Characterization of Group 3 Innate Lymphoid Cell Function in the Innate and Adaptive Immune System

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One never notices what has been done; one can only see what remains to be done.

Marie Skłodowska Curie

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1 Summary

Group 3 innate lymphoid cells (ILC3s) play decisive roles in mammalian physiology including lymphoid tissue development, tissue repair and immune regulation. So far, the functions of ILC3s in the adult immune system have been mainly linked to their capacity to release cytokines in response to microbial or inflammatory signals. It could be demonstrated that ILC3s are indispensable for protective immunity against the mouse intestinal pathogen *Citrobacter rodentium* by the early production of IL-22 in response to IL-23 secreted mainly by dendritic cells (DCs) upon microbial exposure. However, whether ILC3s are able to directly sense and respond to the presence of pathogens thereby contributing to innate immunity is not yet known. Furthermore, whether these cells are capable to interact with cells of the adaptive immune system to meaningfully regulate adaptive immune responses has to be explored.

In the present study, I could show that ILC3s directly responded to microbial products such as the Toll-like receptor (TLR) ligands CpG and Poly I:C *in vitro*. They up-regulated the surface expression of the early activation marker CD69 and secreted IL-22, a cytokine known for its protective immune function in the mucosa. Additionally, I could demonstrate that *in vivo* challenge with TLR ligands CpG and LPS was able to induce ILC3 activation *in vivo*. Furthermore, ILC3s produced high amounts of IL-17 and IL-22 upon exposure to the pro-inflammatory cytokine IL-1β. IL-1β emerged as a strong activator of ILC3s as its presence induced the production of a broad range of cytokines by ILC3s. Altogether, the response of ILC3s varied depending on the nature of innate stimuli.

In addition, I could demonstrate that upon IL-1β exposure, peripheral ILC3s up-regulated the expression of surface major histocompatibility complex class II (MHC II) molecules and expressed co-stimulatory molecules reminiscent of an antigen-presenting cell-like phenotype. Further, I found that ILC3s could take up latex beads, process protein antigen (Ag) and consequently prime $CD4^+$ T cell responses *in vitro*. The cognate interaction of ILC3s and $CD4^+$ T cells led to T cell proliferation both *in vitro* and *in vivo*. By using a mouse model with MHC II deficiency exclusively in ILC3s I could demonstrate that the disruption of Ag-dependent interaction of ILC3s and $CD4^+$ T cells impaired specific T cell and T-dependent B cell responses *in vivo*. In addition, I found that IL-1β-activated peripheral ILC3s were more efficient than non-activated ILC3s in the induction of $CD4^+$ T cell responses. ILC3-$CD4^+$ T cell

interactions turned out to be bidirectional and led to the activation of ILC3s. The activating feedback loop of $CD4^+$ T cells to ILC3s was most likely mediated by soluble factors produced by $CD4^+$ T cells upon Ag encounter. Taken together, my data reveal an activation-dependent function of peripheral ILC3s in eliciting cognate $CD4^+$ T cell immune responses, ascribing to them a novel function in adaptive immunity.

Finally, I found that small intestinal ILC3s and peripheral ILC3s differed from each other in regard to their phenotype, responsiveness to IL-1β and immune function. In contrast to peripheral ILC3s, small intestinal ILC3s expressed high levels of CD69 on their surface suggesting an activated phenotype. I could show that CD69 expression was independent of TLR- and IL-1R signaling, the presence of T and B cells, or the microbiota as well as the availability of IL-23. In addition, small intestinal ILC3s were not able to increase the expression of MHC II molecules and to express co-stimulatory molecules upon IL-1β exposure. Although they were able to take up latex beads and to process exogenous Ag, they were far less efficient in $CD4^+$ T cell activation than peripheral ILC3s. However, they were capable to produce high amounts of IL-22 in response to IL-1β stimulation. Taken together, these data suggest that the immune functions of ILC3s are tissue specific and might be regulated by environmental factors and/or interactions with tissue-specific cells.

2 Introduction

2.1 Innate and adaptive immunity

Humans are daily exposed to a variety of bacteria, viruses, fungi and parasites. Our body has to be protected from all these infectious agents by the highly organized and regulated work of our immune system. To fulfill this difficult task, the immune system has established two different branches, first, the innate immunity and second, the adaptive immunity. The perfect interplay of both of these parts is required to get rid of invading pathogens and to clear ongoing infections.

The innate immunity serves as a first line of defense. It is composed of soluble factors (e.g. complement system) and cells that express so-called pattern recognition receptors (PRRs). These germ-line encoded invariant PRRs are able to recognize molecules and regular patterns that are characteristic components of pathogenic microorganisms, known as pathogen associated molecular patterns (PAMPs). PAMPs are present in many microorganisms but not in the body`s own cells providing already an initial discrimination between self and non-self.

The prototypes of PRRs are so-called Toll-like receptors (TLRs; see chapter 2.3.1 TLR ligands induce early immune responses). The innate immune system mainly contains phagocytic cells like macrophages (MΦ) and neutrophils able to engulf pathogens, to digest and destroy them in intracellular vesicles by the production of degradative enzymes and antimicrobial substances. Natural killer (NK) cells are an additional tool of the innate branch of the immune system. These cells are able to recognize molecules on virus-infected and malignantly-transformed cells via germ-line encoded receptors in order to kill these target cells. Upon recognition of the target cell, NK cells release cytotoxic granzymes and the pore forming protein perforin from their cytoplasmic granules penetrating the cell membrane of the target cell and inducing programmed-cell death (see chapter 2.2.1 Group 1 ILCs). In response to an infectious agent, innate immune cells produce a variety of cytokines and chemokines inducing a process known as inflammation. The induction of an inflammation can be beneficial, as released cytokines and chemokines attract additional effector molecules and immune cells from the blood to the site of infection.

The response of the innate branch of the immune system is extremely fast (within hours) and immediately induced after the recognition of an infectious agent. However, innate defenses are not highly specific and cannot lead to immunological memory, a hallmark of the adaptive immunity.

In contrast to innate immune cells, cells of the adaptive immune system are able to recognize pathogens with high specificity by the expression of antigen (Ag)-specific receptors. Thereby, adaptive immune cells can eliminate those pathogens, which managed to overcome innate immunity. Furthermore, a unique feature of the adaptive immunity is the generation of immunological memory providing faster and enhanced protection against re-infections. The key components of the adaptive branch of the immune system are B and T lymphocytes, which develop in the primary lymphoid organs bone marrow (BM) and thymus, respectively. B cells recognize native Ags from the extracellular environment and consequently secrete antibodies (Abs) promoting the killing of extracellular pathogens (humoral immune response) while T cells are specialized to kill target cells infected with intracellular pathogens or viruses (cytotoxic T cells) and to support the humoral immune response (T helper (Th) cells). T cells only recognize Ag-derived peptides, which are presented to them in context of the major histocompatibility complex (MHC) expressed by Ag-presenting cells (APCs; see chapter 2.4.1 Processing and presentation of Ag to $CD4^+$ T cells). Each lymphocyte of the adaptive immune system bears a unique variant of a receptor on its surface, which is generated by random recombination of variable receptor gene segments and the pairing of distinct variable chains. Thus, the whole population of lymphocytes expresses an enormous repertoire of different receptors with highly diverse Ag recognition sites allowing the specific recognition of a wide variety of different pathogens. Clonal selection and subsequent expansion of the lymphocyte carrying the specific Ag-receptor for the invading pathogen is required for complete elimination of the pathogen. Hence, the adaptive immunity is characterized by its high specificity but requires some time (up to days) to become fully established. Further, adaptive immunity is able to generate immunological memory.[1,2]

2.2 The family of innate lymphoid cells

Innate lymphoid cells (ILCs) are the most recently identified constituents of the innate immune system and represent a novel family of hematopoietic effector cells. In recent years, the family of ILCs has continuously grown and several different cell types with unique effector functions are identified. Previously, it was believed that innate lymphocytes are represented by a single lymphoid lineage, the natural killer (NK) cells. Nowadays, several different innate lymphocytes are described and referred in general to as innate lymphoid cells (ILCs).

All members of the ILC family are characterized by a classical lymphoid morphology and the lack of recombination activating gene (Rag)-dependent rearranged Ag receptors. In addition, all of them lack the expression of cell surface molecules that identify other immune cells and are therefore characterized as cell lineage marker negative (lin^-) cells.[3] Furthermore, all ILCs depend on the expression of the common cytokine receptor γ chain (γ_c chain) and the transcriptional repressor inhibitor of DNA binding 2 (Id2) for their development.[3, 4] The different members of the ILC family are found to play crucial roles in the development of lymphoid tissues, tissue repair and wound healing after injury. They have been reported to regulate commensal bacterial communities, to promote inflammation, to contribute to the resistance to helminthes and bacterial pathogens and to be important for maintenance of organ homeostasis. Extensive research in the past few years has led to the discovery of an unprecedented complexity in the innate lymphocyte lineages collectively referred as ILCs. Therefore, three broad categories of ILCs have been defined based on the expression of different transcription factors and the distinct pattern of effector molecules these cells secrete; i) group 1 ILCs, ii) group 2 ILCs and iii) group 3 ILCs (Fig. 1).[3]

Group 1 ILCs are composed of conventional NK cells and a second subset named ILC1s. Cells within the group 1 ILCs are characterized by their dependency on the T-box transcription factor T-bet and the production of interferon (IFN)-γ, thereby resembling T helper type 1 (Th1) cells. Group 2 ILCs produce Th2 related cytokines such as interleukin (IL)-4, IL-5, and IL-13 and are involved in anti-helminth immunity and the development of allergic diseases such as asthma. So far, within the group 2 ILCs only one cell type, the ILC2, is described. The development of ILC2s depends on the transcription factors retinoic acid related orphan receptor (ROR)-α and Gata3. Group 3 ILCs are identified as the producer of Th17-related cytokines IL-17A, IL17F and IL-22. All subsets within this group depend on the transcription factor RORγt. The group 3 ILCs are composed of the classical lymphoid tissue inducer cells (LTi cells) responsible for lymphoid organogenesis during embryogenesis and in neonates and two other populations with a similar phenotype discovered in adults. Adult ILC3s either express the natural cytotoxicity receptor (NCR) NKp46 or are negative for this molecule. NCR^+ILC3s lack the expression of CD4 whereas within the NCR^-ILC3s a $CD4^+$ and $CD4^-$ subpopulation exists. NCR^+ILC3s mainly produce IL-22 and contribute to immunity against intestinal bacteria. NCR^-ILC3s lacking the expression of NKp46 are able to secrete IL-17 and IL-22, and under certain conditions also IFNγ.

Since, the transcriptional and effector program of the various ILC populations resembles those of T helper (Th) cells, ILCs are believed to be the innate counterparts of the adaptive Th cells.

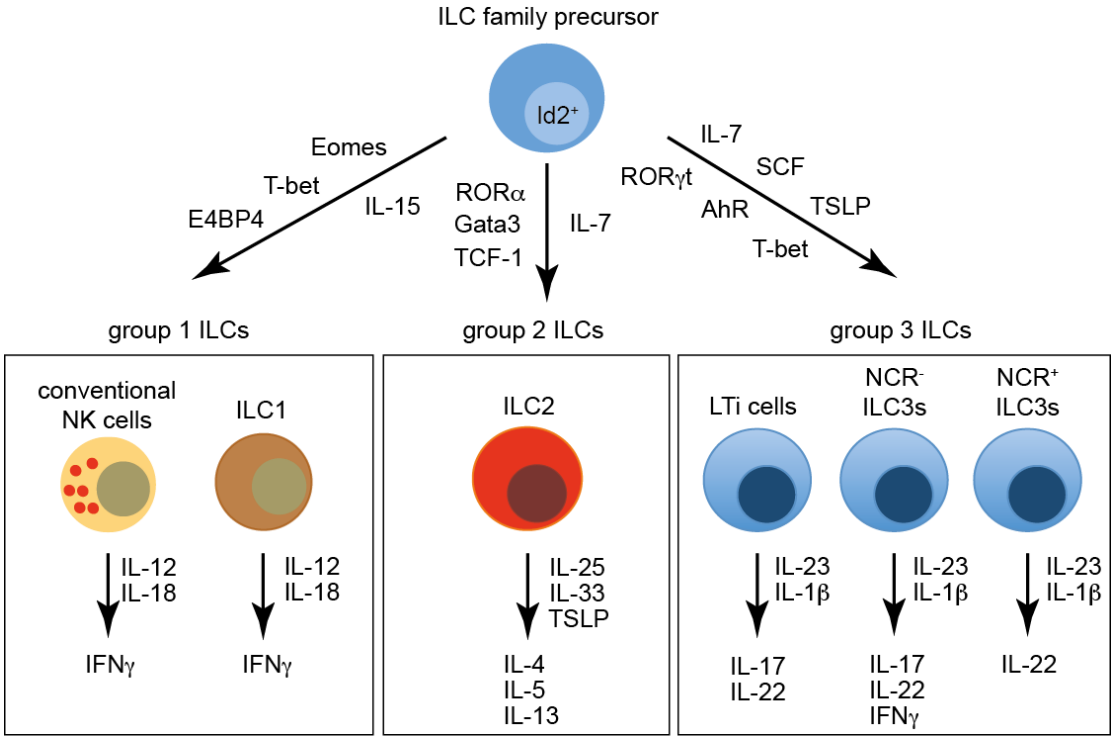


Figure 1: Family of innate lymphoid cells. Simplified illustration of the members of the innate lymphoid cell family, their developmental requirements and cytokine production profiles. AhR: aryl hydrocarbon receptor; ROR: retinoic acid related orphan receptor; SCF: stem cell factor; TCF-1: T cell factor 1; TSLP: thymic stromal lymphopoietin. Adapted from Spits *et al*., Nature Review (2013)[3] and Artis *et al*., Nature (2015).[5]

2.2.1 Group 1 ILCs

Natural killer cells

The first described subset of group 1 ILCs are NK cells. NK cells have been first identified in 1975 as innate effector lymphocytes exhibiting cytotoxic activity against tumor cells.[6, 7] Later, NK cells have been found to be important during viral infections.[8] Mature NK cells can be found in different tissues such as spleen, lymph nodes (LNs), liver, lung, skin and blood. They express several different germline encoded activating receptors such as NKp46 (also known as NCR1, natural cytotoxicity triggering receptor 1) and NK1.1 as well as inhibitory receptors (e.g. Ly49) on their surface. The cytotoxicity of NK cells is regulated by the net signaling of these activating and inhibitory receptors[9] and the cytotoxic activity of NK cells is exhibited via a variety of effector molecules such as perforin, granzymes, Fas ligand

(FasL) and TNF-related apoptosis inducing ligand (TRAIL) inducing apoptosis.[10, 11] Hence NK cells are crucial for the defense against viral infections and tumors and the amplification of inflammatory responses through the induction of TNFα and high levels of IFNγ production.

Conventional NK cells differentiate from the common lymphoid progenitor (CLP) in the BM and their development requires the transcription factor E4BP4 (also known as NFIL3, nuclear factor IL-3)[12, 13] and the cytokine IL-15.[14, 15, 16] NK cells co-express the T-box transcription factors eomesodermin (Eomes) and T-bet, which cooperate in the regulation of the development, maturation and function of NK cells.[17] Although NK cells are the only cytotoxic innate lymphocytes of the ILC family, these cells are classified within the group 1 ILCs based on the expression of T-bet and the production of IFNγ.

ILC1s

So far, ILC1s within the group 1 ILCs are not well defined and characterized. To date, under the term ILC1 several different cell types are described. ILC1s are currently believed to be either intraepithelial ILC1s[18] or so called "ex-RORγt^{+}" ILC3s.[19, 20, 21] Very recently, another distinct ILC1 cell type has been discovered in the small intestine.[22]

Intraepithelial ILC1s are first discovered in human tonsils and small intestine as cells expressing transcription factors Eomes and T-bet, and NK cell surface molecule NKp44 whereas they lack expression of the transcription factors RORγt and aryl hydrocarbon receptor (AhR), which both are crucial for ILC3 development.[18] In addition, intraepithelial ILC1s express intraepithelial lymphocyte markers CD103, CD101 and CD160 and a specific integrin repertoire (e.g. β7 integrin, CD49a) allowing their intraepithelial localization. Further, intraepithelial ILC1s produce IFNγ in response to IL-12 and IL-15 stimulation *in vitro* while lacking IL-22 production. These findings clearly separate intraepithelial ILC1s from ILC3s. Interestingly, some of the intraepithelial ILC1s contain intracellular perforin and granzyme and exhibit lytic activity in presence of a tumor cell line.[18] The murine counterparts of human intraepithelial ILC1s have been identified as cells expressing NKp46 and NK1.1 as well as CD160 distinguishing them from murine splenic NK cells, which do not express CD160 at steady state. Murine intraepithelial ILC1s produce IFNγ in response to IL-12 and IL-15 *in vitro* mirroring the human ILC1 response.[18] Intraepithelial ILC1s are present in RORγt-deficient (*RORγ*$^{-/-}$) and in AhR-deficient (*AhR*$^{-/-}$) mice, both lacking ILC3s, but in contrast, they are absent in mice deficient in

T-bet or NFIL3, two transcription factors previously shown to be master regulators of NK cell development.[17, 23] This suggests a developmental relationship rather between intraepithelial ILC1s and conventional NK cells than between ILC1s and ILC3s. However, analysis of IL-15-receptor-α-chain-deficient (*IL-15Rα*$^{-/-}$) mice does not support this idea. *IL-15Rα*$^{-/-}$ mice show a complete absence of splenic NK cells[24] whereas intraepithelial ILC1s are minimally affected by the lack of IL-15Rα suggesting that intraepithelial ILC1s are a unique ILC1 cell subset distinct from conventional NK cells.[18]

In RORγt-fate map mice, generated by crossing mice expressing Cre recombinase under the control of the Rorc promotor (Rorc(γt)-Cre^{tg} mice)[25] with Rosa26-reporter mice (R26R-EYFP)[26], all cells derived from RORγt^{+} precursors are heritably and permanently labeled by the enhanced yellow fluorescence protein (EYFP)-reporter, even when they loose RORγt expression over further development. By following small intestine-derived RORγt-fate map-positive (RORγt^{fm+}) cells transferred into alymphoid mice, it could be shown that RORγt^{+}NKp46^{+}RORγt^{fm+} cells (RORγt^{+}NKp46^{+} ILC3s) are able to develop into RORγt^{-}NKp46^{+}RORγt^{fm+} cells.[19] These RORγt^{-}NKp46^{+}RORγt^{fm+} cells have lost RORγt expression, however, they are originally derived from RORγt^{+} cells indicated by positive fate-map labeling (RORγt^{fm+}). *In vitro* stimulation with different cytokine (e.g. IL-12 or IL-23) revealed that whereas RORγt^{+}NKp46^{+}RORγt^{fm+} ILC3s produce IL-22 and lack IFNγ production, the RORγt^{-}NKp46^{+}RORγt^{fm+} cells mainly secrete IFNγ while IL-22 production is missing.[19] Therefore, RORγt^{-}NKp46^{+}RORγt^{fm+} cells are categorized as ILC1s but are most probably "ex-RORγt^{+}" ILC3s. Additionally, these ex-RORγt^{+}-ILC3-ILC1s have been suggested to be involved in the pathogenesis of anti-CD40 induced colitis by their massive production of IFNγ.[19]

Another study has identified a cell type with ILC1 phenotype and high IFNγ production accumulating in the gut during dextran sodium sulfate (DSS) colitis in mice with a human immune system or in human patients with Crohn`s disease.[20] Originally, this ILC1 type of cell has been identified in human tonsils characterized by high levels of T-bet expression required for IFNγ production[27] and low levels of RORγt expression suggesting that these cells are originally derived from ILC3s. Supporting this idea, it has been shown that these tonsil ILC1s are distinct from conventional NK cells as they lack perforin and granzyme B. In addition, they do not express NK cell markers CD94 and CD56 and lack the expression of the IL-15Rα chain of the IL-15R, which is essential for the development of NK cells.[14] In patients

with Crohn`s disease the frequency of such ILC1s in the gut is significantly increased compared to controls without inflammatory bowel disease (IBD) and gut ILC1s express high levels of IFNγ.[20] That these ILC1s might also be "ex-RORγt^{+}" ILC3s is additionally supported by the fact that RORγt^{+} fetal gut-derived NKp44^{+} ILC3s are able to differentiate into ILC1s when cultured with IL-2 and IL-12 *in vitro*.

The T-box transcription factor T-bet is a central regulator of type 1 immunity by controlling the expression of IFNγ.[27, 28] Analysis of T-bet-deficient mice (*Tbx21*$^{-/-}$ mice)[29] and adoptive transfer of RORγt^{+} ILCs into alymphoid mice could show that the acquisition of T-bet expression is required for RORγt^{+} ILCs to express NKp46 and to produce IFNγ.[21] That T-bet expression is required for proper IFNγ production by ILCs has been as well described in a mouse model of ulcerative colitis (UC).[30] In so-called TRUC mice (*Tbx21*$^{-/-}$*Rag2*$^{-/-}$ ulcerative colitis mice), which spontaneously develop IBD due to the lack of T-bet,[31] ILCs mainly produce IL-17 promoting the disease whereas their IFNγ production is missing.[30] A report from Klose *et al.* suggested that ex-RORγt^{+}-ILC3-ILC1s develop from RORγt^{+} ILC3s, which expand after birth through AhR signals, up-regulate T-bet expression required for IFNγ production and simultaneously down-regulate RORγt expression.[21] Such ex-RORγt^{+}-ILC3-ILC1s have been identified as the main IFNγ producer in an intestinal infection model of *Salmonella typhimurium*.[21] IFNγ produced by these ILC1s in response to *S. typhimurium* infection has diverse roles. It has been shown to control the mucin release by goblet cells and thereby protecting the epithelial barrier, however, it is as well able to promote enterocolitis.[21]

Recently, Klose *et al*. have identified another ILC1 cell type in the small intestine expressing the activating NK cell receptors NKp46 and NK1.1, and T-bet whereas the expression of Eomes and RORγt is absent.[22] Studies using the RORγt^{fm} mouse showed that these cells are RORγt-fate map negative (RORγt^{fm-}) and therefore do not represent a subset of "ex-RORγt^{+}" ILC3s.[22] Transcriptome analysis of these ILC1s revealed that they have only low cytotoxic activity but, however, show high levels of *IFNγ* and *TNF* gene expression.[22] In an intracellular infection model, in which mice are orally infected with the parasite *Toxoplasma gondii*, ILC1s have been identified as the main producer of IFNγ and TNF promoting the control of parasite replication. Furthermore, adoptive transfer of ILC1s into alymphoid mice after *T. gondii* infection resulted in the production of IFNγ and TNF and subsequently to a substantial reduction of *Toxoplasma* titers.[22] These findings indicate that those recently discovered ILC1s are crucial during intracellular infections. Due to the fact that these

ILC1s lack the expression of Eomes and are RORγt^{fm-} they develop independently of the NK cell as well as the ILC3 lineage suggesting that they are an individual ILC1 lineage.[22]

Collectively, several different cell types with different phenotypic and functional properties are described as ILC1s. In general, ILC1s are cells with a markedly production of IFNγ and the expression of T-bet mirroring Th1 type of cells. The plasticity between ILC1s and ILC3s is still under debate. Moreover, the relationship between ILC1s and conventional NK cells is not fully understood. Further research is required to identify precursor populations and developmental requirements of these cell types.

2.2.2 Group 2 ILCs

Innate lymphocytes capable to produce type 2 cytokines and thereby mirroring Th2 cells are categorized within group 2 ILCs. The existence of innate lymphocytes dedicated to type 2 cytokine production is already reported in 2001. Fort *et al*. demonstrated that *in vivo* IL-25 administration induced the production of IL-5 and IL-13 in *Rag2*$^{-/-}$ mice lacking conventional T and B cells.[32] Subsequently a non-T/non-B FcεR1-negative (non-mast cell) cell population able to produce type 2 cytokines in response to IL-25 was identified and shown to be crucial at the onset of helminth infections.[33] In 2010 three different groups independently of each other identified and further characterized such type 2 cytokine producing innate lymphocytes. These cells, identified by the different groups, have been referred to as natural helper cells,[34] nuocytes[35] and innate helper 2 cells (Ih2 cells).[36] However, recently it has been agreed that these cells can collectively be referred to as ILC2s due to their common production of type 2 cytokines IL-4, IL-5 and IL-13.[3, 34, 35, 36]

ILC2s can be found in mesenteric fat-associated lymphoid clusters (FALCs), a newly identified lymphoid structure associated with adipose tissue in the mouse peritoneal cavity.[34] Additionally, ILC2s are present in mesenteric LNs, liver, spleen and intestine[34, 35, 36] as well as in the airways. [37, 38, 39, 40, 41]

ILC2s can be found in *Rag2*$^{-/-}$ mice whereas they are absent in *Rag2*$^{-/-}\gamma_c^{-/-}$ mice indicating their dependence on γ_c receptor signaling.[34, 35, 36] Additionally, IL-7-deficient (*IL-7*$^{-/-}$) or IL-7-receptor-α-chain-deficient (*IL-7Rα*$^{-/-}$) mice as well as mice with mutations in either SCF or CD117 show reduced numbers or complete absence of ILC2s while the presence of IL-7 in *in vitro* cultures induces proliferation of ILC2s.[34, 42] These findings suggest a role for IL-7 and SCF in the development and maintenance

of ILC2s. Further, ILC2s are present in IL-15-deficient (*IL-15*$^{-/-}$) mice lacking NK cells and in *AhR*$^{-/-}$ mice, which are diminished of ILC3s.[43] In contrast to ILC3s, ILC2s develop independent of RORγt as *RORγ*$^{-/-}$ mice show normal ILC2 numbers.[34] Hence, another member of the ROR family of transcription factors, RORα, plays an essential role in the development and function of ILC2s.[42, 44] Studies with staggerer mice (*RORα*$^{sg/sg}$ mice),[45] which have a spontaneous deletion in *Rora*, showed that ILC2s do not develop in absence of RORα.[42, 44] Additionally, transplantation of total BM cells from *RORα*$^{sg/sg}$ mice into irradiated recipient mice fail to generate ILC2s and recipient mice show an impaired immunity to parasitic helminthes.[42, 44] In addition to Id2, which is absolutely necessary for the development of all innate lymphocytes,[4, 34] the transcription factors Gata3 and TCF-1 (T cell-specific high-mobility group box transcription factor) have been shown to be required for the differentiation of ILC2s.[42, 43, 46, 47] The majority of ILC2s continuously express high levels of Gata3 and mice with a temporally deletion of Gata3 revealed that Gata3 expression is required for the differentiation of ILC2s from their precursors as well as the maintenance of ILC2s.[43] TCF-1-deficient mice (*Tcf7*$^{-/-}$ mice; *Tcf7* is the gene encoding TCF-1) are not able to generate functional ILC2s[46] probably due to the lack of immature ILC2 progenitors[43, 44] in the BM. *Tcf7*$^{-/-}$ mice are unable to mount an efficient immune response upon intranasal challenge with papain (inducing protease-mediated airway inflammation) or upon helminth infection with *Nippostrongylus brasiliensis* that are both infection models in which ILC2s have been demonstrated to promote protective immunity through their production of IL-5 and IL-13.[44, 46] In both infection models, the transfer of *WT* ILC2s into *Tcf7*$^{-/-}$ mice is sufficient to restore ILC2 numbers in the lung and for worm clearance, respectively.[46] Recently, it has been shown that the development of ILC2s also requires Notch signaling.[42, 46] Notch signaling has been reported to support the development of ILC2s *in vitro* as it could be demonstrated that BM-derived common lymphoid progenitors (CLPs) cultured on OP-9 stromal cells expressing the Notch ligand Delta-like 1 (OP9-DL1 cells) can give rise to ILC2 in the presence of IL-7 and IL-33.[42] Normally, OP9-DL1 cells support T cell development from BM-derived CLPs in the presence of IL-7 and fms-like tyrosine kinase 3 ligand (FLT3L).[48] Additionally, multipotent BM precursors in which dominant-negative Mastermind like-1 (dnMAML), a pan-Notch inhibitor is retrovirally induced, fail to differentiate into ILC2s *in vivo*.[46] At the current state of research, ILC2s are phenotypically characterized as cells lacking markers of other lineages (lin$^-$), and simultaneously expressing CD90, IL-7Rα, IL-25R (IL-17BR), IL-33R (T1/ST-2), KLRG1 and high levels of Sca-1 and Gata3.[34, 35, 36]

ILC2s as a prominent source of type 2 cytokines provide protective immunity against helminth infections.[34, 35, 36, 43] ILC2s are able to expand at different anatomical sites in response to IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) mainly produced by epithelial cells during infections. The production of IL-5 and IL-13 by ILC2s results in eosinophilia and goblet cell hyperplasia increasing mucus production. Additionally it has been reported that ILC2s through the production of IL-5 and IL-6 support the self-renewal of B1 B cells *in vitro* and *in vivo*.[34] B1 B cells are most abundant in the peritoneal cavity and play a critical role in innate type immune responses by the production of natural antibodies.[49] Further, ILC2s have been implicated in allergic airway inflammation as well as in lung epithelial tissue repair, thus, playing both damaging and tissue protective roles.[37, 38, 39, 40, 41] A study showed that ILC2s are able to mediate virus-induced airway hyper-reactivity (AHR) through their responsiveness to IL-33, which is produced by alveolar MΦ accumulating during influenza-induced AHR in the lung. Depletion of ILC2s in influenza-infected mice significantly alleviated AHR whereas adoptive transfer of *WT* ILC2s into *IL-13*$^{-/-}$ mice, unable to establish AHR due to the lack of IL-13, is sufficient to induce AHR.[39] In contrast, another study revealed that the depletion of ILC2s in mice infected with another influenza virus strain severely worsened virus-induced AHR. In this study, depletion of ILC2s or blocking of IL-33R signaling through administration of blocking IL-33R mAb, results in diminished lung function and impaired ability to generate hyperplastic epithelial cell responses. It could be shown that the tissue protective role of ILC2s during AHR reported in this study is mediated by the production of amphiregulin, a member of the epidermal growth factor family. Administration of recombinant amphiregulin into ILC2-depleted mice is able to restore airway epithelial integrity and lung function reducing AHR.[41]

ILC2s and T cells

Several reports showed that ILC2s play important roles in a variety of innate immune responses. They are crucial in immunity against helminthes, in allergy and asthma as well as in tissue repair and remodeling mediated via their production of various cytokines such as IL-5 and IL-13, and growth factors (e.g. amphiregulin). It could be shown that in the absence of T and B cells (*Rag2*$^{-/-}$ mice) ILC2s are able to expand and to produce type 2 cytokines upon *N. brasiliensis* infection, although not efficiently enough to expel the worm infection.[34, 35] *N. brasiliensis* infection in *Rag2*$^{-/-}$ mice induce rapid expansion of ILC2s 4 days post infection. However, ILC2 numbers cannot be maintained in the absence of T and B cells.[35] This finding suggests that expanded ILC2s are maintained by the presence of T cells (B cells have been show

to be dispensable for worm expulsion)[50] and gives a first hint for a possible dialog between ILC2s and T cells. In fact, within the last year, several reports demonstrated that ILC2s and T cells interact with each other to promote immunity.[51, 52, 53, 54]

In vitro studies showed that co-culture of ILC2s and $CD4^+$ T cells, which are activated through anti-CD3/anti-CD28 coated on the plate, results in proliferation of ILC2s and enhances their production of Th2 cytokines.[51] This effect is independent of cell-cell contact and occurs over soluble factors shown by the use of a transwell culture system in which ILC2s and $CD4^+$ T cells have been separated by a culture insert.[51] IL-2 produced by activated $CD4^+$ T cells has been reported to be responsible for the proliferation and increased type 2 cytokine production by ILC2s, since addition of neutralizing IL-2 mAb to co-cultures markedly impaired proliferation and cytokine secretion of ILC2s.[51] The crucial role of IL-2 for ILC2 expansion and activation has been confirmed by the finding that *Rag2*$^{-/-}$ mice treated with IL-2/anti-IL-2 complexes are able to efficiently expel worms upon *N. brasiliensis* infection although $CD4^+$ T cells are missing. The rapid worm expulsion correlates with elevated ILC2 numbers.[54] Therefore, the presence of activated $CD4^+$ T cells producing IL-2 induces the expansion of ILC2s and enhances their type 2 cytokine production. Furthermore, it has been shown that the culture of naïve $CD4^+$ T cells in the presence of plate-bound anti-CD3/anti-CD28 results in proliferation of $CD4^+$ T cells and addition of ILC2s even increases the proliferation of $CD4^+$ T cells and also enhances their production of type 2 cytokines.[51, 53] It has been reported that this effect is dependent on cell-cell contact via OX40-ligand (OX40L) expressed on ILC2s.[53] Thus, the presence of ILC2s induces T cell proliferation and supports their differentiation into Th2 cells. These data could be confirmed in *in vivo* studies using two different mouse models with an ablation of ILC2s. Mice lacking ILC2s (iCOS-T or *Ror*$\alpha^{fl/sg}$*Il7r*^{Cre} mice) showed delayed worm expulsion upon *N. brasiliensis* infection and a dramatic reduction of IL-5 and IL-13-producing $CD4^+$ T cells.[54] Furthermore, adoptive transfer of both $CD4^+$ T cells and ILC2s into *IL-7R*$\alpha^{-/-}$ mice lacking ILC2s and T cells results in robust airway eosinophilia upon Ovalbumin (OVA) Ag plus bromelain (a cysteine protease) exposure. Transfer of either $CD4^+$ T cells or ILC2s only shows minimal airway inflammation.[53] Therefore, the *in vivo* interplay between ILC2s and $CD4^+$ T cells is critical for the generation of Th2 cell immunity.

ILC2s are shown to express major histocompatibility complex class II (MHC II) on their surface.[35, 51, 54] This fact raised the question whether ILC2s can act as Ag-presenting cells (APCs). Indeed, ILC2s have been reported to be able to induce $CD4^+$ T cell proliferation in the presence of peptide-Ag *in vitro* and preferentially

induce the production of type 2 cytokines by $CD4^+$ T cells.[51, 54] Detailed analysis showed that ILC2s are able to endocytose soluble Ag and to degrade OVA-DQ, a self-quenched conjugate of Ovalbumin protein that fluoresces when cleaved.[54] Although ILC2s are able to process and present Ag on their surface, confirmed by the presence of E-alpha (Eα)-derived peptides in context with MHC II on the surface of ILC2s 20 h after incubation with the Eα-green fluorescent protein (GFP) fusion protein, ILC2s are not able to induce $CD4^+$ T cell proliferation in the presence of whole protein-Ag *in vitro*.[54] In addition to MHC II, a proportion of ILC2s are reported to express CD80 and CD86 on the surface. Blocking of CD80 and CD86 with neutralizing Abs in co-cultures of ILC2s and $CD4^+$ T cells with peptide has been shown to decrease $CD4^+$ T cell proliferation and production of type 2 cytokines suggesting another cell-cell contact-dependent way of T cell activation by ILC2s. In addition, *in vivo* models demonstrated that IL-13 production by ILC2s is most crucial for the generation of efficient Th2 responses. Intranasal administration of IL-13 or adoptive transfer of IL-13-producing *WT* ILC2s is sufficient to rescue Th2 differentiation of $CD4^+$ T cells *in vivo*.[52] In this study, a role for ILC2-derived IL-13 in the migration of activated DCs to the lung draining LNs and the subsequent support of Th2 differentiation in the LNs is suggested.[52] Collectively, ILC2s and $CD4^+$ T cells are able to interact via soluble factors, surface molecules and Ag-MHC II complexes. The presence of both cell types and their interaction is required to increase ILC2- as well as $CD4^+$ T cell-mediated Th2 immunity. The so far identified immune functions of ILC2s are summarized in figure 2 (Fig. 2).

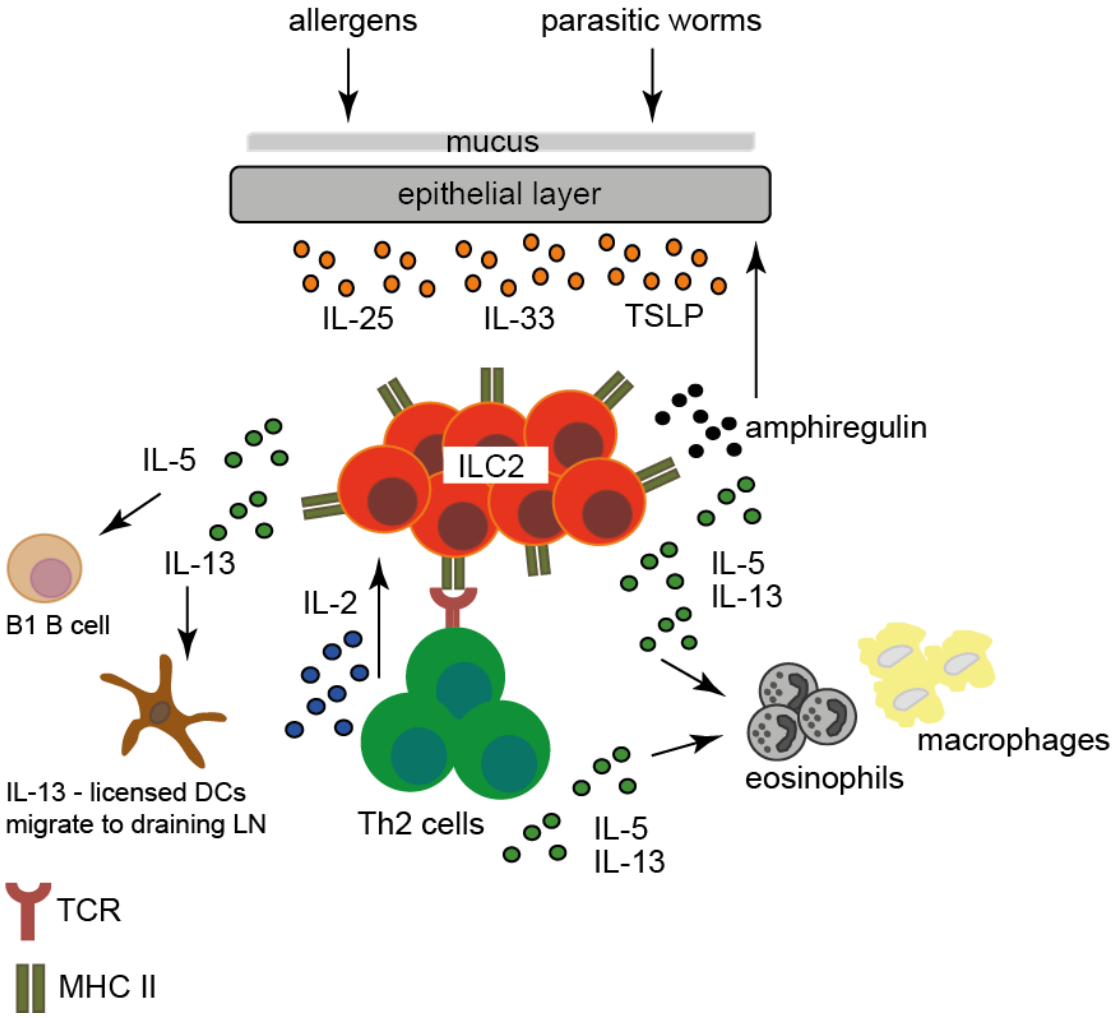


Figure 2: Type 2 immunity and homeostasis mediated by ILC2s. Illustration of the different functions of ILC2s during type 2 immunity and tissue homeostasis. LN: lymph nodes; DC: dendritic cells; TCR: T cell receptor; TSLP: thymic stromal lymphopoietin; MHC II: major histocompatibility complex class II. Adapted from Mc Kenzie *et al*., Immunity (2014).[55]

2.2.3 Group 3 ILCs

Lymphoid tissue inducer cells

The prototypes of the nowadays called ILC3s are lymphoid tissue inducer cells (LTi cells). LTi cells are first discovered in 1992 as a novel population of $CD45^+CD3^-CD4^+$ cells accumulating in the LN during the first days of life.[56] Additionally, these cells are found to be present in the spleen of neonates showing lymphoid size and morphology.

Several years later, $CD45^+CD3^-CD4^+$ cells were identified as the first cells colonizing the fetal LN anlagen, spleen (at embryonic day E13.5), small intestine, stomach and colon, however, they were found to be absent in the fetal liver (FL) and thymus.[57, 58]

$CD45^+CD3^-CD4^+$ cells belong to the hematopoietic lineage ($CD45^+$) and develop independently of the presence of spleen or thymus. A more detailed analysis of the phenotype of these cells revealed that they are negative for other lymphoid, myeloid and erythroid markers. T cell receptor (TCR) and B cell receptor (BCR) genes are found to be in germ line configuration and the mRNA for the recombination activating genes *Rag1* and *Rag2* are missing. However, these cells show expression of the common gamma (γ_c) chain (CD132, IL-2Rγ), CD25 (IL-2Rα), IL-7Rα, CD117, CD90 and lymphotoxin (LT)-β.[57] Additionally, $CD45^+CD3^-CD4^+$ cells express the homing receptor integrin $\alpha_4\beta_7$, which is a ligand for the mucosal vascular addressin cell adhesion molecule (MadCAM-1) that is exclusively expressed on high endothelial venules (HEV) in peripheral and mesenteric LNs until 24 hours after birth.[59] Therefore, $\alpha_4\beta_7$ expression allows the selective entry of LTβ-expressing $CD45^+CD3^-CD4^+$ cells into the developing LN, a fact, which together with the finding that $CD45^+CD3^-CD4^+$ cells are amongst the earliest hematopoietic cells colonizing fetal LN anlagen, the fetal spleen and intestine already suggests a role for these cells in lymphoid tissue development during ontogeny.[57, 58] However, the contribution of $CD45^+CD3^-CD4^+$ cells to lymphoid tissue development and the molecular and cellular events behind it, were still not known at that time.

Origin and differentiation of LTi cells

Two different groups independently of each other could identify a precursor population for LTi cells.[60, 61] Mebius *et al*. found an $IL\text{-}7R\alpha^+Sca\text{-}1^{low}CD117^{low}$ population in the FL at E12.5-E14.5 showing differentiation potential to $CD45^+CD3^-CD4^+$ cells.[60] $IL\text{-}7R\alpha^+Sca\text{-}1^{low}CD117^{low}$ cells, phenotypic analog to the CLP of the BM, additionally showed *in vivo* differentiation potential to B and T cells, NK cells and DCs whereas MΦ only developed from these precursors in *in vitro* studies.[60] In addition, Yoshida *et al*. described a $Lin^-IL\text{-}7R\alpha^+\alpha_4\beta_7^+$ cell population present in the FL (E12.5) as precursor for $CD45^+CD3^-CD4^+$ cells.[61] $Lin^-IL\text{-}7R\alpha^+\alpha_4\beta_7^+$ cells have been reported to lack myeloid and B cell differentiation potential and to loose their T cell differentiation potential after migration to the embryonic intestine where they are suggested to be involved in the formation of Peyer`s Patches (PPs), organized lymphoid tissues containing mainly B lymphocytes. It has been demonstrated that *IL-7R$\alpha^{-/-}$* mice are not able to generate PPs and that the administration of an antagonistic IL-7Rα mAb to pregnant mice completely blocked the generation of PPs.[62, 63] Since $CD45^+CD3^-CD4^+$ cells express IL-7Rα, these findings suggested the involvement of $CD45^+CD3^-CD4^+$ cells in the formation of PPs.

Role of LTi cells in lymphoid tissue development

The identification of molecular and cellular requirements for the formation of lymphoid tissues started with the characterization of the LTα- deficient (*LTα*$^{-/-}$) mice in 1994.[64, 65] *LTα*$^{-/-}$ mice are unable to generate lymphoid tissues and completely lack LNs and PPs. LTα belongs to the tumor necrosis factor (TNF) superfamily and can form heterotrimers with LTβ consisting of one α-chain and two β-chains ($LT\alpha_1\beta_2$). $LT\alpha_1\beta_2$ binds to its own receptor, LTβ – receptor (LTβR).[66, 67] The crucial role of LTβR triggering in the formation of lymphoid tissues has been shown by the fact that administration of soluble LTβR into pregnant *WT* mice blocked the formation of LNs and PPs in the offspring.[68] In 1998 the *LTβR*$^{-/-}$ mice have been described and confirmed the requirement of LTβR triggering for the formation of lymphoid tissues.[69]

The cellular requirements for lymphoid tissue formation are uncovered by studying two different mouse models, *Id-2*$^{-/-}$ mice[4] and *RORγt*$^{-/-}$ mice,[70] respectively. In the absence of either the helix-loop-helix inhibitor of DNA binding 2, Id2, or the orphan nuclear hormone receptor, RORγt, LN and PP development fails completely and subsequent analysis of these mice revealed that they lack $CD45^+CD3^-CD4^+$ cells.

Finally, two *in vivo* adoptive transfer experiments confirmed that $CD45^+CD3^-CD4^+$ cells are indeed able to induce lymphoid tissue formation. First, Finke *et al*. have demonstrated that adoptive transfer of $CD45^+CD3^-CD4^+$ cells isolated from the fetal spleen is able to induce PP development in *CXCR5*$^{-/-}$ mice and secondly, Fukuyama *et al*. adoptively transferred fetal intestinal $CD45^+CD3^-CD4^+$ cells into *Id-2*$^{-/-}$ mice thereby inducing the formation of nasal-associated lymphoid tissue (NALT)-like structures.[71, 72] Based on these findings $CD45^+CD3^-CD4^+$ cells are renamed as lymphoid tissue inducer (LTi) cells.

In general, the generation of lymphoid tissues such as LNs and PPs require the interaction of LTαβ-expressing hematopoietic cells and LTβR-expressing stromal cell.[68] LTβR triggering on stromal cells induces the activation of the nuclear factor-κ-B (NFκB) pathway resulting in the expression of the adhesion molecules vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) as well as the production of the chemokines CXCL12, CXCL13, CCL19 and CCL21. These chemokines are required for the recruitment and retention of additional hematopoietic cells at putative sites for LN or PP development (Fig. 3).[73]

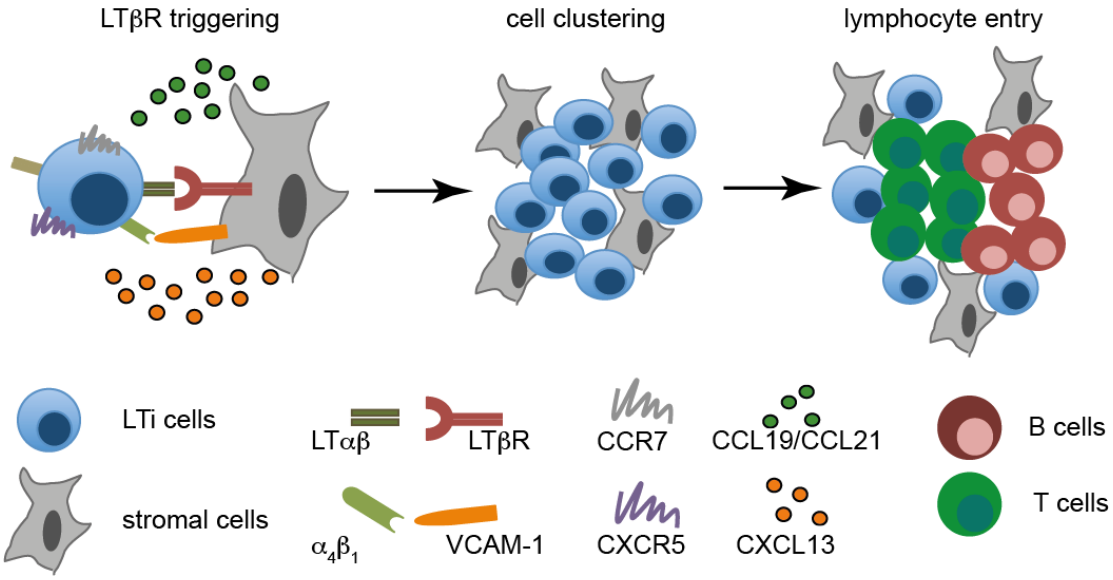


Figure 3: Illustration of lymphoid tissue organogenesis mediated by LTi cells. LTi: lymphoid tissue inducer; LTαβ: lymphotoxin αβ; VCAM-1: vascular cell adhesion molecule-1. Adapted from Mebius, Nature Reviews (2003).[74]

Lymph node organogenesis

LN development starts with the formation of the lymphatic system by the development of the lymph sac at approximately E10.5. *Prox1*, a homeobox gene, is crucial for the budding and sprouting of lymphatic endothelium to form the lymph sacs.[75] Subsequently, from these early lymph sacs lymphatic vessels sprout into tissues and reconnect with other lymphatic vessels. At E15.5 a complete network is formed.[75] At E12.5 – E13.5 the earliest LN anlagen are formed by the colonization with LTi cells that cluster with VCAM-1$^+$ stromal cells. LT$\alpha_1\beta_2$ expression on LTi cells was shown to be induced through IL-7R- and TNF-related activation-induced cytokine receptor (TRANCER)- signaling[76] and allows the cells to interact with LTβR-expressing stromal cells activating their NFκB pathway.[73] Signaling through LTβR on stromal cells interacting with LTαβ-expressing LTi cells is absolutely crucial for LN organogenesis.[68] NFκB pathway activation induces the expression of VCAM-1, ICAM-1 and MAdCAM-1 on the stromal cells amplifying their interaction with LTi cells expressing the corresponding receptors $\alpha_4\beta_1$ and $\alpha_4\beta_7$, respectively.[57] On the other hand, activation of the NFκB pathway of stromal cells triggers secretion of several chemokines including CXCL13 and CCL21 that attract and retain additional LTi cells expressing the respective chemokine receptors CXCR5 and CCR7.[57] The crucial role for NFκB signaling in lymphoid organogenesis has been confirmed by the finding that NFκB-inducing kinase (NIK)-deficient mice fail to develop LNs.[77] Further, interaction of LTi cells and stromal cells via LTαβ and LTβR has been reported to induce the

secretion of IL-7 and TRANCE by the stromal cells.[78] Both IL-7 and TRANCE are able to induce LTαβ expression on newly arriving LTi cells resulting in enhanced LTβR triggering on stromal cells generating a positive feedback loop.[78]

Peyer`s patch organogenesis

For PP development three distinctive stages are described starting with the first at E15.5 involving clustering of VCAM-1$^+$ and ICAM-1$^+$ cells.[58] VCAM-1$^+$ICAM-1$^+$ stromal cells in the developing PP express LTβR and upon LTβR triggering produce the homeostatic chemokines CXCL13, CCL19 and CCL21, which are potent chemoattractants for LTi cells.[79] Thus, subsequently LTi cells colonize these first stromal cell clusters and co-localize with VCAM-1$^+$ICAM-1$^+$ stromal cells in the intestine (E16.5 – E17.5, stage 2).[79] The third stage involves the recruitment of and further colonization by T and B lymphocytes. In addition to the requirement of VCAM-1$^+$ICAM-1$^+$ stromal cells and LTi cells for the development of PPs, another lymphoid cell type has been described to be essential. The so-called lymphoid tissue initiating cell (LTin) is found in the embryonic gut from day E15.5 – E16.5 on and expresses CD11c and CD117 while lacking CD4, CD3 and IL-7Rα expression.[80] In the absence of LTin cells, PP development has been reported to be significantly reduced.[80]

As described for LN development, PP development depends as well on signaling through the LTαβ-LTβR axis. Mice deficient for LTβR, LTα or LTβ are unable to generate PPs.[64, 69, 81] The chemokine CXCL13 is produced by stromal cells in the fetal intestine and is known to direct B cells to lymphoid follicles.[79, 82] Through signaling via CXCR5 expressed on LTi cells, CXCL13 can trigger β1 integrin activation. β1 integrin expression on LTi cells allows the interaction with VCAM-1 expressing stromal cells.[71] The activation of the β1-VCAM-1 axis induces augmented expression of CXCL13 which signals via CXCR5 resulting in the induction of LTαβ expression on LTi cells. Thus, a feedback loop for PP formation is generated via the β1-integrin – VCAM-1 axis.[71] Moreover, signaling via the receptor tyrosine kinase RET is required for the formation of PP anlagen. Despite normal numbers of LTi cells, the lack of RET disrupts the cell cluster aggregation.[80]

Different requirements for LN and PP development

LN and PP organogenesis shows many similarities such as the requirement for the LTαβ-LTβR signaling axis. However, some factors involved in the developmental program differ for LNs and PPs. *TRANCE*$^{-/-}$ mice are shown to lack LNs whereas PPs develop normally.[83] Therefore, TRANCE-TRANCER signaling axis is an additional

example of TNF/TNFR family members crucial for LN development. LTi cells express both TRANCE and TRANCER and the TRANCE/TRANCER signaling axis is required for survival of LTi cells and their clustering at putative sites of LN formation as TRANCE overexpression in *TRANCE*$^{-/-}$ mice is able to rescue LTi cell numbers and LN development.[84] However, TRANCE overexpression in *LT*$\alpha^{-/-}$ mice does not result in rescued LN development suggesting different roles for the TRANCE/TRANCER and the LT$\alpha\beta$/LTβR signaling axis.[84] Moreover, the role of IL-7Rα signaling in PP and LN development differs. While PP development is completely dependent on IL-7Rα-signaling displayed by the lack of PPs in mice with a IL-7Rα deficiency,[62, 85, 86] LN organogenesis does only partially depend on IL-7Rα signaling.[87] As lymphocyte-deficient *Rag1*$^{-/-}$ mice generate LNs normally, the absence of some LNs in *IL-7R*$\alpha^{-/-}$ mice is not due to the role of IL-7R signaling in lymphocyte development.[87] Further, the cytokines IL-7 and SCF (Kit Ligand) differentially regulate LN and PP development. Both IL-7 and SCF are shown to be growth factors for LTi cells and their FL precursors *in vitro* and *in vivo*.[88, 89] However, *IL-7*$^{-/-}$ mice show defects in LN generation, whereas PP development is not perturbed.[88] In contrast, PP development is highly reduced in mice with a deficiency in Kit signaling while all LNs develop normally.[89] In line with this, it could be demonstrated that stromal cells isolated from LNs show high IL-7 and low SCF expression while stromal cells of PPs are characterized by high SCF and low IL-7 expression.[89]

Transgenic overexpression of IL-7 in mice revealed that IL-7 is a survival factor for both LTi cells and their FL precursor by increasing cell survival rather than proliferation. Increased IL-7 availability in these mice induces *de novo* generation of VCAM-1^{+} PP anlagen resulting in increased numbers of PPs in the intestine of adult mice. Furthermore, ectopic lymphoid tissues so-called tertiary lymphoid tissues are formed. The generation of additional PPs and LNs has been reported to depend on the availability of IL-7, LTi cells and LT$\alpha\beta$ signaling.[88] Since *IL-7*$^{-/-}$ mice are unable to form LNs and IL-7 regulates the size of the LTi cell pool *in vivo* it has been suggested that low numbers of LTi cells in *IL-7*$^{-/-}$ mice are responsible for the defects in LN organogenesis.[88] IL-7 and TSLP have been reported to exhibit overlapping biological functions.[90] Transgenic overexpression of TSLP in *IL-7*$^{-/-}$ mice is able to restore LN development due to the induction of an increase in LTi cell numbers. The same is found when TSLP is transgenetically overexpressed in *Rag2*$^{-/-}\gamma_c^{-/-}$ mice demonstrating that peripheral lymphocytes are not required for LN formation.[91] Altogether, IL-7, TSLP and SCF have been shown to regulate the size of the LTi cell pool by

increasing the generation and survival rather than the proliferation of these cells. LN and PP development is clearly regulated by the number of LTi cells, the availability of region-specific cytokines and signaling via LTβR.

LTi cells persist after birth

Beside the existence of fetal LTi cells playing an essential role in lymphoid tissue formation during embryogenesis, several studies showed that LTi-like cells could be found after birth.

RORγt has been described to be essential for the generation of fetal LTi cells and consequently for the formation of lymphoid tissues.[70, 92] In a transgenic mouse model, in which the enhanced green fluorescent protein (EGFP) was used as a reporter gene inserted into the gene encoding RORγt (*Rorc(γt)*$^{+/GFP}$ mouse), it could be shown that RORγt is an exclusive marker for LTi cells during fetal life.[93] Additionally, further analysis of *Rorc(γt)*$^{+/GFP}$ mice revealed that RORγt$^+$ cells could be found in the gut of adult mice.[25] They have been identified within cryptopatches (CPs), isolated lymphoid follicles (ILFs) and PPs, all organized structures in the gut. These RORγt$^+$ cells additionally express IL-7Rα and CD117 and lack the expression of lineage markers suggesting that they are the adult counterparts of the fetal LTi cells.[25] In RORγt-deficient mice, reached by breeding *Rorc(γt)*$^{+/GFP}$ mice to homozygosity (*Rorc(γt)*$^{GFP/GFP}$ *mice*), intestinal RORγt$^+$ cells are absent and CPs as well as ILFs do not develop.[25] Thus, adult RORγt$^+$ cells identified in the adult gut share developmental requirements, phenotype and function with fetal LTi cells and are therefore thought to be the adult equivalents of the fetal LTi cells.

Moreover, Lane *et al*. identified a cell type in the spleen of adult mice with a phenotype closely resembling them of fetal LTi cells. These CD3$^-$CD4$^+$CD11c$^-$ cells are found in the spleen mainly in and around B cell follicles as well as at the T:B cell interface in close proximity to T cells. Detailed analysis revealed that compared to embryonic and neonatal LTi cells these cells express high levels of the T cell co-stimulatory molecules CD30-ligand (CD30L) and OX40L.[94] The receptors for CD30L and OX40L, CD30 and OX40, are only expressed by primed but not by naïve T cells. Therefore, it has been suggested that adult CD3$^-$CD4$^+$CD11c$^-$ splenic LTi-like cells are potential candidates for providing co-stimulatory signals to T cells.[94] Indeed, mice with a deficiency in CD30 and OX40 (*CD30*$^{-/-}$*OX40*$^{-/-}$ mice) are shown to lack proper memory antibody responses due to a failure in the survival of primed CD4$^+$ T cells.[95] It could be shown that this failure was due to the inability of primed CD4$^+$ T cells to interact with CD30L- and OX40L-expressing splenic LTi-like cells.[95] The lack of

CD30L and OX40L revealed as the only difference between embryonic/neonatal LTi cells and adult splenic LTi-like cells. Otherwise embryonic/neonatal LTi cells and $CD3^-CD4^+CD11c^-$ splenic LTi-like cells share a genetic fingerprint, which clearly separates these cells from other lineages.[96]

LTα$^{-/-}$ mice show a disruption of B and T cell segregation in the spleen, which could not be rescued by the transfer of total *WT* splenocytes.[97] However, the transfer of *LTα*$^{-/-}$ splenocytes into *Rag*$^{-/-}$ mice was able to rescue the spleen organization, thereby suggesting a non-T and non-B cell type to be responsible for proper B/T segregation.[98] Indeed, it could be shown that the transfer of either fetal LTi cells or adult LTi-like cells, both expressing high levels of LTαβ, is able to restore B/T cell segregation in the spleen of *LTα*$^{-/-}$ mice within 10 days whereas the transfer of either lymphocytes or DCs fails to restore a correct organization in the spleen.[98] Thus, adult LTi-like cells are important for a correct T and B cell segregation in the adult mouse spleen.[98]

The infection with lymphocytic choriomeningitis virus (LCMV) is characterized by cytotoxic $CD8^+$ T cell-mediated destruction of the T cell zone stromal cell network.[99] It has been shown that the fibroblastic reticular cell (FRC) network is mainly affected by the LCMV infection and consequently hosts are not able any more to mount proper immune responses and loose their immunocompetence. The rebuilding of a correct lymphoid microarchitecture is required to regain immune responsiveness. At the peak of LCMV-mediated tissue destruction, increased numbers of $CD45^+$IL-$7R\alpha^+lin^-$ cells are found in LNs and spleen, most probably through a proliferative accumulation of these cells.[99] These $CD45^+$IL-$7R\alpha^+lin^-$ cells bear a phenotype similar to embryonic LTi cells and could be shown to be able to fully restore the T cell zone stromal network through LTαβ-LTβR-mediated FRC stimulation.[99] In the absence of $CD45^+$IL-$7R\alpha^+lin^-$ cells, the reorganization of the spleen is delayed whereas adoptive transfer of LTi cells into *RORγt*$^{-/-}$ chimeras has been shown to accelerate the restoration of the splenic architecture after LCMV caused destruction.[99] These data indicate that adult LTi-like cells are important for restoration and reorganization of destroyed tissue after birth thereby following the same program as during fetal life.

Moreover, LTi cells have been described to play a role in the generation of T-independent IgA in the gut.[100] T- independent IgA is produced in mainly B cell containing ILFs, which only develop in presence of LTi cells, their LTαβ-LTβR-dependent interaction with stromal cells, and signals from bacteria that enhance the interaction of these two cell types.[100, 101]

As already described for fetal LTi cells and their progenitors,[88, 89] it could be demonstrated that the size of the adult splenic LTi-like cell pool depends on the availability of IL-7. Adult *IL-7*$^{-/-}$, *IL-7R*$\alpha^{-/-}$ and mice lacking the γ_c chain ($\gamma_c^{-/-}$ mice) show reduced numbers of splenic LTi-like cells whereas mice with a transgenic overexpression of IL-7 show increased numbers of LTi-like cells in the spleen.[102, 103] Additionally, the treatment of adult *IL-7*$^{-/-}$ mice with IL-7/anti-IL7 complexes[104] has been shown to be able to rescue numbers of splenic LTi-like cells in adult mice.[103]
Moreover, the transfer of adult splenic LTi-like cells into newborn *CXCR5*$^{-/-}$ mice has been demonstrated to result in the appearance of PP anlagen.[103] This revealed for the first time that adult splenic LTi-like cells are *bona fide* LTi cells able to induce the *de novo* formation of lymphoid tissue. However, compared to fetal LTi cells, adult LTi-like cells have been shown to be less efficient in the induction of PP anlagen.

Development of LTi-like cells found after birth

Whether fetal LTi cells persist or adult LTi cells are newly generated from BM precursors is not fully understood. Fetal LTi cells, which are transferred into adult mice, could be recovered in the spleen expressing CD30L and OX40L.[94, 96] This finding supported the idea that adult LTi cells might develop from fetal LTi cells. Fetal LTi cells have been reported to develop from precursors of the FL.[60, 61] After birth, the BM is the major site of hematopoiesis. Whether a precursor for adult LTi cells exists in the BM was not known. The transfer of total FL cells or total BM cells into *IL-7R*$\alpha^{-/-}$ mice revealed that LTi cells are able to develop either from FL or from BM cells.[103] However, the successful and efficient generation of adult LTi cells from BM cells requires exogenous IL-7.[103] Another study supported the idea that the BM might be the source of adult LTi cells. In this study, the transfer of total BM cells into *ROR*$\gamma t^{-/-}$ mice results in the generation of CP and ILFs.[100]
Collectively, several reports showed that cells with an extremely similar phenotype to fetal LTi cells exist after birth. These cells are called at the time of its discovery LTi-like cells and could be shown to achieve numerous functions. Some of the functions of LTi-like cells after birth are related to their original function as lymphoid tissue inducer,[25, 99, 103] however, LTi-like cells also contribute within several ways to immunity.[94, 95, 100]

NKp46$^-$ and NKp46$^+$ ILC3s

In general, the group 3 ILCs are defined as RORγt^+ IL-17 and/or IL-22-producing cells, thus resembling Th17 cells. The group 3 ILCs are divided into LTi cells

(extensively described before) and a heterogeneous population of RORγt$^+$ ILC3s. This heterogeneous population of RORγt$^+$ ILC3s consists of RORγt$^+$ ILC3s, which additionally express the natural cytotoxicity receptor NKp46 on their surface, here referred to as NCR$^+$ILC3s, and RORγt$^+$ ILC3s lacking NKp46 expression, so-called NCR$^-$ILC3s. NCR$^-$ILC3s are characterized by the expression of IL-17 and IL-22,[105, 106] and under inflammatory circumstances as well IFNγ[107] while NCR$^+$ILC3s exclusively express IL-22 and predominantly exist in the intestine.[108, 109, 110]

The term NCR$^-$ILC3s for adult RORγt$^+$ ILC3s lacking NKp46 was introduced by the new nomenclature of the ILC family[3] and most probably the LTi-like cells (see chapter 2.2.3 Group 3 ILCs, LTi cells persist after birth), which exist after birth exhibiting several functions in tissue organogenesis[25, 99, 103] and immunity,[94, 95, 100, 105] are equivalent to these nowadays called NCR$^-$ILC3s.

The development of NCR$^-$ and NCR$^+$ILC3s depends on the expression of Id2 and RORγt as these cells are missing in *Id2*$^{-/-}$ mice[4] and RORγt$^{GFP/GFP}$ mice.[19, 93, 108, 109, 110]

The aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor, plays a crucial role in the expansion of NCR$^-$ and NCR$^+$ILC3s after birth.[111, 112] It has been reported that *AhR*$^{-/-}$ mice or mice, which only lack AhR on RORγt$^+$ ILCs and T cells (*Ahr*$^{\Delta LTi,\ T}$ mice) are not able to generate CPs and consequently also ILFs are missing, while their prenatal formation of secondary lymphoid tissues is not affected.[111, 112] It could be shown, that ligand binding to AhR inducing AhR signaling is required for the postnatal expansion of ILC3s, which subsequently are able to generate CPs that develop under the influence of signals from the microbiota into ILFs.[111] In contrast, AhR signaling is dispensable for LTi cell development and function during embryogenesis additionally reflected by the presence of PPs in the gut.[112] Kiss *et al*. could show that AhR signaling induces the transcription of Kit (SCF), which has been previously reported to be crucial for ILC3 expansion, survival and maintenance.[89] Therefore, Kiss *et al*. suggested that the postnatal expansion of ILC3s is mediated via the AhR-Kit axis.[111] Furthermore, due to the lack of post-natal occurring IL22- producing ILC3s, *AhR*$^{-/-}$ mice are unable to mount protective immunity against *C. rodentium* infection, an intestinal mouse pathogen that requires IL-22-producing cells for its clearance.[111, 112, 113] AhR is not only responsible for the expansion and maintenance of IL-22-producing ILC3s after birth as it could be demonstrated that AhR influences the IL-22 production on a per cell basis reflected by reduced IL-22 production of remaining ILC3s in *AhR*$^{-/-}$ mice.[114]

A recent study showed that *Tbx21*$^{-/-}$ mice lack NCR$^+$ILC3s while NCR$^-$ILC3s develop normally suggesting that T-bet expression is only required for NCR$^+$ILC3 but not for

NCR$^-$ILC3 development.[22] Further research is required to uncover the precise role for T-bet in the development and function of ILC3s.

The contribution of the microbiota and its microbial signals to the development and maintenance of NCR$^-$ and NCR$^+$ILC3s in the intestine is not fully understood and several reports noted different findings.[108, 109, 112, 115] By the analysis of germfree mice two groups reported that the numbers of RORγt$^+$ NCR$^+$ILC3s are extremely reduced whereas numbers of RORγt$^+$ NCR$^-$ILC3s (or LTi-like cells) and conventional NK cells are normally represented in the intestine.[108, 109] Additionally, the remaining NCR$^+$ILC3s in germfree mice produce lower levels of IL-22. However, it could be shown that the recolonization of germfree mice with normal microbiota is able to rescue the numbers of NCR$^+$ILC3s.[109] In contrast, two other groups reported that the numbers of RORγt$^+$ NCR$^+$ILC3s are not changed in the intestine of either germfree mice or mice treated with antibiotics efficiently eradicating intestinal bacteria.[112, 115] Furthermore, it has been shown that compared to NCR$^+$ILC3s of adult germfree or antibiotic-treated mice, NCR$^+$ILC3s from normal mice produce lower levels of IL-22, suggesting that the presence of the microbiota represses the IL-22 production of NCR$^+$ILC3s. Indeed, it could be demonstrated that the presence of the microbiota induces IL-25 production by epithelial cells, which in turn activates DCs that subsequently inhibit IL-22 production by NCR$^+$ILC3s in a cell-cell contact dependent manner.[115]

RORγt$^+$ ILC3s expressing NKp46 on the surface (NCR$^+$ILC3s) have been discovered by several research groups independently.[108, 109, 110] Up to that time, NKp46 was believed to be highly and specifically expressed by conventional NK cells.[116, 117] In contrast to NK cells, NCR$^+$ILC3s have been reported to develop independent on IL-15 and do not produce perforin or granzymes, therefore lack cytotoxicity.[108, 109, 110] However, NCR$^+$ILC3s have been identified as important producers of IL-22,[108, 109, 110] a cytokine, which is crucial during the infection with the attaching and effacing bacterial pathogen *C. rodentium*.[113] IL-22-deficient (*IL-22*$^{-/-}$) mice infected with *C. rodentium* show increased epithelial damage followed by bacterial dissemination and a high systemic bacterial burden.[113] It could be shown that IL-22 plays a crucial role in the early phase of host defense against *C. rodentium* infection, as administration of neutralizing IL-22 Ab[118] to *WT* mice at day 0 after inoculation with *C. rodentium* results in weight loss, bacterial dissemination and increased mortality whereas *WT* mice, which received the neutralizing IL-22 Ab at day 8 after *C. rodentium* inoculation are able to fully recover from the infection.[113] The receptor of IL-22 (IL-22R) is exclusively expressed by epithelial cells[119] and epithelial cells are able to produce

many anti-microbial proteins such as RegIIIβ and RegIIIγ promoting epithelial cell integrity thereby limiting bacterial dissemination.[113] In addition to the crucial role of NCR$^+$ILC3s during *C. rodentium* infection *in vivo*, another study showed that NCR$^-$ ILC3s (or LTi-like cells) contribute substantially to protective immunity against *C. rodentium*.[106] Sonnenberg *et al*., could show that NCR$^-$ILC3s are able to expand and to produce increased levels of IL-22 upon *C. rodentium* infection in an IL-23-dependent manner.[106] NCR$^-$ILC3s are the main source of IL-22 at early time points of the infection and absolutely required to survive the first days until the adaptive immunity starts to work.[106] Thus, NCR$^-$ and NCR$^+$ILC3s are shown to be the main producers of IL-22 early after infection with *C. rodentium* and are thus indispensable for the clearance of the infection.[106, 108, 109] Another study identified a crucial role for NCR$^-$ILC3s (or LTi-like cells) in innate immunity mediated by their ability to produce high amounts of IL-17 in response to microbial products.[105] Takatori *et al*. reported that NCR$^-$ILC3s are able to produce IL-17, a major mediator of inflammation, in response to the yeast cell wall product zymosan *in vivo*.[105] They could show that NCR$^-$ILC3s found in the spleen of zymosan-treated *Rag2*$^{-/-}$ mice produce high levels of IL-17 and also IL-22. Zymosan treatment induces IL-23 production by DCs, which in turn has been suggested to activate NCR$^-$ILC3s expressing high levels of IL-23R.[105] In line with this, it has been proposed that the protective response of NCR$^-$ and NCR$^+$ILC3s during *C. rodentium* infection depends on IL-23 as mice lacking IL-23 (*IL-23p19*$^{-/-}$ mice) are not able to recover from *C. rodentium* infection.[106, 113] Indeed, it could be shown that IL-22 production by ILC3s is induced by IL-23 secreted by DCs and simultaneously ILC3s enhance the IL-23 production by DCs through activation of LTβR in a positive feedback loop.[120] Accordingly, several *in vitro* studies confirmed that *ex vivo* isolated ILC3s are able to produce IL-22 in response to IL-23 supporting the importance of the IL-23-IL-22 axis.[105, 106, 108]

ILC3s in adaptive immunity

In addition to their crucial role as cytokine-producing cells in innate immunity, NCR$^-$ and NCR$^+$ILC3s have been reported to be involved in adaptive immunity.

Granulocyte-macrophage colony-stimulating factor (GMCSF) has been shown to be essential for the induction and maintenance intestinal tissue-resident DCs,[121] which are implicated, together with MΦ, in the induction and expansion of regulatory T cells (T_{regs}).[122, 123] T_{regs} itself have been reported to regulate intestinal tolerance.[124] Thus, provision of GMCSF is important for the conversion of T cells into T_{regs} in the intestine to balance the intestinal immune response. In addition to epithelial cells, which are

believed to be the source of GMCSF in the gut,[125] a GMCSF-producing cell population expressing RORγt has been found in the small and large intestine of mice at steady state. This cell population contains NCR^- and NCR^+ILC3s and significantly reduced levels of GMCSF in the gut of *RORγt*$^{-/-}$ mice lacking NCR^- and NCR^+ ILC3s confirmed these cells as important GMCSF producer.[126] Since GMCSF is not detectable in newborns but increases with age, commensal bacteria-driven signals have been suggested to induce GMCSF production by ILC3s in the intestine. Indeed, intestinal MΦ could be identified as main source of IL-1β in response to microbial products, which in turn activates ILC3s to produce GMCSF.[126] The importance of IL-1β and IL-1R1 signaling has been confirmed by the fact that ILC3s from *IL-1R1*$^{-/-}$ mice are unable to produce GMCSF. In addition, adoptive transfer of ILC3s from either GMCSF-sufficient (*Csf2*$^{+/+}$) or GMCSF-deficient (*Csf2*$^{-/-}$) mice into *Rag2*$^{-/-}$*γc*$^{-/-}$ mice revealed that only mice, which received GMCSF-sufficient ILC3s are able to produce GMCSF and to convert T cells into T_{regs} upon T cell transfer and immunization.[126] Thus, IL-1β-responsive GMCSF-producing ILC3s are the primary source of GMCSF in the intestine crucial for the induction of T_{regs} mediating intestinal immune tolerance.

ILC3s are also able to interact with B cells. A recent study showed that ILC3s in the spleen are capable to enhance Ag production of marginal zone B cells (MZB cells).[127] NCR^+ILC3s can be found in the marginal zone (MZ) and perifollicular zone areas of the spleen where they are able to interact with marginal reticular cells (MRCs), a MZ subset of stromal cells.[127] The interaction of NCR^+ILC3s with MRCs has been shown to be bidirectional as MRCs promote the survival of NCR^+ILC3s through the release of chemokines and cytokines while NCR^+ILC3s induce up-regulation of ICAM-1 and VCAM-1 on MRCs.[127] In addition to the activation of MRCs, mouse splenic ILC3s express APRIL, a B cell activating factor (BAFF)-related molecule shown to enhance plasma cell survival.[128] Furthermore, ILC3s have been shown to produce GMCSF, which allows the recruitment of neutrophils resulting in enhanced T- independent (TI) production of Abs.[127] In line with this, adoptive transfer of GMCSF-sufficient, but not GMCSF-deficient ILC3s, is able to increase the abundance of splenic neutrophils in *Rag1*$^{-/-}$*γc*$^{-/-}$ mice.[127] Another study could show that intestinal ILC3s promote the IgA production by B cells either through membrane bound lymphotoxin αβ ($LT\alpha_1\beta_2$) or secreted soluble lymphotoxin α ($sLT\alpha_3$).[129] It has been reported that intestinal ILC3s interact with $CD11c^+$ DCs via membrane bound $LT\alpha_1\beta_2$ triggering LTβ receptor (LTβR) expressed on DCs. This interaction controls the expression of inducible nitric oxide synthase (iNOs) by DCs, which is known to be critical for IgA induction.[130, 131]

DCs isolated from mice lacking LTβ expression on ILC3s and T cells ($LT\beta^{\Delta ILC,T}$ mice) are shown to be less potent in inducing IgA *in vitro* when cultured with IgM^+ B cells.[129] Furthermore, in contrast to $LT\beta^{\Delta ILC,T}$ mice, mice with a deficiency of LTα expression on ILC3s and T cells ($LT\alpha^{\Delta ILC,T}$ mice) are not able to produce soluble $LT\alpha_3$ and consequently show highly reduced numbers of CD40L-expressing T cells and reduced IgA levels in the intestine.[129] It could be shown that the provision of CD40L-sufficient, but not CD40L-deficient T cells results in increased IgA levels assuming a crucial role for $sLT\alpha_3$ in promoting the recruitment of T cells to the intestinal lamina propria.[129] Thus, the production of membrane bound $LT\alpha_1\beta_2$ by ILC3s regulates TI IgA induction via controlling iNOS induction by DCs whereas the production of $sLT\alpha_3$ by ILC3s regulates the induction of T-dependent (TD) IgA induction via recruitment of T cells to the gut.[129]

MHC II expression on ILC3s has been reported already some time ago.[57, 93] However, only recently a study suggested that ILC3s regulate $CD4^+$ T cell responses in the intestine most probably through the presentation of commensal bacteria-derived peptides as ILC3s could be shown to produce the Ag-processing machinery for class II presentation.[132] Genome wide transcriptional profiling revealed that ILC3s are highly enriched in transcripts involved in MHC II Ag processing and presentation pathways such as *Cd74*, *H2-Ab1*, *H2-Aa*, *H2-DMb2* and *H2-DMa*.[132] It could be shown that MHC II protein is expressed at highest level on NCR^-ILC3s whereas NCR^+ILC3s only showed minimal expression of MHC II.[132] *In vitro* studies demonstrated that NCR^-ILC3s are able to process exogenous Ag and to present peptide on the cell surface approved by detection of GFP-Eα protein derived peptides (GFP- Eα protein; see chapter 2.2.2 Group 2 ILCs). However, OVA-pulsed ILC3s failed to induce OVA-specific $CD4^+$ T cell proliferation *in vitro* most probably due to the lack of co-stimulatory molecules CD40, CD80 and CD86.[132] As Ag presentation in the absence of co-stimulation has been proposed to limit T cell responses,[133] ILC3s have been suggested to negatively regulate $CD4^+$ T cell responses. Indeed, in a mouse model in which MHC II is exclusively deleted on ILC3s ($MHCII^{\Delta ILC}$ mice) spontaneous inflammation in the intestine as well as significantly increased levels of proliferating T cells with a memory effector phenotype could be observed.[132] The authors of this study claimed that due to the lack of MHC II on ILC3s $CD4^+$ T cell responses to commensal bacteria are dysregulated promoting intestinal inflammation.[132] Some functions of NCR^- and NCR^+ILC3s in innate and adaptive immunity are summarized in figure 4 (Fig. 4).

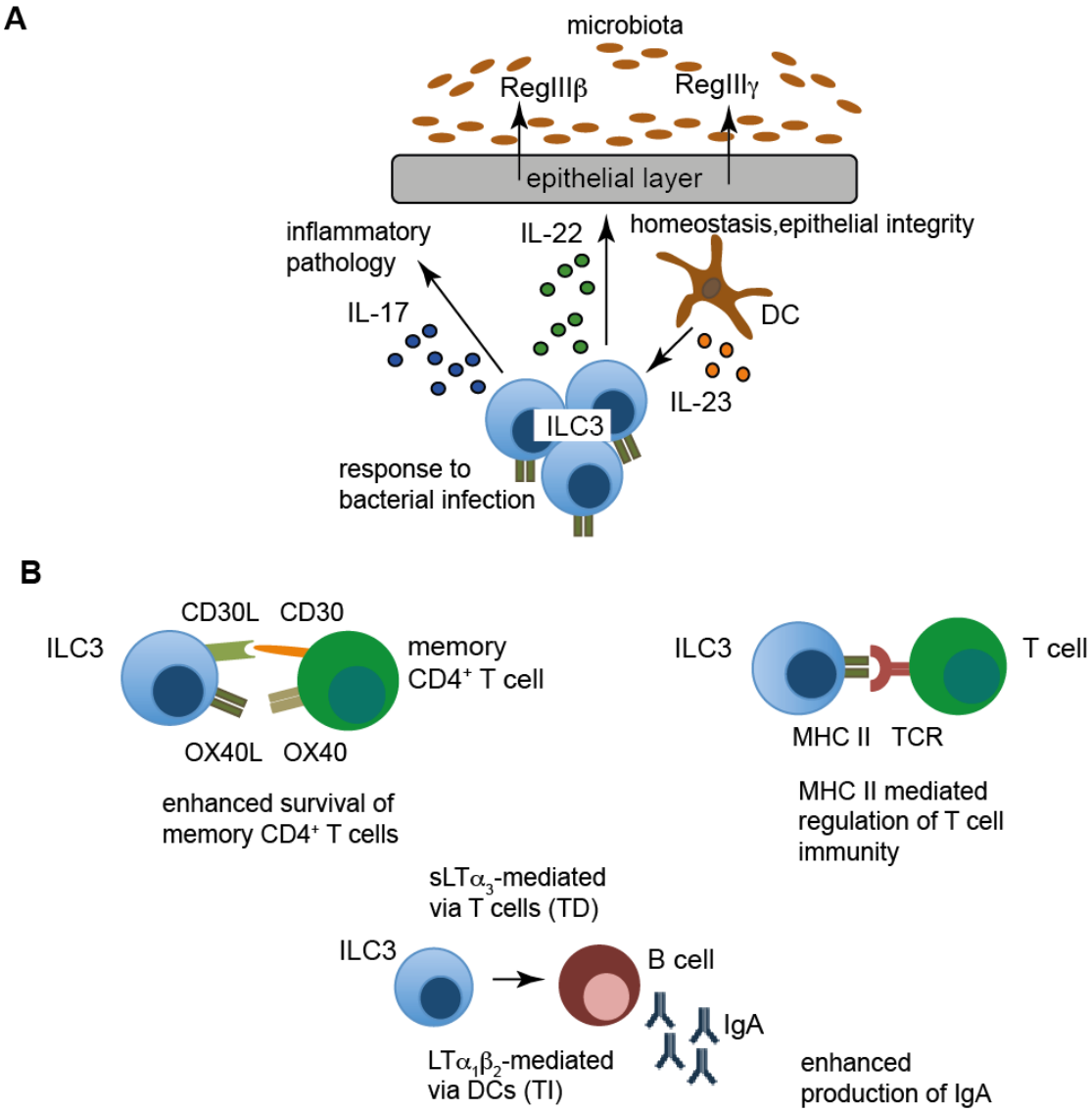


Figure 4: ILC3s in innate and adaptive immunity. A and B) ILC3-mediated innate **(A)** and adaptive **(B)** immunity. DC: dendritic cell; MHC II: major histocompatibility complex class II; TD: T-dependent; TI: T-independent; sLTα_3: soluble lymphotoxin α_3; LT$\alpha_1\beta_2$: lymphotoxin $\alpha_1\beta_2$; IgA: immunoglobulin type A. Adapted from Mc Kenzie *et al.*, Immunity (2014)[55] and Magri *et al.*, Current Opinion in Immunology (2015).[134]

Origin and developmental requirements of ILC2s and ILC3s

During fetal development, LTi cells arise from lin$^-$IL-7R$\alpha^+\alpha_4\beta_7^+$ precursor found in the FL.[60, 61] In adults, ILC2s and ILC3s have been reported to develop from CLPs in the BM.[42, 46, 135] A recent study showed, that for ILC2s a more committed precursor, which is called immature ILC2 (iILC2), exists in the BM.[43] In contrast to CLPs, iILC2s express Id2 as well as high levels of Gata3 and show a close relationship to adult mature ILC2s in genome-wide analysis. It could be shown that iILC2s are able to differentiate successfully into fully functional ILC2s *in vitro* and *in vivo*.[43] Another

research group reported the so-called common helper innate lymphoid progenitor (CHILP; $lin^-Id2^+IL\text{-}7R\alpha^+CD25^-\alpha_4\beta_7^+$), which can be found in the FL as well as in the BM. The CHILP, which is assumed to arise from the CLP, is able to differentiate *in vitro* and *in vivo* into all helper-like ILCs including ILC1s, ILC2s and ILC3s but not into conventional NK cells that are cytotoxic ILCs.[22]

In addition to the expression of the transcriptional repressor Id2, which is required for the development of ILC2s and ILC3s (including LTi cells), Notch signaling has been reported to contribute to ILC2 and ILC3 development.[42, 46, 135, 136] It has been shown that active Notch signaling is required for the development of ILC2s *in vitro* and *in vivo*,[42, 46] whereas the role of Notch signaling in ILC3 development is not completely clear. The development of ILC3s from their precursor appears to depend on Notch signaling that is differentially regulated in fetal and adult precursors.[135] One report claimed, that active Notch signaling is important at very early steps of ILC3 development, but has to be switched off for further differentiation into adult ILC3s.[136]

The development of ILC2s and ILC3s (including LTi cells) requires the cytokine IL-7.[34, 42, 88, 89] Other cytokines such as SCF and TSLP have been reported to play as well a crucial role in the development of LTi cells.[89, 91] Not surprisingly, fetal as well as adult ILC precursors express the respective receptors for the cytokines IL-7 and SCF.[60, 61, 137] Additionally to IL-7Rα (for IL-7) and CD117 (for SCF), they express the cytokine receptor fms-like tyrosine kinase 3 (flt3 also known as CD135).[135, 138, 139] The ligand binding to flt3 is FLT3L, and is a cytokine known to be important for the development of several hematopoietic lineages.[140, 141] The role of FLT3L in the development of ILCs is not yet understood. The effect of FLT3L on ILC precursors, on LTi cells in fetal and neonatal mice as well as on adult ILC populations (including ILC2 and ILC3s) is discussed in the manuscript: *"FLT3L regulates the development of innate lymphoid cells in fetal and adult mice"* attached in the appendix. By the use of FLT3L-deficient mice (*flt3l*$^{-/-}$ mice)[141] and mice with a transgenic overexpression of FLT3L under the control of human β-actin promotor (*flt3l-tg* mice)[142] as well as an approach in which recombinant FLT3L is administrated to adult mice, the role of FLT3L on fetal and adult ILCs and on the development of lymphoid tissues such as PPs has been investigated.

2.3 Activators of immune cells: Toll-like receptor (TLR) ligands and IL-1β

The initiation of an immune response starts with the recognition of the pathogen invading the body. Innate immunity serves as the first line of defense and acts mainly via innate immune cells expressing germ-line encoded pattern-recognition receptors (PRRs) recognizing highly conserved molecular structures of pathogens. Pathogen recognition can induce a variety of effector functions by the innate immune system such as phagocytosis and subsequent digestion of pathogens or the release of cytokines inducing an inflammation. Some pathogens are able to overcome the defense mechanisms of the innate immunity. In this case, the adaptive immunity, the second arm of the immune system, is required for efficient defense against them. A well-orchestrated interplay between the innate and the adaptive immunity is indispensable to mount an efficient immune response. Signals derived from the innate branch have to be integrated by the adaptive branch to fulfill this task. Some of the activators of innate immune cells are described in the following sections.

2.3.1 TLR ligands induce early immune responses

Toll-like receptors (TLRs) are the prototypes of PRRs in mammals able to recognize molecular structures broadly shared by pathogens, the so-called pathogen-associated molecular patterns (PAMPs). Thus, innate immune cells expressing TLRs play an essential role in the early recognition of microbial components and integration of such signals thereby representing an efficient first line defense. TLRs represent an evolutionary ancient host defense system.[143] Nowadays, in mice, 13 different TLRs are identified and each of them recognizes a distinct set of molecular patterns that are not found on healthy host cells allowing the discrimination between self and non-self in a limited way.[144] TLRs are able to recognize various components of the bacteria cell wall such as lipopolysaccharide (LPS) of Gram-negative bacteria, which is exclusively recognized by TLR4.[145, 146] TLR2 is able, together with TLR1 or TLR6, to sense peptidoglycan, lipoproteins and lipopeptides of Gram-positive bacteria.[147, 148, 149, 150, 151] Flagellin, a protein subunit from the bacterial flagella, is recognized by TLR5.[152] TLR3 has been found to recognize double stranded RNA, a major component of many viruses.[153] Additionally, TLR7 is identified as an antiviral TLR, as it senses the synthetic chemical imiquimod, which is known to stimulate antiviral responses.[154] Subsequently, TLR7 and the related TLR8 are shown to

recognize single stranded viral RNA.[155, 156, 157] DNA with unmethylated repeats of the dinucleotide CpG, which are frequent in bacteria but rare in vertebrates, is recognized by TLR9.[158] Furthermore, mouse TLR13 is able to recognize bacterial ribosomal RNA.[159, 160, 161] Thus, the expression of several distinct TLRs allows the recognition of a wide range of different pathogens and viruses. TLRs are either expressed on the surface of the cell (TLR1, TLR2, TLR4, TLR5 and TLR6) and largely recognize microbes on the basis of microbial membrane components in the extracellular space, or they are located within intracellular vesicles (TLR3, TLR7, TLR8 and TLR9) and mainly recognize nucleic acids.[162] TLR engagement activates signaling pathways that provide specific immune responses corresponding to the recognized PAMP. The recruitment of one or a combination of Toll/IL-1R (TIR)-domain containing adaptor proteins (e.g. MyD88, TIRAP, TRIF or TRAM) initiates the specific response.[163] The adaptor molecule, myeloid differentiation primary response protein 88 (MyD88) is used by all TLRs, except by TLR3, which requires TIR-domain containing adaptor inducing interferon beta (TRIF) for downstream signaling. TLR signaling is mediated via the activation of NFκB, mitogen activated protein (MAP) kinases and in case of TLR3 and TLR4 the interferon regulatory transcription factor 3 (IRF3).[163] TLR downstream signaling events culminate in the secretion of inflammatory cytokines, type I IFNs, chemokines as well as antimicrobial peptides leading to activation of MΦ, recruitment of neutrophils and IFN-inducible gene expression.[163] One prominent pro-inflammatory cytokine, which is involved in a variety of effects mediating innate immunity and host responses, is IL-1β described in the next paragraph.

2.3.2 IL-1β production and pro-inflammatory function

IL-1β production and release are mainly initiated through invading microorganisms recognized as PAMPs (described before) or the presence of damage-associated molecular pattern molecules (DAMPs), such as uric acid crystals released by dying cells. Monocytes, MΦ and DCs are the main cell types producing and secreting IL-1β within inflammatory conditions.[164, 165, 166, 167] IL-1β is first synthesized as an inactive form, the so-called pro-IL-1β, and requires its cleavage into a biological active form, IL-1β.[168] This cleavage is mediated by a cysteine protease called caspase-1. Caspase-1 activation requires the assembly and activation of the inflammasome, a multiprotein complex.[168] Subsequently, the biological active form of IL-1β is released into the extracellular milieu. The receptor for IL-1 contains extracellular

immunoglobulin domains and a TIR-domain in the cytoplasmic portion. Signaling via IL-1R starts with the binding of IL-1β to the primary receptor subunit, IL-1R1, which subsequently allows the recruitment of the second receptor subunit, the IL-1R accessory protein (IL-1RAP). The formation of the heterodimer induces the recruitment of signaling intermediates such as MyD88 resulting in the activation of NFκB and MAP kinase (MAPK) pathways.[169] IL-1β released upon microbial triggers or other factors induces a variety of processes in other immune cells. MΦ enhance their cytokine production and phagocytic ability in response to IL-1β.[170] Furthermore, by the induction of the transcription factor IFN regulatory factor 4 (IRF4) IL-1 contributes to the development of Th17 cells, secreting IL-17 and IL-22.[171, 172]

Integration of microbial signals directly via TLRs or indirectly via inflammatory cytokines (e.g. via IL-1β) induces the maturation of immature DCs (iDCs) into mature DCs, which are able to present peptides derived from ingested pathogens to T cells of the adaptive immune system. Before maturation, iDCs are highly phagocytic cells of the innate immune system able to recognize microbial products via TLRs and to ingest pathogens. DC maturation includes up-regulation of MHC II and co-stimulatory molecule (CD40, CD80 and CD86) expression thereby changing from a preferentially phagocytic cell to a professional APC able to activate T cells inducing T cell effector functions.[173, 174] Thus, DCs can be placed at the interface of innate and adaptive immunity, capable of integrating "danger" signals and transmitting them resulting in the induction of a protective adaptive immune response. Ag processing and presentation to $CD4^+$ T cells via MHC II, the role of co-stimulatory molecules and the effector T cell responses, which can be subsequently initiated are described in the following paragraphs.

2.4 Activation of $CD4^+$ T cell responses

2.4.1 Processing and presentation of Ag to $CD4^+$ T cells

T cell-mediated immune responses rely on the recognition of pathogen-derived peptides, which are presented via MHC I or II molecules. Peptides presented via MHC II molecules are recognized by $CD4^+$ T cells whereas peptides presented in context of MHC I molecules are recognized by $CD8^+$ T cells. MHC I molecules are specialized to present intracellular Ags, such as viruses or tumor Ags, while Ags from extracellular sources, such as bacterial Ags, are presented via MHC II.[175] In contrast to MHC I molecules, which are constitutively expressed on almost all nucleated cells,

expression of MHC II molecules is restricted to professional APCs including DCs, MΦ and B cells.

MHC II molecules consist of two transmembrane chains, α- and β-chain, which are synthesized in the endoplasmic reticulum (ER). Stable assembly of the MHC II αβ heterodimer in the ER relies on a specialized type II transmembrane chaperone protein, the invariant chain (Ii, also known as CD74).[176, 177, 178] Ii binds in the MHC II-peptide binding groove, thereby acts like a surrogate peptide, stabilizes the MHC II protein and prevents premature loading of antigenic peptides. The Ii-MHC II heterotrimer is transported via the Golgi apparatus into an endolysosomal compartment, termed MHC II compartment (MIIC). In the MIIC, Ii is degraded by a series of proteolytic cleavage events mediated by the proteases cathepsin S and cathepsin L and a residual class-II associated Ii peptide (CLIP) is left in the peptide-binding groove of the MHC II heterodimer.[179] The CLIP bound to each MHC II heterodimer has to be replaced by peptides arrived in the MIIC via endosomal pathway from the extracellular milieu. Once endocytosed, exogenous proteins are degraded by proteases, the CLIP is replaced by such peptides and MHC II molecules loaded with the specific peptide are transported to the cell surface where they are able to present their loaded peptide to $CD4^+$ T cells.[180] HLA-DM (or in mouse H2-DM) accelerates the exchange of the CLIP with peptides within MIIC.[181, 182, 183]

MHC II expression is mainly restricted to professional APCs like DCs, MΦ and B cells as mentioned before. However, MHC II expression can be induced in non-hematopoietic cell types following the exposure to cytokines, whereby IFNγ is the most potent inducer.[184] For the transcription of MHC II genes the class II transactivator, CIITA, is absolutely required. CIITA is the master regulator of constitutive and inducible expression of all MHC II genes as well as their accessory genes (e.g. the invariant chain Ii).[185, 186, 187] CIITA is recruited to a multiprotein complex referred to as MHC II enhanceosome consisting of several factors (regulatory factor X (RFX), nuclear factor Y (NFY) and cyclic AMP response element binding protein (CREB)) required for MHC II gene expression.[186] The MHC II enhanceosome serves as a "landing pad" for CIITA subsequently inducing the transcription of MHC II genes.[186, 188] In contrast to the components of the MHC II enhanceosome, which are produced in many cell types, the synthesis of CIITA is highly controlled. In mouse, the expression of the gene encoding for CIITA (*MHC2TA*) is regulated by three different promotors (pI, pIII and pIV), which can individually be activated in a cell type- as well as stimulus-specific manner.[189] This

restricted expression of CIITA can act as a molecular switch that controls the specific expression of MHC II.

Before extracellular Ags can be presented via MHC II to $CD4^+$ T cells, these Ags have to be taken up by phagocytic cells. Neutrophils, MΦ and immature DCs are efficient phagocytic cells. Whereas neutrophils and MΦ mainly destroy phagocytosed particles, DCs show reduced degradation and consequently increased conservation of antigenic peptides. Moreover, DCs are the key players in presentation of such antigenic peptides via MHC II and subsequent activation of $CD4^+$ T cells. Immature DCs are known to take up Ag via different types of endocytosis namely phagocytosis or macropinocytosis.[190] Phagocytosis is triggered by the attachment of extracellular particles to surface receptors, such as complement receptors, Fc receptors and mannose receptors.[191] Macropinocytosis is a non-specific process, where large volumes of surrounding fluid containing extracellular Ags are engulfed. Endocytosed extracellular Ags are placed into membrane-delimited compartments, known as phagosomes. Phagosomes finally fuse with lysosomes to form the phagolysosome in which the Ag is degraded by lysosomal proteases. Degraded Ag are loaded on MHC II molecules within the phagolysosome and transported to the cell surface.

As already mentioned before, DCs express TLRs through which they can recognize pathogens and become activated. Exposure to pathogens induces remarkable transformation in DCs. Immature DCs undergo a maturation process and develop from highly endocytic cells into professional APCs. DC activation by stimulation with either TLR ligands or inflammatory cytokines results in migration of DCs to lymphoid organs, redistribution of MHC II molecules from MIIC to the cell surface and expression of co-stimulatory molecules.[192, 193] Although Ag uptake is transiently increased in response to TLR signaling endocytosis is decreased in mature DCs.[194] Furthermore, TLR signaling increases the efficiency of Ag presentation by DCs through phagosome maturation.[195]

2.4.2 $CD4^+$ T cell activation and effector function

The priming of naïve $CD4^+$ T cells to become effector $CD4^+$ T cells carrying out different effector functions requires three distinct signals. The first signal is delivered by the APCs presenting the Ag via MHC II complex to the $CD4^+$ T cells, which recognize the peptide-MHC II complex via its T cell receptor (TCR) inducing TCR downstream signaling. Subsequently the second signal, the co-stimulation, has to be delivered. APCs express co-stimulatory molecules on their surface, which interact

with the corresponding receptors expressed by $CD4^+$ T cells. Finally, the third signal is mediated through cytokines produced by the activated APCs and secreted into the immunological synapse.[196]

$CD4^+$ T cells proliferate and start to produce cytokines when they interact via TCR with APCs presenting their specific Ag in context of MHC II and providing the additional co-stimulation via binding of co-stimulatory molecules to the corresponding receptors on the $CD4^+$ T cells.[197, 198, 199, 200, 201] CD69, a C-type lectin receptor, is one of the earliest glycoproteins acquired during lymphocyte activation.[202] CD69 expression is induced on the surface of T cells upon TCR engagement.[202, 203] The induction of CD69 expression is extremely rapid (within hours) and requires *de novo* synthesis.[204, 205, 206] Thus, T cell proliferation and CD69 up-regulation on the surface of T cells are two parameters to measure early T cell activation upon interaction with the cognate Ag presented by APCs.

APCs are the main drivers of T cell activation and differentiation in lymphoid organs. DC are known as professional APCs and are absolutely crucial for the regulation of T cell activation, the differentiation into a certain T helper subset, and the T cell effector function and survival. In response to microbial simulation, recognized through TLRs, or to inflammatory cytokines, DCs undergo maturation, allowing them to migrate into lymphoid organs where they can interact with T cells, and inducing the expression of the co-stimulatory molecules CD80 and CD86 (also known as B7-1 and B7-2) on their surface.[207] Via CD80 and CD86, DCs are able to deliver the co-stimulation to $CD4^+$ T cells expressing the corresponding receptor, CD28, on the surface.[208, 209] The CD80/CD86 – CD28 co-stimulation pathway is suggested to deliver the most efficient signal, as it could be shown that CD28-deficient cells fail to proliferate in the presence of APCs.[210] CD28 engagement on T cells has several distinct effects. It stimulates the proliferation of T cells and induces up-regulation of high levels of IL-2 secreted by the T cells.[211] Further it promotes T cell survival by enhancing Bcl-XL expression.[212] Those effects have been shown to depend on the engagement of downstream signaling via phosphoinositide-3 kinase (PI3K), NFκB and protein kinase B (PKB).[213] TCR and CD28 triggering induces several signaling cascades, including NFκB, NFAT (nuclear factor of activated T cells), JNK (c-Jun N-terminal kinase) and ERK (extracellular regulated kinase), resulting in the expression of IL-2.[214] IL-2 secretion and IL-2 receptor expression by T cells is increased upon activation thereby IL-2 achieves an autocrine effect and supports the survival of T cells.[215] The main elements of this co-stimulatory pathway, CD80[216] and CD28[217] were already discovered a long time ago and the absolute requirement for co-

stimulation to induce activation and proliferation of T cells could be demonstrated by the fact that T cells were unresponsive upon TCR engagement in the absence of co-stimulation.[218] T cell activation through TCR triggering in the absence of CD80/86-CD28 co-stimulation leads to a state of anergy characterized by severely reduced production of IL-2 and other effector cytokines.[219] CD40, another co-stimulatory molecule, mediates APC-T cell dialog and is expressed on DCs. CD40 can be ligated by its corresponding ligand, CD40L, expressed on T cells. This results in the activation of the NFκB pathway[220] inducing increased DC survival[221] and DC maturation including up-regulation of the co-stimulatory molecules CD80 and CD86.[222]

DCs can provide all three signals required for efficient $CD4^+$ T cell activation. They are able to prime naïve $CD4^+$ T cells inducing activation (signal 1), survival (signal 2) and differentiation (signal 3) into different $CD4^+$ T cell effector subsets. $CD4^+$ T cells are categorized within these Th cell subsets based on the secretion of a distinct set of effector cytokines. Nowadays the following Th subsets are described: Th1 cells, Th2 cells, Th17 cells, T follicular helper cells (Tfh) and inducible regulatory T cells (iT_{regs}).[223, 224] The plasticity between the distinct Th subsets as well as the lineage commitment and differentiation into these subsets, and the categorization based on the effector cytokine repertoire is still under debate.[224] Th1 and Th2 cells, characterized by the expression of IFNγ and IL-4, respectively, have been already identified in 1986.[225] Further research revealed that Th1 cell development depends on the expression of the transcription factor T-bet and the presence of IL-12 and IFNγ produced by other immune cells (e.g. DCs and NK cells, respectively). By the production of IFNγ, Th1 cells are able to promote immunity to intracellular microorganism.[223, 226, 227] Additionally to IL-4 production, Th2 cells are capable to secrete IL-5, IL-9, IL-10, IL-13, IL-25 and amphiregulin thereby contributing to immunity against extracellular parasites and to the induction and persistence of asthma and allergies. Th2 differentiation requires the transcription factor Gata-3 and the cytokines IL-2 and IL-4 whereby IL-4 is a positive feedback loop cytokine in Th2 differentiation produced by the Th2 cell itself.[223, 228, 229, 230] Th17 cells require RORγt and the presence of transforming growth factor (TGF)-β for their development.[231, 232, 233, 234] They are characterized as IL-17 and IL-22 producing cells thereby promoting immunity against extracellular bacteria and fungi.[223, 235] iT_{reg} development depends on the transcription factor FoxP3 and the cytokine TGFβ.[236, 237] Survival of iT_{regs} is mediated via IL-2 as these cells express high levels of CD25, the high affinity receptor for IL-2.[238] iT_{regs} are involved in the regulation and suppression of immune

responses.[238] The cytokines IL-6 and IL-21 are the main cytokines involved in the differentiation of Tfh cells.[239, 240] Recently the transcription factor Bcl-6, which is selectively expressed by Tfh cells, has been identified to play an important role in Tfh cell differentiation.[241] Tfh cells are located in follicular areas of lymphoid tissue and participate in the development of Ag-specific B cell immunity.[242, 243] They are able to interact with Ag-primed B cells in special structures called germinal centers inducing the differentiation of B cells into Ig-producing plasma cells and the development of long-lived memory B cells.[244] Thereby Tfh cells play a significant role in mediating humoral immune responses through the interaction with B cells.

Collectively, DCs are professional APCs and play crucial roles in the induction of adaptive immunity. They are able to integrate innate signals and thereby modulate the outcome of an immune response. They induce the differentiation of $CD4^+$ T cells into distinct helper subsets and promote their survival. Thus, they are able to initiate a pathogen-dependent fine-tuned immune response by the induction of the correct T cell effector subset.

3 Aim of the project

Group 3 innate lymphoid cells (ILC3s) were initially described as key players for the induction of lymphoid tissue formation and organization during embryogenesis. These cells are also found after birth, mainly present at mucosal surfaces in the gastrointestinal tract as well as in peripheral secondary lymphoid organs. An innate immune function has been attributed to ILC3s in the mucosa of adult mice based on their ability to release effector cytokines in response to soluble factors secreted by other cells such as dendritic cells (DCs). However, whether ILC3s are able to directly sense microbial products is not yet known. Additionally, whether ILC3s possess the ability to directly interact with cells of the adaptive immune system and thereby contribute to adaptive immune responses is not fully understood. Finally, the tissue-specific properties of ILC3s have not been investigated until now.

The first aim of the present study was to investigate whether ILC3s are able to directly sense and to respond to innate stimulation. To address this question, I tested the response of ILC3s to different microbial products and inflammatory cytokines. The response of ILC3s to innate challenge was monitored by the examination of changes in the phenotype and cytokine production.

The finding that fetal/neonatal ILC3s can express major histocompatibility complex class II (MHC II) on their surface[57] suggested that ILC3s might be able to interact with $CD4^+$ T cells. Therefore, the second aim of the present study was to uncover the role of ILC3s in T cell and T-dependent B cell responses. To address this question, I tested, whether ILC3s are able to take up antigen (Ag), to process and to present it via MHC II to $CD4^+$ T cells. Subsequently, I investigated whether ILC3s are capable to promote $CD4^+$ T cell responses *in vitro*. To examine the role of ILC3s in $CD4^+$ T cell-mediated immunity *in vivo*, I generated a mouse model with a deficiency of MHC II exclusively in ILC3s, and tested Ag-specific $CD4^+$ T cell and T-dependent B cell responses.

Finally, to uncover tissue-related and environment-dependent ILC3 functions, I compared the phenotype as well as the ability of peripheral spleen-derived ILC3s and small intestine-derived ILC3s to respond to innate challenge and to induce $CD4^+$ T cell responses.

4 Materials and methods

4.1 Materials

4.1.1 Reagents and chemicals

2-mercaptoethanol	Gibco
2-propanol ($CH_3CH(OH)CH_3$)	Merck KgaA
2x SensiMix SYBR Hi-ROX	Bioline
Albumin from bovine serum (BSA)	Sigma-Aldrich
Alum (Aluminium potassium sulphate, $AlK(SO_4)_2$)	Sigma-Aldrich
Ammonium chloride (NH_4Cl)	Sigma-Aldrich
Anti-CD3 soluble Ab (clone 2cII)	A. Rolink, University of Basel
Anti-CD4 microbeads	Miltenyi Biotec
Anti-PE microbeads	Miltenyi Biotec
Brefeldin A (BFA)	Sigma-Aldrich
Carboxyfluorescein succinimidyl ester (CFSE)	Molecular Probes
Ciproxine	Bayer
Collagenase D	Roche
Cytochalasin D (CytD)	AppliChem
D (+) – glucose monohydrate ($C_6H_{12}O_6$ x H_2O)	Merck KgaA
Deoxyadenosine triphosphate (dATP)	Sigma-Aldrich
Deoxycytidine triphosphate (dCTP)	Sigma-Aldrich
Deoxyguanosine triphosphate (dGTP)	Sigma-Aldrich
Deoxythymidine triphosphate (dTTP)	Sigma-Aldrich
Di-ethanolamine ($C_4H_{11}NO_2$)	Sigma-Aldrich
Di-nitrophenyl phosphate (dNPP)	Sigma-Aldrich
Di-potassium hydrogen phosphate (K_2HPO_4)	Merck KgaA
Di-sodium hydrogen phosphate (Na_2HPO_4 x 2 H_2O)	Merck KgaA
DMEM (1x) GlutaMax™-I	Gibco
DNase I	Roche
EDTA disodium salt dihydrate	AppliChem
Ethanol (C_2H_5OH)	Merck KgaA
Fetal calf serum (FCS, heat-inactivated)	Gibco
FluoSpheres® carboxylate-modified microspheres	Molecular Probes

Hoechst 33342	Invitrogen
Hypoxanthine-aminopterin-thymidine (HAT) supplement	Gibco
Intracellular (IC) fixation buffer	eBioscience
Insulin Transferrin Selenium	Gibco
Iscove`s modified Dulbecco`s medium (IMDM)	Sigma-Aldrich
Kanamycin	Sigma-Aldrich
L929	Sigma-Aldrich
Magnesium chloride ($MgCl_2$)	Roche
Magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O)	AppliChem
Non-essential amino acids (NEAA)	Gibco
Oligo dT	Promega
Phosphate buffered saline (PBS)	Biochrom AG
Penicillin Streptomycin (Pen Strep)	Gibco
Percoll	GE Healthcare
Polyethylene Glycol (PEG) 1500	Roche
Potassium bicarbonate ($KHCO_3$)	Sigma-Aldrich
Potassium chloride (KCl)	AppliChem
Primatone	Sigma-Aldrich
Propidium iodide (PI)	Sigma-Aldrich
Proteinase K	Roche
Random hexamers	Sigma-Aldrich
Sodium acide (NaN_3)	Merck KgaA
Sodium chloride (NaCl)	AppliChem
Sodium dodecyl sulfate (SDS, $C_{12}H_{25}NaO_4S$)	BioRad
Sodium hydrogen carbonate ($NaHCO_3$)	Merck KgaA
Sodium hydroxide (NaOH)	Merck KgaA
SuperScript™ III Reverse Transcriptase	Invitrogen
Taq Polymerase	homemade
Tris ($C_4H_{11}NO_3$)	Carl Roth GmbH
Tween-20	AppliChem

4.1.2 Buffers, solutions and media

1x PBS	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 x 2 H_2O

	2 mM KH_2PO_4 in dH_2O
1x TE buffer	10 mM Tris 1 mM EDTA in dH_2O
BMDC medium	IMDM (SF) 5 % FCS 200 ng/ml FLT3L
BMMΦ medium	DMEM (1x) GlutaMax^{TM}-I L929
BW-OTII medium	IMDM (SF) 5 % FCS
DMEM (1x) GlutaMax^{TM}-I	(+) 4.5 g/L D-Glucose (-) Pyruvate
dNTP mix	10 mM dATP 10 mM dCTP 10 mM dGTP 10 mM dTTP in nuclease-free H_2O
ELISA buffer (NP-OVA ELISA)	1 % BSA 0.2 % Tween-20 in 1x PBS
ELISA wash buffer (NP-OVA ELISA)	0.1 % Tween-20 in H_2O
Erythrolysis buffer	0.15 M NH_4Cl 10 mM $KHCO_3$ 0.1 mM EDTA in dH_2O (pH 7.2 – 7.4)

FACS buffer	1x PBS 3 % FCS 2 mM EDTA
HAT medium	IMDM (powder) 3.02 g $NaHCO_3$ 2 % FCS 2 % IL-6 supernatant 1x HAT supplement 0.5 % Ciproxine
IMDM (SF)	IMDM (powder) 3.02 g $NaHCO_3$ 1 % PenStrep 1 % Ciproxine 0.1 % Kanamycin 1 % Insulin Transferrin Selenium 0.3 % Primatone 1 % NEAA 0.1 % 2-mercaptoethanol
IMDM 2 % FCS	IMDM (SF) 2 % FCS
IMDM 5 % FCS	IMDM (SF) 5 % FCS
IMDM 10 % FCS	IMDM (SF) 10 % FCS
lysis buffer (for DNA isolation from mouse biopsies)	1 mM Tris (pH 8.0) 100 mM NaCl 10 mM EDTA (pH 8.0) 0.5 % SDS in dH_2O

substrate buffer (NP-OVA ELISA)	0.1 g $MgCl_2$ x 6 H_2O 10 mM NaN_3 10 % $C_4H_{11}NO_2$ in 1 l dH_2O (pH 9.8)

4.1.3 Cytokines, TLR ligands, peptides, proteins

FMS-like tyrosine kinase ligand (FLT3L)	A. Rolink, University of Basel
IL-1β, recombinant	Biovision
IL-6, supernatant (clone X63)	A. Rolink, University of Basel
IL-7, recombinant	Peprotech
IL-23, recombinant	eBioscience
SCF, recombinant	Peprotech
TNFα, recombinant	R&D systems
IFNβ, recombinant	Sigma-Aldrich
IFNγ, recombinant	R&D systems
CpG (class B)	Trilink Biotechnologies (CpG-ODN1826 InvivoGen)
Flagellin (Flag)	InvivoGen
Imiquimod (Imiqui)	InvivoGen
Lipopolysaccharide (LPS)	Sigma-Aldrich
Pam3Cys (P3C)	InvivoGen
Poly I:C	InvivoGen
Zymosan (Zym)	InvivoGen
$Ovalbumin_{323 - 339}$ ($OVA_{323 - 339}$) peptide	AnaSpec
OVA protein (Imject Ovalbumin)	Thermo Fisher Scientific Inc.
4-hydroxy-3-nitrophenylacetyl NP- (18)-OVAL	Biosearch Technologies Inc.

4.1.4 Antibodies (Abs)

Abs used for flow cytometry analysis (directed against murine proteins)

specificity	**clone**
CD3ε	17A2, 145-2C11
CD4	RM4-4, RM4-5 and GK1.5
CD8α	53-6.7
CD11b	M1/70
CD11c	117310, N418
CD19	6D5
CD21	7G6
CD23	B3B4
CD25 (IL-2Rα)	PC61, 7D4
CD29 (β1 integrin)	HMb1-1
CD40	1C10, HM40-3
CD44	IM7
CD45R (B220)	RA3-6B2
CD54 (ICAM-1)	3E2
CD62L	MEL-14
CD69	H1.2F3
CD80	16-10A1
CD86	GL1
CD90.2 (Thy1.2)	30-H12
CD117 (ckit)	2B8
CD127 (IL-7Rα)	A7R34
CD132 (γ_c chain)	TUGm2
CD184 (CXCR4)	2B11
CD185 (CXCR5)	2G8
CD196 (CCR6)	29-2L17
CD197 (CCR7)	4B12
CD275 (ICOSL)	HK5.3
CD278 (ICOS)	C398.4A
$\alpha_4\beta_7$	DATK32
F4/80	BM8
Gr-1	RB6-8C5
IFNγ	XMG1.2

MHC II	M5/114.15.2, 25/09/2017
NKp46	29A1.4
NK1.1	PK136
RORγt	AFKJS-9, B2D
TCRβ	H57-597
TCRγδ	UC7-13D5

All reagents were purchased from BD Bioscience, eBioscience or Biolegend (all San Diego, CA). Primary monoclonal Abs (mAbs) were conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), brilliant violet 421 (BV421™) or the tandem dyes phycoerythrin cyanine 7 (PECy7), peridinin chlorophyll (PerCP)-eFluor 710 and allophycocyanin cyanine 7 (APCCy7).
Biotin-conjugated primary mAbs were detected using secondary anti-rat polyclonal Abs conjugated either to Streptavidin/PECy7, Streptavidin/APCCy7 or Streptavidin/BV421™ (Biolegend).
Blocking of Fc receptors was performed using anti-mouse FcγRII/III mAb (clone 2.4G2, purified supernatant, home made).

Abs used for Lymphotoxinαβ staining

LTβ-R Fc (human)	J. Browning, Biogen
Biotin-conjugated goat anti-human IgG	Jackson Immunosearch

Abs used for ELISA (NP - OVA)

For coating of plates:

Purified anti-mouse IgM (unlabeled)	M41 (A. Rolink, University of Basel)
Purified anti-mouse IgG3 (unlabeled)	R2-38, BD Bioscience

For detection:

Biotin-conjugated goat anti-mouse IgM	M41 (A. Rolink, University of Basel)
Biotin-conjugated goat anti-mouse IgG	Caltag Laboratories
Biotin-conjugated goat anti-mouse IgG1	Caltag Laboratories
Biotin-conjugated goat anti-mouse IgG2a	Caltag Laboratories
Biotin-conjugated goat anti-mouse IgG2b	Caltag Laboratories
Biotin-conjugated goat anti-mouse IgG3	Caltag Laboratories

Alkaline phosphatase (AKP) – Streptavidin	Roche
AKP- anti-mouse IgG3	R40-82, BD Bioscience

4.1.5 Primers for real time quantitative PCR (RT qPCR)

β-actin:

Fwd: 5' CAATAGTGATGACCTGGCCGT 3'

Rev: 5' AGAGGGAAATCGTGCGTGAC 3'

IL-1β:

Fwd: 5' GACCCCAAAAGATGAAGGGCT 3'

Rev: 5' ATGTGCTGCTGCGAGATTTG 3'

TNFα:

Fwd: 5' CCAAGTACTTAGACTTTGCGG 3'

Rev: 5' CTGAGGAGTAGACAATAAAGGG 3'

IFNγ:

Fwd: 5' CTGAGACAATGAACGCTACAC 3'

Rev: 5' TTTCTTCCACATCTATGCCAC 3'

4.1.6 Kits

Experion RNA HighSens Analysis Kit	BioRad
Foxp3 staining buffer set	eBioscience
LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit	Molecular Probes
mouse IL-17 ELISA MAX™ Standard Set	Biolegend
mouse IL-22 ELISA MAX™ Deluxe Set	Biolegend
RNeasy Micro Kit	Qiagen
RNeasy Mini Kit	Qiagen
SensiMix SYBR Hi-ROX Kit	Bioline
SuperScript™ III Reverse Transcriptase	Invitrogen
Zombie Aqua™ Fixable Viability Kit	Biolegend

4.1.7 Tools and instruments

BD Microtainer SST tubes	BD Bioscience
Cell counter CASY INNOVATIS	Roche
Cell strainer (70 μm / 100 μm)	Falcon
CO_2 - incubator	Binder
Confocal microscope LSM 510 Meta	Zeiss
Cs-137 radiator	Gammacell
Eppendorf centrifuge 5810 R, 5415 R, 5417 R	Eppendorf
Eppendorf Thermomixer Comfort	Eppendorf
Eppendorf Mastercycler Gradient	Eppendorf
ELISA plate reader	ASYS Expert Plus
FastPrep-24 instrument	MP Biomedicals
Flow cytometer BD FACSAria IIu	BD Bioscience
Flow cytometer BD FACSCalibur	BD Bioscience
Flow cytometer BD FACSCanto II	BD Bioscience
MACS® multi stand (magnetic column holder)	Miltenyi Biotec
MACS® separation columns MS/LS	Miltenyi Biotec
MAXI sorb 96 well plates	Nunc
Micro tubes PP (1.5 ml)	Sarstedt
Luminex 100 (LX100) analyzer	Invitrogen
Nanodrop 2000c	Thermo Scientific
Rotorgene RT PCR machine (RG-3000A)	Corbett Research
Shaker Polymax 1040	Heidolph
Syringes 1 ml BD MicroFine$^+$	BD Bioscience
Thermocyler Biometra (PCR)	BIOLABO
Vortex Genie	Scientific Industries
Waterbath	GFL
Zirconia beads	BioSpec Products

4.1.8 Software

Program	**Application**	**Provider**
FlowJo	FACS analysis	Tree Star Inc., USA
Prism	Graphs, calculations	GraphPad Software Inc., USA
ImageJ	Image analysis	NIH, USA

4.1.9 Mice

C57BL/6 (*WT*) mice were purchased from Janvier (Saint Berthevin Cedex, France). *RORγ*$^{-/-}$,[70] *MHCII*$^{\Delta/\Delta}$ (here referred to as *I-ab*$^{-/-}$),[245] *Rag2*$^{-/-}$ (provided by G. Hollaender, University of Basel, Switzerland and Jesus College Oxford, UK),[246] *OT-II*^{tg} (provided by A. Rolink, University of Basel, Switzerland),[247] *I-ab*^{neo} (provided by E. Palmer, University of Basel, Switzerland),[248] *RORc(γt)-Cre*^{tg} mice (provided by A. Diefenbach, University of Mainz, Germany),[25] *MyD88*^{fl},[249] *MyD88*$^{-/-}$ (provided by M. Donath, University Hospital, Basel, Switzerland),[250] *MyD88*$^{-/-}$*Trif*$^{-/-}$ (crossing between *MyD88*$^{-/-}$ and *Trif*$^{-/-}$ mice,[251] provided by B. Holzmann, Technische Universität München, Munich, Germany), *IL-1R1*$^{-/-}$ (provided by M. Manz, University of Zurich, Switzerland),[252] *IL-23p19*$^{-/-}$ (provided by B. Becher, University of Zurich, Switzerland),[253] *I-ab*$^{\Delta ILC3}$ (MHC II deficiency exclusively in RORγt^{+} ILCs) and *MyD88*$^{\Delta ILC3/T}$ mice (MyD88 deficiency exclusively in RORγt^{+} cells) were kept under specific-pathogen free (SPF) conditions. The animal experiments received the approval of the Cantonal Veterinary Office of the city of Basel, Switzerland.

4.2 Methods

4.2.1 Time mating

For timed pregnancies two females and one male were placed in the same cage in the late afternoon. The next morning (approx. 15 h later), male and females were separated again. Females were checked for vaginal plugs. Plug positive females were assumed to be at gestational age 0.5 days.

4.2.2 Generation of I-ab$^{\Delta ILC3}$ and MyD88$^{\Delta ILC3/T}$ mice

I-ab$^{\Delta ILC3}$ mice were generated by crossing *I-ab*^{neo} mice, which contain a floxed *H2-Ab1* allele, with *RORc(γt)-Cre*^{tg} mice. F1 generations were backcrossed to *I-ab*^{neo} mice. *I-ab*$^{\Delta ILC3}$ mice are homozygous for the floxed *H2-Ab1* allele and carry one copy of the Cre transgene.

MyD88$^{\Delta ILC3/T}$ mice were generated by crossing *MyD88*^{fl} mice, which contain a floxed *MyD88* allele, with *RORc(γt)-Cre*^{tg} mice. F1 generations were backcrossed to *MyD88*^{fl} mice. *MyD88*$^{\Delta ILC3/T}$ mice are homozygous for the floxed *MyD88* allele and carry one copy of the Cre transgene.

4.2.3 Genotyping of I-ab$^{\Delta ILC3}$ and MyD88$^{\Delta ILC3/T}$ mice

Isolation of DNA

For isolation of genomic DNA, biopsies were digested in lysis buffer containing 300 μg/ml Proteinase K (at least 3 h at 56 °C while shaking at 700 rpm on a heat block). Saturated NaCl (6 M) was added for 10 min at 4 °C to remove membrane lipids. After centrifugation, supernatant was collected and DNA was precipitated by addition of 2-propanol for 15 min at RT. DNA was recovered in 1x TE buffer and stored at 4 ºC.

Reaction mix for PCR

1. *Cre* - PCR

Primer Cre Fwd: 5' CGTACTGACGGTGGGAGAAT 3'

Primer Cre Rev: 5' TGCATGATCTCCGGTATTGA 3'

PCR reaction mix:

2.5 μl	10x buffer
0.5 μl	Cre Fwd primer (10 μM)
0.5 μl	Cre Rev primer (10 μM)
0.5 μl	dNTPs (10 mM)
0.1 μl	Taq Polymerase (homemade)
1 μl	genomic DNA

fill up to 25 μl final volume with H_2O

PCR program for amplification:

step 1	94 ºC	5 min		
step 2	94 ºC	30 sec		
step 3	58 ºC	30 sec		
step 4	72 ºC	90 sec	**step 2 - 4**	**33 x**
step 5	72 ºC	10 min		
step 6	10 ºC	hold		

product length:

Cre: ~420 base pairs (bp)

2. *MyD88^{fl}* - PCR

Primer MyD88 Fwd: 5' GTT GTG TGT GTC CGA CCG T 3'

Primer MyD88 Rev: 5’ GTC AGA AAC AAC CAC CAC CAT GC 3’

PCR reaction mix:

1.2 µl	10x buffer
1.2 µl	MyD88 Fwd primer (10 µM)
1.2 µl	MyD88 Rev primer (10 µM)
0.24 µl	dNTPs (10 mM)
0.96 µl	$MgCl_2$
1 µl	Taq Polymerase (homemade)
1 µl	genomic DNA

fill up to 12 µl final volume with H_2O

PCR program for amplification:

step 1	94 ºC	3 min		
step 2	94 ºC	30 sec		
step 3	66 ºC	1 min		
step 4	72 ºC	1 min	**step 2 - 4**	**35 x**
step 5	72 ºC	2 min		
step 6	10 ºC	hold		

product length:

homozygote: 353 bp

heterozygote: 266 bp and 353 bp

wild type: 266 bp

3. *I-ab*^{neo} -PCR

Primer common Fwd: 5’ CTC TAC ACC CCC AAC ACA CC 3’

Primer WT Rev: 5’ AGT GAG CGA GCA CAG ACA AG 3’

Primer I-ab Rev: 5’ TCG CCT TCT TGA CGA GTT CT 3’

PCR reaction mix:

1.2 µl	10x buffer
1.2 µl	common Fwd primer (10 µM)
1.2 µl	WT Rev primer (10 µM)
1.2 µl	I-ab Rev primer (10 µM)
0.24 µl	dNTPs (10 mM)

0.96 µl	$MgCl_2$
1 µl	Taq Polymerase (homemade)
1 µl	genomic DNA

fill up to 12 µl final volume with H_2O

PCR program for amplification:

step 1	94 ºC	3 min		
step 2	94 ºC	30 sec		
step 3	62 ºC	30 sec		
step 4	72 ºC	30 sec	**step 2 - 4**	**35 x**
step 5	72 ºC	2 min		
step 6	10 ºC	hold		

product length:

homozygote: 199 bp

heterozygote: 199 bp and 295 bp

wild type: 295 bp

4.2.4 Flow cytometry and cell sorting

Cells derived from various organs were stained with mAbs using standard protocols.[88] Briefly, cells were resuspended in FACS buffer and stained with biotinylated or fluorochrome-conjugated Abs (30 min, 4 ºC). Fc receptors were blocked by incubation of the cells with anti-mouse FcγRII/III mAb (clone 2.4G2). Fluorochrome-conjugated streptavidin was added as a second incubation step (20 min, 4 ºC) for stainings including biotinylated Abs.

Intracellular RORγt staining was carried out using Foxp3 staining buffer set (eBioscience) according to manufacturer`s protocol. For intracellular cytokine staining (IFNγ), IC fixation buffer (eBioscience) was used and cells were incubated with 10 µg/ml Brefeldin A (BFA) for 4 h at 37 °C before analysis.

Dead cells were identified using LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Molecular Probes), Zombie Aqua™ Fixable Viability Kit (Biolegend) or in case of non-fixed cells propidium iodide (PI) solution (Sigma-Aldrich).

Lymphotoxinαβ (LTαβ) staining was performed as previously described.[87] Briefly, cells were treated with anti-FcγRII/III mAb (clone 2.4G2) and 0.5 % mouse and rat serum. LTβR-Fc (J. Browning, Biogen, Cambridge) was added and detected using

biotin-conjugated goat anti-human IgG (Jackson Immunoresearch) pretreated for 30 min with 4 % rat and mouse serum. Finally, fluorochrome-conjugated streptavidin and surface Abs were added.
Data acquisition was realized using FACSCalibur (four color staining) or FACSCanto II (eight color staining). Data were analyzed using FlowJo software (Tree Star).
Cell sorting was done using FACSAria IIu (BD Bioscience, >98 % purity).
Absolute numbers of cells derived from various organs were determined by counting cells with the cell counter CASY INNOVATIS (Roche).

4.2.5 Cell isolation and culture

For *in vitro* generation of FL-derived NCR$^-$ILC3s, total FL cells were isolated from 14.5 days post coitum (dpc) *WT* embryos, stained with PE-conjugated anti-$\alpha_4\beta_7$ Ab (DATK32) and purified with magnetic associated cell sorting (MACS®, Miltenyi Biotec). MACS® - enriched FL-derived $\alpha_4\beta_7^+$ ILC3 precursors were cultured for 5 days in supplemented Iscove's modified Dulbecco's medium containing 2 % FCS (IMDM 2 % FCS) in the presence of 20 ng/ml IL-7 (Peprotech) and 20 ng/ml SCF (Peprotech) at 37 ºC and 10 % CO_2 as described before.[89] Total cells were harvested from cultures and *in vitro* generated NCR$^-$ILC3s were sorted based on CD90.2, CD117 and CD4 expression.
Splenic and small intestinal lamina propria (LP) NCR$^-$ILC3s were isolated from spleen and small intestine (SI) of adult mice. Briefly, SI was opened longitudinally, feces were removed, and SI was cut into small pieces, which were incubated in 1x PBS containing 30 mM EDTA for 30 min at 4 ºC (tubes were laid down horizontally on ice). Afterwards, tissue pieces were washed several times in 1x PBS by vigorous shaking and then incubated in Dulbecco modified eagle's minimal essential medium (DMEM, Gibco) containing 0.025 mg/ml DNAseI (Roche) and 1 mg/ml Collagenase D (Roche) for total 1 h at 37 ºC. Every 15 min, tissue pieces in medium were pipetted up and down 20 times and afterwards the supernatant was collected (by passing through a 100 μm filter). The remaining tissue pieces were re-incubated with fresh pre-warmed medium containing DNAseI and Collagenase D. Cell suspension was pelleted, resuspended in 5 ml 40 % Percoll (GE Healthcare), underlaid with 3 ml 80 % Percoll and centrifuged for 30 min at 20 ºC (1800 rpm, acceleration 4, brake 1). Cells of the interphase were collected (total LP lymphocytes).
Spleens were cut into pieces, washed in 1x PBS by vigorous shaking and digested with DNaseI and Collagenase D as described for SI tissue pieces. After digestion,

spleen cells were pelleted, washed and red blood cells were lysed using erythrolysis buffer (2 min at room temperature (RT)).

Ex vivo isolated splenic or small intestinal LP ILC3s were sorted based on the expression of CD117 or CD90.2 and the lack of the lineage marker CD3ε, CD8α, CD11c, CD19, B220, Gr-1, TCRβ, TCRγδ, NK1.1 and NKp46.

Naïve $CD4^+$ T cells from spleen and LNs of *OT-II*^{tg} mice were magnetically purified by using CD4-beads (LTR4, Miltenyi Biotec) following manufacturer's instruction. Additionally, MACS® - enriched $CD4^+$ T cells were sort-purified based on CD4 expression and the lack of CD11c to reach >98 % $CD4^+$ T cell purity.

BMDCs and BMMΦ were generated as described elsewhere.[254, 255] In brief, femurs and tibias of *WT* mice were collected and the BM was recovered by crushing the bones. Red blood cells were lysed using erythrolysis buffer (2 min at RT). For BMDCs, total BM cells were cultured in a 6 well plate (Nunc) in supplemented IMDM containing 5 % FCS (IMDM 5 % FCS) in the presence of 200 ng/ml FLT3L (A. Rolink, University of Basel) at 37 ºC and 10 % CO_2. For BMMΦ, total BM cells were cultured in a 10 cm petridish (Sarstedt) in DMEM (Gibco) supplemented with L929 (Sigma Aldrich) at 37 ºC and 10 % CO_2. Cells were harvested after 7 days in culture.

4.2.6 Generation of BW - OTII cells

BW-OTII cells were generated as already described.[256] Briefly, total splenocytes of an *OT-II*^{tg} mouse were cultured for 2 days in supplemented IMDM containing 10 % FCS (IMDM 10 % FCS) in the presence of 5 μg/ml soluble anti-CD3 Ab (clone 2cll, A. Rolink, University of Basel). Activated *OT-II*^{tg} splenocytes were fused with the TCRαβ$^-$ BW5147 NFAT-EGFP fusion partner using pre-heated PEG 1500 solution. Cells were plated out at limiting dilution in the presence of HAT (hypoxanthine-aminopterin-thymidine) medium. Grown BW-OTII clones were tested *in vitro* in the presence of irradiated *WT* splenocytes and $OVA_{323-339}$ peptide.

4.2.7 ILC3 stimulation and Ag presentation assay *in vitro*

Sort-purified *in vitro* generated NCR^-ILC3s or e*x vivo* isolated splenic and LP NCR^- ILC3s were cultured in a 96-well plate (Costar, Corning Inc.) in the presence of either TLR ligands (100 ng/ml Pam3Cys, 25 μg/ml Poly I:C, 1 μg/ml Flagellin, 1 μg/ml Imiquimod, 10 μg/ml Zymosan, 1 μg/ml LPS, 1 μM CpG), pro-inflammatory cytokines (20 or 100 ng/ml IL-1β, 20 ng/ml IL-23) or in medium alone for 48 h.

To test Ag presentation and $CD4^+$ T cell activation, 5 x 10^4 sort-purified stimulated (20 ng/ml IL-1β, 24 h) NCR^-ILC3s were co-cultured with either 1.5 x 10^5 *ex vivo* isolated and sort-purified *OT-II*^{tg} $CD4^+$ T cells or 1 x 10^5 cultured BW-OTII cells in the presence of either $OVA_{323-339}$ peptide (5 μg/ml), OVA protein (100 μg/ml) or medium alone (w/o Ag) for 48 - 72 h or 12 - 15 h, respectively.

4.2.8 CFSE labeling and fluorescent latex bead uptake

To follow up proliferation of $CD4^+$ T cells upon antigen (Ag) challenge, *OTII*^{tg} $CD4^+$ T cells were labeled with 7.5 μM carboxyfluorescein succinimidyl ester (CFSE) in 1x PBS for 10 min at 37 °C. The reaction was stopped by addition of 1x PBS containing 80 % FCS.

Fluorescent latex bead uptake was performed as previously described with some adaptations.[257] Briefly, *in vitro* generated NCR^-ILC3s or *ex vivo* isolated splenic and LP NCR^-ILC3s were cultured in a 96-well plate flat bottom over night (o/n) to allow the cells to settle down. Latex beads (FluoSpheres® carboxylate-modified microspheres, 1 μm, red fluorescent (580/605)) were added for 6 h at 37 ºC and 4 ºC. To examine the specificity of bead uptake, ILC3s were pre-incubated for 1 h in the presence of 0.5 μM Cytochalasin D, washed and afterwards incubated with latex beads for 6 h at 37 ºC. To compare bead uptake of BMMΦ and NCR^-ILC3s, cells were harvested after 2 h or 24 h. Bead internalization was analyzed by flow cytometry and immunofluorescence microscopy. For immunofluorescence microscopy, *in vitro* generated NCR^-ILC3s were stained with FITC-conjugated anti-CD90.2 (30-H12, 30 min at 4 °C) and HOECHST dye (Hoechst 33342, 30 min at 37 °C) after incubation with beads. Bead uptake was monitored using a confocal laser-scanning microscope (Zeiss LSM 510 Meta). Images were analyzed with ImageJ (W. Rasband, NIH). An adjustment of brightness and contrast was performed.

4.2.9 Adoptive cell transfer and immunization

To investigate $CD4^+$ T cell proliferation *in vivo*, 3 x 10^6 *OT-II*^{tg} $CD4^+$ T cells ($CFSE^+$) were intravenously (i.v.) injected into *WT*, *I-ab*$^{\Delta ILC3}$ and *I-ab*$^{-/-}$ recipient mice, which additionally were i.v. immunized with $OVA_{323-339}$ peptide (20 μg), OVA protein (100 μg) and CpG (50 μM). 48 h later, *OT-II*^{tg} $CD4^+$ T cell proliferation was examined in the spleen by flow cytometry.

To examine T- dependent (TD) B cell responses *in vivo*, 2 x 10^6 *OT-II*^{tg} CD4$^+$ T cells plus CpG (25 μM) were i.v. injected into *WT*, *I-ab*$^{\Delta ILC3}$ and *ROR*$\gamma^{-/-}$ mice. Mice were immunized intraperitoneally (i.p.) with 100 μg alum-precipitated NP-OVA (NP (18)-OVAL) at day 0. Sera were collected 1 day before (day -1) and at day 5 and 14 after NP-OVA immunization and CD4$^+$ T cell transfer.

To monitor CD4$^+$ T cell – NCR$^-$ILC3 interaction *in vivo*, APCs (either BMDCs or *in vitro* generated NCR$^-$ILC3s) were i.v. injected into *I-ab*$^{-/-}$ mice. 24 h later, *OT-II*^{tg} CD4$^+$ T cells were adoptively transferred (i.v.) and mice were immunized. Different immunization strategies were used; i) alum-precipitated NP-OVA (100 μg), ii) OVA peptide (20 μg) and OVA protein (100 μg), iii) OVA peptide (20 μg), OVA protein (100 μg) and CpG (50 μM). CD4$^+$ T cell proliferation was monitored 48 h after immunization in the spleen, the inguinal LNs and the mesenteric LNs. In a second approach, APCs were injected into the foot pad (f.p.) of *I-ab*$^{-/-}$ mice in a maximal volume of 50 μl and monitored after different time points (3 - 24 h) in the draining LNs (popliteal LNs).

To investigate activation state of NCR$^-$ILC3s upon innate challenge *in vivo*, *WT* or *Rag2*$^{-/-}$ mice were injected i.p. with 100 μM CpG (CpG-ODN1826) or 100 μg LPS. The phenotype of splenic NCR$^-$ILC3s was assessed 6 h later by flow cytometry. To perform RT qPCR analysis of spleen tissue, a small tissue piece was immediately frozen in liquid nitrogen (N_2).

4.2.10 Antibody and cytokine detection by ELISA and Luminex assay

To detect NP-OVA-specific Abs in the serum of immunized mice, NUNC immunoplate Maxisorb F96 plates were coated with 5 μg/ml NP-OVA (Biosearch Technologies Inc.) in 1x PBS at 4 ºC o/n. Sera were incubated for 1.5 h at RT and after washing (H_2O, 0.1 % Tween-20) biotin-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3 (Caltag Laboratories, 1.5 h, RT) were added and detected by alkaline-phosphatase (AKP)-conjugated Streptavidin (Roche, 45 min, RT). Plates were developed with dinitrophenyl phosphate (dNPP, 1 mg/ml, Sigma) in substrate buffer. The reaction was stopped with 1 M NaOH (Fluka). The optical density (OD) was determined at 405 nm with an ELISA reader (ASYS Expert plus).

To detect total IgM and IgG3 levels in the serum of non-immunized mice, NUNC immunoplate Maxisorb F96 plates were coated with either 5 μg/ml purified anti-mouse-IgM (unlabeled, clone M41) or purified anti-mouse IgG3 (clone R2-38) in 1x PBS at 4 ºC o/n. Sera were incubated for 1.5 h at RT and after washing biotin-

conjugated goat anti-mouse IgM was added to IgM coated plates (1.5 h, RT) and detected by AKP-conjugated Streptavidin (45 min, RT). AKP-conjugated anti-mouse IgG3 (R2-38) was added to IgG3 coated plates (1.5 h, RT). Plates were developed and OD at 405 nm was determined as described before.

IL-17 and IL-22 were determined in the cell culture supernatants of NCR^-ILC3s by either using mouse IL-17 ELISA MAX™ Standard Set (Biolegend) or mouse IL-22 ELISA MAX™ Deluxe Set (Biolegend) according to manufacturer's instructions. OD was determined at 450 nm with an ELISA reader (ASYS Expert plus).

In addition, cytokines in cell culture supernatants of *in vitro* generated NCR^-ILC3s were quantified using a multiplex-bead based Luminex assay (mouse cytokine 20-plex panel, Invitrogen, Life technologies) according to manufacturer's protocol. Analysis was performed with a Luminex 100 (LX100) analyzer (Invitrogen, Life technologies).

4.2.11 RNA isolation, cDNA synthesis and RT qPCR

For RNA isolation from whole spleen tissue, a small tissue piece was immediately frozen in liquid N_2 in a 1.5 ml Micro tube PP (Sarstedt) containing Zirconia beads (BioSpec Products). Whole tissue was homogenized using FastPrep-24 instrument (MP Biomedicals) for 1 min at 6.5 m/sec. Afterwards, RNA was isolated with the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. RNA quantification and quality assessment were performed on a Nanodrop 2000c (Thermo Scientific Inc.).

First-strand cDNA synthesis was carried out using Oligo dT (Promega), dNTPs (Roche), random hexamers (Sigma-Aldrich) and Superscript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Real-time qPCR for quantitative expression analysis was performed on a Rotor-Gene RG-3000A (Corbett research) using SensiMix SYBR Hi-Rox Kit (Bioline). The results were normalized to the housekeeping gene β-actin using the comparative threshold cycle method (ΔC_T) for relative quantification.

4.2.12 DNA microarray analysis

The gene expression profiles of *in vitro* generated $CD4^+$ and $CD4^-$ NCR^-ILC3s were assessed using Affymetrix Gene 2.0 ST Array (Affymetrix, Santa Clara, CA). Microarray data were generated by analysis of only one specimen per experimental

condition; i) naïve $CD4^+$ NCR^-ILC3s, ii) naïve $CD4^-$ NCR^-ILC3s, iii) IL-1β-activated $CD4^+$ NCR^-ILC3s and iiii) IL-1β-activated $CD4^-$ NCR^-ILC3s. The activation of cells with IL-1β was carried out in *in vitro* cultures for 1 h at 37 °C. RNA from sort-purified *in vitro* generated $CD4^+$ and $CD4^-$ NCR^-ILC3s (2×10^5 – 1×10^6 cells) was isolated using the RNeasy Micro Kit (Qiagen) according to manufacturer's protocol. High quality of RNA was confirmed with the Experion RNA HighSens Analysis Kit (BioRad). RNA target synthesis starting with 258.7 ng RNA was performed using WT Expression Kit (Ambion). Fragmentation and labeling of amplified cDNA were carried out using GeneChip® WT Terminal Labeling Kit (Affymetrix). DNA was loaded on Mouse Gene 2.0 ST Array (Affymetrix), hybridized for 17 h and afterwards washed and stained using Affymetrix protocol FS450_0007. The GeneChips were processed with an Affymetrix GeneChip® Scanner 3000 7G and DAT images (raw image data from chip scanner) as well as CEL files (intensity values) of the microarray were generated using Affymetrix GeneChip® Command Control® Software (AGCC, version 3.0.0.1214). Affymetrix CEL files were normalized based on the RMA (Robust Multiarray Average)[258] and data were $\log_2$-transformed using Partek® Genomics Suite (version 6.12.0907) software (Partek Inc. St. Louis, MO).

4.2.13 Statistical analysis

Statistical analysis was performed using Mann Whitney U test, unpaired Students t-test and Wilcoxon test with Prism software (GraphPad Software, Inc.).

5 Results

5.1 *In vitro* generation of natural cytotoxicity receptor-negative (NCR^-) ILC3s.

5.1.1 Fetal liver-derived $\alpha_4\beta_7^+$ cells can give rise to $CD4^+$ and $CD4^-$ NCR^- group 3 innate lymphoid cells *in vitro*.

To study the immune response of natural cytotoxicity receptor $(NCR)^-$ group 3 innate lymphoid cells (NCR^-ILC3s) toward innate stimuli *in vitro*, these cells were either *in vitro* generated from $\alpha_4\beta_7^+$ ILC3 precursors or *ex vivo* isolated from different organs of adult mice. The $\alpha_4\beta_7^+$ ILC3 precursors could be found in the fetal liver (FL) of *WT* embryos 14.5 dpc and were known to give rise to NCR^-ILC3s *in vitro*.[60, 61] Therefore, for *in vitro* generation of NCR^-ILC3s, I cultured FL-derived $\alpha_4\beta_7^+$ ILC3 precursors for 5 days in the presence of IL-7 and SCF. These two factors were known to be essential for the survival and maintenance of NCR^-ILC3s *in vitro* and *in vivo*.[88, 89, 91, 103] By the use of these culture conditions the majority of $\alpha_4\beta_7^+$ ILC3 precursors differentiated into FL- derived NCR^-ILC3s expressing characteristic phenotypic markers of NCR^- ILC3s like CD90.2, CD127 (IL-7Rα) and CD117 (Fig. 5 A).

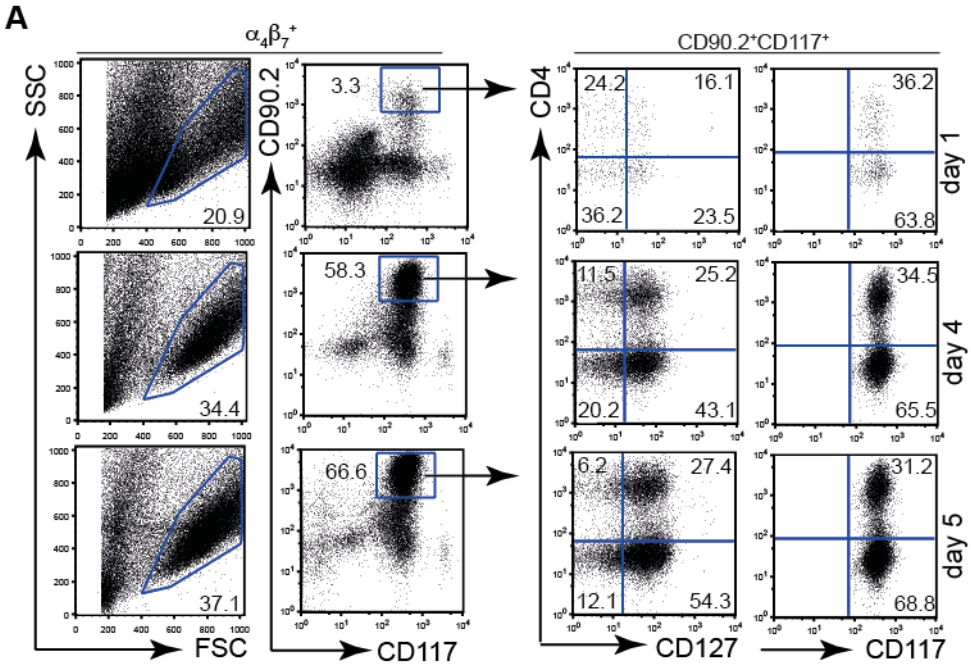


Figure 5: *In vitro* generation of NCR$^-$ILC3s from $\alpha_4\beta_7^+$ ILC3 precursors isolated from the FL of 14.5 dpc *WT* embryos. A) Phenotype of $\alpha_4\beta_7^+$ cells isolated from the FL of 14.5 dpc *WT* embryos 1, 4 and 5 days in culture with IL-7 and SCF. Representative dot plots are shown. Numbers in dot plots show the percentage of cells in each gate.

Phenotypic analysis of *in vitro* generated NCR$^-$ILC3s revealed that within the CD90.2$^+$CD117$^+$ population of NCR$^-$ILC3s two different subpopulations of cells existed (Fig. 6 A). One subpopulation showed CD4 expression on the surface (CD4$^+$ NCR$^-$ILC3s, approximately 30 % of total NCR$^-$ILC3s), whereas the other lacked the expression of CD4 (CD4$^-$ NCR$^-$ILC3s, approximately 70 % of total NCR$^-$ILC3s) (Fig. 6 A). To examine whether differences in the phenotype of these two subpopulations exist, I performed a detailed analysis of *in vitro* generated NCR$^-$ILC3s.

The retinoic acid related orphan receptor RORγt is a transcription factor, which is absolutely required for the development of all ILC3 subsets.[70, 93] In line with this, both *in vitro* generated CD4$^+$ and CD4$^-$ NCR$^-$ILC3s expressed comparable high levels of RORγt (Fig. 6 B). Since IL-7 and SCF are both important cytokines required for the survival and maintenance of ILC3s and their precursors,[88, 89, 91, 103] I investigated the expression of their according receptors on *in vitro* generated NCR$^-$ILC3s. Both CD4$^+$ and CD4$^-$ NCR$^-$ILC3s showed high expression of the γ_c chain, which builds together with the IL-7Rα chain the receptor for IL-7 (Fig. 6 B).[259] The IL-7Rα chain was as well expressed on both CD4$^+$ and CD4$^-$ NCR$^-$ILC3s (Fig. 5 A). Additionally, high expression levels for CD117, the receptor for SCF, could be detected on both subsets of *in vitro* generated NCR$^-$ILC3s (Fig. 6 B). During embryogenesis, ILC3s

play a crucial role in lymphoid tissue formation and organization.[4, 70, 71, 72] This process involves the interaction of LTαβ-expressing ILC3s and LTβR-expressing stromal cells.[68, 260] In line with this, both $CD4^+$ and $CD4^-$ NCR^-ILC3 subsets expressed comparable high levels of LTαβ (Fig. 6 B). In addition to LTαβ-LTβR signaling, several integrins and adhesion molecules ($\alpha_4\beta_7$ and β1 integrin, ICAM-1) as well as chemokine and cytokine receptors (CXCR4, CXCR5, CCR6 and CCR7) are involved in the process of lymphoid tissue formation mediated by ILC3s.[73, 79] In accordance, both $CD4^+$ and $CD4^-$ NCR^-ILC3 subsets expressed $\alpha_4\beta_7$ and β1 integrin as well as ICAM-1 (Fig. 6 B). Further, CXCR4, CXCR5, CCR6 and CCR7 were expressed on $CD4^+$ and $CD4^-$ NCR^-ILC3s (Fig. 6 B). Additionally, $CD4^+$ and $CD4^-$ NCR^-ILC3s expressed comparable high levels of CD44 whereas they both lacked the expression of CD62L and NKp46 (Fig. 6 B). The expression of NK1.1 and CD69 was low on both subpopulations of *in vitro* generated NCR^-ILC3s (Fig. 6 B). Altogether, these data demonstrated that *in vitro* generated NCR^-ILC3s display a characteristic NCR^-ILC3 phenotype already described in 1997.[57] Furthermore, the *in vitro* generated $CD4^+$ and $CD4^-$ NCR^-ILC3 subsets show an equal phenotype.

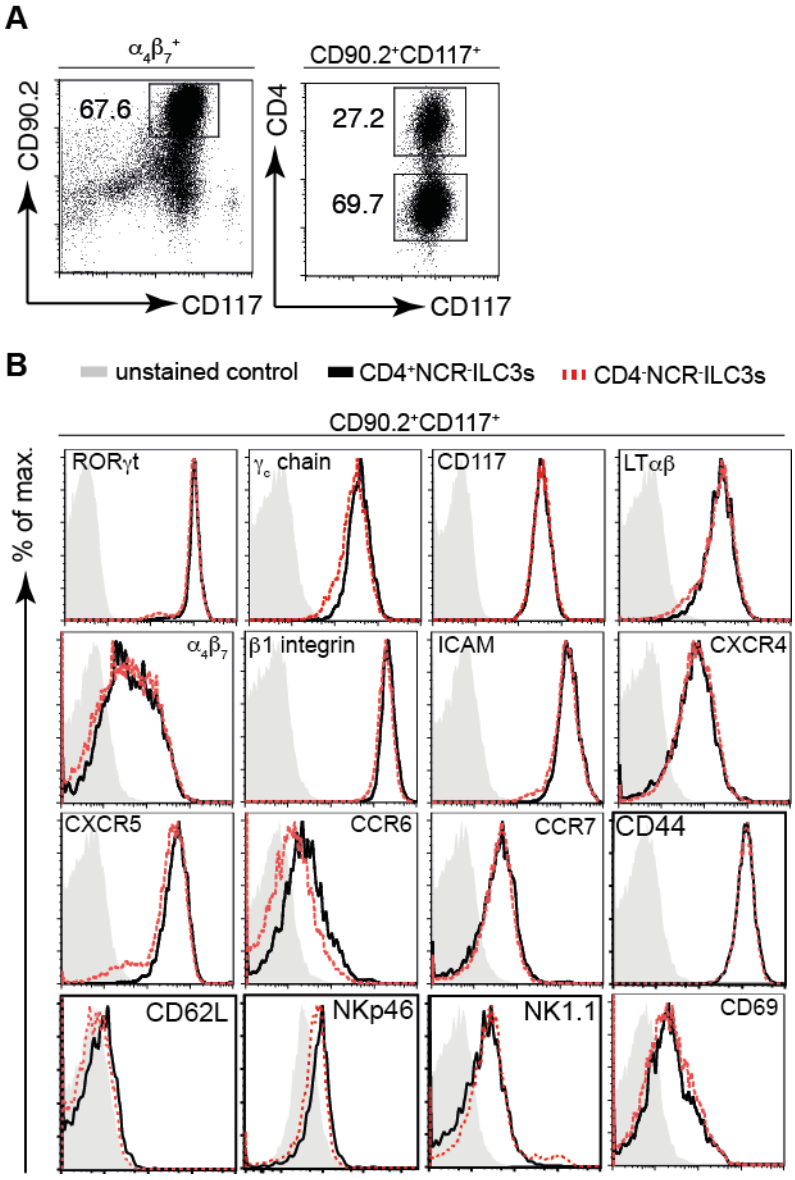


Figure 6: Phenotype of *in vitro* generated NCR$^-$ILC3s. A) Phenotype of *in vitro* generated NCR$^-$ILC3s derived from $\alpha_4\beta_7^+$ ILC3 precursors. First dot plot shows total cells differentiated from $\alpha_4\beta_7^+$ ILC3 precursors after 5 days in culture. Second dot plot shows CD4$^+$ and CD4$^-$ subsets within the CD90.2$^+$CD117$^+$ NCR$^-$ILC3 population. Representative dot plots are shown. **B)** Representative histograms of intracellular RORγt expression and the expression of several surface molecules (indicated in the figure) by CD4$^+$ and CD4$^-$NCR$^-$ILC3 subsets.

5.2 *In vitro* generated NCR$^-$ILC3s produce cytokines and show an activated phenotype after innate stimulation *in vitro*.

5.2.1 Innate stimulation of *in vitro* generated NCR$^-$ILC3s induces cytokine secretion.

Innate immunity is mainly mediated by immune cells directly recognizing microbial products through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors.

To examine whether *in vitro* generated NCR$^-$ILC3s were either able to directly sense the presence of pathogens or to respond to inflammatory cytokines produced by other immune cells, I cultured NCR$^-$ILC3s for 48 h in the presence of several different pro-inflammatory cytokines or TLR ligands. Cytokines and TLR ligands were used at concentrations already described for *in vitro* stimulation.[30, 261, 262]

IL-22 is a cytokine, which is able to directly induce antimicrobial host defense. It stimulates epithelial cells to produce antimicrobial peptides and contributes to epithelial cell integrity.[113] Therefore, I measured whether innate stimulation of NCR$^-$ ILC3s was able to induce IL-22 production. Upon stimulation with the pro-inflammatory cytokine IL-1β, *in vitro* generated NCR$^-$ILC3s produced high levels of IL-22 whereas non-stimulated naïve NCR$^-$ILC3s did not show IL-22 secretion (Fig. 7 A). IL-22 secretion upon IL-1β stimulation even exceeded those induced in response to IL-23 stimulation, a factor which was already reported to induce IL-22 production by ILC3s (Fig. 7 A).[105, 106, 108, 120] Both CD4$^+$ and CD4$^-$ NCR$^-$ILC3 subpopulations produced comparable levels of IL-22 in response to IL-1β stimulation (Fig. 7 A).

IL-17 is a major mediator of inflammation and plays a critical role in host defense against extracellular bacteria and fungi.[263, 264] Therefore, I additionally measured whether *in vitro* generated NCR$^-$ILC3s were able to produce IL-17 in response to innate stimulation. Indeed, *in vitro* generated NCR$^-$ILC3s produced IL-17 upon exposure to IL-1β, although at lower levels than IL-22 (Fig. 7 B). *In vitro* IL-23 stimulation did not induce IL-17 production by *in vitro* generated NCR$^-$ILC3s although it was previously reported that IL-17 production of ILC3s upon *Candida albicans* infection depends on IL-23 (Fig. 7 B).[265]

Furthermore, I measured cytokine production of NCR$^-$ILC3s after 48 h *in vitro* stimulation with TLR ligands. In response to Poly I:C, the TLR ligand for TLR3, NCR$^-$ ILC3s secreted significantly higher amounts of IL-22 compared to naïve NCR$^-$ILC3s.

Other TLR ligands such as Imiquimod and CpG only led to low or negligible production of IL-22 (Fig. 7 C). The addition of TLR ligands to *in vitro* cultures was unable to induce IL-17 secretion by *in vitro* generated NCR$^-$ILC3s (Fig. 7 C). Notably, IL-1β was an extremely strong inducer of IL-17 and IL-22 production by NCR$^-$ILC3s. Basal and IL-23-induced IL-22 production by ILC3s was reported to be dependent on IL-1R1,[266, 267, 268] but whether ILC3s release other cytokines in response to IL-1β was not known so far. Therefore, I screened for additional cytokines produced by *in vitro* generated NCR$^-$ILC3s after 48 h IL-1β exposure. IL-1β-exposed NCR$^-$ILC3s secreted IL-2, IL-6, MIP-1α, IFNγ and TNFα, all known for their capacity to promote T cell responses (Fig. 7 D). In addition, IL-1β induced NCR$^-$ILC3s to secrete interferon-induced protein of 10 kDa (IP-10), a chemoattractant for mononuclear cells and CXCR3$^+$ effector T cells (Fig. 7 D).[268] Reminiscent of its RORγt-dependent expression in T cells,[269] GMCSF was already produced by naïve NCR$^-$ILC3s and its secretion was enhanced upon IL-1β exposure (Fig. 7 D).

Collectively, these data demonstrated that the response of NCR$^-$ILC3s to innate stimulation differed dependent on the nature of the stimuli. IL-1β acted as a strong activator of NCR$^-$ILC3s inducing the production of a wide range of cytokines, many of which were known to alter T cell behavior. Moreover, NCR$^-$ILC3s responded directly to TLR3 stimulation with IL-22 secretion.

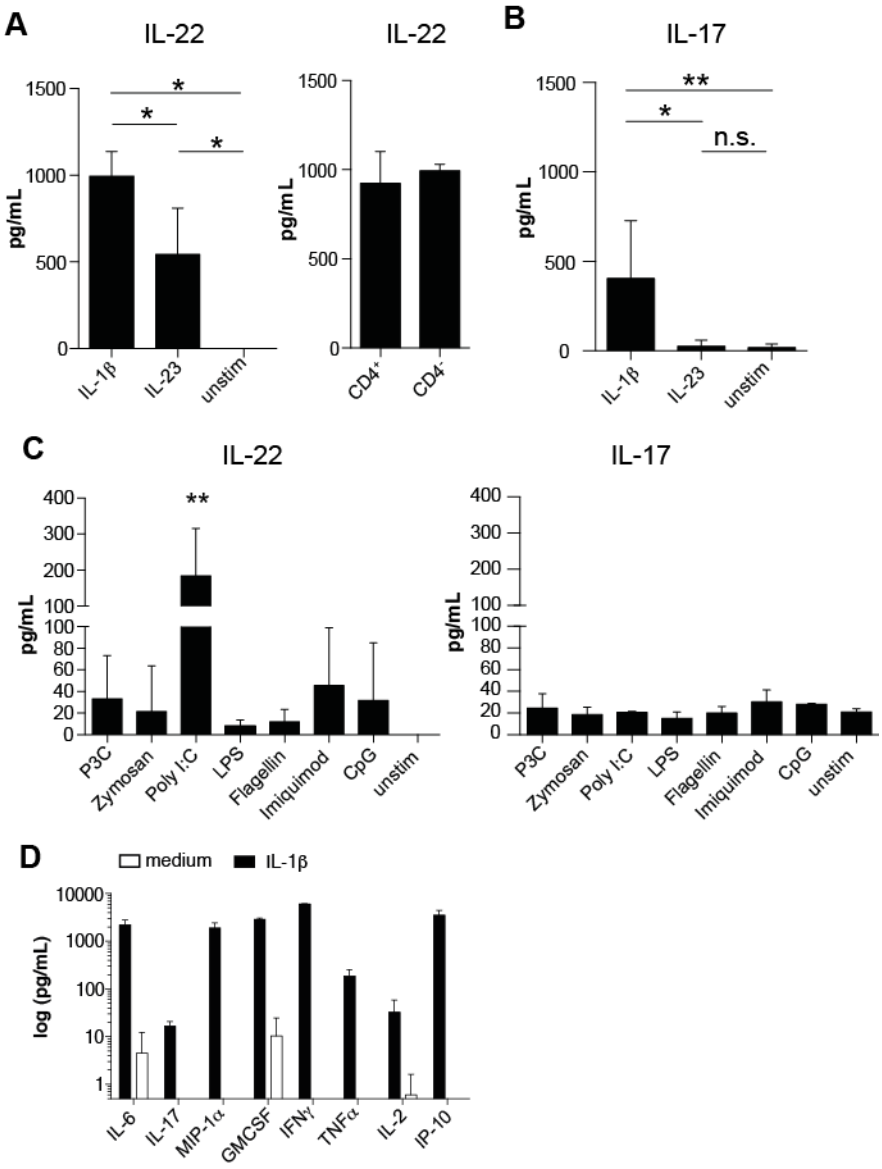


Figure 7: Cytokine secretion of activated NCR$^-$ILC3s. A) IL-22 secretion by *in vitro* generated total NCR$^-$ILC3s upon 48 h exposure to IL-1β, IL-23 or medium alone (unstim) and by CD4$^+$ and CD4$^-$NCR$^-$ ILC3 subsets upon 48 h exposure to IL-1β. Data are shown as mean values + SD (n = 3 - 7, 3 independent experiments; *P ≤ 0.05). **B)** IL-17 secretion by *in vitro* generated total NCR$^-$ILC3s upon 48 h exposure to IL-1β, IL-23 or medium alone (unstim). Data are shown as mean values + SD (n = 3 - 6, 2 independent experiments; n.s. = not significant; *P ≤ 0.05; **P ≤ 0.01). **C)** IL-22 and IL-17 secretion by *in vitro* generated total NCR$^-$ILC3s upon 48 h exposure to TLR ligands (indicated in the figure) or medium alone (unstim). Data are shown as mean values + SD (n = 3 - 7, at least 2 independent experiments; **P ≤ 0.01). **D)** Cytokine production by *in vitro* generated NCR$^-$ILC3s after 48 h exposure to IL-1β or medium alone. Results are shown as mean values + SD.

5.2.2 *In vitro* generated NCR$^-$ILC3s show an activated phenotype upon innate stimulation.

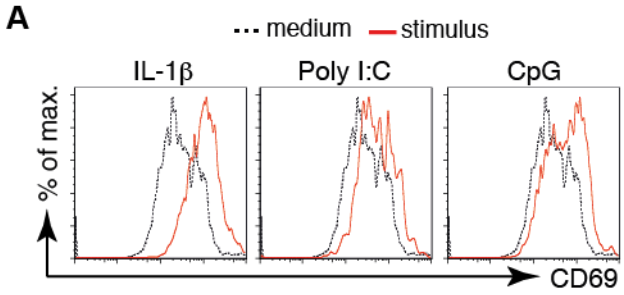
Based on the findings that *in vitro* generated NCR$^-$ILC3s were able to respond to innate stimulation with the production of cytokines, I next investigated, whether stimulation with microbial products or inflammatory cytokines induces changes in the phenotype of NCR$^-$ILC3s. How dendritic cells (DCs) undergo maturation and activation upon exposure to signals associated with infection and inflammation is well documented.[173] However, the mechanism by which NCR$^-$ILC3s undergo activation and change in phenotype and function was still not known. I therefore tested pro-inflammatory cytokines and TLR ligands for their ability to induce phenotypical changes resembling activation of NCR$^-$ILC3s.

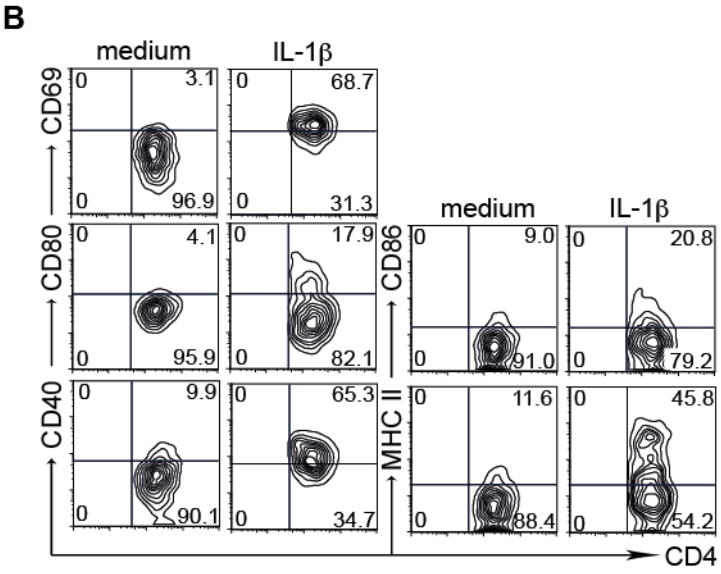
CD69 is known as the earliest inducible cell surface glycoprotein acquired during lymphocyte activation.[202] Thus, I screened for CD69 expression on NCR$^-$ILC3s cultured in the presence of either pro-inflammatory cytokines or TLR ligands. Indeed, IL-1β, Poly I:C and CpG were able to induce surface CD69 expression on NCR$^-$ILC3s indicating their activation (Fig. 8 A).

MHC class II (MHC II) expression is required for Ag presentation to CD4$^+$ T cells. Additionally, the expression of co-stimulatory molecules such as CD80/CD86 and CD40 on professional APCs, like DCs, provide accessory signals to CD4$^+$ T cells, which are required for complete CD4$^+$ T cell activation, proliferation and increased survival.[207] Therefore, I asked whether, analog to DCs, activated NCR$^-$ILC3s expressed co-stimulatory molecules and up-regulated MHC II molecule expression on the surface. When cultured in medium alone, *in vitro* generated sort-purified CD4$^+$ NCR$^-$ILC3s showed a naïve phenotype displayed by the absence of CD69 and co-stimulatory molecules (Fig. 8 B). Upon exposure to IL-1β for 48 h, sort-purified *in vitro* generated CD4$^+$ NCR$^-$ILC3s expressed the co-stimulatory molecules CD80 and CD86 and up-regulated the expression of CD40, CD69 and MHC II (Fig. 8 B). Hence, upon IL-1β stimulation, *in vitro* generated NCR$^-$ILC3s acquired an APC-like phenotype, reminiscent of activated DCs.

Further, I determined the gene expression profile of CD4$^+$ and CD4$^-$ NCR$^-$ILC3s by performing a whole transcript array (Affymetrix Mouse Gene 2.0 ST Array). First, sort-purified *in vitro* generated naïve and activated CD4$^+$ and CD4$^-$ NCR$^-$ILC3s were analyzed for the expression of genes related to their phenotype. It has to be noted that CD4$^+$ and CD4$^-$ NCR$^-$ILC3s were activated *in vitro* only for 1 h in the presence of IL-1β. Genes related to the phenotype of NCR$^-$ILC3s such as *Cd4*, *Rorc*, *Cd90*,

Cd117 and *Il1r1* were highly expressed by $CD4^+$ and $CD4^-$ NCR^-ILC3s shown by an expression value of around 12.0 (log_2 values of signal intensity measured in the array; Fig. 8 C). Expression values around 5.0 were measured for genes like *Cd3* and *Cd19*, which are related to T and B cells, respectively (Fig. 8 C). Based on these findings, I assumed that genes with an expression value below or around 5.0 were not expressed by NCR^-ILC3s while genes with an expression level of around 12.0 were highly expressed by these cells. Therefore, both naïve and IL-1β-activated $CD4^+$ and $CD4^-$ NCR^-ILC3s showed expression of genes related to their phenotype at high levels (Fig. 8 C). No differences in genes related to the phenotype of NCR^- ILC3s were found when naïve and IL-1β-activated NCR^-ILC3s were compared (Fig. 8 C). The expression value of *Cd4* in naïve and IL-1β-activated $CD4^+$ NCR^-ILC3s was 12.8 and 12.7, respectively. $CD4^-$ NCR^-ILC3s showed *Cd4* expression values clearly lower than that of $CD4^+$ NCR^-ILC3s (8.4 and 8.0; Fig. 8 C). The fact that *in vitro* generated NCR^-ILC3s expressed an APC-like phenotype upon IL-1β exposure indicated, that these cells might be involved in Ag presentation. Therefore, I examined the expression of genes related to MHC II-dependent Ag-presentation in $CD4^+$ and $CD4^-$ NCR^-ILC3s. Genes related to Ag-presentation such as *Cd74*, *H2-Ab1* and *H2-Aa* were expressed by both naïve and IL-1β-activated $CD4^+$ and $CD4^-$ NCR^- ILC3s at high levels (expression values in the range of 9.3 – 11.7; Fig. 8 C). Other genes related to Ag-presentation like *H2-DMb2* and *H2-DMa* were expressed at slightly lower levels (7.7 – 8.6; Fig. 8 C). No differences in transcript levels of genes related to Ag-presentation could be observed when naïve NCR^-ILC3s were compared to IL-1β-activated NCR^-ILC3s (Fig. 8 C).





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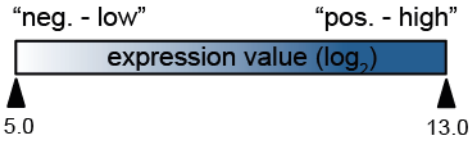


	naïve		activated (IL-1β, 1h)		
genes	CD4⁺	CD4⁻	CD4⁺	CD4⁻	
Cd4	12.8	8.4	12.7	8.0	genes related to NCR⁻ILC3 phenotype
Rorc	13.0	12.8	12.9	12.7	
Cd90	11.9	11.7	11.8	11.6	
Cd117	12.1	12.0	12.1	12.2	
Il1r1	12.6	12.5	12.6	12.5	
Cd74	11.5	10.8	11.7	11.2	genes related to Ag-presentation
H2-Ab1	11.5	10.9	11.2	10.8	
H2-DMb2	8.6	7.8	8.4	8.0	
H2-Aa	10.1	9.3	10.0	9.4	
H2-DMa	8.1	7.7	8.1	7.9	
Cd3	5.5	5.3	5.8	5.6	genes related to T and B cell phenotype
Cd19	5.4	5.1	5.4	5.2	

Figure 8: Innate stimulation of *in vitro* generated NCR$^-$ILC3s induces changes in the expression of surface molecules. A) Representative histograms of CD69 expression on *in vitro* generated NCR$^-$ ILC3s after 48 h stimulation with IL-1β, Poly I:C, CpG or medium alone. **B)** Expression of CD69, CD80, CD86, CD40 and MHC II on sort-purified *in vitro* generated CD4$^+$ NCR$^-$ILC3s cultured for 48 h with IL-1β or medium alone as indicated in the figure. Numbers in contour plots show the percentage of cells in each quadrant. Data are representative of 3 independent experiments. **C)** Expression levels of individual genes expressed in naïve and IL-1β-activated (1 h) CD4$^+$ and CD4$^-$ NCR$^-$ILC3s. The expression values are shown as log_2 transformed values of signal intensities measured in the array. Expression values are depicted as numbers (5.0 = negative or low expression; 13.0 = positive or high expression) and in a color code (white = neg. or low expression; blue = pos. or high expression). Genes were clustered as i) genes related to the phenotype of NCR$^-$ILC3s, ii) genes related to Ag-presentation, and iii) genes related to T and B cell phenotype. Data shown were generated by the analysis of DNA isolated from one individual specimen of two independent rounds of *in vitro* NCR$^-$ILC3 generation. 90 - 120 embryos collected from 13 - 15 pregnant female *WT* mice were used to isolate $\alpha_4\beta_7^+$ ILC3 precursors differentiating into NCR$^-$ILC3 *in vitro*.

Altogether, I could show that $\alpha_4\beta_7^+$ ILC3 precursors isolated from the FL of *WT* embryos could give rise to NCR$^-$ILC3s *in vitro*. CD4$^+$ and CD4$^-$ NCR$^-$ILC3s, sharing an identical phenotype, were both able to respond to innate stimulation. They produced IL-17 and IL-22 upon exposure to the pro-inflammatory cytokine IL-1β. Additionally, remarkable IL-22 secretion by NCR$^-$ILC3s could be induced in the presence of the cytokine IL-23 and the TLR ligand Poly I:C. Furthermore, the TLR ligands CpG and Poly I:C as well as the pro-inflammatory cytokine IL-1β induced the up-regulation of the early activation marker CD69 on NCR$^-$ILC3s indicating that NCR$^-$ ILC3s are able to directly respond to innate stimuli. Finally, IL-1β turned out to be a remarkable strong activator of *in vitro* generated NCR$^-$ILC3s, capable to induce the production of a broad repertoire of cytokines and the expression of co-stimulatory molecules and MHC II molecules. All findings observed by *in vitro* stimulation of *in vitro* generated NCR$^-$ILC3s are summarized in Table 1.

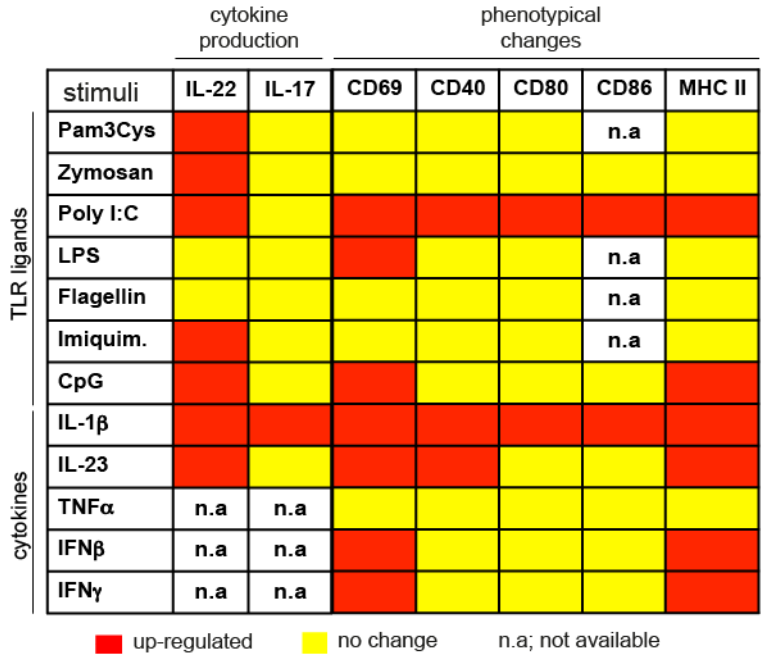


Table 1: Summary: Innate stimulation of *in vitro* generated NCR$^-$ILC3s induces cytokine secretion and changes in the phenotype. Summary of changes in *in vitro* generated NCR$^-$ILC3s in response to innate stimulation with different TLR ligands and pro-inflammatory cytokines as indicated. Red: up-regulation of cytokine production or surface marker expression; yellow: no changes observed; n.a: not available.

5.3 Splenic NCR$^-$ILC3s can become activated through innate stimulation *in vivo* and acquire an APC-like phenotype upon IL-1β exposure *in vitro*.

5.3.1 Stimulation with TLR ligands induces activation of splenic NCR$^-$ILC3s *in vivo*.

Our research group and others have previously shown that FL-derived and adult ILC3s share phenotypic and functional properties such as lymphotoxin β-dependent formation of lymphoid tissues and its organization.[96, 103] I could show that FL-derived NCR$^-$ILC3s produced cytokines and changed their phenotype upon *in vitro* exposure to either microbial products or pro-inflammatory cytokines. However, whether adult NCR$^-$ILC3s *in vivo* were able to sense innate stimulation was not known.

In vivo, NCR$^-$ILC3s could be identified in the spleen of adult mice by the lack of all lymphoid lineage markers (lin$^-$) and the expression of CD90.2 and CD117 at high levels (Fig. 9 A). The lineage cocktail used to distinguish lineage positive (lin$^+$) and

lineage negative (lin^-) cells contained antibodies against CD3ε, CD8α, CD11c, CD19, B220, Gr-1, TCRβ, TCRγδ, NK1.1 and NKp46. Similar to *in vitro* generated NCR^- ILC3s, *ex vivo* isolated splenic NCR^-ILC3s could be subdivided into two subpopulations, $CD4^+$ and $CD4^-$ NCR^-ILC3s, respectively (Fig. 9 A). Approximately 70 % of all NCR^-ILC3s expressed CD4 whereas approximately 30 % lacked CD4 expression (Fig. 9 A). Both subpopulations expressed comparable high levels of the transcription factor RORγt (Fig. 9 A). Hence, considering phenotypic markers that are characteristic for NCR^-ILC3s, *ex vivo* isolated splenic and *in vitro* generated NCR^- ILC3s were identical (Fig. 9 A). For identification and phenotypic characterization of splenic NCR^-ILC3s, I used *Rag2*$^{-/-}$ mice since these mice harbor increased numbers of NCR^-ILC3s compared to *WT* mice. The phenotype of splenic NCR^-ILC3s of *Rag2*$^{-/-}$ mice did not differ from that of *WT* mice.

In order to test whether *in vivo* exposure to innate stimuli was able to induce activation of splenic NCR^-ILC3s, *WT* mice were injected intraperitoneally (i.p.) with a single dose of 100 μM CpG, the ligand for TLR9. Control mice were injected with a single dose of PBS. First of all, I monitored the effects of *in vivo* CpG treatment on the whole splenic tissue of *WT* mice. 6 h after CpG injection the relative expression of the pro-inflammatory cytokines IL-1β, TNFα and IFNγ was significantly increased compared to PBS treated mice (Fig. 9 B). Thus, CpG was able to generate an inflammatory milieu in the spleen of treated *WT* mice including elevated levels of IL-1β, a factor, which was shown to act as a strong activator of *in vitro* generated NCR^- ILC3s.

Therefore, I next investigated the phenotype of NCR^-ILC3s *ex vivo* isolated from the spleen of either CpG or PBS treated mice. Indeed, i.p. injection of CpG induced activation of splenic NCR^-ILC3s displayed by high surface expression of CD69 in CpG compared to PBS treated *WT* mice (Fig. 9 C) consistent with the data observed for *in vitro* generated NCR^-ILC3s after CpG stimulation *in vitro* (Fig. 8 A, Table 1). Whether splenic NCR^-ILC3 activation resulted from a direct effect of CpG or indirectly through the established inflammatory milieu (e.g. IL-1β) could not be determined.

Microbial products as well as sterile host-derived danger molecules released upon injury are able to induce the inflammasome complex, which promotes the cleavage of pro-IL-1β into bioactive IL-1β.[270, 271] One of these microbial products is Lipopolysaccharide (LPS), the component of the outer membrane of gram-negative bacteria. LPS, the ligand of TLR 4, is known to induce IL-1β production upon *in vivo* challenge.[167] Therefore, I additionally tested whether *in vivo* treatment with LPS was able to induce activation of splenic NCR^-ILC3s in adult mice. Injection of 100 μg LPS

resulted in high expression of CD69 and up-regulation of MHC II expression on splenic $CD4^+$ NCR^-ILC3s examined 6 h after treatment (Fig. 9 D). *In vivo* LPS treatment did not affect the amount of splenic NCR^-ILC3s nor the ratio between $CD4^+$ and $CD4^-$ NCR^-ILC3s (Fig. 9 D). Whether activation of NCR^-ILC3s was mediated directly by LPS or by LPS-induced IL-1β has to be explored.

Collectively these data demonstrated that *in vivo* treatment of adult mice with TLR ligands CpG or LPS resulted in activation of splenic NCR^-ILC3s monitored by increased CD69 and MHC II expression. However, from these experiments it could not be revealed whether the TLR ligands acted directly on the cells or indirectly by generating an inflammatory environment.

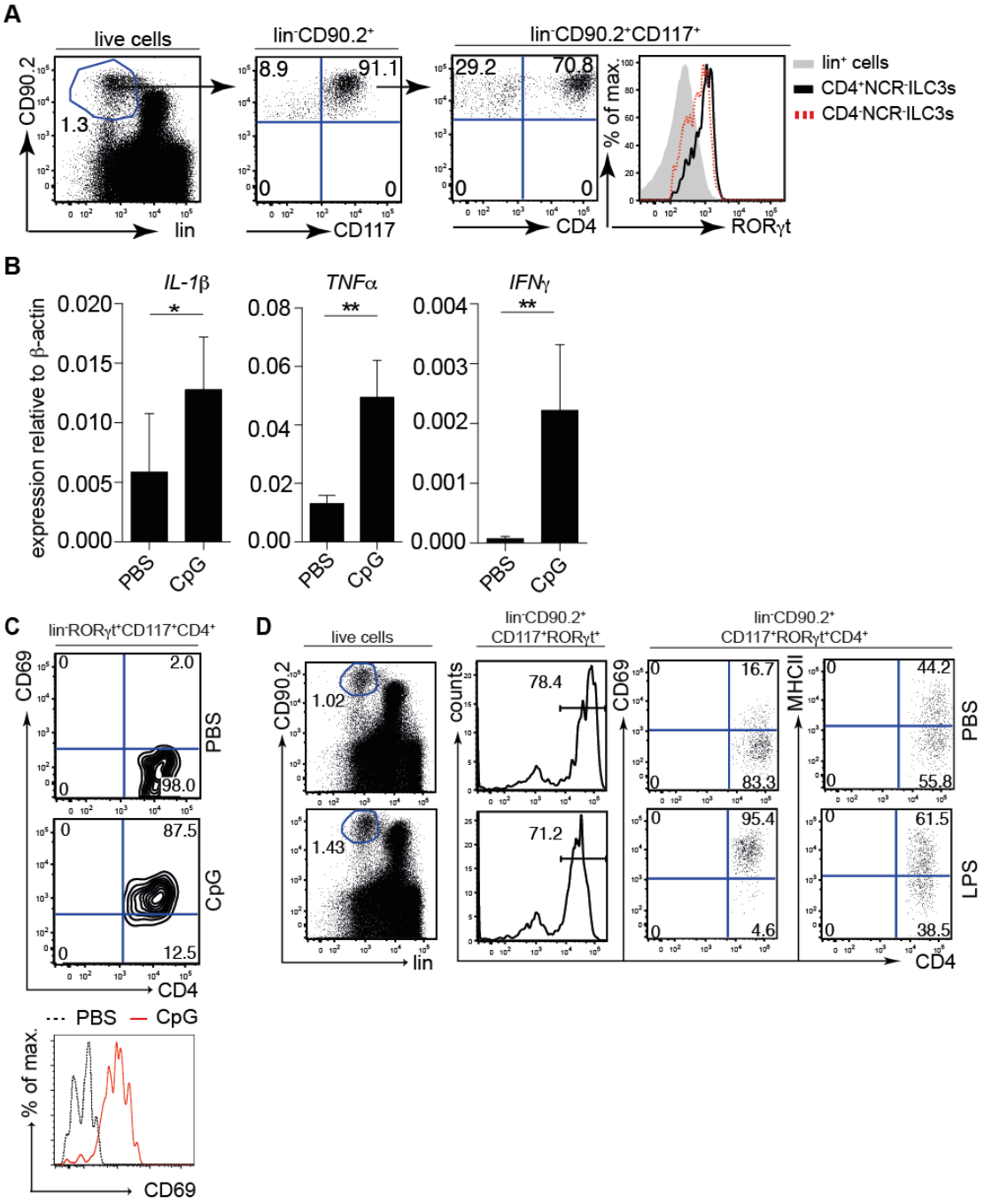


Figure 9: ***In vivo*** **treatment with TLR ligands induces an inflammatory milieu in the spleen and activates splenic NCR$^-$ILC3s. A)** Phenotype of NCR$^-$ILC3s *ex vivo* isolated from the spleen of adult *Rag2*$^{-/-}$ mice. Numbers in dot plots show the percentage of cells in each gate. Representative dot plots and histogram are depicted. **B)** qRT-PCR of *IL-1β, TNFα* and *IFNγ* transcripts in total spleen tissue from *WT* mice 6 h after i.p. injection of either PBS or CpG. Expression is shown relative to the house keeping gene *β-actin*. Data are representative for 1 of 2 independent experiments (n = 5; mean values + SD; *P ≤ 0.05; **P ≤ 0.01). **C)** CD69 expression of splenic CD4$^+$ NCR$^-$ILC3s of *WT* mice 6 h after i.p. injection of PBS or CpG as indicated in the figure. Representative dot plots and a histogram of 3 independent experiments is shown. **D)** CD69 and MHC II expression of splenic NCR$^-$ILC3s of *Rag2*$^{-/-}$ mice 6 h after i.p. injection of PBS or LPS as indicated in the figure. First dot plots represent live cells. Histograms show CD4$^+$ and CD4$^-$ subsets within the lin$^-$CD90.2$^+$CD117$^+$RORγt$^+$ population. Dot plots are additionally gated on CD4$^+$ NCR$^-$ILC3s. Numbers show the percentage of cells in each gate. Data are representative of at least 3 independent experiments.

5.3.2 IL-1β activates splenic NCR$^-$ILC3s and induces up-regulation of MHC II and co-stimulatory molecule expression.

My previous findings showed that *in vitro* generated NCR$^-$ILC3s were able to respond to innate stimulation *in vitro*. Further, *in vivo* challenge of adult mice with TLR ligands induced activation of splenic NCR$^-$ILC3s. However, whether splenic NCR$^-$ILC3s isolated from adult mice were able to express co-stimulatory molecules and MHC II upon IL-1β exposure, as it has been demonstrated for *in vitro* generated NCR$^-$ILC3s (Fig. 8 B), remained to be determined. To test this, I isolated NCR$^-$ILC3s from the spleen of adult *Rag2*$^{-/-}$ mice. As mentioned before, *Rag2*$^{-/-}$ mice harbor increased numbers of ILC3s compared to *WT* mice while lacking T and B cells. Therefore, the usage of *Rag2*$^{-/-}$ mice facilitated the enrichment of a high number of ILC3s without contaminating T or B cells. *Ex vivo* isolated splenic NCR$^-$ILC3s of *Rag2*$^{-/-}$ mice were sort-purified based on the expression of CD90.2/CD117 and the lack of lineage markers (lin$^-$). 48 h after IL-1β exposure, splenic NCR$^-$ILC3s up-regulated the expression of CD69 and co-stimulatory molecules (Fig. 10 A). Interestingly, compared to naïve *in vitro* generated NCR$^-$ILC3s, which lacked MHC II expression (Fig. 8 B), *ex vivo* isolated NCR$^-$ILC3s already expressed MHC II on their surface in the absence of innate stimulation. Furthermore, MHC II expression was increased upon IL-1β exposure and exceeded those of IL-β-activated *in vitro* generated NCR$^-$ILC3s (Fig. 10 A and Fig. 8 B). Altogether, *ex vivo* isolated splenic NCR$^-$ILC3s were able to respond to IL-1β stimulation thereby acquiring an APC-like phenotype.

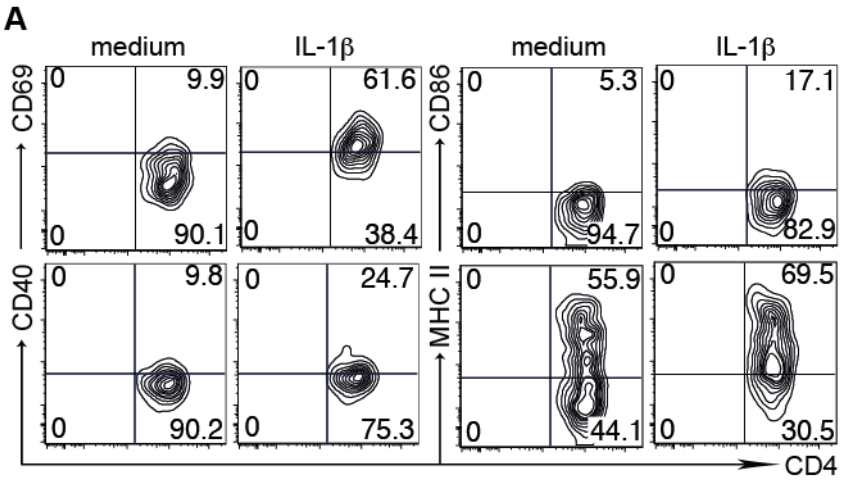


Figure 10: IL-1β induces the expression of MHC II and co-stimulatory molecules on splenic NCR$^-$ ILC3s. A) Expression of CD69, CD40, CD86 and MHC II on sort-purified *ex vivo* isolated splenic CD4$^+$ NCR$^-$ILC3s cultured for 48 h in the presence of IL-1β or medium alone as indicated in the figure.

Numbers in contour plots show the percentage of cells in each quadrant. Data are representative of 3 independent experiments.

5.4 Peripheral NCR$^-$ILC3s are able to take up latex beads, to process protein Ag and to promote CD4$^+$ T cell responses *in vitro.*

5.4.1 NCR$^-$ILC3s can internalize latex beads.

Based on the notion that inflammatory stimulation of NCR$^-$ILC3s induced the acquisition of an APC-like phenotype, I wondered whether ILC3s could function as APCs. Therefore, I asked whether NCR$^-$ILC3s were able to internalize and process exogenous Ag in order to present it to naïve CD4$^+$ T cells. First of all, I measured the capacity of NCR$^-$ILC3s to take up exogenous Ag using red fluorescent latex beads with a size of 1 μm. Sort purified *in vitro* generated and *ex vivo* isolated splenic NCR$^-$ ILC3s were cultured for 6 h in the presence of red fluorescent latex beads. Both *in vitro* generated and *ex vivo* isolated NCR$^-$ILC3s were able to internalize red fluorescent latex beads at 37 °C (Fig. 11 A - C). Bead uptake was severely inhibited at 4 ºC or in the presence of 0.5 μM Cytochalasin D (CytD), an inhibitor of actin polymerization (Fig. 11 B and C). Those two controls showed the specificity of bead internalization by NCR$^-$ILC3s. As previously described, *in vitro* generated NCR$^-$ILC3s could be subdivided into CD4$^+$ and CD4$^-$NCR$^-$ILC3 subsets (Fig. 6 A and B). Both CD4$^+$ and CD4$^-$ NCR$^-$ILC3 subsets were able to internalize latex beads, although CD4$^+$ NCR$^-$ILC3 were slightly more efficient in taking up Ag than their CD4$^-$ counterpart (Fig. 11 D). However, compared to BM-derived macrophages (BMMΦ), bead uptake by NCR$^-$ILC3s occurred with slower kinetics (Fig. 11 E). Altogether, I could show that NCR$^-$ILC3s were able to internalize exogenous Ags.

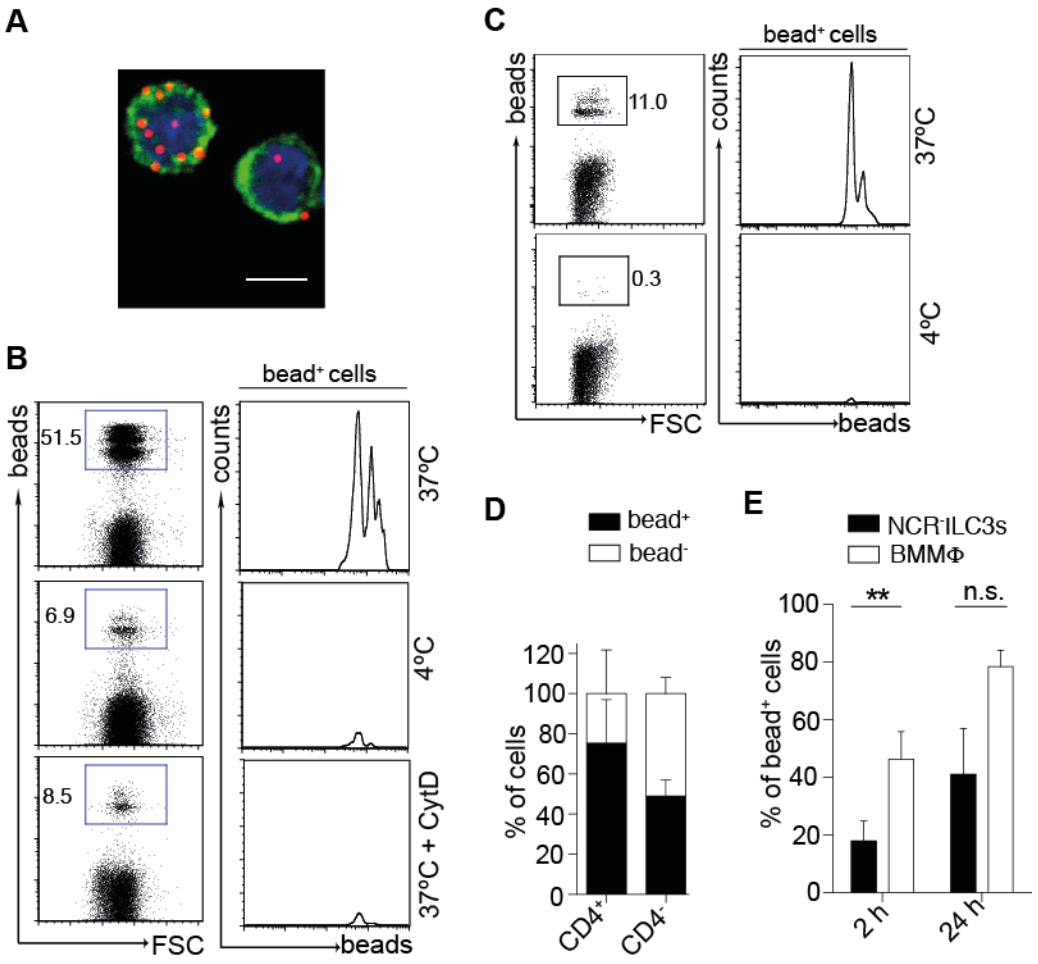


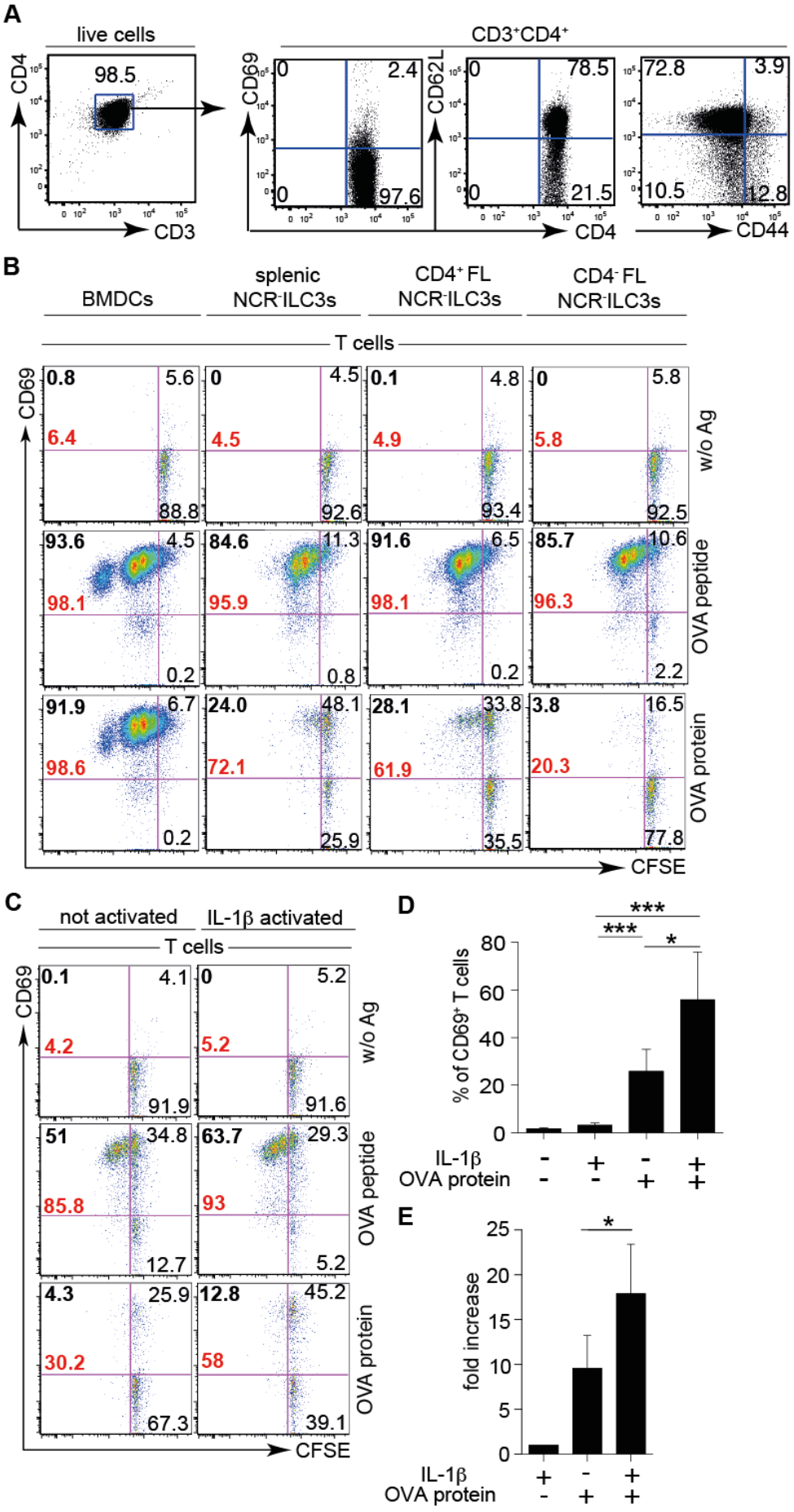
Figure 11: Naïve NCR$^-$ILC3s can internalize latex beads. A) Representative immunofluorescence image of red fluorescent latex bead uptake by sort-purified *in vitro* generated NCR$^-$ILC3s. Scale, 5 µm. **B - C)** Representative plots of sort-purified **(B)** *in vitro* generated or **(C)** *ex vivo* isolated splenic NCR$^-$ ILC3s cultured with beads for 6 h at either 37 °C or 4 °C, or at 37 °C in presence of 0.5 µM Cytochalasin D (CytD) as indicated in the figure. Histograms show bead$^+$ cells. Data are representative of at least 3 independent experiments (n = 3 - 5). **D)** Percentage of bead$^+$ and bead$^-$ cells within *in vitro* generated CD4$^+$ and CD4$^-$ NCR$^-$ILC3s after 6 h incubation with beads (mean values + SD). Data shown are representative of at least 3 independent experiments (n = 3 - 5). **E)** Bead internalization by NCR$^-$ILC3s and BMMΦ. Percentage of bead$^+$ cells 2 and 24 h after addition of beads (mean values + SD; n = 3 - 5; n.s. = not significant; **P $\leq$ 0.01).

5.4.2 Activated NCR$^-$ILC3s can induce Ag-specific CD4$^+$ T cell activation and proliferation.

To further investigate Ag processing and presentation by NCR$^-$ILC3s as well as the induction of CD4$^+$ T cell priming *in vitro*, I made use of a T cell transgenic mouse model, the OT-II transgenic (*OT-II^{tg}*) mouse.[247] All CD4$^+$ T cells found in homozygous *OT-II^{tg}* mice bear a transgenic T cell receptor (TCR) on their surface that specifically recognizes chicken Ovalbumin ($OVA_{323-339}$) in the context of MHC II (*H-2^b*). OT-II

transgenic $CD4^+$ T cells were isolated from LNs and spleen of naïve *OT-II*^{tg} mice, enriched with magnetically associated cell sorting and sort-purified before use for *in vitro* and *in vivo* experiments. Sort-purified *OT-II*^{tg} $CD4^+$ T cells showed a naïve phenotype displayed by the lack of CD69 expression and simultaneously high levels of CD62L expression (Fig. 12 A). CD44, a marker indicating an activated memory-like phenotype for T cells was only expressed by a minority of sorted *OT-II*^{tg} $CD4^+$ T cells (Fig. 12 A). To test the ability of NCR^-ILC3s to prime $CD4^+$ T cells *in vitro*, sort-purified NCR^-ILC3s (*H-2*^{b}) were first *in vitro* stimulated with IL-1β for 24 h and afterwards incubated for 72 h with sort-purified carboxyfluorescein succinimidyl ester (CFSE)-labeled *OT-II*^{tg} $CD4^+$ T cells in the presence of OVA peptide$_{323-339}$ or whole OVA protein (Fig. 12 B). CFSE labeling of *OT-II*^{tg} $CD4^+$ T cells allowed me to monitor T cell proliferation under different co-culture conditions. BM-derived dendritic cells (BMDCs), known as professional APCs, were used as a positive control for efficient Ag presentation and $CD4^+$ T cell activation. The activation of $CD4^+$ T cells was monitored by surface CD69 expression and the CFSE proliferation profile. *Ex vivo* isolated and *in vitro* generated $CD4^+$ and $CD4^-$ NCR^-ILC3s were able to activate the majority of $CD4^+$ T cells in the presence of OVA peptide considering surface CD69 expression (Fig. 12 B). When naïve *OT-II*^{tg} $CD4^+$ T cells and IL-β-activated NCR^- ILC3s were co-cultured without Ag, neither $CD4^+$ T cell activation (CD69 expression) nor proliferation could be observed (Fig. 12 B). *In vitro* generated FL-derived $CD4^+$ and *ex vivo* isolated splenic NCR^-ILC3s were able to induce several rounds of OVA-specific $CD4^+$ T cell proliferation when pulsed with OVA peptide or, to a lesser extent, with OVA protein (Fig. 12 B). $CD4^-$ NCR^-ILC3s were considerably less efficient in inducing protein Ag-specific $CD4^+$ T cell responses (Fig. 12 B).

To further examine the effect of pre-activation of NCR^-ILC3s on their capacity to elicit T cell responses, I stimulated *ex vivo* isolated splenic NCR^-ILC3s with IL-1β for 24 h or left them untreated, and co-cultured them for additional 48 h with naïve *OT-II*^{tg} $CD4^+$ T cells in the presence or absence of whole OVA protein (Fig. 12 C - E). 30.2 % of the $CD4^+$ T cells in culture with untreated NCR^-ILC3s and OVA protein expressed CD69 and only 4.3 % of the T cells proliferated (Fig 12 C). IL-1β-activated NCR^-ILC3s increased the percentage of both $CD69^+$ and proliferating T cells (Fig. 12 C - E). Together, these results showed that NCR^-ILC3s were able to induce $CD4^+$ T cell activation and proliferation *in vitro* and that pre-activation of NCR^-ILC3s with IL-1β increased their efficiency to induce $CD4^+$ T cell activation.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live cells
CD3+CD4+
CD4
CD3
98.5
CD69
CD62L
CD4
CD44
0
2.4
97.6
78.5
21.5
72.8
3.9
10.5
12.8
B
BMDCs
splenic NCR-ILC3s
CD4+ FL NCR-ILC3s
CD4- FL NCR-ILC3s
T cells
CD69
w/o Ag
OVA peptide
OVA protein
CFSE
0.8
5.6
6.4
88.8
0
4.5
4.5
92.6
0.1
4.8
4.9
93.4
0
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0.2
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98.6
0.2
24.0
48.1
72.1
25.9
28.1
33.8
61.9
35.5
3.8
16.5
20.3
77.8
C
not activated
IL-1β activated
T cells
CD69
w/o Ag
OVA peptide
OVA protein
CFSE
0.1
4.1
4.2
91.9
0
5.2
5.2
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51
34.8
85.8
12.7
63.7
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93
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4.3
25.9
30.2
67.3
12.8
45.2
58
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D
% of CD69+ T cells
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OVA protein
*

Figure 12: NCR$^-$ILC3s can induce Ag-specific CD4$^+$ T cell activation and proliferation. A) Representative dot plots of *ex vivo* isolated sort-purified *OT-II^{tg}* CD4$^+$ T cells. Live cells after isolation and sort-purification are represented in the first dot plot. Surface expression of CD69, CD62L and CD44 is shown gated on CD3$^+$CD4$^+$ T cells. Numbers in dot plots show the percentage of cells in each gate. **B)** Naïve CFSE-labeled *OT-II^{tg}* CD4$^+$ T cells were cultured with either BMDCs, IL-1β-activated *ex vivo* isolated splenic NCR$^-$ILC3s or *in vitro* generated CD4$^+$ or CD4$^-$ NCR$^-$ILC3s in the presence of OVA peptide, OVA protein or medium alone (w/o Ag) as indicated in the figure. Representative plots of CD69 and CFSE expression by CD4$^+$ T cells 72 h later. Bold black numbers: % of proliferating T cells. Bold red numbers: % of total CD69$^+$ T cells. **C)** Naïve CFSE-labeled *OT-II^{tg}* CD4$^+$ T cells were cultured with non- or IL-1β-activated splenic NCR$^-$ILC3s in the presence of OVA peptide, OVA protein or medium alone (w/o Ag) for 72 h as indicated in the figure. Representative dot plots are shown. **D)** Percentage of CD69$^+$ T cells upon co-culture with non- or IL-1β-activated splenic NCR$^-$ILC3s in the presence or absence of Ag as indicated in the figure. Data are shown as mean values + SD (n = 3 - 7; *P $\leq$ 0.05; ***P $\leq$ 0.001). **E)** Fold increase of percentage of CD69$^+$ T cells upon co-culture with non- or IL-1β-activated splenic NCR$^-$ILC3s in the presence of OVA protein relative to co-culture of IL-1β- activated splenic NCR$^-$ILC3s and T cells in the absence of Ag as indicated in the figure. Data are shown as mean values + SD (n = 3 - 7; *P $\leq$ 0.05). **D – E)** Data are representative of at least 3 - 7 independent experiments.

5.4.3 Ag-dependent interaction of CD4$^+$ T cells and splenic NCR$^-$ ILC3s induces *de novo* activation of splenic NCR$^-$ILC3s.

In co-cultures of naïve *OT-II^{tg}* CD4$^+$ T cells and untreated splenic NCR$^-$ILC3s, the surface expression of CD69 on NCR$^-$ILC3s was increased approximately 7-fold when Ag (OVA protein) was present compared to co-cultures without Ag (Fig. 13 A). This phenomenon was not further increased by previously activating NCR$^-$ILC3s with IL-1β (Fig. 13 A). It is important to note, that pre-activation of NCR$^-$ILC3s (IL-1β, 24 h) induced CD69 expression, which peaked early after activation and later decreased in co-cultures, unless T cells and Ag were added. To exclude that the presence of whole OVA protein was responsible for CD69 up-regulation on NCR$^-$ILC3s in co-cultures with CD4$^+$ T cells and OVA protein, I cultured sort-purified *ex vivo* isolated splenic NCR$^-$ILC3s in the presence or absence of whole OVA protein for 48 h and examined their CD69 expression. The presence of whole OVA protein did not induce CD69 expression on NCR$^-$ILC3s (Fig. 13 B). Thus, this experiment showed that Ag alone was not able to activate naïve NCR$^-$ILC3s.

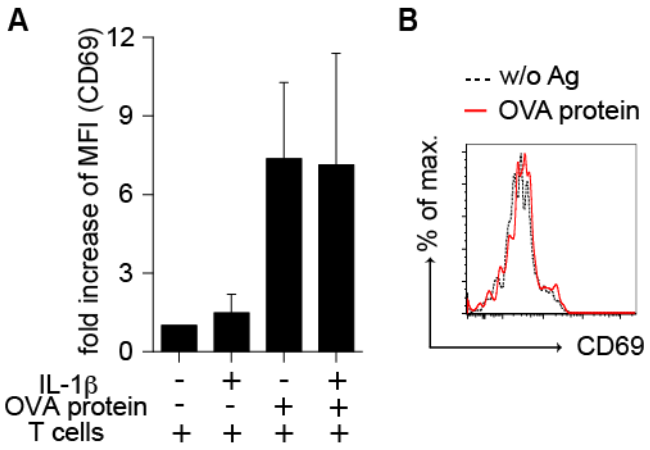


Figure 13: Ag-dependent CD4$^+$ T cell activation induces activation of splenic NCR$^-$ILC3s. A) Fold increase of mean fluorescent intensity (MFI) of CD69 expression on non- or IL-1β-activated (24 h) splenic NCR$^-$ILC3s co-cultured with *OT-II^{tg}* CD4$^+$ T cells in the presence or absence of OVA protein (48 - 72 h) relative to non-activated splenic NCR$^-$ILC3s co-cultured with *OT-II^{tg}* CD4$^+$ T cells in the absence of Ag. Data are shown as mean values + SD (3 - 7 independent experiments). **B)** Representative histogram of CD69 expression on non-activated splenic NCR$^-$ILC3s in presence of OVA protein or in medium alone.

5.4.4 Activation of NCR$^-$ILC3s can be induced by soluble factors produced in co-cultures of APCs and CD4$^+$ T cells in the presence of cognate Ag.

My previous observations showed that the presence of cognate Ag in co-cultures of *OT-II^{tg}* CD4$^+$ T cells with NCR$^-$ILC3s induced and sustained activation of NCR$^-$ILC3s (Fig. 13 A). This activation of NCR$^-$ILC3s reached an equal level regardless of whether NCR$^-$ILC3s were pre-activated with IL-1β or not (Fig. 13 A). Further, the presence of Ag alone was not able to induce activation of NCR$^-$ILC3s *in vitro* (Fig. 13 B). Thus, I assumed that *OT-II^{tg}* CD4$^+$ T cells produced some soluble factors after Ag-dependent interaction with NCR$^-$ILC3s, which were able to activate NCR$^-$ILC3s. However, one could not exclude that NCR$^-$ILC3s themselves also secreted soluble factors upon Ag-dependent interaction with CD4$^+$ T cells. To further test the effect of soluble factors produced by activated CD4$^+$ T cells on the activation of NCR$^-$ILC3s, cell culture supernatant (SN) of activated CD4$^+$ T cells had to be produced. The following possibilities existed; i) co-cultures of NCR$^-$ILC3s and CD4$^+$ T cells with Ag, ii) co-cultures of BMDCs and CD4$^+$ T cells with Ag, or iii) CD4$^+$ T cell activation in presence of anti-CD3/anti CD28 Abs. In my previous *in vitro* assay, I could show that *OT-II^{tg}* CD4$^+$ T cells were fully activated in the presence of BMDCs and OVA protein

(Fig. 12 B). Therefore, I decided to use the SN of co-cultures of BMDCs with $CD4^+$ T cells and Ag to further test soluble factor-mediated activation of splenic NCR^-ILC3s.
Sort-purified *ex vivo* isolated splenic NCR^-ILC3s showed increased expression of CD69 when cultured for 48 h in the presence of SN collected from co-cultures of BMDCs and $CD4^+$ T cells with Ag (Fig. 14 A). The CD69 expression level was compared to that of NCR^-ILC3s, which were either cultured in cell culture medium alone or in SN collected from co-cultures of BMDCs and $CD4^+$ T cells without Ag (Fig. 14 A). In addition to increased CD69 expression, only SN collected from co-cultures of BMDCs and $CD4^+$ T cells with Ag induced a slight up-regulation of CD80 and CD40 on splenic NCR^-ILC3s (Fig. 14 B). Together, these data showed that soluble factors secreted upon Ag-dependent interaction of $CD4^+$ T cells and BMDCs were able to induce the activation of splenic NCR^-ILC3s. Moreover, these soluble factors assumed to support the efficiency of Ag-dependent splenic NCR^-ILC3-$CD4^+$ T cell interaction by the induction of co-stimulatory molecule expression.
$CD4^+$ T cells are known to produce cytokines such as IFNγ upon encounter of Ag presented via MHC II on APCs.[272] My previous data showed that *in vitro* generated NCR^-ILC3s increased CD69 and MHC II expression upon exposure to IFNγ (Table 1). Thus, IFNγ might be a potential soluble factor inducing and sustaining activation of NCR^-ILC3s during Ag-dependent interaction with $CD4^+$ T cells. In line with this, intracellular staining of *OT-II*^{tg} $CD4^+$ T cells revealed that $CD4^+$ T cells produced IFNγ only in the presence of BMDCs and OVA protein (Ag) while IFNγ production was low or not detectable in $CD4^+$ T cells co-cultured with BMDCs in the absence of Ag (Fig. 14 C). The role for $CD4^+$ T cell-derived IFNγ in NCR^-ILC3 activation needs to be further explored.
$CD4^+$ T cells are known to produce IL-2 as a consequence of activation through their antigen receptor.[211] IL-2 secreted by activated $CD4^+$ T cells was shown to directly induce the proliferation of group 2 ILCs (ILC2s).[51] Moreover it was reported that the presence of IL-2 in co-cultures of $CD4^+$ T cells and ILC2s enhanced the ability of ILC2s to produce type 2 related cytokines.[51] Although the interplay of IL-2 and ILC2s was well described, a role for IL-2 in ILC3-mediated $CD4^+$ T cell responses was so far not known. Therefore, I examined whether naïve splenic NCR^-ILC3s express the receptor for IL-2. CD25, the IL-2 receptor alpha chain (IL-2Rα), builds together with the IL-2Rβ and IL-2Rγ chain the high affinity receptor for IL-2.[273] Analysis of naïve *ex vivo* isolated splenic NCR^-ILC3s revealed that CD25 was highly expressed on the surface of these cells (Fig. 14 D). The presence of SN collected from co-cultures of BMDCs and $CD4^+$ T cells with Ag even increased expression of CD25 on splenic

NCR$^-$ILC3s (Fig. 14 D). Thus, NCR$^-$ILC3s expressed the high affinity receptor for IL-2 providing the possibility to respond to IL-2. However, the effects of T cell-derived IL-2 on NCR$^-$ILC3s have to be explored in further experiments.

The inducible T cell co-stimulator ICOS belongs to the CD28/CTLA-4 family and is mainly *de novo* expressed on T cells upon activation.[274] ICOS acts, like CD28, as a co-stimulatory signal for T cell proliferation, however it is not able to induce IL-2 production.[275] Interestingly, whereas naïve splenic NCR$^-$ILC3s expressed low levels, the expression of ICOS was increased in the presence of SN collected from co-cultures of BMDCs and CD4$^+$ T cells with Ag (Fig. 14 D). Furthermore, the ligand for ICOS (ICOSL) was also expressed on naïve *ex vivo* isolated splenic NCR$^-$ILC3s but contrary to ICOS its expression was diminished in the presence of SN from co-cultures with Ag (Fig. 14 D). The precise role for ICOS and its ligand expressed on NCR$^-$ILC3s is completely unknown and requires further investigation. Collectively, these data showed that NCR$^-$ILC3s underwent different phenotypical changes reflecting their activation in the presence of soluble factors produced by activated CD4$^+$ T cells.

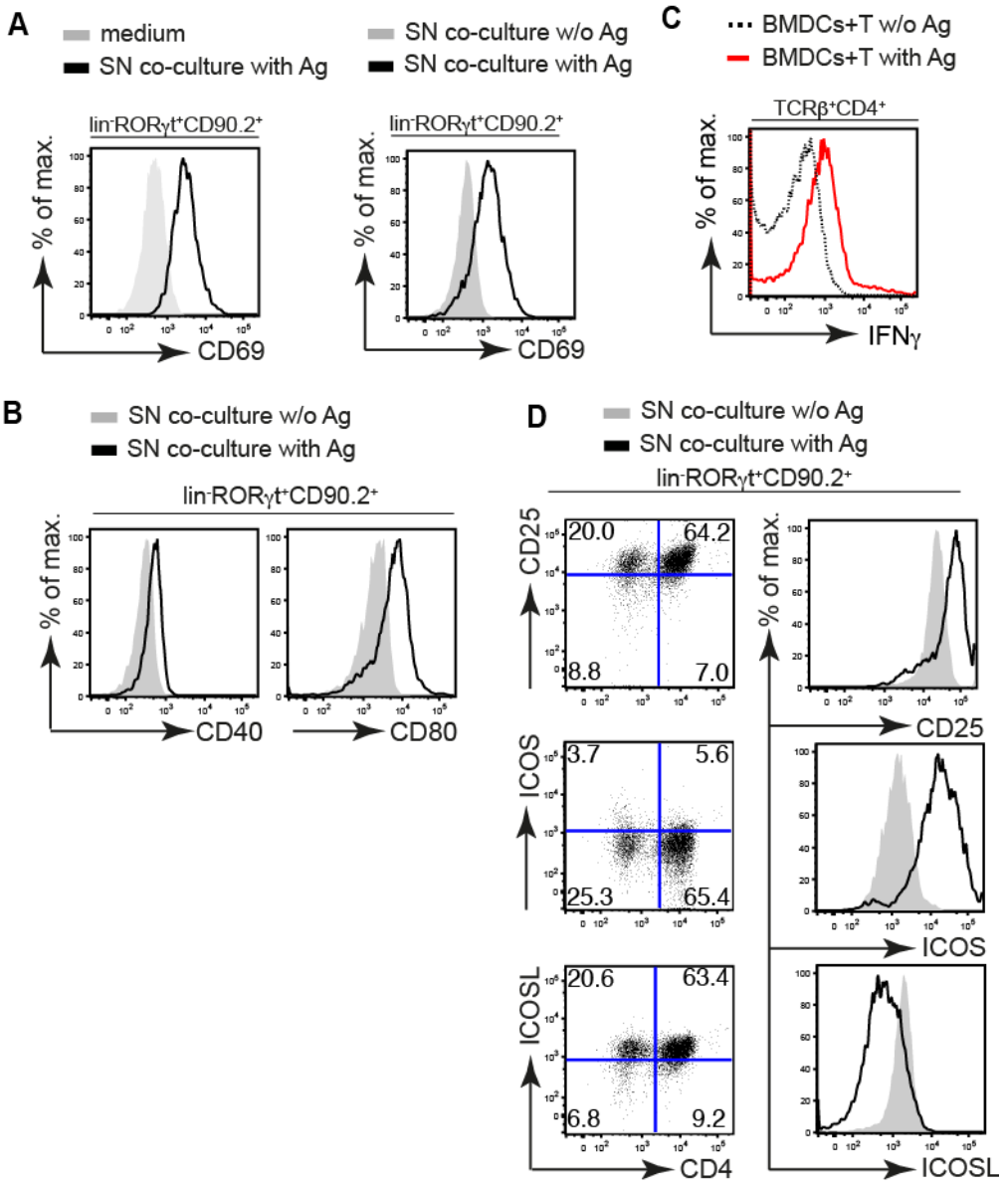


Figure 14: Splenic NCR$^-$ILC3s show changes in the phenotype in the presence of soluble factors produced in co-cultures of BMDCs, CD4$^+$ T cells and the cognate Ag. A) Representative histograms of CD69 expression on sort-purified *ex vivo* isolated splenic CD4$^+$ NCR$^-$ILC3s in the presence of co-culture SN (BMDCs and CD4$^+$ T cells with or without Ag). **B)** Histograms of CD40 and CD80 expression on splenic NCR$^-$ILC3s in the presence of co-culture SN. **C)** Representative histogram of IFNγ expression by *OTII*^{tg} CD4$^+$ T cells upon 48 h co-culture with BMDCs in the presence or absence of OVA protein. **D)** Representative dot plots of CD25, ICOS and ICOSL expression of naïve sort-purified *ex vivo* isolated splenic NCR$^-$ILC3s upon 48 h culture in medium alone. Representative histograms of CD25, ICOS and ICOSL expression of CD4$^+$ NCR$^-$ILC3s upon 48 h culture in the presence of co-culture SN. Data shown are representative of at least 2 independent experiments. SN; supernatant.

5.5 NCR$^-$ILC3s elicit CD4$^+$ T cell immunity *in vivo* by promoting Ag-dependent CD4$^+$ T cell proliferation and enhancing T-dependent B cell responses.

5.5.1 *I-ab$^{\Delta ILC3}$* mice – a model to study Ag presentation by ILC3s *in vivo*.

I could show that NCR$^-$ILC3s, either generated *in vitro* from a FL-derived precursor or isolated *ex vivo* from the spleen of adult mice, were able to elicit CD4$^+$ T cell responses *in vitro* by processing and presenting Ag via MHC II as well as by providing co-stimulatory signals. To examine the role of NCR$^-$ILC3s in CD4$^+$ T cell responses *in vivo*, I generated a new mouse model in which MHC II expression was deleted exclusively in ILC3s. For this reason, I crossed mice expressing Cre recombinase under the control of the RORc promoter (*RORc(gt)-Cre^{tg}*)[25] to mice carrying a floxed *H2-Ab1* allele (*I-ab^{neo}*).[248] Mice homozygous for the floxed *H2-Ab1* allele and carrying one copy of the Cre transgene are here referred as *I-ab$^{\Delta ILC3}$* mice. *I-ab$^{\Delta ILC3}$* mice were healthy, did not show signs of spontaneous inflammation and had a normal distribution of T and B lymphocytes, macrophages (MΦ), dendritic cells (DCs) and natural killer (NK) cells in the spleen (Fig. 15 A and B). Numbers of splenic ILC3s were also similar in *WT* and *I-ab$^{\Delta ILC3}$* mice (Fig. 15 B). Within the splenic B cell compartment, absolute numbers of splenic marginal zone B cells (MZB; CD19$^+$CD21^{high}CD23^{low}) and follicular B cells (FolB; CD19$^+$CD21^{low}CD23^{high}) did not differ in *I-ab$^{\Delta ILC3}$* compared to *WT* mice (Fig. 15 C). *I-ab$^{\Delta ILC3}$* mice displayed a small reduction in absolute numbers of naïve CD4$^+$ and CD8$^+$ T cells (TCRβ$^+$CD62L^{high}CD44$^{-/low}$) compared to *WT* mice whereas numbers of central memory (TCRβ$^+$CD62L^{high}CD44^{high}) as well as effector memory (TCRβ$^+$CD62L$^{-/low}$CD44^{high}) CD4$^+$ and CD8$^+$ T cells were comparable between *I-ab$^{\Delta ILC3}$* and *WT* mice (Fig. 15 D). MHC II was highly expressed on splenic B cells, DCs and MΦ of *I-ab$^{\Delta ILC3}$* and *WT* mice whereas splenic NCR$^-$ILC3s of *I-ab$^{\Delta ILC3}$* mice completely lacked MHC II (Fig. 15 E).

Furthermore, I tested splenic NCR$^-$ILC3s isolated from adult *I-ab$^{\Delta ILC3}$* mice for their ability to respond to innate stimulation. Therefore, *ex vivo* isolated sort-purified splenic NCR$^-$ILC3s of *I-ab$^{\Delta ILC3}$* mice were cultured in the presence of IL-1β or in medium alone. 48 h later, *I-ab$^{\Delta ILC3}$* derived splenic CD4$^+$ NCR$^-$ILC3s showed increased levels of CD69 expression (Fig. 15 F) and up-regulated the expression of

the co-stimulatory molecule CD86 on the surface (Fig. 15 F). Only a slight increase of CD40 expression (from 6.7 % to 10.0 %) on IL-1β-activated $CD4^+$ NCR^-ILC3s could be observed (Fig. 15 F). As expected, MHC II expression was absent on splenic $CD4^+$ NCR^-ILC3s isolated from *I-ab*$^{\Delta ILC3}$ mice, however, 5 % of IL-1β-activated splenic NCR^-ILC3s were found to express MHC II at low levels (Fig. 15 F). It cannot be excluded, that blast formation induced by IL-1β activation was responsible for this slight shift in MHC II expression (from 0 % to 5.0 %). As a comparison, 55.9 % of naïve splenic $CD4^+$ NCR^-ILC3s of *WT* mice expressed MHC II and increased their MHC II expression to 69.5 % upon 48 h exposure to IL-1β (Fig. 10 A). Therefore, one could assume that the 5 % of $CD4^+$ NCR^-ILC3s expressing low levels of MHC II in *I-ab*$^{\Delta ILC3}$ mice were not relevant for Ag presentation. Altogether, *I-ab*$^{\Delta ILC3}$ mice displayed completely normal immune compartments, lacked the expression of MHC II only on ILC3s and IL-1β stimulation was able to induce activation of splenic NCR^-ILC3s *in vitro*.

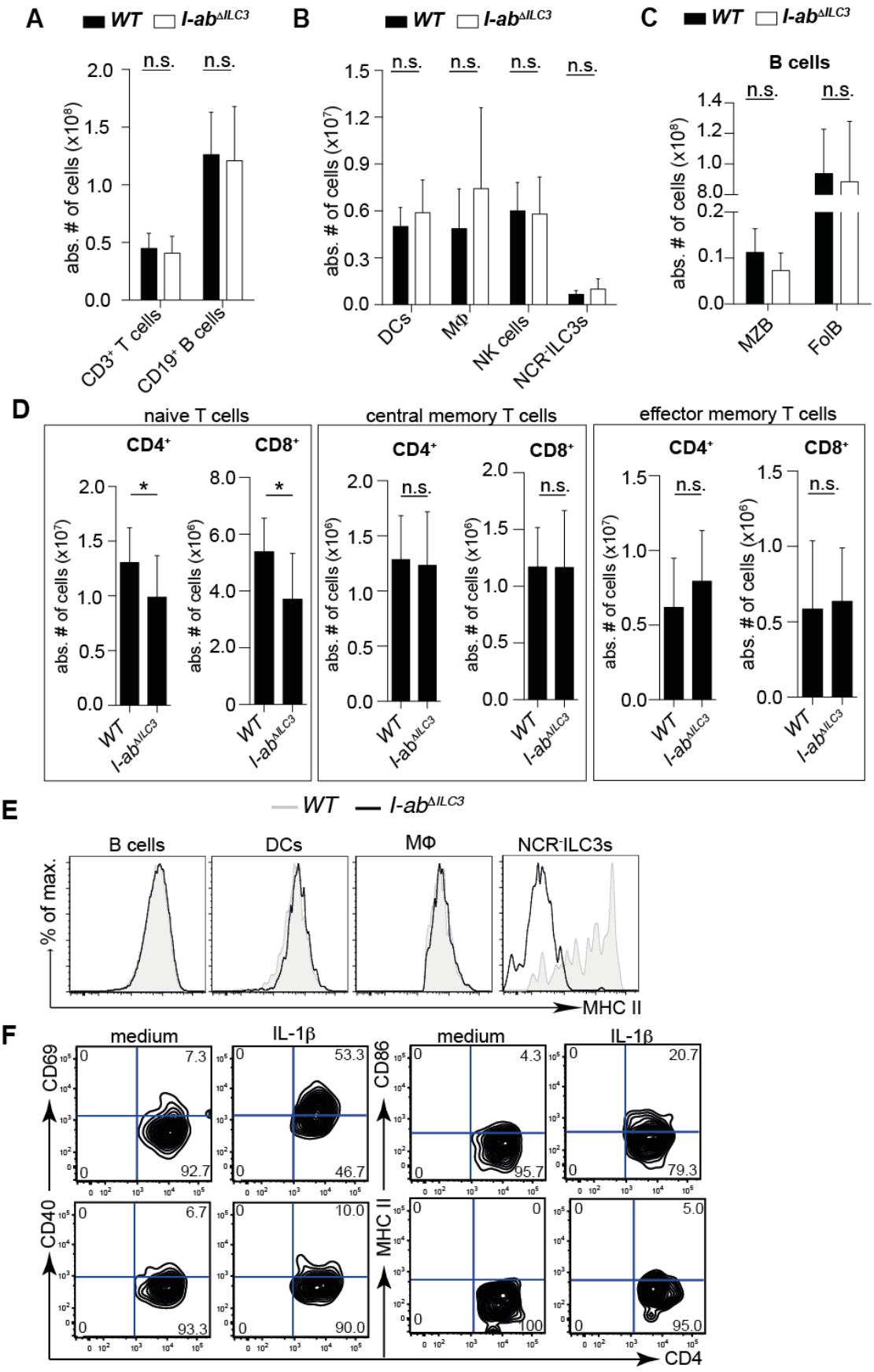


Figure 15: *I-ab*$^{\Delta ILC3}$ mice show normal numbers of lymphocytes in the spleen and lack MHC II expression exclusively on RORγt^{+} ILC3s. A – C) Absolute numbers of **(A)** T cells (CD3^{+}) and B cells

($CD19^+$), **(B)** DCs ($CD11c^+$), MΦ ($CD11b^+F4/80^+$), NK cells ($NK1.1^+NKp46^+$) and NCR^-ILC3s (lin^- $ROR\gamma t^+CD117^+NCR^-$), and **(C)** marginal zone B cells (MZB; $CD19^+CD21^{high}CD23^{low}$) and follicular B cells (FolB; $CD19^+CD21^{low}CD23^{high}$) in the spleen of *WT* and *I-ab*$^{\Delta ILC3}$ mice. **D)** Absolute numbers of naïve ($TCR\beta^+CD62L^{high}CD44^{-/low}$), central memory ($TCR\beta^+CD62L^{high}CD44^{high}$) and effector memory ($TCR\beta^+CD62L^{-/low}CD44^{high}$) $CD4^+$ and $CD8^+$ T cells in the spleen of *WT* and *I-ab*$^{\Delta ILC3}$ mice. **E)** Representative histograms of MHC II expression on splenic B cells, DCs, MΦ and $CD4^+$ NCR^-ILC3s of *WT* and *I-ab*$^{\Delta ILC3}$ mice. **F)** Expression of CD69, CD40, CD86 and MHC II on sort-purified *ex vivo* isolated splenic $CD4^+$ NCR^-ILC3s of *I-ab*$^{\Delta ILC3}$ mice cultured for 48 h in the presence of IL-1β or medium alone. Numbers in contour plots show the percentage of cells in each quadrant. Data are representative of 2 independent experiments. **A – D)** All data are shown as mean + SD (n = 9 - 12; 3 - 4 independent experiments; n.s. = not significant; *$P \leq 0.05$).

5.5.2 ILC3s elicit Ag-specific T cell proliferation *in vivo.*

To examine the ability of NCR^-ILC3s to act as APCs *in vivo*, I first tested whether NCR^-ILC3s contribute to Ag-specific $CD4^+$ T cell proliferation *in vivo*. In order to address this question, I compared the proliferation of adoptively transferred *OT-II*^{tg} $CD4^+$ T cells after *in vivo* immunization with the cognate Ag (OVA) in the following recipients; *WT, I-ab*$^{\Delta ILC3}$ and *I-ab*$^{-/-}$ mice. *I-ab*$^{-/-}$ mice have a complete deficiency of all *MHC II* genes and were therefore used as negative controls unable to present Ag via MHC II to $CD4^+$ T cells.[245] *I-ab*$^{-/-}$ mice displayed a normal distribution of total $CD3^+$ T and $CD19^+$ B lymphocytes in the spleen compared to *WT* mice (Fig. 16 A). Absolute numbers of MZB cells ($CD19^+CD21^{high}CD23^{low}$) and FolB cells ($CD19^+CD21^{low}CD23^{high}$) in *I-ab*$^{-/-}$ mice were similar to *WT* mice (Fig. 16 B). However, *I-ab*$^{-/-}$ mice showed significantly reduced relative and absolute numbers of $CD4^+$ T cells compared to *WT* mice (Fig. 16 C). MHC II expression is involved in the selection process and maturation of $CD4^+$ T cells in the thymus whereas T cell differentiation into the $CD8^+$ lineage is not affected by the loss of *MHC II* genes in *I-ab*$^{-/-}$ mice.[245, 276] In line with this, I found significantly increased relative and absolute numbers of $CD8^+$ T cells in *I-ab*$^{-/-}$ mice, probably as a result of expansion in the absence of $CD4^+$ T cells (Fig. 16 C). Detailed characterization of the T cell subsets in *I-ab*$^{-/-}$ mice revealed that the remaining $CD4^+$ T cells mainly showed an activated memory-like phenotype. Absolute and relative numbers of naïve $CD4^+$ T cells ($T_{naïve}$; $TCR\beta^+CD62L^{high}CD44^{-/low}$) were significantly reduced whereas relative numbers of $CD4^+$ T cells with an effector memory-like phenotype (T_{EM}; $TCR\beta^+CD62L^{-/low}CD44^{high}$) were significantly increased compared to *WT* mice (Fig. 16 D). However, absolute numbers of central memory (T_{CM}; $TCR\beta^+CD62L^{high}CD44^{high}$) and effector memory $CD4^+$ T cells were both significantly lower in *I-ab*$^{-/-}$ compared to *WT* mice (Fig. 16 D). Analysis of the $CD8^+$ T cell subsets in *I-ab*$^{-/-}$ mice revealed that relative and absolute numbers of naïve and central memory $CD8^+$ T cells were comparable to *WT* mice

and only a significant increase in relative and absolute numbers of effector memory CD8$^+$ T cells could be observed (Fig. 16 D). Altogether these data showed that although *I-ab*$^{-/-}$ mice had a normal distribution of CD8$^+$ T cells they harbored a disrupted CD4$^+$ T cell compartment with a severe reduction of naïve CD4$^+$ T cells. Therefore, to proper compare CD4$^+$ T cell responses in *WT, I-ab*$^{\Delta ILC3}$ and *I-ab*$^{-/-}$ mice, adoptive transfer of Ag-specific *OT-II*^{tg} CD4$^+$ T cells was performed providing each recipient with the same amount of naïve CD4$^+$ T cells capable to respond to OVA immunization.

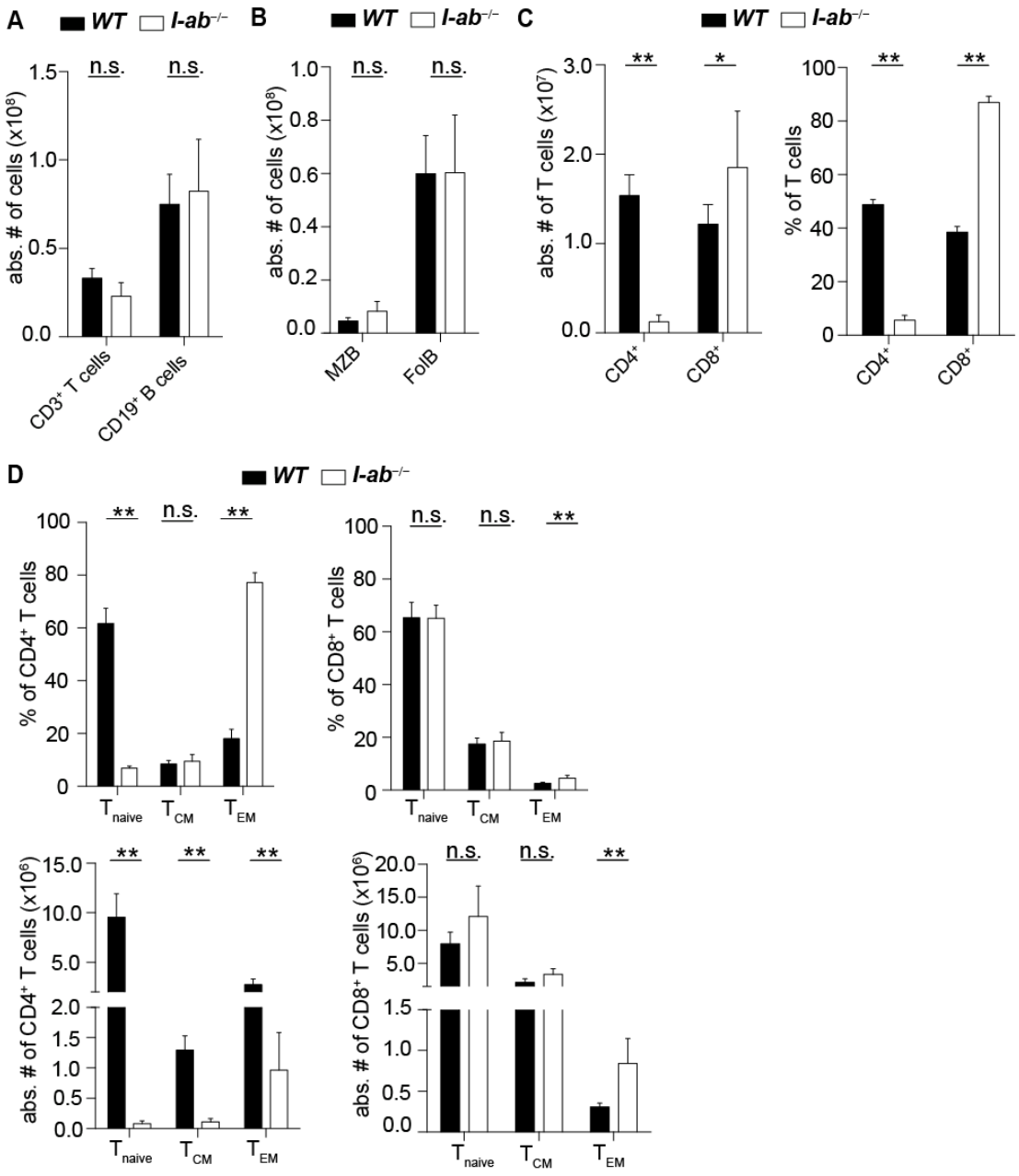


Figure 16: *I-ab*$^{-/-}$ mice show reduced numbers of CD4$^+$ T cells in the spleen. A - B) Absolute numbers of **(A)** T cells (CD3$^+$), B cells (CD19$^+$) and **(B)** marginal zone B cells (MZB;

$CD19^+CD21^{high}CD23^{low}$) and follicular B cells (FolB; $CD19^+CD21^{low}CD23^{high}$) in the spleen of *WT* and *I-ab*$^{-/-}$ mice. **C - D)** Absolute and relative numbers of $CD4^+$ and $CD8^+$ T cell subsets in the spleen of *WT* and *I-ab*$^{-/-}$ mice as indicated in the figure. **A – D)** All data are shown as mean + SD (n = 5; n.s. = not significant; *P ≤ 0.05; **P ≤ 0.01). $T_{naïve}$: naïve T cells; T_{CM}; central memory T cells; T_{EM}: effector memory T cells.

To examine the contribution of NCR^-ILC3s to $CD4^+$ T cell proliferation *in vivo*, 3 x 10^6 CFSE-labeled *OT-II*^{tg} $CD4^+$ T cells were adoptively transferred into *WT, I-ab*$^{\Delta ILC3}$ or *I-ab*$^{-/-}$ mice followed by an immunization with ovalbumin (OVA) $peptide_{323-339}$ and OVA protein in combination with CpG (Fig. 17 A). CpG was additionally used for the immunization as an adjuvant since it induced activation of splenic NCR^-ILC3s and an inflammatory milieu in the spleen of *WT* mice upon *in vivo* treatment (Fig. 9 B and C). The proliferation of adoptively transferred *OT-II*^{tg} $CD4^+$ T cells in the different recipient mice was examined 2 days later to uncover the contribution of NCR^-ILC3s to transient $CD4^+$ T cell responses.[277] As expected, CFSE-labeled *OT-II*^{tg} $CD4^+$ T cells proliferated in *WT*, but not *I-ab*$^{-/-}$ mice (Fig. 17 B). In *I-ab*$^{\Delta ILC3}$ mice, *OT-II*^{tg} $CD4^+$ T cell proliferation was significantly reduced demonstrating that ILC3s were able to present Ag and to meaningfully alter OVA-specific $CD4^+$ T cell responses *in vivo* (Fig. 17 B).

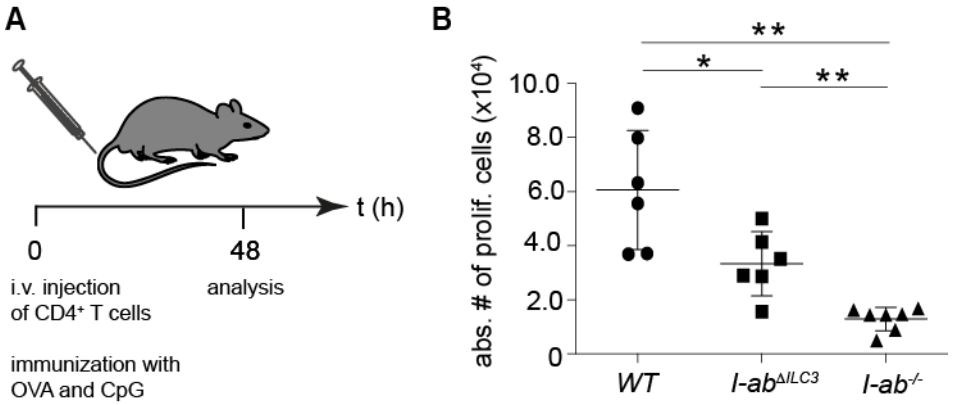


Figure 17: ILC3s elicit Ag-specific T cell proliferation *in vivo*. A) Schematic representation of the experimental performance of the *in vivo* experiment. t = time (h). **B)** Sort-purified CFSE-labeled *OT-II*^{tg} $CD4^+$ T cells were injected i.v. into *WT* (•), *I-ab*$^{\Delta ILC3}$ (▪) and *I-ab*$^{-/-}$ (▲) mice immunized with OVA peptide, OVA protein and CpG. Absolute numbers of proliferating *OT-II*^{tg} $CD4^+$ T cells recovered from the spleen 2 days later (mean values + SD; 4 independent experiments; n = 6 – 7; *P ≤ 0.05; **P ≤ 0.01).

5.5.3 ILC3s support Ag-specific T-dependent B cell responses *in vivo.*

My data could demonstrate that ILC3s were able to elicit Ag-dependent $CD4^+$ T cell proliferation *in vivo*. However, whether ILC3s contribute to T dependent (TD) B cell responses was not yet investigated. To study the role of ILC3s in TD B cell responses, I compared B cell responses in *WT, I-ab*$^{\Delta ILC3}$ and *ROR*$\gamma^{-/-}$ mice. *ROR*$\gamma^{-/-}$ mice are described to completely lack ILC3s and consequently LNs and PPs the places where immune responses can be generated.[25, 70] Therefore, the spleen, which develops independently of ILC3s, serves as the main place to generate adaptive immune responses in *ROR*$\gamma^{-/-}$ mice. Further, *ROR*$\gamma^{-/-}$ mice show a diminished T cell pool.[70] It has been reported that RORγt regulates the survival of double positive ($CD4^+CD8^+$) thymocytes during T cell development in the thymus by enhancing the expression of the anti-apoptotic factor Bcl-xL.[70] In line with this, analysis of *ROR*$\gamma^{-/-}$ mice revealed significantly reduced relative numbers of $CD3^+$ T cells compared to *WT* mice (Fig, 18 A). This reduction could not be reflected in absolute cell numbers, probably due to the fact that T cells in *ROR*$\gamma^{-/-}$ mice only have the possibility to accumulate in the spleen while T cells in *WT* mice can be distributed within other secondary lymphoid organs (Fig. 18 A). The development of B cells was not affected by RORγt deficiency shown by increased relative and absolute numbers of total $CD19^+$ B cells in the spleen of *ROR*$\gamma^{-/-}$ mice compared to *WT* mice (Fig. 18 A). Within the splenic B cell compartment, a normal distribution of MZB cells ($CD19^+CD21^{high}CD23^{low}$) and FolB cells ($CD19^+CD21^{low}CD23^{high}$) was observed (Fig. 18 B). The significant increased in B cell numbers in *ROR*$\gamma^{-/-}$ mice might be the consequence of accumulation of these cells in the spleen. Furthermore, *ROR*$\gamma^{-/-}$ mice showed a decrease in relative numbers of $CD4^+$ but not $CD8^+$ T cells compared to *WT* mice (Fig. 18 C). This reduction was probably induced by the lack of a subset of $CD4^+$ T cells, the Th17 cells, whose differentiation is known to be regulated by RORγt expression.[231] However, the decreased relative numbers of $CD4^+$ T cells in *ROR*$\gamma^{-/-}$ mice could not be reflected in absolute cell numbers (Fig. 18 C). The majority of $CD4^+$ and $CD8^+$ T cells found in the spleen of *ROR*$\gamma^{-/-}$ mice showed a memory-like phenotype (Fig. 18 D - E). Relative and absolute numbers of naïve $CD4^+$ T cells ($T_{naïve}$; $TCR\beta^+CD62L^{high}CD44^{-/low}$) were significantly reduced in *ROR*$\gamma^{-/-}$ mice whereas effector memory $CD4^+$ T cells (T_{EM}; $TCR\beta^+CD62L^{-/low}CD44^{high}$) were increased

compared to *WT* mice. Collectively, these data showed that *RORγ*$^{-/-}$ mice harbored a diminished T cell pool.

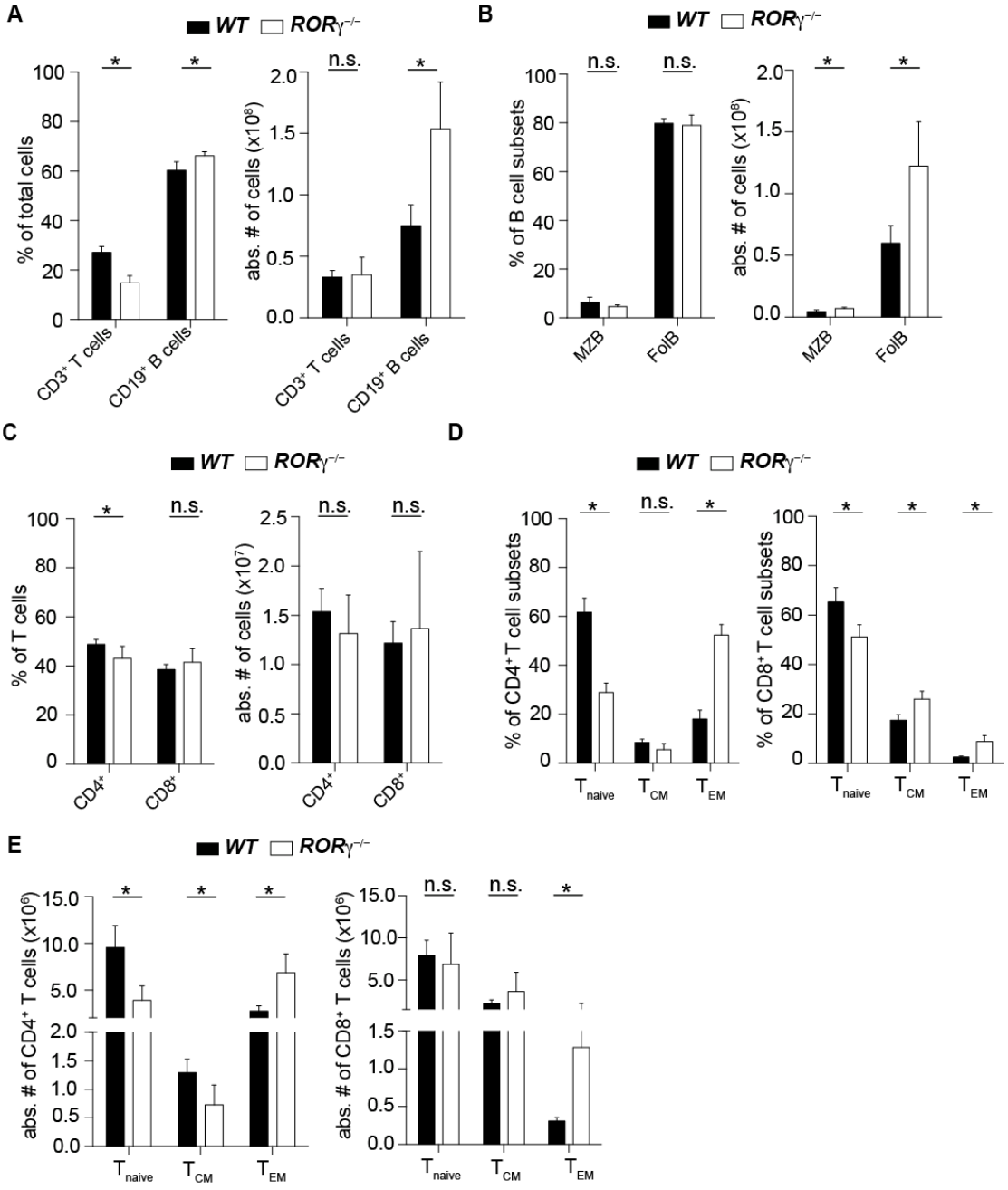


Figure 18: *RORγ*$^{-/-}$ mice show a diminished T cell repertoire with a bias to memory-like phenotype T cells. A - C) Relative and absolute numbers of **(A)** T cells (CD3$^+$) and B cells (CD19$^+$), **(B)** marginal zone B cells (MZB; CD19$^+$CD21^{high}CD23^{low}) and follicular B cells (FolB; CD19$^+$CD21^{low}CD23^{high}), and **(C)** CD4$^+$ and CD8$^+$ T cells in the spleen of *WT* and *RORγ*$^{-/-}$ mice. **D - E)** Relative **(D)** and absolute **(E)** numbers of CD4$^+$ and CD8$^+$ T cell subsets in the spleen of *WT* and *RORγ*$^{-/-}$ mice. **A – E)** All data are shown as mean + SD (n = 5; n.s. = not significant; *P $\leq$ 0.05). $T_{naïve}$: naïve T cells; T_{CM}; central memory T cells; T_{EM}: effector memory T cells.

Since RORγ$^{-/-}$ mice only showed low numbers of naïve T cells, adoptive transfer of *OT-II*^{tg} CD4^{+} T cells was required to provide a pool of naïve CD4^{+} T cells able to achieve CD4^{+} T cell help to B cells. Hereby, RORγ$^{-/-}$ mice could be used to study TD B cell responses in the complete absence of ILC3s.

To examine the contribution of ILC3s to TD B cell responses, 2 x 10^{6} *OT-II*^{tg} CD4^{+} T cells were adoptively transferred into *WT, I-ab*$^{\Delta ILC3}$ and *RORγ*$^{-/-}$ mice at day 0. Subsequently, all mice were immunized i.p. with a single dose of Alum-precipitated 4-hydroxy-3-nitrophenyl-acetyl (NP)-OVA (100 μg per mouse) plus CpG at day 0. Mice were bleed one day before adoptive transfer and immunization (day -1; naïve), and 5 (day 5) as well as 14 (day 14) days after adoptive transfer and immunization (Fig. 19 A). Each time the serum was collected to measure immunoglobulin (Ig) levels in the different recipients.

Naïve *WT* and *I-ab*$^{\Delta ILC3}$ *mice* showed similar levels of total IgM measured in the serum at day -1 whereas total IgM levels in naïve *RORγ*$^{-/-}$ mice were slightly higher (Fig. 19 B). Furthermore, IgG3, the Ig subtype often produced in response to T independent (TI) Ags, could be detected in *I-ab*$^{\Delta ILC3}$ and *RORγ*$^{-/-}$ mice at day -1 at slightly higher levels than in *WT* mice (Fig. 19 C). These findings indicated that naïve *I-ab*$^{\Delta ILC3}$ and *RORγ*$^{-/-}$ mice were able to mount immune responses against TI Ags and to secrete normal or even higher levels of IgG3 than *WT* mice.

The establishment of a humoral immune response to TD Ags includes isotype class switch, a process that takes place within germinal centers (GC), which are especially formed in response to the encountered Ag.[278, 279] Therefore it was not surprising that the level of total IgG produced against nitrophenylacetyl (NP)-OVA was low in the serum of mice only 5 days upon immunization and CD4^{+} T cell transfer (Fig. 19 D). 14 days after immunization, NP-OVA-specific total IgG was increased in all three different recipient mouse strains (Fig. 19 E). The loss of MHC II on ILC3s (*I-ab*$^{\Delta ILC3}$) resulted in a significant reduction of NP-OVA-specific IgG titers compared to IgG titers measured in *WT* mice (Fig. 19 E). In *RORγ*$^{-/-}$ mice, which lack ILC3s, Th17 cells, LNs and PPs, NP-OVA-specific IgG titers were even more reduced (Fig. 19 E). Additionally, a more detailed analysis of IgG subtypes revealed that NP-OVA specific IgG1, IgG2a, IgG2b and IgG3 levels were all low or not detectable 5 days after immunization (Fig. 19 F). However, the IgG subtypes IgG1, IgG2a, IgG2b and IgG3 were found to be produced against NP-OVA 14 days after immunization (Fig. 19 G). MHC II deficiency on ILC3s in *I-ab*$^{\Delta ILC3}$ mice resulted in a significant reduction of all NP-OVA-specific IgG subtypes compared to *WT* mice (Fig. 19 G). *RORγ*$^{-/-}$ mice showed again an even higher reduction of NP-OVA specific IgG subtypes (Fig. 19

G). Collectively, these data showed that Ag-presentation by ILC3s contributed to T cell priming *in vivo,* and that TD B cell responses were impaired when Ag-presentation was abolished in ILC3s.

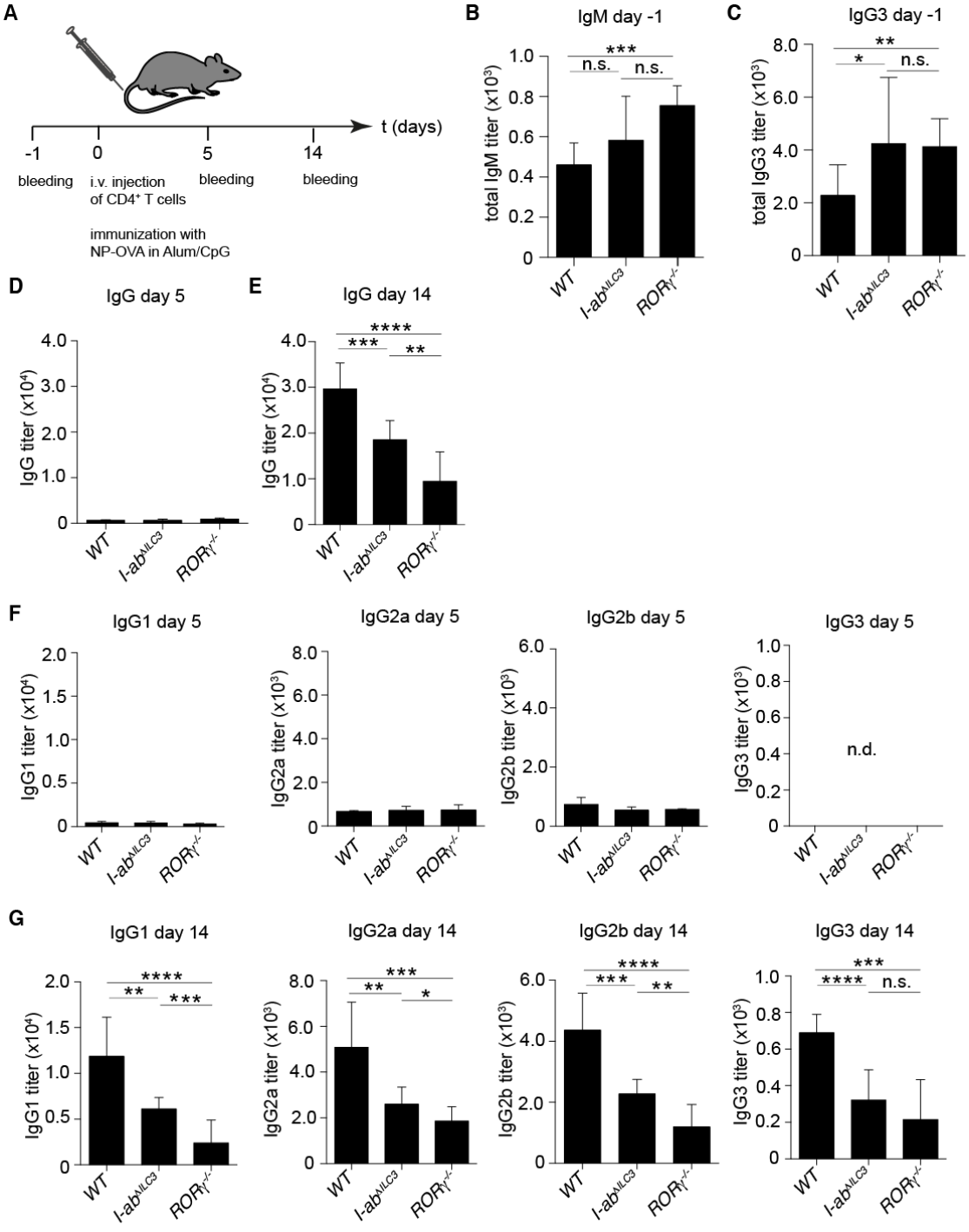


Figure 19: ILC3s support Ag-specific TD B cell responses *in vivo*. A) Schematic representation of the experimental performance of the *in vivo* experiment. t = time (days). **B - C)** Total IgM **(B)** and total IgG3 **(C)** levels in naïve *WT*, *I-ab*$^{\Delta ILC3}$ and RORγ$^{-/-}$ mice measured 1 day before immunization (day -1). Data are shown as mean values + SD (n = 9 – 10, n.s. = not significant; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001). **D - E)** *WT*, *I-ab*$^{\Delta ILC3}$ and RORγ$^{-/-}$ mice were i.p. immunized with 100 µg Alum-precipitated NP-OVA after i.v. injection of *OT-II*^{tg} CD4^{+} T cells plus CpG. NP-OVA-specific total IgG level 5 days **(D)** and 14 days **(E)** after immunization are shown. Data are representative as mean values + SD (n = 9 – 10)

from 3 independent experiments (**P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001). **F - G)** NP-OVA-specific IgG1, IgG2a, IgG2b and IgG3 levels in *WT*, *I-ab*$^{\Delta ILC3}$ and ROR$\gamma^{-/-}$ mice measured 5 days **(F)** or 14 days **(G)** after i.p. immunization with 100 μg NP-OVA in Alum and i.v. injection of *OT-II*^{tg} CD4$^+$ T cells plus CpG. Data are depicted as mean values + SD (n = 9 – 10; n.d. = not detectable; n.s. = not significant; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).

5.5.4 CD4$^+$ T cell - NCR$^-$ILC3 interaction *in vivo*.

My previous data showed that NCR$^-$ILC3s contribute to CD4$^+$ T cell responses *in vitro* as well as *in vivo* by direct interaction with CD4$^+$ T cells in an Ag-dependent manner. For the *in vivo* experiments I made use of the *I-ab*$^{\Delta ILC3}$ mouse model, in which ILC3s lacked MHC II expression and were therefore not able to contribute with Ag-presentation to CD4$^+$ T cell responses.

To gain more insight into the interaction of NCR$^-$ILC3s and CD4$^+$ T cells *in vivo*, the next approach was to monitor Ag-dependent interaction of CD4$^+$ T cells and NCR$^-$ ILC3s directly *in vivo*. Adoptive transfer of Ag-specific *OTII*^{tg} CD4$^+$ T cells and NCR$^-$ ILC3s into *I-ab*$^{-/-}$ mice followed by immunization with the cognate Ag should allow the monitoring of CD4$^+$ T cell - NCR$^-$ILC3 interaction *in vivo*. Therefore, I started to establish a protocol for adoptive transfer of *OTII*^{tg} CD4$^+$ T cells in combination with APCs (BMDCs or NCR$^-$ILC3s) into *I-ab*$^{-/-}$ recipients.

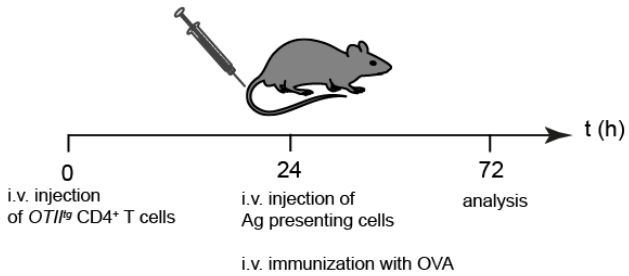
First, I adoptively transferred 3 x 10^6 naïve CFSE-labeled *OTII*^{tg} CD4$^+$ T cells i.v. at time point 0 h into *I-ab*$^{-/-}$ mice to allow the migration of transferred cells into secondary lymphoid tissues. 24 h later, 1 x 10^6 APCs were additionally injected i.v. and the recipient mice were immunized with the cognate Ag. The proliferation of adoptively transferred *OTII*^{tg} CD4$^+$ T cells was examined 48 h later as a first indication for Ag-dependent CD4$^+$ T cell – NCR$^-$ILC3 interaction (Fig. 20 A).[280] According to my previous *in vivo* experiments, the following different immunization strategies were tested; i) alum precipitated NP-OVA, ii) OVA peptide/OVA protein or iii) OVA peptide/OVA protein plus CpG. These immunization strategies should provide the cognate Ag as well as additional stimulation of recipient mice.

Analysis of *I-ab*$^{-/-}$ mice 72 h after the start of the *in vivo* experiment revealed that i.v injected *OTII*^{tg} CD4$^+$ T cells were detectable in the spleen as well as in the inguinal and mesenteric LNs (mLNs; Fig. 20 B and C). However, independent of the immunization strategy, the recovered *OTII*^{tg} CD4$^+$ T cells did not proliferate in the recipient mice (Fig. 20 B and C). No differences in CD4$^+$ T cell proliferation in *I-ab*$^{-/-}$ recipients were observed by injection of either BMDCs or NCR$^-$ILC3s as APCs (Fig. 20 C and D). The absence of CD4$^+$ T cell proliferation could be due to the inability of APCs to reach the areas where interaction with CD4$^+$ T cells would take place within

the secondary lymphoid organ. Thus, further experiments are required to examine whether transferred APCs were localized in close proximity to adoptively transferred $CD4^+$ T cells.

CXCR5 was shown to be crucial for B cell homing to peripheral LNs.[281] Since NCR^- ILC3s express high levels of CXCR5 (Fig. 6 B) I assumed that these cells were able to reach LNs upon adoptive transfer in order to interact with $CD4^+$ T cells in such organized structures. Therefore, in a second approach, I tried to limit the migration of NCR^-ILC3s and $CD4^+$ T cells to the popliteal LNs in order to increase the possibility of interaction of these two cell types (Fig. 20 E). The popliteal LNs are the draining LNs upon injection into the foot pad of mice. Thus, I first injected different amounts (3 x 10^5 – 1 x 10^6 cells) of *in vitro* generated NCR^-ILC3s into the foot pad of *I-ab*$^{-/-}$ mice and screened for the presence of transferred NCR^-ILC3s after different time points by flow cytometry. NCR^-ILC3s were only detectable in very low numbers 3 h after injection. At later time points after injection, I could not detect NCR^-ILC3s in the draining LNs indicating that these cells either migrate further or die. Since, with this approach it was not possible to provide the popliteal LN of *I-ab*$^{-/-}$ mice with MHC II-expressing NCR^-ILC3s, there was no need for further transfer of $CD4^+$ T cells and immunization of recipient mice in this experimental approach. Thus, by the use of the different approaches described before it was not possible to monitor $CD4^+$ T cell – NCR^-ILC3 interaction directly *in vivo*.

A

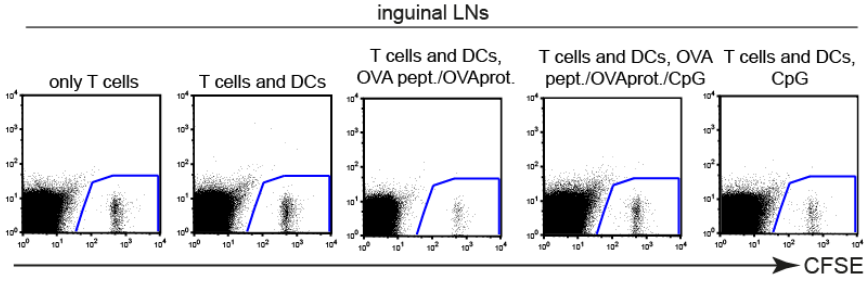


B

immunization	NP/OVA in Alum		OVA pept. / OVA prot.		OVA pept. / OVA prot./CpG	
organ	*OTII*^{tg} CD4$^+$ T cells recovered	*OTII*^{tg} CD4$^+$ T cell proliferation	*OTII*^{tg} CD4$^+$ T cells recovered	*OTII*^{tg} CD4$^+$ T cell proliferation	*OTII*^{tg} CD4$^+$ T cells recovered	*OTII*^{tg} CD4$^+$ T cell proliferation
spleen	✔	✖	✔	✖	✔	✖
inguinal LNs	✔	✖	✔	✖	✔	✖
mLNs	✔	✖	✔	✖	✔	✖

✔ = yes ✖ = no

C



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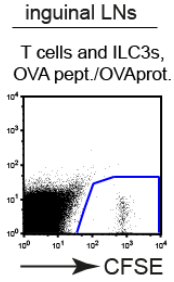

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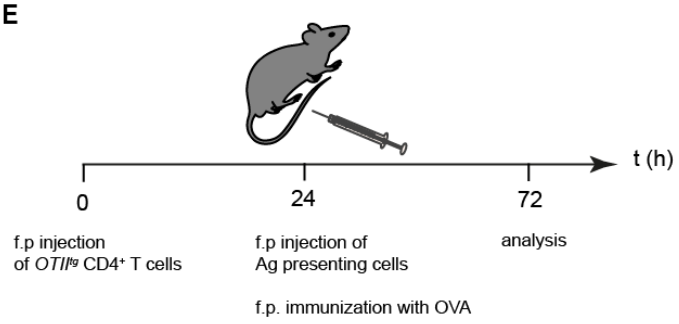

Figure 20: Establishment of adoptive transfer of APCs and *OTII*^{tg} CD4$^+$ T cells *in vivo*. A) Schematic representation of the experimental performance. **B)** Summary of the outcome of different approaches carried out to establish a protocol for adoptive transfer of APCs and *OTII*^{tg} CD4$^+$ T cells in order to monitor NCR$^-$ILC3s-CD4$^+$ T cell interactions *in vivo*. ✔ = yes; *OTII*^{tg} CD4$^+$ T cells could be recovered, ✖ = no; recovered *OTII*^{tg} CD4$^+$ T cells did not proliferate. **C)** Representative dot plots of adoptively transferred CFSE-labeled CD4$^+$ T cells (blue gate) recovered in the inguinal LNs of *I-ab*$^{-/-}$ mice 72 h after adoptive transfer either alone or in combination with BMDCs (DCs) and immunization as indicated in the figure. **D)** Representative dot plot of adoptively transferred CFSE-labeled CD4$^+$ T cells recovered in the inguinal LNs of *I-ab*$^{-/-}$ mouse, which received *in vitro* generated NCR$^-$ILC3s followed by immunization indicated in the figure. **E)** Schematic representation of the experimental performance of the second approach. i.v.: intravenous; f.p.: foot pad.

5.6 Small intestinal NCR$^-$ILC3s differ from splenic NCR$^-$ILC3s in regard to their phenotype, response to innate stimulation and ability to induce CD4$^+$ T cell responses.

5.6.1 The phenotype of small intestinal lamina propria-derived NCR$^-$ ILC3s differs from that of splenic NCR$^-$ILC3s.

In vivo, ILC3s are predominantly found in mucosa-associated tissues such as the gastrointestinal tract.[108, 109, 110, 266] How intestinal NCR$^-$ILC3s contribute to innate and adaptive immune responses was not fully understood. Therefore, I focused more detailed on NCR$^-$ILC3s found in the lamina propria (LP) of the small intestine of adult mice. First of all, I examined the phenotype of *ex vivo* isolated small intestinal LP NCR$^-$ILC3s of adult *WT* mice. LP NCR$^-$ILC3s could be identified as lin$^-$ CD90.2$^+$CD117$^+$RORγt$^+$ cells (Fig. 21 A). Within this cell population, both CD4$^+$ and CD4$^-$ subpopulations could be found (Fig. 21 A). The CD4$^+$ population represented approximately 10 % of total LP NCR$^-$ILC3s whereas 90 % of the cells were CD4$^-$ (Fig. 21 A). Both CD4$^+$ and CD4$^-$ LP NCR$^-$ILC3s expressed comparable high levels of RORγt (Fig. 21 A). The expression profile of the chemokine receptor CCR6 differed in CD4$^+$ and CD4$^-$ LP NCR$^-$ILC3s. Whereas all CD4$^+$ LP NCR$^-$ILC3s expressed CCR6, within the CD4$^-$ LP NCR$^-$ILC3s subset 60 % of the cells was CCR6$^-$ (counting for 70 % of total CD4$^-$ LP NCR$^-$ILC3s; Fig. 21 B). This indicated that the CD4$^-$ LP NCR$^-$ILC3 subset represented a more heterogeneous population. MHC II expression could be observed on both CD4$^+$ and CD4$^-$ LP NCR$^-$ILC3s (Fig. 21 B). Whereas around 40 % of all CD4$^+$ LP NCR$^-$ILC3s expressed MHC II only approximately 20 % of all CD4$^-$ LP NCR$^-$ILC3s were MHC II$^+$.

The intestine contains a large number of commensal bacteria as well as potential pathogens,[282] which might have an influence on the activation state of LP NCR$^-$ ILC3s. Therefore, I investigated the expression of co-stimulatory molecules CD40 and CD86 as well as the early activation marker CD69 on the surface of LP NCR$^-$ ILC3s *ex vivo* isolated from naïve *WT* mice. Both subsets of LP NCR$^-$ILC3s lacked the expression of CD40 and CD86 (Fig. 21 C). CD69 was highly expressed on both CD4$^+$ and CD4$^-$ LP NCR$^-$ILC3 subsets isolated from naïve *WT* mice (Fig. 21 D). LP NCR$^-$ILC3s expressed CD69 two to three fold higher compared to *ex vivo* isolated splenic NCR$^-$ILC3s, which lack CD69 expression (Fig. 21 E). It was possible that either bacteria, endogenous IL-1 or IL-23 present in the small intestine induced the

expression of CD69 on LP NCR$^-$ILC3s of naïve *WT* mice. Therefore, I investigated *IL-1R1*$^{-/-}$ mice, which lack the receptor for IL-1 and hence are unresponsive to IL-1. LP NCR$^-$ILC3s isolated from naïve *IL-1R1*$^{-/-}$ mice showed CD69 expression at similar levels to *WT* mice (Fig. 21 F). Thus, signaling via IL-1R1 was not responsible for the high expression of CD69 on LP NCR$^-$ILC3s. Microbial products and components of bacteria are mainly sensed through TLRs. Therefore, I investigated CD69 expression on LP NCR$^-$ILC3s *ex vivo* isolated from *MyD88*$^{\Delta ILC3/T}$ mice, which lack the expression of MyD88 on RORγt-expressing cells. MyD88 is an adaptor molecule used by almost all TLRs for activation of NFκB downstream signaling transmitting the recognized stimulus.[163] However, CD69 was highly expressed on LP NCR$^-$ILC3s lacking MyD88. Furthermore, *ex vivo* isolated LP NCR$^-$ILC3s of mice with a complete deficiency of MyD88 (*MyD88*$^{-/-}$) also showed high expression of CD69 (Fig. 21 F). These findings indicated that the absence of MyD88-mediated TLR signaling in ILC3s as well as in all other immune cells did not influence CD69 expression on LP NCR$^-$ILC3s. TLR3 and TLR4 are known to signal independent of MyD88 by the use of another adaptor molecule, named TRIF.[283, 284] Therefore, I tested mice with a deficiency in both MyD88 and TRIF (*MyD88*$^{-/-}$*Trif*$^{-/-}$) for the expression of CD69 on LP NCR$^-$ILC3s. Naïve *MyD88*$^{-/-}$*Trif*$^{-/-}$ mice showed as well high levels of CD69 expression on LP NCR$^-$ILC3s (Fig. 21 F). Thus, bacteria and microbial products sensed through TLRs were not responsible for CD69 expression by LP NCR$^-$ILC3s. My previous *in vitro* stimulation data suggested IL-23 as a potential activator of ILC3s (Fig. 7 A and Table 1). Furthermore, IL-22 production by ILC3s in a model of *C. rodentium* infection, an intestinal pathogen, has been reported to be IL-23 dependent.[106] Thus, I investigated the phenotype of LP NCR$^-$ILC3s *ex vivo* isolated from *IL-23p19*$^{-/-}$ mice. However, LP NCR$^-$ILC3s isolated from *IL-23p19*$^{-/-}$ mice showed CD69 expression similar to *WT* (Fig. 21 F). Additionally, LP NCR$^-$ILC3s isolated from *germfre*e mice expressed CD69 excluding the microbiota to be responsible for this phenomenon (Fig. 21 F). CD69 expression on LP NCR$^-$ILC3s was also not the consequence of the presence of T and B lymphocytes, as *Rag2*$^{-/-}$ mice contained LP NCR$^-$ILC3s expressing CD69 at levels comparable to *WT* (Fig. 21 F). Altogether, compared to splenic NCR$^-$ILC3s, LP NCR$^-$ILC3s continuously expressed CD69 independent of IL-1R1- and TLR signaling, the presence of T and B cells, as well as the microbiota or the availability of IL-23.

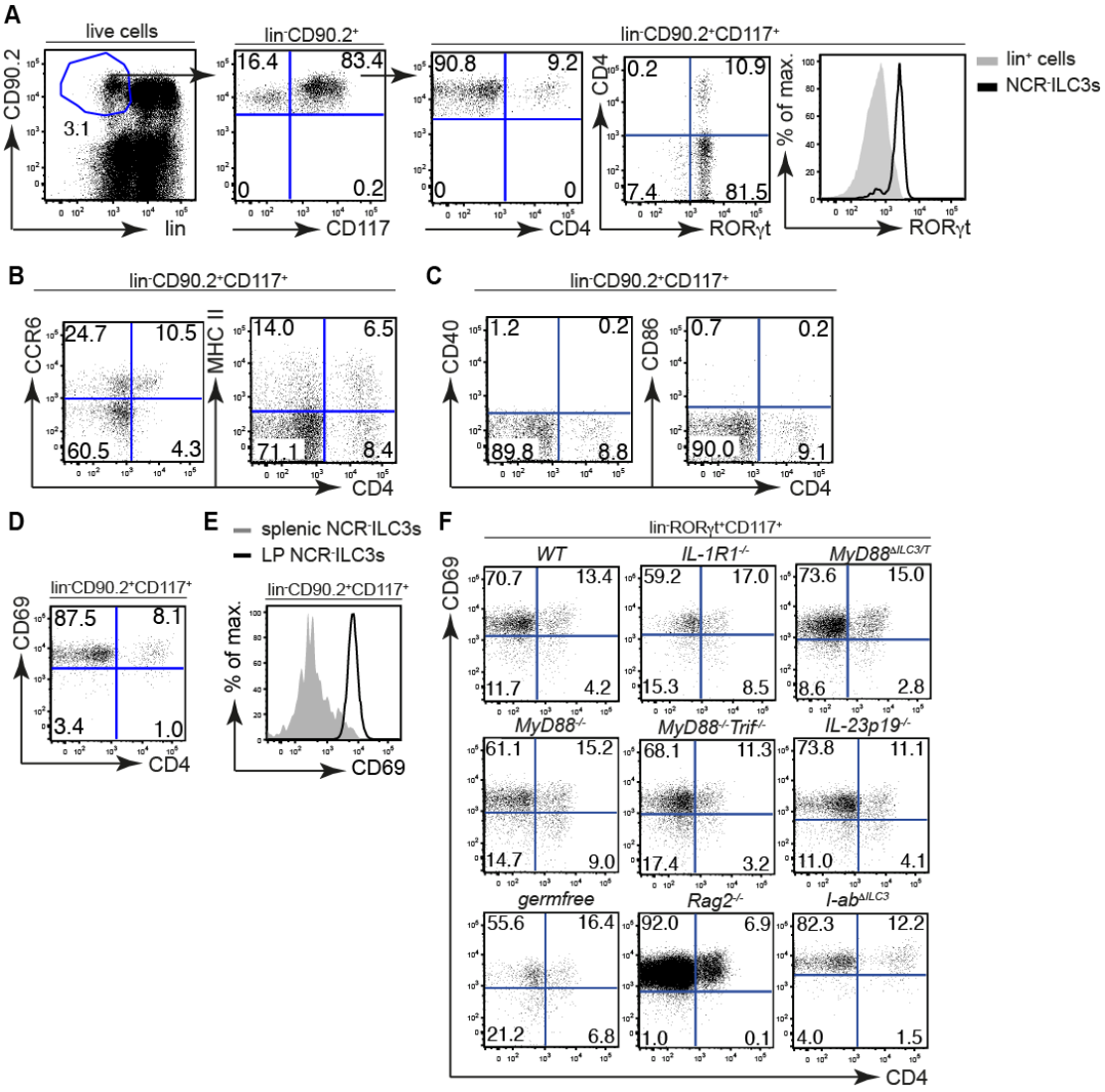

Figure 21: Naïve LP NCR$^-$ILC3s express CD69 independent on IL-1R1 and TLR signaling, IL-23, the microbiota and the presence of T and B lymphocytes. A) Phenotype of *ex vivo* isolated LP NCR$^-$ ILC3s of *WT* mice. RORγt expression of LP CD4$^+$ and CD4$^-$ NCR$^-$ILC3s is shown. RORγt expression of total LP NCR$^-$ILC3s (black line) is displayed in the histogram. Grey solid line corresponds to lin$^+$ cells. Numbers in dot plots show the percentage of cells in each gate. Representative dot plots and histogram are illustrated. **B - C)** Expression of **(B)** CCR6 and MHC II, and **(C)** CD40 and CD86 on naïve *ex vivo* isolated LP NCR$^-$ILC3s of adult *WT* mice. Numbers in dot plots show the percentage of cells in each gate. Representative dot plots are shown. **D)** Representative dot plot of CD69 expression of LP NCR$^-$ ILC3s. **E)** Representative histogram of CD69 expression of LP compared to splenic NCR$^-$ILC3s. **F)** Representative dot plots of CD69 expression on CD4$^+$ and CD4$^-$ LP NCR$^-$ILC3s isolated from different mouse strains as indicated in the figure.

5.6.2 Small intestinal LP NCR$^-$ILC3s do not express co-stimulatory molecules upon IL-1β stimulation.

It has been previously reported that mucosa-associated intestinal ILC3s rather limit CD4$^+$ T cell responses to commensal bacteria than promote CD4$^+$ T cell responses due to the lack of co-stimulatory molecules.[132] However, whether these cells express

co-stimulatory molecules under inflammatory condition similar to splenic NCR$^-$ILC3s has never been investigated. Therefore, I isolated LP NCR$^-$ILC3s from adult *Rag2*$^{-/-}$ mice as these mice harbor increased numbers of ILC3s and simultaneously lack T and B cells facilitating the enrichment of high numbers of ILC3s without contaminating T or B cells. Similar to *WT* derived LP NCR$^-$ILC3s (Fig. 21 A and B) *Rag2*$^{-/-}$ derived LP NCR$^-$ILC3s could be identified as lin$^-$CD90.2$^+$CD117$^+$ cells expressing high levels of the transcription factor RORγt (Fig. 22 A). Sort-purified *ex vivo* isolated NCR$^-$ILC3s were then cultured for 48 h in the presence of IL-1β or in medium alone. *In vitro* exposure to IL-1β induced blast formation of LP NCR$^-$ILC3s (Fig. 22 B). However, despite increasing amount of IL-1β in the *in vitro* cultures, neither the expression of the co-stimulatory molecules CD40 and CD86 nor the up-regulation of MHC II could be detected on LP NCR$^-$ILC3s (Fig. 22 C). Furthermore, LP NCR$^-$ILC3s showed high levels of IL-22 production in the absence of IL-1β, and IL-22 secretion was increased in the presence of IL-1β (Fig. 22 D). IL-22 secretion by IL-1β-activated LP NCR$^-$ILC3s exceeded those of IL-1β-activated splenic NCR$^-$ILC3s. In contrast to LP NCR$^-$ILC3s, splenic NCR$^-$ILC3s did not secrete IL-22 in the absence of innate stimulation (Fig. 22 D). Therefore, the response of LP NCR$^-$ILC3s to innate stimulation differed from that of splenic NCR$^-$ILC3s as such as LP NCR$^-$ILC3s predominantly produced IL-22 and did not up-regulate expression of MHC II and co-stimulatory molecules upon IL-1β exposure.

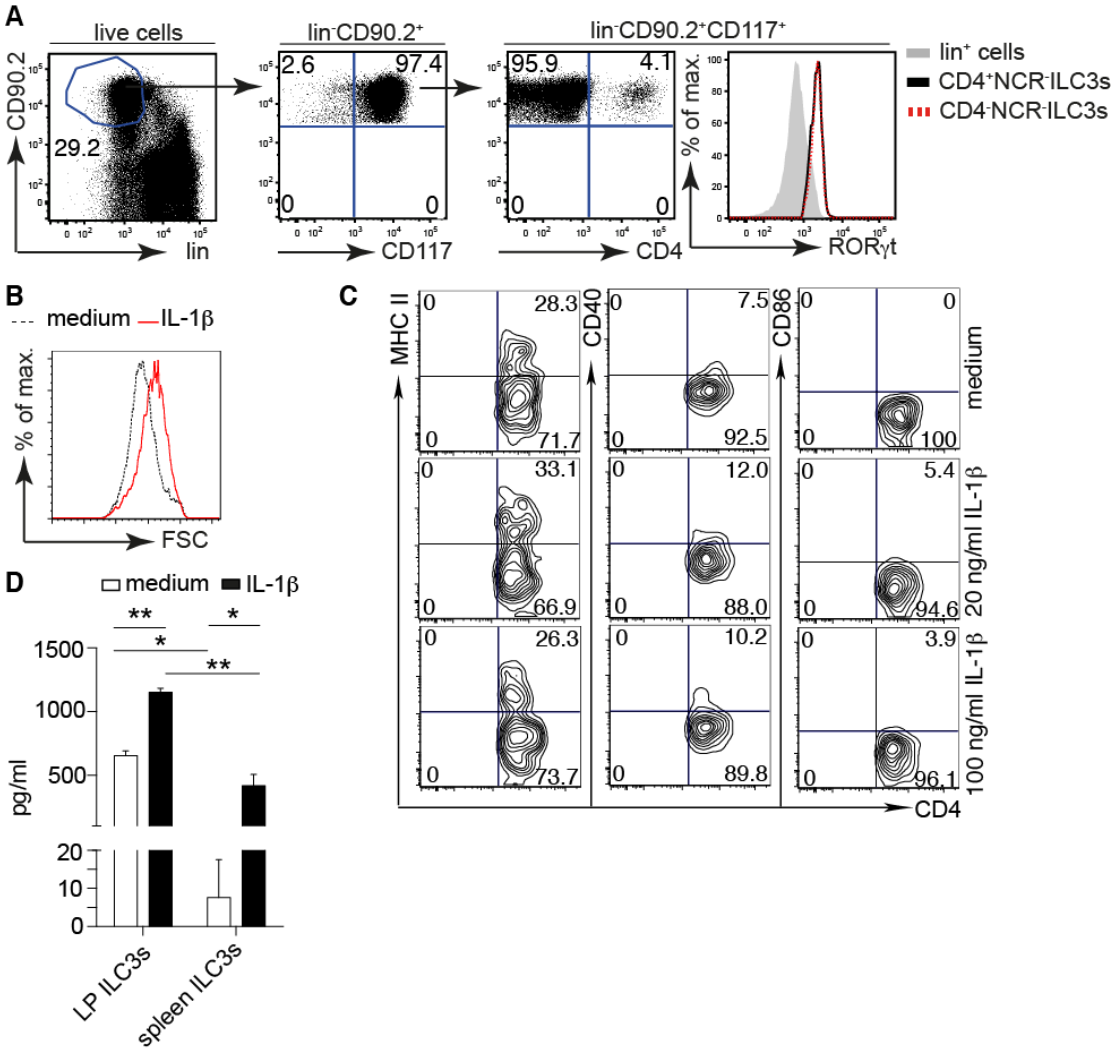

Figure 22: Small intestinal LP NCR⁻ILC3s do not express co-stimulatory molecules upon stimulation with IL-1β. A) Phenotype of NCR⁻ILC3s *ex vivo* isolated from the lamina propria (LP) of the small intestine of *Rag2*$^{-/-}$ mice. Numbers in dot plots show the percentage of cells in each gate. Representative dot plots and histogram are depicted. **B)** Representative histogram of forward scatter (FSC) level in LP NCR⁻ILC3s cultured for 48 h in the presence of IL-1β or in medium alone. **C)** Expression of MHC II, CD40 and CD86 by LP NCR⁻ILC3s cultured for 48 h in the presence of different concentrations of IL-1β or in medium alone as indicated in the figure. Data are representative of 4 independent experiments. Numbers in dot plots show the percentage of cells in each quadrant. **D)** IL-22 secretion by either *ex vivo* isolated LP or splenic NCR⁻ILC3s upon 48 h exposure to IL-1β or medium alone. Data are shown as mean values + SD (n = 3 - 6, at least 3 independent experiments; *P ≤ 0.05; **P ≤ 0.01).

5.6.3 Small intestinal LP NCR⁻ILC3s are able to internalize exogenous Ag.

Naïve LP NCR⁻ILC3s showed MHC II expression on their surface (Fig. 21 B and Fig. 22 C). Therefore, I tested the capacity of LP NCR⁻ILC3s to take up exogenous Ag. Sort-purified *ex vivo* isolated LP NCR⁻ILC3s were cultured for 6 h in the presence of red fluorescent latex beads at 37 °C and 4 °C. Whereas LP NCR⁻ILC3s were able to

internalize latex beads at 37 °C, bead uptake was severely impaired at 4 °C as expected (Fig. 23 A). Thus, naïve *ex vivo* isolated LP NCR$^-$ILC3s, similar to splenic NCR$^-$ILC3s, were capable to internalize exogenous Ag.

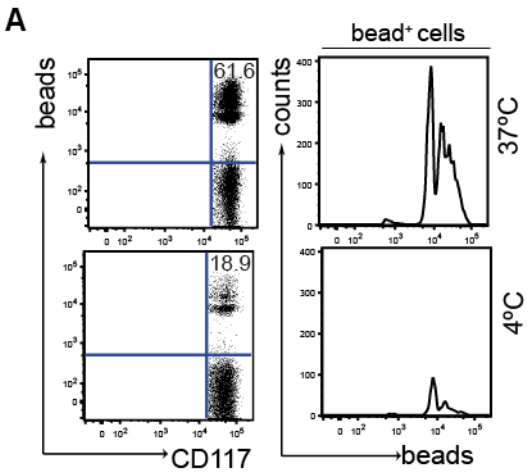

Figure 23: LP NCR$^-$ILC3s are able to take up exogenous Ag. A) Representative plots and histograms of sort-purified *ex vivo* isolated LP NCR$^-$ILC3s incubated for 6 h with red fluorescent latex beads at 37 ºC or 4 ºC. Number in the dot plots shows percentage of bead$^+$ cells (4 independent experiments).

5.6.4 Small intestinal LP NCR$^-$ILC3s are unable to efficiently induce CD4$^+$ T cell activation.

Upon IL-1β exposure, LP NCR$^-$ILC3s were unable to express co-stimulatory molecules (Fig. 22 C). Interaction of T cells with APCs lacking co-stimulatory molecules rather limits T cell responses and induces T cell anergy.[133, 218] Whether LP NCR$^-$ILC3s were able to either elicit CD4$^+$ T cell responses or limit them was not fully understood. Therefore, I tested the ability of pre-activated LP NCR$^-$ILC3s to induce CD4$^+$ T cell responses *in vitro* by the use of so-called BW-OTII cells. BW-OTII cells were generated by the fusion of activated *OT-II^{tg}* T cells (activated in the presence of soluble anti-CD3 Ab) with TCRαβ$^-$ BW-5147 NFAT-EGFP fusion partner (see 4.2.6 Generation of BW-OTII cells).[256] TCRαβ$^-$ BW-5147 NFAT-EGFP hybridoma cells contain nuclear factor of activated T cells (NFAT)-binding sites inserted upstream of the enhanced green fluorescent protein (EGFP) coding sequence. Thus, TCRαβ$^-$ BW-5147 NFAT-EGFP hybridoma cells fused to *OT-II^{tg}* CD4$^+$ T cells (BW-OTII cells) provide a tool to study Ovalbumin-MHC II dependent TCR engagement (Fig. 24 A and B). *Ex vivo* isolated sort-purified LP or splenic NCR$^-$ILC3s were activated with IL-

1β for 24 h and subsequently co-cultured with BW-OTII cells in the presence or absence of OVA peptide (Fig. 24 A and B). The percentage of GFP^+ BW-OTII cells in the co-culture reflected the efficiency of Ag presentation by NCR^-ILC3s. IL-1β-activated LP NCR^-ILC3s were less efficient in BW-OTII activation in the presence of OVA peptide compared to splenic NCR^-ILC3s (Fig. 24 A and B). GFP^+ BW-OTII cells were almost not detectable in co-cultures in the absence of Ag (Fig. 24 A and B). To test whether LP NCR^-ILC3s were able to process also whole OVA protein in order to interact with $CD4^+$ T cells, IL-1β-activated LP NCR^-ILC3s were co-cultured with sort-purified *ex vivo* isolated *OT-II*^{tg} $CD4^+$ T cells either in the presence or absence of whole OVA protein (Fig. 24 C and D). LP NCR^-ILC3s were 3 times less efficient to induce cognate T cell activation *in vitro* compared to splenic NCR^-ILC3s (Fig. 24 C and D). Thus, these data demonstrated that although NCR^-ILC3s were able to process exogenous Ag and to interact with $CD4^+$ T cells, they showed only low $CD4^+$ T cell activation efficiency. Collectively, these data showed that LP NCR^-ILC3s responded differentially than splenic NCR^-ILC3s to innate simulation and were less efficient in triggering $CD4^+$ T cell responses.

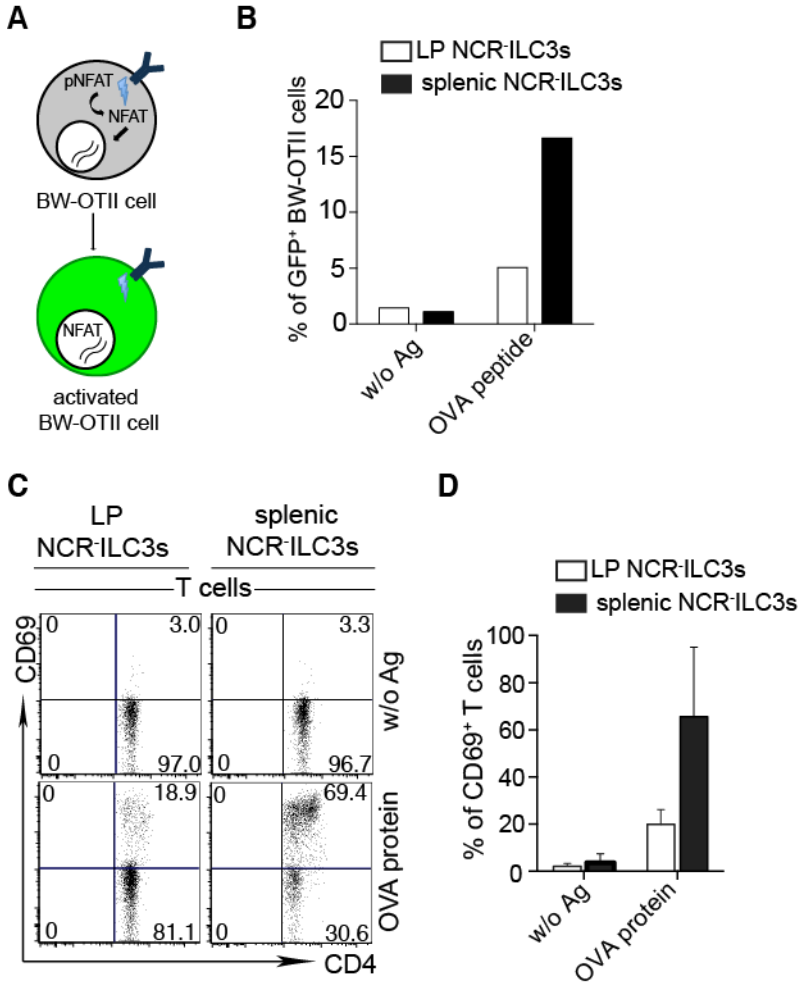

Figure 24: Activated LP NCR$^-$ILC3s do not induce efficient CD4$^+$ T cell activation. A) Schematic representation of naïve GFP$^-$ BW-OTII cells (fusion of TCR$\alpha\beta^-$ BW5147 NFAT-EGFP cells with *OT-II^{tg}* CD4$^+$ T cells) and activated GFP$^+$ BW-OTII cells upon TCR engagement. **B)** Percentage of GFP$^+$ BW-OTII cells upon co-culture with IL-1β-activated LP or splenic NCR$^-$ILC3s in the presence or absence of OVA peptide. Data are shown as mean values (n= 2-3). **C)** Naïve sort-purified *OT-II^{tg}* CD4$^+$ T cells were cultured with IL-1β-activated LP or splenic NCR$^-$ILC3s in presence of OVA protein or medium alone (w/o Ag) as indicated in the figure. Representative plots of CD69 expression by *OT-II^{tg}* CD4$^+$ T cells 48 h later. Data are representative of 4 independent experiments. **D)** Percentage of CD69$^+$ *OT-II^{tg}* CD4$^+$ T cells upon co-culture with IL-1β-activated LP or splenic NCR$^-$ILC3s in the presence or absence of OVA protein. Data are shown as mean values + SD (4 independent experiments).

6 Discussion

Beside the role as key players in the induction of lymphoid tissue formation and organization during embryogenesis, ILC3s are found after birth, mainly accumulating in the intestine and are as well present in peripheral organs. So far, the immune functions described for NCR^- and NCR^+ILC3s in the adult immune system are based on their ability to secrete effector cytokines in response to soluble factors derived from other immune cells. I show here that NCR^-ILC3s were able to directly sense and respond to microbial products and pro-inflammatory cytokines. Upon IL-1β exposure, peripheral NCR^-ILC3s expressed MHC II and co-stimulatory molecules and became *bona fide* APCs, as they were able to take up exogenous Ag, to process it and consequently promoted OVA-specific $CD4^+$ T cell proliferation *in vitro* and *in vivo*. In addition, activated NCR^-ILC3s expressed an unexpected repertoire of cytokines known to alter T cell responses. Ag-specific T cell proliferation and IgG-mediated humoral immunity were impaired in animals in which Ag-presentation was abolished exclusively in ILC3s. Moreover, in the presence of Ag, the T cell priming led to an extended activation of ILC3s suggesting a reciprocal crosstalk between NCR^-ILC3s and $CD4^+$ T cells. Indeed, soluble factors most likely produced by $CD4^+$ T cells upon Ag encounter were able to induce *de novo* activation of NCR^-ILC3s. These novel data suggest that under inflammatory conditions the cognate interaction of NCR^- ILC3s and $CD4^+$ T cells contributes to adaptive immunity. Furthermore, I show here that small intestinal NCR^-ILC3s differed phenotypically and functionally from peripheral NCR^-ILC3s. Small intestinal NCR^-ILC3s produced high amounts of IL-22 upon exposure to IL-1β, but were not able to up-regulate MHC II molecule expression or to express co-stimulatory molecules. Moreover, although they were able to take up, process and present exogenous Ag, the induction of $CD4^+$ T cell responses was less efficient compared to peripheral NCR^-ILC3s. These data suggest that NCR^-ILC3 immune function might be tissue-specific and depend on environmental signals that are differentially active under homeostatic and inflammatory conditions.

Innate stimulation of NCR$^-$ILC3s induces cytokine production and changes in the phenotype.

The FL harbors a population of $\alpha_4\beta_7^+$ cells, which were described to give rise to ILC3s lacking the expression of the natural cytotoxicity receptor (NCR) NKp46 *in vitro*.[60, 61] I line with this, I was able to generate NCR$^-$ILC3s from the $\alpha_4\beta_7^+$ ILC3 precursor population isolated from the FL of 14.5 dpc *WT* embryos. These *in vitro* generated NCR$^-$ILC3s showed a phenotype (Fig. 6 A - B), which is similar to the classical phenotype described for NCR$^-$ILC3s.[57] The existence of a CD4$^+$ and a CD4$^-$ NCR$^-$ILC3 subpopulation in embryonic tissues was reported in several studies[61, 63, 93] and CD4$^+$ and CD4$^-$ NCR$^-$ILC3s showed nearly identical phenotype and gene expression patterns.[285] In line with this, I could demonstrate that *in vitro* generated NCR$^-$ILC3s can be subdivided into CD4$^+$ and CD4$^-$ NCR$^-$ILC3s possessing an identical phenotype except the surface expression of CD4 (Fig. 6 A - B). Altogether, the *in vitro* generation of NCR$^-$ILC3s provided me with sufficient numbers of cells to study the function of CD4$^+$ and CD4$^-$ NCR$^-$ILC3s in innate and adaptive immune responses.

NCR$^-$ILC3s were reported to produce IL-17 and IL-22 upon *in vivo* challenge with zymosan, a yeast cell wall product.[105] Furthermore, NCR$^-$ILC3s could be shown to provide protective immunity during fungal infection with *Candida albicans*[265] or bacterial infection with *Citrobacter rodentium*[106] by massive production of IL-17 and IL-22, respectively. The secretion of IL-17 and/or IL-22 was dependent on the presence of IL-23 produced by other immune cells in response to the pathogens or microbial products.[105, 106] Consistent with these findings, I could show that *in vitro* generated NCR$^-$ILC3s were able to produce high amounts of IL-22 in response to IL-23 stimulation *in vitro*. In addition, I could demonstrate that NCR$^-$ILC3s secreted IL-22 upon exposure to several TLR ligands whereby Poly I:C induced the production of significantly high levels of IL-22 (Fig. 7 A and C). Thus, NCR$^-$ILC3s are as well capable to directly sense and respond to the presence of microbial products without the involvement of other immune cells suggesting a rapid and independent cytokine-mediated role for NCR$^-$ILC3s in protective immunity. Only few studies reported the expression of TLRs on ILC3s.[286, 287, 288] TLR4 was shown to be expressed on murine ILC3s whereas TLR2 expression was reported on murine and human ILC3s.[287, 288] Nevertheless, my data suggest, that NCR$^-$ILC3s express at least some TLRs allowing the direct response to microbial products.

IL-17 secretion could not be observed in the presence of the tested TLR ligands (Fig. 7 C). Whether the exposure of NCR$^-$ILC3s to a combination of several TLR ligands is required and sufficient to induce IL-17 secretion remains to be investigated. Moreover, whether ligands for the aryl hydrocarbon receptor (AhR), which are reported to be crucial for the secretion of IL-17 and IL-22 by Th17 cells,[289] are potential inducer of IL-17 and/or IL-22 production by NCR$^-$ILC3s remains to be explored. In contrast to other studies, which reported IL-23-dependent IL-17 production by NCR$^-$ILC3s,[105, 265] I could not observe IL-17 secretion of *in vitro* generated NCR$^-$ILC3s in response to IL-23 stimulation *in vitro*. However, the presence of the pro-inflammatory cytokine IL-1β induced IL-17 secretion (Fig. 7 B). Considering IL-17 production by ILC3s in different *in vivo* infection models, my *in vitro* data hence suggest that NCR$^-$ILC3s might require IL-23 and additional factors, such as IL-1β, to secrete IL-17.

The pro-inflammatory cytokine IL-1β was able to induce remarkably high levels of IL-22 secretion by NCR$^-$ILC3s even exceeding those induced by IL-23 (Fig. 7 A). Both CD4$^+$ and CD4$^-$ NCR$^-$ILC3 subsets were capable to secrete IL-22 at similar levels, indicating that in addition to similarities in their phenotype they shared the capacity to secrete typical ILC3 cytokines. In line with the responsiveness to IL-1β, ILC3s were reported to express the appropriate receptor, IL-1R1 and MyD88 allowing IL-1R1 downstream signaling.[266, 267, 268, 288] In addition, it was shown that basal and IL-23- as well as IL-1β-induced production of IL-22 by ILC3s was dependent on IL-1R1.[266, 267, 268] Blockade of IL-1β *in vivo* resulted in a decreased production of IL-17 and IFNγ by *ex vivo* isolated ILC3s upon IL-23 stimulation consistent with the fact that levels of IL-23R expression by ILC3s were reduced.[290] Similarly, the production of IL-17, IL-22 and IFNγ by ILC3s, their expansion *in vitro* and their accumulation at sites of infection *in vivo* was partially triggered through IL-1R signaling.[262, 291] Altogether, these data suggest that IL-1β triggers ILC3 responses. IL-1β and/or IL-23 are produced by DCs and MΦ under steady state and upon microbial challenge.[126, 164, 165, 166, 167, 170, 292] Considering my findings that NCR$^-$ILC3s are responsive to both, microbial products and cytokines, one could assume that their full activation might be reached by a combination of direct pathogen recognition and indirect sense of a cytokine milieu provided by other immune cells. Further, it could be shown that IL-1β is able to increase IL-23R expression on NCR$^-$ILC3s,[290] a fact, which might enhance their responsiveness to IL-23 and therefore suggests a role for IL-1β in orchestrating IL-23-dependent NCR$^-$ILC3 responses. Several TLR ligands such as Poly I:C and LPS were previously reported to induce pro-IL1β.[293] However, Poly I:C-induced direct

NCR$^-$ILC3 activation was not mediated through IL-1β secretion of NCR$^-$ILC3s as addition of neutralizing IL-1β Ab was not able to reduce activation in the presence of Poly I:C (data not shown). This suggests, that TLR ligand-induced NCR$^-$ILC3 activation was at least not mediated through IL-1β secretion of NCR$^-$ILC3s.

Moreover, I could show that the exposure of *in vitro* generated NCR$^-$ILC3s to IL-1β induced the secretion of an unexpected broad repertoire of different cytokines, such as IL-2, IL-6, MIP-1α, IP-10, IFNγ, TNFα and GMCSF, all known for their capacity to alter T cell responses (Fig. 7 D). Whereas IL-2 and IL-6 are involved in T cell activation and proliferation,[211, 294, 295, 296] MIP-1α and IP-10 can act as chemoattractants for mononuclear cells and effector T cells.[297, 298] IFNγ has been shown to induce Th1 cell differentiation,[299] whereas GMCSF plays an important role in the induction and maintenance of intestinal tissue resident DCs,[121] which are in turn crucial for the induction of regulatory T cells (T_{regs}) responsible for the regulation of intestinal tolerance.[122, 123, 124] In line with this, it could recently be shown that IL-1β-induced secretion of GMCSF by ILC3s in the intestine indirectly regulates the differentiation and number of T_{regs} suggesting a cytokine-mediated regulatory role for ILC3s in the intestinal immune system.[126] However, the role of the different cytokines released by NCR$^-$ILC3s in response to IL-1β stimulation needs to be further explored. T cell activation, proliferation, survival, differentiation or migration could be examined in the presence of soluble factors produced by IL-1β - exposed NCR$^-$ILC3s *in vitro*. Further, one has to consider that most likely not each cell of the *in vitro* generated NCR$^-$ILC3 population was able to produce the entire repertoire of cytokines. It might be possible that this population is functionally heterogeneous containing several different cell clones capable of producing one or more cytokines. In response to IL-1β, or to other pro-inflammatory cytokines and microbial products, those different cell clones might be able to secrete one single or even several effector cytokines as described for human ILC and CD4$^+$ T cell clones.[20, 287, 300, 301] Altogether, these data suggested that activation-induced and stimuli-dependent cytokine production by NCR$^-$ILC3s might play different roles in immunity.

Beside cytokine production upon direct exposure to microbial products and pro-inflammatory cytokines, I examined whether one can phenotypically distinguish naïve and “experienced” NCR$^-$ILC3s. In order to answer this question, I measured the surface expression of CD69, which is reported to be the earliest inducible glycoprotein during lymphocyte activation.[202] *In vitro* generated NCR$^-$ILC3s expressed low to negligible levels of CD69, indicating their naïve phenotype obtained upon differentiation from $\alpha_4\beta_7^+$ ILC3 precursors residing in the sterile environment of

the FL (Fig. 6 B and Fig. 8 B). However, *in vitro* exposure to TLR ligands Poly I:C and CpG, as well as the pro-inflammatory cytokine IL-1β, increased the expression of CD69 indicating the activation of NCR$^-$ILC3s (Fig. 8 A). Further, I could demonstrate that *in vivo* challenge with CpG or LPS induced activation of NCR$^-$ILC3s residing in the spleen of adult mice. Splenic NCR$^-$ILC3s up-regulated the expression of CD69 and MHC II (Fig. 9 C and D). It has been reported, that CpG is able to induce activation and maturation of DCs resulting in the up-regulation of the expression of certain surface molecules, such as MHC I and II, and co-stimulatory molecules.[302, 303] In line with the finding that CpG stimulates APCs to secrete cytokines like IL-1, TNFα, IL-12 and IFNγ,[302, 303, 304, 305] I could demonstrate that *in vivo* administration of CpG induced an inflammatory milieu in the spleen with significantly increased levels of the pro-inflammatory cytokines IL-1β, TNFα and IFNγ (Fig. 9 B). Similar to CpG, it has been reported that *in vivo* challenge of mice with LPS induces the production of IL-1β.[167] Regarding the fact that the pro-inflammatory cytokine IL-1β appeared to be a strong activator of NCR$^-$ILC3s *in vitro* able to induce the production of various cytokines and the up-regulation of CD69 expression, one can assume that the activation of splenic NCR$^-$ILC3s observed upon *in vivo* challenge of adult mice with microbial products was at least partially mediated by the induction of a pro-inflammatory milieu.

Altogether, my data showed that direct recognition of certain microbial products and sensing of pro-inflammatory cytokines induced activation of NCR$^-$ILC3s *in vitro* and *in vivo*. Activation and cytokine production of NCR$^-$ILC3s was dependent on the nature of the present stimuli suggesting that NCR$^-$ILC3s might be able to differentially regulate and adapt their responses to innate stimulation.

Activated NCR$^-$ILC3s acquire an APC-like phenotype.

In addition to CD69 up-regulation, I could demonstrate that *in vitro* generated and *ex vivo* isolated splenic NCR$^-$ILC3s increased the expression of MHC II molecules and acquired the expression of co-stimulatory molecules CD80/86 and CD40 upon IL-1β exposure *in vitro* (Fig. 8 B and 10 A). These data showed that peripheral NCR$^-$ILC3s were able to acquire an APC-like phenotype upon exposure to inflammation-associated signals. This phenomenon was already well documented for DCs, which were able to mature into professional APCs fully capable of initiating efficient CD4$^+$ T cell immunity.[173] Expression of MHC II by a fraction of fetal/neonatal ILC3s was already reported.[57] Here, I could show that whereas naïve splenic NCR$^-$ILC3s

already expressed MHC II molecules on their surface, naïve *in vitro* generated NCR$^-$ ILC3s only showed low levels of MHC II expression. This low expression level might arise due to the fact that these cells differentiated from $\alpha_4\beta_7^+$ ILC3 precursors residing in the sterile environment of the FL whereas splenic NCR$^-$ILC3s of adult mice might have already been exposed to factors able to induce MHC II expression. The determination of the gene expression profile of *in vitro* generated CD4$^+$ and CD4$^-$ NCR$^-$ILC3 subsets revealed the expression of gene-transcripts related to MHC II expression and Ag presentation at high levels under steady state (Fig. 8 C). This suggests that already naïve NCR$^-$ILC3s possess the machinery for MHC II-dependent Ag presentation. Surprisingly, no differences in the transcript levels of these genes could be observed upon exposure to IL-1β *in vitro* for 1 h suggesting that this short stimulation might not be sufficient to induce substantial changes. Hence, these data might imply that NCR$^-$ILC3s require a prolonged exposure to inflammatory signals to acquire an APC-like phenotype.

NCR$^-$ILC3s take up latex beads, process protein Ag and promote CD4$^+$ T cell responses *in vitro.*

Having demonstrated that peripheral NCR$^-$ILC3s were able to acquire an APC-like phenotype upon innate stimulation, I intended to examine their ability to take up exogenous Ag and to induce CD4$^+$ T cell responses. Indeed, I could show that peripheral NCR$^-$ILC3s were capable to internalize latex beads although with slower kinetics than MΦ (Fig. 11 A - E). This could be either due to less efficient internalization of beads by or due to limitations in the experimental technique. In fact, MΦ were adherent in *in vitro* cultures facilitating the uptake of soluble beads compared to NCR$^-$ILC3s, which were in suspension. Moreover, I could demonstrate the specificity of bead internalization by peripheral NCR$^-$ILC3s as bead uptake was severely inhibited in the presence of Cytochalasin D, reported to inhibit phagocytosis,[257, 306] and when bead uptake was performed at 4 °C, a condition shown to prevent ingestion of targets.[307]

In addition, I could show that peripheral NCR$^-$ILC3s were able to prime naïve Ag-specific CD4$^+$ T cells. CD4$^+$ T cell proliferation could be observed in the presence of activated peripheral NCR$^-$ILC3s and peptide-Ag, and to a lesser extent with entire protein-Ag (Fig. 12 A - E). Not surprisingly, peripheral NCR$^-$ILC3s were less potent in priming naïve T cells than BMDCs, probably because DCs were more efficient in processing of protein Ag and expressed higher levels of co-stimulatory and MHC II

molecules. Despite their similarities in phenotype (Fig. 6 B), cytokine production (Fig. 7 A) and gene expression (Fig. 8 C),[285] *in vitro* generated CD4$^+$ NCR$^-$ILC3s had a greater potential to induce protein Ag-specific T cell proliferation than CD4$^-$ NCR$^-$ ILC3s (Fig. 12 B). These data were in line with a previous finding that embryonic CD4$^+$ NCR$^-$ILC3s were more differentiated than their CD4$^-$ counterpart[308] and my data showing that CD4$^+$ NCR$^-$ILC3s were slightly more efficient in bead uptake (Fig. 11 D). Although activation of splenic NCR$^-$ILC3s led to significantly increased CD4$^+$ T cell activation, I unexpectedly observed that naïve splenic NCR$^-$ILC3s without prior activation were able to induce CD4$^+$ T cell responses (Fig. 12 C - E). However, I could show that Ag-dependent interaction of NCR$^-$ILC3s and CD4$^+$ T cells resulted in NCR$^-$ILC3 activation (Fig. 13 A), which might explain why naïve NCR$^-$ILC3s could trigger T cell priming in the absence of previous activation by IL-1β. These data suggested that T cell-derived signals might be able to enhance and/or prolong NCR$^-$ ILC3-mediated T cell activation. A similar crosstalk has been reported for ILC2-T cell interactions whereby CD4$^+$ T cells activated through TCR engagement produced cytokines able to reciprocally induce ILC2 proliferation and to increase their Th2 type cytokine production.[51] Another study suggested an additional reciprocal effect in which ILC2s induced T cell proliferation and increased Th2 cytokine production in a cell-cell contact-dependent manner via OX40L.[53] Collectively, this illustrates a multifaceted crosstalk between ILCs and T cells, which most likely acts on T as well as ILC responses. A role for OX40L expressed on adult ILC3s in CD4$^+$ T cell responses was already proposed a decade ago.[94, 95] It could be shown that adult ILC3s expressed OX40L and CD30L providing the possibility to interact within secondary lymphoid organs with primed CD4$^+$ T cells expressing CD30 and OX40.[94, 95] Thereby, it was suggested that ILC3s promoted the survival of CD4$^+$ memory T cells by providing accessory signals, at least via OX40L.[94, 95, 309] The expression of CD30L and OX40L on adult ILC3s was reported to be the only difference between fetal and adult ILC3s,[102] which otherwise were shown to share phenotypic and functional properties.[96, 103] In line with this, I found that splenic NCR$^-$ILC3s were phenotypically similar to FL-derived NCR$^-$ILC3s (Fig. 6 A - B and Fig. 9 A).

Having demonstrated that the presence of Ag could not be responsible for NCR$^-$ILC3 activation upon Ag-dependent interaction with CD4$^+$ T cells (Fig. 13 B), I assumed that a positive feedback loop based on secreted cytokines from T cells to ILC3s might exist as it was already demonstrated for ILC2-T cell interactions.[51] Therefore, I examined the ability of soluble factors produced by CD4$^+$ T cells upon cognate Ag-encounter to reciprocally induce NCR$^-$ILC3 activation. The following approaches were used to generate cell culture supernatant (SN) containing CD4$^+$ T cell-derived

cytokines; i) co-culture of NCR$^-$ILC3s and CD4$^+$ T cells with Ag, ii) co-culture of BMDCs and CD4$^+$ T cells with Ag, or iii) CD4$^+$ T cell activation in presence of anti-CD3/anti-CD28 Abs coated on a plate. Indeed, I could show that naïve splenic NCR$^-$ ILC3s up-regulated the expression of CD69 and slightly increased the expression levels of CD40 and CD80 in response to soluble factors produced in the co-culture of BMDCs and CD4$^+$ T cells with Ag (Fig. 14 A and B) suggesting that these soluble factors induced activation of NCR$^-$ILC3s, which might increase their efficiency to promote CD4$^+$ T cell responses. In line with the previous finding that CD4$^+$ T cells secrete IFNγ upon Ag encounter,[272] I could show that CD4$^+$ T cells in *in vitro* cultures with BMDCs and Ag produced IFNγ (Fig. 14 C). IFNγ was previously reported to enhance MHC II expression either on cells constitutively expressing MHC II molecules, such as B cells and DCs[310] or on cells, which do not constitutively express MHC II.[311] In addition, IFNγ was shown to increase the expression of other genes involved in the class II Ag presentation pathway overall suggesting that IFNγ indirectly promotes peptide-specific activation of CD4$^+$ T cells.[312, 313, 314] As I could demonstrate that NCR$^-$ILC3s up-regulated MHC II expression in response to IFNγ stimulation *in vitro* (Table 1), IFNγ might mediate NCR$^-$ILC3 activation observed during Ag-dependent NCR$^-$ILC3-CD4$^+$ T cell interactions. To further test the involvement of IFNγ during ILC3-CD4$^+$ T cell interactions one could examine NCR$^-$ ILC3 activation in the presence of neutralizing IFNγ Ab. IL-2, another cytokine produced by CD4$^+$ T cells upon Ag encounter,[211] was previously reported to promote survival, proliferation and type 2 cytokine secretion by ILC2s.[51] Furthermore, treatment of *Rag2*$^{-/-}$ mice with IL-2/anti-IL-2 complexes upon infection with *Nippostrongylus brasiliensis* was reported to increase the ILC2 numbers and the production of Th2 type cytokines in absence of CD4$^+$ T cells sufficient to overcome the infection.[54] I could show that CD25, the high affinity receptor for IL-2, was expressed at high levels on naïve NCR$^-$ILC3s and was even more increased upon their activation (Fig. 14 D). Moreover, I found that NCR$^-$ILC3s were able to secrete IL-2 upon IL-1β exposure *in vitro* (Fig. 7 D). These data suggest a role for IL-2, either NCR$^-$ILC3- or T cell-derived, on the survival, proliferation or cytokine production of both cell types. I could show that the ligand for ICOS, ICOSL, was expressed on naïve NCR$^-$ILC3s (Fig. 14 D). ICOSL binding to ICOS, which is *de novo* expressed on CD4$^+$ T cells upon activation,[274] has previously been reported to be involved in the induction CD40L expression on T cells allowing the interaction with CD40$^+$ B cells.[315] This is crucial for the induction of T-dependent B cell responses.[316, 317] This finding suggested that NCR$^-$ILC3s can provide an additional co-stimulatory signal promoting

$CD4^+$ T cell-mediated immunity. In line with a previous finding, that ICOS is able to down-regulate ICOSL expression,[318] I observed decreased levels of ICOSL expression on NCR^-ILC3s in the presence of soluble factors produced by $CD4^+$ T cells upon Ag-encounter. At the same time ICOS expression on NCR^-ILC3s was increased *in vitro* (Fig. 14 D). It is possible that the level of ICOS expression on NCR^- ILC3s induced by the integration of signals from $CD4^+$ T cells regulates ICOSL expression and thereby NCR^-ILC3 immune function. However, the precise role for ICOS-ICOSL interactions of NCR^-ILC3s with $CD4^+$ T cells *in vivo* remains to be explored.

The data regarding the positive feedback loop from $CD4^+$ T cells to NCR^-ILC3s were based on *in vitro* experiments transferring the SN of co-cultures from BMDCs and $CD4^+$ T cells with Ag to naïve NCR^-ILC3s. It could not be excluded that in addition to $CD4^+$ T cells, BMDCs produced soluble factors upon Ag-dependent interaction with $CD4^+$ T cells leading to NCR^-ILC3 activation. Therefore, the SN of $CD4^+$ T cells, which are activated by anti-CD3/anti-CD28 Abs should be used to confirm these findings. Secondly, it is possible that NCR^-ILC3s secrete cytokines itself in response to Ag-dependent interaction with $CD4^+$ T cells, which might further affect NCR^-ILC3 activation in an autocrine manner. Finally, one has to consider that the *in vitro* findings of Ag-dependent NCR^-ILC3-T cell crosstalk might not fully reflect the *in vivo* situation.

The disruption of Ag-dependent interaction of NCR^-ILC3s and $CD4^+$ T cells impairs $CD4^+$ T cell and T-dependent B cell responses *in vivo*.

By using *I-ab$^{\Delta ILC3}$* mice, I studied the role of MHC II-mediated Ag presentation by ILC3s in T cell responses *in vivo*. In contrast to a previous study in which the *I-ab$^{\Delta ILC3}$* mouse strain has been reported to spontaneously develop signs of systemic inflammation,[132] our *I-ab$^{\Delta ILC3}$* mouse colony kept under strict specific pathogen free conditions did not show any pathology or abnormal lymphocyte numbers (Fig. 15 A - D). In the study mentioned above, *I-ab$^{\Delta ILC3}$* mice developed intestinal inflammation at 12 weeks of age, which could be attenuated by antibiotic treatment demonstrating the dependence on intestinal pathogens.[132] Thus, the discrepancy between our mice and the mice reported by Hepworth *et al*., might be due to exposure to local intestinal pathogens in different animal facilities. This assumption was in line with another study, reporting as well the absence of intestinal inflammation in the same mouse strain up to the age of 30 weeks.[319] In contrast to the finding of Hepworth *et al.* that T

cells in *I-ab*$^{\Delta ILC3}$ mice preferentially possess an activated memory-like phenotype,[132] I could show that our *I-ab*$^{\Delta ILC3}$ mice had normal numbers of CD4$^+$ and CD8$^+$ memory T cells and only a slight reduction in the naïve T cell compartment (Fig. 15 D) suggesting that these mice were overall comparable to naïve *WT* mice. Using these mice, I could demonstrate that the specific lack of MHC II on ILC3s drastically impaired CD4$^+$ T cell responses in the spleen. The proliferation of adoptively transferred CD4$^+$ T cells upon immunization was significantly reduced in *I-ab*$^{\Delta ILC3}$ mice when compared to *WT* mice demonstrating the crucial role of MHC II-mediated Ag presentation of ILC3s *in vivo* (Fig. 17 B). This substantial reduction in CD4$^+$ T cell proliferation in *I-ab*$^{\Delta ILC3}$ mice was not expected, as these mice showed normal numbers of DCs with high expression of MHC II (Fig. 15 B and E). Moreover, my *in vitro* data demonstrated that BMDCs were more efficient in the induction of CD4$^+$ T cell activation than NCR$^-$ILC3s *in vitro* (Fig. 12 B). Despite this, the *in vivo* data suggested that the ability of NCR$^-$ILC3s to present Ag might be increased in the splenic microenvironment. It is possible that the splenic cytokine milieu or the positioning of ILC3s within the spleen increases their efficiency in CD4$^+$ T cell activation. In line with this, NCR$^-$ILC3s were previously reported to reside at the interface between B and T cell zones and it was shown that they were placed in the spleen around blood vessels and in LNs around high endothelial venules (HEVs), providing the possibility to directly capture Ag or to interact with incoming lymphocytes.[94, 98, 102] Thus, NCR$^-$ILC3s were located strategically within the lymphoid microenvironment to efficiently promote immune responses *in vivo*. Another possibility would be that splenic NCR$^-$ILC3s are able to crosstalk with other APCs within the splenic compartment. They might for example provide cytokine- or cell-cell contact-dependent signals to DCs thereby enhancing DC immune function. This might explain the discrepancy between the data observed *in vitro* and *in vivo*. My data showed that MHC II-mediated Ag presentation by peripheral ILC3s was crucial for the induction of CD4$^+$ T cell activation and proliferation within transient T cell responses (2 days). The expected peak of T cell proliferation is around day 8 upon immunization.[277] Therefore, one should additionally examine the contribution of peripheral ILC3s to persisting CD4$^+$ T cell responses (day 8) in comparison to DCs.

I also investigated the role of peripheral ILC3s in the induction of TD B cell responses *in vivo*. I could show that immunization of *I-ab*$^{\Delta ILC3}$ mice with a TD Ag (NP-OVA in Alum) after adoptive transfer of Ag-specific CD4$^+$ T cells resulted in drastically impaired TD B cell responses compared to *WT* mice. NP-OVA-specific IgG levels were significantly reduced in *I-ab*$^{\Delta ILC3}$ mice (Fig. 19 E and G). The overall decrease in NP-OVA-specific Ab isotypes suggested that cognate ILC3-T cell interactions did not

significantly affect T helper cell polarization. *RORγ*$^{-/-}$ mice, completely lacking ILC3s, Th17 cells, LNs and PPs,[25, 70, 231] showed even more reduced NP-OVA-specific IgG levels (Fig. 19 E and G). This might either be due to the absence of LNs, normally providing a site for germinal center formation required for isotype class switch, or due to the absence of NCR$^-$ILC3s, which were reported to promote the survival of memory T cells within the spleen.[309] T-independent (TI) Ags, which do not require the help of primed T cells, stimulate marginal zone B (MZB) cells to produce IgM and IgG3 class Abs.[320, 321] In line with increased numbers of MZB cells, *RORγ*$^{-/-}$ mice showed the highest levels of total IgM and IgG3 compared to *WT* and *I-ab*$^{\Delta ILC3}$ mice measured before immunization (Fig. 19 B and C). In contrast, another study suggested that elevated levels of IgM observed in *RORγ*$^{-/-}$ mice were caused by an increase in B1 B cells migrating from the peritoneal cavity to the spleen due to the lack of gut-associated lymphoid tissues,[127] a fact, which cannot be exclude as I did not examine B1 B cell numbers. The role of ILC3s in TI immune responses is not fully understood, as splenic ILC3s could also be shown to enhance Ab production of MZB cells through GMCSF secretion.[127] Further, IgA production in the gut was reported to indirectly depend on ILC3s.[100, 129]

Collectively, my *in vivo* data demonstrated that peripheral NCR$^-$ILC3s were able to support Ag-specific CD4$^+$ T cell and TD B cell responses. One has to consider that in *I-ab*$^{\Delta ILC3}$ mice MHC II expression was deleted not only on NCR$^-$ILC3s but also on ILC3s expressing the natural cytotoxicity receptor NKp46 (NCR$^+$ILC3s). However, MHC II was reported to be substantially expressed on NCR$^-$ILC3s when comparing MHC II expression on the different ILC3 subsets suggesting that NCR$^-$ILC3s were mainly affected by the deficiency of MHC II in the *I-ab*$^{\Delta ILC3}$ mouse model.[132, 319]

Small intestinal NCR$^-$ILC3s differ from splenic NCR$^-$ILC3s in regard to their phenotype, response to innate stimulation and ability to induce CD4$^+$ T cell responses.

ILC3s predominantly accumulate in gut-associated lymphoid tissues, such as the lamina propria (LP) of the small and large intestine, ILFs, PPs and mLNs.[108, 266] Having demonstrated that activated peripheral ILC3s expressed both MHC II and co-stimulatory molecules and were fully capable of inducing T cell responses *in vitro* and *in vivo*, I intended to examine the role of NCR$^-$ILC3s residing in the LP of the small intestine of adult mice in CD4$^+$ T cell responses. Small intestinal NCR$^-$ILC3s could be identified based on the expression of classical ILC3 markers (Fig. 21 A). However,

they differed in the expression of the early activation marker CD69. Whereas naïve splenic NCR$^-$ILC3s did not express CD69, its expression was high on naïve small intestinal NCR$^-$ILC3s (Fig. 21 D - E). It could be excluded that the slightly different experimental procedure used to isolate splenic and small intestinal NCR$^-$ILC3s (see 4.2.5 Cell isolation and culture) was responsible for the expression of CD69 on small intestinal NCR$^-$ILC3s (data not shown). A recent study associated the function of intestinal ILC3s to an inhibition of CD4$^+$ T cell responses.[132] The fact that small intestinal NCR$^-$ILC3s were highly positive for CD69 let me ask the question whether intestinal CD69$^+$ NCR$^-$ILC3s have a repressive immune function. None of the tested mouse strains contained CD69$^-$ NCR$^-$ILC3s in the gut. CD69 expression appeared to be independent of TLR- and IL-1R signaling as well as the presence of the microbiota or IL-23 (Fig. 21 F). Thus, these findings excluded that commensal bacteria, microbial products, or IL-1 and IL-23 secreted by intestinal DCs or MΦ at steady state or upon microbial stimulation,[126, 165, 292, 322] led to activation-induced up-regulation of CD69. Which other factors, not investigated in this thesis, were responsible for CD69 expression on small intestinal NCR$^-$ILC3s remains to be explored. Previous reports demonstrated that CD69 regulates lymphocyte egress from lymphoid organs.[323, 324] Therefore CD69 expression might retain Ag-loaded NCR$^-$ILC3s in the lamina propria and prevent T cell priming in other lymphoid organs. In addition, CD69 deficiency was previously reported to be associated with increased production of pro-inflammatory cytokines by intestinal CD4$^+$ T cells and reduced induction of T_{regs} suggesting an important role for CD69 expression in the regulation of mucosal immune responses.[325] Furthermore, a recent study demonstrated that a fraction of T_{regs} expressed CD69 at steady state enhancing their expression of suppression-associated markers (e.g. CTLA-4) and their suppressive function compared to T_{regs} lacking CD69.[326] However, the precise function of CD69 expression by small intestinal NCR$^-$ILC3s remains to be explored. Preliminary data of adoptive transfer experiments showed that NCR$^-$ILC3s were able to regulate their CD69 expression dependent on the lymphoid tissue they have colonized suggesting as well an organ-dependent regulation of CD69.

The regulatory role of ILC3s in CD4$^+$ T cell immunity in the intestine was supported by the idea that ILC3s limit T cell responses to commensal bacteria in the intestine at steady state through Ag presentation in the absence of co-stimulation.[132] However, the role of small intestinal NCR$^-$ILC3s in CD4$^+$ T cell immunity under inflammatory conditions was not known. I could show that small intestinal NCR$^-$ILC3s were fully capable to internalize latex beads (Fig. 23 A) and expressed MHC II molecules on their surface (Fig. 21 B and Fig. 22 C) suggesting that they were able to present

exogenous Ag to $CD4^+$ T cells. However, in contrast to peripheral NCR^-ILC3s, I found that small intestinal NCR^-ILC3s were unable to express co-stimulatory molecules after *in vitro* stimulation with IL-1β (Fig. 22 C). In addition, I could show that in contrast to peripheral NCR^-ILC3s, small intestinal NCR^-ILC3s were far less efficient in promoting peptide- or protein-specific $CD4^+$ T cell responses (Fig. 24 A - D). Preliminary data suggested that $CD4^+$ and $CD4^-$ NCR^-ILC3 subsets of either splenic or small intestinal NCR^-ILC3s did not differ in their ability to induce $CD4^+$ T cell activation, as it was shown for FL-derived *in vitro* generated NCR^-ILC3s (Fig. 12 B), ruling out that the relatively high ratio of $CD4^+$ NCR^-ILC3s in the spleen (Fig. 9 A) compared to the small intestine (Fig. 21 A) accounts for the higher efficiency in $CD4^+$ T cell activation. Hence, it appeared that while splenic NCR^-ILC3s were capable of priming T cell responses, mucosal NCR^-ILC3s might rather prevent T cell responses through the absence of co-stimulation. It remains to be investigated whether small intestinal NCR^-ILC3s can become efficient APCs when they were *ex vivo* cultured for several days before co-culture with Ag and T cells. Long-term *ex vivo* culture of small intestinal NCR^-ILC3s might lead to the loss of their organ-dependent phenotype, activation state and function. It has to be noted that the exposure of small intestinal NCR^-ILC3s to IL-1β indeed induced further activation of these cells. For example, small intestinal NCR^-ILC3s produced high levels of IL-22, a cytokine involved in intestinal homeostasis, in response to IL-1β (Fig. 22 D). Altogether, my findings supported the idea that IL-1β is a strong activator of ILC3s, and that the outcome of ILC3 effector functions depends on additional tissue-specific cells and cytokines. Hence, the microenvironments in which ILC3s reside might profoundly alter their function and responsiveness to stimulatory factors, as suggested by the different expression signature profiles displayed by ILC3s isolated from various tissues.[327, 328]

7 Conclusion

Innate lymphoid cells are crucial for tissue formation and remodeling, protective innate immunity and immune regulation. The data of my PhD thesis work shown here demonstrate that mouse ILC3s are able to directly sense and recognize pathogen-derived products and pro-inflammatory cytokines. Naïve ILC3s can be generated from fetal liver progenitors. Upon challenge with TLR ligands or cytokines such as IL-1β, they show an activated phenotype. Moreover, in response to innate challenge ILC3s produce different cytokines dependent on the nature of the stimuli. My findings further show that innate stimulation induces ILC3s to acquire an APC-like phenotype. Thereby, I could demonstrate that ILC3s meaningfully regulate $CD4^+$ T cell responses *in vitro* and *in vivo*. ILC3s are able to take up, process and present Ag to $CD4^+$ T cells. Results of several *in vivo* models reveal that ILC3s are able to contribute to T cell and TD B cell responses ascribing to them a novel function in adaptive immunity. Furthermore, I could show that during Ag-dependent interaction of ILC3s and $CD4^+$ T cells a positive feedback loop exists inducing further activation of ILC3s. Finally, my data point out tissue-specific immune functions of ILC3s, namely peripheral ILC3s differ from small intestinal ILC3s with respect of their phenotype, response to innate stimulation and induction of $CD4^+$ T cell immunity. Intestinal ILC3s release cytokines in response to innate activation but lack co-stimulatory molecules and are less efficient in T cell stimulation.

Altogether, my findings show that ILC3s are capable to contribute to innate and adaptive immunity by the production of cytokines and the ability to present Ag and to directly interact with $CD4^+$ T cells. Not only the response of ILC3s to innate challenge seems to be fine-tuned dependent on the stimuli, but also the tissue in which ILC3s reside appears to alter their immune function.

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9 Appendix

9.1 Abbreviations and symbols

9.1.1 Abbreviations

Ab	antibody
Ag	antigen
AhR	aryl hydrocarbon receptor
AHR	airway hyper-reactivity
AKP	alkaline phosphatase
Alum	aluminium potassium
APC	antigen presenting cell
APC	allophycocyanin
APC Cy7	allophycocyanin cyanine 7
BCR	B cell receptor
BFA	Brefeldin A
BM	bone marrow
BMDCs	bone marrow derived dendritic cells
BMMΦ	bone marrow derived macrophages
bp	base pair
BSA	bovine serum albumin
BV	brilliant violet
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CHILP	common helper innate lymphoid progenitor
CIITA	class II transactivator
CLIP	class II associated invariant chain peptide
CLP	common lymphoid progenitor
CP	cryptopatch
CpG	cytosine phosphatidyl guanine
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
CytD	cytochalasin D

DAMP	danger associated molecular pattern
DC	dendritic cell
DMEM	Dulbecco`s modified eagle minimal essential medium
DNA	deoxyribonucleic acid
dNPP	dinitrophenyl phosphate
dpc	days post coitum
DSS	dextran sodium sulfate
E	embryonic day
EDTA	ethylenediamine-tetraacetic acid (disodium salt dehydrate)
e.g.	[exempli gratia], for example
EGFP	enhanced green fluorescent protein
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
et *al.*	[et alii], and others
EYFP	enhanced yellow fluorescent protein
FACS	fluorescence-activated cell sorting
FALC	fat associated lymphoid cluster
FcγR	Fc gamma receptor chain
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	fetal liver
Flag	flagellin
Flt3	fms-like tyrosine kinase 3
FLT3L	fms-like tyrosine kinase 3 ligand
fm	fate map
FolB	follicular B cell
FoxP	forkhead box protein P3
f.p.	foot pad
FRC	fibroblastic reticular cell
Fwd	forward
GALT	gut-associcated lymphoid tissue
GATA3	gata binding protein 3
GC	germinal center
γ_c	common cytokine gamma chain
GFP	green fluorescent protein

GMCSF	granulocyte macrophage colony stimulating factor
HAT	hypoxanthine-aminopterin-thymidine
HEV	high endothelial venule
IBD	inflammatory bowel disease
IC	intracellular
ICAM-1	intracellular adhesion molecule 1
ICOS	inducible T cell co-stimulator
ICOSL	inducible T cell co-stimulator ligand
Id2	inhibitor of DNA binding 2
IFNγ	interferon gamma
IFNβ	interferon beta
Ig	immunoglobulin
Ii	invariant chain
iILC2	immature group 2 innate lymphoid cell
IL	interleukin
IL-1R1	interleukin 1 receptor type 1
IL-7R	interleukin 7 receptor
IL-23R	interleukin 23 receptor
ILC	innate lymphoid cell
ILF	isolated lymphoid follicle
IMDM	Iscove`s modified Dulbecco`s medium
Imiqui	Imiquimod
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IP-10	IFNγ-induced protein 10
IRF3	interferon regulatory transcription factor 3
i.v.	intravenous
JNK	c-Jun N-terminal kinase
LCMV	lymphocytic choriomeningitis virus
lin	lineage
LN	lymph node
LP	lamina propria
LPS	lipopolysaccharide
LTαβ	lymphotoxin αβ
LTβR	lymphotoxin β receptor
LTi	lymphoid tissue inducer

LTin	lymphoid tissue initiating
mAb	monoclonal antibody
MACS®	magnetic associated cell sorting
MadCam-1	mucosal addressin cell adhesion molecule 1
MAPK	mitogen associated protein kinase
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
MIIC	major histocompatibility complex class II compartment
MIP-1α	macrophage inflammatory protein 1 alpha
mLN	mesenteric lymph node
MRC	marginal reticular cell
Myd88	myeloid differentiation primary response gene 88
MZ	marginal zone
MZB	marginal zone B cell
MΦ	macrophage
NALT	nasal-associated lymphoid tissue
NCR	natural cytotoxicity receptor
NEAA	non-essential amino acid
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor-kappa B
NIK	nuclear factor-kappa B inducing kinase
NK	natural killer
NODR	nucleotide binding oligomerization domain-like receptor
NP	4-hydroxy-3-nitrophenyl-acetyl
OD	optical density
OVA	Ovalbumin
o/n	over night
PAMP	pathogen associated molecular pattern
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PE	phycoerythrin
PECy7	phycoerythrin cyanine 7
PEG	polyethylene glycol
PerCP	peridinin chlorophyll

pH	[pondus hydrogenii], potential hydrogen
PI	propidium iodide
Poly I:C	polyinosinic:polycytidylic acid
PP	Peyer`s patch
Prox1	prospero homeobox gene 1
PRR	pattern recognition receptor
P3C	Pam3Cys
RAG	recombination activating genes
RegIIIβ	regenerating islet-derived protein III beta
RegIIIγ	regenerating islet-derived protein III gamma
Rev	reverse
RNA	ribonucleic acid
RORα	retinoic acid related orphan receptor alpha
RORγt	retinoic acid related orphan receptor gamma
RT	room temperature
RT qPCR	real time quantitative PCR
SCF	stem cell factor
SD	standard deviation
SF	serum free
sg	staggerer
SI	small intestine
SN	supernatant
SPF	specific pathogen free
TCF-1	T cell factor 1
T_{CM}	central memory T cell
TCR	T cell receptor
TD	thymus dependent
T_{EM}	effector memory T cell
Tfh	T follicular helper
Tg	transgenic
TGF	tumor growth factor
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TI	thymus independent

TIR	Toll/Interleukin-1 receptor homology
TIRAP	TIR-domain containing adaptor protein
TLR	Toll-like receptor
$T_{naïve}$	naïve T cell
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TRAIL	TNF related apoptosis inducing ligand
TRAM	TRIF-related adaptor molecule
TRANCE	TNF related activation induced cytokine
TRANCER	TNF related activation induced cytokine receptor
T_{reg}	regulatory T cell
TRIF	TIR-domain containing adaptor inducing interferon beta
TRUC	*Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ ulcerative colitis mouse model
TSLP	thymic stromal lymphopoietin
UC	ulcerative colitis
VCAM-1	vascular cell adhesion molecule 1
WT	wild type
w/o	without
Zym	zymosan

9.1.2 Symbols

%	percent
°C	degree Celsius
#	number
cm	centimeter
g	gram
h	hour
kDA	kilo dalton
l	liter
m	meter
M	molar
mg	milligram
min	minutes
ml	milliliter
mM	millimolar

ng	nanogram
nm	nanometer
pg	picogram
rpm	rounds per minute
sec	seconds
U	Unit
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar

9.2 Publication/Manuscript submitted/in preparation

1. Activated group 3 innate lymphoid cells promote T-cell-mediated immune responses

Nicole von Burg, Stéphane Chappaz, Anne Baerenwaldt, Edit Horvath, Somdeb Bose Dasgupta, Devika Ashok, Jean Pieters, Fabienne Tacchini-Cottier, Antonius Rolink, Hans Acha-Orbea, and Daniela Finke

***Proc Natl Acad Sci U S A*. 2014, 111: 12835-12840**

2. FLT3L regulates the development of innate lymphoid cells in the fetal and adult mice

Anne Baerenwaldt, Matthias Kreuzaler, **Nicole von Burg**, Edit Horvath, Annick Peter, David Voehringer, Antonius Rolink, and Daniela Finke
(submitted)

3. Maintenance of immune homeostasis through ILC/T cell interactions

Nicole von Burg and Daniela Finke
(peer reviewed Frontiers in Immunology review article, in preparation)

von Burg N *et al*., 2014

Activated group 3 innate lymphoid cells promote T-cell-mediated immune responses

Nicole von Burg, Stéphane Chappaz, Anne Baerenwaldt, Edit Horvath, Somdeb Bose Dasgupta, Devika Ashok, Jean Pieters, Fabienne Tacchini-Cottier, Antonius Rolink, Hans Acha-Orbea, and Daniela Finke

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PNAS

Activated group 3 innate lymphoid cells promote T-cell–mediated immune responses

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Group 3 innate lymphoid cells (ILC3s) have emerged as important cellular players in tissue repair and innate immunity. Whether these cells meaningfully regulate adaptive immune responses upon activation has yet to be explored. Here we show that upon IL-1β stimulation, peripheral ILC3s become activated, secrete cytokines, up-regulate surface MHC class II molecules, and express costimulatory molecules. ILC3s can take up latex beads, process protein antigen, and consequently prime CD4$^+$ T-cell responses in vitro. The cognate interaction of ILC3s and CD4$^+$ T cells leads to T-cell proliferation both in vitro and in vivo, whereas its disruption impairs specific T-cell and T-dependent B-cell responses in vivo. In addition, the ILC3–CD4$^+$ T-cell interaction is bidirectional and leads to the activation of ILC3s. Taken together, our data reveal a novel activation-dependent function of peripheral ILC3s in eliciting cognate CD4$^+$ T-cell immune responses.

T-cell activation | antigen presentation

Innate lymphoid cells (ILCs) are a group of lymphocytes that play a critical role in immediate immune host defense as well as mucosal and lymphoid tissue homeostasis. Although they lack somatically rearranged antigen (Ag) receptors, they exhibit a transcription factor and cytokine profile similar to T helper (Th) cells. Therefore, ILCs are classified into three major families (1). Reminiscent of Th1 cells, ILC1s are characterized by Interferon (IFN)γ production and developmental regulation by T-bet. ILC2s secrete interleukin (IL)-5, IL-9, and IL-13 and, analogous to Th2 cells, depend on Gata3. ILC3s produce IL-22 and IL-17A and together with Th17 cells express the retinoic acid receptor-related orphan receptor RORγt (2, 3). ILC3s can be fractioned into NKp46$^+$ and NKp46$^-$ subsets including lymphoid tissue inducer (LTi) cells. Here, lin$^-$NKp46$^-$CD4$^{+/-}$RORγt$^+$ ILCs are referred to as natural cytotoxicity receptor (NCR)$^-$ILC3s. ILCs express Toll-like receptors (TLRs) (4) and IL-1R, indicating that they can directly sense microbial products and inflammatory signals (5–7). The ability to rapidly release cytokines upon microbial challenge fostered the idea that ILCs may bias the outcome of T-cell responses. In addition, both ILC2 and ILC3s were recently shown to modulate CD4$^+$ T-cell responses through Ag-peptide presentation by MHC class II (8, 9). In line with this, it was proposed a decade ago that ILC3s interact with T cells in secondary lymphoid organs and thereby promote CD4$^+$ T-cell memory responses (10, 11). Whether peripheral ILC3s can mature into Ag-presenting cells (APCs) providing costimulation for T-cell–mediated immunity has never been explored. In the present study, we show that in mice where MHC class II is specifically deleted in ILC3s, Ag-specific T-cell and T-dependent (TD) B-cell responses are impaired, demonstrating that ILC3s present Ag to CD4$^+$ T cells in vivo. IL-1β strongly activates fetal liver (FL)-derived and splenic NCR$^-$ILC3s and induces both the expression of costimulatory molecules and the up-regulation of MHC class II molecules, thereby enhancing their T-cell priming potential. Finally, we show that in the presence of Ag, the cognate interaction between CD4$^+$ T cells and NCR$^-$ILC3s leads to the activation of the latter, suggesting an unexpected crosstalk between these two cell types. Altogether, we show that peripheral ILC3s can mature upon activation into bona fide APCs shaping T-cell–mediated immune responses.

Results

ILC3s Elicit Ag-Specific T-Cell Proliferation and TD B-Cell Responses in Vivo. To investigate whether ILC3s can initiate CD4$^+$ T-cell responses in vivo, we generated mice with a deletion of *Iab* exclusively in ILC3s by crossing mice expressing Cre recombinase under the control of the RORc promoter [*RORc(γt)-Cre^{tg}*] (12) to mice with a floxed *H2-Ab1* allele (*I-ab^{neo}*) (13). Mice homozygous for the floxed *H2-Ab1* allele and carrying one copy of the Cre transgene, here referred to as *I-ab$^{\Delta ILC3}$* mice, were healthy, did not show signs of spontaneous inflammation, and had a normal distribution of lymphocytes, macrophages (MΦ), and dendritic cells (DCs) (Fig. 1*A*). Numbers of ILC3s were also similar in *WT* and *I-ab$^{\Delta ILC3}$* mice (Fig. 1*A*). MHC class II expression was normal on splenic B cells, DCs, and MΦ, whereas NCR$^-$ILC3s completely lacked MHC class II (Fig. 1*B*). To examine Ag-specific CD4$^+$ T-cell proliferation in vivo, 3×10^6 carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4$^+$ T cells from *OT-II (H-2^b)* T-cell receptor transgenic mice (*OT-II^{tg}*) were adoptively transferred into *WT*, *I-ab$^{\Delta ILC3}$*, or *I-ab$^{-/-}$* mice. Following immunization with ovalbumin (OVA) peptide$_{323–339}$ and OVA protein plus CpG, labeled *OT-II^{tg}* T cells proliferated in *WT*, but not in *I-ab$^{-/-}$* mice (Fig. 1*C*). In *I-ab$^{\Delta ILC3}$* mice, *OT-II^{tg}*

Significance

Group 3 innate lymphoid cells (ILC3s) play decisive roles in mammalian physiology including tissue repair, lymphoid tissue development, and immune regulation. So far, the functions of ILC3s in the adult immune system have been mainly linked to their capacity to release cytokines in response to microbial or inflammatory signals. The results presented here show that activated ILC3s can alter the outcome of adaptive immune responses by directly stimulating CD4$^+$ T cells. Indeed, IL-1β–activated ILC3s express costimulatory molecules and induce cognate CD4$^+$ T-cell responses. More importantly, antigen-driven T- and B-cell responses are impaired in vivo when this cellular interaction is disrupted. Overall, our data show that peripheral ILC3s play a yet unappreciated role in T-cell–mediated immunity.

Author contributions: N.v.B., S.C., and D.F. designed research; N.v.B., S.C., E.H., D.A., H.A.-O., and D.F. performed research; A.B., S.B.D., J.P., F.T.-C., A.R., and H.A.-O. contributed new reagents/analytic tools; A.R. performed cell sorting; N.v.B. and D.F. analyzed data; and N.v.B., S.C., and D.F. wrote the paper.

The authors declare no conflict of interest.

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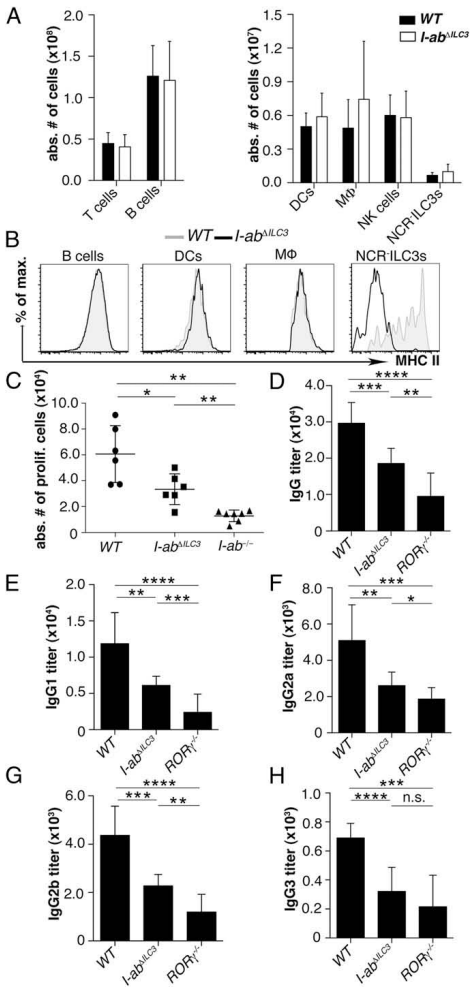

Fig. 1. ILC3s elicit Ag-specific T-cell proliferation and TD B-cell responses in vivo. (*A*) Absolute numbers of T cells ($CD3^+$), B cells ($CD19^+$), DCs ($CD11c^+$), MΦ ($CD11b^+$ $F4/80^+$), natural killer (NK) cells ($NK1.1^+NKp46^+$), and NCR^-ILC3s ($lin^-ROR\gamma t^+$ $CD117^+NCR^-$) in the spleen of *WT* and *I-ab*$^{\Delta ILC3}$ mice. Data are shown as mean ± SD ($n = 9$; three independent experiments). (*B*) Representative histograms of MHC class II expression on splenic B cells, DCs, MΦ, and $CD4^+NCR^-$ILC3s of *WT* and *I-ab*$^{\Delta ILC3}$ mice (three independent experiments). (*C*) Sort-purified CFSE-labeled *OT-II*^{tg} $CD4^+$ T cells were injected i.v. into *WT* (filled circle), *I-ab*$^{\Delta ILC3}$ (filled square), and *I-ab*$^{-/-}$ (filled triangle) mice immunized with OVA peptide, OVA protein, and CpG. Absolute numbers of proliferating *OT-II*^{tg} $CD4^+$ T cells recovered from the spleen 2 d later (mean values ± SD; four independent experiments; $n = 6$–7; $^*P \leq 0.05$; $^{**}P \leq 0.01$). (*D–H*) *WT*, *I-ab*$^{\Delta ILC3}$, and *ROR*$\gamma^{-/-}$ mice were i.p. immunized with 100 μg alum-precipitated NP-OVA after i.v. injection of *OT-II*^{tg} $CD4^+$ T cells plus CpG. NP-OVA–specific IgG (*D*), IgG1 (*E*), IgG2a (*F*), IgG2b (*G*), and IgG3 (*H*) levels 14 d after immunization. Data are presented as mean values ± SD ($n = 9$–10) from three independent experiments (n.s., not significant; $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$).

T-cell proliferation was significantly reduced, demonstrating that ILC3s were able to present Ag and to meaningfully alter OVA-specific T-cell responses in vivo. To study the role of ILC3s in TD B-cell responses, we immunized *WT*, *I-ab*$^{\Delta ILC3}$, and *ROR*$\gamma^{-/-}$ mice intraperitoneally (i.p.) with a single dose of Alum-precipitated nitrophenylated-OVA (100 μg) and adoptively transferred 2×10^6 *OT-II*^{tg} $CD4^+$ T cells plus CpG. The loss of MHC class II on ILC3s (*I-ab*$^{\Delta ILC3}$) resulted in a significant reduction of 4-hydroxy-3-nitrophenyl-acetyl (NP)-OVA–specific IgG 14 d after immunization (Fig. 1*D*). Additionally, a more detailed analysis of IgG subtypes revealed that specific IgG1, IgG2a, IgG2b, and IgG3 levels were significantly reduced (Fig. 1 *E–H*). In *ROR*$\gamma^{-/-}$ mice, where ILC3s, Th17 cells, lymph nodes, and Peyer's patches were completely absent, NP-OVA–specific IgG titers were even more reduced. Collectively, these data unambiguously show that Ag presentation by ILC3s contributes to T-cell priming in vivo and that $CD4^+$ T-cell proliferation and TD B-cell responses were impaired when Ag presentation was abolished in ILC3s.

NCR^-ILC3s Can Internalize Latex Beads. Based on the in vivo finding that the lack of MHC class II molecules by ILC3s impaired T-cell–mediated immune responses, we tested the capacity of NCR^-ILC3s generated from $\alpha_4\beta_7^+$ FL progenitor cells in vitro (14) or ex vivo isolated from the spleen of adult mice to take up red fluorescent latex beads. We and others have previously shown that FL-derived and adult ILC3s share phenotypic and functional properties such as lymphotoxin β-dependent induction of lymphoid tissue formation (15, 16). Both in vitro-generated and ex vivo-isolated NCR^-ILC3s were capable of internalizing red fluorescent latex beads, although with slower kinetics than bone marrow (BM)-derived MΦ (BMMΦ) (Fig. 2 *A* and *B* and Fig. S1). Bead uptake was severely inhibited at 4 °C or in the presence of 0.5 μM Cytochalasin D (CytD), an inhibitor of actin polymerization, showing the specificity of internalization (Fig. 2*C*). In vitro-generated NCR^-ILC3s could be subdivided into $CD4^+$ and $CD4^-NCR^-$ILC3 subsets (Fig. S2 *A* and *B*). Both subsets had a $CD69^-$ naïve phenotype and expressed comparable levels of RORγt, LTαβ, common gamma chain (γc), CD117, integrins, and chemokine receptors (Fig. S2*C*). $CD4^+$ NCR^-ILC3s were slightly more efficient in taking up Ag than their $CD4^-$ counterpart (Fig. S2*D*). Thus, NCR^-ILC3s can internalize and process exogenous Ags.

Upon Proinflammatory Stimulation NCR^-ILC3s Become Activated and Secrete Cytokines. How DCs undergo maturation and activation upon exposure to signals associated with infection and inflammation is well documented (17). We therefore tested proinflammatory cytokines and TLR ligands for their ability to activate NCR^-ILC3s. IL-1β, Poly I:C, and CpG up-regulated the expression of surface CD69 on NCR^-ILC3 (Fig. 3*A*). Similar results were obtained from ex vivo-isolated splenic NCR^-ILC3s of *WT* mice 6 h after i.p. injection with CpG (Fig. S3). Upon stimulation with IL-1β, in vitro-generated NCR^-ILC3s produced high levels of IL-22, exceeding those induced upon IL-23 stimulation (Fig. 3*B*). In vitro stimulation with TLR ligands revealed that Poly I:C could induce IL-22 production by NCR^-ILC3s (Fig. 3*C*). Thus, these data show that ligands for TLR 3 and 9 have the ability to directly activate NCR^-ILC3s and that IL-1β is a remarkably strong inducer of IL-22 secretion. In addition, IL-1β–exposed NCR^-ILC3s secreted IL-2, IL-6, macrophage inflammatory protein 1 (MIP-1)α, IFNγ, and tumor necrosis factor (TNF)α, all known for their capacity to promote T-cell responses (Fig. 3*D*). IL-1β also induced the secretion of IFN-induced protein of 10 kDa (IP-10), a chemoattractant for mononuclear cells and $CXCR3^+$ effector T cells (18). Collectively, these data demonstrate that IL-1β and TLR ligands can activate NCR^-ILC3s, remarkably altering the repertoire of cytokines they produce.

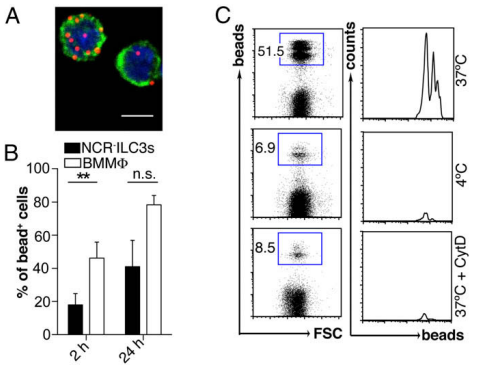

Fig. 2. Naïve NCR⁻ILC3s can internalize latex beads. (*A*) Representative immunofluorescence image of red fluorescent latex bead uptake by sort-purified in vitro-generated NCR⁻ILC3s. (Scale bar, 5 μm.) (*B*) Bead internalization by NCR⁻ILC3s and BMMΦ. Percentage of bead⁺ cells 2 and 24 h after addition of beads (mean values ± SD; n.s., not significant; $^{**}P \leq 0.01$). (*C*) Representative plots of NCR⁻ILC3s cultured with beads for 6 h at either 37 °C or 4 °C, or at 37 °C in the presence of 0.5 μM CytD. Data are representative of at least three independent experiments ($n = 3$–5).

IL-1β Induces the Expression of MHC Class II and Costimulatory Molecules on Peripheral NCR⁻ILC3s. We further asked whether, analogous to DCs, activated NCR⁻ILC3s express costimulatory molecules and up-regulate MHC class II molecules. Both in vitro-generated and ex vivo-isolated CD4⁺NCR⁻ILC3s had a naïve phenotype shown by the absence of CD69 and costimulatory molecules (Fig. 4 *A* and *B*). Upon stimulation with IL-1β for 48 h, both sort-purified in vitro-generated CD4⁺ NCR⁻ILC3s and ex vivo-isolated CD4⁺NCR⁻ILC3s expressed the costimulatory molecules CD80 and CD86 and up-regulated the expression of CD40, CD69, and MHC class II (Fig. 4 *A* and *B*). Hence, upon IL-1β stimulation, NCR⁻ILC3s acquire an APC-like phenotype, reminiscent of activated DCs. It has been reported that mucosa-associated ILC3s were unable to express costimulatory molecules (9). Whether these cells could respond to inflammatory stimulation has never been investigated. Therefore, we stimulated ex vivo-isolated small intestinal lamina propria (LP)-derived NCR⁻ILC3s with IL-1β for 48 h. Interestingly, although IL-1β stimulation resulted in blast formation, even with high concentrations of IL-1β, we were unable to detect up-regulation of MHC class II or expression of CD40 and CD86 (Fig. S4 *A* and *B*). These data unambiguously show that peripheral but not LP NCR⁻ILC3s express costimulatory molecules upon innate activation.

Activated NCR⁻ILC3s Can Induce Ag-Specific CD4⁺ T-Cell Activation and Proliferation. We next examined whether peripheral NCR⁻ILC3s could trigger naïve T-cell priming in vitro. NCR⁻ILC3s (*H-2*^{b}) were in vitro stimulated with IL-1β and incubated with CFSE-labeled *OT-II*^{tg} CD4⁺ T cells in the presence of OVA peptide$_{323–339}$ or OVA protein. Ex vivo-isolated and in vitro-generated CD4⁺ and CD4⁻ NCR⁻ILC3s were able to activate the majority of CD4⁺ T cells in the presence of OVA peptide monitored by CD69 expression (Fig. 5*A*). In vitro-generated CD4⁺ and ex vivo-isolated NCR⁻ILC3s were able to induce several rounds of OVA-specific CD4⁺ T-cell proliferation when pulsed with OVA peptide or, to a lesser extent, with OVA protein. CD4⁻NCR⁻ILC3s were considerably less efficient in inducing protein Ag-specific CD4⁺ T-cell responses (Fig. 5*A*). To further examine the effect of activation of NCR⁻ILC3s on the capacity to elicit T-cell responses, we stimulated ex vivo-isolated splenic NCR⁻ILC3s with IL-1β or left them untreated and cocultured them with *OT-II*^{tg} CD4⁺ T cells and OVA protein (Fig. 5 *B*–*D*). A total of 30.2% of the T cells in culture with untreated NCR⁻ILC3s and OVA protein expressed CD69, and only 4.3% of the T cells proliferated (Fig. 5*B*). IL-1β–activated NCR⁻ILC3s increased the percentage of both CD69⁺ and proliferating T cells (Fig. 5 *B*–*D*). Compared with splenic NCR⁻ILC3s, LP NCR⁻ILC3s were three times less efficient to induce cognate T-cell activation in vitro (Fig. S5 *A* and *B*). Interestingly, we noted that the CD69 surface expression level of NCR⁻ILC3s increased approximately sevenfold in the presence of *OT-II*^{tg} CD4⁺ T cells and OVA protein, compared with cocultures without Ag and without previous IL-1β stimulation. This phenomenon was not further increased by previously activating NCR⁻ILC3s with IL-1β (Fig. S6*A*). It is important to note that CD69 expression on NCR⁻ILC3s peaked early after activation and decreased in cocultures, unless T cells and Ag were added. Ag alone was not able to sustain CD69 expression (Fig. S6*B*). Together, these results show that IL-1β increased the capacity of NCR⁻ILC3s to induce CD4⁺ T-cell activation and that the cognate ILC3–CD4⁺ T-cell interaction led to the activation of NCR⁻ILC3s.

Discussion

We show here that upon IL-1β stimulation NCR⁻ILC3s expressed MHC class II and costimulatory molecules and became bona fide APCs as they were able to promote OVA-specific CD4⁺ T-cell proliferation in mice. In addition, activated NCR⁻ILC3s expressed an unexpected repertoire of cytokines known to alter T-cell responses. Ag-specific T-cell proliferation and IgG-mediated humoral immunity were impaired in animals in which Ag presentation was abolished exclusively in ILC3s. Finally, in the presence of Ag, the T-cell priming led to an extended activation of ILC3s. These novel data suggest that upon

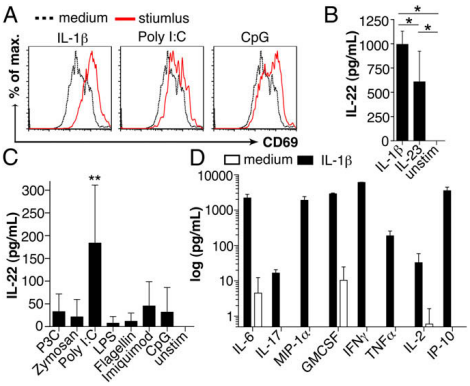

Fig. 3. Cytokine secretion of activated NCR⁻ILC3s. (*A*) Representative histograms of CD69 expression on in vitro-generated NCR⁻ILC3s after 48 h stimulation with IL-1β, Poly I:C, CpG, or in medium alone (four independent experiments). IL-22 secretion by in vitro-generated NCR⁻ILC3s upon 48 h exposure to IL-1β, IL-23 (*B*), TLR ligands (*C*), or medium alone (mean values ± SD, $n = 6$–7, three independent experiments; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$). (*D*) Cytokine production by in vitro-generated NCR⁻ILC3s after 48 h culture with IL-1β or medium alone. Results are shown as mean values ± SD.

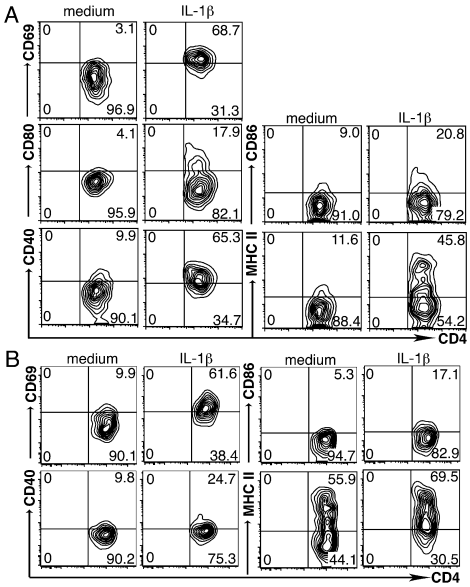

Fig. 4. IL-1β induces the expression of MHC class II and costimulatory molecules on peripheral NCR$^-$ILC3s. Expression of CD69, CD40, CD80, CD86, and MHC class II on sort-purified in vitro-generated CD4$^+$NCR$^-$ILC3s (*A*) and ex vivo-isolated splenic CD4$^+$NCR$^-$ILC3s (*B*) cultured for 48 h with IL-1β or medium alone. Numbers in contour plots show the percentage of cells in each quadrant. Data are representative of three independent experiments.

inflammation the cognate interaction of NCR$^-$ILC3s and CD4$^+$ T cells contributes to adaptive immunity.

Our in vivo data demonstrate that peripheral NCR$^-$ILC3s process protein Ags and stimulate Ag-specific CD4$^+$ T-cell responses. Whether the cognate interaction between NCR$^-$ILC3s and CD4$^+$ T cell leads directly to the priming of T cells or whether this interaction rather polarizes or enhances the T-cell response has yet to be established. In either case, the cytokines that are secreted by NCR$^-$ILC3s upon activation are likely to decisively impact the outcome of T-cell responses. Because NCR$^-$ILC3s reside at the interface between B- and T-cell zones (10, 19), they are located strategically within the lymphoid microenvironment to efficiently promote immune responses in vivo. Considering their 10 times lower numbers compared with DCs in the spleen of *WT* mice, our in vivo data emphasize the potential of ILC3s to induce CD4$^+$ T-cell proliferation. The immunization of *I-ab*$^{\Delta ILC3}$ mice indeed showed that the specific lack of MHC class II on NCR$^-$ILC3s drastically impaired T and TD B-cell responses in the spleen. The overall decrease in Ag-specific Ab isotypes suggests that cognate ILC3–T cell interactions did not significantly affect Th cell polarization. In vitro, NCR$^-$ILC3s were less potent than BM-derived DCs in priming naïve T cells, probably because DCs expressed higher levels of costimulation and MHC class II molecules. Despite this, our data show that ILC3s are crucial for mounting adaptive immune responses in vivo, suggesting that their ability to present Ag is increased in the splenic microenvironment. This could be either due to the splenic cytokine milieu or the crosstalk between ILC3s and other immune cells. In line with the finding that CD4$^+$NCR$^-$ILC3s are more differentiated than their CD4$^-$ counterpart (20), CD4$^+$ NCR$^-$ILC3s had a greater potential of inducing Ag protein-specific T-cell proliferation.

The *I-ab*$^{\Delta ILC3}$ mouse strain generated by others has been reported to spontaneously develop signs of systemic inflammation (9). In our *I-ab*$^{\Delta ILC3}$ mouse colony that we kept under strict specific pathogen-free conditions, we did not observe any pathology or abnormal lymphocyte numbers. The discrepancy between our mice and the mice reported by Hepworth et al. might be due to microbial exposure in different animal facilities.

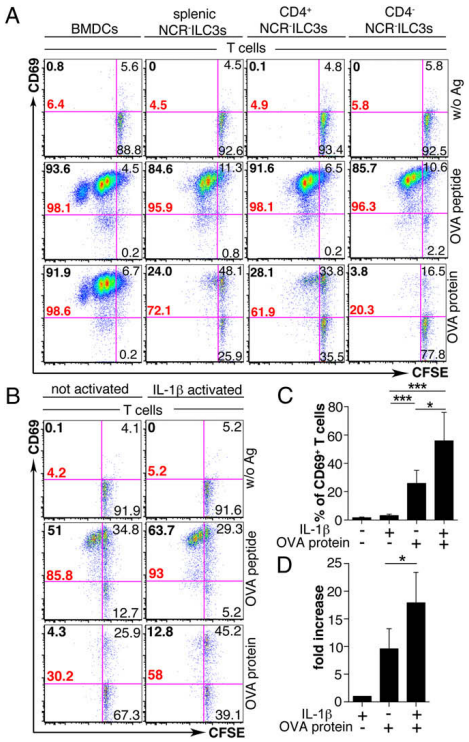

Fig. 5. NCR$^-$ILC3s can induce Ag-specific CD4$^+$ T-cell activation and proliferation. (*A*) Naïve CFSE-labeled *OT-II*^{tg} CD4$^+$ T cells were cultured with either BMDCs, IL-1β–activated ex vivo-isolated splenic NCR$^-$ILC3s, or in vitro-generated sorted CD4$^+$ or CD4$^-$NCR$^-$ ILC3s in the presence of OVA peptide, OVA protein, or medium alone (without Ag). Representative plots of CD69 and CFSE expression by CD4$^+$ T cells 72 h later. Bold black numbers, percentage of proliferating T cells; bold red numbers, percentage of total CD69$^+$ T cells. (*B*) Naïve CFSE-labeled *OT-II*^{tg} CD4$^+$ T cells were cultured with non- or IL-1β–activated splenic NCR$^-$ILC3s in the presence of OVA peptide, OVA protein, or medium alone (without Ag) for 72 h. (*C*) Percentage of CD69$^+$ CD4$^+$ T cells upon coculture with non- or IL-1β–activated splenic NCR$^-$ILC3s in the presence or absence of Ag. Data are shown as mean values ± SD ($n = 3$–7; $^*P \leq 0.05$; $^{***}P \leq 0.001$). (*D*) Fold increase of percentage of CD69$^+$ CD4$^+$ T cells upon coculture with non- or IL-1β–activated splenic NCR$^-$ILC3s in the presence of OVA protein relative to coculture of IL-1β–activated NCR$^-$ILC3s and T cells in the absence of Ag. Data are shown as mean values ± SD ($n = 3$–7; $^*P \leq 0.05$). Data are representative of at least three independent experiments.

Whereas ILC3s appear to limit T-cell responses to commensal bacteria in the intestine through Ag presentation in the absence of costimulation (9), we show here that splenic NCR$^-$ILC3s have the ability to promote T- and B-cell responses in the periphery. Interestingly, we were unable to detect costimulatory molecules on LP NCR$^-$ILC3s ex vivo or after in vitro stimulation with IL-1β. Hence, it appears that the microenvironments in which ILC3s reside profoundly alter their function, as suggested by the different expression signature profiles displayed by ILC3s isolated from various tissues (21, 22). We observed that ex vivo-isolated splenic ILC3s from naïve mice lacked costimulatory molecules but a substantial fraction expressed MHC class II. Activated NCR$^-$ILC3s, however, expressed both MHC class II and costimulatory molecules and were fully capable of inducing T-cell responses in vitro and in vivo. In the mucosa, the microbiota-driven release of IL-1β promotes the production of granulocyte macrophage colony-stimulating factor (GM-CSF) by ILC3s. This triggers the release of retinoic acid and IL-10 by mucosal DCs and MΦ, leading to the expansion of regulatory T cells (23). These data together with our findings support the idea that IL-1β is a strong activator of ILC3s and that the outcome of ILC3 effector functions depends on additional tissue-specific cells and regulatory cytokines. Hence, splenic ILC3s are fully capable of priming T-cell responses, whereas mucosal ILC3s may prevent T-cell responses through absence of costimulation and induction of tolerogenic cytokine responses by other cells. Because of their remarkable ability to secrete cytokines, ILCs are emerging as important cellular players, actively modulating immune responses. For instance, human splenic ILC3s enhance the survival of marginal zone B cells through the release of B-cell activating factor (BAFF) and stimulate perifollicular neutrophils through GM-CSF (24). In humans, IL-1R signaling was shown to induce NCR$^-$ILC3s to produce IL-17, IL-22, and IFNγ (25, 26). We show here that IL-1β increased the secretion of IL-2, IL-6, MIP-1α, IP-10, IFNγ, TNFα, and GM-CSF in vitro. Although our in vitro data suggest that IL-1β is likely to play an important role in ILC activation and immunity, the actual molecular and cellular events leading to NCR$^-$ILC3 activation in vivo are still unknown. Ag-dependent cognate interaction between ILC3s and T cells also led to NCR$^-$ILC3 activation reflected by the upregulation of CD69 (Fig. S6*A*). This result may explain why naïve NCR$^-$ILC3 could trigger T-cell priming in the absence of previous activation by IL-1β (Fig. 5*B*). A similar TCR-dependent crosstalk occurs during ILC2–T cell interactions (8), illustrating that T cells or T-cell–derived cytokines may reciprocally act on ILC responses. Collectively, our data reveal an important function for group 3 ILCs in promoting peripheral T-cell–mediated responses and improve our understanding of how these cells may link innate and adaptive immune responses.

Materials and Methods

Mice. *C57BL/6* were purchased from Janvier (Saint Berthevin). *RORγ*$^{-/-}$ (3) and *MHCII*$^{\Delta/\Delta}$ (here referred to as *I-ab*$^{-/-}$) (27) were described elsewhere. *OT-II*^{tg} mice were kindly provided by A. Rolink, *Rag2*$^{-/-}$ mice by G. Hollaender (University of Basel, Basel, and Jesus College, Oxford), *I-ab*^{neo} (13) mice by E. Palmer (University of Basel, Basel), and *RORc(γt)-Cre*^{tg} mice (12) by A. Diefenbach (University of Freiburg, Freiburg im Breisgau, Germany). All mice were bred and maintained under specific pathogen-free conditions. *I-ab*$^{\Delta ILC3}$ mice were generated by crossing *I-ab*^{neo} mice, which contain a floxed *H2-Ab1* allele, with *RORc(γt)-Cre*^{tg} mice. F1 generations were backcrossed to *I-ab*^{neo} mice. The animal experiments received the approval of the Cantonal Veterinary Office of the city of Basel.

Antibodies. FITC, Phycoerythrin (PE), PerCP–eFluor 710, PE–Cy7, Allophycocyanin (APC), APC–Cy7, BV421 or biotin-conjugated anti-CD3 (17A2), anti-CD4 (RM4-5), anti-CD11b (M1/70), anti-CD11c (117310, N418), anti-CD19 (6D5), anti-CD29 (HMb1-1), anti-CD40 (1C10), anti-CD45R (RA3-6B2, B220), anti-CD69 (H1.2F3), anti-CD90.2 (30-H12), anti-CD117 (2B8), anti-CD196 (29-2L17), anti-CD197 (4B12), anti–Gr-1 (RB6-8C5, Ly-6G), anti-TCRβ (H57-597), anti-TCRγδ (UC7-13D5), anti-MHC II (M5/114.15.2), anti-$\alpha_4\beta_7$ (DATK32), and anti-NKp46 (29A1.4) antibodies (Abs) were purchased from BioLegend; anti-CD54 (3E2), anti-CD132 (TUGm2, γ_c chain), anti-CD184 (2B11, CXCR4), anti-CD185 (2G8, CXCR5), and anti-MHC II (25-9-17) Abs from BD Bioscience; and anti-CD3 (145-2C11), anti-CD8α (53-6.7), anti-CD11c (N418), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-F4/80 (BM8), anti-NK1.1 (PK136), NKp46 (29A1.4), and anti-RORγt (AFKJS-9, B2D) Abs from eBioscience. As secondary reagent, fluorochrome-conjugated streptavidin (BioLegend) was used.

Flow Cytometry and Cell Sorting. Flow cytometry stainings were performed using standard protocols (28). Intracellular RORγt staining was performed using a fixation/permeabilization kit (FoxP3 staining buffer kit, eBioscience). Dead cells were identified using fixable Aqua Live/Dead cell staining kit (Molecular Probes, Life Technologies) or propidium iodide solution (Sigma-Aldrich). Lymphotoxin staining was performed as previously described (29). Data acquisition was performed with a FACSCalibur or FACSCanto II (BD Bioscience), and data were analyzed using FlowJo software (Tree Star). Cell sorting was done using a FACS Aria (BD Bioscience, >98% purity).

Cell Isolation and Culture. BMDCs and BMMΦ were generated as described elsewhere (30, 31). Cells were harvested after 7 d in culture at 37 °C, 10% CO_2. NCR$^-$ILC3s were generated in vitro from $\alpha_4\beta_7^+$ precursors as described before (14) and sorted based on CD90.2, CD117, and CD4 expression. Splenic and small intestinal LP NCR$^-$ILC3s were isolated from the spleen and small intestine of adult *Rag2*$^{-/-}$ mice. The small intestine was opened longitudinally, cut into pieces, incubated in 1× PBS containing 30 mM EDTA (30 min, 4 °C), washed several times in 1× PBS, and then incubated in DMEM containing 0.025 mg/mL DNaseI (Roche) and 1 mg/mL Collagenase D (Roche) for 1 h at 37 °C. Every 15 min, the supernatant was collected, and tissue pieces were washed and reincubated with medium containing DNaseI and Collagenase D. Cell suspension was pelleted, resuspended in 5 mL 40% Percoll (GE Healthcare), underlayed with 3 mL 80% Percoll, and centrifuged at 20 °C and 630 × *g* for 30 min. Cells of the interphase were collected. Spleens were also cut into pieces, washed in 1× PBS, and digested with DNaseI and Collagenase D as described above. After digestion, spleen cells were washed and erythrolysis was performed. Ex vivo-isolated splenic or LP NCR$^-$ILC3s were sorted based on the expression of CD117 and the lack of the lineage marker CD3, CD8α, CD11c, CD19, B220, Gr-1, TCRβ, TCRγδ, NK1.1, and NKp46. CD4$^+$ T cells from the spleen and lymph nodes of *OT-II*^{tg} mice were magnetically purified by using CD4 beads (LTR4, Miltenyi Biotec) following the manufacturer's instruction. MACS-enriched CD4$^+$ T cells were sorted to reach >98% purity and labeled with 7.5 μM CFSE (Molecular Probes) in 1× PBS (8 min, room temperature).

NCR$^-$ILC3 Stimulation and Ag Presentation Assay. Sort-purified in vitro-generated NCR$^-$ILC3s or ex vivo-isolated splenic or LP NCR$^-$ILC3s were cultured in a 96-well plate (Costar, Corning, Inc.) in the presence of either TLR ligands [100 ng/mL Pam3Cys, 25 μg/mL Poly I:C, 1 μg/mL Flagellin, 1 μg/mL Imiquimod (InvivoGen), 10 μg/mL Zymosan, 1 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich), 1 μM CpG (Trilink Biotechnologies, ODN1826 sequence InvivoGen)], proinflammatory cytokines [20, 100 ng/mL IL-1β (Biovision, Inc.), 20 ng/mL IL-23 (eBioscience)], or in medium alone for 48 h. To test Ag presentation and T-cell activation, sort-purified stimulated (20 ng/mL IL-1β, 24 h) NCR$^-$ILC3s were cocultured in the presence of *OT-II*^{tg} CD4$^+$ T cells and either $OVA_{323–339}$ peptide (5 μg/mL, AnaSpec), OVA protein (100 μg/mL, Imject Ovalbumin, Thermo Fisher Scientific, Inc.), or medium alone (without Ag) for 48–72 h.

Fluorescent Latex Bead Uptake. Fluorescent latex bead uptake was performed as previously described (32) with some adaptations. Briefly, in vitro-generated or ex vivo-isolated splenic NCR$^-$ILC3s were cultured in a 96-well plate, and latex beads [FluoSpheres carboxylate-modified microspheres, 1 μm, red fluorescent (580/605), Molecular Probes, Life Technologies] were added for 6 h at 37 °C, 4 °C, or 37 °C in the presence of 0.5 μM CytD (Applichem). To compare bead uptake of BMMΦ and NCR$^-$ILC3s, cells were harvested after 2 h or 24 h. Bead internalization was analyzed by flow cytometry. For immunofluorescence microscopy, in vitro-generated NCR$^-$ILC3s were stained with FITC-conjugated anti-CD90.2 (30-H12, 30 min at 4 °C) and HOECHST dye (Hoechst 33342, Invitrogen, 30 min at 37 °C) after incubation with beads. Bead uptake was monitored using a confocal laser-scanning microscope (Zeiss LSM 510 Meta). Images were analyzed with ImageJ (W. Rasband, National Institutes of Health, Bethesda). An adjustment of brightness and contrast was performed.

Adoptive Cell Transfer and Immunization. We injected 3×10^6 *OT-II*^{tg} CD4$^+$ T cells (CFSE$^+$) intravenously (i.v.) into *WT*, *I-ab*$^{\Delta ILC3}$, and *I-ab*$^{-/-}$ recipient mice i.v. immunized with $OVA_{323-339}$ peptide (20 μg/mL), OVA protein (100 μg/mL), and CpG (50 μM). Forty-eight hours later, *OT-II*^{tg} CD4$^+$ T-cell proliferation was examined in the spleen. We i.v. injected 2×10^6 *OT-II*^{tg} CD4$^+$ T cells plus CpG (25 μM) into *WT*, *I-ab*$^{\Delta ILC3}$, and *ROrγ*$^{-/-}$ mice. Mice were immunized i.p. with 100 μg alum-precipitated NP-OVA [NP (18)-OVAL, Biosearch Technologies, Inc.]. Sera were collected 14 d after NP-OVA immunization.

Ab and Cytokine Detection by ELISA and Luminex Assay. To detect NP-OVA–specific Abs in the serum of immunized mice, NUNC immunoplate Maxisorb F96 plates (Thermo Scientifics) were coated with 5 μg/mL NP-OVA (Biosearch Technologies, Inc.) in 1× PBS at 4 °C overnight. Sera were incubated for 1.5 h at room temperature, and after washing [H_2O 0.1% Tween-20 (AppliChem)] biotin-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgG3 (Caltag Laboratories) was added, incubated for 1.5 h at room temperature, and detected by alkaline-phosphatase–conjugated Steptavidin (Roche, 45 min, room temperature). Plates were developed with dinitrophenyl phosphate (1 mg/mL, Sigma) in substrate buffer [0.1 g $MgCl_2 \times 6$ H_2O (Merck), 10 mM NaN_3 (Sigma), and 10% diethanolamine (Sigma) at pH 9.8 filled up to 1 L]. The reaction was stopped with 1 M NaOH (Fluka). OD was determined at 405 nm with an ELISA reader (ASYS Expert plus). IL-22 was determined in the supernatant of NCR$^-$ILC3s cultures by using mouse/rat ELISA MAX Deluxe Set (Biolegend) according to the manufacturer's instructions. OD was determined at 450 nm with an ELISA reader (ASYS Expert Plus). In addition, cytokines were quantified using a multiplex-bead–based Luminex assay (mouse cytokine 20-plex panel, Invitrogen, Life Technologies) according to the manufacturer's protocol. Analysis was performed with a Luminex 100 (LX100) analyzer (Invitrogen, Life Technologies).

Statistical Analysis. Statistical analysis was performed using Mann–Whitney *U* test, unpaired Student *t* test, and Wilcoxon test with Prism software (GraphPad Software, Inc.).

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Supporting Information

von Burg et al. 10.1073/pnas.1406908111

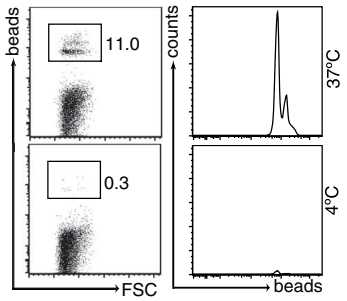

Fig. S1. Sort-purified ex vivo-isolated splenic NCR$^-$ group 3 innate lymphoid cells (ILC3s) incubated for 6 h with red fluorescent latex beads at 37 °C or 4 °C.

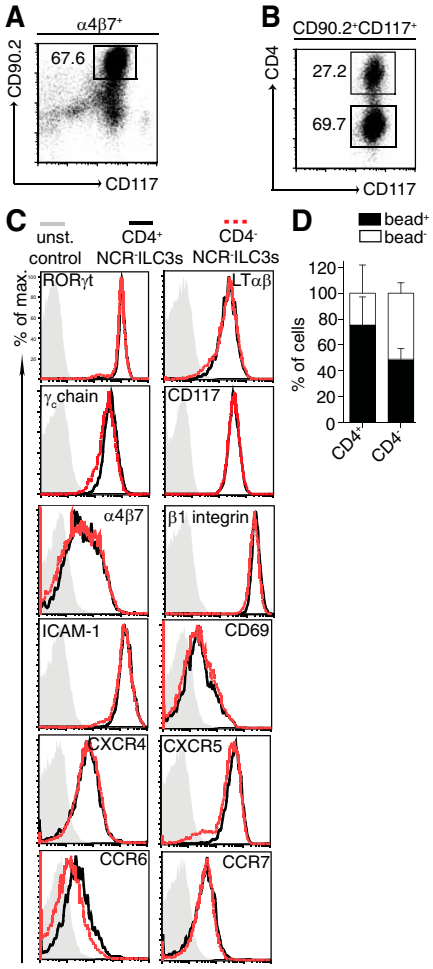

Fig. S2. (*A* and *B*) Phenotype of in vitro-generated NCR$^-$ILC3s derived from $\alpha_4\beta_7^+$ fetal liver (FL) precursors. Representative plots are shown. (*C*) Representative histograms of RORγt, LTαβ, γ_c chain, CD117, $\alpha_4\beta_7$, β1, ICAM1, CD69, CXCR4, CXCR5, CCR6, and CCR7 expression by CD4$^+$ and CD4$^-$NCR$^-$ILC3 subsets. (*D*) Percentage of bead$^+$ and bead$^-$ cells within CD4$^+$ and CD4$^-$NCR$^-$ILC3s after 6 h incubation with beads (mean values ± SD). Data shown are representative of at least three independent experiments (n = 3–5).

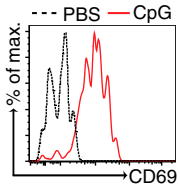

Fig. S3. CD69 expression of splenic NCR$^-$ILC3s (lin$^-$RORγt$^+$CD117$^+$CD4$^+$) of *WT* mice 6 h after i.p. injection of CpG or PBS. Representative histogram of three independent experiments is shown.

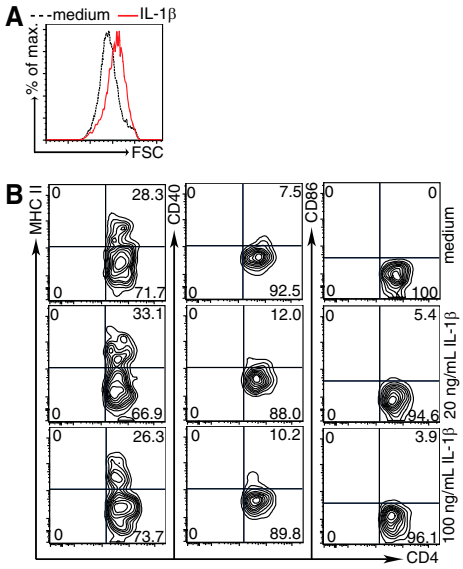

Fig. S4. (*A*) Representative histogram of forward scatter (FSC) level in lamina propria (LP) NCR$^-$ILC3s of adult *Rag2*$^{-/-}$ mice cultured for 48 h in the presence of IL-1β or in medium alone. (*B*) Expression of MHC class II, CD40, and CD86 by LP NCR$^-$ILC3s (lin$^-$RORγt$^+$CD117$^+$CD4$^+$) cultured for 48 h in the presence of IL-1β or in medium alone. Data are representative of four independent experiments.

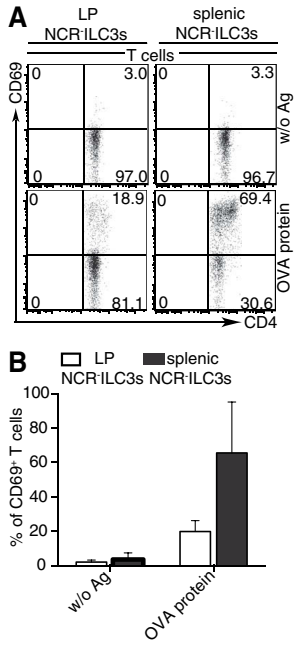

Fig. S5. (*A*) Naïve sort-purified CFSE-labeled *OT-II*^{tg} CD4$^+$ T cells were cultured with IL-1β–activated splenic or LP NCR$^+$ILC3s in the presence of ovalbumin (OVA) protein or medium alone [without antigen (Ag)]. Representative plots of CD69 expression by *OT-II*^{tg} CD4$^+$ T cells 48–72 h later. Data are representative of four independent experiments. (*B*) Percentage of CD69$^+$*OT-II*^{tg} CD4$^+$ T cells upon coculture with IL-1β–activated splenic or LP NCR$^+$ILC3s in the presence or absence of OVA protein. Data are shown as mean values ± SD (four independent experiments).

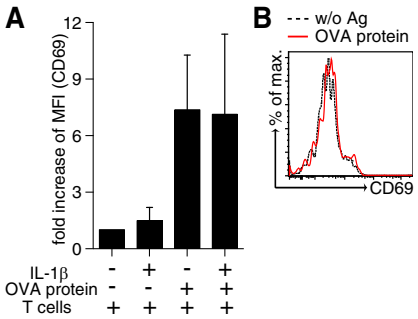

Fig. S6. (*A*) Fold increase of mean fluorescent intensity of CD69 expression by non- or IL-1β–activated (24 h) splenic NCR$^+$ILC3s cocultured with CD4$^+$ T cells in the presence or absence of OVA protein (48–72 h) compared with nonactivated NCR$^+$ILC3s cocultured with CD4$^+$ T cells in the absence of Ag. Data are shown as mean values ± SD (3–7 independent experiments). (*B*) Representative histogram of CD69 expression by nonactivated splenic NCR$^+$ILC3s in the presence of OVA protein or in medium alone.

Baerenwaldt A *et al.*

FLT3L regulates the development of innate lymphoid cells in the fetal and adult mice

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(submitted)

Flt3L regulates the development of innate lymphoid cells in fetal and adult mice

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Short title: **Flt3L promotes ILC development**

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Key points:

1. Flt3L controls intestinal ILCs in fetal and adult mice by regulating the progenitor pool in the fetal liver and bone marrow.
2. Development of ILC2s and ILC3s is independent of the presence of dendritic cells.

Abstract

Fms-like tyrosine kinase 3 ligand (Flt3L) promotes survival of lymphoid progenitors in the bone marrow (BM) and differentiation of dendritic cells (DCs), but its role in regulating innate lymphoid cells (ILCs) during fetal and adult life is not understood. By using Flt3L knockout and transgenic mice we could demonstrate that Flt3L controlled ILC numbers by regulating the pool of $\alpha4\beta7^-$ and $\alpha4\beta7^+$ lymphoid tissue inducer (LTi) cell progenitors in the fetal liver (FL) and common lymphoid progenitors in the BM. Deletion of *flt3l* severely reduced the number of FL progenitors and LTi cells in the neonatal intestine resulting in impaired development of Peyer's patches (PPs). In the adult intestine, natural killer cells and group 2 and 3 ILCs were severely reduced. This effect occurred independently of DCs as ILC numbers were normal in mice in which DCs were constitutively deleted. Finally**,** we could show that administration of recombinant Flt3L increased the number of $NKp46^-$ ILC3s in *WT* and even in $Il7^{-/-}$ mice, which generally have reduced numbers of ILCs. Taken together, Flt3L is essential for ILC and PP development by targeting lymphoid progenitor cells during fetal and adult life.

Introduction

Innate lymphoid cells (ILCs) are a family of immune cells that participate in the early response to infections at mucosal surfaces.[1,2] Beside their abundance in mucosa-associated tissues like gastrointestinal tract, skin and lung,[2-5] ILCs are found in lymphoid organs such as spleen, lymph nodes (LNs), Peyer's patches (PPs) and tonsils.[6,7] Recently, ILCs were categorized into three groups due to their transcriptional regulation of development and cytokine production.[8] Group 1 ILCs consist of natural killer (NK) cells and ILC1s. They are characterized by the expression of NK1.1 and NKp46,[2,9] require the transcription factor T-bet [10,11] and produce interferon gamma.[12,13] Group 2 ILCs (ILC2) are identified by the expression of Sca1, CD25 and CD127.[14] They depend on the transcription factors Gata3 [15] and RORα [14,16] and produce T helper cell type 2 cytokines like interleukin (IL)-5 and IL-13.[14,17] Group 3 ILCs (ILC3s) include lymphoid tissue inducer (LTi) cells that are important for the development of LNs and PPs [7,18-20] and adult ILC3s. The latter are divided into several subsets according to their expression of CD4 and NKp46.[21,22] They depend on the expression of RAR-related orphan receptor gamma (RORγt) [20] and produce IL-17 and/or IL-22.[23-25]

Cytokines play an important role during hematopoietic development, either by supporting proliferation, survival or lineage commitment.[26] Only few cytokines are described that are required for the development and maintenance of ILCs under steady state conditions. NK cell development depends on IL-15 but not on IL-7,[27] which instead is crucial for ILC3s [13,28-30] and ILC2s.[15,17] In addition, we have shown that stem cell factor (SCF) [29] and thymic stromal lymphopoietin [31] are important for LTi cell development. During fetal development, LTi cells arise from $\alpha4\beta7^+$ $CD127^+$ fetal liver (FL) progenitors.[32] In adults, ILCs develop from common lymphoid progenitors (CLPs) in the bone marrow (BM).[16,33,34] Fetal and adult ILC progenitor express receptors for cytokines that are important for ILC development, like the receptors for IL-7 (CD127) and SCF (CD117).[32,35,36] Additionally, they express the cytokine receptor fms-like tyrosine kinase 3 (flt3 or CD135).[33,37,38] Evidence for a role of flt3 ligand (Flt3L) for ILC development came from a study of Yang and colleges, who reported a diminished development of lung ILC2s after transfer of BM cells from *flt3*$^{-/-}$ mice into irradiated *WT* mice.[39] Another study, however, reported normal ILC

numbers in the small intestine (SI) of *flt3l*$^{-/-}$ mice.[40] In order to clarify the effect of Flt3L on ILC development, we analyzed *flt3l*$^{-/-}$ and *flt3l-tg* mice as well as mice treated with recombinant Flt3L. In addition, we compared fetal and adult ILC development in *flt3l*$^{-/-}$ and *Il7*$^{-/-}$ mice. We show that Flt3L controls intestinal LTi cell numbers in neonatal mice and ILC numbers in the SI of adult mice by regulating the ILC progenitor pool in FL and BM.

Materials and Methods

Mice

C57BL/6 mice were obtained from Janvier. *Rag2*$^{-/-}$*Il2rg*$^{-/-}$,[31] *Il7*$^{-/-}$,[41] *flt3l*$^{-/-}$,[42] *flt3l-tg*,[43] CD11c-Cre[44] and R-DTA[45] mice were on a *C57BL/6* background. All mice were bred and maintained under SPF conditions according to the guidelines of the cantonal veterinarian office of Basel or the animal care and use committees of Lower Franconia, Germany. LN numbers of mice were determined by injection of 1% Chicago sky Blue 6B ink (Sigma-Aldrich) subcutaneously into the footpads of mice 2 days before analysis. For Flt3L treatment mice were injected intraperitoneally (i.p.) for 10 days with 20µg recombinant Flt3L per day and sacrificed 1 day after the last treatment.

Antibodies, intracellular staining, flow cytometry and cell sorting

The following antibodies (Abs) used for flow cytometry were purchased from Biolegend: FITC-conjugated anti-CD3 (145-2C11), anti-CD8 (53-6.7), anti-CD11c (M1/70), anti-CD19 (6D5), anti-Gr-1 (RB6-8C5), anti-TCRβ (h57-597), anti-TCRγδ (UC7-13D5); Alexa-488 conjugated anti-CD103 (2E7); PE-conjugated anti-CD3 (145-2C11), anti I-A (MHC II) (M5/114.15.2); PE-Cy7-conjugated anti-CD11b (M1/70); APC-conjugated anti-CD11c (N418), anti-CD25 (PC61); anti-CD117 (2B8), anti-KLRG1 (2F1/KLRG1); Alexa-647 conjugated anti-CD45.2 (104); APC-Cy7-conjugated anti-CD4 (GK1.5) and streptavidin; Brilliant Violet 510 conjugated anti-Thy1 (53-2.1) and anti-Sca1 (D7). The following Abs used for flow cytometry were purchased from eBioscience: FITC-conjugated anti-B220 (RA3-6B2), anti-NK1.1 (PK136); PE-conjugated anti-CD135 (A2F10); PE-Cy7-conjugated anti-CD45.1 (A20), anti-CD127 (A7R34); eFluor660-conjugated anti-NKp46 (29A1.4);

biotinylated anti-Sca-1 (D7). Brilliant Violet 421-conjugated anti-CD117 (2B6) was purchased from Biolegend or BD Biosciences. PE-conjugated anti-α4β7 (DATK32) was purchased from BD Biosciences.

The lineage cocktail used for the identification of ILCs consisted of: anti-CD3, anti-CD8, anti-CD11c, anti-CD19, anti-B220, anti-NK1.1, anti-Gr-1, anti-TCRβ and anti-TCRγδ. For intracellular staining of RORγt and Gata3, the FoxP3 staining buffer kit with PE-conjugated anti-RORγt (AFKJS-9) and eFluor660-conjugated anti-Gata3 (TWAJ) (eBioscience) was used. Cells were acquired using a FACS Canto II or LSR Fortessa (BD Biosciences) and analyzed with Flow Jo software (Tree star). Cell sorting was done using a FACS Aria II.

Whole mount immunohistochemistry

Whole mount VCAM staining of small intestines (SIs) from 1-2 day old mice was performed as described before.[18] Briefly, after removing the serosa, intestines were fixed with 4% paraformaldehyde. Free aldehyde groups were quenched with 4% glycin in phosphate-buffered saline (PBS) and tissue was rehydrated using 50%, 70% and 100% methanol. Endogenous peroxidase was blocked by incubation with 30% H_2O_2 in methanol. After blocking with PBS, 1.5% skim milk, 0.1% Triton X-100 (PBSMT), intestines were incubated with biotinylated anti-VCAM Abs (eBioscience, clone 429) over night at 4ºC. After extensive washing with PBSMT, intestines were incubated with horseradish peroxidase-conjugated streptavidin (Biolegend) for 3h at RT. After washing with PBS and tris-buffered saline, 3,3'-Diaminobenzidine substrate (Sigma) was added to visualize Ab binding.

Cell isolation

BM cells were obtained by crushing bones in a mortar. Cells from the spleen, mesentery and FL were obtained by gently pressing the organs between two glass slides. Lamina propria (LP) preparation from SI of adult mice was done as described before.[21] Briefly, SIs were collected, PPs were removed, SIs were opened longitudinally, cut into 1-2cm pieces and incubated in Ca^{2+}- and Mg^{2+}- free PBS containing 30mM EDTA (Ethylenediaminetetraacetic acid) for 30 min on ice. Epithelial cells and intra-epithelial cells were removed by shaking vigorously and repeated washing of the tissue with PBS. Intestinal pieces were incubated 4 times

with DMEM containing 1mg/ml Collagenase D (Roche) and 0,025mg/ml DNAse I (Roche) for 15 min at 37ºC. After each digestion, supernatant was collected in DMEM, 5% FCS, 2mM EDTA. LP cells were purified using a Percoll (GE Healthcare) gradient at 20ºC and 1800rpm for 30min. Cells of the interphase were collected.

SI from 0.5 days old mice were collected and mesenteric tissue was removed under the microscope. Intestines were opened longitudinally and washed with PBS by vigorous shaking. E15.5 intestines were collected under the microscope and 3 intestines were pooled. For digestion of fetal and neonatal tissue, intestines were cut into small pieces and digested 2 times with DMEM containing 1mg/ml Collagenase D (Roche) and 0,025mg/ml DNAse I (Roche) for 30 min at 37ºC. Supernatant was collected in DMEM, 5% FCS, 2mM EDTA.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 for Macintosh OS X. All data were tested for normal distribution using the Shapiro-Wilk test. If data were normal distributed, differences between groups were calculated using a two-tailed unpaired Student's T-Test. If data were not normal distributed differences between groups were calculated using the Mann-Whitney-U test. Statistical significant differences are depicted as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

Flt3L controls LTi cell and Peyer's patch development

During fetal development LTi cells arise from FL progenitors.[32,35] They can be derived either from $CD117^+$ $CD127^+$ $\alpha4\beta7^-$ or from $CD117^+$ $CD127^+$ $\alpha4\beta7^+$ progenitors,[33,46] which were reported to express CD135.[33,37] In line with this, we found that the majority of both $\alpha4\beta7^-$ and $\alpha4\beta7^+$ $Thy1^-$ progenitors in the FL were $CD135^+$ (Figure 1A). We have previously shown that IL-7 is crucial for LTi cell and LN development and that in addition to IL-7 other cytokines promote intestinal LTi cell and PP development.[29,31,47] To understand the influence of Flt3L on LTi cell development we analyzed fetal and neonatal *flt3l*$^{-/-}$ mice. Absolute numbers of $\alpha4\beta7^-$

and $\alpha4\beta7^+$ progenitors were reduced in the FL of E14.5 *flt3l*$^{-/-}$ mice as compared to *WT* and *Il7*$^{-/-}$ mice (Figure 1B). PP development was significantly impaired in *flt3l*$^{-/-}$ mice (Figure 1C). Accordingly, we detected a 3-fold reduction of LTi cells in the SI of day 0.5 *flt3l*$^{-/-}$ mice (Figure 1D). It was reported that a CD11c$^+$ lymphoid cell population called lymphoid tissue initiating cell (LTin) contributes to PP development.[48] We found that the number of LTin cells (gating see Figure S1B) was reduced in the intestine of E15.5 *flt3l*$^{-/-}$ embryos (Figure 1E). In contrast to *Il7*$^{-/-}$ mice, almost all LNs were present in *flt3l*$^{-/-}$ mice (Figure 1F). Accordingly, LTi cell numbers in the mesenteries of 0.5 days old neonatal mice (see gating in Figure S1A) were slightly reduced in *Il7*$^{-/-}$ mice but normal in *flt3l*$^{-/-}$ mice (Figure 1G). Since LTi cells were negative for flt3 (Figure S2A), we conclude that Flt3L deficiency mainly affected LTi progenitors in the FL. Together, these data show that Flt3L regulates PP development by controlling the number of intestinal LTi and LTin cells.

Flt3L controls ILC3 numbers in the lamina propria of adult mice

To investigate the role of Flt3L for adult ILCs, we compared the number of NK cells, ILC2s and ILC3s (see gating in Figure S1C-E) in the SI of *WT*, *Il7*$^{-/-}$, *flt3l*$^{-/-}$ and *flt3l-tg* mice. As already reported, NK cell numbers were normal in the SI of *Il7*$^{-/-}$ mice, while ILC2s and ILC3s were reduced (Figure 2A).[13,15] In contrast, loss of Flt3L led to a significant reduction of NK cell, ILC2 and ILC3 numbers in the SI compared to *WT* controls (Figure 2A). While ILC2 reduction was comparable in *Il7*$^{-/-}$ and *flt3l*$^{-/-}$ mice, ILC3 numbers were lower in *flt3l*$^{-/-}$ mice compared to *Il7*$^{-/-}$ mice (Figure 2A). Transgenic over-expression of Flt3L under the control of the human β-actin promoter led to increased numbers of NK cells, ILC2s and ILC3s (Figure 2A). To further characterize the influence of Flt3L on ILC3s in the SI, we analyzed the different ILC3 subsets, namely CD4$^+$ ILC3s, NKp46$^+$ ILC3s and CD4$^-$ NKp46$^-$ (DN) ILC3s (gating see Figure S1E). In *flt3l*$^{-/-}$ mice, we detected a 4-fold reduction of DN and NKp46$^+$ ILC3 and a 8-fold reduction of CD4$^+$ ILC3 numbers compared to *WT* mice (Figure 2B). CD4$^+$ and DN ILC3 numbers were significantly lower as in *Il7*$^{-/-}$ mice. In *flt3l-tg* mice CD4$^+$ ILC3s were 20-fold, DN ILC3s were 16-fold and NKp46$^+$ ILC3s were 9-fold increased as compared to *WT* controls. Together, these data show that Flt3L controls the number of ILCs in the adult gut, preferentially targeting CD4$^+$ and DN ILC3s.

Administration of recombinant Flt3L increases ILC numbers in *WT* and *Il7*$^{-/-}$ mice

The results obtained from *flt3l-tg* mice prompted us to ask whether the treatment of adult mice with recombinant Flt3L could increase SI ILC numbers in *WT* mice and restore SI ILCs in *Il7*$^{-/-}$ and *flt3l*$^{-/-}$ mice. Therefore, we injected recombinant Flt3L over a period of 10 days into adult mice. As a control, we determined the number of CD11c^{+} DCs that are known to expand during Flt3L administration.[49] DCs in the SI were highly increased after Flt3L treatment in all three strains (Figure 3A). In *WT* mice, the number of NK cells and ILC2s was significantly higher in Flt3L-treated mice compared to PBS-treated controls (Figure 3B). Amongst ILC3s, the CD4^{+} and DN subsets were mainly responding to Flt3L treatment in *WT* mice (Figure 3C), confirming our previous data. In *Il7*$^{-/-}$ mice, NK cells and ILC3s expanded upon Flt3L treatment while ILC2s were not changed in numbers (Figure 3B-C). Flt3L treatment was unable to expand the number of NK cells, ILC2s and ILC3 in *flt3l*$^{-/-}$ mice (Figure 3B-C). These data show that short-term administration of Flt3L increases the number of several ILCs in the SI of *WT* and *Il7*$^{-/-}$ mice. However, ILC numbers could not be restored in *flt3l*$^{-/-}$ mice.

CD103^{+} DCs are not required for ILC development

Flt3L is an important cytokine for the development of DCs.[42,50] In the intestine, three subsets of DCs can be discriminated by their expression of CD103 and CD11b.[51] CD103^{+} DCs were described to be responsive to Flt3L.[51] Indeed, CD11c^{high} DCs, which contain mainly CD103^{+} DCs, were dramatically reduced in the SI of *flt3l*$^{-/-}$ mice, while numbers of CD11c^{low} cell including CD103^{-} DCs and monocytes were comparable to *WT* mice (Figure 4A-B). Recently, it has been reported that CX_3CR1^{+} DCs can regulate ILC3 numbers in the SI.[52] To investigate whether the effect of Flt3L on ILCs was indirectly mediated by DCs, we analyzed mice in which DCs were constitutively ablated by crossing CD11c-Cre mice with mice expressing Diphtheria toxin A (DTA) under the control of a loxP flanked stop cassette in the Rosa26 locus (R-DTA). The resulting CD11c-Cre/R-DTA (*ΔDC*) mice [53] lack more than 90% of conventional DCs including myeloid, lymphoid and plasmacytoid DCs in thymus, spleen and LNs.[53] In the SI, *ΔDC* mice showed a severe reduction of CD11c^{high} DCs with an almost complete absence of CD103^{+} DCs (Figure 4C-D). In contrast,

CD11c^{low} macrophages (MΦ) showed a moderate reduction (Figure 4C-D). Despite the notion that *ΔDC* mice and *flt3l*$^{-/-}$ mice had reduced DC numbers in the SI, *ΔDC* mice had normal numbers of PPs (Figure 4E) suggesting that CD103^{+} DCs are not required for LTi cell development. While NK cell numbers were normal in the SI of *ΔDC* mice, ILC2 numbers were increased (Figure 4F). Analysis of the different ILC3 subsets revealed that the number of NKp46^{+} ILC3s but not CD4^{+} or DN ILC3s was elevated (Figure 4G). Thus, we could show that ILCs in the SI were not dependent on CD103^{+} DCs.

Flt3L affects CLP numbers but not their differentiation into ILC3s

Since we neither found Flt3 protein nor transcript expression in ILC2s or ILC3s (Figure S2B-C), we focused on ILC progenitors in the BM. Therefore, we analyzed the number of CLPs and immature ILC2s (iILC2s) in the BM, which are progenitors for all ILCs and for ILC2s, respectively.[15,33] Previous studies have shown that CLP numbers are reduced in *flt3l*$^{-/-}$ mice.[54] In line with this, we found a 4.5-fold reduction of CLPs in the BM of *flt3l*$^{-/-}$ mice while CLP numbers were normal in the BM of *Il7*$^{-/-}$ mice as compared to *WT* controls (Figure 5A). In contrast to CLPs, iILC2s were only reduces 2-fold in *flt3l*$^{-/-}$ mice (Figure 5B), while *Il7*$^{-/-}$ mice showed a severe loss of these cells.

Although CLP numbers are reduced in *flt3l*$^{-/-}$ mice, not all CLP-derived lymphocytes were affected to the same degree in the periphery. In the spleen, NK cells were severely reduced, while B cells were only diminished 2-fold and T-cells did not differ from *WT* numbers (Figure 5C). In the SI no difference in IgM^{+} B cell and T cell numbers was detectable in *WT* and *flt3l*$^{-/-}$ mice (Figure 5D). This prompted us to ask whether the loss of Flt3L might impair the ability of CLPs to generate ILCs. Therefore, we adoptively transferred FACS sorted Lin^{-} CD117^{low} CD127^{+} Sca1^{low} CD135^{+} CLPs (gating see Figure S1F) from *WT* mice (CD45.1) and from *flt3l*$^{-/-}$ mice (CD45.2) in a 1:1 ratio into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (CD45.2). As controls, we reconstituted *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice with CD45.1 and CD45.2 *WT* CLPs and analyzed the mice 6 weeks later. The frequency of ILC3s derived from *WT* and *flt3l*$^{-/-}$ CLPs was comparable (Figure 5E-F) indicating that *flt3l*$^{-/-}$ CLPs had no intrinsic defect in ILC lineage commitment.

Discussion

Flt3L plays an essential role in survival of lymphoid progenitors in the BM [54] and differentiation of DCs.[42,50] In this study we show that Flt3L is important to control ILC numbers in the SI during fetal and adult life and the ILC progenitor pool in the FL and BM.

During fetal development, LTi cells are crucial for the development of LNs and PPs. Both organs are differently regulated by cytokines such as IL-7, SCF and tumor necrosis factor-family members like receptor activator of NFκB ligand (RANKL).[29,55-57] Our data demonstrate that in the absence of Flt3L, PP development was impaired while LN development was normal. This was reflected by normal LTi cell numbers in the mesenteries and highly reduced LTi cell numbers in the SI of neonatal mice. In addition, the reduced number of LTin cells in *flt3l*$^{-/-}$ mice probably contributed to the almost complete abrogation of PP development. Since FL progenitors expressed CD135 and were severely reduced in *flt3l*$^{-/-}$ mice, Flt3L controls the size of the fetal ILC progenitor pool. Why Flt3L is dispensable for LN anlagen remains to be investigated.

In the adult system, we could show that Flt3L controls the number of NK cells, ILC2s and ILC3s. A former study by Kinnebrew and colleges postulated that ILCs are not diminished in *flt3l*$^{-/-}$ mice.[40] In their study the frequency of CD3^{-} CD4^{-} Thy1^{+} cells amongst CD45^{+} cells was equal in *WT* and *flt3l*$^{-/-}$ mice. Since a change in the number of other cell types influences the frequency of the analyzed population, it is possible that the loss of DCs led to normal ILCs frequencies in *flt3l*$^{-/-}$ mice although total cell numbers may be reduced. In contrast, we determined the absolute number of the different ILC groups in *flt3l*$^{-/-}$ mice. We observed a significant reduction of NK cells in the SI of *flt3l*$^{-/-}$ mice. This is in line with the observation of reduced NK cell numbers in the spleen of *flt3l*$^{-/-}$ mice.[42] ILC2s were reduced in the SI of adult *flt3l*$^{-/-}$ mice, which is in agreement with previous data showing that Flt3L is important for ILC2 development.[39] CD4^{+} and DN ILC3s were mainly affected by the loss of Flt3L. This was also obvious in *flt3l-tg* mice as well as in *WT* and *Il7*$^{-/-}$ mice treated with recombinant Flt3L, in which the CD4^{+} and DN ILC3s were the main ILC3 subsets responding to Flt3L.

Administration of recombinant Flt3L was previously shown to increases the number of DCs.[49] In addition, NK cells and regulatory T cells were reported to expand after

Flt3L treatment.[58-62] The increase of these two cell types was shown to be a result of the Flt3L-driven expansion of DCs and their secretion of IL-2 and IL-15. [59-62] As the expansion of DCs could be responsible for the increase of ILC2s and ILC3s after Flt3L treatment we analyzed ΔDC mice, which mainly lack the Flt3L-responsive $CD103^+$ DCs in the SI. ILC numbers were normal or even increased in ΔDC mice, strongly arguing against the hypothesis that ILC expansion is driven by DCs. The inability of Flt3L treatment to increase ILCs in *flt3l*$^{-/-}$ mice, albeit increasing DC numbers, also supports our conclusion. It was recently shown that the number of $NKp46^+$ ILC3s depends on CX_3CR1^+ cells, which contain $CD103^-$ DCs and resident MΦ.[52] In ΔDC mice, $NKp46^+$ ILC3s were even increased, which might be explained by the relative enrichment of $CD11c^{low}$ $CD103^-$ MΦ.

As ILCs are negative for CD135, the effect of Flt3L on ILC numbers was most likely mediated by regulating the $CD135^+$ progenitor pool (CLPs) of ILCs in the BM. In agreement with this hypothesis, we found a strong reduction of CLP numbers in *flt3l*$^{-/-}$ mice, which was comparable to the reduction of ILC3s in the SI (Figure 2A and 5A). The reduction of ILC2s and iILC2s was less severe in the absence of Flt3L (Figure 2A and 5F) probably because other cytokines such as IL-7 can rescue ILC2 development. The important role of IL-7 for ILC2 development is also reflected by the finding that Flt3L treatment did not increase ILC2 numbers in *Il7*$^{-/-}$ mice.

Despite reduced CLP numbers in *flt3l*$^{-/-}$ mice, competitive transfer experiments showed that CLPs from *flt3l*$^{-/-}$ mice have the same ability as *WT* CLP to give rise to ILC3s if transferred at the same number into Flt3L-sufficient hosts. Considering the clear dependence of CLP numbers on Flt3L, the unequal number of CLPs in *WT* and in *flt3l*$^{-/-}$ mice might explain why Flt3L-treatment in *flt3l*$^{-/-}$ mice was not able to increase ILC numbers as seen in *WT* mice. Thus, it is likely that an extended treatment with recombinant Flt3L can compensate for low CLP numbers and can increase ILC numbers in *flt3l*$^{-/-}$ mice. In addition, we cannot exclude that *flt3l*$^{-/-}$ mice mounted an immune response against the recombinant protein, which led to the neutralization and subsequently lower abundance of Flt3L.

Taken together, our study demonstrates that the presence of Flt3L is important to generate ILCs during fetal development as well as during adult life. Our data offer the possibility to use Flt3L as therapeutic approach for restoring intestinal ILCs in patients with severe combined immunodeficiency and for immune protection of

mucosal surfaces. Since Flt3L treatment is already approved for treatment of cancer patients [63] beneficial effects of this cytokine for mucosal immunity could be tested in human trials.

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Author Contributions

A.B. and D.F. designed experiments and wrote the paper. A.B., M.K., N.v.B., E.H. and A.P. performed experiments. A.B. analyzed the data. D.V. provided mice. A.G.R. provided mice and experimental protocols.

Conflicts of interest

The authors have no conflicting financial interests.

Figure legends

Figure 1. Flt3L controls Peyer's patch development. (A-B) Analysis of $\alpha4\beta7^-$ and $\alpha4\beta7^+$ progenitors in the fetal liver (FL) of E14.5 embryos. (A) Surface expression of flt3 (CD135) on *WT* cells (black), grey shaded: unstained. (B) Number of progenitors (n=10-12). (C) Analysis of PP anlagen in the SI of 0.5 days old mice by VCAM-1 whole mount staining. left: Representative pictures. $VCAM^+$ spots are indicated by arrows. Right: Quantification of PP anlagen. (D) LTi cell number in the SI of 0.5 days old mice (n=14-16). (E) LTin cell numbers in the gut of E15.5 embryos (n=6). (F) Presence of lymph nodes (LN) in adult *Il7*$^{-/-}$ and *flt3l*$^{-/-}$ mice compared to *WT* set as 100% (n=6-8). (G) LTi cell number in the mesenteric region of 0.5 days old mice (n=17-28). **P* < .05; ** *P* < .01; *** *P* < .001

Figure 2. Flt3L controls ILC numbers in the intestine. (A-B) Number of NK cells, ILC2s and ILC3s (A) as well as ILC3 subsets (B) in the SI of indicated mice. DN: $CD4^-$ $NKp46^-$ (double negative) (n=6-18). Bars show the mean with SEM. **P* < .05; ** *P* < .01; *** *P* < .001

Figure 3. Flt3L treatment increases ILC numbers in the adult intestine. Mice were treated with 20µg recombinant Flt3L for 10 days (grey bars). Controls were injected with PBS (white bars). Numbers of $CD11c^{high}$ DCs (A), NK cells and ILC2s (B) as well as ILC3 subsets (C) in the SI are shown (n=5-6). Bars show the mean with SEM. **P* < .05; ** *P* < .01; *** *P* < .001

Figure 4. Loss of DCs does not reduce ILCs in the intestine. (A-D) Analysis of macrophages (MΦ) and DC subsets in the SI of adult mice. (A and C) Representative FACS plots of MΦ and DC subsets in *WT* and *flt3l*$^{-/-}$ mice (A) and ΔDC mice with littermate controls (litters) (C). (B and D) Number of $CD11c^{high}$ DCs and $CD11c^{low}$ MΦ in *WT* and *flt3l*$^{-/-}$ mice (n=6) (B) and in ΔDC mice and litters (n=4-8) (D). (E) Number of PPs in adult ΔDC mice and litters. Lines show the mean. (F-G) Number of NK cells, ILC2 (F) and ILC3 subsets (G) in the SI of ΔDC mice and litters (n=4-8). Bars show the mean with SEM. **P* < .05; ** *P* < .01

Figure 5. Flt3L affects CLP numbers but not their differentiation into ILC3s. (A) Number of CD135$^+$ CLP per 1 Mio. lineage$^-$ (Lin-) BM cells (n=6-8). (B) Number of iILC2s per 1 Mio. Lin$^-$ BM cells (n=6-8). (C) Number of NK cells, CD19$^+$ B cells and CD3$^+$ T cells in the spleen (n=6). (D) Number of CD19$^+$ IgM$^+$ B cells and CD3$^+$ T cells in the SI (n=6-12). (E-F) *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice were injected with a 1:1 mixture of Ly5.1 *WT* CLPs and Ly5.2 *Flt3l*$^{-/-}$ (n=8) or Ly5.2 *WT* CLPs (n=4). Frequency of CD45.1$^+$ and CD45.2$^+$ ILC3s (lineage$^-$ Thy1$^+$ RORγt$^+$) in the SI was analyzed 6 weeks later. Representative FACS plots (E) and pie charts for all mice (F) are shown. Bars and pie charts show the mean with SEM. **P* < .05; ** *P* < .01; *** *P* < .001

Supplementary figure legends

Figure S1. Gating strategies. Gating strategy for: (A) LTi cells from the mesentery of 0.5 days old mice. (B) LTin cells in the gut of E15.5 embryos. (C-E) NK cells (C), ILC2s (D) and ILC3s with their subsets (E) in the SI of adult mice. (F-G) CLPs (F) and iILC2s (G) in the BM.

Figure S2. CD135 expression on ILCs. (A) CD135 expression on LTi cells (CD117$^+$ CD3$^-$ Thy1$^+$ CD127$^+$) in the mesentery of 0.5 days old mice (gray). As control CD117$^+$ CD3$^-$ Thy1$^-$ cells were used (black). Light grey: unstained. CD135$^+$ cells are marked with an arrow. (B) CD135 expression on lineage$^-$ CD117$^+$ KLRG1$^+$ RORγt$^-$ ILC2s (light gray) and lineage$^-$ CD117$^+$ KLRG1$^-$ RORγt$^+$ ILC3s (dark gray) from the SI of *WT* mice in comparison to lineage$^+$ CD117$^+$ DCs (black). (C) qRT-PCR of flt3 transcripts in FACS sorted ILC3s from the SI of *WT* mice compared to *WT* splenocytes. Bars show characteristic data from 1 of 2 independent experiments.

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Figure 1.: Flt3L controls Peyer's patch development.

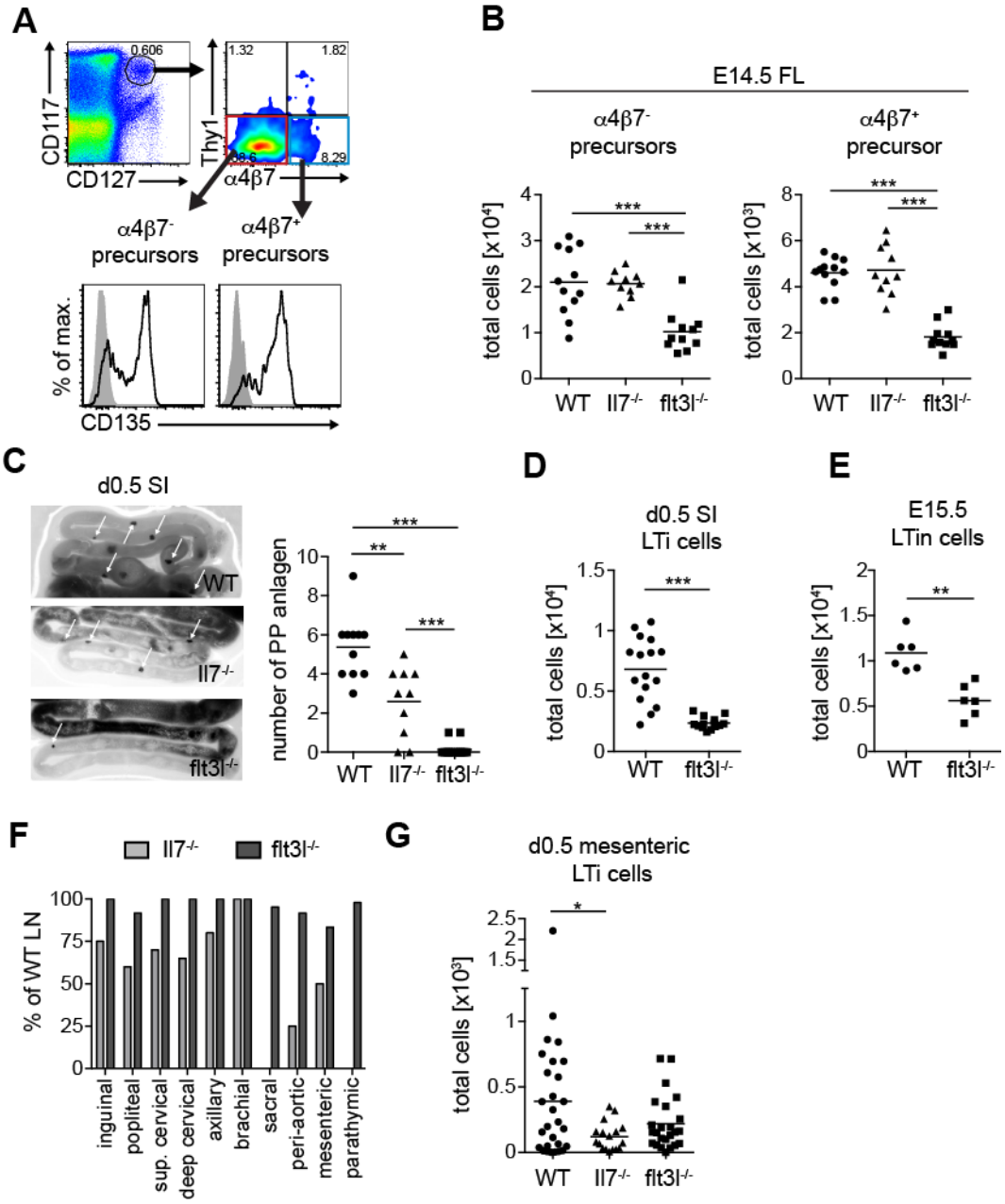

Figure 2.: Flt3L controls ILC numbers in the intestine.

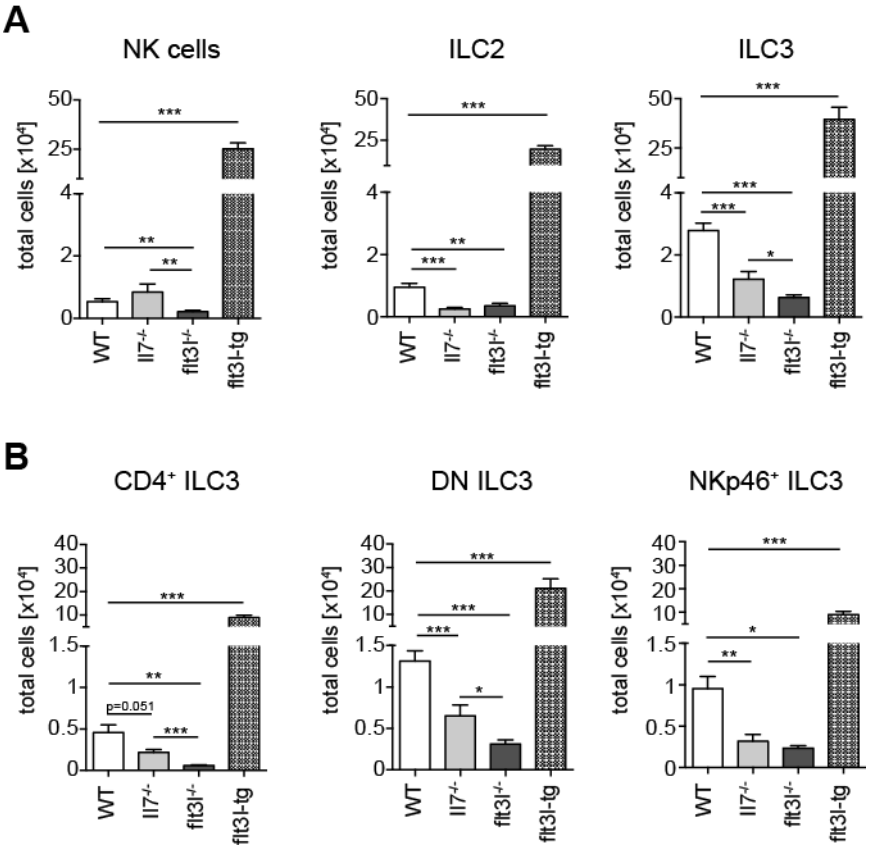

Figure 3.: Flt3L treatment increases ILC numbers in the adult intestine.

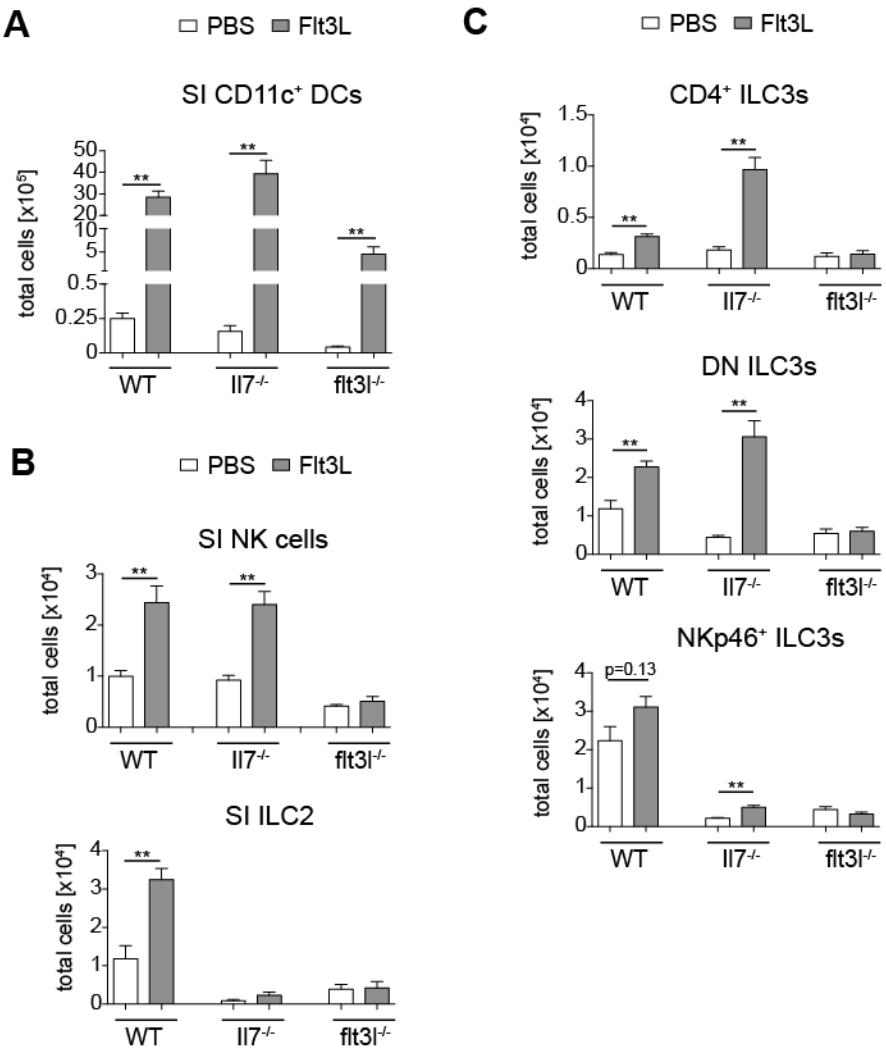

Figure 4.: Loss of DCs does not reduce ILCs in the intestine.

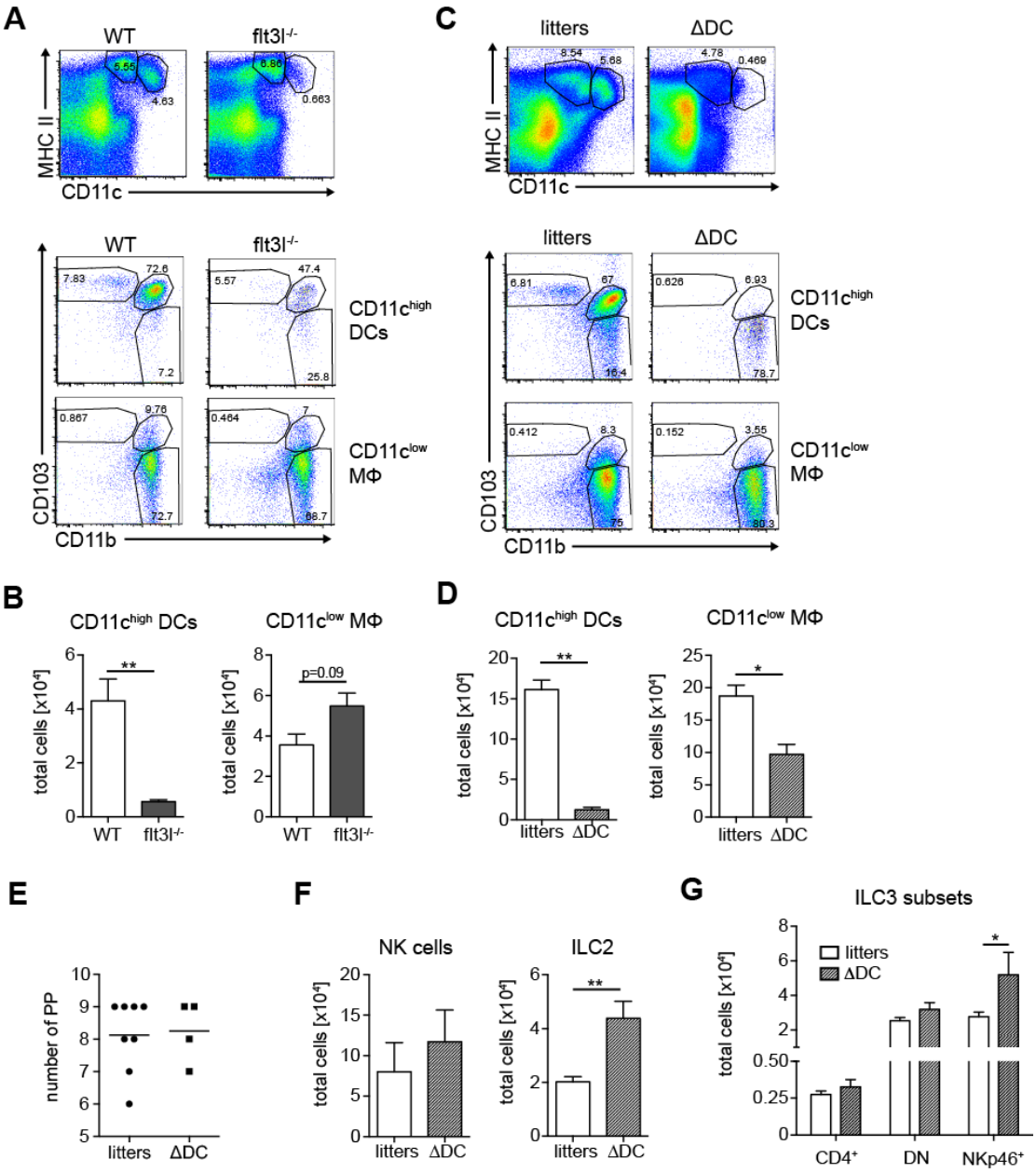

Figure 5.: Flt3L affects CLP numbers but not their differentiation into ILC3s.

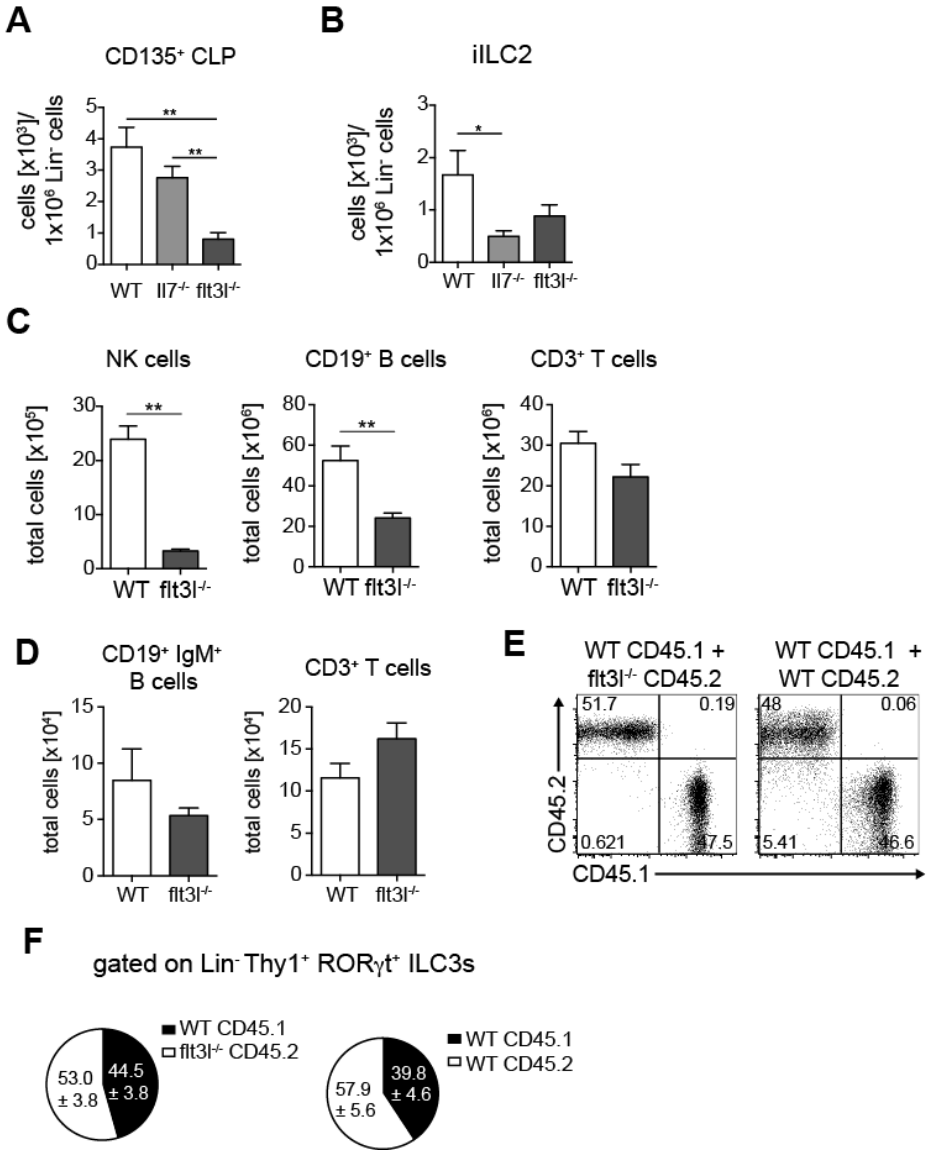

Figure S1.: Gating strategies.

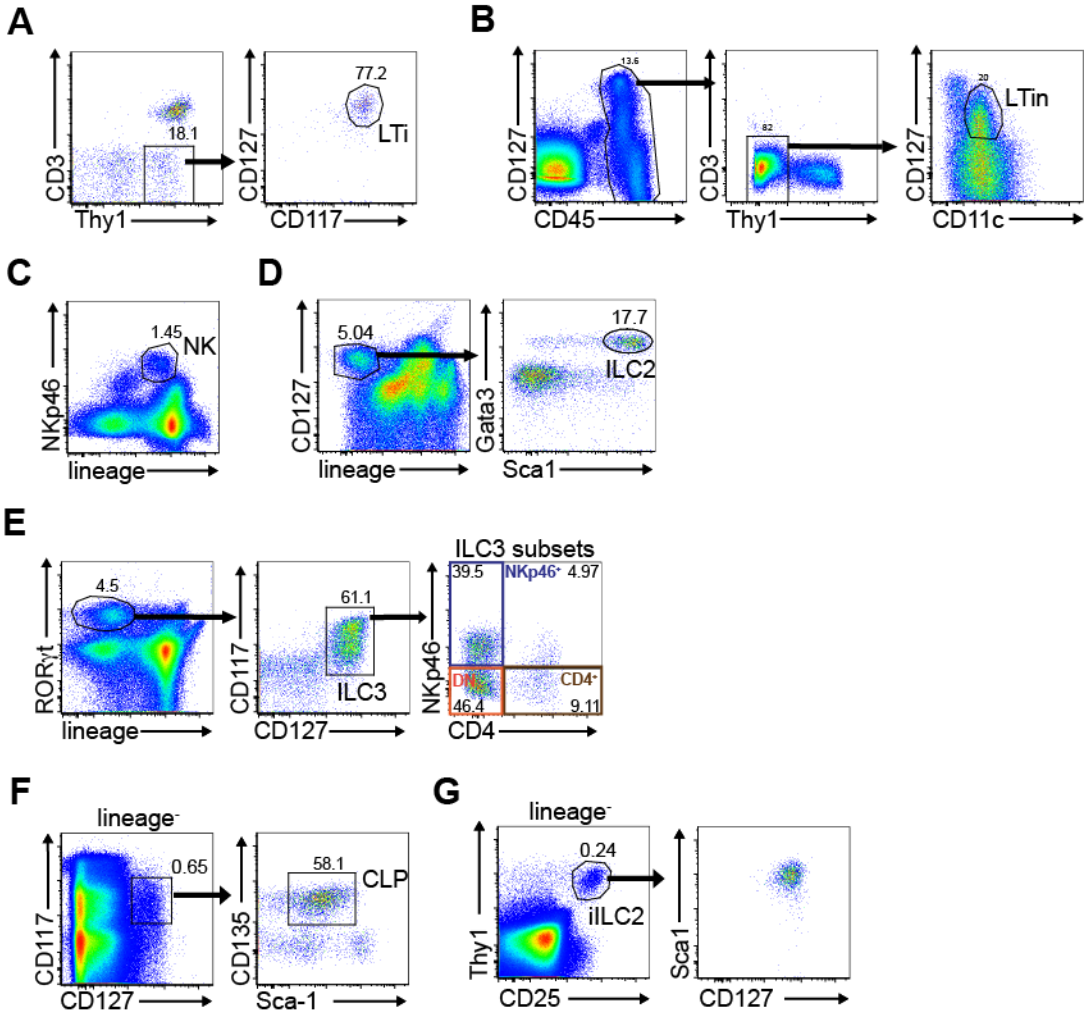

Figure S2.: CD135 expression on ILCs.