencies in this axis can lead to bacterial dissemination[152]. Therefore, ILC3 as a major cellular source of IL-22, play an essential role in bacteria containment and maintenance of intestinal homeostasis.

ILC3 IN INTESTINAL INFLAMMATION

ILC3 in experimental colitis and intestinal inflammation

Several intestinal infections models are used to study ILC3 function. Enteropathogenic bacterial infection and DSS-colitis models have unveiled the protective role of ILC3 from bacteria dissemination by maintaining the epithelial barrier. Although IL-22 plays an essential role in the antimicrobial response and mucus production[25,153], other ILC-derived factors including GM-CSF and LT have been shown to play a role in intestinal damage responses.

IL-22-mediated responses

A commonly used enterogenic bacterium is *Citrobacter rodentium*, a murine-specific enteric pathogen that causes acute colitis, used to model human enteropathogenic and entherohemorrhagic *Escherichia coli*. Studies with *C. rodentium* have shown the importance of ILC3-derived IL-22 in mediating early anti bacterial defenses and in avoiding bacterial dissemination[4,133,141]. IL-22R signaling on epithelial cells contributed to epithelial integrity and caused up-regulation of genes encoding the antimicrobial peptides RegIIIβ, RegIIIγ and S100a.

IL-22 producing ILC3 increased in number during the immediate response to *C. rodentium* infection[74,132,142]. The importance of these cells in immunity against this bacterium was shown by the finding that infected Rag2$^{-/-}$ IL2Rγ$^{-/-}$ mice, which have less ILC3, produce much lower levels of IL-22 as compared to Rag2$^{-/-}$ and Rag2$^{-/-}$ IL2Rβ$^{-/-}$ mice, resulting in the inability to clear the pathogen and increased mortality[4,132,133]. In this setting, only the CD4^{+}NKp46^{-} ILC3 subset and not the NKp46^{+} ILC3 subset was essential for *C. rodentium* clearance[152]. Moreover, in the presence of pathogens, IL23R signaling in CD4^{+} ILC3 was an essential prerequisite for IL-22 secretion and bacterial clearance[133].

The importance of IL-22 in immunity to *C. rodentium* is further underscored by the finding that IL-22 deficient mice fail to recover from *C. rodentium* inoculation and succumb to the infection by day 9[133]. More precisely, experiments using neutralizing antibodies against IL-22 show that this cytokine is essential at the early stages of infection[133,154]. When IL-22 is neutralized at the time of infection, mice fail to clear the bacterium and die. In contrast, neutralizing IL-22 at later time points is progressively less deteriorating with 40% of the animal surviving IL-22 neutralization at day 4. After 4 days, IL-22 is no longer necessary as neutralization after this point does not affect bacterial clearance or survival of the mice[133].

IL-17 and IFNγ-mediated responses

A second experimental model of enteric inflammation in which ILC3 are implicated is the colitis induced by *Helicobacter hepaticus* in Rag1-deficient mice. In these mice, in the

absence of adaptive immunity, *H. hepaticus* induces the production IL-17a and IFNγ by ILC3 in an IL-23 dependent manner[155]. The pathogenic ILC3 in this model share several phenotypic characteristics with classical LTi cells except for the absence of CD117 (c-kit). These CD117^{low} ILC3 accumulate in the inflamed colon and are directly responsible for the chronic pathology[155]. In an independent study, the developmental relationship of IFNγ producing ILC was addressed. In line with T helper cell differentiation, ILC3 expressing NKp46 can downregulate Rorc and NKp46[156].

This is accompanied by a loss of IL-22 production and the gain of IFNγ secretion. This conversion from a cell type actively involved in intestinal homeostasis to a more inflammation prone cell is driven by a loss of IL-7R signaling, which in the intestine can be the result of lower epithelial-derived IL-7 levels due to changes in the commensal microbiota[156]. Indeed, in germfree mice this conversion occurred much more frequently. Again this highlights the importance of the homeostatic equilibrium encompassing ILC3, epithelial cells, lymphoid tissues and the commensal microbiota and the fact that disruption of this balance can result in pathology driven by a variety of mechanisms.

Lymphotoxin-mediated response

Besides IL-22R signaling, the LTβR pathway is also important for the clearance of *C. rodentium.* Ligation of this receptor on intestinal epithelial cells induces the production of the chemokines CXCL1 and CXCL2 resulting in the recruitment of neutrophils to the site of the infection. Inhibiting LTβR signaling provokes colonic pathology and early death in mice upon *C. rodentium* infection[70].

Again ILC3 are important in these models, being the cellular source of membrane LTα1β2 (LT), the ligand for the LTβR. Mice that specifically lack LT in ILC3 show bacterial dissemination, disruption of the epithelial layer and high morbidity after *C. rodentium* infection[70]. Importantly, there is also a role for hematopoietic cells expressing LTβR as transfer of bone marrow-derived cells from LTβR$^{-/-}$ mice into wild type mice leads to susceptibility to infection and subsequent mortality in 60% of the infected mice. In addition, in mice that specifically lack LTβR in intestinal epithelium LTβR expression on hematopoietic cells can partially protect against *C. rodentium*-induced mortality. This shows the relevance of LTβR signaling in both epithelial cells and hematopoietic cells to orchestrate an efficient response against *C. rodentium* infection[70].

Graft versus Host Disease

In addition to their well defined roles in mucosal homeostasis and early stages of enteric infections, recent studies have highlighted novel functions of ILC3 in tissue regeneration after radiation damage in the thymus[157] and the protection of intestinal stem cells from graft versus host disease (GvHD)[61].

Allogeneic hematopoietic stem cell (HSCT) transplantation is used to treat several hematological malignancies. One of the major side effects limiting successful stem cell transplantation is the occurrence of intestinal GvHD[158] mediated by alloreactive T cells that recognize recipient tissues as foreign and cause severe inflammatory disease. Reasoning that factors important during wound healing might also be involved in limiting the extent of damage provoked by intestinal GvHD, experiments were initiated in experimental GvHD models to define a possible role for IL-22 in GvHD severity[61] As it turned out, bone marrow transplant (BMT) recipient mice that lacked IL-22 suffered from increased GvHD severity and significantly increased GvHD mortality[61]. On the other hand, transplantation with IL22-deficient donor marrow did not have any impact on GvHD-associated organ pathology. These findings implied the post-transplant presence of a subset of cells elementary for IL-22 production and limiting GvHD-induced inflammatory damage[61]. Using bone-marrow chimeras as secondary transplant recipients it became clear that recipient-derived hematopoietic cells were responsible for the production of protective IL-22. Detailed analysis revealed that IL-22 production was restricted to a radio-resistant population of $CCR6^+$ ILC3 that survived pre-transplant conditioning. In addition, IL-22R was found to be present on epithelial stem and progenitor cells in the small intestinal crypts and IL-22 production by ILC3 was positively regulated by IL-23 derived from intestinal DC[61]

Importantly, intestinal stem cells were a direct target of the GvHD and crypt cell apoptosis was increased during GvHD. Of note, also host-derived ILC3 appeared to be targeted by the GvHD as their numbers subsided during disease, concomitant with an increase in the number of apoptotic crypt cells[61]. Combined these data suggest a role for ILC3-derived IL-22 in protecting epithelial stem cells from GvHD-induced cell death.

The fact that intestinal stem cells (ISCs) as well as their downstream progenitors express IL-22R[61,159,160] has led to the perception that IL-22 could act as an instructor of epithelial cell function at the level of progenitor cells. ILC3, by virtue of their high radiation tolerance and IL-22 production, protect the progenitor compartment from GvHD-induced cell death ensuring proper epithelial regeneration and barrier function.

Taken together, it is evident that ILC3-derived cytokines play an essential role in intestinal homeostasis and damage responses in the epithelium. The production of these cytokines must however be tightly control as overproduction may lead to chronic inflammation or malignant transformation[150]. Similarly, lack of negative regulators can also promote disease. For example, lack of IL-22 binding protein (IL-22BP), a soluble receptor that neutralizes secreted IL-22, facilitates tumor development in the gut[161].

Understanding the mechanisms that control ILC3 activation and function is therefore crucial to fully comprehend homeostasis and damage responses in mucosal surfaces.

AIMS AND OUTLINE OF THIS THESIS

Type 3 innate lymphoid cells (ILC3) have emerged as important players in mucosal immunology where they are an essential source of IL-22, critical in the early stages of bacterial infection. The discovery of ILC3 raised many questions regarding their effector functions and mechanisms of activation. While the protective role during enteropathogenic infections has been characterized, the function of ILC3 during tissue damage is poorly understood. Understanding tissue damage responses such as those occurring during chemotherapy or radiotherapy induced-mucositis is essential for the design of new strategies in anti-cancer treatments. Recent findings in a graft versus host disease model identified IL-22 as an important factor that protected stem cells from T cell-mediated killing. Determining the contribution of ILC3 to epithelial protection after intestinal insult and understanding the crosstalk of ILC3 with the local microenvironment is therefore of great interest.

During intestinal damage responses, activating signals may regulate activation of ILC3. It is currently known that APC-derived cytokines including IL-23 and IL-1β promote cytokine production by ILC3. Nevertheless, murine intestinal ILC3 subsets are found at different compartment within the lamina propria and local signals may drive their activation. $CCR6^{+}$ILC3 localize in the cryptopatches close to the intestinal crypts where they may communicate with stem cells as suggested by the observations in the model of GvHD. In contrast, $NKp46^{+}$ILC3 are closer to the differentiated epithelial cells. Expression of NCRs by human and murine ILC3 raises the possibility of an additional mechanism of activation. Indeed, NKp44 ligation on human ILC3 triggered TNFα production by ILC3. However, further investigation is needed to understand the role of these receptors as well as their contribution to ILC3 function *in vivo*.

The overall aim of this thesis is to investigate the role of ILC3 after acute intestinal damage. We set up a Methotrexate-induced intestinal damage model to gain insight into the contribution of ILC3 to tissue damage responses. In **chapter 2**, we studied the intestinal epithelial damage response after MTX treatment in the absence of ILC3. Using $Ror\gamma t^{-/-}$ and anti-Thy1-treated $Rag1^{-/-}$ mice we demonstrated that ILC3 protect epithelial cells from chemotherapy-induced pathological damage. To understand the effector functions of ILC3 after cytostatic insult, we isolated lamina propria $CCR6^{+}$ILC3 and $NKp46^{+}$ILC3 and analyzed their cytokine production.

In **chapter 3**, we further examined the role IL-22 in the epithelial response after MTX-induced damage. By examining the tissue healing-associated pathway of STAT3 activation, antimicrobial responses and maintenance of goblet cells in the absence of IL-22, we gained knowledge on the effects of IL-22 on differentiated epithelial cells. Importantly,

we demonstrate an essential function for IL-22 in intestinal stem cell maintenance.

While activation of ILC3 by APC-derived cytokines has been characterized, the role of NCR as a potential activating mechanism remains poorly understood. Analysis of lamina propria ILC3 revealed an increase in frequency and activating profile of NKp46^{+} ILC3 after MTX (chapter 2). In **chapter 4**, we aimed to clarify the mechanism of activation of ILC3 and the function of NCR expression on mucosal ILC3. This chapter describes the co-activating function of NCR on human ILC3.

In **chapter 5** we further gained knowledge on the in *vivo role* of NKp46 in radiation and chemotherapy-induced intestinal damage.

Finally, in **chapter 6** I discuss the main conclusions of this thesis and present the data in a broader perspective.

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Patricia Aparicio-Domingo[1]*, Monica Romera-Hernandez[1]*, Julien J. Karrich[1], Ferry Cornelissen[1], Natalie Papazian[1], Dicky Lindenbergh-Kortleve[2], James Butler[3], Louis Boon[4], Mark C. Coles[3], Janneke N. Samsom[2] and Tom Cupedo[1]

[1] Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands
[2] Department of Pediatrics, division of Gastroenterology and Nutrition, Erasmus University Medical Center, Rotterdam, The Netherlands
[3] Centre for Immunology and Infection, Department of Biology and Hull York Medical School, York, UK
[4] Bioceros, Utrecht, The Netherlands

*Equal contribution

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CHAPTER 2

Type 3 innate lymphoid cells maintain intestinal epithelial stem cells after tissue damage

SUMMARY

Disruption of the intestinal epithelial barrier allows bacterial translocation and predisposes to destructive inflammation. To ensure proper barrier composition, crypt-residing stem cells continuously proliferate and replenish all intestinal epithelial cells within days. As a consequence of this high mitotic activity, mucosal surfaces are frequently targeted by anti-cancer therapies, leading to dose-limiting side effects. The cellular mechanisms that control tissue protection and mucosal healing in response to intestinal damage remain poorly understood. Type 3 innate lymphoid cells (ILC3) are regulators of homeostasis and tissue responses to infection at mucosal surfaces and we hypothesized that ILC3 are involved in intestinal epithelial recovery after insult. We demonstrate that ILC3 are required for epithelial activation in response to small intestinal tissue damage induced by the chemotherapeutic agent methotrexate (MTX). Multiple subsets of ILC3 were activated rapidly after intestinal tissue damage and in the absence of ILC3 epithelial activation was lost, correlating with increased pathology and severe damage to the intestinal crypts. Using ILC3-deficient Lgr5 reporter mice we show that maintenance of intestinal stem cells after damage is severely impaired in the absence of ILC3. These data unveil a novel function of ILC3 in limiting tissue damage by preserving tissue-specific stem cells

INTRODUCTION

The intestinal epithelium consists of a single layer of cells that combines the efficient uptake of nutrients and water with providing a physical barrier between the intestinal microbiota and our body[1]. Damage sustained by intestinal epithelial cells needs to be swiftly and efficiently repaired to prevent inappropriate immune responses to commensal bacteria. Because all intestinal epithelial cells are renewed within 3-5 days the intestines are one of the most mitotically active organs in the body[2,3]. The combination of this high mitotic index and the high antigenic pressure make the intestines sensitive to damage-induced inflammatory conditions. Intestinal damage is an early event in the development of both Graft-versus-Host disease (GvHD)[4] and alimentary mucositis[5] and a driver of bacterial translocation and T cell activation in inflammatory bowel disease (IBD)[6].

Mucositis is a major dose-limiting side effect of anti-cancer chemo and radiotherapy that is directly related to the sensitivity of highly proliferative intestinal epithelial cells to these cytotoxic treatments.

A major pathway involved in the intestinal epithelial response to damage is mediated by activation of STAT3, which is expressed along the crypt-villus axis of the intestinal epithelium[7,8]. Phosphorylated STAT3 translocates to the nucleus to activate genes involved in proliferation, survival and mucosal defense[9-11]. In humans, mutations in *STAT3* have been identified as susceptibility factors for IBD[9,12,13]. In mice, upon DSS-induced colitis, STAT3 activation in intestinal epithelial cells is required to promote mucosal wound healing[10].

Intestinal regeneration depends on the continuous differentiation of epithelial cells from crypt residing intestinal stem cells (ISC)[3,14,15]. Even though multiple intestinal progenitor cells have been described, the best-characterized population are the Lgr5 expressing cells that reside at the crypt bottom, interspersed with Paneth cells. These stem cells have the ability to give rise to all intestinal epithelial cells ex vivo[16]. Similar to its role in differentiated epithelial cells, STAT3 activation has also been recognized as an important pathway involved in intestinal epithelial stem cell survival[17].

Type 3 innate lymphoid cells (ILC3) are innate immune cells that reside in the lamina propria of both the small and large intestines and are involved in tissue homeostasis, early defense against enteric pathogens and containment of microbiota[18,19]. In the intestines, multiple ILC3 subsets exist, two of which can be distinguished by mutual exclusive expression of the natural cytotoxicity receptor NKp46 and the chemokine receptor CCR6[20,21]. Most of the Nkp46$^+$ ILC3 are found dispersed throughout the lamina propria, and this localization depends on their expression of CXCR6[22]. In contrast, the majority of NKp46$^-$CCR6$^+$ ILC3 are located in anatomically defined sites known as cryptopatches[23]. Cryptopatches are located in close proximity to the intestinal crypts and can evolve into

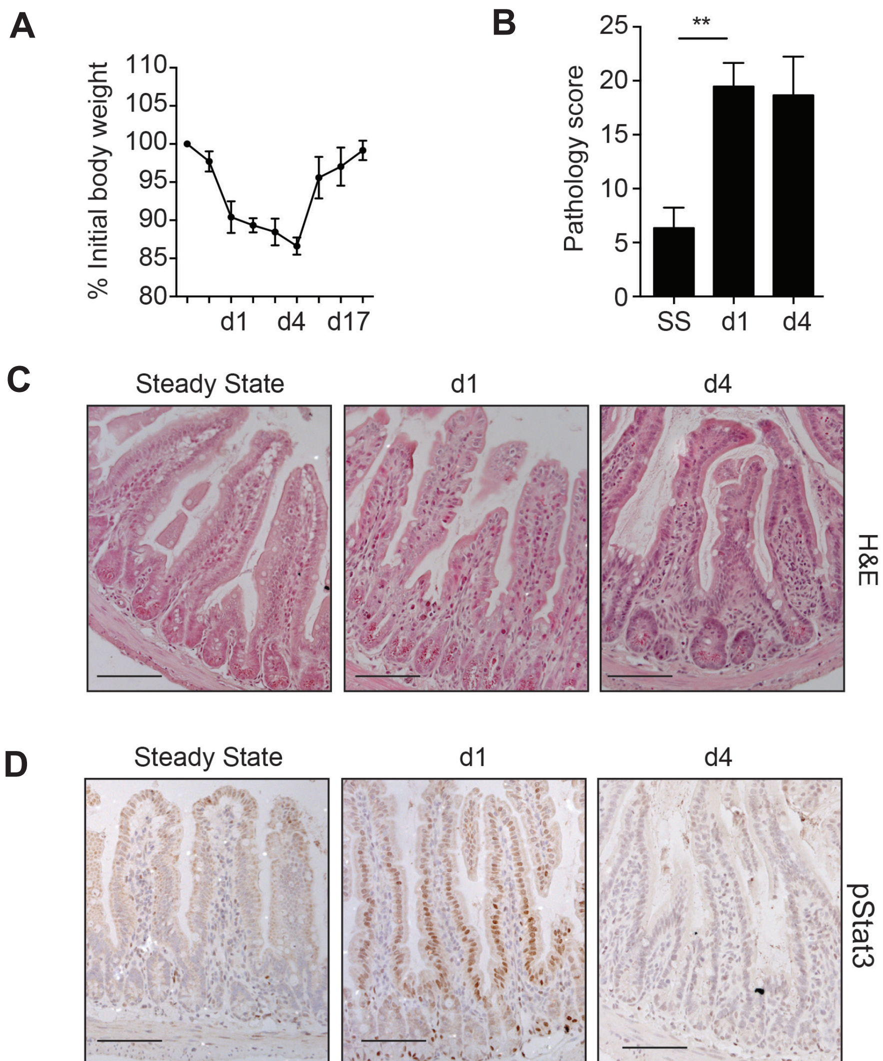
isolated B cell follicles (ILF) to serve as inductive sites for IgA[24]. Recent findings indicate that under inflammatory conditions, such as experimental GvHD, ILC3 have the capacity to interact with the epithelial stem cells in the crypts, protecting them from T cell-mediated killing[25].

The well-known ability of ILC3 to condition their local microenvironment, the close proximity of ILC3 to intestinal crypts and the ability of ILC3 to communicate with epithelial stem cells led us to hypothesize that ILC3 are involved in directing intestinal epithelial responses to tissue damage. Using the Methotrexate (MTX)-model of small intestinal damage we now show that ILC3 are activated immediately after MTX-induced damage leading to a rapid activation of epithelial STAT3. In the absence of ILC3, MTX treatment leads to severe pathology, mainly localized in the intestinal crypts and the inability to preserve $Lgr5^+$ intestinal stem cells (ISC). Our data reveal a novel function for ILC3 as organizers of the intestinal epithelial response to tissue damage through activation of epithelial cells and maintenance of ISC and suggest that ILC3s might in future be therapeutically harnessed to prevent stem cell loss during chemotherapy.

RESULTS AND DISCUSSION

Damage-induced epithelial activation depends on Thy1$^+$ cells

To study tissue damage responses in the small intestine, where most ILC3 reside, we exposed mice to MTX, an anti-metabolite that inhibits folic acid metabolism and targets cells in S-phase[26]. MTX-induced intestinal damage is a well-established, self-resolving model of small intestinal damage that has been used to dissect epithelial responses to sterile insult[27-29]. Upon MTX administration, mice lost weight until day 4, after which they fully recovered by day 7 (Figure 1A). The rapid weight loss correlated with intestinal pathology, which peaked as early as day 1 after the last MTX injection and subsequently stabilized at day 4 (Figure 1B) before recovering by day 7 (not shown). Pathological examination of the small intestine showed villus flattening and crypt hyperplasia with an overall loss of epithelial architecture (Figure 1C). To visualize damage responses by epithelial cells we assessed phosphorylation of epithelial STAT3, since activation of this pathway in intestinal epithelial cells (IEC) plays a central role in mucosal wound healing[11]. Phosphorylation of STAT3 was induced early after MTX application, peaking at day 1 (Figure 1D). Four days after the last MTX injection, the phosphorylation of STAT3 had already returned to baseline (Figure 1D). These data show that MTX-induced small intestinal damage induces rapid and transient epithelial activation visualized by phosphorylation of STAT3. Immediate MTX-induced pathology has been attributed mainly to direct effects on intestinal epithelial cells, leading to epithelial cell-intrinsic responses to tissue damage[29]. To determine whether the early STAT3 phosphorylation at day 1 is indeed epithelial cell intrinsic or whether immune cells are involved, we administered MTX to Rag1$^{-/-}$ mice, which lack adaptive immunity and to Rag1$^{-/-}$ mice pre-treated with Thy1-depleting antibodies to also remove innate immune cell subsets. One day after MTX administration, STAT3 phosphorylation was induced in IEC of Rag1$^{-/-}$ mice treated with isotype control antibodies, confirming that adaptive immune cells are dispensable for this induction (Figure 1E). In contrast, depletion of Thy1$^+$ cells in Rag1$^{-/-}$ mice strongly impaired STAT3 phosphorylation in IEC in response to tissue damage (Figure 1E). To validate the reduction in phosphorylation of STAT3 we quantified the intensity of pSTAT3 staining on sections using semi-automated analysis and found a significant reduction of pSTAT3 intensity at day 1 after the last MTX administration (Figure 1F). In addition, transcript analysis of total ileum revealed a significant reduction in levels of the STAT3 target gene *Socs3*, indicative of reduced STAT3 signaling (Figure 1G). Of note, pSTAT3 positive hematopoietic cells were found in the lamina propria of both control Rag1$^{-/-}$ and Thy1-depleted Rag1$^{-/-}$ mice (Figure 1E), suggesting differential regulation of STAT3 activation in epithelial and hematopoietic cells. In conclusion, these experiments establish that epithelial phosphorylation of STAT3 after tissue damage is not cell intrinsic and requires the presence of Thy1$^+$ cells.


A
% Initial body weight
110
105
100
95
90
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80
d1
d4
d17
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Pathology score
25
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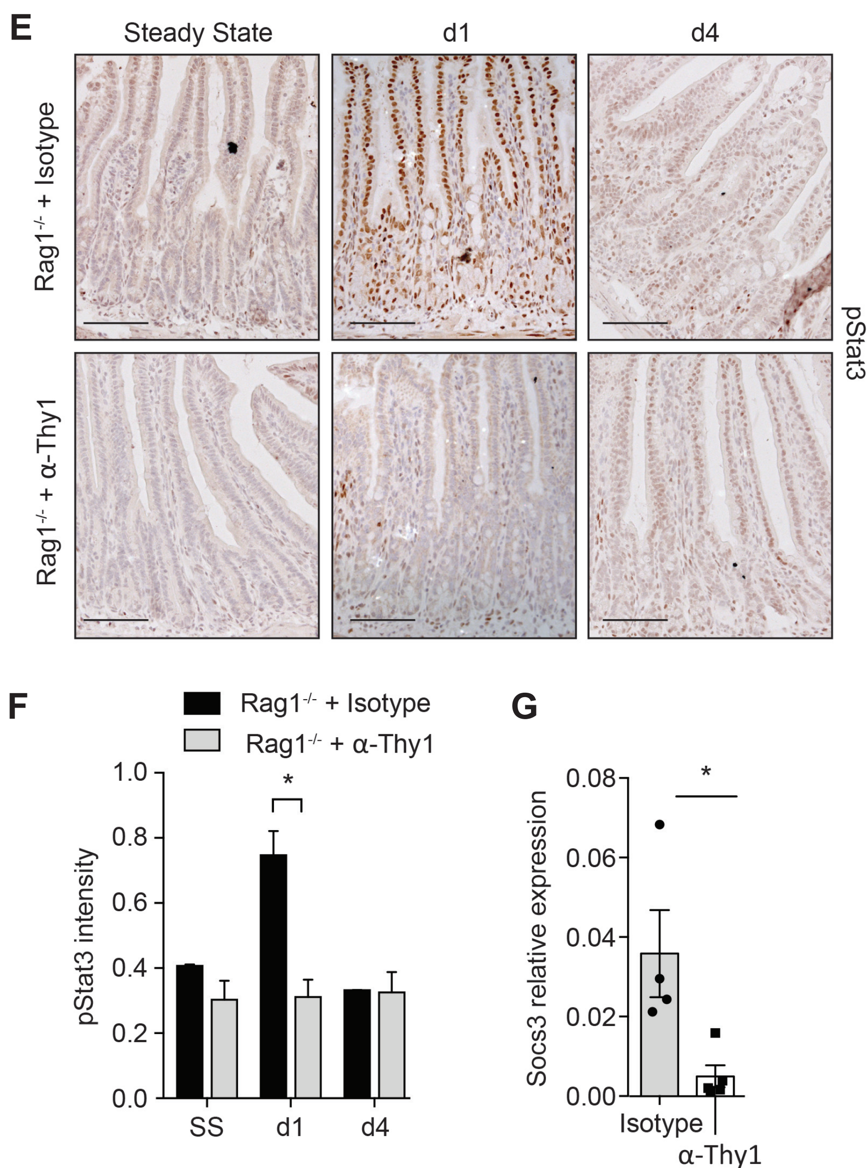


Figure 1. Epithelial STAT3 phosphorylation after MTX depends on Thy1⁺ cells. (A) Body weight of wild-type (WT) mice (n=7) treated with MTX. (percentage of initial weight) (B) MTX-induced pathology as described in materials and methods. (n>5 per time point) (C) Representative H&E staining (D) and immunostaining of pSTAT3 in ileal sections from WT animals at the indicated time points.(n>5 per time point) (E) Representative immunostaining of pSTAT3 in ileal sections from isotype and α-Thy1 treated Rag1$^{-/-}$ mice at the indicated time points. (F) pSTAT3 intensity in ileal sections at the indicated time points. (n=4 per group) (G) *Socs3* transcript levels relative to *Gapdh* from ileum at day 1 after MTX. (n>4) *, P<0.05 . Bars, 50µm.

ILC3 are activated in response to intestinal tissue damage

The finding that phosphorylation of epithelial STAT3 depended on Thy1$^+$ cells led us to hypothesize that Thy1$^+$ ILC3 are involved in the intestinal response to MTX-induced tissue damage. To determine whether ILC3 are activated in response to intestinal damage, CCR6$^+$ and NKp46$^+$ lamina propria ILC3 were analyzed for expression of transcripts associated with activation at 1 day after the last MTX injection (Figure 2A and B). Compared to homeostasis, CCR6$^+$ ILC3 maintained transcription of *Ltb*, *Rorc*, *Ifng*, *Il22* and *Csf2* but showed increased transcription of *Tnf* and *Il17a* (Figure 2A). NKp46$^+$ ILC3 significantly increased transcription of *Ltb*, *Rorc*, *Il22*, *Ifng*, *Csf2* and *Tnf* (Figure 2B). These data show that in response to intestinal tissue damage, both NKp46$^+$ and CCR6$^+$ ILC3 are activated, albeit in a differential manner. In line with the activation of ILC3 at the cytokine level, we also noted that expression of NKp46 was increased on ILC3 at day 1 after insult. This increased expression was apparent both at the protein (Figure 2C) and transcript level (Figure 2D). Collectively this shows that immediately after tissue damage, lamina propria ILC3 are in an activated state. To confirm the transcription of these cytokines in an *in vivo* setting we analyzed the intestines of Rag1$^{-/-}$ mice in the presence or absence of Thy1$^+$ cells. Transcript analysis of total ileum, or protein analysis after overnight ileal explant cultures clearly showed that the presence of Thy1$^+$ cells was essential for generation of transcripts for *Csf2*, *Tnf*, *Il22*, *Ltb* and *Ifng*, (Figure 2E) as well as protein for both IL-22 and GM-CSF (Figure 2F) in response to tissue damage. These experiments unveil the rapid activation of ILC3 subsets in response to intestinal tissue damage.

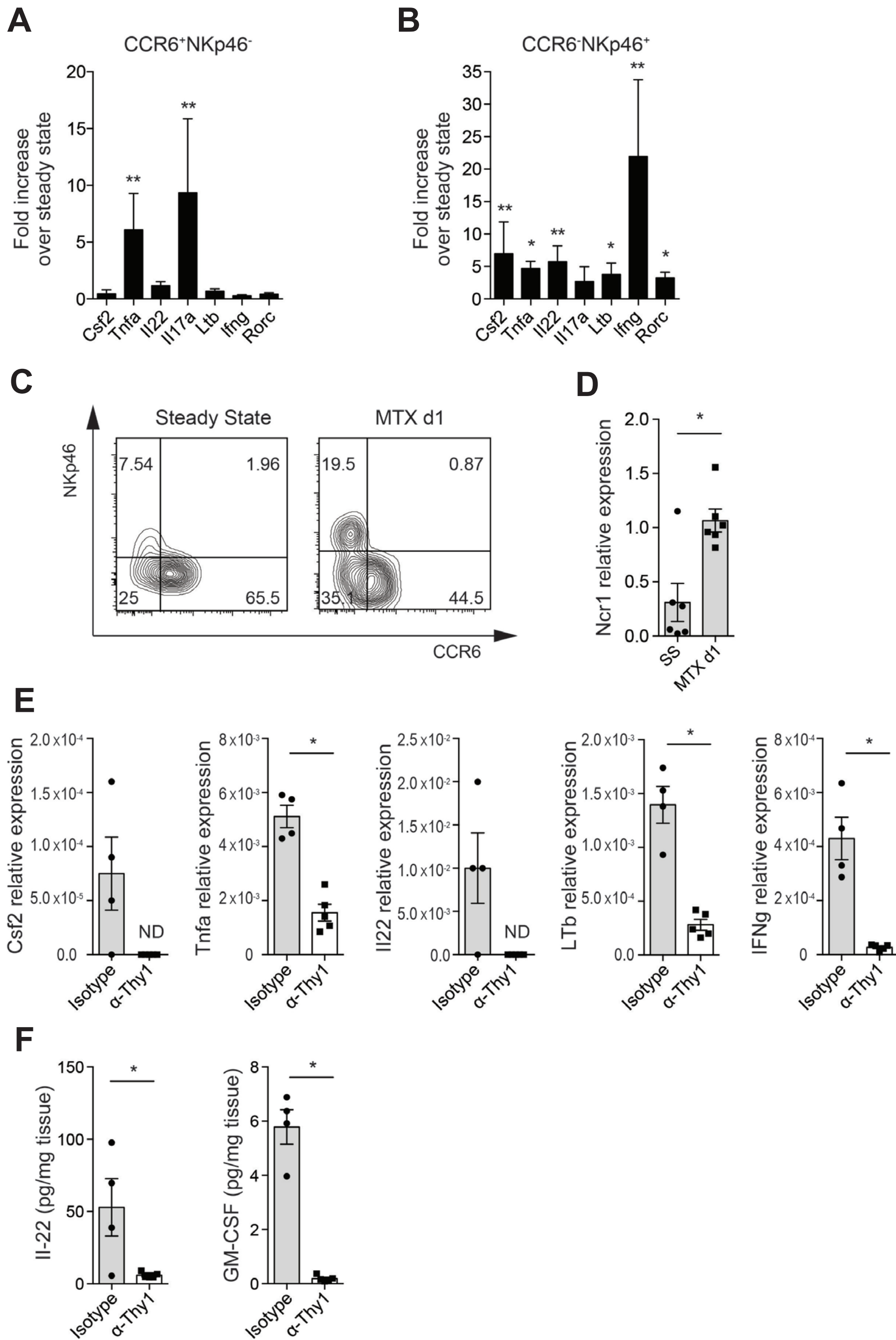


Figure 2. ILC3 are activated upon MTX-induced damage. Fold induction of indicated transcripts relative to steady state, of lamina propria CCR6⁺NKp46⁻ILC3 (A) and CCR6⁻NKp46⁺ ILC3 (B) at day 1 after MTX. N=6 data points. Each data point represents pooled ILC3 from five to seven mice. (C) Representative flow cytometry plot of NKp46 and CCR6 expression on lamina propria ILC3 at steady state and at day 1 after MTX. (D) Transcript levels of *Ncr1* relative to *Gapdh* from CCR6⁻NKp46⁺ ILC3. (n=6 per group) (E) Transcriptional analyses of indicated genes relative to *Gapdh* from total ileal mRNA of isotype control or Thy1–depleted Rag1$^{-/-}$ mice at day 1 after MTX. (F) Protein levels of IL-22 and GM-CSF after overnight ileal explant cultures isolated at day 1 after MTX. (n>4) *, P<0.05; **, P<0.01. ND, not detected.

ILC3 deficiency aggravates MTX-induced damage

Depletion of $Thy1^+$ cells diminished activation of epithelial STAT3 in response to MTX and ILC3 were activated early after tissue damage. However, because Thy1 antibodies do not specifically target ILC3 we next exposed ILC3-deficient $Ror\gamma t^{-/-}$ mice to MTX to confirm that ILC3 are responsible for the observed damage-associated epithelial activation. In contrast to WT animals, intestinal epithelial cells in $Ror\gamma t^{-/-}$ mice did not show phosphorylation of STAT3 in IEC in response to tissue damage (Figure 3A) and consequently had reduced STAT3 signaling as evidenced by lower levels of *Socs3* transcripts (Figure 3B).

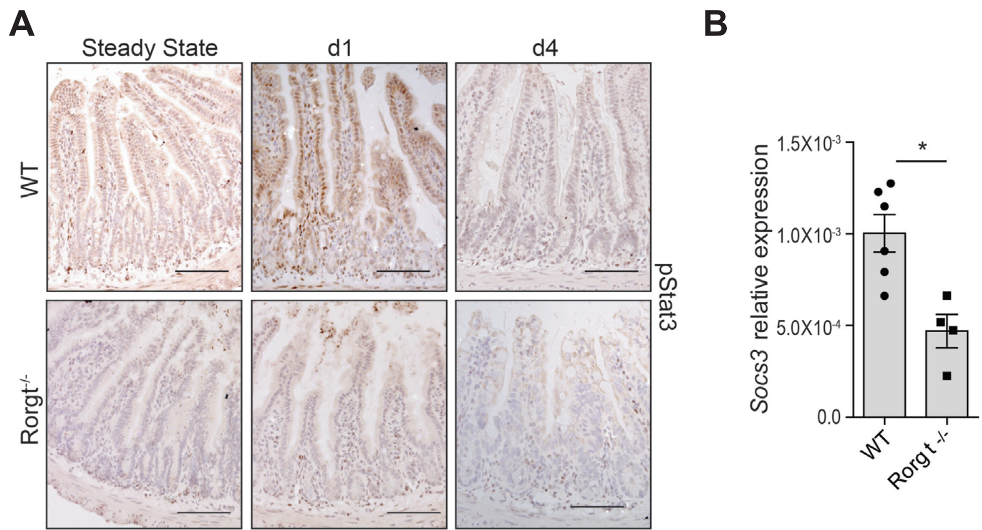
Histological analysis of the small intestines of MTX-exposed $Ror\gamma t^{-/-}$ mice revealed a slight increase in overall pathology at day 4 after MTX (Figure 3C). The differences between overall pathology in WT and $Ror\gamma t^{-/-}$ mice did not reach statistical significance, even though there seemed to be a trend towards increased of pathology in $Ror\gamma t^{-/-}$ mice at day 4 (Figure 3D).

To understand this trend, we performed differential pathology scoring on intestinal villi and crypt compartments. This revealed a significant increase in the damage sustained by the small intestinal crypts in $Ror\gamma t^{-/-}$ mice compared to WT control mice (Figure 3E).The extent of crypt epithelial flattening, the presence of crypt abscesses and the eventual loss of crypts were all significantly increased in the absence of Rorγt (Figure 3F).

To further evaluate epithelial fitness, we analyzed mucin-producing goblet cells as maintenance of the mucus layer is a requisite to ensure a proper epithelial barrier[30,31]. Histological analysis of small intestine at day 4 after MTX revealed reduced numbers of goblet cell in $Ror\gamma t^{-/-}$ mice compared to WT mice (Fig. 3G and H). While proximal and distal small intestine showed significantly less goblet cells in $Ror\gamma t^{-/-}$ mice compared to WT animals, jejujunm showed similar numbers (Fig. 3H)

Survival of epithelial cells in response to tissue damage is regulated by the balance between several pro- and anti- apoptotic molecules[2]. To assess whether this balance was shifted in the absence of Rorγt we determined transcript levels of the pro-apoptotic gene *Bax* and the anti-apoptotic gene *Bcl2l1* (BclXL) in ileum (Figure 3I). In $Ror\gamma t^{-/-}$ mice, one day after MTX, the *Bax* - *Bcl2l1* ratio was significantly increased, indicating a shift towards a more pro-apoptotic program in the absence of ILC3.

Combined, these data suggest that the absence of $Ror\gamma t^+$ ILC3 augments tissue damage to the stem cell-containing intestinal crypts in response to MTX.


A
Steady State
d1
d4
WT
Rorgt-/-
pStat3
B
Socs3 relative expression
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*
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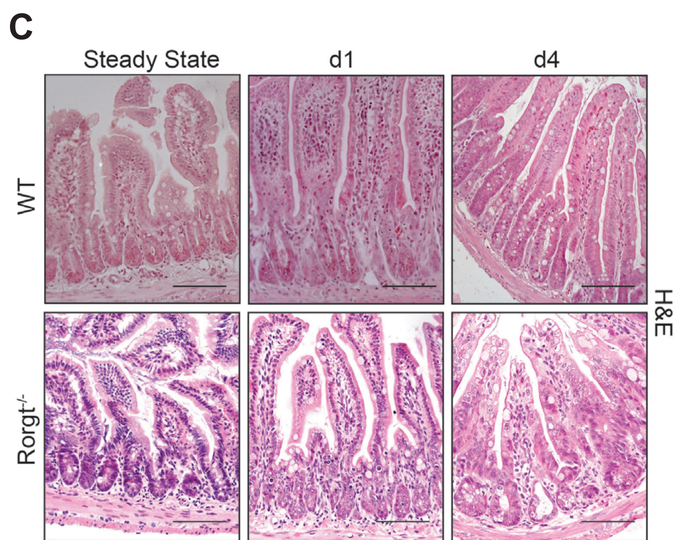
Rorgt -/-


C
Steady State
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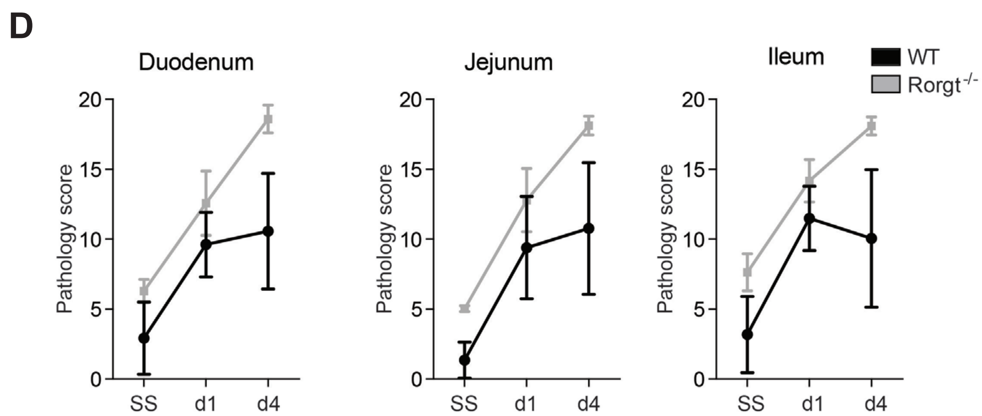
D
Duodenum
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d1
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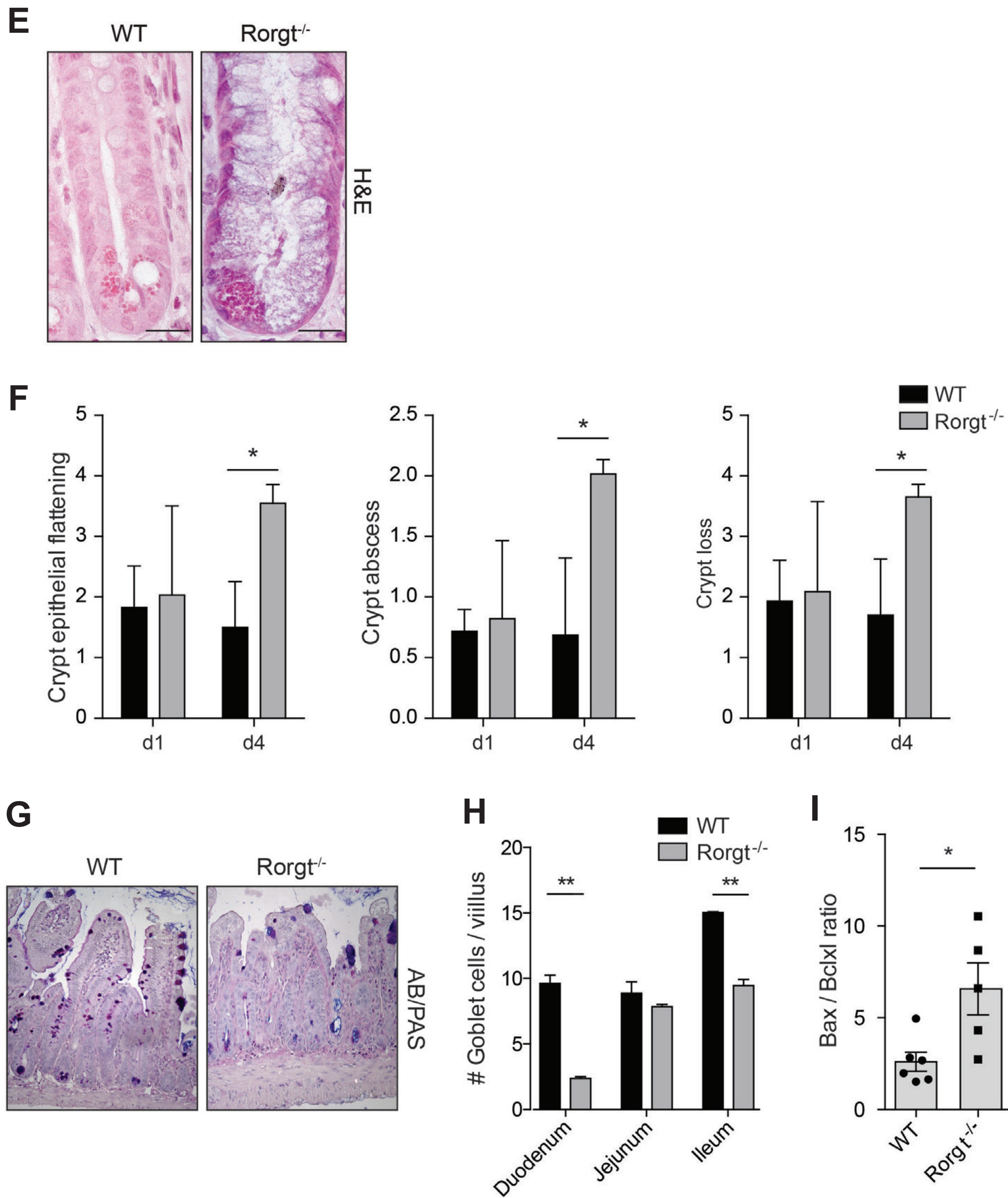


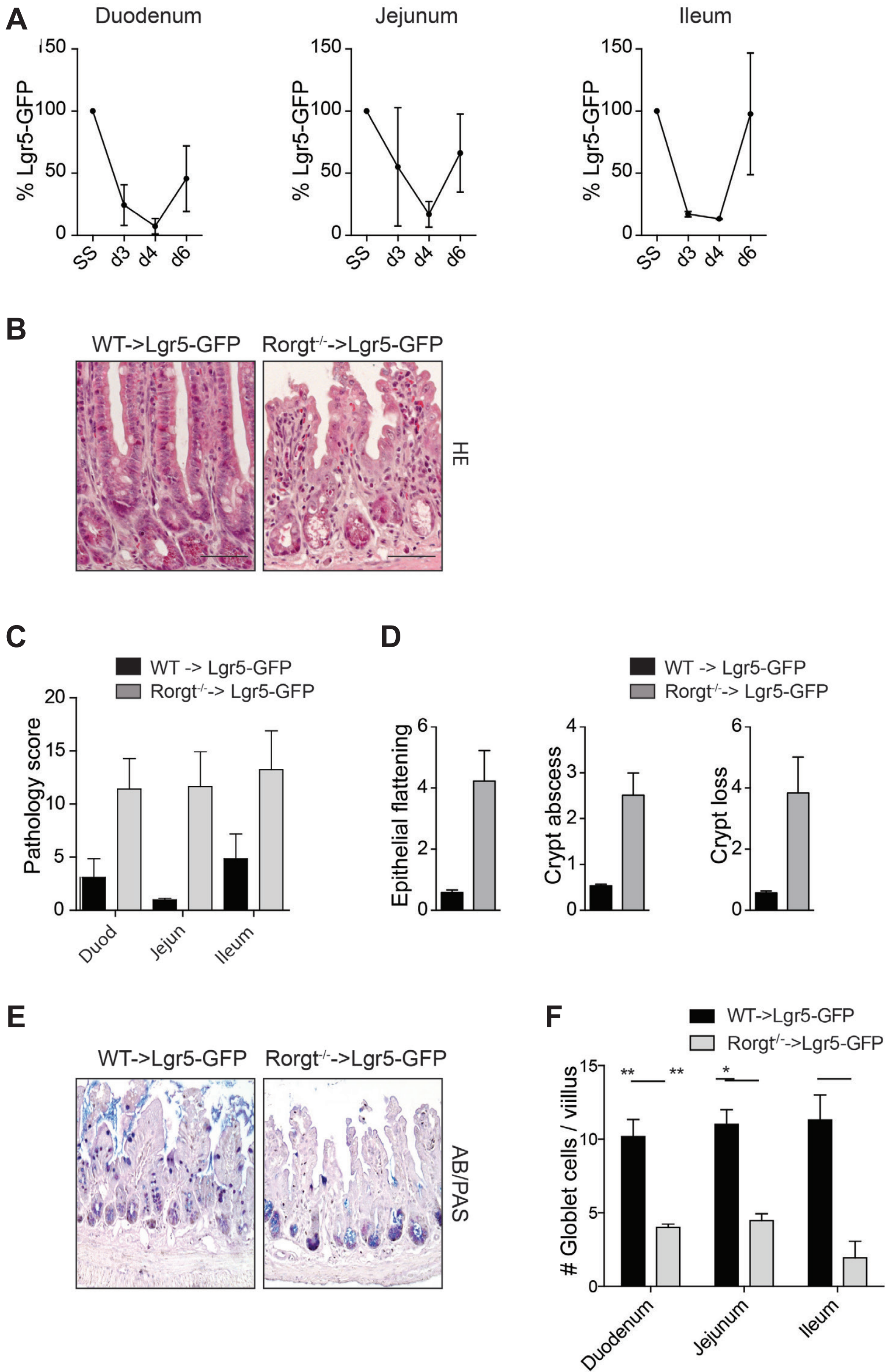
Figure 3. Absence of Rorγt augments crypt damage after MTX. (A) pSTAT3 in ileum sections from WT and Rorγt$^{-/-}$ mice at the indicated time points. (B) *Socs3* transcripts relative to *Gapdh* from total ileal mRNA of WT (n=6) and Rorγt$^{-/-}$ (n=4) mice at day 1 after MTX. (C) Representative H&E staining of ileal sections at the indicated time points. (D) MTX-induced small intestinal damage in WT and Rorγt$^{-/-}$ mice as specified in materials and methods. (SS:Steady State) (E) Representative high-power magnification of ileal crypts of WT and Rorγt$^{-/-}$ mice 4 days after MTX (F) Crypt damage in WT and Rorγt$^{-/-}$ mice at day 1 and day 4. (n>4 per group per time point) (G) AB//PAS staining showing goblet cells of small intestinal sections of WT and Rorγt$^{-/-}$ mice at 4 after MTX. (H) Goblet cell number per villus of duodenum, jejunum and ileum at day 4 after MTX. (I) Ratio between *Bax* and *Bcl2l1* transcript levels in ileum of WT (n=6) and Rorγt$^{-/-}$ (n=5) mice at day 1 after MTX. *, P<0.05. Bars, 50µm (A and C), 10µm (E).

ILC3 preserve intestinal stem cells after tissue damage

A prerequisite for intestinal epithelium regeneration is the survival of crypt-residing ISC[32]. Because Rorγt$^{-/-}$ mice exposed to MTX had increased crypt pathology, we investigated the fate of Lgr5^{+} ISC in response to MTX. In order to determine whether Lgr5^{+} ISC are sensitive to MTX, we exposed Lgr5-GFP reporter mice to MTX and assessed the percentage of GFP^{+} cells in isolated crypts at several time points post treatment (Figure 4A). Lgr5^{+} ISC numbers declined directly after treatment, indicating that ISC were targeted by MTX. Stem cell recovery started after day 4 and numbers normalized by day 6 (Figure 4A) To investigate the role of Rorγt-expressing ILC3 in epithelial stem cell maintenance, we generated radiation bone-marrow chimeras by transferring either wild type or Rorγt-deficient bone marrow into lethally irradiated Lgr5-GFP recipient mice. To eliminate host-derived radio-resistant ILC3, both groups were treated with depleting αThy1 antibodies, after which de-novo generation of ILC3 could occur from the WT but not from the Rorγt$^{-/-}$ bone marrow. Thus, Rorγt$^{-/-}$-Lgr5-GFP chimeric mice provide a model to study GFP-labeled Lgr5^{+} ISC in the absence of Rorγt^{+} hematopoietic cells. In response to MTX, Rorγt$^{-/-}$-Lgr5-GFP chimeric mice developed increased intestinal pathology compared to WT chimeric mice (Figure 4B and C) and this was characterized by crypt epithelial flattening, crypt abscesses and crypt loss. (Figure 4D). In agreement with these results, histological analysis of small intestinal samples revealed reduced numbers of goblet cells in Rorγt$^{-/-}$-Lgr5-GFP chimeric mice as compared to WT-Lgr5-GFP chimeras (Fig. 4 E and F), resembling the pathology in full Rorγt-deficient mice.

To determine the effects of absence of Rorγt^{+} lymphocytes on ISC we purified small intestinal crypts from Rorγt sufficient and deficient chimeric mice. At day 4 after MTX, flow cytometric analyses revealed a significant reduction in the percentage of Lgr5-GFP^{+} ISC within the EpCAM-1^{+} crypt epithelial cell fraction (Figure 4E). This reduction in Lgr5^{+} ISC occurred along the entire length of the small intestine. In duodenum, jejunum and ileum Lgr5^{+} ISC were reduced by 68% (68.3 ±3.1), 71% (71.2±1.4) and 52% (52.1±8.1) respectively (Figure 4E and F), highlighting the importance of Rorγt^{+} ILC3 as guardians of intestinal stem cells after damage. At steady state, the percentages of Lgr5-GFP^{+} cells were comparable between both groups (not shown).

Collectively our findings reveal that ILC3 preserve organ-specific stem cells in response to tissue insult. MTX application evokes a rapid and transient activation of epithelial STAT3 that depends on Thy1^{+}Rorγt^{+} cells. Multiple subsets of ILC3 respond to intestinal tissue damage and absence of ILC3 aggravates pathology in intestinal crypts. Importantly, we could show that the maintenance of intestinal stem cells after cytotoxic therapy is regulated by ILC3. Our findings thus highlight a previously unappreciated feature of ILC3 in coordinating epithelial responses to tissue damage in the small intestine. Their location in close proximity to the crypts and their resistance to chemo- and radiotherapy-induced cell death put ILC3 in the ideal position to minimize tissue damage after cytotoxic insult and controlling ILC3 responses might hold the key to designing future therapeutic strategies aimed at minimizing intestinal damage in patients undergoing anti-cancer therapies.


A
Duodenum
Jejunum
Ileum
% Lgr5-GFP
150
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SS
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d4
d6
B
WT->Lgr5-GFP
Rorgt-/- ->Lgr5-GFP
HE
C
WT -> Lgr5-GFP
Rorgt-/- -> Lgr5-GFP
Pathology score
Duod
Jejun
Ileum
D
Epithelial flattening
Crypt abscess
Crypt loss
E
AB/PAS
F
Globlet cells / villus
Duodenum
Jejunum
Ileum

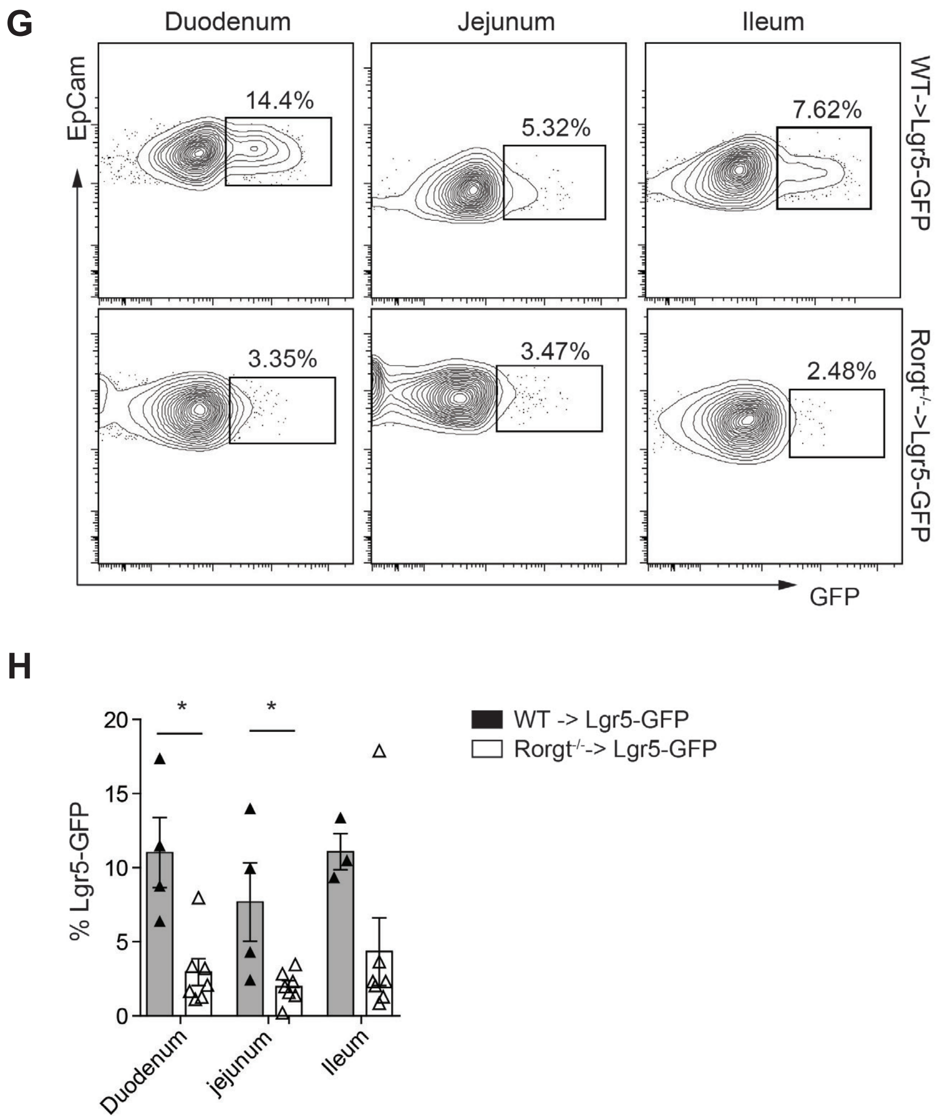

Figure 4. ILC3 preserve intestinal stem cell after MTX-induced damage (A) Percentage of GFP+ stem cells within EpCAM1+ cells from purified intestinal crypts at the indicated time points. (Percentage Lgr5 relative to steady state) (B) Representative H&E staining of ileal sections of WT and Rorγt-/- chimeras at day 4 after MTX. (C) Small intestinal damage in WT and Rorγt-/- chimeras at day 4 after MTX as described in materials and methods (n=3). (D) Crypt pathology score for crypt epithelial flattening, crypt abscess and crypt loss. (E) Goblet cell staining of ileal section of WT and Rorγt-/- mice at 4 after MTX. (F) Counting of goblet cells per villus from small intestinal sections at day 4 after MTX. (G) Frequency of GFP+ from WT and Rorγt-/- chimeras at day 4 after MTX. Numbers adjacent to outlined areas indicate percent of EpCAM+Lgr5-GFP+ cells. (H) Percent of EpCAM+ Lgr5-GFP+ cells in duodenum, jejunum and ileum of WT (n=4) and Rorγt-/- (n=7) chimeric mice . *, P<0.05. Bars, 50µm

MATERIALS AND METHODS

Mice. C57BL/6, Rorγt$^{-/-}$, Rag1$^{-/-}$ and Lgr5-GFP mice were bred in the animal facility of the Erasmus University Medical Center Rotterdam. Animal experiments were approved by the relevant authorities and procedures were performed in accordance with institutional guidelines. Age and gender-matched littermates were used whenever possible.

Thy1^{+} cells were depleted using α-Thy1 antibodies (clone YTS154, provided by Herman Waldmann, Cambridge) or isotype controls (a-Phytochrome AFRC MAC5.1). Antibodies were diluted in saline and mice were injected i.p. with 200ug every other day, for 2 weeks.

Methotrexate. 8-12 weeks old mice were injected i.p. with 120mg/kg MTX (PCH) at day-1 and with 60mg/kg at day 0. Body weight was monitored daily and tissues were collected at day 1 and day 4 after the last MTX injection.

Radiation chimeras. 8 weeks old Lgr5-GFP mice were irradiated at 9Gy and subsequently reconstituted by i.v injection of 1-2.10^{6} bone marrow cells from either WT or Rorγt$^{-/-}$ mice. Mice were under antibiotic water for 2 weeks after bone marrow transplantation. To eliminate radio-resistant ILC3, 2 weeks after reconstitution, Lgr5-GFP chimeras received 3 i.p. injections with 200μg of α-Thy1 antibody (YTS154, provided by Herman Waldmann, Cambridge) during one week. Four weeks after Thy1 depletion, Lgr5-GFP chimeras were exposed to MTX.

Crypt isolation. Isolation of intestinal crypts was performed as previously described [16]. Briefly, isolated small intestines were opened longitudinally and washed with cold PBS. Tissues were cut into 5mm pieces and subsequently washed by mechanical pipetting with cold PBS until supernatant was clear. Tissue were incubated with EDTA (2mM) in PBS at 4°C for 30 min. Tissues were then washed several times with cold PBS and suspended by vigorous pipetting. Crypt-enriched sediments were passed through a 70μm cell strainer and centrifuge at 600rpm for 3 min to separate the crypts from single cells. Crypts were incubated with 1 ml of TrypLE Express (Gibco) at 37°C for 10-15 min until crypt dissociation was observed. Single cell suspensions were stained with the conjugated antibodies EpCAM-1 (G8.8 Biolegend) and CD45 (30F11, Invitrogen) and analyzed for the expression of GFP by flow cytometry (FACSCantoII, BD).

Histology. Small intestinal tissue pieces (5mm) were fixed in 4% PFA and embedded in paraffin. Four-μm sections were deparaffinized and stained with hematoxylin (Vector Laboratories) and eosin (Sigma-Aldrich). For pSTAT3 detection endogenous peroxidases were blocked and antigen retrieval was achieved by microwave treatment in citrate

buffer (10mM, pH 6.0). Prior to staining, Fc receptors were blocked in blocking solution (Supplementary materials and methods). Tissue sections were incubated overnight at 4°C with rabbit pSTAT3 primary antibody (D3A7, Cell signaling). Immunoreactions were detected using biotinylated goat-anti-rabbit (Vector Laboratories) and incubated with the Vectastin ABC Elite Kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were counterstained with hematoxylin.

Pathology was scored blinded by at least two independent analysts by quantification of damage in villus, crypts, epithelium, inflammation and bleeding as previously described [27]. Measurement of pSTAT3 intensity in IEC from sections was determined using HistoQuest software (TissueGnostics).

Explant cultures. Isolated small intestine was opened longitudinally and cleaned with cold PBS. A piece of 1cm length was cultured in RPMI with 10%FCSi and 1%P/S at 37°C for 24 hours. Protein content of supernatants was determined by enzyme-linked immunosorbent assay (eBioscience) and absorbance was measured at 450nm using Victor X4 (Perkin Elmer). Protein content present in the supernatants was calculated relative to the tissue weight.

Isolation of lamina propria lymphocytes. Isolated small intestine was opened longitudinally and washed with cold HBSS containing Hepes (15 mM), pH 7.2. Tissues were cut in 1cm pieces and incubated in HBSS buffer containing EDTA (Supplementary materials and methods) at 37°C, two times for 20 min to remove epithelium and intraepithelial lymphocytes. The tissues were digested with Collagenase VIII (100U/ml, Sigma) in RPMI at 37°C in a shaker, two times for 1 hour. Supernatants were passed through a 70µm cell strainer and washed in cold HBSS. Pellets were suspended in 90% percoll , overlaid with 40% percoll and centrifuge at 1800rpm for 20 min to allow separation of mononuclear cells (MNC) by density gradient. Interphase was washed and stained with conjugated antibodies (CD45 (30F11, Invitrogen); Lin-biotin (eBioscience): CD19 (1D3), CD3 (145-2C11), CD11c (N418), CD11b (M1/70), Gr1 (RB6-8C5); Streptavidin (Biolegend), NK1.1 (PK136, eBioscience); CD127 (A7R34, eBioscience); CD117 (2B8, BD), NKp46 (2941.4, eBioscience); CCR6 (29-2L17, Biolegend)). Lamina propria lymphocytes were analyzed by flow cytometry (Facs ARIAIII, BD) and ILC3 were sorted as $CD45^{+}Lin^{-}NK1.1^{-}CD127^{+}CD117^{+}CCR6^{+/-}NKp46^{+/-}$

Transcript analysis. RNA was extracted using the RNA-XS kit (Machery Nagel) followed by reverse-transcription with random hexamer primers. RNA from sorted cells was amplified according to the manufacturer's protocol (Ovation PicoSL WTA System V2, NuGen). For quantitative PCR, a Neviti Thermal Cycler (Applied Biosystems) and DyNAmo Flash SYBR

Green qPCR kit (Finnzymes) were used, with the addition of MgCl2 to a final concentration of 4 mM. All reactions were done in duplicate and are normalized to the expression of Gapdh. Relative expression was calculated by the cycling threshold (CT) method as $2^{-\Delta CT}$. The primers sequences can be found in Table 1.

Statistical analysis. Samples were analyzed using unpaired Mann-Whitney test. P values < 0.05 were considered significant. Data are shown as mean ± SEM.

Table 1. Primer sequences

Gene name	Forward primer	Reverse primer
mGapdh	TCAACGGCACAGTCAAG	GCTCCACCCTTCAAGTG
mSocs3	GGAGCCCCTTTGTAGACT	CGGGAAACTTGCTGTG
mBcl2l1(Bclxl)	CGTGGCCTTTTTCTCC	GGCTGCTGCATTGTTC
mBax	AAGGCCCTGTGCACTAA	GAGGCGGTGAGGACTC
mIl22	CTCCCCCAGTCAGACAG	CAATCGCCTTGATCTCTC
mCsf2 (Gm-csf)	GACCCGCCTGAAGATATT	ATCCGCATAGGTGGTAACT
mTnf (Tnfa)	GGGGGCTTCCAGAACT	GGGCCATAGAACTGATGAG
mIl17	CTTGGCGCAAAAGTGA	TTGCTGGATGAGAACAGAA
mIfng	CAAAAGGATGGTGACATGA	GGGTTGTTGACCTCAAACT
mRorc	GTGGGGACAAGTCATCTG	CGGCCAAACTTGACAG
mLtb	ACGTCGGGTTGAGAAGA	GGATGTGGAGGCTAGATTC
mNcr1 (NKp46)	CCCCCTGAAACTGGTAGTA	GTGGCAGTCTTCAGTTGG

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Patricia Aparicio-Domingo[1], Monica Romera-Hernandez[1], Natalie Papazian[1], Julien J. Karrich[1], Ferry Cornelissen[1], James Butler[2], Mark C. Coles[2] and Tom Cupedo[1]

[1] Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands
[2] Centre for Immunology and Infection, Department of Biology and Hull York Medical School, York, UK

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CHAPTER 3

Role of ILC3-derived IL-22 in epithelial responses after intestinal damage

SUMMARY

The epithelium of the small intestine contains the highest number of rapidly cycling cells in the human body and as a result is very sensitive to anti-cancer drugs that target cycling cells. In the intestines, most ILC3 are localized in cryptopatches in close proximity to the crypts that contain the intestinal stem cells. ILC3 are an important source of IL-22, which has a pivotal function in intestinal homeostasis. Recent studies highlighted the important role of the IL-22-STAT3 axis in controlling tissue damage in the colon. Our previous findings demonstrated that ILC3 are essential for protection of small intestinal epithelial cells after chemotherapy insult but the mechanisms by which ILC3 shield intestinal epithelium remain unknown. Therefore, we hypothesized that ILC3-derived IL-22 is involved in protection of intestinal epithelial cells after chemotherapy-induced damage. To test this hypothesis, we induced intestinal toxicity by administering the cytostatic drug methotrexate (MTX) in IL-22$^{-/-}$ mice and studied epithelial responses. IL-22 deficient mice showed a conserved intestinal architecture and goblet cell numbers after MTX-induced damage that was comparable to WT animals. In contrast to ILC3 deficient mice, IL-22$^{-/-}$ mice expressed higher levels of pSTAT3. Although activation of STAT3 was observed in IL-22$^{-/-}$ mice, a decrease in antimicrobial responses suggested reduced epithelial barrier function. Importantly, analysis of the progenitor compartment of mice exposed to MTX unveiled a role for IL-22 in maintaining stem cells after chemotherapy insult. Collectively these data identify IL-22 as an important factor to preserve the regenerative responses of damaged epithelium.

INTRODUCTION

ILC3 play a pivotal role in coordinating epithelial responses after administration of the cytostatic drug methotrexate (MTX) (Chapter 2). Immediately after intestinal damage, epithelial STAT3 is activated and ILC3 transcribe a set of genes involved in epithelial homeostasis and tissue repair. However, the exact mechanisms by which ILC3 limit tissue damage after chemotherapy insult remain unclear.

ILC3-derived cytokines, including IL-22 and GM-CSF, contribute to the homeostatic cytokine pool that is necessary to maintain epithelial integrity.

In the murine intestine, ILC3 are the main cellular source of IL-22 and directly contribute to bacterial clearance. Studies with the murine enteropathogenic bacteria *Citrobacter rodentium* identified IL-22 as they key cytokine for bacterial containment and epithelial integrity[1,2]. Ligation of the IL-22R expressed by intestinal epithelial cells (IEC) mediates an antimicrobial response by promoting the expression of Reg3β, Reg3γ and S100a proteins[2-4]. Upon colonic damage induced by dextran sulfate sodium (DSS), IL-22R ligation on epithelial cells leads to phosphorylation of STAT3, which translocates to the nucleus to activate genes implicated in proliferation, survival and mucosal integrity[5-7]. While the role of IL-22 in inflammatory bowel disease (IBD) remains controversial, as both pathogenic and protective roles have been described[8,9], in experimental colitis IL-22 has a protective function by promoting proliferation and reducing apoptosis in IEC[10]. In the DSS-induced colitis model, IL-22 directly regulated epithelial STAT3 activation that in turn promoted intestinal wound healing[10].

Conversely, uncontrolled IL-22 production and inappropriate STAT3 activation predisposes to tumorigenesis in the intestinal tract. In humans, *STAT3* genes were identified as susceptibility loci for IBD[7,11,12]. In mice, models using *Helicobacter hepaticus* and the mutagen AOM in Rag1$^{-/-}$ mice unveiled that IL-22 produced by ILC3 was causative for carcinoma progression[13]. Therefore, regulation of the IL-22-STAT3 axis is essential for controlling tissue damage while preventing inflammation and inflammation-related cancer development.

In addition, the effector function of IL-22 on proliferation and cell survival of epithelial cells suggest that ILC3-derived IL-22 could contribute to tissue maintenance and regeneration. In a model of graft versus host disease (GvHD), host-derived radio resistant CCR6^{+}ILC3 were shown to be the source of IL-22, which protected intestinal crypts from allogenic T cell-mediated killing[14]. These findings highlight the important function of IL-22 in maintaining integrity of the differentiated epithelium as well as fitness in the progenitor compartment.

Thus, emerging evidence indicates a pivotal role for IL-22 in epithelial homeostasis and bacterial containment. However little is known with regard to the role of IL-22 in the

pathobiology of small intestinal mucositis and in mucosal healing.

ILC3 secrete large amounts of IL-22 in the small intestine and upregulate *Il22* transcription after MTX-induced damage. Since pSTAT3 was expressed at lower levels in the absence of ILC3, and IL-22 can signal trough STAT3, we investigated the potential role of IL-22 in epithelial activation of STAT3 and its possible function in epithelium protection from chemotherapeutic damage.

Therefore, the aim of this study was to understand whether the mechanisms underlying the protective functions of ILC3 are driven by IL-22. We addressed this question using IL-22 deficient mice exposed to the cytostatic drug MTX and studying epithelial activation, barrier function and fitness of intestinal stem cells.

RESULTS

Intestinal epithelial expression of pSTAT3 in the absence of IL-22

To study the role of IL-22 in STAT3 regulation after acute cytostatic damage, wild type (WT) and IL-22$^{-/-}$ mice were treated with MTX and the small intestinal epithelium was analyzed for STAT3 activation before and after MTX administration.

In contrast to WT mice, IL-22$^{-/-}$ mice exhibited higher levels of pSTAT3 at homeostatic conditions (Fig. 1A). One day after MTX, IL-22$^{-/-}$ and WT mice expressed similar levels of phosphorylated STAT3 but in contrast to WT mice, pSTAT3 expression in IL-22$^{-/-}$ intestinal epithelial cells was preserved four days after MTX administration (Fig. 1A). In line with these results, quantification of pSTAT3 intensity demonstrated the constitutive and stable phosphorylation of epithelial STAT3 in IL-22$^{-/-}$ mice before and after MTX administration (Fig. 1B). Strikingly, although IL-22$^{-/-}$ and WT mice expressed similar levels of pSTAT3 one day after MTX, transcript levels of the downstream target gene *Socs3* in IL-22$^{-/-}$ mice seemed lower than in WT mice (p-value 0.22) (Fig. 1C). Similarly, at day 4 after MTX *Socs3* transcript levels in IL-22$^{-/-}$ remained reduced compared to WT animals (p-value 0.42) despite the higher pSTAT3 expression observed in IL-22 deficient mice (Fig. 1C).

In contrast, transcript levels of the STAT3 target gene *Bcl2l1 (*Bclxl*)*, a pro-survival factor, were similar to WT at day 1 (p-value 0.63) after the last injection and at day 4 after MTX (p-value 0.42) (Fig. 1D).

Although a role for IL-22 in epithelial STAT3 activation has been identified, here we show that epithelial phosphorylation of STAT3 can occur independently of this cytokine suggesting that additional signals are driving its activation.

The higher pSTAT3 levels observed in IL-22$^{-/-}$ mice at homeostatic conditions and at the latest time points indicate a deregulated STAT3 activation and suggests a pre-inflammatory state in IL-22$^{-/-}$ mice.

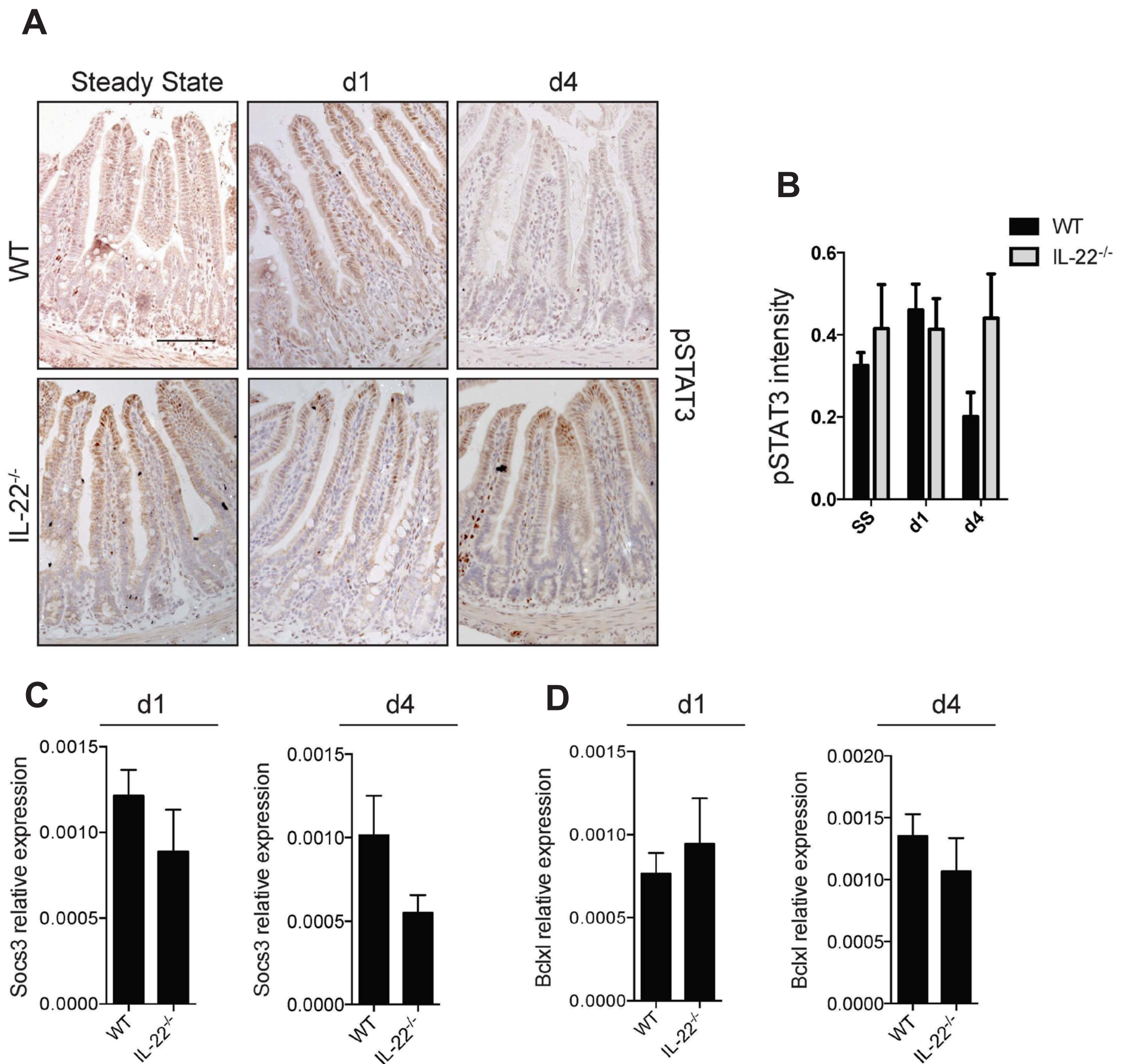

Figure 1. Constitutive expression of pSTAT3 in IL-22 deficient mice. (A) Immunohistochemistry for pSTAT3 in ileal sections of WT and IL-22$^{-/-}$ mice at steady state (SS) and at day 1 and 4 after the last MTX injection. (B) Quantification of pSTAT3 intensity in intestinal epithelial cells in WT and IL-22$^{-/-}$ mice measured with semi-automated software. (C) Transcript analysis of the STAT3 downstream target gene *Socs3* from ileal samples of WT and IL-22$^{-/-}$ mice at day 1 and day 4 after MTX administration. (D) Relative expression of *Bcl2l1* (Bclxl) in ileum of WT and IL-22 deficient mice at day 1 and 4 after MTX-induced damage. Scale bar: 50 um; (n=3-4 per group).

Intestinal pathology in IL-22 deficient mice exposed to MTX

Intestinal damage was evident one day after MTX administration in IL-22$^{-/-}$ and WT mice and damage-induced morphological changes remained visible at day 4 after MTX treatment (Fig. 2A). Intestinal damage in IL-22 deficient mice was similar to WT mice and consisted of increased villus atrophy, decreased epithelial thickness and increased lamina propria infiltration. In contrast to WT mice, IL-22$^{-/-}$ mice did not lose weight during MTX treatment (Fig. 2B). The kinetics of the pathology in IL-22 deficient mice resembled those observed in MTX-treated WT mice (Fig 2C and Chapter 2), with a significant increase of intestinal damage one day after the last MTX injection and stabilized levels by day 4 (Fig. 2C).

Detailed examination of small intestinal sections of duodenum, jejunum and ileum revealed comparable intestinal damage in IL-22$^{-/-}$ and WT mice after MTX treatment, and only slightly higher pathology was observed in duodenum of IL-22$^{-/-}$ mice at day 1 after cytostatic insult (p-value 0.22) (Fig. 2D) These results are in line with previous reports that identified the proximal small intestine as the most affected region upon cytostatic treatment[15]. Specific analysis of the crypt compartment showed comparable pathology in WT and IL-22$^{-/-}$ mice, with a significant increase of damage at day 1 that consisted of crypt abscesses, epithelial flattening and some crypt loss (Fig. 2E). In accordance with these data, expression of the pro-apoptotic gene *Bax* in small intestinal samples from IL-22$^{-/-}$ mice was comparable to WT animals. (Fig. 2F)

Overall, intestinal architecture and morphology of IL-22 deficient mice was comparable to WT animals after administration of the cytostatic drug MTX.

Epithelial barrier after MTX-induced damage in the absence of IL-22

To determine whether the preservation of epithelial barrier functions after chemotherapy insult is altered in the absence of IL-22, antimicrobial response and mucus producing goblet cells were analyzed from small intestinal samples. Transcriptional analysis of total RNA from ileum of IL-22$^{-/-}$ mice revealed lower levels of the antimicrobial peptides *Reg3*γ and *Defensin α5* (Fig. 3A).

Decreased mRNA levels were found at steady state and remained unchanged after MTX administration. In contrast, in WT mice, transcript levels of *Reg3γ* rapidly decreased one day after MTX administration and subsequently recovered by day 4. *Defensin α5* transcript levels progressively decreased after MTX, both at day 1 and day 4. Despite the aberrant antimicrobial response. goblet cells were present in IL-22$^{-/-}$ mice and no alterations were observed with regard to the numbers, distribution or morphology of the mucus producing cells (Fig. 3B and C).

These data show the dependency for antimicrobial responses on IL-22 and the dispensable role of IL-22 in the generation and maintenance of mucus-producing goblet cells.

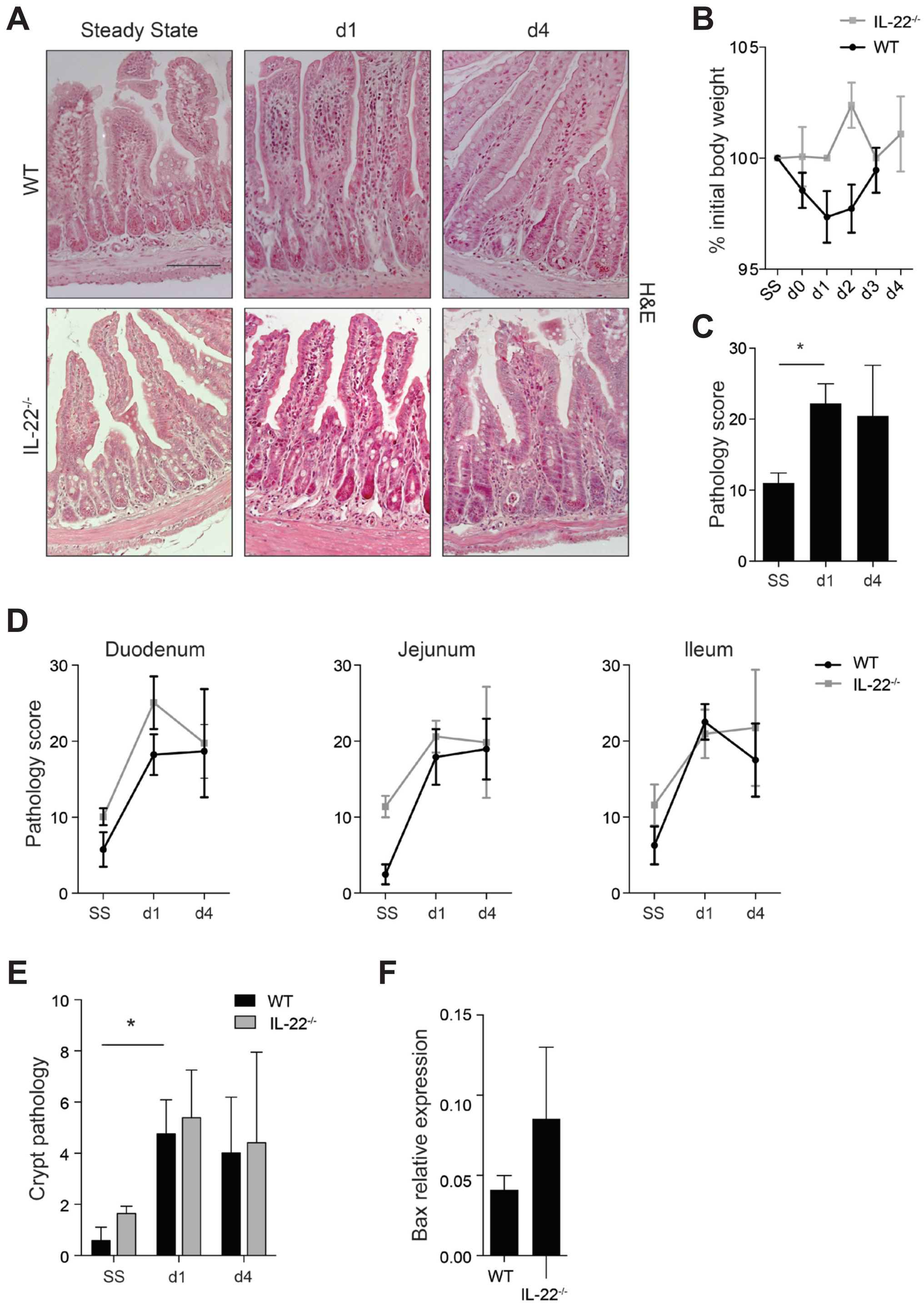

Figure 2. Conserved pathology in MTX-treated IL-22$^{-/-}$ mice. (A) Representative H&E staining of WT and IL-22$^{-/-}$ after MTX-induced damage at the indicated time points. (B) Body weight of WT and IL-22$^{-/-}$ mice over the course of the MTX treatment relative to the initial body weight. (C) Overall intestinal pathology in IL-22 deficient mice at steady state conditions (SS) and after MTX-induced damage. (D). Pathology score of duodenum, jejunum and ileum of WT mice compared to IL-22$^{-/-}$ mice during the course at steady state, and day 1 and 4 after MTX. (E) Analysis of the crypt compartment in IL-22 deficient mice. (F) *Bax* transcript levels of ileums of IL-22$^{-/-}$ and WT mice at day 1 after MTX. *, P<0.05; Scale bar: 50 um ; (n=3-4 per group).

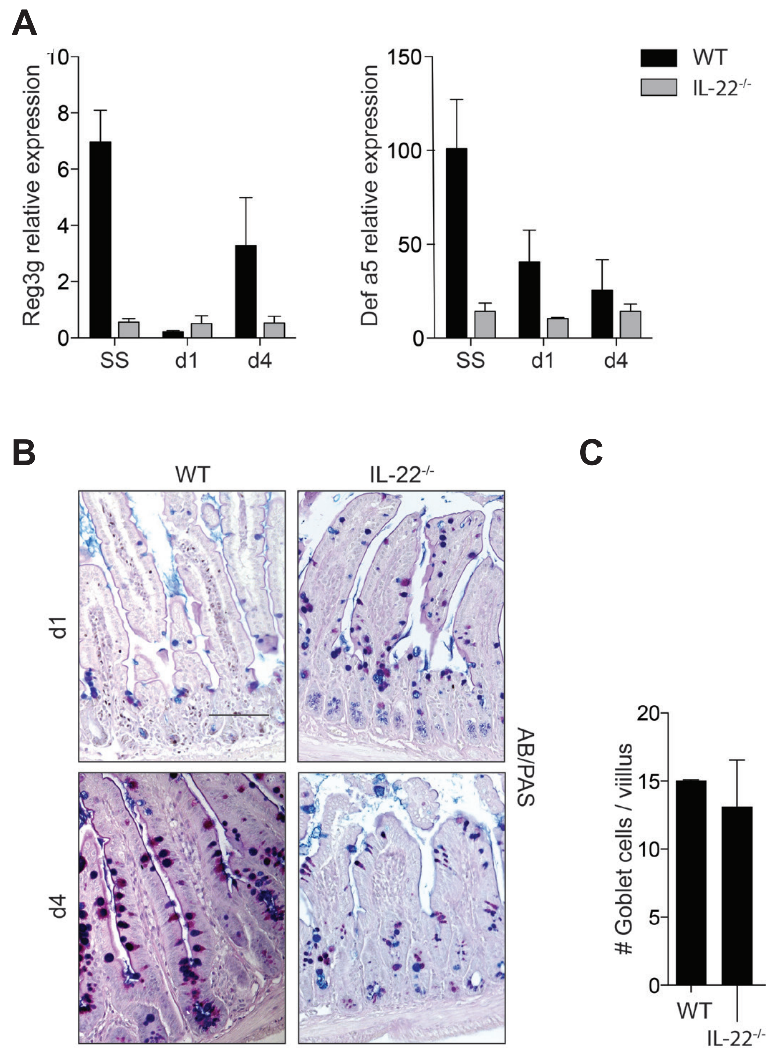

Figure 3. Decreased antimicrobial response in IL-22 deficient mice. (A) Transcriptional analysis of ileum of WT and IL-22$^{-/-}$ mice at steady state, day 1 and 4 after MTX administration. Relative expression of the antimicrobial peptides Reg3γ and Defensin-α5 is shown. (B) Alcian blue & PAS staining for goblet cells in WT and IL-22 deficient mice at day 1 and 4 after MTX-induced damage. (C) Goblet cell counting in ileal sections from WT and IL-22 deficient mice four days after MTX-administration. .

IL-22 is necessary for stem cell maintenance after MTX-induced damage

Cryptopatch ILC3 are strategically located in the vicinity of intestinal crypts, suggesting a possible crosstalk between epithelial cells and ILC3. Indeed, in the absence of ILC3, the frequency of intestinal stem cells decreased after MTX-induced damage (Chapter 2). To determine whether IL-22 contributed to stem cells maintenance after cytostatic insult, we neutralized IL-22 in MTX-treated Lgr5-GFP mice. We used neutralizing antibodies to block IL-22 prior to MTX exposure and during drug administration (Fig. 4A). Analysis of purified crypts from Lgr5-GFP mice at day 4 after MTX revealed a decreased in the frequency of EpCAM^{+} GFP^{+} stem cells in small intestine of IL-22 neutralized animals (Fig. 4B). This reduction was approximately 2 fold in duodenum and ileum, whereas the frequency in jejunum was unaltered (Fig 4C). These results revealed the role of IL-22 in maintenance of stem cell numbers during MTX-induced damage.

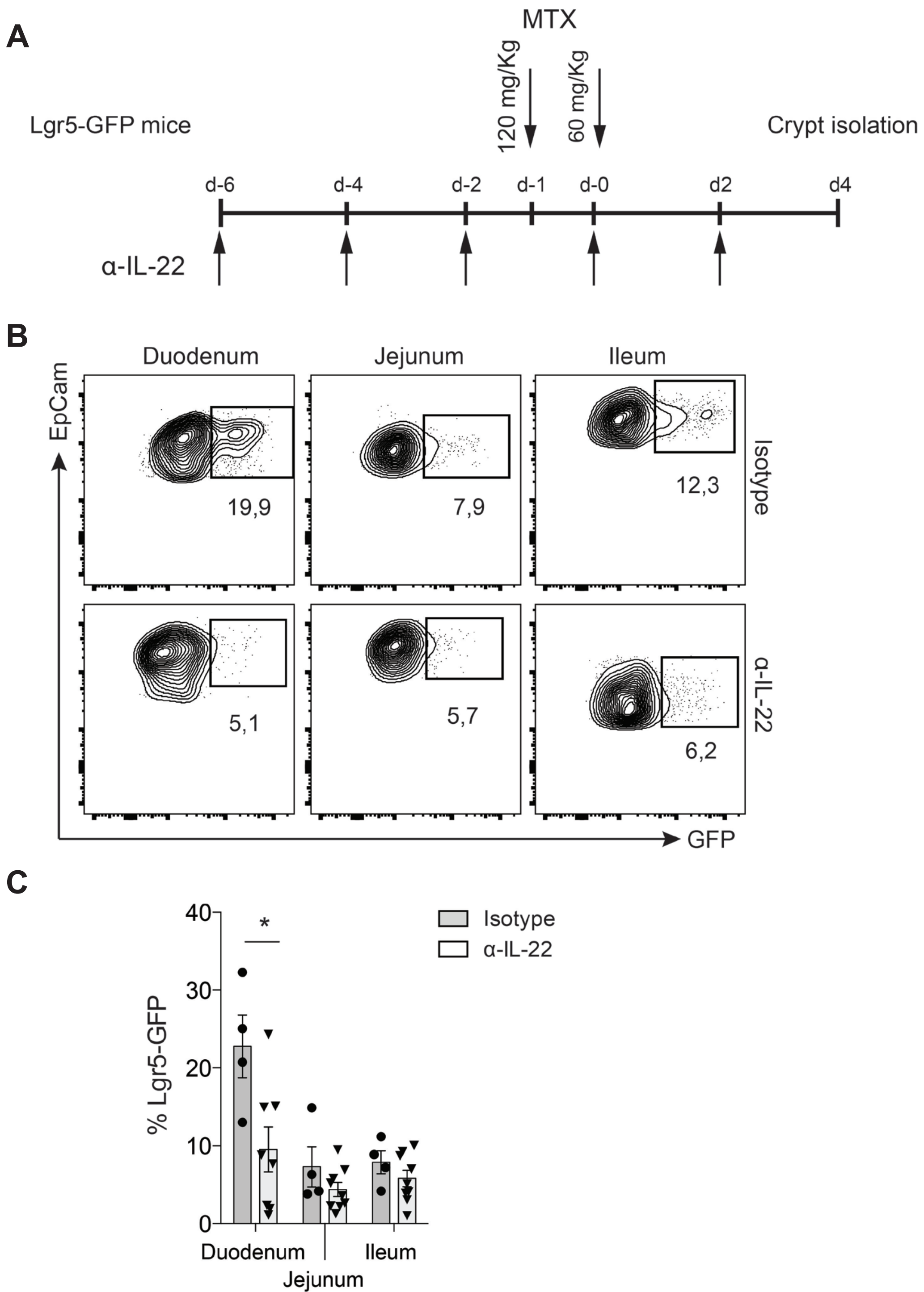

Figure 4. IL-22 protects intestinal stem cells from chemotherapy-induced damage. (A) Setup of IL-22 blocking experiments in Lgr5-GFP reporter mice treated with MTX. IL-22 neutralizing or isotype control antibodies were administered i.p. every other day before and during MTX administration. Four days after the last MTX injection, intestinal crypts were isolated. (B) Representative plots of single cell suspensions of crypts of isotype and α-IL22 treated animals. Percentage of GFP^{+} cells in duodenum, jejunum and ileum at day 4 after MTX are shown. (C) Frequency of Lgr5-GFP^{+} cells within the EpCAM population isolated from crypts of IL-22 neutralized animals and isotype controls. *, P<0.05; (n=6-7 per group).

DISCUSSION

Damage to mucosal surfaces can set the stage for pathological inflammation and needs to be repaired in a rapid and robust manner. Cytostatic drugs such as those used during chemotherapy as part of anti-cancer treatments, damage the mucosal epithelium and are causative for the development of mucositis. Mucositis occurs in approximately half of the patients receiving standard chemo or radiotherapy, and in almost all patients receiving high dose of chemotherapy as conditioning for HSC transplantation[16,17]. A multistep model of the pathobiology of mucositis has been proposed[18]. During the initiation phase, DNA breaks and reactive oxygen species (ROS) are generated causing cell death and injury. These events lead to a second phase of primary damage responses, in which the NF-kB pathway is activated and pro-inflammatory cytokines are secreted. During the third phase, pro-inflammatory signals including TNFα and IL1β amplify the NF-kB pathway in a positive-feedback loop. As a result of the inflammation, the ulcerative phase begins. This phase is characterized by the loss of epithelial integrity and subsequent bacterial translocation, inducing monocyte infiltration and further inflammation. Finally, healing occurs after cessation of the damaging agent through regeneration of the epithelium[18,19]. Although the pathobiology of mucositis has been extensively studied, the exact mechanisms behind mucosal wound healing and maintenance are incompletely understood.

The STAT3 pathway has been linked to mucosal wound healing and defects in activation of this transcription factor lead to aberrant epithelial responses in colitis models[5-7]. We have shown that during cytostatic-induced damage, intestinal ILC3 were required for phosphorylation of epithelial STAT3 at the peak of damage (Chapter 2). At this time point, ILC3 secreted an array of cytokines that are thought to activate the STAT3 pathway and are known to be involved in tissue homeostasis and repair. Lack of ILC3 during cytostatic treatment led to decreased expression of epithelial pSTAT3 and correlated with increased intestinal damage and loss of stem cells. However, the mechanisms by which ILC3 protected epithelium remained unknown. In the murine intestines, ILC3 represent the major source of IL-22[20]. Although IL-22 can also signal through activation of STAT1 and STAT5 pathways[21,22], STAT3 phosphorylation appears to be the primary mediator of IL-22 signaling. Because ILC3 transcribed high levels of IL-22, we next investigated whether IL-22 linked the phenotype observed in MTX-treated mice to the absence of ILC3. To address this question, we measured STAT3 phosphorylation and analyzed intestinal integrity and fitness in $IL22^{-/-}$ mice exposed to MTX. Interestingly, the kinetics of STAT3 phosphorylation in IL-22 deficient mice were different from WT animals. While WT animals exhibited a peak of pSTAT3 expression at day 1 after MTX, pSTAT3 in

IL-22$^{-/-}$ remained at constant levels during the entire analysis. Due to the pivotal role of IL-22 in epithelial homeostasis, it is likely that compensatory mechanisms are acting in IL-22$^{-/-}$ mice leading to STAT3 activation to maintain epithelial integrity. Moreover, the lack of IL-22 can lead to subclinically inflamed intestines and an increase in inflammatory cytokines that activate STAT3.

Alternatively, impaired function of negative regulators of STAT3 such as Socs3[23], could cause an impaired hyperactivation of this pathway. Supporting this concept, *Socs3* transcripts seemed lower in IL-22$^{-/-}$ mice compared to WT animals. In contrast, analysis of *Bcl2l1* transcripts appeared to be similar to WT mice at day 1 and 4 after MTX treatment. Of note, the STAT3 target gene *Bcl2l1 (*Bclxl*)* is a pro-survival factor[24,25], which may be induced as a result of the pre-inflammatory state suffered by IL-22$^{-/-}$ mice. Therefore, further experiments are needed to understand the activated state of STAT3 pathway in IL-22$^{-/-}$ mice, for instance by analyzing additional downstream target genes and negative regulators. Nevertheless, these results suggest that additional cytokines are involved in the activation of STAT3 in the absence of IL-22.

In agreement with the prolonged phosphorylation of epithelial STAT3, IL-22 deficient mice showed no significant differences in MTX-induced pathology when compared to WT animals. In addition, goblet cells necessary for the generation of the mucus-based glycocalix that protects the intestinal epithelium[26,27], were conserved in the absence of IL-22 and distributed throughout the villi similar to WT mice. Recent findings highlighted the role of IL-22 in tissue healing by promoting mucus production[27]. Our findings showed that IL-22 was dispensable for goblet cell maintenance after acute intestinal damage. Nevertheless, pSTAT3 was constitutively active in IL-22$^{-/-}$ mice, suggesting that STAT3 rather than IL-22 might control goblet cell maintenance. Therefore, it is necessary to further analyze STAT3-activating cytokines present at baseline levels in IL-22$^{-/-}$ mice, such as the well-studied IL-6 or IL-11[7,28]. These studies could reveal compensatory mechanism that may be acting in the absence of IL-22.

A role for epithelial STAT3 activation in antimicrobial responses has also been described[29,30]. Epithelial cell-specific STAT3 was essential for epithelial protection from *C. rodentium* infection by regulating AMP expression[29]. Interestingly, in our MTX model, antimicrobial responses in IL-22 deficient mice were reduced and remained at low levels despite the fact that STAT3 appeared to be activated at the same extent as in WT animals. These findings indicate that even in the presence of STAT3, IL-22 is a master regulator of AMP production.

During cytostatic treatment highly proliferating stem cells located at the base of

the crypts are targeted ([31] and Chapter 2). Therefore the intestinal crypts represent a very vulnerable compartment that needs to be maintained to guarantee epithelial regeneration and integrity. A recent studied reported the protective role of IL-22 in the crypt compartment in a GvHD model and showed IL-22 receptor expression by Lgr5$^+$ stem cells[14]. We revealed that ILC3 were required for stem cell maintenance after MTX-induced damage (Chapter 2). In the current study we show the crucial role of IL-22 in stem cell maintenance after chemotherapy insult. In the absence of IL-22, the frequency of Lgr5$^+$ stem cells is decreased and Lgr5 transcript levels are reduced. The reduction of Lgr5$^+$ stem cells in the presence of IL-22 neutralizing antibody resembles the results observed in Lgr5-GFP chimeric mice (Chapter 2). Moreover, Rag1$^{-/-}$ mice treated with anti-thy1 antibody but not the littermate controls exhibited a reduction in IL-22 production. Therefore the findings of this chapter indicate that ILC3 rather than T cells are the main source of IL-22 contributing to the maintenance of intestinal epithelial stem cells after MTX-induce damage. Nevertheless, whether STAT3 plays a role in the loss of stem cells after MTX-induced intestinal damage remains unknown. For instance, pSTAT3 blocking strategies in Lgr5-GFP reporter mice might help to fully understand the role of this pathway in stem cell maintenance.

In conclusion, IL-22$^{-/-}$ mice maintained expression of epithelial pSTAT3 during the course of the MTX treatment, which correlated with a normal pathology and goblet cell numbers and distribution. The constitutive expression of pSTAT3 suggests that other cytokines contribute to the activation of this pathway in the absence of IL-22 and suggest a possible pre-inflammatory state of IL-22 deficient mice. Nevertheless, antimicrobial responses were reduced in the absence of IL-22 indicating reduced fitness of the epithelial barrier. Importantly, we showed that IL-22 contributed to stem cell maintenance after cytostatic insult. Therefore, this study identifies IL-22 as an essential factor for stem cell protection against MTX-induced damage.

MATERIALS AND METHODS

Mice. C57BL/6, IL-22$^{-/-}$ (W. Ouyang, Genentech) and Lgr5-GFP mice were housed in the animal facility of the Erasmus University Medical Center. Animal experiments were approved by the relevant authorities and procedures were performed in accordance with institutional guidelines. Age and gender-matched littermates were used whenever possible.

MTX model. 8-12 weeks mice were injected i.p. with 120mg/Kg MTX (PCH) at day-1 and with 60mg/Kg at day 0. Body weight was daily monitored and tissues were collected at day 1 and day 4 after the last MTX injection.

Histology. Small intestine sections (5 mm) were fixed in 4% PFA and embedded in paraffin. Four-μm sections were deparaffinized and stained with hematoxylin (Vector Laboratories) and eosin (Sigma-Aldrich). For detection of goblet cells sections were stained with Alcian-Blue (Fluka) /PAS (Leica). For P-STAT3 detection endogenous peroxidases were block and antigen retrieval was achieved by microwave treatment in citrate buffer (10mM, pH 6.0). Prior to staining FC receptors were block in blocking solution consisting of 10% mouse serum and 10% goat serum. Tissue sections were incubated overnight at 4°C with rabbit P-STAT3 primary antibody (D3A7, Cell signaling). Immunoreactions were detected using biotinylated goat-anti-rabbit (Vector Laboratories) and incubated with the Vectastin ABC Elite Kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were counterstained with hematoxylin. For counting of positive cells, 5-10 villus were analyzed per section per mouse and n=3-5 mice were used per experiment. Intensity of pSTAT3 staining on sections was quantified using a HistoQuest software (TissueGnostics)

Crypt isolation. Isolation of intestinal crypts was performed as previously described[32]. Briefly, isolated small intestines were opened longitudinally and washed with cold PBS. The tissue was cut into 5 mm pieces and consequently washed by mechanical pipetting with cold PBS until supernatant was clear. The tissue was incubated in EDTA (2mM) in PBS at 4°C for 30 min. The tissues were then washed several times with cold PBS and suspended by vigorous pipetting, removing each time the supernatant containing the villous fraction. The sediment enriched in crypts was passed through a 70µm cell strainer and centrifuge at 600 rpm for 3 min to separate the crypts from the single cells in suspension. The crypts were incubated with 1 ml of TrypLE Express (Gibco) at 37°C for 10-15 min until crypt dissociation was observed. Single cell suspension was stained with conjugated antibodies (Table 2) and EpCAM^{+} crypt cells were analyzed for the expression of GFP by flow cytometry (FACS CantoII, BD)

PCR. RNA was extracted using the RNA-XS kit (Machery Nagel) followed by reverse-transcription with random hexamer primers. For quantitative PCR, a Neviti Thermal Cycler (Applied Biosystems) and DyNAmo Flash SYBR Green qPCR kit (Finnzymes) were used, with the addition of MgCl2 to a final concentration of 4 mM. All reactions were done in duplicate and are normalized to the expression of GAPDH. Relative expression was calculated by the cycling threshold (CT) method as $2^{-\Delta t}$.
The primers sequences can be found in Table 1

Statistical analysis. Samples were analyzed using unpaired Mann-Whitney's test. P values < 0.05 were considered significant.

Table 1. Primer sequences

Gene name	Forward primer	Reverse primer
mGapdh	TCAACGGCACAGTCAAG	GCTCCACCCTTCAAGTG
mSocs3	GGAGCCCCTTTGTAGACT	CGGGAAACTTGCTGTG
mBcl2l1(Bclxl)	CGTGGCCTTTTTCTCC	GGCTGCTGCATTGTTC
mBax	AAGGCCCTGTGCACTAA	GAGGCGGTGAGGACTC
mLgr5	GGGGGTGTGAGAATGTCT	AGGGCCTTCAGGTCTTC
mReg3γ	CCATCTTCACGTAGCAGC	CAAGATGTCCTGAGGGC
mDefensin α5	CCACAAAACAGATGAAGAGAC	TCTTTTGCAGCCTCTTATTC

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Patricia Aparicio-Domingo, Ferry Cornelissen, Natalie Papazian,
Monica Romera-Hernandez, Julien J. Karrich and Tom Cupedo[1]

Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands

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CHAPTER 4

Activation of human ILC3: A role for Natural Cytotoxicity Receptors

SUMMARY

Group 3 innate lymphoid cells (ILC3) play a pivotal role in homeostasis, tissue repair and early innate defense in mucosal tissues. An important ILC3 effector cytokine is IL-22. *In vitro*, IL-22 secretion by human ILC3 is induced by combinations of cytokines and TLR ligands, which are all soluble factors. However, *in vivo* ILC3 activation will be a local process occurring at the site of damage and we thus hypothesize that local cell surface ligands will drive activation of ILC3. To identify receptors for cell surface molecules capable of activating ILC3 we analyzed the ability of Natural cytotoxicity receptors (NCR), NKp30, NKp44 and NKp46 to induce IL-22 production. Using agonist antibodies against individual NCRs in combination with the TLR2 ligand Pam3Cys we show that NCRs are co-stimulatory receptors on ILC3 in the context of TLR co-stimulation. In addition, NCR-ligands expressed on intestinal epithelial cell lines, are capable of activating ILC3 in combination with the TLR2 ligand Pam3Cys. As NCR ligands are stress-induced molecules on normal cells, NCR ligation could allow for spatial control of ILC3 activation in response to intestinal damage.

INTRODUCTION

In the last few years, IL-22 producing ILC3 have emerged as important players in early immunity against enteric infections[1,2]. Activation and cytokine production of ILC3 is regulated by cytokines produced by dendritic cells. IL-23 and IL-1β are potent activators of ILC3 and are essential to ensure proper ILC3 activation after infection, leading to IL-22 mediated epithelial activation and innate immune responses[1,3-5]. In addition, ILC3 can directly sense environmental signals by TLRs and the aryl hydrocarbon receptor (AHR) [6,7].

However, this model of ILC3 activation does not allow for spatially controlled activation of ILC3 at sites of tissue damage. Controlling ILC3 activation is of relevance as evidence is accumulating that uncontrolled or deregulated production of IL-22 is sufficient to induce malignant transformation of the intestinal epithelium[8]. On the contrary, lack of IL-22 exacerbates intestinal bacterial infections[1,9] and compromises maintenance of intestinal stem cells[10] (Chapter 3 of this thesis). Thus, additional mechanisms must be involved to ensure the local function of ILC3 and prevent uncontrolled activation by soluble factors.

Production of IL-22 by ILC3 was originally reported to be induced by ligation of the IL-23 receptor[9]. However, experiments using expanded or cloned human tonsilar ILC3 indicated that after expansion with IL-2 and feeder cells, IL-23 is not sufficient to activate IL-22 production and that an additional co-stimulatory signal is needed[11]. Co-stimulation can consist of either a second cytokine such as IL-1β or a Toll-like receptor ligand[11-13]. Similarly, microbial products in the absence of cytokine stimulation also lead to suboptimal ILC3 activation[11]. This suggests that ILC3, which are strategically located at the mucosal surfaces, have the capacity to sense microbial patterns and thus changes in microbial composition, but will only respond to these signals in the context of the correct environmental or inflammatory cues.

Interestingly, mucosal ILC3 can express the natural cytotoxicity receptors (NCRs), NKp30, NKp40 and NKp46[14,15]. NCRs were originally described as activating receptors expressed on NK cells with the capacity to trigger robust cytotoxic activity[16]. While humans can express the three NCRs, only Nkp46 is conserved in mice[17]. NKp44 ligation on human ILC3 leads to TNFα production and synergizes with other cytokines to induce IL-22 secretion[18].

NCRs are thought to recognize ligands expressed on malignant and stressed cells, nevertheless, only very few NCR ligands have been identified. Some of the NCR ligands discovered are of viral origin[19] and bind to one or more NCRs triggering NK cell function[20]. Ligands for NKp30[21] and NKp44[22] are also expressed by tumor cells and can trigger NK cell activity. These include a membrane bound form of MLL5, a ligand for NKp44, and B7H6, a ligand for NKp30[23] [21]. B7H6 can also be expressed on non-transformed cells upon stimulation with TLR ligands or pro-inflammatory cytokines[21,24].

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Thus, NCR activation of ILC3 by membrane bound ligands may represent a mechanism that in contrast to soluble factors such as cytokines and TLR ligands, could control activation of ILC3 in a local manner in response to stress or damage-induced ligands.

This chapter describes the mechanisms of activation of human ILC3 and identifies the NCR as co-activating receptors on human ILC3 in the presence of TLR2 ligands and independently of cytokines. In addition, we demonstrated that B7H6 expressing epithelial cells co-activate human ILC3 and that its expression can be modulated by damage induced by cytostatic drugs.

RESULTS

Activation of ILC3 by soluble cytokines and TLR ligands

Although recent findings have demonstrated the capacity of ILC3 to respond to cytokine stimuli and TLR ligands, it is still unclear whether a single signal is sufficient to fully activate ILC3 to produce cytokines. To address this question, we isolated ILC3 from pediatric tonsils and performed various *in vitro* stimulations with either a single signal or a combination of signals. Analysis of supernatants of cultured ILC3 stimulated with IL-23 alone revealed no significant induction of IL-22 protein compared to cultures without stimulation (Fig. 1A). In contrast, stimulation of ILC3 with the combination of IL-23 and IL-1β fully activated ILC3 as measured by the higher concentrations of IL-22 protein in the supernatant of the cultures (Fig. 1A). Transcript analysis of culture ILC3 did show higher levels of *Il22* mRNA in ILC3 cultured in the presence of IL-23 compared to non-stimulated ILC3, nevertheless stimulation of ILC3 with IL-23 and IL-1β greatly increased *Il22* transcript levels by ILC3 (Fig. 1B). Similarly, stimulation of ILC3 with the TLR2 ligand Pam3Cys (P3C) in combination with IL-23 promoted IL-22 production by ILC3, but either P3C or IL-23 alone failed to do so (Fig. 1C). We further validated these results performing intracellular staining on stimulated ILC3. Indeed, stimulation with IL-23 and P3C together promoted full activation of ILC3 as seen by the higher frequency of IL-22^{+} ILC3 (15.5%) as compared to ILC3 stimulated with IL-23 (6.6%) or P3C (0.9%) alone (Fig. 1D).

To further gain knowledge on the co-activating functions of additional TLR ligands on ILC3, we performed in vitro stimulations of ILC3 with several agonist TLR ligands. As a second signal, we used IL-23 as this appeared to be an efficient co-activator of ILC3. As observed before, stimulation with the TLR2 ligand P3C in combination with IL-23 induced robust IL-22 protein production by ILC3 (Fig. 1E). Although co-stimulation of ILC3 with IL-23 and the TLR4 ligand lipopolysaccharide (LPS) promoted some IL-22 production, lower levels were observed as compared to co-stimulation with P3C. Stimulation with Poly I:C and CpG, the synthetic ligands of TLR3 and TLR9 respectively, had only a minor effect on

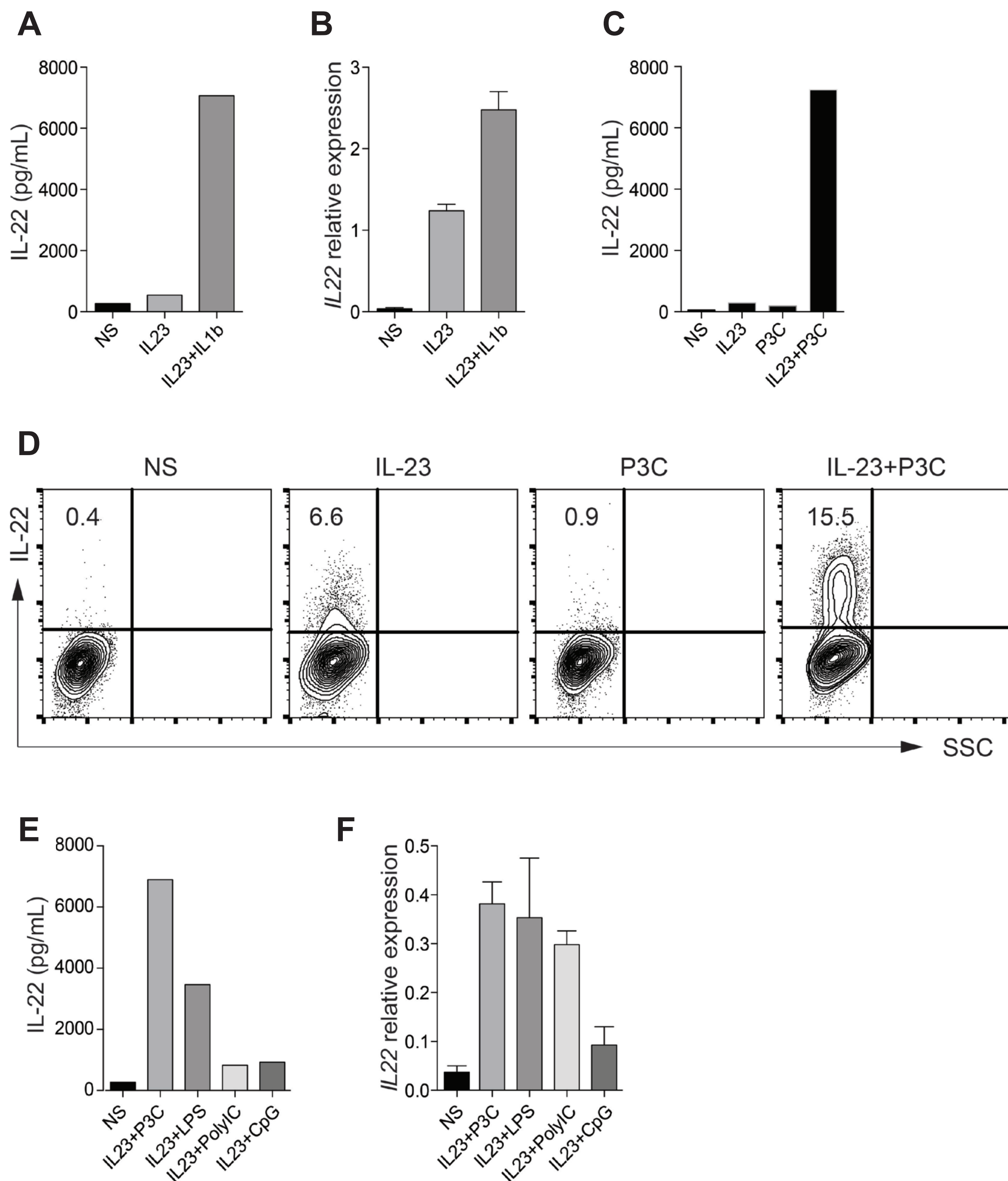

Figure 1. Activation of ILC3 by cytokines and TLRs. (A) IL-22 protein levels of cultures of tonsilar NCR⁺ ILC3 stimulated for 4 days with IL-23 alone or combined with IL-1β, compared to not stimulated NCR⁺ ILC3 (NS) measured by ELISA. (B) *IL-22* transcripts levels of NCR⁺ILC3 stimulated with IL-23 for 4 days in the presence or absence of IL-1β. (C) IL-22 protein levels measured in cultures of tonsilar NCR⁺ ILC3 stimulated for 4 days with IL-23, Pam3Cys (P3C) or the combination of both (D) IL-22 intracellular staining of NCR⁺ILC3 cultured for 4 days in the presence of IL-23, P3C or the combination of both. (E) IL-22 protein secretion of NCR⁺ ILC3 stimulated for 2 days with IL-23 combined with the synthetic ligands for TLR2, 4, 3 and 9, P3C, LPS, Poly I:C and CpG respectively. (F) mRNA levels of *IL-22* in NCR⁺ILC3 stimulated with IL-23 in the presence of various TLR ligands.

IL-22 production by tonsil derived ILC3. (Fig.1E). Transcription of *Il22* was also detected in ILC3 stimulated with TLR ligands (Fig. 1F). *Il22* transcripts were detected in ILC3 stimulated with P3C, LPS and Poly I:C in combination with IL-23, but only a small increase in IL-22 transcription was found in the presence of CpG as compared to non-stimulated ILC3 (Fig. 1F). Although co-activation with different TLRs drove *Il22* transcription by ILC3, co-stimulation with P3C appeared to be the strongest microbial co-activator in these experiments.

These data demonstrated that one signal is sub-optimal to drive full ILC3 activation and cytokine protein production and that for full activation of ILC3 a second signal is needed.

NCR are co-activating receptors on human ILC3

The functional role of the NCRs expressed by ILC3 is still poorly understood. Recent studies showed that ligation of NKp44 in combination with IL-23 triggered TNFα production, whereas IL-22 production was mainly driven by cytokine stimulation[18]. Since TLR2 can also functions as a co-activating factor for ILC3, and TLR ligand are likely present in the intestinal microenvironment, we next explored the ability of human ILC3 to be activated through NCRs in the presence of P3C. To address this question, NCR$^+$ ILC3 were isolated from human pediatric tonsils and activated with plate-bound agonist antibodies against NKp30, NKp44 and NKp46 in combination with the synthetic TLR2 ligand Pam3Cys (Fig. 2A-I). Ligation of either one of the NCRs in combination with P3C resulted in increased production of TNFα (Fig 2A-C), in line with previously published data on cytokine co-stimulation[18].

In contrast to previous work that showed that co-activation of ILC3 through cytokines and NCRs rather than NCR ligation alone induced IL-22 production, our experiments showed that engagement of individual NCRs in combination with P3C induced high levels of IL-22 (Fig. 2D-F). Furthermore, GM-CSF protein was also produced by ILC3 in response to NCR/TLR2 co-stimulation. (Fig. 2G-I). When combined with cytokines NKp44 is the main activating NCR expressed by ILC3[18]. Our results revealed the co-activating role of NKp30, NKp44 and NKp46 expressed on ILC3 in the presence of TLR ligands. Since NCR ligands are considered to be damage or stress-induced ligands, this could mimic a situation in which bacterial translocation occurs in the context of tissue damage.

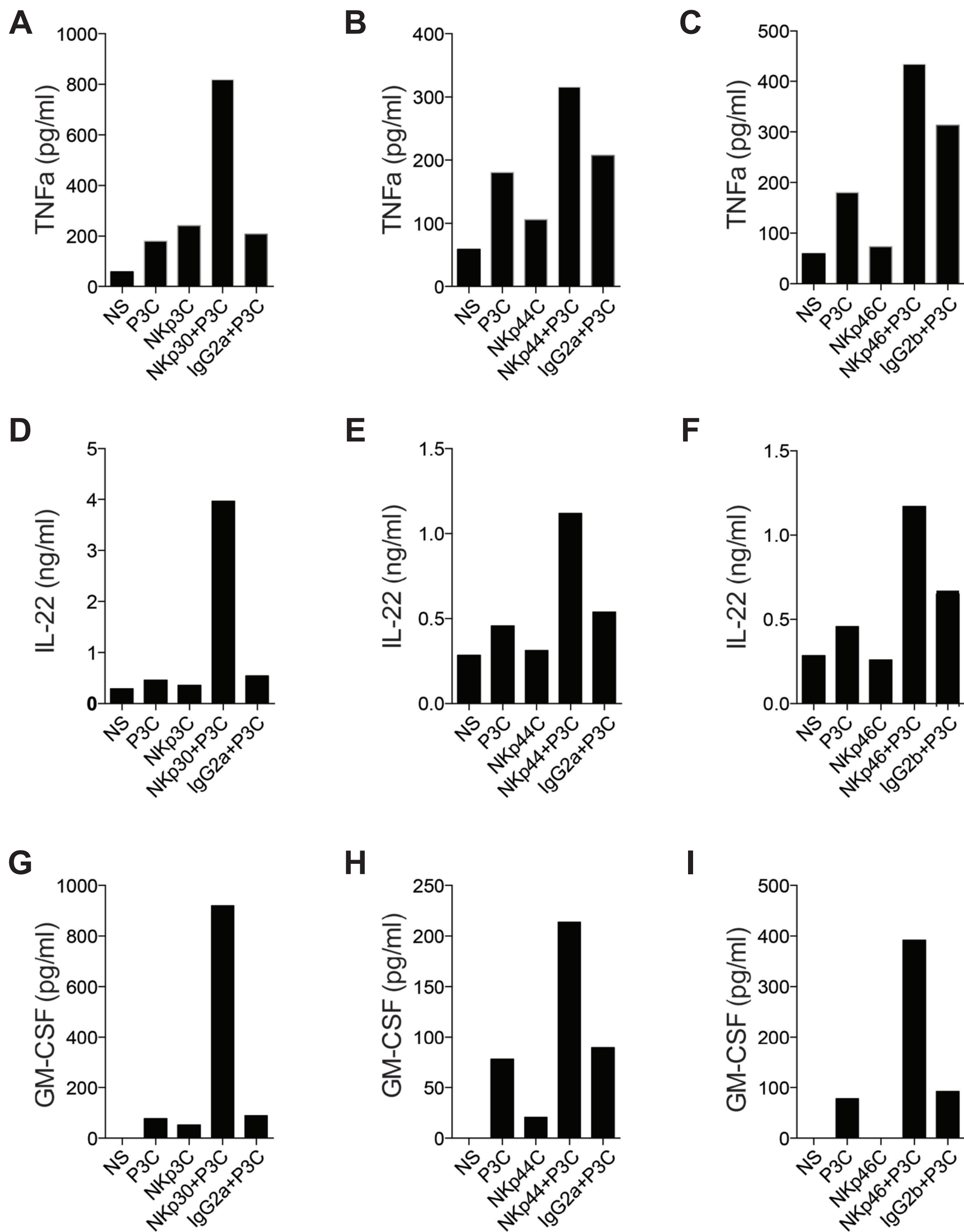

Figure 2. NCR are co-activating receptors in human ILC3. (A-I) Protein levels in supernatants of tonsilar NCR$^+$ ILC3 cultured for 4 days with the TLR2 ligand Pam3Cys (P3C) and NCR (α-NKp30, α-NKp44 and α-NKp46)-agonist antibodies as measured by ELISA. Isotype controls for NKp30, NKp44 (IgG2a) and NKp46 (IgG2b) were used in combination with P3C. (A-C) TNFα protein levels secreted to culture supernatant. (D-F) IL-22 secretion of NCR$^+$ ILC3 (G-I) GM-CSF protein levels of NCR$^-$ stimulated ILC3 in the presence of P3C. Data are representative of three independent experiments

Intestinal epithelial cell contact co-activates human ILC3

To study human ILC3 activation in more physiological conditions, we established co-cultures of primary ILC3 and intestinal epithelial cell lines. ILC3 from pediatric tonsils were cultured together with the epithelial cell line Caco-2 in the presence or absence of P3C (Fig. 3A). Co-culture of ILC3 and epithelial cell lines did not promote IL-22 production whereas addition of P3C to the co-culture system greatly increased IL-22 levels (Fig. 3A). To identify whether the epithelial-derived co-activating signal was a soluble or a membrane bound factor, co-cultures were established using a transwell insert that prevented cell-cell contact between ILC3 and Caco-2 cells (Fig. 3B). Analysis of the transwell co-culture of ILC3 and Caco-2 cells showed that indeed direct contact between ILC3 and epithelial cells was necessary to fully activate ILC3 as reduced *Il22* transcript levels were found in the presence or absence of P3C, indicating that epithelial-derived activating signal is a cell-bound factor. In contrast, stimulation with the soluble cytokines IL-23 and IL-1β promoted *Il22* transcription by ILC3 (Fig. 3B) confirming the requirement of two co-activating signals for optimal cytokine production by ILC3. Similarly, co-culture of ILC3 with the epithelial cell line LoVo led to IL-22 production, that was cell-contact dependent as shown by the absence of IL-22 protein in the transwell co-cultures (Fig. 3C). In line with these results, supernatant from co-cultures of ILC3 and Caco-2 in the presence of P3C promoted only very little production of IL-22 when added to ILC3 in the presence of a TLR2 ligand (Fig. 3D). These data reveal that the epithelial-derived co-activating signal for ILC3 is a cell-bound factor and that in this context ILC3 activation requires cell-cell contact with ligand-expressing epithelial cells.

Since epithelial cells also express TLR2, we next investigated whether P3C in the co-culture was acting on ILC3 or on the epithelium. To study the effect of P3C on epithelial cells, TLR2 on epithelial cells was neutralized by a blocking antibody before addition of ILC3 (Fig. 3F, condition a) or simultaneously with ILC3 and P3C administration (Fig. 3F, condition b). Blocking TLR2 signaling on epithelial cell lines only, did not influence IL-22 production by ILC3 compared to co-culture in the presence of P3C, whereas TLR2 blocking on both epithelial cells and ILC3, drastically reduced IL-22 protein levels (Fig. 3F).

These data show that epithelial cell lines co-activate ILC3 in a cell-cell contact dependent manner. This activation required a TLR2 co-activating signal on ILC3 and occurred independent of exogenously added cytokines.

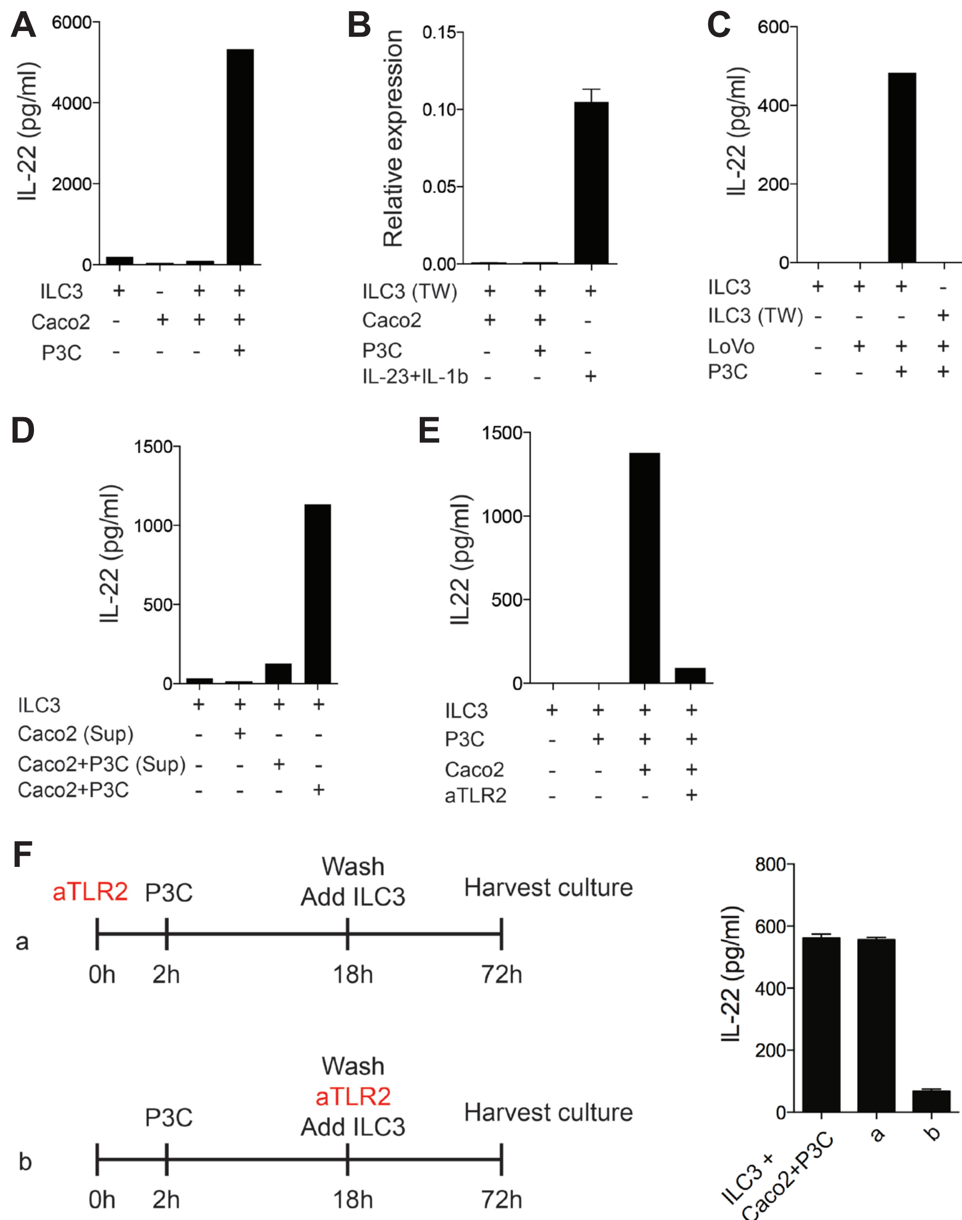

Figure 3. Epithelial cells co-activate mucosal NCR+ ILC3 (A) ELISA of IL-22 protein levels in supernatant of tonsilar NCR$^+$ ILC3 co-cultured with epithelial cell lines for 48 h in the presence or absence of the TLR2 ligand Pam3Cys (P3C). (B) *IL-22* transcript levels of NCR$^+$ ILC3 cultured 48 h in a transwell system (TW) in the presence of Caco-2 epithelial cell with or without P3C stimulation, and compared to ILC3 stimulated with IL-23 and IL-1β. (C) Co-culture of NCR$^+$ ILC3 and LoVo epithelial cells for 48 h in the presence or absence of P3C compared to culture of ILC3 in a transwell system (TW). (D) IL-22 protein levels of culture of NCR$^+$ILC3 without stimuli, with supernatant of co-culture of ILC3 and Caco-2 (Sup) in the presence or absence of P3C for 48 h and ILC3 cultured with Caco-2 in the presence of P3C. (E) IL-22 protein secretion in P3C stimulated NCR$^+$ILC3 co-cultured with Caco-2 in the presence of TLR2 neutralizing antibody. (F, a and b) Experimental setup of TLR2 blocking strategies in ILC3-Caco-2 co-culture. Caco-2 cells were cultured for 16h with P3C before addition of ILC3 (F, a) Caco-2 epithelial cells were pre-treated with anti-TLR2 antibody for 18 hours before addition of ILC3 (F, b) TLR2 blocking antibody was added to the co-culture in combination with ILC3. Supernatant was harvested 2 days after ILC3 addition and IL-22 protein content was measured in the culture supernatants and compared to ILC3 co-cultured with Caco-2 in the presence of P3C .

ILC3 are co-activated by membrane-bound NCR ligands

Since epithelial cells have the capacity to co-activated ILC3 we next determined whether epithelial cell lines expressed membrane-bound endogenous NCR ligands. One of the prototypic NCR ligands is B7H6, a membrane bound NKp30 ligand [21,25]. Analysis of different epithelial cell lines showed expression of B7H6 both at mRNA (Fig. 4A) and protein level (Fig. 4B). LoVo and Caco-2 cell lines were analyzed and compared to K562 and Raji cells as a positive control[21]. LoVo cells expressed highest levels of B7H6 at the transcriptional and protein levels (Fig. 4 A and B). Because co-culture of ILC3 and epithelial cells in combination with the TLR2 ligand P3C led to IL-22 production by ILC3 in a cell-cell contact dependent manner (Fig. 3A), we next determined whether the epithelial-derived co- activating signal involved NCR ligands. Co-culture of ILC3 with LoVo or Caco-2 cell lines barely induced IL-22 production by ILC3 (Fig. 4C and D). As observed before (Fig. 3A), addition of P3C to the cultures strongly induced IL-22 in both co-culture systems (Fig. 4C and D). Interestingly, when endogenous NCR ligands were blocked using soluble NCR-Fc proteins (combination of NKp30-Fc, NKp44-Fc and NKp46-Fc), IL-22 production was diminished both in Caco-2 and LoVo co-cultures (Fig. 4C and D). In contrast, even though co-activation of ILC3 by NCR ligand-expressing cell lines promoted GM-CSF secretion, levels of GM-CSF were only modestly reduced in the presence of NCR-Fc proteins (Fig. 4E) In sum, this set of data revealed that NCR-ligands on epithelial cells can co-activate human ILC3.

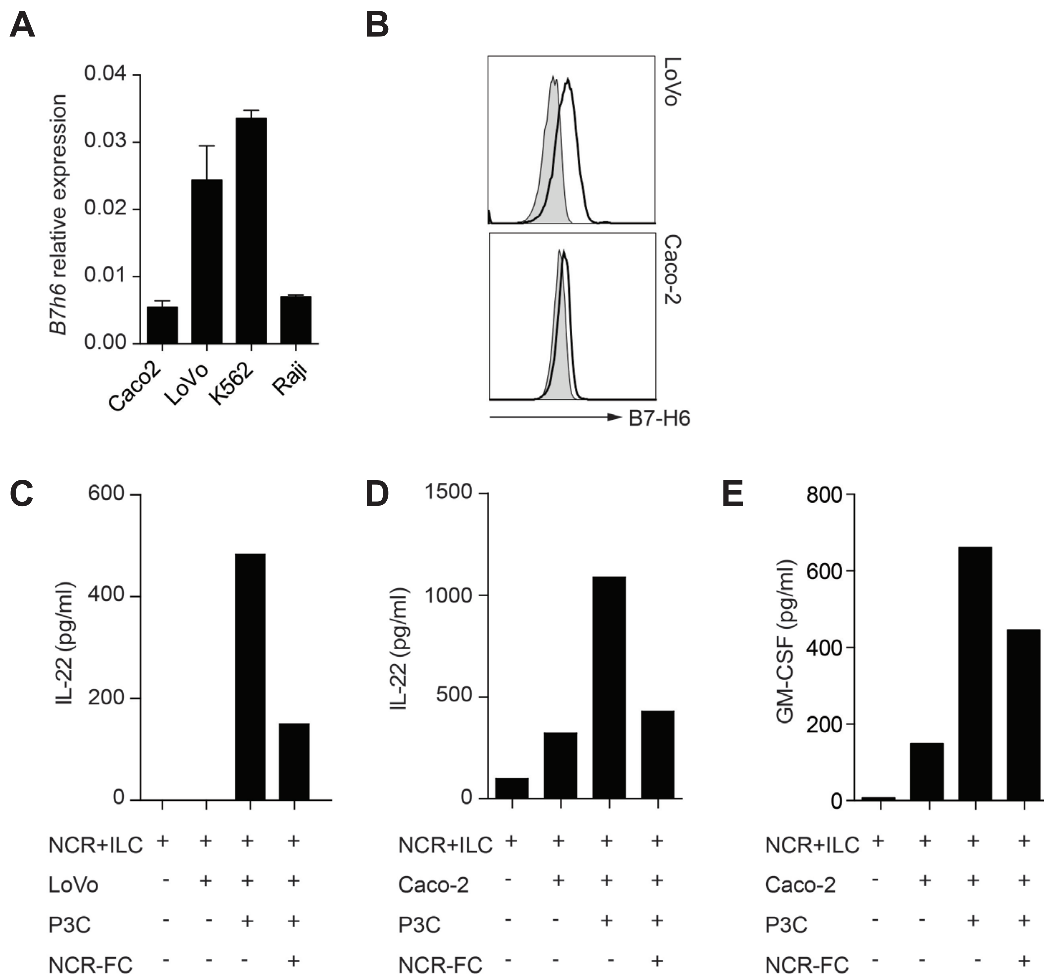

Figure 4. Epithelial cells activate ILC3 via NCR ligation. (A) Transcriptional analysis of Caco-2 and LoVo epithelial cells for the NKp30 ligand, B7H6. K562 and Raji cells were used as a positive control as expression of this ligand has been previously shown[21]. (B) B7H6 protein expression in LoVo and Caco-2 cells compared to the isotype control. (C-D) IL-22 protein levels in supernatant of ILC3 co-cultured with epithelial cell lines for 48 h in the presence or absence of P3C and NCR-FC (NKp30-FC, NKp44-FC and NKp46-FC). Data are representative of three independent experiments. (E) GM-CSF production in co-culture of P3C stimulated LC3 and Caco-2 in the presence of NCR-FC antibodies.

Modulation of B7H6 expression in epithelial cells upon cytostatic insult

Functional activation of ILC3 by membrane bound NCR ligands suggests the possibility of active crosstalk between ILC3 and epithelial cells. Intestinal ILC3 are frequently found close to epithelial cells[9] where they can secrete an array of effector cytokines. Recognition of ligands expressed by epithelia cells, could therefore modulate ILC3 responses in a spatially controlled manner. To further understand the interactions between ILC3 and epithelial cells, we explored the influence of ILC3-derived cytokines and bacterial products in the regulation of epithelial B7H6 expression. Stimulation of Caco-2 epithelial cell lines with P3C, IL-22 and TNFα failed to regulate B7H6 expression as compared to non-stimulated cells yet a small reduction in B7H6 transcripts was found in epithelial cells stimulated with recombinant human IL-22 protein (p-value 0.05) (Fig. 5A). Similarly, protein levels of B7H6 in stimulated Caco-2 epithelial cells were comparable to non-stimulated cells (Fig. 5B, upper panel). Similarly, stimulation of LoVo epithelial cells with P3C and IL-22 did not modulate B7H6 transcription and only a mild upregulation was observed following TNFα stimulation (p-value 0.07) (Fig. 5C). Similar to transcript levels, B7H6 protein levels in LoVo cells in the presence of each of the stimuli were comparable to non-stimulated LoVo epithelial cells (Fig. 5B, lower panel). Since NCR ligands are thought to be induced in response to stress conditions, we next explored the effect of the cytostatic drug MTX on expression of NCR ligands. Transcript analysis for B7H6 in Caco-2 cells did not change in response to MTX (Fig. 5D) whereas LoVo cells upregulated B7H6 expression in a dose-dependent manner (Fig. 5E).

These experiments show that cytokines and TLR ligands fail to regulate B7H6 expression whereas the cytostatic drug MTX is capable of inducing B7H6 expression by epithelial cells and suggest a potential mechanism of ILC3 activation under cytostatic-induced damage.

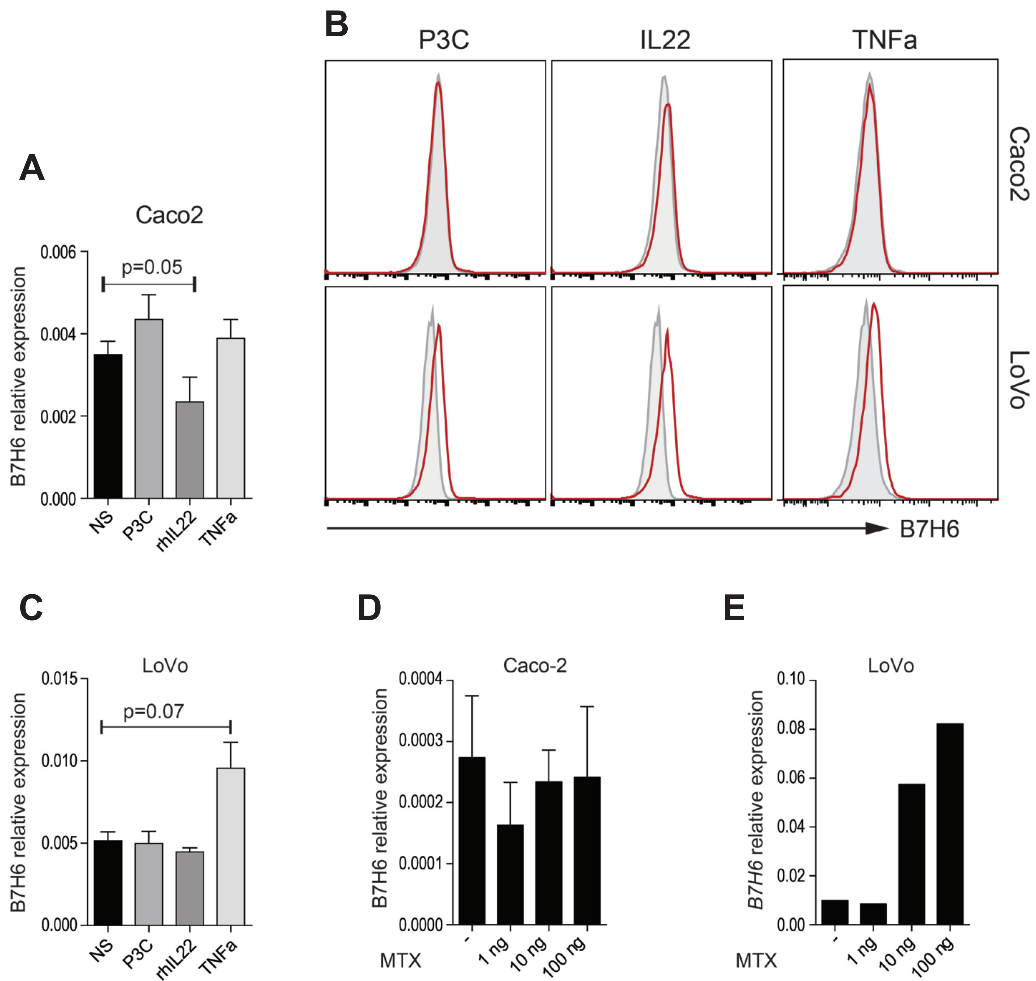

Figure 5. Modulation of NCR ligands by cytostatic drugs. (A) *B7h6* transcript levels of Caco-2 epithelial cells stimulated for 48 h with either the TLR2 ligand Pam3Cys (P3C), recombinant human IL-22 (rhIL22) or TNFα, compared to not stimulated epithelial cells (NS) (B) B7H6 protein expression in Caco-2 and LoVo epithelial cells stimulated with P3C, rhIL22 and TNFα for 48 hours (red) compared to not stimulated epithelial cells (grey). (C) *B7h6* transcript levels of LoVo epithelial cells stimulated for 48 h with P3C, rhIL22 or TNFα, compared to not stimulated epithelial cells (NS) (D-E) Transcript levels of *B7h6* in Caco-2 and LoVo epithelial cell lines stimulated overnight with the indicated concentrations of the cytostatic drug methotrexate (MTX).

DISCUSSION

In the previous chapters of this thesis, I described the protective functions of ILC3 after acute intestinal damage. Nevertheless, the mechanisms by which ILC3 sense tissue damage and carry out effector functions remain poorly understood. Here we set out to gain knowledge on the factors that drive activation of mucosal ILC3.

Previous studies had shown the activating role of cytokines such as IL-23 and IL-1β. Whether a single cytokine stimulus promotes cytokine production by ILC3 or whether a combination of signals are required, it is still unclear. To gain knowledge on the signals that lead to cytokine production by mucosal ILC3, we performed *in vitro* stimulations of tonsil-derived ILC3 with cytokines and TLR ligands, which are found under physiological conditions. These experiments showed that in our culture system, two signals are required for full activation of ILC3 as stimulation with a single signal led to suboptimal cytokine production.

Besides the well-studied role of cytokines, expression of NCRs by ILC3 may represent an additional activating mechanism on ILC3. Indeed, human tonsil-derived ILC3 produced TNFα upon NKp44 ligation with agonist antibody-coated beads[18]. On the contrary, IL-22 and GM-CSF production were induced by cytokines including IL-23 and IL-1β. Although NKp44 ligation alone failed to trigger IL-22 production by ILC3, NKp44 engagement synergized with cytokine stimulation to induce IL-22[18]. Here we show that in addition to cytokines, NCRs also function as co-stimulatory signals that synergise with TLR signals to promote IL-22, GM-CSF and TNFα production.

In contrast to previous findings that used agonist antibody-coated beads and identified NKp44 as the major activating NCR on ILC3[18], our experiments were based on plate-bound NCR agonist antibodies and showed that in this context NKp30 was the major activating NCR as measured by higher levels of secreted IL-22, GM-CSF and TNFa. To further understand whether this discrepancy is due to the different methodology used between the two labs or whether it reflects the preferential response of specific NCRs according to the second signals, a cytokine or a TLR ligand, additional experiments are needed that directly compare the two methodologies.

The few endogenous NCR ligands identified are cell surface proteins often induced by cellular stress or transformation[22,24-26]. Based on this we hypothesized that activation of ILC3 via NCR ligation by membrane bound ligands would be a potential system to control ILC3 activation at sites of tissue damage. Indeed, intestinal epithelial cell lines expressing B7H6 co-activate human NCR^{+} ILC3 in the presence of a TLR2 ligand. In this context, full activation of ILC3 required TLR2 ligation and cell contact with epithelial cells. Blocking NCR binding in the ILC3-epithelial cell culture system showed a reduced activation of ILC3 revealing an NCR-dependent activation of ILC3. Since human ILC3 can be activated

through NCRs, it is tempting to speculate that similar mechanisms might be operational in human intestine after tissue damage induced during for instance anti-cancer chemo or radio therapy, conditioning for hematopoietic stem cell transplantation or as the result of uncontrolled immunity in inflammatory bowel diseases. This notion might be in line with recent findings showing the appearance of NCR$^+$ ILC3 in circulation of leukemia patients undergoing induction chemotherapy prior to hematopoietic stem cell transplantation (HSCT)[27]. The presence of NCR$^+$ ILC3 correlated with reduced development of GvHD after subsequent HSCT, which might possibly be the result of increased mucosal tissue repair.

Expression of NCR ligands can be induced by pro-inflammatory cytokines and TLRs on non-transformed cells[21,24]. Our data showed that cytokines and TLR ligands do not regulate expression of the NKp30 ligand B7H6 on epithelial cells lines. In contrast, we observed that the cytostatic drug MTX was capable of inducing B7H6 expression on LoVo cells in a dose-dependent manner. These data suggest that B7H6 may be a damage-induced protein used by ILC3 to switch on a tissue-protective program. Although ILC3-derived cytokines had only a minor effect on expression of B7H6 by epithelial cell lines, further experiments are needed to exclude the role of ILC3-derived cytokines in NCR ligands expression and to clarify the role of NCR-dependent activation of ILC3 as well as its effector function on epithelial cells.

Collectively, these data show that ILC3 can respond to bacterial products and stress-induced ligands in a spatially controlled manner leading to secretion of molecules involved in mucosal tissue protection.

MATERIALS AND METHODS

Isolation of human ILC3 from tonsils

Tonsils from routine pediatric tonsillectomies were kindly provided by patients at the Sophia children's hospital (Rotterdam) contingent on informed consent and after approval by the Medical Ethical Commission of the Erasmus University Medical Center Rotterdam. Tissue was dissociated using Gentle MACs (Miltenyi Biotec) and digested with Colagenase IV (1mg/ml, Roche) for 15 min at 37°C. Cell suspensions were passed through a 100µm cell strainer and washed with DMEM containing 10% FCS and P/S (100U/ml). Mononuclear cell (MNC) fractions were isolated by ficoll density gradient centrifugation. $CD117^{+}$ cells were enriched from the MNC fraction using CD117 MicroBeads (A3C6E2, MACS separation, Miltenyi Biotec) and subsequently stained with conjugated antibodies. CD117-enriched MNCs were analyzed by flow cytometry (ARIA, BD) and ILC3 were sorted as $CD45^{+}Lin^{-}$ $CD56^{-}CD127^{+}CD117^{+}CD69^{+}NKp44^{+}$. Sorted ILC3 were rested for 7 days in IL-7 and SCF prior to use.

List of monoclonal antibodies

Human monoclonal antibodies: CD45 (HI30, eBioscience); Lin (ExBio): CD3 (Mem57), CD19 (LT19), CD14 (Mem15), CD34 (4H11); CD94 (DX22, eBioscience); CD127 (104D2, Biolegend); CD117 (RDR5, eBioscience); CD69 (FN50, Biolegend); NKp44 (P448, Biolegend)

NCR stimulation of human ILC3

Culture-treated round-bottom 96 well-plates were coated with agonist antibodies, or their isotypes, to NKp30 (5ug/ml, R&D systems, IgG2a), NKp44 (5ug/ml, R&D systems, IgG2a) and NKp46 (5ug/ml, R&D systems, IgG2b) in PBS for 3 hours at 37°C. Plates were washed and sorted tonsilar ILC3 were added in the presence or absence of Pam3Cys (P3C, 1ug/ml, Invivogen) for 24h. Cytokine content in supernatants was determined by enzyme-linked immunosorbent assay (DuoSet ELISA, R&D systems) and absorbance was measured at 450nm using Victor X4 (Perkin Elmer).

Culture and analysis of intestinal epithelial cell lines

Intestinal epithelial cell lines were cultured with DMEM containing 10% FCSi and P/S (100U/ml). Caco-2 and LoVo epithelial cells were seeded in a 96-well plates and stimulated for 48 h with the following TLR ligands; TLR2 ligand P3C (1ug/ml), TLR3 ligand Poly I:C (1ug/ml), TLR4 ligand LPS (1ug/ml; all from Invivogen); Similarly, epithelial cells were cultured for 48 h in the presence of cytokines TNFα (10 ng/ml; R&D systems) or rhIL-22 (10 ng/ul; Peprotech). Stimulation with the cytostatic drug methotrexate was performed overnight at 1-100 ng/ml. Cells were either lysed for RNA isolation and transcript analysis

of B7H6 or stained with B7H6 antibody (kindly provided by E. Vivier, CIML, Marseille) for flow cytometric analysis.

Co-culture of intestinal epithelial cell lines and ILC3

Caco-2 and LoVo cell lines were co-cultured with sorted tonsilar ILC3 (ratio 2:3) in the presence or absence of Pam3Cys (P3C, 1ug/ml) and NCR-Fc fusion proteins (NKp30-Fc, NKp44-Fc and NKp46-Fc, all at 5ug/ml, R&D systems) for 48 hours.

For TLR2 blocking experiments, either ILC3 or Caco-2 epithelial cells were treated with TLR2 neutralizing antibody (10 ug/ml, 383936, R&D systems) as described in figure 3F.

Cytokine content in supernatant was determined by enzyme-linked immunosorbent assay (DuoSet ELISA, R&D systems) and absorbance was measured at 450nm using Victor X4 (Perkin Elmer).

PCR

RNA was extracted using the RNA-XS kit (Machery Nagel) followed by reverse-transcription with random hexamer primers. For quantitative PCR, a Neviti Thermal Cycler (Applied Biosystems) and DyNAmo Flash SYBR Green qPCR kit (Finnzymes) were used, with the addition of MgCl2 to a final concentration of 4 mM. All reactions were done in duplicate and were normalized to the expression of *Gapdh*. Relative expression was calculated by the cycling threshold (CT) method as $2^{-\Delta t}$.
Primers sequences can be found in Table 1

Table 1. List of primers

Gene name	Forward Primer	Reverse Primer
hGAPDH	GTCGGAGTCAACGGATT	AAGCTTCCCGTTCTCAG
hB7H6	CCCAACCCCTCAACAT	GGTCGGAATGCCTCTT
hIL22	GCTGCCTCCTTCTCTTG	TCCGTGGAACAGTTTCTC

Statistical analysis

Samples were analyzed using unpaired Mann-Whitney test. P values < 0.05 were considered significant.

4

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Patricia Aparicio-Domingo[1], Monica Romera-Hernandez[1], Natalie Papazian[1], Papazian1, Ferry Cornelissen[1], Julien J. Karrich[1], James Butler[2], Mark C. Coles[2] and Tom Cupedo[1]

[1] Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands
[2] Centre for Immunology and Infection, Department of Biology and Hull York Medical School, York, UK

Manuscript in preparation

CHAPTER 5

In vivo function of Natural Cytotoxicity Receptors expressed by ILC3

SUMMARY

In the past few years, several studies have identified ILC3 and ILC3-derived cytokines as important players in mucosal protection against different pathogens. During enteric infections, activation of ILC3 is driven by APC-derived factors including IL-23 and IL-1β. These are all soluble factors, which do not allow for spatial control of immune cell activation. However, during epithelial damage, ILC3 activation should be a local process occurring only at the site of damage and thus cell surface ligands are expected to drive activation of ILC3 in response to damage. In the small intestine, the NKp46^{+}ILC3 subset is dispersed in the lamina propria close to the differentiated epithelium. Although *in vitro* assays have identified Natural cytotoxicity receptors (NCRs) as co-activating receptors on human ILC3, the *in vivo* role for NCRs remains unclear. Studies with the enterobacterium *C. rodentium* showed that NCRs were dispensable for bacterial containment. Nevertheless, NCR ligands are considered to be damage induced-ligands, and therefore they could control ILC3 activation upon intestinal stress in the absence of pathogens. Since NKp46^{+}ILC3 responded to acute epithelial damage induced by MTX, we next explored the role of NCRs in epithelial responses after MTX-induced damage. Indeed, the absence of NCRs aggravated epithelial pathology upon chemotherapeutical insult and radiation-induced damage. The protective functions mediated by NKp46 appeared to be independent of IL-22 and GM-CSF. Collectively these results identify an *in vivo* role for NCRs after MTX-induced damage and suggest that activation of ILC3 during infection or tissue damage is controlled by separate mechanisms.

INTRODUCTION

Over the last few years, intestinal ILC3 have gained appreciation in mucosal immunology due to their ability to rapidly respond to microbiota and carry out effector functions, mainly through production of IL-22[1-5]. Examination of ILC3 within the small intestine revealed the existence of multiple subsets that locate in different compartments[6,7], suggesting spatially controlled functional specialization of ILC3. NKp46^{-}CCR6^{+} ILC3 are mainly found within cryptopatches, small cell clusters located in the deeper parts of the lamina propria close to the intestinal crypts, while NKp46^{+}CCR6^{-} ILC3 are dispersed throughout the lamina propria[8,9]. The NKp46^{+} ILC3 population is thus placed in close proximity to the differentiated epithelium, which represents the first line of defense against enteric pathogens. In line with these observations, a recent report has shown that the NKp46^{+} ILC3 population contributed to the mucosal barrier by producing IFNγ upon *Salmonella* infection, important for formation of the mucus layer[6]. During enteric infections such as with *C. rodentium*, the epithelial barrier gets compromised and bacterial translocation can occur. Several findings have highlighted the pivotal function of ILC3-derived IL-22 in providing a rapid response to infections to ensure intestinal homeostasis and prevent bacterial dissemination[10-12]. In this scenario, activation of ILC3 is driven by APC-derived cytokines, including IL-23[13] and IL-1β[7]. Since NKp46^{+}ILC3 are located close to the epithelium and have the capacity to rapidly respond by production of effector cytokines one can envision that they also contribute to epithelial protection upon tissue damage in the absence of enteric infections, when integrity of the mucosal barrier is once again essential to prevent bacterial translocation.

In the previous chapter of this thesis I discussed the functional role for NCR as co-activating receptor on human ILC3, which confirms and extends published data[14]. Moreover, we demonstrated the co-activating role of NKp30-ligands expressed by epithelial cell lines on cytokine-stimulated ILC3 *in vitro*.

At present, only few NCR ligands have been characterized, yet NCRs are presumed to be damage- and pathogen-associated molecular pattern recognition receptors[15-19]. Most of the NCR ligands identified are virus hemagglutinins or tumor-expressed ligands[20,21], which trigger proliferation and cytotoxicity of NK cells[22,23]. Indeed, studies on NK cells identified a role for NKp46 in tumor surveillance, both *in vivo* and *in vitro*[24,25] Moreover, NKG2D, a functionally similar receptor expressed by NK cells for which more ligands have been identified, plays an important role in recognition of damaged and transformed cells[22,23,26]. However, whether NCRs expressed on ILC3 have a functional role *in vivo* is still unknown.

In contrast to humans that express NKp30, NKp44 and NKp46, NKp46 is the only NCR conserved in mice[27]. The functional role of NKp46 expression on murine ILC3 remains elusive as NKp46 cross-linking does not induce production of cytokines[14]. Nevertheless, NKp46-mediated activation of ILC3 may require co-stimulatory signals that are only found *in vivo* under certain (patho-) physiological conditions. Conversely, expression of NKp46 is dispensable for ILC3-mediated control of enteric infections with the murine enterobacterium *Citrobacter rodentium*[28], indicating that NCR signaling on ILC3 is not required for innate immune responses against this pathogen.

Since NCRs are thought to recognize stress-induced ligands and intestinal NKp46$^+$ ILC3 responded to intestinal damage induced by the cytostatic drug methotrexate (MTX), we here dissected the function of this receptor in controlling epithelial responses to acute insults. By using NKp46 deficient mice and NKp46-targeting antibodies, we show that the absence of NKp46 aggravates intestinal epithelial damage upon MTX insult. NKp46 appeared to mediate the protective functions by factors different from IL-22, GM-CSF or TNFα.

RESULTS

Nkp46-deficient mice are more susceptible to MTX-induced damage

To determine the role of NCRs in tissue damage responses, intestinal damage was induced in Ncr1-deficient mice that lack NKp46, the only NCR expressed in mice, by administration of the cytostatic drug methotrexate (MTX). Histological examination of small intestinal tissue from NKp46 deficient mice and littermate controls revealed no differences in lamina propria infiltration, epithelium flattening and crypt abscesses characteristic of MTX-induced damage (Fig. 1A). Body weight dropped in both groups one day after the last MTX injection, however NKp46 deficient mice exhibited different kinetics with the maximal body weight loss six days after MTX in contrast to NKp46 sufficient mice that showed the peak of body weight loss at day four (Fig. 1B). Body weight loss in NKp46 deficient mice stabilized after the first MTX injection and showed statistically significant differences compared to NKp46 sufficient mice at day 2 and day 3 after the last MTX injection.

Although pathology analysis of the separate crypt and villi compartments of NKp46 sufficient and deficient mice did not show statistically significant differences, we observed a trend towards an increased crypt damage in the absence of NKp46. (Fig. 1C) Indeed, overall small intestinal damage at this time point was increased in Ncr1$^{-/-}$ mice compared to littermate controls (Fig. 1D) and detailed examination revealed higher pathological score in jejunum and ileum of Ncr1$^{-/-}$ mice compared to littermate controls (Fig. 1E).

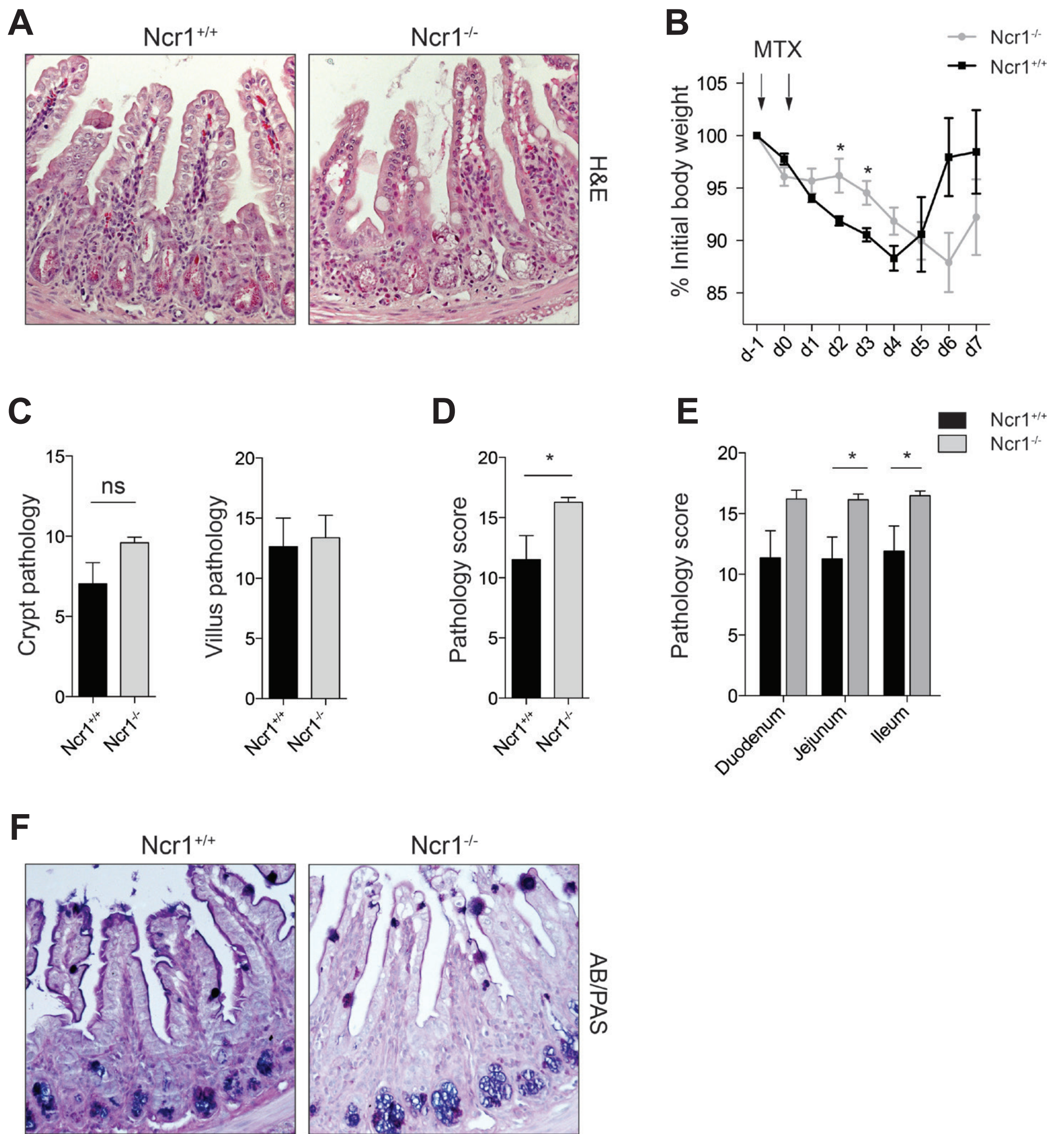

Figure 1. NKp46-deficiency aggravates MTX-induced intestinal damage. (A) Representative H&E staining of ileal sections of Ncr1$^{+/+}$ and Ncr1$^{-/-}$ mice four days after the last MTX injection. (B) Body weight changes in Ncr1$^{+/+}$ and Ncr1$^{-/-}$ mice during and after MTX administration relative to the initial body weight. (C) Small intestinal pathology at day 4 after MTX, as described in material and methods. (D) Pathology score of duodenum, jejunum and ileum in Ncr1$^{+/+}$ and Ncr1$^{-/-}$ mice at day 4 after the last MTX injection. (E) Pathology score in the intestinal crypts of Ncr1$^{+/+}$ and Ncr1$^{-/-}$ mice four days after MTX administration. (F) Ileal section of Ncr1$^{+/+}$ and Ncr1$^{-/-}$ mice at day 4 after MTX showing AB/PAS staining for goblet cells. (n=4 per group); *, P<0.05; ns, not significant.

Maintenance of the mucus layer is of great importance to safeguard epithelial homeostasis and guarantee barrier functions. We previously observed that in the absence of ILC3, goblet cell numbers are reduced. To address the role of NKp46 in maintenance of mucus producing cells, goblet cells from $Ncr1^{-/-}$ mice and $Ncr1^{+/+}$ mice were stained with AB/PAS four days after the last MTX injection (Fig. 1F). Histological examination showed the presence of goblet cells in both Ncr1-sufficient and deficient mice, suggesting that NKp46 is dispensable for goblet cell maintenance after MTX-induced damage.

In contrast to bacterial infection models that failed to identify a role for NKp46 *in vivo*, the aggravated intestinal pathology observed in $Ncr1^{-/-}$ mice indicated a protective role of NKp46 expression after MTX-induced intestinal damage.

Reduced epithelial pSTAT3 in NKp46-deficient mice

Epithelial activation after chemotherapeutic insult results in phosphorylation of STAT3 early after damage. To assess the role of NKp46 in the early epithelial STAT3 activation, we exposed $Ncr1^{-/-}$ mice to MTX and examined activation of the STAT3 pathway. Wild type (WT) mice, as well as $Ncr1^{+/-}$ mice showed phosphorylation of epithelial STAT3 one day after the last MTX injection (Fig. 2A). Analysis of small intestinal sections from NKp46 sufficient mice revealed STAT3 phosphorylation in contrast to NKp46 deficient mice that showed reduced levels of epithelial pSTAT3 (Fig. 2A). The lower pSTAT3 levels in $Ncr1^{-/-}$ mice were specific for epithelial cells as pSTAT3 positive cells were still present in the lamina propria of these mice (Fig. 2A). Quantification of pSTAT3 intensity in small intestinal sections demonstrated significantly reduced pSTAT3 intensity in epithelial cells from NKp46 deficient mice compared to WT controls (Fig. 2B). Despite the reduced levels of pSTAT3 in epithelium of $Ncr1^{-/-}$ mice, transcriptional analysis of total mRNA from small intestinal tissues showed similar expression of the downstream target gene *Socs3* in NKp46 sufficient and deficient mice one day after the last MTX injection (Fig. 2D). Similarly, transcript levels of *Bclxl*, a pro-survival gene and known STAT3 target gene, were also comparable between WT and $Ncr1^{-/-}$ mice and no significant differences were observed at this time point and (Fig. 2D). Nevertheless, since transcriptional analysis was performed in total mRNA from small intestinal samples, any difference may have been masked by the higher number of infiltrating cells in the lamina propria of NKp46 deficient mice that expressed pSTAT3 and therefore may transcribe downstream genes. Nevertheless, these data show a robust reduction of epithelial pSTAT3 in $Ncr1^{-/-}$ mice at the protein level and suggest a role for NKp46 in intestinal epithelial activation in response to tissue damage.

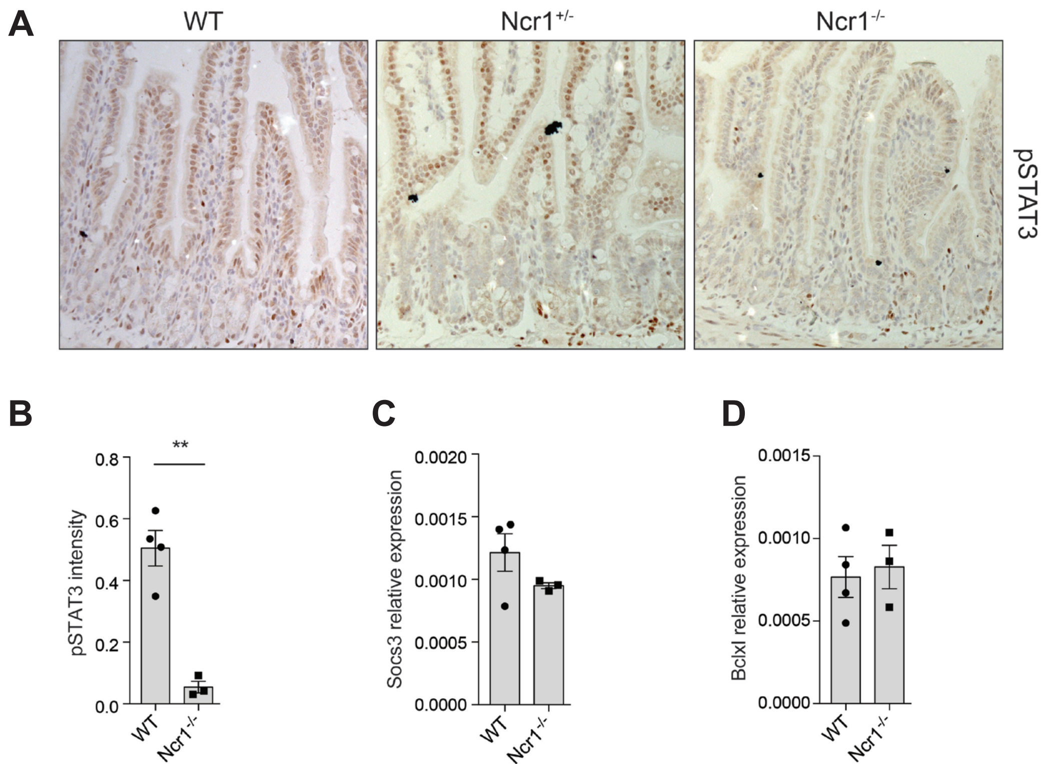

Figure 2. Reduced pSTAT3 expression in the absence of NKp46. (A) Immunohistochemical staining for pSTAT3 in ileal sections of WT, Ncr1$^{+/-}$ and Ncr1$^{-/-}$ mice one day after the last MTX injection. (B) Quantification of epithelial pSTAT3 intensity in WT and Ncr1$^{-/-}$ mice at day 1 after MTX administration. (C) Transcript analysis of *Socs3* from total mRNA in ileal samples from WT Ncr1$^{-/-}$ mice one day after the last MTX injection, relative to *Gapdh* (D) Transcript analysis of *Bclxl* relative to *Gapdh***,** from total mRNA in ileal samples from WT and Ncr1$^{-/-}$ mice at day 1 after MTX. (n=3-4 per group); **, P<0.01.

Cytokine production in NKp46 deficient mice

ILC3-derived cytokines have been implicated in epithelial responses after acute damage and bacterial infections. To study whether NKp46 is involved in the production of effector cytokines after epithelial damage, we performed a transcriptional analysis of small intestinal samples from NKp46 sufficient and deficient mice one day after MTX administration. Analysis of total mRNA levels from ileum showed similar expression levels of *Il22* in the presence or absence of NKp46 (Fig. 3A). *Csf2* (GM-CSF*)* transcript levels appeared to be reduced in NKp46 deficient animals compared to WT controls, but no significant differences were observed (Fig. 3B). In contrast, small intestinal transcript levels of *Tnf* (TNFα*)* were significantly reduced in Ncr1$^{-/-}$ mice compared to Nc1 sufficient mice (Fig. 3C).

In addition, we examined cytokine protein production in small intestinal explants after overnight culture. Surprisingly, analysis of explant cultures one day after the last MTX injection revealed significantly higher levels of IL-22 in NCR deficient mice compared to WT controls (Fig. 3D). GM-CSF protein levels however, were similar in NCR deficient and sufficient mice at day 1 after MTX administration (Fig. 3D).

To specifically study the role of NKp46 in cytokine production of ILC3, we isolated NKp46^{+} ILC3 from lamina propria of WT mice, and the corresponding GFP^{+} ILC3 population from NKp46-deficient, Ncr1$^{gfp/gfp}$ mice. Transcriptional analysis of lamina propria NKp46^{+}ILC3 one day after the last MTX injection showed a reduction in *Il22* transcript levels although no statistically differences (p-value 0,06) were reached (Fig. 3F) Differences in expression levels of *Csf2* and *Tnf* were modest and no significant differences were observed between NKp46 deficient mice and WT animals (Fig. 3G and H). These results suggest that regulation of additional cytokines may underlie the effects observed in NKp46$^{-/-}$ mice.

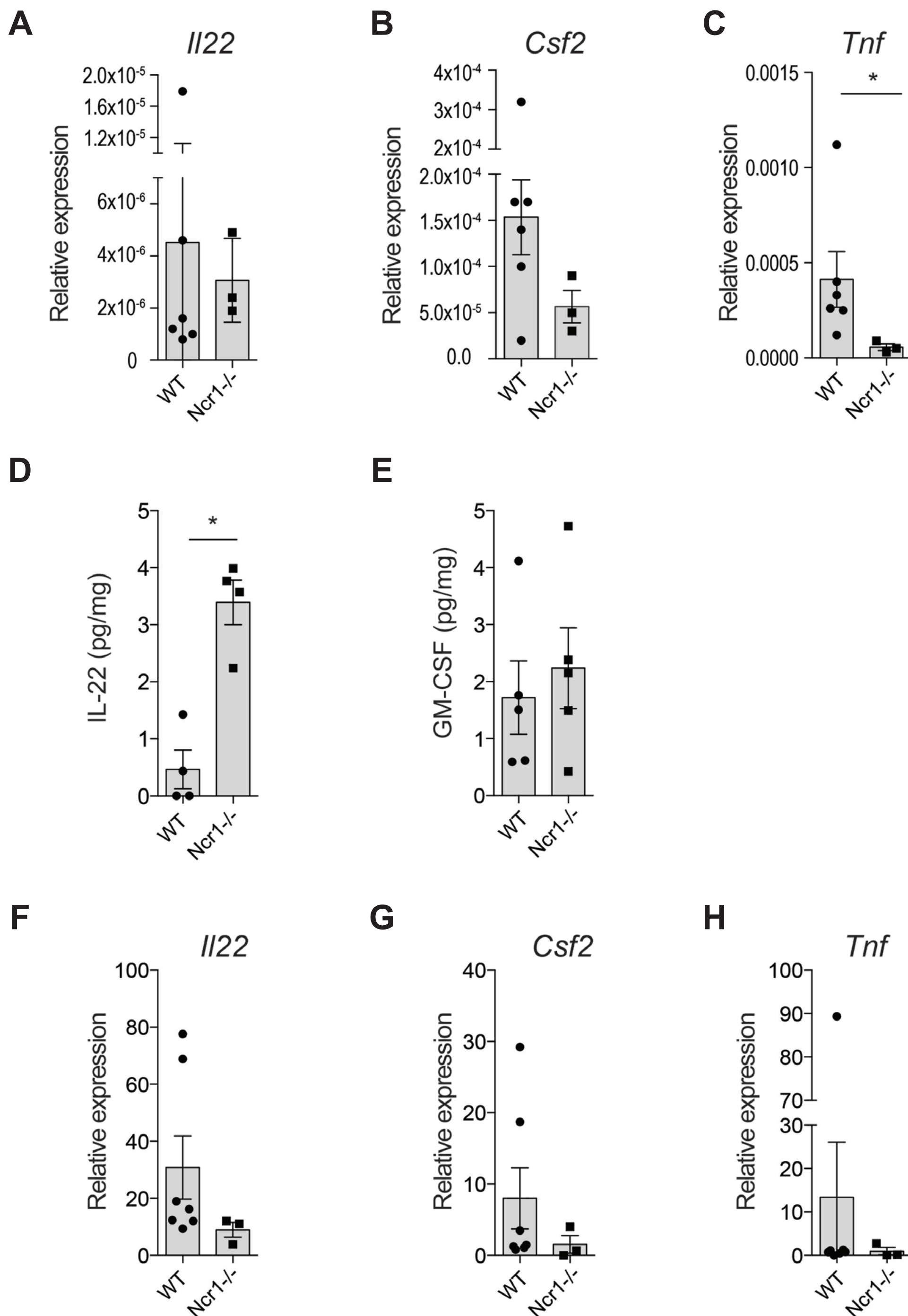

Figure 3. Cytokine production in the small intestine of NKp46-deficient mice after MTX-induced damage. (A-C) Transcript levels of *Il22*, *Csf2* and *Tnf* relative to *Gapdh*, in total ileal mRNA from WT and Ncr1$^{-/-}$ mice at day 1 after MTX administration. (D-E) Protein levels of IL-22 and GM-CSF after overnight ileal explant cultures isolated at day 1 after MTX. (F-H) Transcript levels of *Il22*, *Csf2* and *Tnf* relative to *Gapdh*, from NKp46^{+}ILC3 isolated from the lamina propria of WT and Ncr1$^{-/-}$ mice at day 1 after MTX. (n=3-5 per group); *, P<0.05.

In vivo targeting of NKp46+ ILC3 during MTX-induced damage

To further investigate the *in vivo* role of NKp46 after small intestinal damage, we treated Rag1$^{-/-}$ mice with an NKp46 targeting antibody and induced intestinal damage by administration of MTX (Fig. 4A). Small intestinal tissue samples were analyzed one day after administration of the cytostatic drug for expression of ILC3-derived cytokines. Transcript levels from total ileal mRNA indicated similar levels of *Il22*, *Csf2* and *Tnf* in αNKp46-treated mice and isotype control (Fig. 4B). In addition, IL-22 and GM-CSF protein secretion was analyzed from ileal explants at day 1 after MTX administration (Fig. 4C). Similar to gene expression, IL-22 and GM-CSF protein production was comparable between αNKp46-treated mice and isotype control and no statistically significance was observed (Fig. 4C). To specifically study the effect of NKp46 targeting on ILC3, we isolated lamina propria NKp46^{+} ILC3 from αNKp46-treated mice and isotype controls one day after MTX administration (Fig. 4D). To assess the effectiveness of the binding of the αNKp46 antibody, we stained the *in vivo* αNKp46 antibody with a detection antibody (Donkey-aRat). NKp46^{-} ILC3 represented almost half of the total population of lamina propria ILC3 in the control group (57%±3.8) whereas αNKp46-treated mice exhibited an increased in the frequency of NKp46^{-} ILC3 (77.2%±8.9) (Fig. 4D). Staining for αNKp46 antibody did not show positive cells in the isotype-treated group, whereas analysis of the αNKp46-treated group revealed approximately two thirds of the NKp46^{+} ILC3 population bound to the NKp46-targeting antibody (16.3%±5.4). Strikingly, αNKp46-treated mice also showed a number of ILC3 that were positive for the *in vivo* antibody as well as for NKp46 (6.4%±3.8) (Fig. 4D).

Transcriptional analysis of lamina propria NKp46^{+} ILC3 one day after MTX treatment did not show significant differences and revealed similar expression of *Il22*, *Csf2* and *Tnf* in αNKp46-treated mice compared to control littermates (Fig. 5E).

Collectively these results suggest that the production of IL-22, GM-CSF and TNFα by ILC3 does not require NKp46 expression and are in line with the findings observed in Ncr1 deficient mice. Alternatively, NKp46 may control the production of different factors by ILC3 and thus the production of additional cytokines in the absence of NKp46 remains to be investigated.

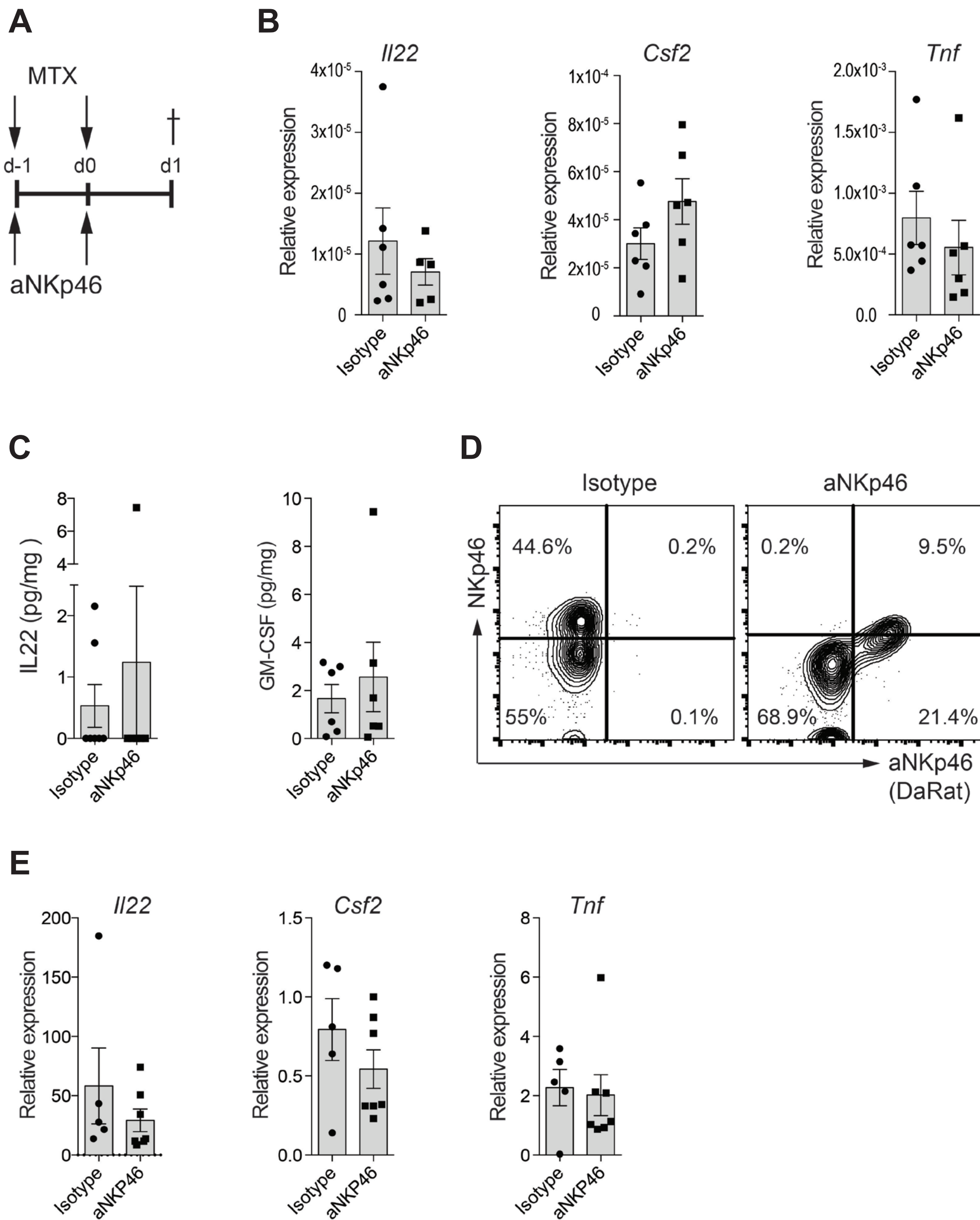

Figure 4. Targeting of NKp46 during MTX-induced damage. (A) Schematic overview of the experimental setup. Rag1$^{-/-}$ mice received MTX and NKp46-targeting antibody or isotype control at day -1 and day 0 as described in material and methods. At day 1 after the last injection, mice were sacrificed. (B) Transcript levels of *Il22*, *Csf2* and *Tnf* relative to *Gapdh*, in total ileal mRNA from NKp46 and isotype treated mice at day 1 after MTX administration. (C) Protein levels of IL-22 and GM-CSF after overnight ileal explant cultures isolated at day 1 after MTX. (D) Representative flow cytometry plots of lamina propria ILC3 from NKp46 and isotype-treated mice at day 1 after MTX (DaRat (Donkey a-Rat)=detection antibody for NKp46-targeting antibody). (E) Transcript levels of *Il22*, *Csf2* and *Tnf* relative to *Gapdh*, from NKp46^{+}ILC3 isolated from the lamina propria of NKp46 and isotype-treated mice at day 1 after MTX. (n=6-7 per group)

NKp46-targeted mice succumb to radiation-induced damage

Besides studying the role of NKp46 in intestinal damage induced by cytostatic drugs, we explored the role of this receptor in radiation-induced tissue damage responses. In this model, WT mice were sublethally irradiated followed by administration of the αNKp46-targeting antibody (Fig. 5A). Body weight was monitored daily for 12 days in αNKp46-treated mice and isotype control-injected mice (Fig. 5B). Isotype control and αNKp46-treated mice exhibited similar body weight kinetics in the first week but by day 8, αNKp46-treated mice developed significant weight loss and continued to loose weight over the course of the experiment (Fig. 5B). Importantly, αNKp46-treated displayed reduced survival and succumbed to radiation-induced damage by day 10 while isotype control-treated littermates survived and all the experimental animals were sacrificed at the latest time point (Fig. 5C)

Histological examination of small intestinal sections from αNKp46-treated mice and isotype controls showed similar intestinal damage in both groups that increased at later time points and was characterized by lamina propria infiltration and bleeding (Fig. 5D). Goblet cells appeared to be conserved and distributed similarly in αNKp46-treated mice and the control group after radiation-induced damage (Fig. 5E). Transcript analysis of total mRNA from small intestinal tissues showed higher levels of *Csf2* in αNKp46-treated mice compared to control group at day 4 (Fig. 5F). Nevertheless these differences did not reach significance and were absent at day 12. In contrast, *Tnf* levels at day 4 were similar in αNKp46-treated mice and isotype-injected animals and only differed at day 12, when αNKp46-treated mice exhibited slight reduced levels (Fig. 5F). Although the factors involved in tissue protection remain to be identified, these data indicate a potential role of NKp46 in responses to radiation-induce damage.

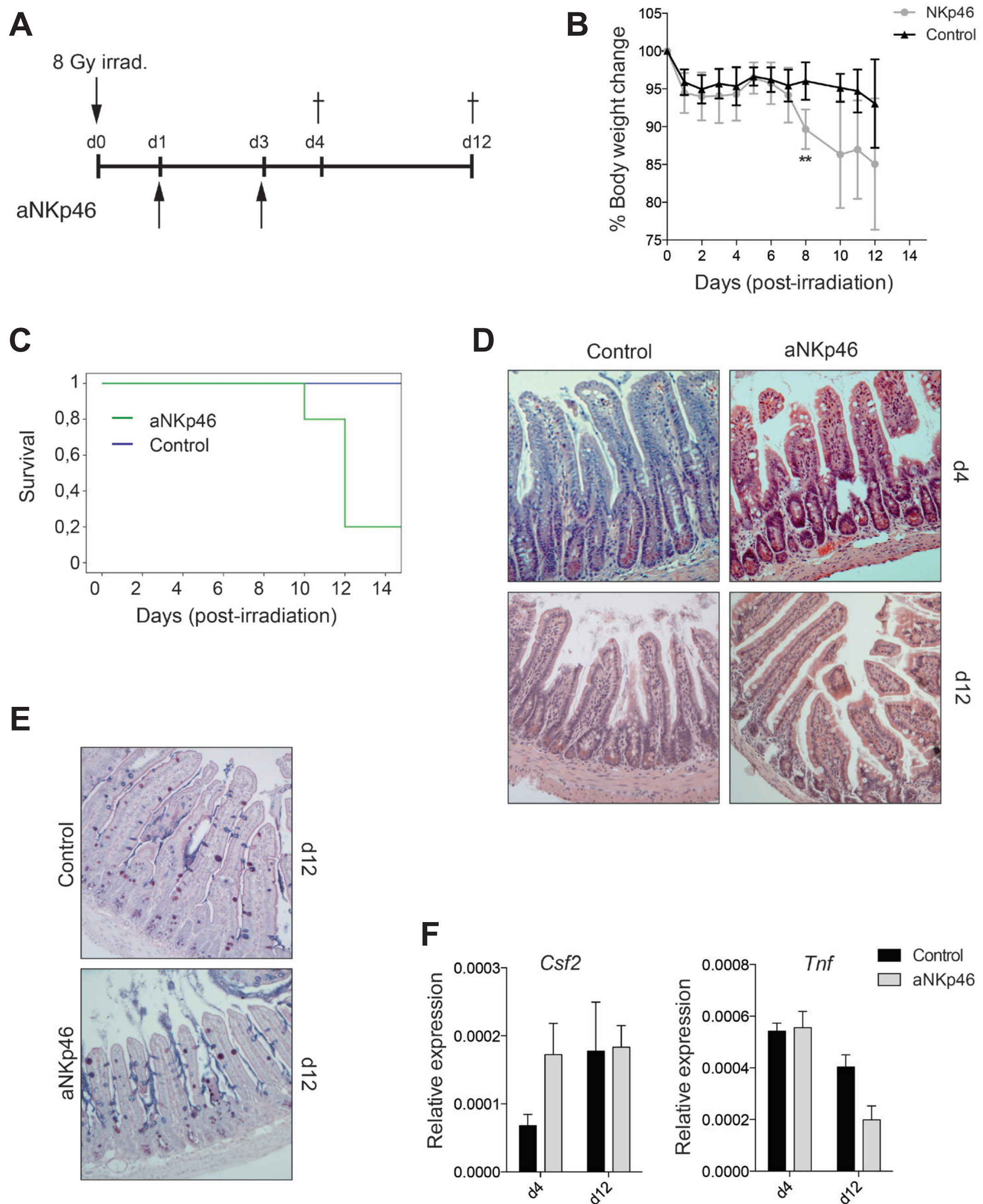

Figure 5. NKp46-treated mice succumbed to radiation-induced damage. (A) Schematic overview of the experimental setup. WT mice were sublethally irradiated at day 0 followed by administration of NKp46-targeting antibody or isotype control at day 1 and day 3 after irradiation. At day 4 and 12 after irradiation mice were sacrificed. (B) Body weight change over the course of the experiment, relative to the initial body weight. (C) Survival curve of NKp46-treated mice (green line) and isotype control (blue line). (n=5 per group, per time point) (D) Representative H&E staining of ileal section at day 4 and 12 after irradiation. (E) Ileal section 12 days after irradiation showing AB/PAS staining for goblet cells. (F). *Csf2* and *Tnf* transcript levels relative to *Gapdh*, from total small intestinal mRNA at day 4 and 12 after irradiation.; **, P<0.01.

DISCUSSION

In the previous chapters of this thesis I discussed the protective role of ILC3 in mucositis induced damage by administration of cytostatic drugs. Because NCR ligands are induced upon stress or damage[17,18] and NCRs are co-activating receptors on ILC3[14] (and chapter 4), we next investigated whether the protective functions carried out by ILC3 during MTX-induced damage involved NCR signaling. Indeed, the absence of NKp46 aggravated intestinal pathology after administration of MTX. Although examination of the crypt and villus compartment separate did not show significant differences in NKp46 deficient mice compared to littermate controls, overall intestinal pathology was more severe in Ncr1$^{-/-}$ mice and reached significance when detailed analysis was performed in individual regions of the small intestine.

Of note, monitoring body weight after treatment revealed different kinetics in NKp46 deficient and sufficient mice. While NKp46 sufficient mice loss weight immediately after the first injection and continued loosing weight until day 4, Ncr1$^{-/-}$ mice stabilized their body weight after the first MTX injection and maintained it stable for two days. The delayed kinetics in the early time points was translated into a delayed peak of weight loss at day 6. A possible explanation of this behavior could be that the Ncr1$^{-/-}$ mice are pre-sensitized or pre-disposed to inflammation, resulting in an effective initial response to epithelial damage that is no longer contained at the later stages. Analysis of Ncr1$^{-/-}$ mice at steady state might reveal whether the absence of NKp46 induces a pre-inflammatory state.

Since ILC3 deficient mice displayed reduced epithelial pSTAT3 levels, we next questioned the role of NKp46 in the activation of this pathway. Intestinal epithelium of NKp46-deficient mice after MTX treatment revealed lower expression of pSTAT3 compared to NKp46 sufficient mice, which recapitulated the phenotype observed in Rorγt deficient mice (chapter 2). Because transcription of ILC3-derived cytokines including *Il22*, *Csf2* and *Tnf* was reduced in Rorγt deficient mice after MTX treatment, we next explored whether similar differences occurred in the absence of NKp46 and could explain the lower levels of pSTAT3. However, transcript levels of *Il22* and *Csf2* from small intestinal tissues were comparable in the presence or absence of NKp46 and only *Tnf* was significantly reduced in Ncr1$^{-/-}$ mice. Additionally, analysis of small intestinal explants revealed significantly higher levels of IL-22 protein in Ncr1$^{-/-}$ deficient mice when compared to NKp46 sufficient mice, suggesting that additional factors may contribute to epithelial activation and phosphorylation of STAT3. Indeed, the concept that cytokines different from IL-22, GM-CSF and TNFα may control epithelial activation after MTX was subsequently validated, as transcript analysis of NKp46 deficient or sufficient ILC3 revealed comparable cytokine levels. As discussed in chapter 3 of this thesis, which revealed normal levels of pSTAT3 in

IL-22$^{-/-}$ after MTX treatment, the fact that reduced pSTAT3 was observed in the presence of high levels of IL-22 favors the idea that during MTX-induced damage, activation of STAT3 is independent of IL-22.

An important notion is that during histological analysis of small intestinal tissues, lamina propria infiltration was observed in both NKp46 deficient mice and littermate controls. Since lamina propria infiltrating cells can produce IL-22 and GM-CSF among others[29-31], the differences that might be present in Ncr1$^{-/-}$ mice may be masked by the cytokines produced by infiltrating cells. It is possible that infiltrating cells present in the lamina propria of NKp46 deficient mice are different from those found in NKp46 sufficient mice with regard to their kind or cytokine production. Therefore, exhaustive quantification of lamina propria infiltrating cells as wells as their cytokine profile would further clarify epithelial responses in the absence of NKp46.

Similar to Ncr1$^{-/-}$ mice, Rag1$^{-/-}$ mice treated with aNKp46-targeting antibody demonstrated a comparable *Il22*, *Csf2* and *Tnf* transcription in total ileal mRNA and purified intestinal ILC3. Although the majority of NKp46^{+} ILC3 were targeted by the *in vivo* monoclonal antibody, NKp46 was still detectable as seen by the fraction of ILC3 stained with the antibody against this receptor, indicating that a fraction of NKp46 was still available on ILC3 and thus partial activation through these receptors may have occurred. Despite the fact that NKp46-targeting antibody has been described as an NKp46-blocking antibody[32], the precise function of this antibody *in vivo* is still unclear. Studies performed on cultured NK cells demonstrated that blockade of activating receptors including NKG2D[33] or NKp46[32] resulted in counterintuitive enhanced NK cell responses. Strikingly, short term blocking, consisting of 1-3 days of treatment with NKp46-targeting antibody, which was sufficient to saturate NKp46 receptors, failed to boost NK cell activity[32]. By contrast, longer treatments lasting up to two weeks enhanced NK cell responses. Our experiments resembled the results observed in the short-term treatment performed on NK cells, in which receptors are targeted but effector responses are lacking. Therefore, it would be interesting to study ILC3 responses after MTX-induced damage with longer exposure to the NKp46-targeting antibody.

To further study the role of NKp46 upon epithelial insult we set up a model of radiation-induced damage. Sublethally irradiated WT mice treated with NKp46-targeting antibody significantly loss weight over the second week of treatment and succumbed to radiation-induced damage by day 10. Although histological examination of small intestinal tissues did not reveal clear differences between NKp46-treated mice and isotype-injected group, this could be explained by the fact that only the mice that survived the treatment

were analyzed. One may expect that the mice that survived were able to cope with the radiation-induced damage and therefore histological and transcriptional profile may not significantly differ from the control group. While radiation-mediated mortality in mice treated with NKp46-targeting antibody was envisioned as a fatal wasting disease of the gastrointestinal tract, additional experiments are needed to exclude other radiation-mediated pathologies such as bone marrow failure.

Collectively these data identify an *in vivo* role for NKp46 and suggest that activation of ILC3 after enteric infection or tissue damage is controlled by separate mechanisms. However, the NKp46-derived factors involved in tissue protection remain unknown and additional studies are required to uncover the signals by which NKp46^{+}ILC3 drive epithelial protection.

MATERIALS AND METHODS

Mice. C57BL/6 wild type (WT), Rag1$^{-/-}$ and Ncr1$^{gfp/gfp}$ (kind gift from Ofer Mandelbaum, Jerusalem) were housed in the animal facility of the Erasmus University Medical Center. Animal procedures were approved by the relevant authorities and performed in accordance with institutional guidelines. Age and gender-matched littermates were used whenever possible.

MTX model. 8-12 weeks mice were injected i.p. with 120mg/Kg MTX (PCH) at day-1 and with 60mg/Kg at day 0. Body weight was daily monitored and tissues were collected at day 1 and day 4 after the last MTX injection.

Radiation model. 8-12 weeks mice were sublethally irradiated at 800cGy. NKp46-targerting antibody (29A1.4, eBioscience) or isotype control (Ig2a) was administered at day 1 (100ug) and day 3 (50ug) after total body irradiation. Body weight was daily monitored and tissues were collected at day 4 and day 12 after irradiation.

NKp46 targeting. 8-12 weeks mice were injected i.p. with 150ug of aNKp46 (29A1.4,eBioscience) or isotype control (IgG2a). Body weight was daily monitored and tissues were collected at the indicated time points.

Histology. Small intestinal tissue pieces (5mm) were fixed in 4% PFA and embedded in paraffin. Four-µm sections were deparaffinized and stained with hematoxylin (Vector Laboratories) and eosin (Sigma-Aldrich). For pStat3 detection endogenous peroxidases were blocked and antigen retrieval was achieved by microwave treatment in citrate buffer (10mM, pH 6.0). Prior to staining, Fc receptors were blocked in blocking solution (Supplementary materials and methods). Tissue sections were incubated overnight at 4°C with rabbit pStat3 primary antibody (D3A7, Cell signaling). Immunoreactions were detected using biotinylated goat-anti-rabbit (Vector Laboratories) and incubated with the Vectastin ABC Elite Kit (Vector Laboratories) and 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were counterstained with hematoxylin.
Pathology was scored blinded by at least two independent analysts by quantification of damage in villus, crypts, epithelium, inflammation and bleeding as previously described [34]. Measurement of pStat3 intensity in IEC from sections was determined using HistoQuest software (TissueGnostics).

Explant cultures. Isolated small intestine was opened longitudinally and cleaned with cold PBS. A piece of 1cm length was cultured in RPMI with 10%FCSi and 1%P/S at 37°C for 24 hours. Protein content of supernatants was determined by enzyme-linked immunosorbent assay (eBioscience) and absorbance was measured at 450nm using Victor X4 (Perkin Elmer). Protein content present in the supernatants was calculated relative to the tissue weight.

Isolation of lamina propria lymphocytes. Isolated small intestine was opened longitudinally and washed with cold HBSS containing Hepes (15 mM), pH 7.2. Tissues were cut in 1cm pieces and incubated in HBSS buffer containing EDTA (Supplementary materials and methods) at 37°C, two times for 20 min to remove epithelium and intraepithelial lymphocytes. The tissues were digested with Collagenase VIII (100U/ml, Sigma) in RPMI at 37°C in a shaker, two times for 1 hour. Supernatants were passed through a 70µm cell strainer and washed in cold HBSS. Pellets were suspended in 90% percoll, overlaid with 40% percoll and centrifuge at 1800rpm for 20 min to allow separation of mononuclear cells (MNC) by density gradient. Interphase was washed and stained with conjugated antibodies (Supplementary materials and methods). Lamina propria lymphocytes were analyzed by flow cytometry (Facs ARIAIII, BD) and ILC3 were sorted as $CD45^{+}Lin^{-}NK1.1^{-}CD127^{+}CD117^{+}CCR6^{+/-}NKp46^{+/-}$

List of monoclonal antibodies

CD45 (30F11, Invitrogen); Lin-biotin (eBioscience): CD19 (1D3), CD3 (145-2C11), CD11c (N418), CD11b (M1/70), Gr1 (RB6-8C5); Streptavidin (Biolegend), NK1.1 (PK136, eBioscience); CD127 (A7R34, eBioscience); CD117 (2B8, BD), NKp46 (2941.4, eBioscience); CCR6 (29-2L17, Biolegend)

Transcript analysis. RNA was extracted using the RNA-XS kit (Machery Nagel) followed by reverse-transcription with random hexamer primers. RNA from sorted cells was amplified according to the manufacturer's protocol (Ovation PicoSL WTA System V2, NuGen). For quantitative PCR, a Neviti Thermal Cycler (Applied Biosystems) and DyNAmo Flash SYBR Green qPCR kit (Finnzymes) were used, with the addition of MgCl2 to a final concentration of 4 mM. All reactions were done in duplicate and are normalized to the expression of Gapdh. Relative expression was calculated by the cycling threshold (CT) method as $2^{-\Delta CT}$.

List of Primers

Gene name	Forward Primer	Reverse Primer
mGapdh	TCAACGGCACAGTCAAG	GCTCCACCCTTCAAGTG
mSocs3	GGAGCCCCTTTGTAGACT	CGGGAAACTTGCTGTG
mBcl2l1	CGTGGCCTTTTTCTCC	GGCTGCTGCATTGTTC
mIl22	CTCCCCCAGTCAGACAG	CAATCGCCTTGATCTCTC
mGmcsf	GACCCGCCTGAAGATATT	ATCCGCATAGGTGGTAAC T
mTnfa	GGGGGCTTCCAGAACT	GGGCCATAGAACTGATGAG

Statistical analysis

Samples were analyzed using unpaired Mann-Whitney test. P values < 0.05 were considered significant. Data are shown as mean ± SEM.

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CHAPTER 6

General discussion

INTRODUCTION

The intestinal barrier is formed by a single layer of specialized epithelial cells that segregates the host from the external environment through a highly regulated process[1]. Maintenance of intestinal homeostasis requires a well-balanced interaction between the microbiota, epithelial cells and the underlying immune system[2,3]. Epithelial breakdown can occur as a consequence of pathogenic infections, intestinal inflammation[4,5] or as a side effect of anti-cancer treatments, which are causative of epithelial damage and often lead to mucositis[6]. Although the events taking place during mucositis have been extensively studied[7], the precise mechanisms behind mucosal healing are still unknown.

The family of innate lymphoid cells (ILCs) has emerged as important players at barrier surfaces where they represent the innate counterpart of T helper cells but without the requirement for specific antigen recognition[8,9], resulting in much faster responses. Expression of the transcription factor Rorγt and the transcriptional inhibitor Id2 delineates type 3 innate lymphoid cells (ILC3)[10-13]. ILC3 are found along the entire intestinal tract[14] where they secrete IL-22, GM-CSF and TNFα among others cytokines in response to APC-derived IL-23 and IL-1β, resembling Th17 effector functions[15-18]. Different subsets of ILC3 have been identified within the small intestine, including lamina propria ILC3, characterized by the expression of natural cytotoxicity receptors (NCRs) and cryptopatch ILC3 that express CCR6 and localize close to intestinal crypts[19-21].

Over the last few years, type 3 innate lymphoid cells (ILC3) have gained interest in mucosal immunology due to their ability to protect epithelial surfaces from bacterial insults[16,19,22]. ILC3 are strategically located close to epithelia cells where they can rapidly mediate innate immune responses[15,16]. Based on the location and the effector functions, we hypothesized that ILC3 contribute to the activation of tissue healing programs after intestinal epithelial damage. The studies described in this thesis aimed to validate this hypothesis and to further understand how ILC3 sense tissue damage and carry out effector functions, with the overall aim to provide knowledge on the mechanisms underlying mucosal healing.

Type 3 innate lymphoid cells in epithelial damage responses

Damage to the intestinal epithelium needs to be repaired in a rapid and efficient manner to guarantee the uptake of water and nutrients while preventing bacterial dissemination[1,5]. Due to their high turnover, epithelial cells from the gastrointestinal tract are frequently targeted by anti-cancer treatments with chemo or radiotherapy[23-25]. Epithelial damage induced by anti-cancer therapies can result in the development of mucositis, which affects

nearly all patients receiving high intensity chemotherapy and limits the effectiveness of cancer treatments[7,26,27]. Although the use of cytotoxic treatments has been present in medical practice for more than six decades[28-30], still little is known about possible ways to ameliorate mucositis and promote mucosal wound healing.

To gain knowledge on the mechanism protecting epithelial barrier after acute intestinal damage, we used methotrexate (MTX), which is a well-established model of intestinal epithelial damage[31,32] to interrogate the role of ILC3 in epithelial responses.

ILC3 promote STAT3 activation and protect intestinal epithelial cells

At present, ILC3-specific KO models are lacking and therefore several complementary models are required to study ILC3 function. Using Rorγt$^{-/-}$ mice and Thy1-depleting strategies in Rag1$^{-/-}$ mice we demonstrated the role of ILC3 in mediating epithelial responses after intestinal damage. In **chapter 2** we showed that after MTX-induced damage, the STAT3 pathway, which is involved in epithelial wound healing[33], is activated. Activation of epithelial STAT3 correlated with the peak of intestinal damage that occurred one day after drug administration, suggesting that after cyclostatic treatment protective programs are rapidly activated in epithelial cells.

When ILC3-deficient Rorγt$^{-/-}$ mice were treated with MTX, pSTAT3 expression was reduced compared to WT animals indicating the need for Rorγt^{+} cells to activate STAT3. However, Rorγt is also expressed by some T cells including mucosal Th17 cells[12] and MTX-induced pathology has been attributed to direct effects on intestinal epithelial cells leading to cell-intrinsic responses[32]. Hence, we next determined whether the effects observed in Rorγt$^{-/-}$ mice were epithelial cell intrinsic and occurred in the absence of adaptive immune cells. Using Rag1$^{-/-}$ mice treated with MTX we showed that activation of STAT3 early after damage is not epithelial cell intrinsic and does not require adaptive immune cells. In contrast, depletion of Thy1^{+} cells in Rag1$^{-/-}$ mice resulted in impaired activation of STAT3, confirming the role of Thy1^{+} cells in early epithelial activation after damage. Because expression of Thy1 has also been described in intestinal stromal cells[34,35] Thy1-depleting strategies could also impact the stromal compartment and in that way influence the results. However, before performing Thy1-depleting experiments in Rag1$^{-/-}$ mice treated with MTX, we validated that the dose and time frame of Thy1-injections were adequate for ILC3 depletion and although we did not observed major morphological changes, specific analysis of intestinal stromal cells after Thy1 depletion would be necessary to clarify whether that is indeed the case.

Since STAT3 is essential for epithelial survival[36] and colonic tissue repair after DSS-induced colitis[37], we next determined whether the impaired STAT3 activation observed in ILC3 deficient mice after MTX treatment translated into increased intestinal pathology. Indeed, histological analysis revealed aggravated pathology in Rorγt$^{-/-}$ mice that was

especially obvious at day 4 after MTX, which is the time point where WT animals started to show signs of recovery. Of note, the number of mucus-producing goblet cells, essential to maintain the mucus layer that covers and protects the epithelium[38], was also decreased in Rorγt$^{-/-}$ mice compared to WT animals, suggesting that the epithelial barrier might be compromised in the absence of ILC3.

Moreover, during damage responses, survival of epithelial cells is regulated by the balance between pro- and anti-apoptotic molecules[5]. In line with the increased pathology observed in Rorγt$^{-/-}$ mice, transcript analysis of small intestinal tissues revealed a shift towards a pro-apoptotic program when compared to WT animals, in line with the aggravated intestinal pathology in Rorγt$^{-/-}$ mice.

Collectively these experiments demonstrated that Rorγt^{+} Thy1^{+} innate lymphoid cells are required for proper epithelial responses after MTX damage. Since ILC3 are amongst the few cells that fit in this category, these findings strongly favored the hypothesis that ILC3 are involved in tissue protection after intestinal damage.

ILC3 response to MTX-induced epithelial damage

To further demonstrate the role of ILC3 in intestinal responses after MTX treatment, we isolated intestinal CCR6^{+} and NKp46^{+} ILC3 at the peak of MTX-induced damage.

Transcript analysis of intestinal ILC3 showed that both subsets of intestinal ILC3, CCR6^{+} and NKp46^{+} ILC3 are activated and transcribe an array of effector cytokines including *Il22*, *Csf2* and *Tnf* indicating the responsiveness of ILC3 to epithelial damage. Similar results were observed in Thy1-depleted Rag1$^{-/-}$ mice that compared to isotype-treated controls expressed significantly lower levels of ILC3-derived cytokines, indicating that the effects observed in Thy1-treated Rag1$^{-/-}$ are likely to be ILC3-mediated.

Since IL-22 and GM-CSF are involved in maintenance of intestinal homeostasis and tissue defense[39,40], expression of these genes by ILC3 after MTX-treatment strongly suggests their direct contribution in epithelial responses. In contrast to IL-22 that mainly signals through receptors expressed by intestinal epithelial cells[39], GM-CSF receptors are expressed by both epithelial and myeloid cells[40] and thus provides two complementary yet distinct regulatory mechanisms to guarantee epithelial homeostasis. On one hand, during infection and injury, GM-CSF controls activation, differentiation and proliferation of myeloid cells[41,42]. In addition, during homeostatic conditions, ILC3 represent the major source of GM-CSF, which activates DCs and macrophages to produce immune-regulatory factors including IL-10 and RA to promote homeostasis and prevent intestinal inflammation[43].

On the other hand, in an acute DSS damage model, GM-CSF has been shown to play an important role in epithelial protection as GM-CSF deficient mice exhibited impaired barrier function and increased vulnerability to colitis[44]. In agreement with these results,

GM-CSF$^{-/-}$ mice exhibited reduced bacterial clearance following enteric bacterial infection with *C. rodentium*[45] and *S. typhymurium*[46]. Moreover, after DSS-induced colitis GM-CSF can be secreted by epithelial cells[40].

Because ILC3 are rapid responders to tissue damage, it seems logical to expect that besides their role during homeostatic conditions, ILC3-derived GM-CSF may play a role in epithelial protection after tissue damage, by either activating DCs or by acting directly on the epithelium to promote tissue repair.

ILC3 maintain intestinal stem cells after MTX-induced damage

Maintenance of fitness in intestinal crypts is of crucial importance as it represents the site where intestinal stem cells reside and proliferate to give rise to progeny that eventually will differentiate into specialized epithelial cells[47]. The high proliferation rate of crypt-residing cells make them highly susceptible to cyclostatic treatments[23]. Under inflammatory conditions such as those occurring during experimental GvHD, crosstalk between ILC3 and intestinal crypts has been proposed[48]. In this GvHD model, ILC3-derived IL-22 protected intestinal crypts from T cell-mediating killing. Considering that ILC3 residing in cryptopatches are located close to intestinal crypts[49] and that ILC3-derived cytokines are known to regulate epithelial homeostasis, one may envision that an active interaction between these two compartments might also take place during tissue injury.

In response to MTX, intestinal pathology in Rorγt$^{-/-}$ mice was especially obvious in the crypt compartment as compared to WT animals. Hence, we performed a detailed examination of the intestinal crypts to further understand the role of ILC3 in crypt protection. Histological analysis of small intestinal tissues of MTX-treated Rorγt$^{-/-}$/Lgr5-GFP chimeras revealed a more severe pathology and a reduction in goblet cell numbers as compared to WT chimeras, recapitulating the phenotype observed Rorγt$^{-/-}$ mice. Importantly, analysis of purified crypts from WT and Rorγt$^{-/-}$ chimeras showed that in the absence of Rorγt^{+} cells, the number of stem cells was compromised after MTX treatment. These findings revealed a previously unappreciated role for ILC3 in preserving intestinal stem cells after tissue insult and established a link between intestinal stem cells and ILC3 after chemotherapeutical-induced intestinal damage. Nonetheless, the precise mechanisms by which ILC3 safeguard intestinal stem cells remains to be identified. In this context, there is increasing awareness that during intestinal epithelial injury such as that occurring during radiation or chemotherapy regimens, loss of Lgr5^{+} stem cells is compensated by the regenerative response of neighboring damage-resistance epithelial cells[23,47,50,51]. Intestinal injury models have shown that quiescent stem cells located at the position +4, including BMI1^{+} cells, are activated after epithelial damage and repopulate Lgr5^{+} stem cells[50,52]. Moreover, additional studies have identified the regenerative capacity of committed label-retaining cells (LRC) including Paneth cells, which upon

epithelial insult convert into Lgr5$^+$ stem cells[53-55]. Therefore, several complementary mechanisms operate during intestinal damage to guarantee epithelial regeneration, and hence additional experiments are required to further understand the contribution of ILC3 in tissue protection and mucosal wound healing. Analysis of single cells by lineage tracing experiments may help to solve some of these issues.

Based on the specific distribution of ILC3 within the small intestine, it is reasonable to speculate that cryptopatch-residing CCR6$^+$ ILC3, that are located close to the intestinal crypts are responsible for stem cell maintenance after MTX-treatment as indicated by the loss of Lgr5$^+$ cells in ILC3-deficient Rorγt$^{-/-}$/Lgr5-GFP chimeras. Although the mechanisms behind stem cell protection remain uncovered, it has been shown that STAT3 is required for crypt stem cell survival[56], which would be in agreement with the impaired STAT3 activation observed in Rorγt$^{-/-}$ mice. Although significant differences in pSTAT3 intensity were not found in the crypt compartment of Rorγt$^{-/-}$ mice compared to WT animals (data not shown), additional experiments are needed to clarify the contribution of STAT3 in Lgr5$^+$ stem cells survival. For instance, analysis of MTX-treatment in epithelial-specific STAT3 KO mice would help to answer this question. Alternatively, *in vitro* culture of intestinal crypts, which are being extensively used to study regenerative response of intestinal tissue[57,58] could be of great benefit to study the effects of ILC3 and ILC3-derived factors on crypt-residing epithelial cell responses.

The role of IL-22 in MTX-induced damage

From all the cytokines present in mucosal tissues, IL-22 plays an outstanding role due to its direct function on epithelial cell homeostasis and tissue defense[39,59,60]. Intestinal ILC3 constitute a major source of IL-22 in the mouse intestine[19,22,60] and hence in the last years efforts have been made to understand the role of ILC3 in mucosal tissues. Studies with the enterogenic bacterium *C. rodentium* have demonstrated the crucial role of ILC3-derived IL-22 in induction of antimicrobial peptides, which are essential to prevent bacterial dissemination[19,61]. In contrast, the role of IL-22 in intestinal damage responses is still poorly understood. However, recent findings have linked STAT3 and IL-22 to mucosal wound healing[62,63].

In our MTX model, epithelial STAT3 was activated at the peak of damage and its impaired activation correlated with an increased pathology. Interestingly, at the peak of damage, intestinal ILC3 are activated and transcribe several genes including IL-22. Therefore, we hypothesized that the effects observed in the absence of ILC3 after MTX treatment could be the result of reduced IL-22 levels. In **chapter 3** we tested this hypothesis and treated IL-22$^{-/-}$ mice with MTX to study epithelial responses.

Histological analysis of IL-22 deficient mice after MTX-induced damage showed prolonged activation of epithelial STAT3 in contrast to WT animals that as explained above, had a transient activation of this pathway one day after treatment and returned to baseline levels by day 4. These experiments seem to indicate that IL-22 is not required for STAT3 activation after MTX. However, the fact that STAT3 was persistently activated could suggest a pre-inflammatory state in IL-22$^{-/-}$ mice, with additional STAT3-activating cytokines being secreted.

It is known that hyperactivation of STAT3 promotes development of gastric cancer[64,65] and colitis-associated cancer[37]. If IL-22$^{-/-}$ mice are pre-disposed to inflammation, they might display activated epithelial responses that mask MTX effects. While at these early time points activated STAT3 appeared to have a protective role, prolonged and exaggerated STAT3 activity can however translate into malignant transformation at later time points[37,66].

In agreement with the maintained pSTAT3 expression, MTX-induced pathology and goblet cell numbers were comparable between IL-22 deficient mice and WT animals.

As expected, antimicrobial responses were impaired in IL-22 deficient mice, confirming previous publications[16,59,67].

Although recent findings have shown the importance of the IL-22-STAT3 axis in mucosal wound healing[62], our data indicate that additional signals can regulate epithelial activation of STAT3. Indeed, published reports have demonstrated the role of IL-6 in STAT3 activation[37]. As IL-6 is involved in inflammatory responses[68,69], it would be interesting to study the contribution of this cytokine to STAT3 activation in IL-22$^{-/-}$ mice. Alternatively, the impaired expression of negative regulators of STAT3 like SOCS3[69] or TGFβ [66] may contribute to the phenotype observed in IL-22$^{-/-}$ mice. Indeed, transcript analysis of *Socs3* although did not reach statistically differences, seemed lower in IL-22$^{-/-}$ mice than in WT animals at day 1 and 4 after MTX, suggesting the impaired function of STAT3 negative regulators. Since pSTAT3 is present before and after MTX treatment in IL-22$^{-/-}$ mice as compared to WT animals, analysis of this and other negative regulators under homeostatic conditions is necessary to clarify STAT3 regulation in IL-22$^{-/-}$ mice.

Nevertheless, these data highlight the importance of a well-balanced IL-22-STAT3 axis in intestinal epithelial responses.

IL-22 controls intestinal stem cell maintenance after MTX-induced damage

Using Rorγt$^{-/-}$/Lgr5-GFP chimeras we showed a loss of stem cells after MTX treatment. The role of IL-22 in protecting intestinal stem cells from immune-mediate killing has been demonstrated in an experimental GvHD model[48]. To determine whether the loss of stem cells observed after MTX in the absence of ILC3 resulted from reduced levels of IL-22, we

neutralized IL-22 in Lgr5-GFP mice during MTX treatment. Indeed, analysis of purified intestinal crypts after MTX treatment revealed reduced numbers of Lgr5-GFP$^+$ cells in IL-22 blocked animals compared to isotype-treated mice. Moreover, analysis of intestinal crypts showed reduced transcription of Lgr5 in α-IL22 treated mice compared to isotype control, confirming the protective role of IL-22 in stem cell maintenance after tissue damage. Nevertheless, whether the protective effects of IL-22 acted directly on stem cells or indirectly on other cell types remains to be investigated. Since Paneth cells are considered to be the niche of stem cells providing important factors for their proliferation and survival[70], they represent a potential candidate to study the IL-22 indirect effects.

Maintenance of intestinal stem cells is of great importance to guarantee tissue regeneration and hence preservation of epithelial barrier properties. Collectively these results unveil the protective role of ILC3-derived IL-22 in tissue protection from chemotherapeutic-mediated intestinal damage by means of stem cell maintenance. Importantly, these findings open a window of opportunity for the design of new strategies in anti-cancer therapies, aimed to minimizing mucosal injury and promoting tissue repair.

Activation of ILC3

As described throughout this thesis, ILC3 have a pivotal role in mucosal tissues due to their capacity to produce effector cytokines both under homeostatic conditions and upon tissue damage. After MTX treatment, intestinal ILC3 respond to intestinal damage by producing several effector cytokines, of which IL-22 plays an outstanding role in tissue protection at the progenitor level. Understanding the signals that drive ILC3 activation are therefore of great interest to further comprehend ILC3 protective functions.

Activation of human ILC3 requires two signals

In the last few years extensive research has shown that production of IL-22 by ILC3 is strongly induced by DC-derived IL-23[16,17]. However, ligation of IL-23R on ILC3 is apparently not sufficient to drive IL-22 production and a second signal is required to promote full activation of ILC3. This second signal can be either cytokines such as IL-1β[71,72] or TLR ligands such as the TLR2 ligand Pam3Cys (P3C)[17].

Using human tonsil-derived ILC3, we showed in **chapter 4** that stimulation with IL-23 leads to suboptimal activation of ILC3 and that indeed addition of either IL-1β or the TLR2 ligand P3C induced higher levels of IL-22, confirming previous findings. Activation of ILC3 by APC-derived cytokines has been mostly studied in the context of bacterial infection, during which DCs release cytokines in response to pathogenic insult. However, during tissue damage different mechanism may influence ILC3 activation. In favor of this idea, it was recently shown that epithelial-derived IL-23 can trigger IL-22 production by ILC3 in a DSS-induced colitis model[73]. Similarly, IL-1β can be secreted by intestinal epithelial cells in

response to DNA damage[74]. Therefore, further experiments are necessary to understand whether epithelial-derived cytokines promote ILC3 function after tissue damage.

Stimulation by soluble cytokines is nevertheless a risky mechanism since it may lead to uncontrolled activation of ILC3 and could promote inflammation by activating effector Th17 cells. Hence, additional mechanisms must exist to ensure local activation of ILC3 at sites of tissue damage only.

NCR are co-activating receptors on human ILC3

After MTX treatment, both CCR6$^+$ and NKp46$^+$ ILC3 were activated, yet their cytokine profiles were different.. At the peak of damage, NKp46$^+$ ILC3 but not CCR6$^+$ ILC3, significantly up-regulated transcription of *Il22* and *Csf2* (chapter 2). These findings corroborate previous publications that demonstrated that NKp46$^+$ ILC3 are the major source of IL-22 in the mucosal tissues[19,22,75]. Importantly these results uncovered for the first time the differential cytokine profile of lamina propria NKp46$^+$ILC3 versus cryptopatch-residing CCR6$^+$ILC3 after intestinal epithelial damage, suggesting a functional dichotomy.

Interestingly, after MTX treatment lamina propria ILC3 increased the expression of NKp46, both at protein and mRNA levels (chapter 2), suggesting that this receptor may be involved in ILC3 activation after epithelial damage. From studies on NK cells we know that NCRs recognize stress-induced ligands which trigger NK cell activity[76]. Therefore it seems logical to think that during intestinal damage, NKp46$^+$ ILC3 may sense similar ligands and become activated. Using human tonsil-derived ILC3 we showed that ligation of NKp30, NKp44 or NKp46 in combination with the TLR2 ligand P3C triggered cytokine production by ILC3, including IL-22, GM-CSF and TNFα. These findings support the results of Glatzer and colleagues that showed that engagement of NKp44 on human ILC3 leads to TNFα production[77]. Moreover, co-culture of epithelial cells expressing the membrane bound NKp30 ligand B7H6 and P3C-stimulated NCR$^+$ ILC3 further validated the co-activating role of NCR on ILC3 in a more physiological scenario.

It is thus tempting to speculate that similar mechanisms may occur during epithelial damage responses *in vivo*. Supporting this concept, we showed that stimulation of epithelial cells with MTX induces B7H6 expression. Therefore, during MTX-induced damage ILC3 may sense stress-induced epithelial NCR ligands, which in turn will drive their activation leading to cytokine production and the subsequently initiation of tissue protective programs.

NKp46 contributes to epithelial protection after MTX-induced damage

Although recent studies have demonstrated that NKp46 is not involved in tissue protection from enteropathogenic bacterial infection[61], our data strongly suggest that during epithelial damage this receptor may be important to regulate ILC3-mediated epithelial activation. Hence in **chapter 5** we tested this hypothesis in an intestinal damage model in

the absence of NKp46. Analysis of MTX-treated Ncr1$^{-/-}$ mice revealed impaired epithelial activation as compared to WT animals that translated into reduced STAT3 activation and increased intestinal pathology. Moreover, blocking of NKp46 in sublethally irradiated mice led to fatal wasting disease.

Surprisingly, NKp46-mediated effects appeared to be independent of IL-22. These findings are in agreement with the results observed in MTX-treated IL-22$^{-/-}$ mice, which showed epithelial STAT3 activation despite the lack of IL-22 (chapter 3). Although both IL-22 and STAT3 are shown to be involved in epithelial responses and mucosal wound healing[62], our data suggest that during epithelial damage such as after MTX treatment both these factors may be required but they function independently of each other.

Nevertheless, the fact that STAT3 levels were reduced in the presence of IL-22 indicates that activation of this pathway during epithelial damage depends on additional signals. Of note, NKp46-mediated activation of NK cells drive IFNγ secretion[78,79] and interestingly IFNγ is secreted by activated NKp46$^+$ILC3 after MTX treatment (chapter 2). Although IFNγ has been implicated in mucosal barrier protection by inducing mucus secretion by goblet cells[21], this cytokine can have adverse effects as it alters tight junction permeability decreasing epithelium resistance[80,81] and can promote intestinal inflammation[82,83].

Concluding remarks

The results of this thesis bring me to propose a model of intestinal damage responses in which ILC3 mediate protective epithelial activation by mechanisms different from those occurring during infectious events (Fig. 1). In this model, cryptopatch-residing CCR6$^+$ILC3 and lamina propria NKp46$^+$ ILC3 respond to epithelial insults yet they are activated and achieve effector functions by distinctive means, indicating a functional dichotomy of intestinal ILC3. Importantly, we demonstrated that ILC3-mediated protective functions are partly carried out by IL-22, which contributed to intestinal stem cell protection after cyclostatic treatment. Although ILC3 contributed to epithelial STAT3 activation, we showed that IL-22 is not required to trigger activation of this pathway during epithelial insult and that additional factors may contribute to epithelial protection. In this context, several ILC3-derived factors are potentially good candidates. For instance, ILC3-derived lymphotoxin β (LTβ) may have a protective role during epithelial insult as it was up-regulated by intestinal ILC3 after MTX-induced damage. This is also suggested by the fact that signaling through the LTβ receptor expressed by intestinal epithelial cells promotes the secretion of the ILC3-activating cytokine IL-23[73] and the recruitment of effector cells[84]. Moreover, it has been shown that in the liver, which is an epithelial organ that shares some features with mucosal organs[85], tissue regeneration depends on LTβ signaling, which promoted STAT3 phosphorylation[86]. One can thus envision that during intestinal damage responses the epithelial-ILC3 crosstalk may require ILC3-derived LTβ and therefore it deserves further exploration.

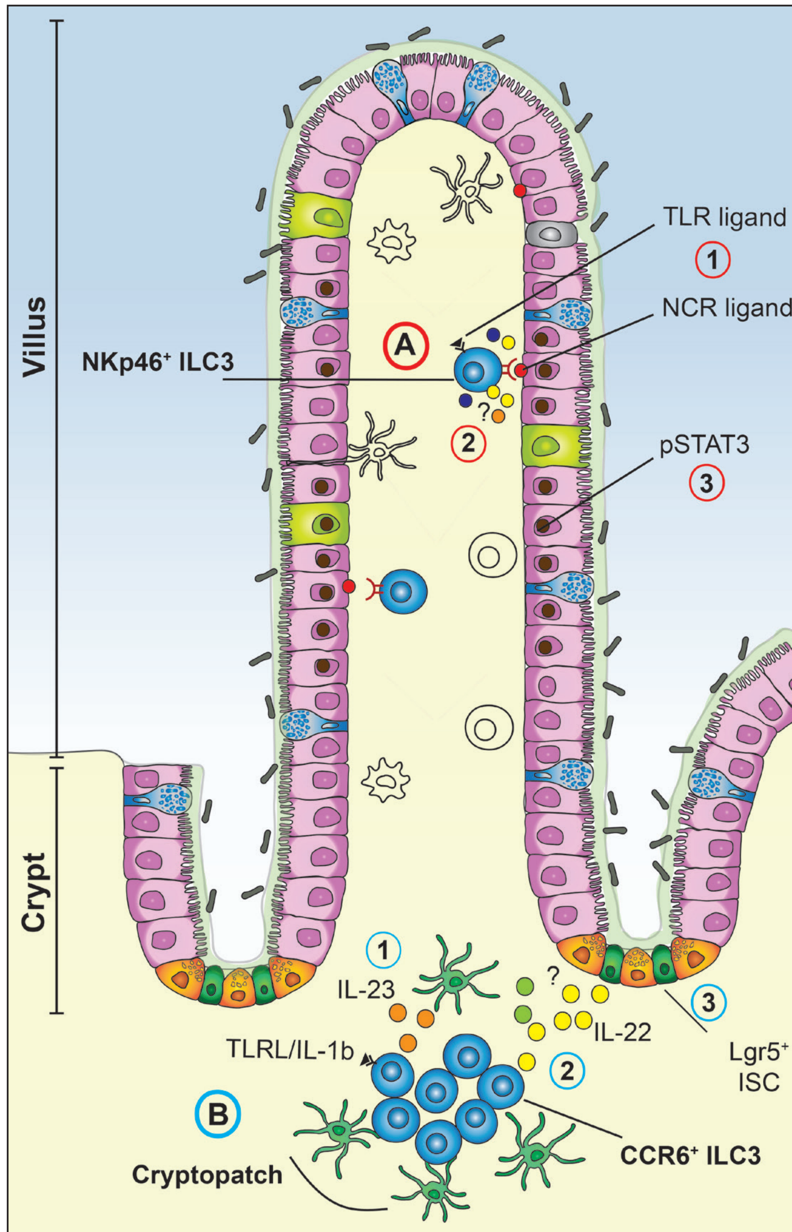

Figure 1. Model of intestinal ILC3 during tissue damage: Functional dichotomy. After epithelium damage, such as that occurring upon cyclostatic insult, both CCR6⁺ ILC3 and NKp46⁺ respond to tissue damage by secreting effector cytokines including IL-22. **(A)** Stress-induced NCR ligands expressed as a result of the epithelial damage are recognized by lamina propria NKp46⁺ ILC3 that in combination with a second signal such as the TLR2 ligand Pam3Cys would drive activation of ILC3 (1). Secretion of effector cytokines by NKp46⁺IL3 would trigger activation of epithelial STAT3 (2). Activation of STAT3 would promote initiation of tissue protective programs in intestinal epithelial cells contributing to mucosal wound healing and tissue repair (3). Exact NKp46⁺ILC3-derived protective signals remain to be determined. **(B)** On the other hand, DC-derived signals, such as IL-23, activates cryptopatch-residing CCR6⁺ILC3 that in combination with TLR ligands or IL-1β promote cytokine production by ILC3 (1). CCR6⁺ ILC3-derived IL-22 contributes to intestinal Lgr5⁺ stem cells protection after intestinal damage (2), which is essential to guarantee epithelium integrity (3). Despite IL-22, additional signals may participate in stem cell maintenance.

Collectively the findings described in this thesis expand our knowledge of ILC3 biology. Besides the well study role of ILC3 in anti-microbial responses we showed that ILC3 preserve organ-specific stem cells in response to tissue insult and that mechanistically, IL-22 is one of the effector molecules involved. Nevertheless, the protective effects observed appeared to be only partially dependent on this cytokine and the question of which additional factors or cell types contribute to stem cell protection remains unsolved.

Decreased STAT3 expression in intestinal epithelial cells occurred in the absence of ILC3 independently of IL-22. Thus it is still unclear which ILC3-derived factors trigger this pathway and whether STAT3 directly participates in the fitness of intestinal epithelial stem cells in chemotherapy-induced damage. STAT3-blocking strategies in MTX treated-Lgr5-GFP mice or experiments neutralizing individual ILC3-derived factors, e.g. LTβ or GM-CSF, during MTX-induced damage might help to answer this question. Moreover establishing co-cultures of intestinal crypts in the presence or absence of ILC3 or ILC3-derived signals and analyzing stem cell activity could contribute to a better understanding of the role of ILC3 in the preservation of intestinal stem cells in response to tissue damage.

Interestingly, ligation of NCR can trigger cytokine production by ILC3. Since ligands for these receptors are thought to be induced upon tissue damage, it is important to further investigate their expression upon chemo- or radiotherapy as a possible mechanism of ILC3 activation upon intestinal damage and whether its expression correlates with ILC3 activity.

In sum, ILC3 location and their resistance to chemo- and radiotherapy-induced cell death put ILC3s in the ideal position to minimize tissue damage after cytotoxic insult. Controlling ILC3 responses, for instance through NCR ligation or through Rorγt agonists, might hold the key to designing future therapeutic strategies aimed at minimizing intestinal damage in patients undergoing anticancer therapies.

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ADDENDUM

SUMMARY

The work described in this thesis had as goal to gain knowledge on the role of ILC3 in mucosal tissues after epithelial damage. In these studies we aimed to characterize the different ILC3 subset present in the small intestine during epithelial injury as well as their mechanisms of activation an effector functions. **Chapter 1** gives an overview of the current knowledge of the cells present in the small intestine and their interactions. Specialized epithelial cells constitute a single layer of cells that separates the host from the external environment. Maintenance of epithelial integrity is of great importance to keep bacteria at bay while providing an adequate absorption of nutrients and water. Breakdown of the epithelial barrier can lead to bacterial translocation and inflammation and therefore different mechanisms have evolved to guarantee efficient regeneration upon tissue damage. Underneath the epithelium, highly regulated crosstalk between multiple types of hematopoietic and epithelial cells ensures a healthy barrier under homeostatic conditions. In the last few years, ILC3 residing in the intestinal tract have gained appreciation as they represent an essential source of IL-22, which is crucial to deal with bacterial infections. Nevertheless, the role of ILC3 in circumstances where infections are not the main driver of mucosal insult is still poorly understood.

In chapter 2, we investigated the role of ILC3 in tissue protection using the cytostatic drug methotrexate (MTX) as an agent to induced small intestinal damage. As a result of cytostatic treatment, damage to epithelium occurs, which can ultimately result in development of mucositis, resembling the complications suffered by patients enrolled in chemo- or radiotherapy regimens. Using Rorγt$^{-/-}$ and Thy1-treated Rag1$^{-/-}$ mice we demonstrated an important role for ILC3 in intestinal tissue protection after MTX treatment. We showed that ILC3 promote activation of epithelial STAT3, which has been implicated in mucosal wound healing. Lack of ILC3 translated into reduced pSTAT3 levels and aggravated intestinal pathology, which was especially obvious in the crypt compartment. Detailed analysis of intestinal crypts after MTX treatment revealed a loss of Lgr5^{+} stem cells in the absence of ILC3, indicating that an active crosstalk between ILC3 and the crypt compartment occurs in response to epithelial insult.

We further validated the functional role of ILC3 during epithelial damage by transcriptional analysis of purified lamina propria CCR6^{+} and NKp46^{+}ILC3, which revealed increased transcription of genes involved in tissue protection, including the cytokine IL-22. Because, IL-22 is known to have an important role in mucosal protection, we next investigated the functional consequence of the absence of this cytokine during epithelial responses to MTX treatment. In **chapter 3**, we addressed this question by treating IL-

$22^{-/-}$ mice with MTX. Surprisingly, analysis of epithelial STAT3 showed comparable levels between WT and L-$22^{-/-}$ mice. In line with the activation of STAT3, intestinal pathology was also not increased in the absence of IL-22. Nevertheless, using IL-22 neutralizing antibodies we showed that IL-22 contributes to stem cell maintenance, as $Lgr5^{+}$ stem cell numbers were decreased in IL-22 blocked animals as compared to isotype-treated mice after MTX administration. However, the results showed that IL-22 is only partially contributing to the phenotype observed in ILC3 deficient mice and hence additional factors are likely to participate in stem cell maintenance after MTX treatment.

Having demonstrated the response of intestinal ILC3 to MTX-mediated epithelial damage and their contribution to epithelial activation, we next explored the mechanisms by which ILC3 sense tissue damage and mediate these effector functions. We noticed that at the peak of damage, intestinal ILC3 up-regulated the expression of NKp46 and interestingly at this time point the population of $NKp46^{+}$ILC3 exhibited a remarkable activated phenotype, suggesting that this receptor may be involved in ILC3 activation after cytostatic treatment. Therefore we next explored the requisites for ILC3 activation and whether the expression of this receptor could confer effector capacity to intestinal ILC3. As described in **chapter 4**, using human tonsil-derived ILC3 we interrogated the role of natural cytotoxicity receptors (NCRs) on human ILC3. *In vitro* assays demonstrated that NKp30, NKp44 and NKp46 function as co-activating receptors that trigger full activation of ILC3 in combination with a second signal that consisted of cytokines such as IL-23, IL-1βb or the TLR2 ligand Pam3Cys (P3C). We further validated these findings using a co-culture system of human epithelial cells and ILC3, in which epithelial cells expressing the membrane bound NKp30 ligand B7H6 co-activated NCR^{+}ILC3 in the presence of P3C. These experiments confirmed the co-activating function of NCRs on ILC3 in the presence of TLR ligands and indicated that activation of NCR^{+}ILC3 can occur in a spatially controlled manner.

Because NCR ligands are induced upon stress conditions, one may envision that these mechanisms take place after epithelial damage, and that $NKp46^{+}$ILC3 would recognize these ligands to become activated and provide protective functions. In **chapter 5,** we determined whether NCRs participate in the response to intestinal damage by studying epithelial responses after MTX treatment in the absence of NKp46, the only NCR expressed by murine ILC3. Using MTX-treated $Ncr1^{-/-}$ mice we showed that NKp46 contributes to epithelial activation after tissue damage. This was illustrated by impaired STAT3 activation and increased intestinal pathology in $Ncr1^{-/-}$ mice as compared to WT controls. Moreover, the protective function of NKp46 was subsequently confirmed using total body irradiation as an additional model for intestinal damage. Using NKp46 targeting

antibodies we showed the protective function of this receptor, as antibody-treated mice lost significantly more weight and ultimately succumbed to radiation-induced damage. However, the NKp46-mediated protective effects appeared to be independent of IL-22, indicating that additional ILC3-derived protective signals are involved after NKp46-mediated activation.

Finally, **chapter 6** discusses the main findings of the studies described in this thesis and places them in a broader perspective.
In sum, we demonstrated an important role for ILC3 in intestinal epithelial responses to tissue damage. Importantly, we identified some of the mechanisms driving ILC3 activation and characterized ILC3 effector functions. Collectively the work in this thesis contributes to the understanding of mucosal wound healing and opens new research opportunities to study tissue protection and repair during anti-cancer treatments.

SAMENVATTING

Het doel van het onderzoek beschreven in dit proefschrift was het verkrijgen van meer inzicht in de rol van ILC3 in mucosaal-weefsel na schade aan het darmepitheel. Tijdens deze studies hebben we de verschillende ILC3 populaties gekarakteriseerd welke aanwezig zijn in de dunne darm tijdens epitheelschade, evenals de wijze van hun activatie en hun effector functies. **Hoofdstuk 1** beschrijft de huidige kennis van de cellen aanwezig in de dunne darm en hun interacties. Gespecialiseerde epitheel cellen vormen een één enkele cellaag welke de gastheer van het externe milieu scheidt. Het onderhouden van de integriteit van deze cellaag is van vitaal belang om bacteriën buiten de gastheer te houden, zonder dat dit ten koste gaat van de normale absorptie van water en voedingsstoffen. Afbraak van de epitheliale barrière kan leiden tot bacteriële translocatie en ontstekingen. Om dit te voorkomen bestaan er verscheidene mechanismen om efficiënte weefsel regeneratie te bevorderen na schade. Onder de epitheel laag zorgt een nauwkeurig gereguleerde communicatie tussen verschillende typen hematopoëtische en epitheliale cellen voor een gezonde barrière tijdens homeostase. In de afgelopen jaren zijn ILC3 welke zich in de darm bevinden, met veel interesse onderzocht vanwege hun rol als essentiële bron van IL-22, een cruciaal cytokine in het verweer tegen bacteriële infecties. Desondanks wordt de rol van ILC3 in omstandigheden waarbij infecties niet de voornaamste oorzaak van mucosale schade zijn, slechts in mindere mate begrepen.

In **hoofdstuk 2** hebben we de rol van ILC3 als beschermers tegen weefselschade onderzocht door gebruik te maken van het cytostaticum methotrexaat (MTX), een medicijn wat weefselschade veroorzaakt in de dunne darm. Als gevolg van de toediening hiervan treedt er schade op aan het epitheel wat uiteindelijk resulteert in het ontstaan van mucositis. Dit hebben wij gebruikt als model voor de complicaties die patiënten ondervinden wanneer zij worden onderworpen aan chemo- of radiotherapie. Door gebruik te maken van Rorγt$^{-/-}$ en Thy1-behandelde Rag1$^{-/-}$ muizen konden we aantonen dat ILC3 een belangrijke rol spelen in de bescherming van het darmweefsel na MTX behandeling. We hebben bewezen dat ILC3 de activatie van STAT3 in epitheel cellen bevorderen, een proces wat geïmpliceerd is in mucosale wond genezing. Het ontbreken van ILC3 leed tot verminderde pSTAT3 en verhoogde darmpathologie, wat zich met name uitte in de crypten. Gedetailleerde analyse van darmcrypten na MTX behandeling toonde een verlies van Lgr5^{+} stamcellen in de afwezigheid van ILC3, suggererend dat ILC3 met het crypt-compartiment communiceert in reactie op schade aan het epitheel.

We hebben vervolgens de functionele rol van ILC3 tijdens epitheelschade gevalideerd aan de hand van transcriptie-analyse van uit de lamina propria gezuiverde CCR6^{+} en

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NKp46$^+$ ILC3. Dit onthulde verhoogde expressie van genen betrokken bij bescherming tegen weefselschade, waaronder het cytokine IL-22. Omdat het bekend is dat IL-22 een belangrijke beschermende rol vervult tijdens epitheelschade hebben we de functionele gevolgen van de afwezigheid van IL-22 onderzocht na MTX behandeling. Dit hebben we in **hoofdstuk 3** gedaan door IL-22$^{-/-}$ muizen te behandelen met MTX. Analyse van epitheliale STAT3 expressie liet geen verschil zien tussen WT en IL-22$^{-/-}$ muizen. In lijn met de activatie van STAT3 observeerde we geen toegenomen darmpathologie in de afwezigheid van IL-22. Desondanks, door gebruik te maken van IL-22-neutraliserende antilichamen konden we aantonen dat IL-22 bijdraagt aan het onderhouden van stamcellen aangezien het aantal Lgr5$^+$ stamcellen verminderd was in dieren waarin IL-22 was geneutraliseerd vergeleken met isotype-controle behandelde dieren, na MTX. Echter, de resultaten toonden dat IL-22 slechts ten dele bijdraagt aan het fenotype zoals geobserveerd in ILC3-deficiente muizen, en dat er dus waarschijnlijk additionele factoren betrokken zijn bij het onderhoud van het aantal stamcellen na MTX behandeling.

Nadat we de effecten van ILC3 op MTX-geïnduceerde epitheelschade en hun bijdrage aan epitheelactivatie hebben laten zien, zijn we vervolgens de mechanismen gaan onderzoeken hoe ILC3 weefselschade herkennen en hierdoor worden geactiveerd. Op het hoogtepunt van schade aan het epitheel vonden we dat ILC3 in de dunne darmen NKp46 opreguleerde en, opvallend genoeg, de populatie NKp46$^+$ ILC3 toonde een geactiveerd fenotype op dit tijdspunt. Dit suggereerde dat deze receptor betrokken is bij de activatie van ILC3 na behandeling met cytostatica. Hierom zijn we gaan onderzoeken welke signalen ILC3 nodig hebben voor hun activatie en of de aanwezigheid van deze receptor betrokken is bij het induceren van ILC3 effector functies. Zoals beschreven in **hoofdstuk 4**, door gebruik te maken van ILC3 geïsoleerd uit humane tonsillen, hebben we de rol van 'natural cytotoxicity' (natuurlijk cytotoxische) receptoren (NCR) op humane ILC3 onderzocht. *In vitro* analyses hebben laten zien dat NKp30, NKp44 en NKp46 functioneren als co-activerende receptoren die volledige activatie van ILC3, wanneer in combinatie met een tweede signaal (bijvoorbeeld IL-23, IL-1beta of de TLR2-ligand Pam3Cys (P3C), induceert. We hebben deze observaties gevalideerd aan de hand van een co-kweek systeem van humane epitheelcellen met ILC3. Deze epitheelcellen, die de membraan-gebonden NKp30-ligand B7H6 tot expressie brengen, co-activeerde NCR$^+$ ILC3 in de aanwezigheid van P3C. Deze experimenten bevestigde de co-activerende functie van NCR receptoren op ILC3 in de aanwezigheid van TLR-liganden en dat NCR$^+$ ILC3 geactiveerd kunnen worden door hun omgeving.

Omdat NCR-liganden worden geïnduceerd door stress is het aannemelijk dat deze mechanismen worden geïnduceerd na epitheelschade, en dat NCR$^+$ ILC3 deze

liganden vervolgens herkennen worden geactiveerd om bescherming te bieden aan het beschadigde epitheel. In **hoofdstuk 5** hebben we onderzocht of NCR receptoren betrokken zijn bij de bescherming tegen epitheelschade door de respons van het epitheel op MTX te bestuderen in de afwezigheid van NKp46, de enige functionele NCR in de muis. Door gebruik te maken van MTX-behandelde Ncr1$^{-/-}$ muizen hebben we kunnen laten zien dat NKp46 bijdraagt aan de activatie van het epitheel na weefselschade. Dit werd geïllustreerd door verminderde STAT3 activatie en verhoogde darmpathologie in Ncr1$^{-/-}$ muizen ten opzichte van WT controles. De beschermende functie van NKp46 is bevestigd in een model van totale lichaamsbestraling als een tweede model voor dunne darm schade. Met anti-NKp46 antilichamen hebben we laten zien dat deze receptor een beschermende rol vervult aangezien antilichaam-behandelde muizen significant meer lichaamsgewicht verloren en uiteindelijk stierven aan de bestralings-geïnduceerde schade. Echter, het NKp46-gemedieerde beschermende effect bleek IL-22 onafhankelijk wat aangeeft dat additionele signalen betrokken zijn bij NKp46-gereguleerde activatie van ILC3.

Als laatst bediscussieert **hoofdstuk 6** de belangrijkste bevindingen van de studies beschreven in dit proefschrift en plaatst deze in een breder perspectief.

In dit proefschrift beschrijven we een belangrijke rol voor ILC3 in de response van het darmepitheel na weefselschade. We hebben enkele mechanismen geïdentificeerd die leiden tot de activatie van ILC3 en hebben hun effector functies gekarakteriseerd. Het werk in dit proefschrift draagt bij aan de kennis over mucosale wondgenezing en opent nieuwe onderzoekswegen met betrekking tot de bescherming en het herstel van het darmepitheel tijdens anti-kanker behandelingen.

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PHD PORTFOLIO

Name PhD student: Patricia Aparicio Domingo
Erasmus MC department: Hematology
Research School: Molecular Medicine
PhD period: 15 August 2010 – 15 April 2015
Promoter: Prof. Dr. J.J. Cornelissen
Co-promoter: Dr. T. Cupedo

Courses & Seminars	ECTS
2011	
Advanced Immunology course Faculty of Medicine, VU University Medical Center and Sanquin Research, Amsterdam.	4,2
Animal Experimentation course Erasmus University Medical Center and Utrecht University.	4,2
The 4th Symposium and master classes on Mucosal Immunology "Adaptive immune response in the mucosa: B cells and beyond" Erasmus Postgraduate School of Medicine, Rotterdam	0,5
2012	
Workshop on Photoshop and Illustrator CS5 Erasmus Postgraduate School of Medicine, Rotterdam	0,3
2013	
The 5th Symposium and master classes on Mucosal Immunology "Cytokines and border patrol" Erasmus Postgraduate School of Medicine, Rotterdam	0,5
Mucosal Immunology symposium AMC, Amsterdam	0,3
2012-2013	
Organizing invited speaker lunch	0,1

2014

Immunology mini-Symposium and master classes on Mucosal Immunology " Immune regulation by dendritic cells at epithelial interfaces" Erasmus Postgraduate School of Medicine, Rotterdam	0,3
Mucosal Immunology Symposium Dutch society for Immunology. Lunteren, The Netherlands	0,5

2010-2015

Erasmus Hematology Lectures	2
AIO/Postdoc presentations	1,5
Journal Clubs	1,5
Work-discussions	7,2

Teaching activities

2013-2014

Supervising and teaching a Master Student	10

Scientific meetings & Conferences

2011

Molecular Medicine Day Oral presentation; Rotterdam, The Netherlands	2
International Congress Mucosal Immunology (ICMI) Oral presentation; Paris, France	2
Annual meeting of the Dutch Society for Immunology (NvvI) Oral presentation; Noordwijkerhout, The Netherlands	2

2012

Molecular Medicine Day Poster presentation; Rotterdam, The Netherlands	1
Annual meeting of the Dutch Society for Immunology (NvvI) Oral presentation; Noordwijkerhout, The Netherlands	2

2013

Molecular Medicine Day Poster presentation; Rotterdam, The Netherlands	1
International Congress Mucosal Immunology (ICMI) Poster presentation; Vancouver, Canada	1
Lymphoid Tissue meeting (LT3) Oral Presentation; Rotterdam, The Netherlands	2
Annual meeting of the Dutch Society for Immunology (NvvI) Poster presentation (1st prize); Noordwijkerhout, The Netherlands	1

2014

Molecular Medicine Day Poster presentation; Rotterdam, The Netherlands	1
International Conference Innate Lymphoid cells (ILC1) Poster presentation; Institute Pasteur. Paris, France	1
Annual meeting of the Dutch Society for Immunology (NvvI) Oral presentation; Efteling, The Netherlands	2

Total ECTS 52

PUBLICATIONS

Aparicio-Domingo P*, Romera-Hernandez M*, Karrich J.J, Cornelissen F, Papazian N, Lindenbergh-Kortleve D, Samsom J.N, Cupedo T. Type 3 innate lymphoid cells maintain intestinal stem cells after tissue damage. J Exp Med. 2015 Oct 19;212(11):1783-91

Romera-Hernandez M, Aparicio-Domingo P, Cupedo T. Damage control: Rorγt+ innate lymphoid cells in tissue regeneration. Curr Opin Immunol. 2013 Apr;25(2):156-60

Hoorweg K, Peters CP, Cornelissen F, Aparicio-Domingo P, Papazian N, Kazemier G, Mjosberg JM, Spits H, Cupedo T. Functional differences between human NKp44- and NKp44+ RORC+ innate lymphoid cells. Front Immunol. 2012 Apr 9;3:72

Aparicio-Domingo P, Cupedo T. Rorγt+ innate lymphoid cells in intestinal homeostasis and immunity. J Innate Immun. 2011;3(6):577-84

Cornelissen F, Aparicio-Domingo P, Reijmers RM, Cupedo T. Activation and effector functions of human RORC+ innate lymphoid cells. Curr Opin Immunol. 2011 Jun; 23(3):361-7

* Equal contribution

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ABOUT THE AUTHOR

Patricia Aparicio Domingo was born on April 25th, 1983 in Madrid, Spain. In 2003 she started her studies in Biology at Alcala University, Madrid. In 2007 she joined the Molecular Genetics Unit at the Spanish Research Council (CSIC)-Biomedicine Institute of Valencia, Spain, for a three-month internship as a research trainee. In 2009 she completed the five-year program of Biological Sciences with a major in Molecular Biology at Alcala University. She studied the last year of her degree under the Erasmus exchange program at the University of Leiden, where she carried out a 9 months internship in the department of Molecular Biology under the supervision of Dr. O. Stockhammer studying the role of the TLR-adaptor protein Sarm in innate immunity and embryogenesis. After completing her internship she enrolled in the Biomolecular Sciences Master's program at the Vrije University of Amsterdam where she graduated in 2010. During her master program, she joined the department of Molecular genetics at the Netherlands Cancer Institute (NKI), Amsterdam, for a 6 months internship where she studied the function of *ERAS* as novel mammary oncogene in the group of Dr. J. Hilkens. In August 2010, she started her PhD studies in the lab of Dr. T. Cupedo in the department of Hematology at the Erasmus University Medical Center, Rotterdam. Her 4.8 years as a PhD candidate led to the studies described in this thesis. In August 2015 she started her postdoctoral studies about the role of stroma cells in secondary lymphoid organs during viral infections, in the group of Dr. S. Luther in the department of Biochemistry at the University of Lausanne in collaboration with the group of Prof. Dr. med. D.D. Pinschewer in the department of Biomedicine at the University of Basel, Switzerland.

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