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Cross-talk between ILC3 and BDCA1^{+} DC results in bi-directional activation via IL-1β

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Abstract

Innate lymphoid cells (ILCs) are crucial effectors of innate immunity and tissue remodelling but the physiological signals required by ILCs to exert their functions are only partially elucidated. The present study aimed at analysing the role of the interplay between ILCs and myeloid dendritic cells (DCs) in the contest of human tonsil inflammation.

We found that, in vitro, tonsil-derived mDCs efficiently stimulated the proliferation of ILC3, but not ILC1 and ILC2, as assessed by Ki67 staining. In particular, between the two mDC subsets analysed, BDCA1$^+$ DCs are mainly responsible of ILC3 proliferation; conversely, BDCA3$^+$ DCs have only a modest effect. Upon co-culture with BDCA1$^+$ DCs, ILC3 rapidly acquired the expression of natural cytotoxic receptors (NCR). Accordingly, the amount of Nkp44^{pos} ILC3 in human tonsil correlated with the degree of inflammation. In addition, BDCA1$^+$ DCs induced the production of large amount of cytokines, including IL-22, IL-8 and GM-CSF by ILC3. When compared to BDCA3$^+$ DCs, BDCA1$^+$ DCs were much stronger inducers of both ILC3 activation and cytokine production. On the other hand, ILC3 enhance the maturation of BDCA1$^+$ DCs and stimulate them to produce IL-1β that appears to be critically involved in DC-mediated ILC3 proliferation, IL-22 production and, likely in a autocrine way, DC maturation. Finally, human ILC3 express DNAM-1 receptor that is involved in DC-mediated GM-CSF production by ILC3, upstream signal of IL-1β production by DCs. Altogether these data clearly shown that BDCA1$^+$ DCs are particularly well suited to support ILC3 expansion and cytokine release. ILC3, in turn, activate mDCs suggesting a broader role of ILC3/mDC cooperation in regulating mucosal homeostasis or promoting pathological processes.

INTRODUCTION

1.1 Development of Innate lymphoid cells (ILC)

The term innate lymphoid cells (ILCs) is collectively used to identify a variety of cell types that share common phenotypic and functional features (1, 2). All ILCs have morphological characteristics of lymphoid cells and lack rearranged antigen-specific receptors. The ILC family is heterogeneous and includes, along with classic cytotoxic natural killer (NK) cells and lymphoid tissue inducer (LTi) cells (which were discovered in 1997 and well known to promote the formation of lymph nodes during embryogenesis), the more recently described non-cytotoxic ILC populations.

The non-cytotoxic ILCs consist of three distinct groups: group 1 ILC (ILC1), group 2 ILC (ILC2) and group 3 ILC (ILC3) including LTi cells (3, 4-6). As will be described later, the non-cytotoxic ILC subsets are defined on the basis of their differential requirements for transcription factors during their development, the patterns of expression of effector cytokines and the acquisition of other distinct effector functions (7, 3).

The ILC development takes place first in the fetal liver and later in the adult bone marrow from common lymphoid progenitors, CLPs, that give rise also to B cell and T cell precursors, NK cell precursors (NKPs), and the more recently described common helper ILC precursors (ChILPs) that express Id2 and variable levels of pro-myelocytic

leukemia zinc finger (PLZF) (Fig. 1). ChILPs give rise to all ILC groups but not NK cells, whereas $PLZF^+$ ILC precursors generate all ILC subsets but not NK cells or LTi cells. Therefore, ILC development from CLPs (via NKP or ChILP) involves progressive lineage restrictions, in which B and T cell potentials are lost in aid of reinforce ILC potential.

Differentiation of all ILCs from a CLP requires the transcription factors inhibitor of DNA binding 2 (Id2), nuclear factor interleukin-3 regulated (NFIL3) (3, 4, 8, 9–13), and thymocyte selection-associated high mobility group box (Tox) (14, 15), and it involves additional precursor cell populations. Among these, NK cell precursors (NKps) that give rise to NK cells, as well as a common helper innate lymphoid precursor (ChILP) that gives rise to all other defined ILCs in a process in which T cell factor 1 (TCF1) (16, 17) and GATA binding protein 3 (GATA3) (4, 18) are required.

Genetic ablation of GATA3 in haematopoietic stem cells (HSCs) resulted in the inhibition of all ILC subsets and T cells but not of NK cells or B cells (4). While, GATA3 deletion in mature ILCs affected ILC2 subset, but not ILC3 populations (4, 19, 20, 21), suggesting that GATA3 plays a crucial role in both post-development maintenance and survival of ILC2s. Other recent data indicate that multiple transcription factors are involved in the regulation of ILC subset development and function. For instance, the transcription factor GFI1 might contribute to the function of GATA3 in ILC2s, throught Gata3 gene regulation. GATA3 level expression is reduced in ILC2s deficient in GFI1 (22), thus providing a potential mechanism through which GATA3 expression is maintained in mature ILC2s.

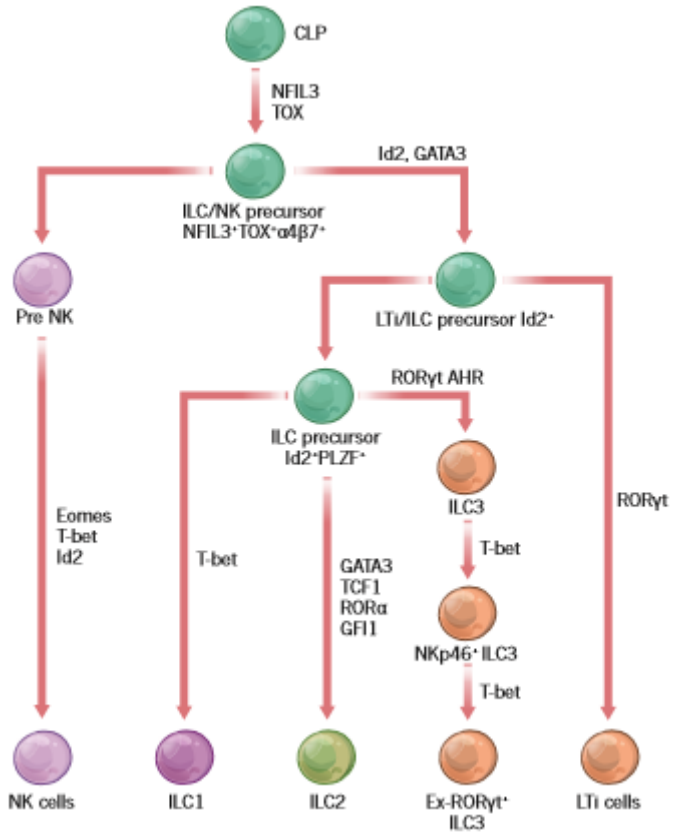
The transcription factor Nfil3 has been proposed as a central orchestrator of the differentiation of NK cells and ILCs. James Di Santo et al. have studied Nfil3 reporter mice, providing evidences for the expression of this transcription factor in various ILC subsets and for the regulation of Nfil3 expression by cytokines, including IL-2 and IL-7.

Depletion of Nfil3 affects the homeostasis of all ILC subsets via ChILPs and controls PLZF expression in a subset of ChILPs via Id2. Moreover, it has been described that germline ablation of Nfil3 has a significant effect on ILC function, in particular on the ability of mice to defend against infection with Citrobacter rodentium.

Eomesodermin and T-bet have both redundant and non-redundant roles in the development, function and migration of NK and $CD8^+$ T cells (23), whereas the master transcription factor of TH1 cells, T-bet (24), is crucial for the development and IFNγ-production by non-cytotoxic ILC1. It should be taken into account that T-bet is required for upregulation of NKp46 expression and IFNγ production by CCR6− ILC3s (8, 25, 26). In addition, the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor that senses multiple exogenous and endogenous compounds, including toxins, tryptophan metabolites, dietary products and bacterial pigments, is able to control both survival and function of ILC3 subsets (27, 28) and TH17 cells (29). Finally, Notch signalling, which is required for T-cell development, has also been involved in the development of ILC3s (16, 30, 31) and ILC2s (21, 32). Notably, there are also many transcription factors that act on ILC subsets but not on the corresponding T-helper cell subsets. For instance, RORα is important for ILC2s but does not seem to play a T-cell essential role in TH2 cell development and function (32,33). The mechanism underlying the fine regulation of ILC, attained by these transcription factor, remains to be clarified, but one emerging concept involves obligate suppression of alternative lymphoid cell fates, on the basis of reciprocal repression as a means by virtue of to control binary cell fate decisions. Id2 is a transcriptional repressor that reduces the activity of E-box transcription factors (E2A, E2-2, and HEB), which play a critical role in early B and T cell development. Thus, increasing expression of Id2 in CLP promotes ILC development to the detriment of B and T cell fates (34, 35). Accordingly, NKP and ChILP express variable levels of Id2, whereas CLPs do not

express Id2 (8, 36). In a similar fashion, Gata3 represses B cell fate by blocking EBF1 and thereby facilitates T and ILC differentiation from CLPs (4, 18, 37). Once generated, mature ILCs exit these sites, circulate in the blood, and enter tissues following codes based on adhesion molecules and chemokines. ILC precursors may leave the fetal liver or the bone marrow and complete their maturation in response to local signals.

In support of this hypothesis, NKP and ILC3 precursors are found in human tonsils (38). In mouse, ILC3 precursors are found in the fetal gut (30) **(Fig.1)**



|Model of developmental pathways of innate lymphoid cells and conventional natural killer cells

1.2 ILC subsets

1.2.1 ILC1 and NK cells

ILC1s and NK cells are subsets of ILCs that produce interferon-g (IFN-g). Although ILC1 cells are still not well defined, many also express the pan–NK cell marker NKp46 in both humans and mice. Because of these shared features, NK cells and ILC1 cells were classified together as group 1 ILCs in the original proposal for a uniform nomenclature of ILCs1.

Transcription factors that influence ILC1 commitment include T-bet, EOMES, ETS1 and TOX (15, 23, 39, 40) T-bet (encoded by Tbx21) represents the signature transcription factor for mature ILC1s that produce IFN-γ. Committed precursors for $EOMES^+$ ILC1s (that is NK cells) are well characterized and include NKP subsets that express T-bet and EOMES (36). Committed precursors for $EOMES^-$ ILC1s are proposed to include $CD127^+NKp46^+NK1.1^+$ bone marrow cells, but these cells have not been strictly described yet. TOX-deficient mice lack $NKp46^+$ NK cells in the bone marrow and spleen, suggesting that this transcription factor plays an important role in ILC1 development, at least for $EOMES^+$ ILC1s (15). The additional effect of TOX deficiency on ILC3 development could indicate a role for this transcription factor in a subset of multipotent ILCPs.

ETS1 deficiency affects pre-NKP numbers and, as indicated by transcriptional analyses, ETS1 sustains Id2 and T-bet expression in these progenitors1.

ILC1s are discernible from NK cells by their expression of CD127 (IL-7Ra), furthermore, they depend on the transcription factor T-bet, whereas NK cells depend

mainly on the transcription factor Eomes2. Moreover, NK cells can exert granule-dependent cell cytotoxicity, whereas ILC1s use the lymphocyte cytotoxic molecule TRAIL to mediate their cytolytic function.

In the context of the Immunological Genome Project, Marco Colonna and colleagues (Washington University, St. Louis) have addressed the precise molecular definition of ILC identity through the use of whole-genome microarray data sets. From these data, it appears that the genes expressed in ILC1 cells, but not those expressed in NK cells, define a 'core ILC signature' that includes transcripts that encode the chemokine receptor CXCR6, IL-7R and the transmembrane protein TMEM176A. However, targeting of Tgfbr2, which encodes receptor II for transforming growth factor-b (TGF-b), in NKp46^{+} cells revealed that TGF-b can repress Eomes expression and consequently drives NK cells toward ILC1s, thus suggesting that there is a plasticity of ILC subsets, also found in humans. The group of Hergen Spits (Academic Medical Center Amsterdam) demostrated that tissue-infiltrating monocytes promote the differentiation of ILC1 cells via IL-12, whereas tissue-resident CD103^{+} dendritic cells (DCs) can convert ILC3s into ILC1s.

Human group 1 ILC includes 2 ILC1 subsets that can be distinguished from NK cells and each other based on their intestinal anatomical site and on the expression of certain surface markers.

Intraepithelial CD127^{low} ILC1s express CD103, CD56, CD94, and NKp44 and are responsive to IL-12 and IL-15.(41). Furthermore, the intraepithelial CD127^{low} ILC1s are CD56 (42) and express perforin, which is essential for cytotoxicity, thus resemble to NK cells.

Human CD127^{high} ILC1s are mainly located in the lamina propria, lack CD56, CD94, and NKp44 expression, and respond to IL-12 and IL-18 by producing IFN-g (43). It is to be confirmed whether human CD127^{high} ILC1s depend on IL7 for their development.

1.2.2 ILC2

Group 2 ILCs require IL-7 for their development (44) and produce TH2 cell-associated cytokines. ILCs that produce IL-5 in response to IL-25 stimulation, were discovered in 2002 (45, 46). Beside IL-25, ILC2s are responsive to IL-33 (44, 47-49) and thymic stromal lymphopoietin (TSLP) (21, 50) and produce type 2 cytokines, mainly IL-5 and IL-13, but also amphiregulin, which is important for tissue repair, (51) and IL-9 and IL-4. Several transcription factors have been identified to be crucial for ILC2 subset differentiation, including GATA3, GFI1, TCF1 and RORα (4, 16, 19, 20, 22, 32, 50). GATA3 is considered a signature transcription factor for mature ILC2s, and cell populations expressing high levels of GATA3 are highly enriched for these cells (4, 18, 19, 20). GATA3 transcriptional activity leads to activation of type 2 cytokine transcription and is necessary for both survival (52) and effector functions in mature ILC2s. ILC2s can be found in the bone marrow and are, for their noteworthy proliferative capacity, considered as ILC2 precursors (19). However, bone marrow ILC2s produce type 2 cytokines (19), so they cannot be considered to be functionally immature.

Notch has been shown to have a role in triggering ILC2 development in vitro (16, 32) its role *in vivo* in this process is still unclear. By contrast, TCF1 is essential for ILC2 development *in vivo* (16, 17). RORα is a transcription factor of the nuclear hormone receptor superfamily. Rorα is highly expressed by multiple ILC subsets (32, 33, 53) and its ablation causes a selective defect in ILC2 numbers, especially in the bone

marrow(32, 33.) The factors that induce RORα in the ILC2 lineage are not known, but could include GATA3 (20). The downstream targets of RORα that promote ILC2 differentiation are similarly undefined.

A decade later, several research groups (44, 47, 49) simultaneously reported the same populations (Lin^{-} $sca1^{+}$ $Thy1^{+}$ $T1/ST2^{+}$) but named them differently. All these cells, which are at the final stage of differentiation, produce IL-5 and IL-13 in response to IL-25 and IL-33 (IL-1-like cytokine). They were first identified in mice and named natural helper cells (NHCs), in view of their ability to help B1 cells produce antibody (44) nuocytes (47) and IL-13 production (nu = the 13th letter of the Greek alphabet), and type 2 innate helper cells (Ih2) (49). Nuocytes and Ih2 cells have similar surface markers, with the exception of Ih2 cells being Sca-1 negative, suggesting a close relationship between them. Similar cell populations with CD161 and CRTH2 as surface markers, a high affinity for PGD2-R, and in their final differentiation state, were described in human (54). It is not clear whether these different reported cell types are truly the same or belong to distinct populations. Finally, ILC2s denote the populations of ILCs that produce TH2 cell- associated cytokines. Further characterization of natural helper cells, nuocytes and IH2 cells may eventually justify subdivision of the group 2 ILC population in the future.

1.2.3 ILC3

ILC3 cells were originally described as lymphoid tissue–inducer cells in the fetus and were required for the development of lymph nodes and Peyer's patches. It was later established that expression of the nuclear hormone receptor RORgt is essential for the generation of lymphoid tissue–inducer cells, as well as of another subset of ILC3 cells, not involved in lymphoid tissue formation, that express natural cytotoxic receptors (4). Expression of RORγt in early haematopoietic precursors marks cells that have committed to the ILC3 lineage; among these, fetal liver $\alpha4\beta7^+$ cells in mice50 as well as a subset of $CD34^+CD117^+$ cells in humans (55). RORγt expression in both mouse and human ILC3 precursors is associated with several hallmarks of mature ILC3s.

Notch signals have been proposed to contribute to the development, especially in fetal liver, of ILC3 precursor (26, 56). Consistent with Notch signaling in ILC3-restricted precursors, the transcription factor TCF1 is also important for generating mature ILC3 subsets(17). Notch signaling is also required for the development of $NKp46^+$ ILC3s (26, 57) Another transcription factor required for the generation of some ILC3 subsets (for instance, LTi cells) is TOX; whereas $NKp46^+$ ILC3s are TOX-independent (15). Many ILC3 subsets, including LTi cells and $NKp46^+$ ILC3 subsets, are affected by AHR deficiency (28, 33, 58). ILC3s respond to IL-23 (59-63)and IL-1b (63, 64)by secreting IL-22.

It is well established that the group 3 ILC population is heterogeneous and includes cell types that produce either IL-17A and/or IL-22, or IL-17A, IL-22 and IFNγ.

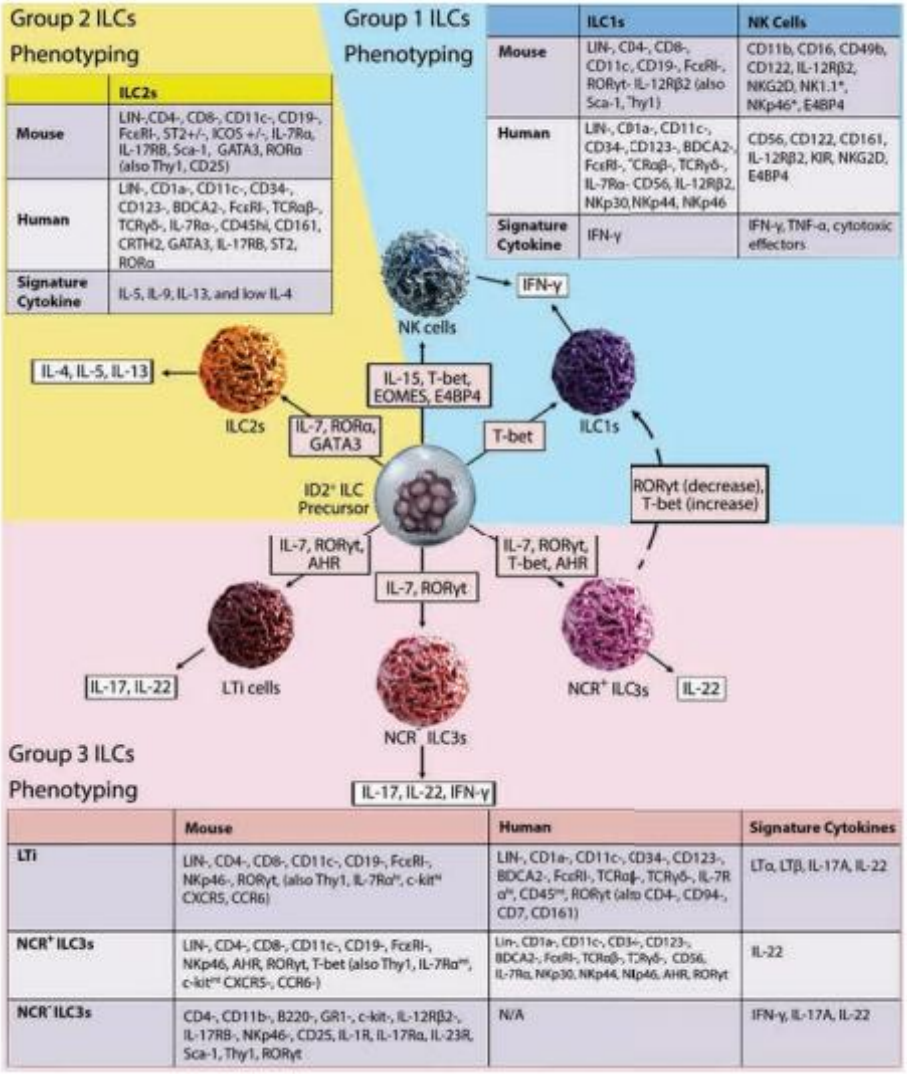
The prototypical group 3 ILCs are LTi cells, which are crucial for the formation of secondary lymphoid organs during embryogenesis. An effector role for LTi cells in innate immunity has also been suggested, as LTi cells are capable of producing IL-17A and IL-22 following stimulation4. More recently, other RORγt-dependent ILC3 subsets were identified, such as NCR^+ cells, which express NKp46 in mice and NKp44 in humans. These NCR^+ ILC3s lack cytotoxic ability and do not produce IFNγ (thereby distinguishing them from ILC1s), but they are potent producers of IL-22 following stimulation with IL-23 and/or IL-1β. Moreover, it has been described that upon stimulation with IL-1b and IL-23, human RORgt $^+$ $NKp44^{neg}$ ILC3s can differentiate *in vitro* into NCR^{pos} ILC3s and under the influence of IL-12 into $CD127^+$ ILC1(43). NCR1 ILC3s can also differentiate into ILC1s upon stimulation with IL-12. During this process, these cells downregulate RORgt and upregulate Tbet.(43). A analogous conversion of NCR1 ILC3s into INF-γ–producing $Tbet^{high}$ $ROR\gamma t^{low}$ ILCs has been observed *in vivo* in a mouse model.(25). These data indicate that ILC3s are cells with plastic features that can adopt an ILC1 fate depending on environment stimuli. In both mice and humans, NCR^+ ILC3s are enriched in mucosal sites (including the intestinal lamina propria and Peyer's patches), but they are rare in other lymphoid tissues, such as the spleen and peripheral lymph nodes, in steady state condition (25, 60, 65-67).

ILC3s in both human and mouse, constitutively express the receptors for IL-7 (IL-7R or CD127) and TSLP, IL-15/IL-2 (IL-2Rβγ), IL-23 (IL-23R), IL-1 (IL-1R), and SCF (c-kit). Among these cytokines, IL-7, TSLP, and SCF are mainly required for ILC3 development and induce proliferation of ILC3s, together with IL-2/IL-15 and IL-1 stimulation. On the contrary, IL-23 and IL-1 play a pivotal role in inducing ILC3 effector functions *in vitro* and *in vivo* (59, 61, 68-71). Apart from cytokine receptors, ILC3s are capable of directly recognizing environmental cues. The main receptors that enable ILC3s to exert their function were described.

Both mouse and human ILC3s express the transcription factor aryl hydrocarbon receptor (AhR); several endogenous molecules, such as metabolites of tryptophan and arachidonic acid, are able to activate AhR. AhR ligands, derived from bacterial metabolites, can modulate ILC3 functions. For instance, it was recently shown that under conditions of unrestricted tryptophan availability, Lactobacilli species can produce an AhR ligand, indole-3-aldehyde, ables to enhance IL-22 expression in ILC3, and as a consequence allows the survival of mixed microbial communities and provides colonization resistance to Candida albicans. Interestingly, Crellin et al (72). observed, by using quantitative RT-PCR, broad expression of many Toll like receptor (TLR) transcripts, including TLR1, 2, 5, 6, 7, and 9 by *ex vivo* isolated human ILC3 as well as by cloned ILC3 lines, although the degree of expression was still lower than on monocytes. Among these, only TLR2 agonists were able to induce cytokine production by human ILC3 in the presence of cytokines like IL-2, IL-15, and IL-23 (72). Thus, TLR2 engagement on ILC3 seems to act as a costimulus, rather than as a trigger on its own, as is the case when TLR agonists stimulate myeloid cells. As previously mentioned, both human and mouse ILC3 subsets express NK cell activating Receptors (actR). A consistent fraction of human ILC3s derived from tonsils and gut LP expresses NKp44, NKp46, and NKp30, although to lower levels compared to NK cells. NKG2D or CD94/NKG2C is conversely not expressed by human ILC3s. Recent studies show that among the different NK cell actR expressed by ILC3s, such as NKp46, NKp30, and CD2, only engagement of NKp44 results in a strong cytokine response by ILC3s (73). Thus, the biology of NKp44 and its role on human ILC3 need to be further investigated.

Engagement of NKp44 in *ex vivo* isolated ILC3s is sufficient to induce cytokine production, demonstrating that ILC3s can directly sense the environment and be activated in the absence of pro-inflammatory cytokines (73). NKp44 triggering in *ex*

vivo isolated ILC3 selectively induces the expression of TNF and IL-2, while cytokine stimulation, for instance by IL-23, IL-1, and IL-7, preferentially induces IL-22 and GM-CSF expression. Thus, ILC3 are able to switch between IL-22 or TNF production, depending on the triggering stimuli. However, combined engagement of NKp44 and cytokine receptors results in a strong synergistic effect both at transcriptome and protein level (73) **(Fig.2).**



The innate lymphoid cell family

1.3 Tissue Distribution

In healthy individuals, about 0.01% to 0.1% of circulating lymphocytes express a CD127+ ILC phenotype. The majority of $CD127^{+}$ ILCs found in peripheral blood are group 2 ILCs, whereas $NKp44^{pos}$ ILC3s (74) and CD1272 ILC1s (41) are nearly absent

The composition of human ILC subsets depends on the tissue type. For instance, whereas group 2 ILCs and $NKp44^{neg}$ ILC3s are the most prevalent ILC subsets in healthy human skin tissue (75, 76) in other tissues such as thymus, tonsils, and bone marrow and in the gut, $NKp44^{pos}$ ILC3 is the prominent ILC subset. In 2009, in parallel with the identification of human fetal LTi, localized in fetal mesenteric lymph nodes, and postnatal tonsillar LTi-like cells (61) It has been reported the first study where described the presence of human IL-22–producing ILC3s in the healthy gut (Figure 3A) (59). These $NKp44^{+}$ ILC3s, which were originally called NK22 cells, produce IL-22 that signals to epithelial cells where it promotes proliferation, IL-10 and antimicrobial peptide production, and mucus production. The human gut also contains $CD127^{high}$ ILC1s and CD127low ILC1s. (41, 43) CD127low ILC1s respond to danger signals originating from epithelial cells and myeloid cells, suggesting that they play a role in the immune response against pathogens that elicit these danger signals. Although ILC2 are present in the human gut, most prevalently in the fetal gut (74) and in adult human intestinal tissues, their role of in gut homeostasis and immunity has received considerably less attention.

ILC2s are most thoroughly studied in the context of lung immune cell homeostasis and immunity. CRTH2+ ST2+ ILC2s have been identified in healthy human lung (51, 74). Besides ILC2s, ILC1s and ILC3s were detected in the human lung, but their function remains poorly understood. Several ILC subsets have been characterized in the skin of healthy wild-type mice and humans. Kim et al were the first to identify an ILC subset (ILC2) in human skin, which expressed ST2, a component of the IL-33 receptor, but not CRTH2. (77). Other groups revealed the presence of ILC2 in healthy skin, but in these studies the dermal ILC2s were found to express CRTH2. (78) It has been also detected ILC1 and NCR^+ ILC3s but not NCR^- ILC3s in human skin. Within ILC2s, natural helper cells (NHCs) were first observed in fat-associated lymphoid clusters (FALCs) of the intestinal mesentery, in fatty deposits in the peritoneal cavity, and surrounding the kidneys (44). In another study, it was found that NHCs are also resident in lung tissue of mice (42). Nuocytes were identified in mesenteric lymph nodes (mLNs), spleen, intestine, and in low abundance in peripheral blood (47). Type 2 innate helper cells (Ih2) have a broad tissue distribution but are abundant in mLNs, liver, and spleen

The liver contains cNK cells as well as ILC1s, the two of which can be distinguished by their different expression of surface markers and transcription factors. In mice, liver ILC1s are $CD3\epsilon^-$ $NK1.1^+$ $DX5^-$ cells with high expression of both the integrin CD49a (VLA-1) and the cytotoxicity-inducing ligand TRAIL. In mice, a unique subset of NK-like cells resides in salivary glands (79-81). While these cells closely resemble cNK cells in both surface receptor and transcription factor expression, they also share several features with unconventional NK cells, and in particular, with liver ILC1s. Most notably, both salivary gland ILC1s and liver ILC1s express TRAIL and CD49a (79, 80). Unlike liver ILC1; however, salivary gland ILC1 express DX5 and Eomes.

1.4 Heterogeneous function of ILC

1.4.1 Role of ILCs in immunity

Each cell type in an organism is expected to have a specific function that justifies its evolutionary conservation**.**

Within ILC3, LTi cells play a pivotal role during prenatal organogenesis of LN and PP. To initiate lymphoid organogenesis, LTi cells activate the local mesenchyme at predestined locations by ligating the LTβR expressed on these stromal cells. Once activated, stromal cells will secrete chemokines (e.g., CXCL13, CCL21, and CCL19) and will increase their expression of adhesion molecules such as VCAM-1, ICAM-1, and MAdCAM-1. As a result, additional LTi cells are attracted to the primordial organ. In addition, ILC3s are critically involved for post-natally developing intestinal lymphoid organs such as cryptopatches (CP) and isolated lymphoid follicles. $NKp46^{+}$ ILCs mainly reside in mucosal tissues (82) and are the major source of IL-22 in the mouse intestine (60, 62, 69, 83). In humans, IL-22-producing ILCs are also present in adult intestines and palatine tonsils and are characterized by expression of the NCR NKp44 (59, 61, 84). Intestinal ILCs are in a continuous cross talk with epithelial cells, immune cells, and the commensal microflora acting mainly in the maintenance of epithelial integrity during homeostasis, but on the other hand, Rorγt + ILCs are essential for the early phases of immunity in response to enteric pathogens. In particular, IL-22 is a cytokine that acts directly on intestinal epithelial cells and increases the production of antimicrobial peptides such as β-defensins and

antimicrobial proteins RegIIIβ and RegIIIγ, which play critical roles in the immune response against the gut pathogen Citrobacter rodentium-

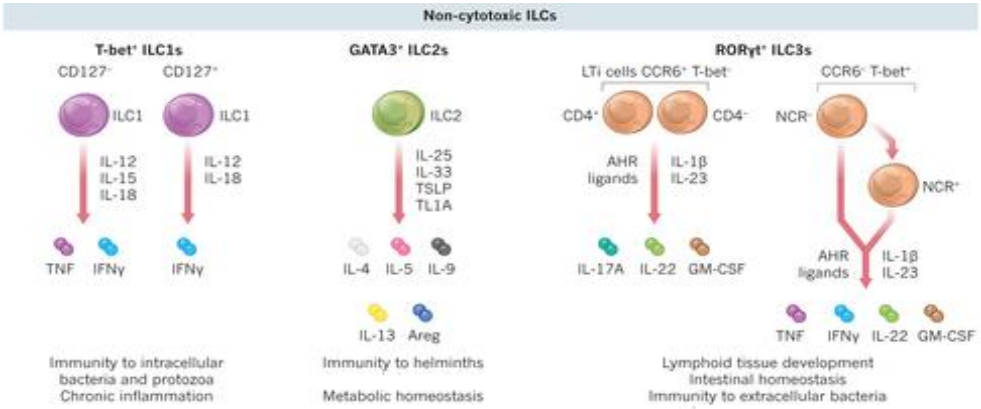
ILC3 promote tissue protective and repair responses through the production of Lymphotoxin (LT)α1β2 and IL-22. Infection of lymph nodes with lymphocytic choriomeningitis virus leads to the destruction o lymphoid stromal cells (LSCs). ILC3s restore LSCs through LTαβ2 and activation of LTβ receptor on LSCs (85) A similar ILC3-mediated mechanism was found to protect the thymus from the consequences of full-body irradiation. ILC2s are also involved in tissue-repair responses through the production of amphiregulin and IL-13. Upon infection of mouse lungs with the H1N1 influenza virus, ILC2s contribute to tissue repair through the expression of amphiregulin (51). Furthermore, injury to the bile duct, which can lead to severe liver disease, leads to the IL- 33–mediated activation of ILC2s that promote cholangiocyte proliferation and epithelial restoration through the release of IL-13 A similar ILC2-mediated protective mechanism is induced in the intestines upon damage of the epithelium induced by dextran sodium sulfate. An other important aspect of ILC2 function is their capability to mediate resistance to the helminth Nippostrongylus brasiliensis (85). These cells are critical components of the innate immune response to parasitic worm infections since represent an early source of IL-13, which is critical in driving the physiological 'weep and sweep' processes such as goblet cell mucus secretion and contraction of intestinal smooth muscle which are required to effect worm expulsion T

Type 2 responses, including ILC2s, are also required to avoid the induction of type 3 responses that lead to metabolic syndrome, insulin resistance, diabetes, as well as obesity-associated asthma (86). Evidences also exist for a cooperation between T cells and ILC2s. In particular, mice deficient for one component of the IL-25R (IL-17RB)

have only a few ILCs and also show diminished IL-13-producing T cells (47) and transfer of ILC2 into these mice restored antigen-specific IL-13 production by T cells.

The prototypic function of group 1 ILCs is potent expression of IFN-γ upon activation with cytokines or surface receptor crosslinking

Recent studies have focused on the potential roles of intestinal ILC1s during protective responses to intestinal pathogens. New findings in this respect suggest that following oral infection of mice with the intracellular pathogen *Toxoplasma gondii*, ILC1s produce the majority of IFN-γ, as well as TNF-α, while cNK cells and ILC3s contribute to a lesser extent (8). In this particular infection model, ILC1 were identified as the cell type responsible for controlling infection, since they promoted by their rapid attraction of inflammatory monocytes to the site of infection. Unfortunately, due to the lack of specific ILC1-knockout mice or depleting antibodies, it is currently impossible to unequivocally attribute a particular function to either ILC1s or cNK cell. Nevertheless, in other infection model, ILC1s represent the major innate cell subset to respond with IFN-γ production in the LP, epithelium, and MLN of infected mice (87). Consistent with this finding, Pamer and colleagues further demonstrated that mice lacking ILC1s as well as mice deficient in IFN-γ were more susceptible to lethal *C. difficile* infection. However, in this study, it was concluded that converted ILC3s, rather than true ILC1s, were responsible for the majority of the produced IFN-γ (25). There is emerging evidence that intestinal ILC1s contribute to the immune defense to microbial pathogens. However, to date, the exact role of ILC1s has not clearly demonstrated because an accurate assessment of the individual roles of ILC1s, cNK cells, and converted ILC3 remains impossible **(Fig.3)**



Effector functions of ILCs

1.4.2 ILCs in pathology

A dysregulated response of ILCs were associated with inflammatory pathology **(Fig.4)** Inflammatory conditions of the lung are characterized by a type 2 signature. It has been recognized that type 2 cytokines are critical for pulmonary recruitment of type 2 effector cells, such as eosinophils (IL-5 and granulocyte-macrophage colony-stimulating factor), mast cells (IL-9), and immunoglobulin E–producing B cells (IL-4 and IL-13), and cytokines that directly affect target tissue (eg, IL-13–induced fibrosis). CD342 CRTH2+ ILC2s were identified in nasal polyp tissues of patients suffering from chronic rhinosinusitis (CRS), a typical type 2 inflammatory disease characterized by eosinophilia and high immunoglobulin E level. Interestingly, TSLP activates human ILC2 by directly upregulating GATA3 via STAT5, resulting in the production of high amounts of type 2 cytokines. TSLP protein expression was significantly increased in patients with severe asthma (88). Taken together, these data indicate that human ILC2s are involved in lung inflammation and pathology. This conclusion is confirmed by numerous studies in mouse models of type 2 inflammatory diseases, in particular of allergic asthma (89-92)

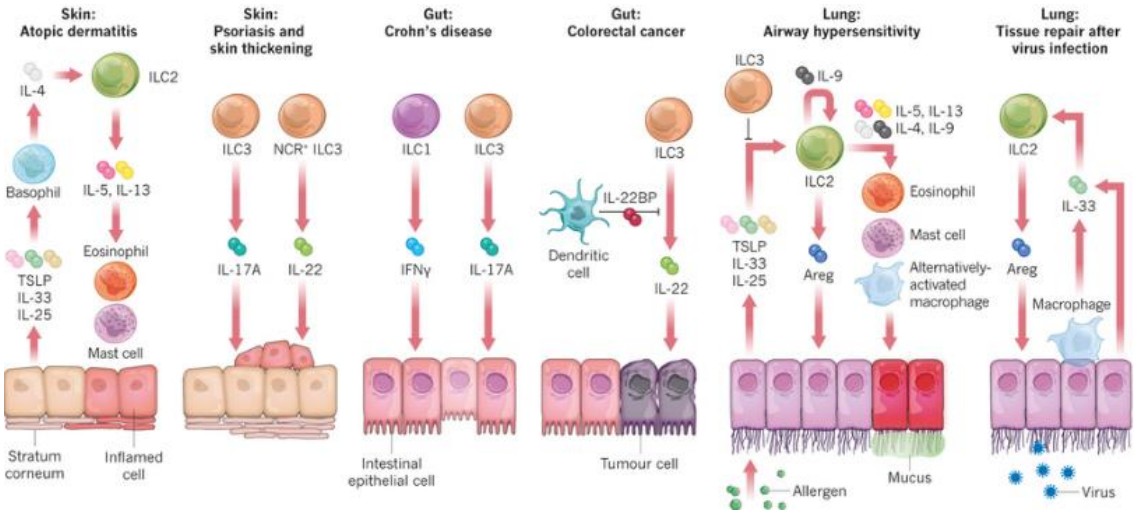
ILC2s and IL-13 are also associated with hepatic fibrosis induced in mice by thioacetamide, carbontetrachloride, and Schistosoma mansoni (93), and with

pulmonary fibrosis (94), chronic rhinosinusitis (74), and atopic dermatitis (77, 78), as well as allergen- (78, 95) and rhinovirusinduced asthma exacerbation in patients (96, 97). Last, ILC2s are proposed to play a central role in asthma-induced obesity. In the diseased skin of human atopic dermatitis patients, increased numbers of ILC2s were observed compared with healthy controls (77, 78), suggesting that ILC2s play a role in atopic dermatitis. Interestingly, interaction of ILC2-expressed killer lectin-like receptor G1 with E-cadherin widely expressed on keratinocytes and Langerhans cells suppressed IL-33–induced production of IL-5 and IL-13 by dermal ILC2. This is suggest an involvement of ILC2s in atopic dermatitis, because interrupted E-cadherin signaling may be a key factor in the development of atopic dermatitis (56). In addition, transgenic overexpression of IL-33 in keratinocytes results in an atopic dermatitis-like syndrome that correlated strong infiltration of ILCs.

Several studies demonstrated that inflamed intestinal tissues from patients with Crohn's disease harbor larger numbers of ILC1s, suggesting a role for ILC1s in inflammatory pathology (41, 43). in a mouse model of colitis induced by anti-CD40 injection into Rag-deficient mice. In this model, IFN-γ produced by ILC1s is known as the major factor driving wasting disease and systemic inflammation (98). An other group demonstrated that in a model of inflammatory bowel disease, intestinal ieILC1s contribute to intestinal pathology through production of IFN-γ (41). Accordingly, mice with a human immune system show accumulation of IFN-γ-producing human ILC1s in the inflamed intestine upon challenge with the colitis-inducing agent dextran sodium sulfate (41, 43). A recent study demonstrated that ILC1s contribute to organ dysfunction seen in a mouse model of ischemic kidney injury. The authors of this study identified $NK1.1^{+}$ non-T cells as the major responsible for kidney dysfunction following ischemia–reperfusion injury, and showed that depletion with anti-NK1.1, which depletes both cNK cells and ILC1s in kidneys, ameliorated disease, whereas

anti-asialo-GM1 treatment, which preferentially depletes cNK cells, did not protect from disease (99). The exact mechanism for this ILC1-mediated tissue damage awaits further investigation. IBD patients have also been reported to exhibit significant alterations in intestinal ILC3 subset. (43, 100); Due to their ability to modulate epithelial cell functions as well as to respond against commensal bacteria and pathogens, ILC3 also participate in the complex regulation of inflammatory bowel disease (IBD), displaying rather a dual role. Indeed, several investigators have suggested a protective role for IL-22, likely produced by ILC3s, in innate and adaptive IBD models (101, 102). On the other hand, expression of IL-17 and IFN-γ from ILC3s has been implied to drive inflammation in innate IBD models, such as anti-CD40 or Helicobacter hepaticus-induced colitis (66, 103). IL-22-producing NKp44^{+} ILC3 were found to be reduced in patients with Crohn's disease and were preserved in ankylosing spondylitis patients exhibiting subclinical intestinal inflammation (104), suggesting that this population of cells may play a role in limiting the development of clinical GI disease. The loss of a potentially 'protective' ILC3 population in IBD was also associated with an increase in potentially 'pathologic' ILCs (43, 100, 105). The cells of this subset lack expression of NKp46 and secrete both IFNγ and IL-17A in addition to IL-22 and accumulated in the intestinal lamina propria of Crohn's disease patients. IL-22 appears to have a critical role in driving keratinocyte hyperproliferation, a major feature of psoriasis. A recent report revealed the ability of ILC3s in psoriasis skin and blood to produce both IL-17A and IL-22. Experiments performed using fresh psoriasis patient skin, has validated the increased presence (B4-fold) of IL-22-producing ILC3s. The frequency of circulating NKp44^{pos} ILC3s was also increased in the blood of psoriasis patients compared with healthy individuals or atopic dermatitis patients. A detailed time course of a psoriasis patient treated with anti-tumor necrosis factor α (TNFα) showed a close association between therapeutic response, decrease in

inflammatory skin lesions, and decrease of circulating NKp44^{pos} ILC3s. Overall, these findings suggest a potential role for NKp44^{pos} ILC3s in psoriasis pathogenesis.



Pro-inflammatory functions of innate lymphoid cells.

AIM OF THE STUDY

Innate lymphoid cells (ILC) are an emerging field in immunology. They are crucial effectors of innate immunity and tissue remodelling for their ability to produce cytokines, playing a central role in supporting mucosal homeostasis. Interestingly, high frequencies of ILCs have been found in several pathology states and recent findings demonstrated that signals from injured or infected tissues expand and activate ILC1s, ILC2s, and ILC3s. Therefore, ILCs act promptly in response to infection and injury, and modulate type 1, type 2, and type 3 responses but the physiological signals required by ILCs to exert their functions are only partially elucidated. . Studying the mechanism underlying ILC function not only increase the knowledge about the biology of ILCs but may have a great potential for therapeutic benefit in several human diseases since may be targeted to critically enhance or block early immune responses. In view of this consideration, The present study aimed at analysing the role of the interplay between ILCs and dendritic cells (DCs), main cellular source of the cytokines involved in ILC activation, in the contest of human tonsil inflammation.

3 Methods

3.1 Tissue Collection and Cell Purification

All tissues were collected after subjects provided informed consent, with approval of tissue-specific protocols by the Medical Ethical Committee. Tonsils were obtained from pediatric tonsillectomies.

Tonsil tissue was cut in small pieces, mechanically disrupted and then digested for 30 min in 1mg/ml collagenase IV (Invitrogen) and 10µg/ml DNasi (Roche) at 37°C to form a single cell suspension. Cell suspension was passed through a 70-µm cell strainer and mononuclear cells were isolated with Ficoll-Paque.

3.2 Cell Sorting

Tonsil mononuclear cells were enriched by using fluorescence cell sorting (FACS): B cells were depleted by labeling with FITC-conjugated anti-CD19. All enriched cell fractions were subsequently labelled with PE-Dazzle-conjugated anti-CD127, PercpCy5.5-conjugated anti-CD117, and FITC-conjugated anti-CD3 anti-CD19, CD14, CD94, CD34, BDCA2, BV421-coniugated BDCA1, PeCy7-coniugated BDCA3, APC-H7-coniugated

HLA-DR, PE-coniugated CD11c. Cells were FACS sorted to high purity (above 95%) by using FACSaria II cell sorting (BD). Total ILCs were sorted as lineage^{-} (CD3,CD19,CD94,CD14,CD34,BDCA2,) CD127^{high}, NKp44^{pos} ILC3 were sorted as Lin^{-} CD127^{high} CD117^{+} NKp44^{+}. NKp44^{-} ILC3 were sorted as Lineag^{-} CD127^{high}CD117^{+} Nkp44^{-} while ILC1 as Lineage^{-} CD127^{high}CD117^{-} NKp44^{neg} (ILC1). NK cells were sorted as Lin^{+} NKp46^{+}. Dendritic cells were sorted as Lin^{-} HLA-DR^{+} CD11c^{+}or Lin^{-} HLA-DR^{+} BDCA1^{+} or Lin- HLA-DR^{+} BDCA3^{+}. A detailed description of the antibodies used for flow cytometry is given in the table 1.

Table 1

Antigen	**Clone**	**Fluorochrome**	**Company**
hCD3	SK7	FITC	BD
hCD14	MOP9	FITC	BD
hCD19	4G7	FITC	BD
hCD34	581	FITC	BD
hCD94	HP-3D9	FITC	BD
hBDCA2	AC144	FITC	Miltenyi
hCTRH2	REA598	PE	Miltenyi
hNKp44	P44-8	AF647	BD
hCD117	104D2	PerCP-cy5.5	BD
hCD45	2D1	APC-H7	BD
hNKp46	9E2	Pe-cy7	Biolegend
hHLA-DR	G46-6	APC-H7	BD
hBDCA1	L161	BV421	Biolegend
hBDCA3	REA674	Pe-Cy7	Miltenyi

hIFNg	B27	PE	BD
hIL-8	G265-8	PE	BD
hGM-CSF		PE	Biolegend
hTNFα	6401.1111	PE	eBioscience,
hIL-2	5344.111	PE	BD
hIL-17	CZ8-23G1	APC	Miltenyi
hCD80	L307.4	PE	BD
hCD83	HB15e	FITC	BD
hCD40	TRAP1	Pe-cy5.5	BD
hIl-1β	AS10	FITC	BD
hKi67	B56	BV421	Biolegend
hCD127	A019D5	PE-DAZZLE	Biolegend
hIl-22	22URTI	PE	eBioscience
hPVR	L95	Unconiugated-Ab	
hNectin-2	L14	Unconiugated-Ab	
hDNAM-1	F22	Unconiugated-Ab	

3.3 Intracellular cytokine staining

Intracellular staining for the detection of cytokines was performed on freshly isolated tonsillar ILC3 and cNK cells stimulated for 18 h with autologous total DCs, autologous tonsil or allogeneic blood derived $BDCA1^+$ or $BDCA3^+$ DCs at ratio 2:1 and monensin (2 uM) and brefeldin (10ug/ml) were added during the last 6 hour of culture. Alternatively blood $BDCA1^+$ DCs were co-cultured for 36h with tonsillar $NKp44^{pos}$ILC3s and monesin and brefeldin were added during the last 12h hours of culture. Cells were then fixed in 1% paraformaldehyde, permeabilized with saponin 0.1% in PBS and stained with the

following antibodies: PE-conjugated anti-IL-22, PE-conigated anti-GM-CSF PE-conjugated anti-IFNγ, PE coniugated anti-TNFα,PE-conjugated anti-IL-8 and anti-IL-2, FITC-coniugated anti-IL-1β. Samples were then acquired using FACS Canto II (BD) cytometer and data analysed by FlowJo xV.0.7.

3.4 Quantitative PCR Real Time

mRNA was isolated from cells by using RNeasy MicroKit (Quiagen). cDNA was synthesized by using Quantitect Reverse Transcription Reagents (Quiagen) and assayed by qPCR in duplicates by using a Quant studio DX Real-time PCR system. IL-1β TaqMan Gene expression assays (all Applied Biosystems) was employed and mRNA content was normalized to GAPDH expression. Mean relative gene expression was determined by using the DDCT method.

3.5 Cell Culture Assays

Freshly isolated FACS-sorted human ILC were plated at 5x104 cells/ml, in 96-well round bottom plates in the presence of 50 ng/ml of IL-7. For DC-mediated ILC proliferation, purified Lin^- $CD127^{high}$ were cultured with autologous Lin^- $HLADR^+$ $CD11c^+$ DCs for 6 days. For ILC3 and NK cell proliferation experiments, cells were stimulated with autologous tonsillar $BDCA1^+$ DCs, $BDCA3^+$ DCs or alternatively blood $BDCA1^+$ DCs at ratio 2:1 for 6 days. Where indicated, cells were stimulated with recombinant IL-1β (50ng/ml) and IL-23 (50ng/ml). Proliferation was assessed by flow cytometry using CFSE dye or alternatively Ki67 staining. For $BDCA1^+$ DC maturation, purified $BDCA1^+$ DCs from blood were cultured with freshly isolated $Nkp44^{pos}$ ILC3 or NK cells for 24h and then analysed for the expression of CD80, CD83, CD40. The level of expression is measured as the geometric mean of fluorescence (MFI). IL-1β production were measured on purified blood $BDCA1^+$ DCs left untreated, stimulated with $NKp44^{pos}$ ILC3 or recombinant GM-CSF (25ng/ml). In selected experiments neutralizing anti-human IL-1β antibody and anti-

human DNAM-1 blocking antibodies, or isotype control, were added to the cultures at the beginning.

4 Results

4.1 Myeloid dendritic cells are strong activators of human ILC3s

In human tonsil, ILC3s represent the most prominent subset among CD127^{high} ILCs, while ILC2 are barely detectable (106). Within ILC3 subsets, ILC3 that express NKp44 is the most abundant subset (Fig.1A) and, interestingly, we observed that their amount correlated with that of B cells as well as with the frequency of proliferating B cells (Fig.1B)

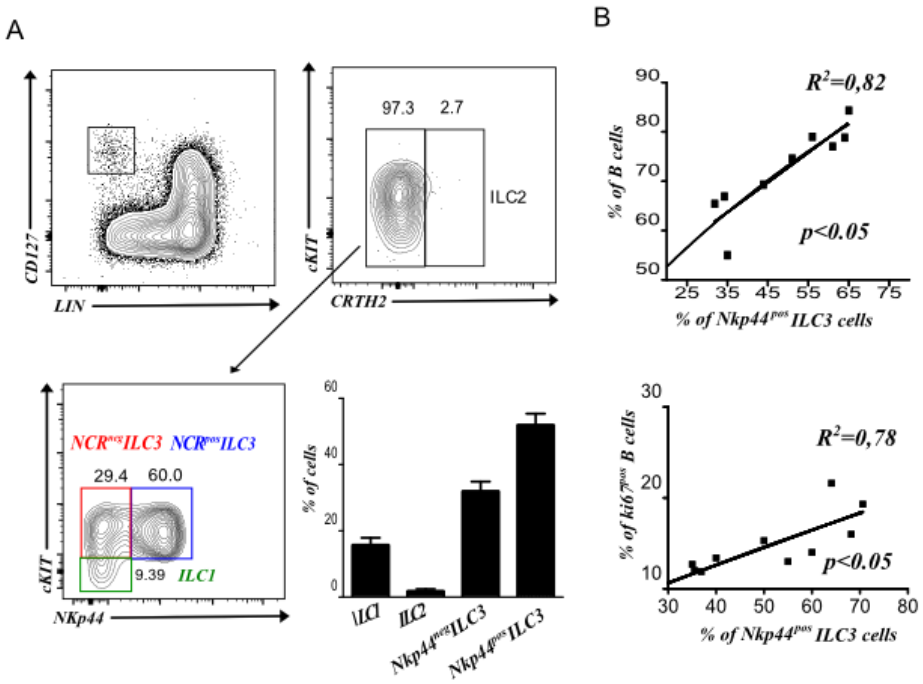


Fig.1 Distribution of ILC populations in human inflamed tonsil and correlation with B cells.

A. Flow cytometry analysis of the expression of CRTH2, c-KIT and NKp44 in tonsil mononuclear cell populations depleted of B cells (CD19), followed by gating on Lin− (CD3,CD14, CD19, BDCA2, CD34, CD94) and CD127 ^{high} cells: c-KIT^{+}NKp44^{pos}cells are called 'NKp44^{pos} ILC3 cells' here; CRTH2^{+} cells are called 'ILC2 cells' c-KIT^{+} NKp44^{neg} are called "NKp44^{neg} ILC3" here, cells c-KIT^{-} NKp44^{neg} are called "ILC1 cells". Numbers in gates (outlined areas) or quadrants indicate percent cells in each
B Correlation between the frequency of NKp44^{pos} ILC3 and the frequency of of B cells (upper panel) or the frequency of KI67^{+} B cells among CD45^{+} cells (lower panel)

Because of this apparent association between inflamed tonsil reactivity and the frequency of NKp44^{pos}ILC3, we hypothesized that accessory cells, such as myeloid dendritic cells (mDCs) might play a role in ILC3 activation and/or proliferation. To evaluate the ability of tonsil-derived mDCs to instruct ILCs, Lin^{neg} HLA-DR^{+} CD11c^{+} cells were sorted from tonsil tissue and co-cultured with autologous CD127^{high} ILCs. Upon 6 day co-culture of total CD127^{high} ILC population with autologous tonsil-derived mDCs, the frequency of NKp44^{pos}ILC3s was significantly increased to the detriment of NKp44^{neg} ILC3s, whereas no difference was observed for ILC1s (Fig.2A). In agreement with this data, only NKp44^{pos}ILC3 acquired the expression of Ki67 proliferation marker (Fig.2B). Confirming their activating ability, mDCs derived from tonsil induced an elevated production of IL-22 in autologous ILC3 (Fig.2C). On the other hand, tonsillar mDCs were, at least in this setting, poor stimulators of IFNγ by tonsillar ILC1s, while they could, as expected by previous reports, efficiently induce IFNγ release by autologous tonsillar NK cells (Fig.2D).

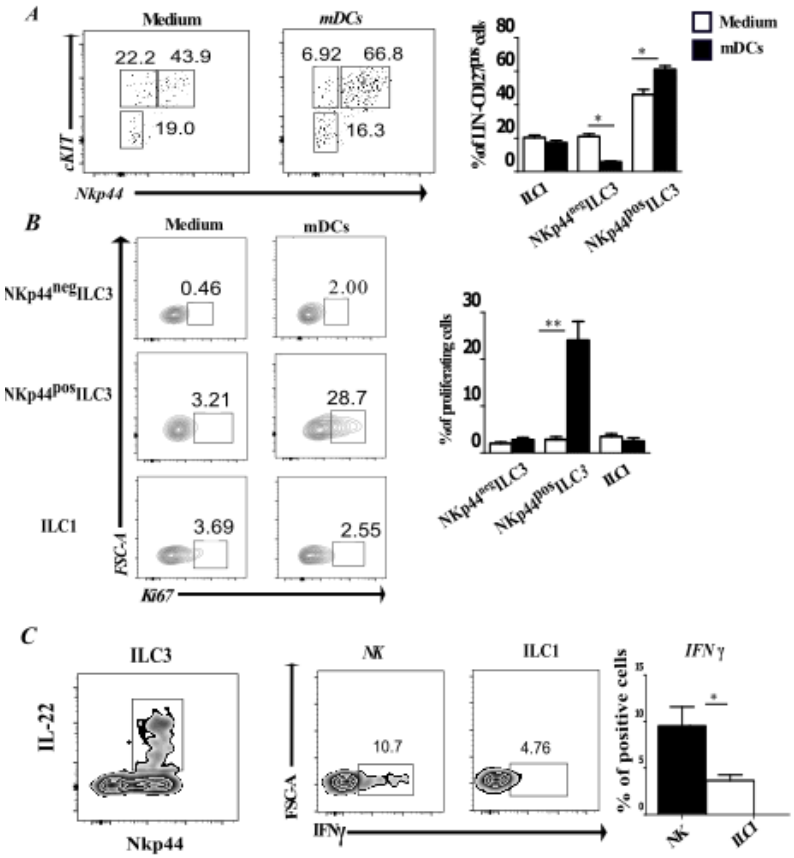


Fig. 2 Tonsillar mDCs selectively induce activation of NKp44^{pos} ILC3
Analysis of phenotype and cytokine profile of human tonsil-derived ILC subsets following mDC stimulation

A) Freshly isolated Lin$^-$ CD127^{high} from tonsil are cultured for 6 days with LIN$^-$ HLA-DR$^+$ CD11C$^+$. ILCs were phenotyped for the expression of c-Kit and NKp44 for the evaluation of ILC subset distribution and numbers in quadrants indicate percent cells in each. One representative experiment (left) and on the right, bar diagrams indicate percentage of ILC1s, Nkp44^{neg} ILC3s and Nkp44^{pos}ILC3s cultured alone (white bar) or with mDCs (black bar), mean percentage ± SEM (right) are shown (n = 6).
B) FACS-sorted CD127^{high} Lin$^-$ cells were stimulated with mDCs for 6 days. Intranuclear expression of Ki67 was measured by FC after gating on different Lin$^-$ subsets, as described in figure 1A and mean percentage ± SEM of KI67+ cells is shown (n = 3)
C) Intracellular cytokine expression of FACS-sorted ILC3, ILC1 and NK cells stimulated with mDCs. Mean percentage ± SEM of IFNγ-producing cells (n = 6)

4.2 BDCA1+ dendritic cells are mainly responsible of mDC-induced ILC3 activation

Considering the ability of tonsil derived-mDCs to induce, among total tonsil ILCs, a selective activation of ILC3s, NKp44^{pos}ILC3 were sorted with high purity from tonsil and cultured with mDCs. We confirmed the capability of mDCs to stimulate ILC3 proliferation and activation as assessed by CFSE dilution of NKp44^{pos}ILC3s and acquisition of activation markers during co-culture (Fig.3A,B). Because both BDCA1$^+$ and BDCA3$^+$ DCs are harboured in human tonsil, we evaluated the role of these distinct myeloid subsets in supporting ILC3 functions. Highly purified BDCA1$^+$ and BDCA3$^+$ DCs were isolated from tonsillar tissues and co-cultured with autologous NKp44^{pos} ILC3s. BDCA1$^+$ DCs were much more potent inducers of ILC3 proliferation and of activation (Fig.3D,E) compared to BDCA3$^+$ DC subset.

The high frequency of NKp44^{pos}ILC3 observed in human inflamed tonsil (Fig.1A) might result from the locoregional proliferation of this ILC subset but also by conversion of NKp44^{neg} ILC3 into NKp44^{pos} cells. Thus, we investigated whether the two distinct subsets of mDCs might also display the potential to drive differentiation from NKp44^{neg} ILC3 to the NKp44-expressing counterpart. NKp44^{neg} ILC3s were sorted and cultured for 6 days with mDCs or, alternatively, with each of the two DC subsets. mDCs efficiently induced

the expression of NKp44 on ILC3s, and, again, BDCA1^{+} DCs were mainly responsible for this induction. Remarkably, the higher acquisition of NKp44 induced by BDCA1^{+}DCs were accompanied by an up-regulation of NKp46 expression on ILC3. (Fi.3F-I).

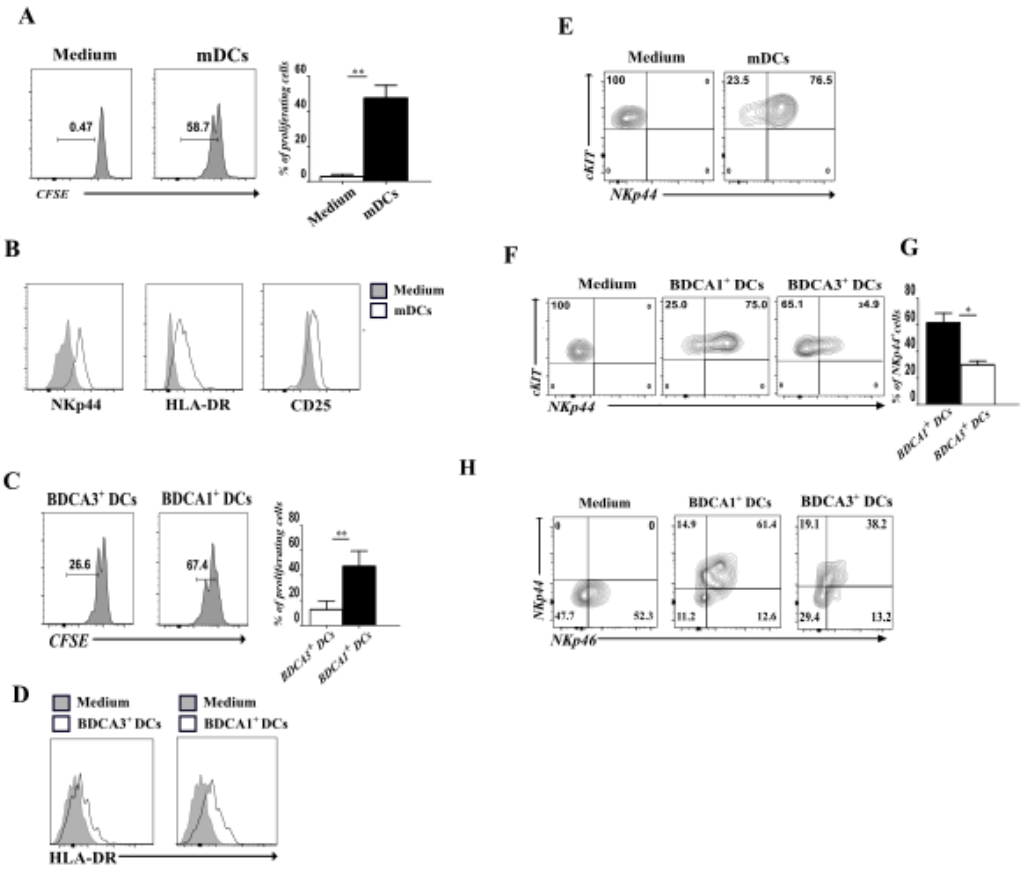


Fig.3 BDCA1+ DCs are potent stimulators of NKp44^{pos} ILC3 proliferation and activation

Analysis of proliferation and activation of human tonsil-derived NKp44^{pos} ILC3 subsets following stimulation with BDCA1+ or BDCA3+ DC subsets

A) Flow cytometry of purified NKp44^{pos}ILC3 from tonsil, stimulated for 6 days with mDCs, and stained with the CFSE proliferation dye. Data shown is representative of 3 experiments

B Surface expression of the indicated activation markers was measured by FC on FACS sorted NKp44^{pos}ILC3 either on unstimulated (gray filled) and stimulated with mDCs (empty).

C) Flow cytometry of purified NKp44^{pos} ILC3 from tonsil, stimulated for 6 days with BDCA1^{+} or BDCA3^{+} DCs, and stained with the CFSE proliferation dye. Data shown is representative of 3 experiments

D Surface expression of the indicated activation marker was measured by FC on FACS sorted NKp44 ILC3 after stimulation with BDCA1+ or BDCA3 DCs

(E-H) Freshly isolated NKp44^{neg} ILC3, from tonsil are cultured for 6 days with mDCs, BDCA1+ or BDCA3+ DCs. Cells were phenotyped for the expression of c-Kit, NKp44 and NKp46, numbers in quadrants indicate percent cells in each. Bar diagrams indicate percentage of NKp44+ cells.

Remarkably, and in agreement with these latter results, BDCA1^{+} DCs were also more effective than BDCA3^{+} DCs in inducing the release of IL-22, IL-8 and GM-SCF, while IL-2, TNFα and IL-17 were not produced by ILC3 upon interaction with DCs (Fig 4).

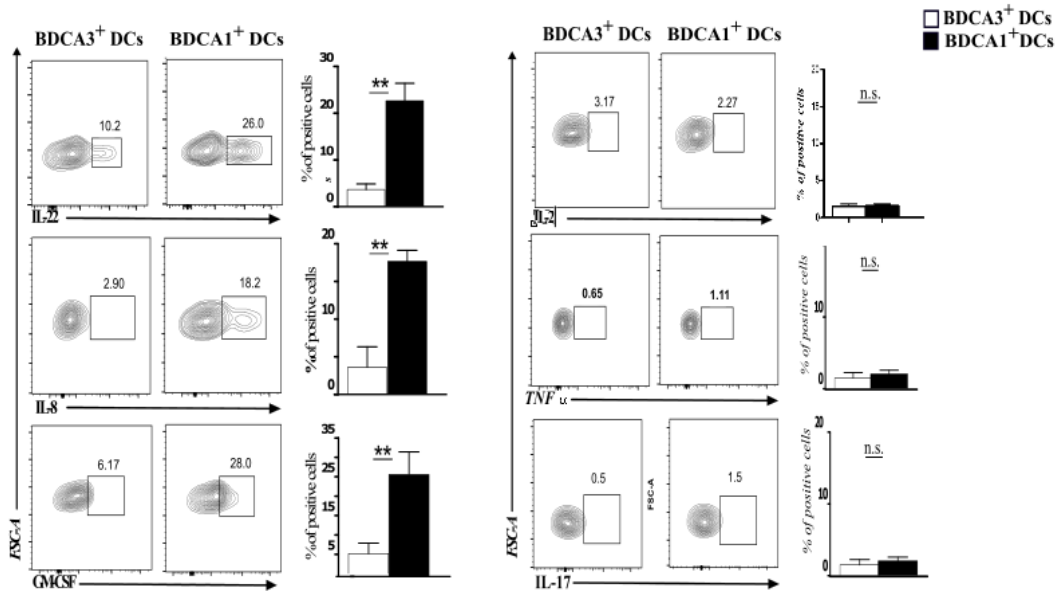


Fig. 4 Stimulation with BDCA1+ induces high level of IL-22, IL-8 and GM-CSF but only very low TNF, IL-2 and IL-17 in NKp44 ILC3.
Ex vivo analysis of cytokine profile of FACS-sorted NKp44^{pos} ILC3 derived from human tonsil after stimulation with autologous myeloid subsets
Intracellular cytokine expression of FACS-sorted nkp44 ILC3 cells stimulated as indicated, was measured by FC. One representative experiment (A) and mean percentage ± SEM of cytokine producing cells are shown (n = 6).

In our experimental conditions, DCs were able to activate ILC3 in absence of exogenous stimuli able to trigger DC activation. We thus wondered whether a possible explanation

could rely on the activated status of DCs, which were isolated from inflamed tonsils. To address this question, BDCA1^{+} DCs were sorted also from peripheral blood and co-cultured with tonsillar NKp44^{pos}ILC3. Also in this case BDCA1^{+} DCs could potently activate NKp44^{pos}ILC3, inducing both high amount of cytokines and cell proliferation (Fig.5A). Interestingly, in the presence of peripheral blood BDCA1^{+}DCs, ILC3s proliferate much more efficiently than autologous tonsillar NK cells, which are known to proliferate in the presence of activated DCs (107) (Fig.5B).

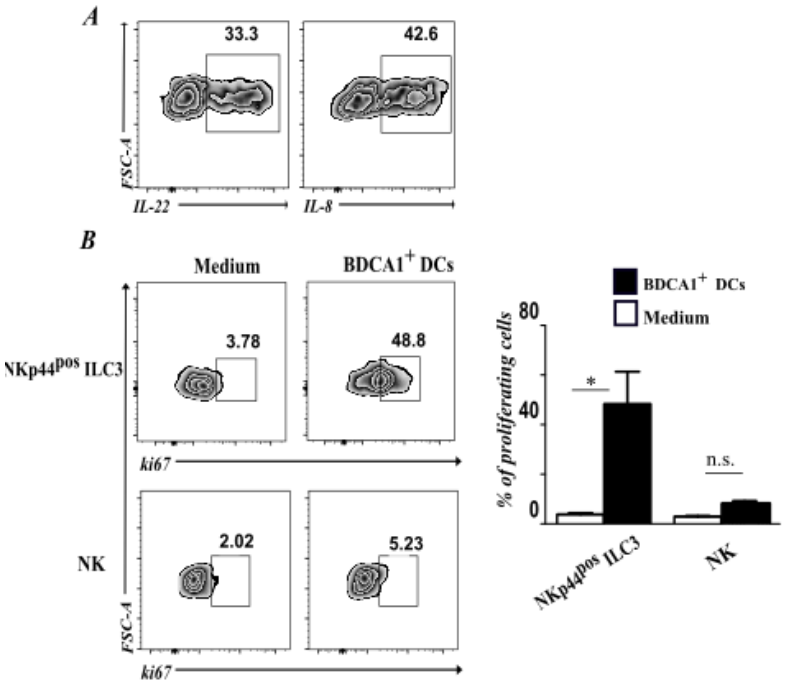


Fig. 5 Circulating BDCA1^{+} DCs efficiently activate NKp44^{pos} ILC3
Ex vivo analysis of cytokine profile and proliferation of FACS-sorted NKp44^{pos} ILC3 derived from human tonsil after stimulation with blood BDCA1^{+} DCs
A Intracellular cytokine expression of FACS-sorted nkp44 ILC3 cells stimulated with blood BDCA1+ DCs was measured by FC.
B) Flow cytometry of purified NKp44^{pos} ILC3 and NK cells from tonsil, stimulated for 6 days with BDCA1^{+} DCs, and stained with the Ki67 proliferation dye. Data shown is representative of 3 experiments

4.3 NKp44^{pos}ILC3 induce activation and production of IL-1β in BDCA1+ DCs

The evidence that DCs freshly isolated from peripheral blood could specifically and strongly activate ILC3s, raised the question whether a specific crosstalk between DCs and ILC3s can occur and, similarly to NK cells, results in a bidirectional activation. Thus, both NKp44^{pos}ILC3 and NK cells were isolated from tonsils and cultured for 24h with BDCA1^{+}DCs directly isolated from peripheral blood. NKp44^{pos}ILC3 could induce the maturation of BDCA1^{+} DCs, as shown by the upregulation of CD40, CD80 and CD86 (Fig. 6A) and appeared much more potent than NK cells in inducing this effect (Fig.6B).

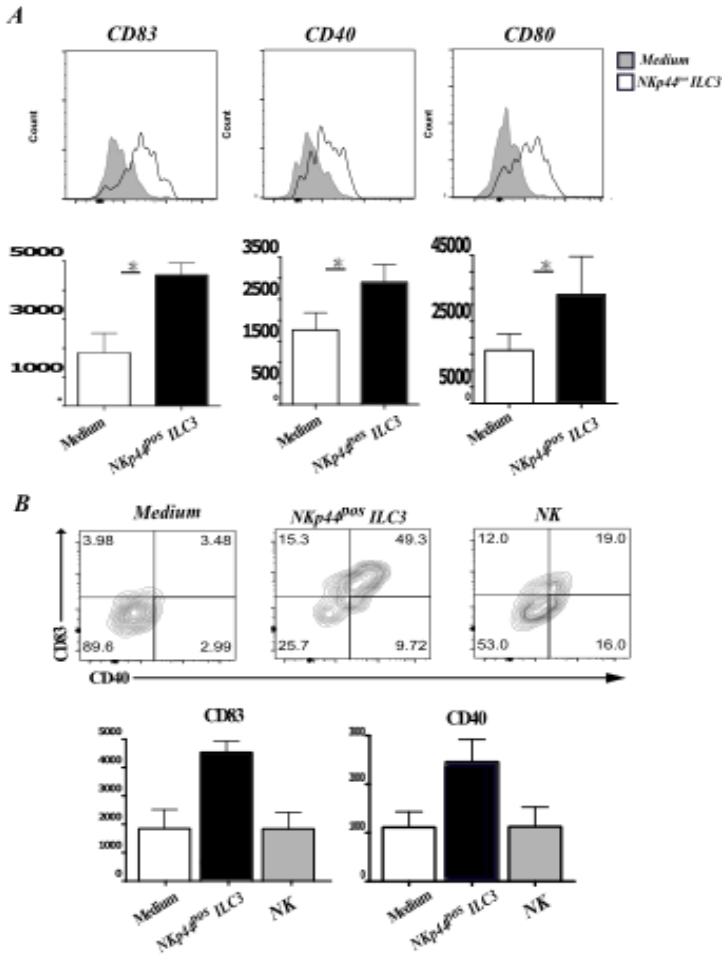


Fig.6 NKp44^{pos} ILC3 induce maturation of BDCA1^{+} DCs
Phenotypical analysis of FACS-sorted BDCA1^{+} DCs derived from blood after stimulation with tonsillar NKp44^{pos} ILC3
A) FACS-sorted peripheral blood BDCA1^{+} DCS cells were cultured with NKp44^{pos} ILC3 and surface expression of CD80, CD40 and CD84 was measured by FC either on BDCA1^{+} DCs left untreated (gray filled) or stimulated with NKp44^{pos} ILC3 (empty). One representative experiment out of three is shown. Bar diagrams indicate geometrical mean fluorescence intensity (MFI) ± SEM (n = 3) of unstimulated (white bar) or stimulated cells (black bar).
B) FACS-sorted blood BDCA1^{+} DCS cells were cultured with tonsillar NKp44^{pos} ILC3 and NK cells and surface expression of CD40 and CD83 was measured by FC on BDCA1+ DCs. One representative experiment out of three is shown. Bar diagrams indicate geometrical mean fluorescence intensity (MFI) ± SEM (n = 3) of unstimulated (white bar), stimulated with NKp44^{pos} ILC3 cells (black bar) or stimulated with NK cells (gray bar)

Therefore, since ILC3s could activate DCs, which in turn could induce ILC3s to proliferate (Fig.3D), we envisaged that NKp44^{pos}ILC3 could stimulate BDCA1^{+} DCs to release soluble factors able to trigger ILC3 proliferation.

It has been previously shown that proliferation of ILC3s requires the presence of definite cytokines (ref). Among the potential cytokines that could participate in DC-induced ILC3 proliferation, IL-7(108, 109), IL-23 (110) and IL-1β (111, 112) were suitable candidates. However, the specific contribution of each of these cytokines on human ILC3 proliferation remained to be clearly determined. To better address this issue, NKp44^{pos} ILC3 cells were sorted from tonsils and, in order to dissect the unique contribution of each single cytokine, they were stimulated with IL-1β, IL-23 or IL-7 alone or in combination. The three cytokines had different impacts on the proliferation of human NKp44^{pos}ILC3. IL-7 was able to mediate the survival but acted as weak inducer of proliferation; IL-23 had no or very limited effect while IL-1β promote strong expansion of NKp44^{pos}ILC3 (Fig.7A).

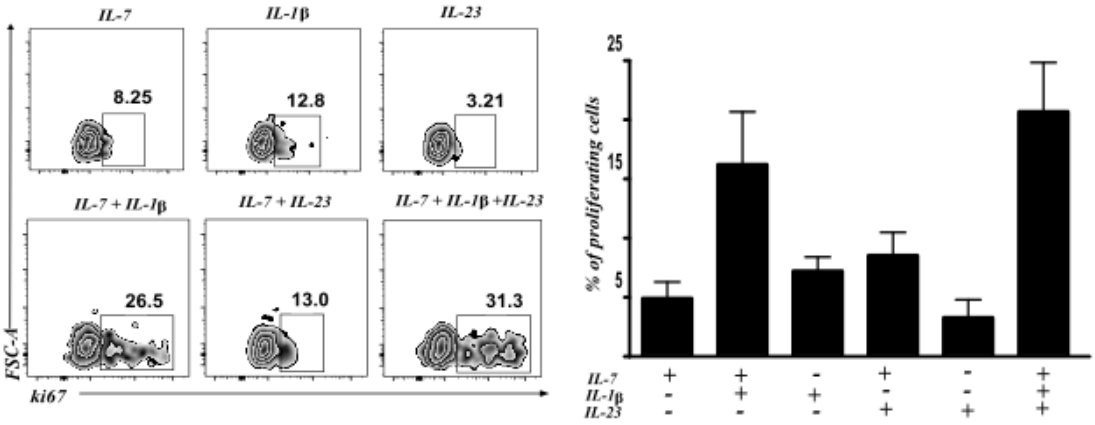


Fig.7 IL-1β strongly promotesNKp44^{pos}ILC3 expansion
Ex vivo analysis of proliferation of FACS-sorted NKp44^{pos} ILC3 derived from human tonsil after stimulation with different cytokine combinations
A. Flow cytometry of purified NKp44^{pos}ILC3and NK cells from tonsil, stimulated for 5 days with IL-7, IL-23 and IL-1b alone or in combination and stained with the KI67 proliferation dye. Data shown is representative of 3 experiments. One representative experiment (A) and mean percentage ± SEM of proliferating cells are shown (n = 6).

Thus, considering that ILC3s could significantly proliferate in the presence of BDCA1^{+}DCs, we investigated whether they could stimulate the release of IL-1β by DCs.

Upon co-culture with NKp44^{pos} ILC3 (36h), BDCA1^{+} DCs produced IL-1β, as evidenced by both intracellular staining and increased content of IL1 mRNA (Fig.7B). Remarkably, DC-mediated proliferation of ILC3 was dependent on IL1ββ production by DCs, since the addition of IL-1b blocking mAbs during co-culture was able to inhibit ILC3 proliferation (Fig.7C).

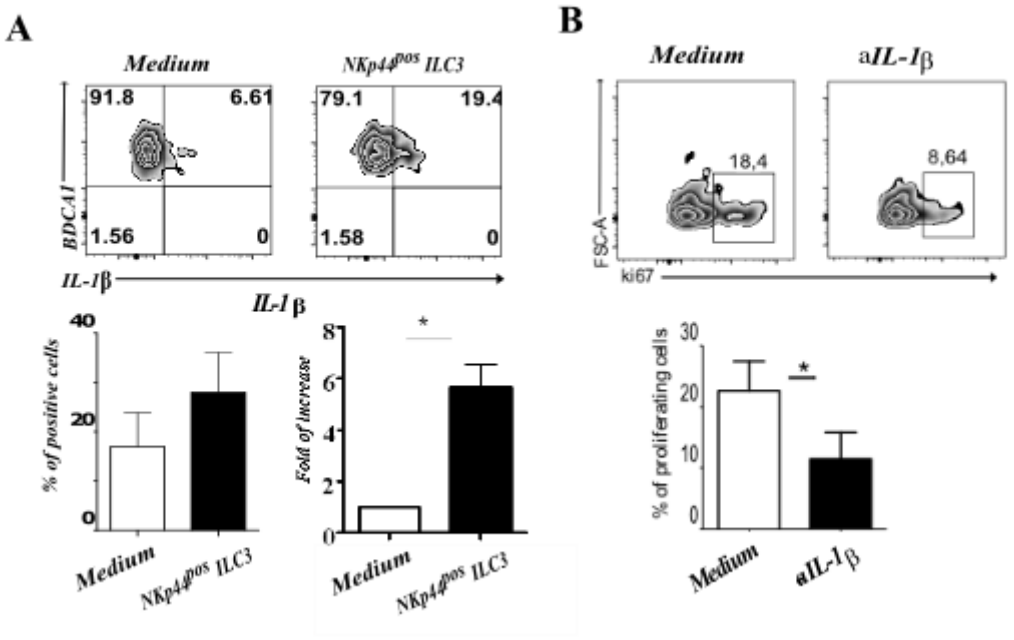


Fig.8 NKp44^{pos} ILC3 induce the release of IL-1β by BDCA1^{+} DCs

Ex vivo analysis of IL-1β production of blood BDCA1+ DCs upon stimulation with NKp44 ILC3

A) Intracellular cytokine expression of FACS-sorted blood BDCA1+ DCs cells stimulated with Nkp44^{pos} ILC3 was measured by FC. Bar indicate mean percentage ± SEM of cytokine producing cells (left). On the right IL-1β mRNA was detected by quantitative reverse transcription–PCR in BDCA1+ DCs alone (white bar) or stimulated with Nkp44 ILC3 (black bar). Bars represent mean fold change± s.e.m. relative to BDCA1+ DCs alone of three independent experiments, after normalizing to glyceraldehyde 3-phosphate (GAPDH)

B) Flow cytometry of purified NKp44^{pos} ILC3 from tonsil, stimulated for 4 days with BDCA1+ DCs, in the presence of IL-1β blocking antibody and stained with the Ki67 proliferation dye.

4.4 IL-1β is a key cytokine during ILC3/DC cross-talk

In addition to proliferation, IL-1β plays pivotal role in other ILC3 functions, including the production of relevant cytokines, We therefore analyzed whether IL-1β released by DCs upon interaction with ILC3 could also affect IL-22 production. Blocking IL-1b during co-culture was able to inhibit not only the expression of proliferation marker Ki67 (Fig.7C) but also the production of IL-22 (Fig.8A). Thus, BDCA1+DC-mediated activation of ILC3 appears to be IL-1β-dependent since it could be inhibited by addition of IL-1β-blocking mAb. Interestingly, phenotypical analysis of BDCA1+DCs, co-cultured in the presence of IL-1β-blocking mAb, revealed a more immature phenotype compared to control, as evidenced by the lower level of CD40 and CD83 markers (Fig.8B). Overall, we concluded that IL-1b is a key cytokine during DC/ILC3 cross-talk, since it promotes ILC3 proliferation, IL-22 production and, likely through the formation of autocrine loops, DC maturation.

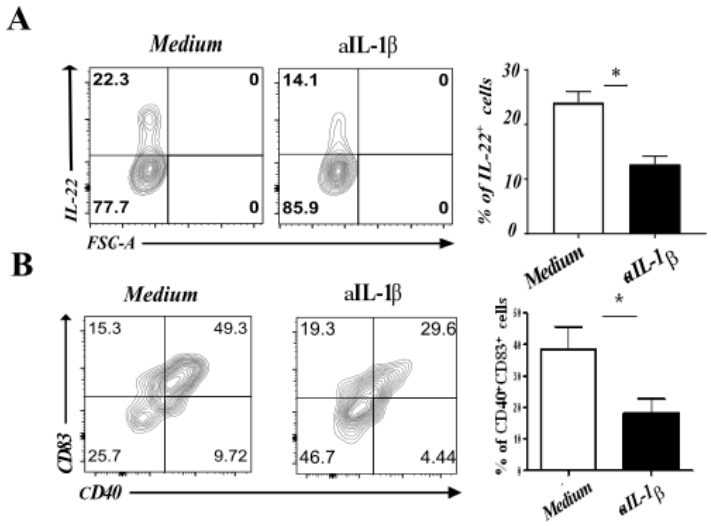


Fig. 9 IL-1β is crucial in supporting NKp44 ILC3/BDCA1+ DCs cross-talk

A) Intracellular cytokine expression of FACS-sorted nkp44 ILC3 cells stimulated with blood BDCA1+ DCs in the presence of aIL-1β was measured by FC

B) FACS-sorted BDCA1+ DCs were analysed for the expression of CD40 and CD83 upon co-culture with NKp44 ILC3 in the presence of aIL-1β .

4.5 IL-1β is released by mDCs following their recognition by ILC3 via DNAM-1 receptor

Having shown that mDCs release IL-1β upon interaction with ILC3s and that IL1β plays a main role during this cellular interaction, it remained to be determined the mechanism by which ILC3 could trigger IL-1b production by mDCs. Interestingly, following interaction with mDCs, ILC3s produce GM-CS which is known to induce IL-1β release by mDCs. Having confirmed that BDCA1^{+} DCs produce IL-1β in the presence of GM-CSF (Fig.9A) (113, 114, 115), we investigated the suitable activating signal able to trigger GM-CSF release by ILC3 upon their interaction with mDCs.

Considering that blood DCs express both PVR and Nectin-2 in immature state and upregulate them during the maturation and DNAM-1 has been described in mouse ILC3s, we analysed the expression of DNAM-1 on human NKp44^{pos}ILC3. We confirmed the expression of DNAM-1 on human ILC3s as well as the expression of PVR and nectin-2 on BDCA1^{+} DCs (fig.9B).

Flow cytometry analysis reveals the DNAM-1 receptor was expressed on human ILC3s to a level similar to that observed in NK cells. Next, hypothesing a possible involvement of DNAM-1 in inducing GM-CSF production by ILC3s, we added a DNAM-1 blocking antibody to the co-culture ILC3/BDCA-1^{+} DCs and evaluated the intracellular content of GM-CSF. Intracellular staining revealed that engagement of DNAM-1 receptor is required for GM-CSF production by ILC3s since this production was importantly reduced in the presence of aDNAM-1 blocking antibody. Altogether this data reveal a novel mechanism of activation of ILC3 mediated by DNAM-1 that may trigger ILC3/mDCs cross-talk generating a self-prepetuating- cycle.

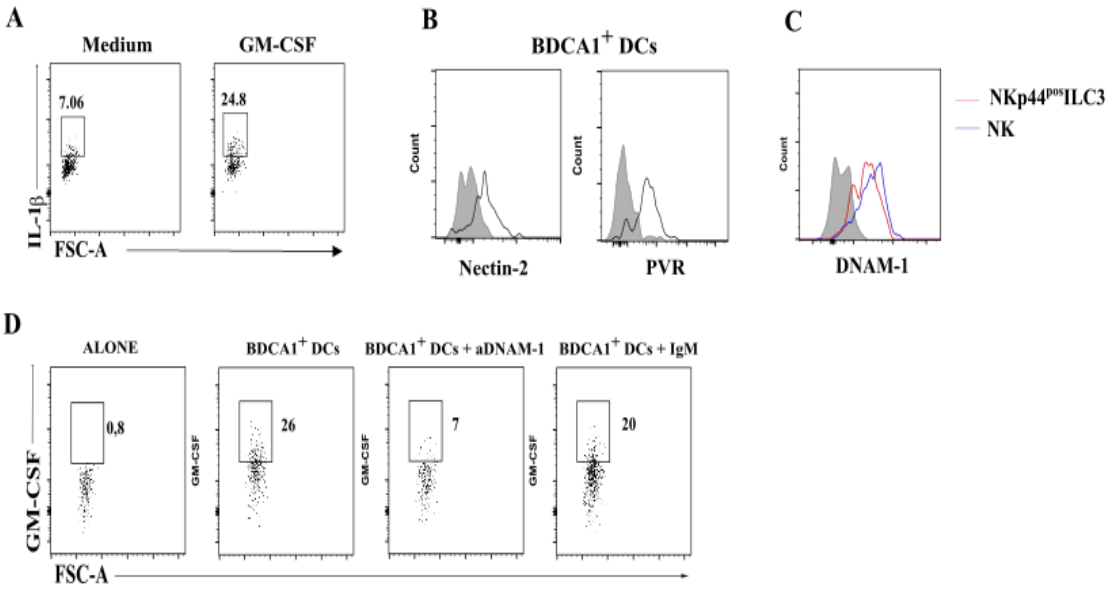


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Fig. 10 Engagement of DNAM-1 receptor is required for initiating ILC3/BDCA1^{+} DC cross-talk

A) Intracellular IL-1β cytokine expression of FACS-sorted BDCA1+ DCs cells left untreated or stimulated with GM-CSF.

B) FACS-sorted BDCA1^{+} DCs were analysed for the expression of PVR and Nectin-2

C) Tonsil- derived Nkp44^{pos} ILC3 and NK cells were analysed for the expression of DNAM-1.

D) Flow cytometry of purified NKp44^{pos} ILC3 from tonsil, stimulated with BDCA1^{+} DCs, in the presence of DNAM-1 blocking or mAb or control IgM and intracellular stained with GM-CSF.

DISCUSSION

ILCs are a population of innate lymphocytes that are relatively rare in comparison to adaptive lymphocytes in lymphoid tissues, but are enriched at barrier surfaces of the mammalian body, such as the skin, lung and intestine, as well as adipose and some mucosal-associated lymphoid tissues 3–6.

ILC1s, ILC2s, and ILC3s mirror the cytokine production and effector functions of CD8+ T cells, TH1, TH2, and TH17 cells. Nevertheless, in contrast to T cells, ILCs do not undergo antigen-driven clonal selection and expansion, and therefore, ILCs act promptly like a population of memory T cells. As a consequence, within hours after infection or injury, the effector cytokines IFNg, IL-5, and IL-13, or IL-17 and IL-22, which can be produced by both ILCs and T cells, are produced mostly by ILCs

Research on the biology of ILCs has already advanced our understanding of their development and the role they play in regulating acute and chronic inflammation as well as tissue repair

Given the fundamental role of ILC3 in the immune system by initiating, regulating and resolving inflammation, a dysregulated response may lead to a variety of pathologies. Further, studies in mouse and in humans have revealed that ILC responses are significantly altered in several disease states.

An important question to clarify with regard to ILCs is how they integrate into the general immune system, how these cells interact with other cells in their surrounding environment to promote, limit or resolve inflammation. Much remains to be uncovered on the activation and function of ILCs

Although many reports have clarified the functions of ILCs and their role in the defense against pathogens, their receptor repertoire and the main signaling pathways

able to trigger effector functions in ILC3s have not been extensively investigated. It was shown that, similar to NK cells, ILC3s can be mainly activated by cytokines

The cellular sources of these cytokine are predominantly activated macrophages and DC. In our project we analyzed the interplay between ILCs and dendritic cells in the context of human inflamed tonsil

We first evaluated the potential of myeloid DCs to interact with ILCs in human tonsil by co-culturing them with autologous LIN- $CD127^{High}$ cells and we observed an accumulation of $NKp44^{pos}$ ILC3 that express KI67 proliferation marker and produce high level of IL-22. Then. we isolated $Nkp44^{pos}$ ILC3 and assessed the contribution of each myeloid DC subsets to ILC3 activation. In human tonsil, there are two main subset of myeloid DCs expressing BDCA or BDCA3 markers.

The results obtained reveal that, between the two subsets of mDCs analysed, a close cooperation occurs between ILC3 and $BDCA1^{+}$ DCs. In particular, we demonstrated that BDCA1+ DCs can increase the pool of $NKp44^{pos}$ILC3 by inducing their proliferation and by promoting the differentiation from $Nkp44^{neg}$ILC3. Moreover, $BDCA1^{+}$ DCs induce also the release of high amount of cytokines, including IL-22, GM-CSF and IL-8 by ILC3s confirming the capability of this subset of DC to finely regulate ILC function. On the other hand, ILC3 enhance the maturation of BDCA1+ DCs and stimulate them to produce IL-1β that appear to be critically involved in BDCA1+ DCs/ILC3 cross-talk since IL-1β blocking antibody could inhibit DC-mediated ILC3 proliferation, IL-22 production and DC maturation. Finally, we described a novel mechanism of activation of human ILC3 that involve DNAM-1 receptor. The presence of DNAM-1 ligand on DCs can trigger GM-CSF production by ILC3, that is an important upstream signal of IL-1β production by DCs. Therefore, activation of $NKp44^{pos}$ ILC3 via DNAM-1 may represent the starting signal able to trigger the bidirectional cytokine cascade generating a self-perpetuating cycle.

Altogether these data clearly shown that myeloid dendritic cells represent a crucial regulator of ILC3 expansion and cytokine release. ILC3, in turn, activate DCs suggesting that the bidirectional interactions between DCs and ILC3 cells result in reciprocal effects on both cell types and may have a critical influence on the outcome of immune responses

In particular, the capability of myeloid DCs to drive expansion and accumulation of Np44^{pos}ILC3 is an information that should be taken in account in all the pathologies where it can observe significantly elevated numbers of NKp44^{pos} ILC3.

Functional analyses of ILC populations have mainly been restricted to cytokine production. ILC3s are able to switch between IL-22 or TNF production, depending on the triggering stimulus. NKp44 triggering in ex vivo isolated ILC3 selectively induces the expression of TNF and IL-2 as well as a coordinated pro-inflammatory program, while cytokine stimulation (IL-23, IL-1, and IL-7) preferentially induces IL-22 and GM-CSF expression. Thus, in according to our previous data, BDCA1^{+} DCs resulted more potent than BDCA3 in inducing cytokine production by NKp44^{pos} ILC3. In particular, upon stimulation with BDCA1^{+} DCs, ILC3 produce high amount of IL-22, GM-CSF and IL-8 but very low level of TNFα, IL-2,and IL-17. This specific pattern of cytokine triggered by mDCs is similar to that induced by cytokine stimulation, and in line with this data, Il-1β represent a key signal in the dialogue between BDCA1^{+} DCs and Nkp44^{pos}ILC3. Beside IL-22, GM-CSF play broad roles in maintaining mucosal homeostasis, but sustained production of GM-CSF by ILCs in inflammatory foci could recruit inflammatory progenitor cells and contribute to differentiation of myeloid effector cells. GM-CSF has been shown to control the differentiation of inflammatory monocytes **(**116**),** and this population was markedly reduced following anti-GM-CSF treatment. GM-CSF may also altered haematopoiesis and accumulation of granulocyte macrophage progenitors as described in T cell dependent colitis (117). In addition,

increases in GM-CSF secreting ILCs are a feature of IBD. However, targeting GM-CSF may not be straightforward in IBD as there are clear host protective functions of GM-CSF in the intestine. Given the multiple activities of GM-CSF, we focused our efforts to improve the understanding of this response identifying DNAM-1 receptor as GM-CSF upstream pathway that may drive a positive feedback loop between ILC3 cells and DCs and generate the proinflammatory cycle within the tissue.

CONCLUSIONS

The present study aimed at better clarify the complex network of ILC interaction with dendritic cells in order to increase the knowledge about ILC roles in both healthy and diseased tissues. In particular, a subset of myeloid DCs, expressing BDCA1 marker, appears to be well suited to support ILC3 activation and proliferation. ILC3s in turn, activate DCs by inducing their maturation and IL-1β release, cytokine critically involved in DC-mediated ILC3 proliferation, IL-22 production and, likely in autocrine way, DC maturation. Also, we described a new mechanism of DC-mediated ILC3 activation involving DNAM-1 receptor. Blocking of DNAM-1 receptor on ILC3 results in a significant less production of GM-CSF by ILC3 that is pivotal for IL-1β release by DCs. Finally, myeloid DCs are able to finely regulate ILC3 function, and the cross-talk ILC3/mDCs may influence the outcome of immune responses. In particular, this cooperation may have broad roles in maintaining mucosal homeostasis, but a sustained bidirectional cytokine cascade may result in a dysregulated response generating a pro-inflammatory cycle within the tissue.

Such kind of knowledge could lead to a new type of immunotherapies based on the manipulation of ILCs that could allow the optimal shaping of immune responses in prevention and therapy while in the context of immunopathology, the manipulation of ILCs may allow blocking the development of detrimental types of immune responses.

BIBLIOGRAPHY

1. Spits H, Di Santo JP. 2011. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. Nat. Immunol. 12:21–27 668 Spits

2. Vivier E, Spits H, Cupedo T. 2009. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? Nat. Rev. Immunol. 9:229–34

3. Spits, H. et al. Innate lymphoid cells – a proposal for uniform nomenclature. Nature Rev. Immunol. 13, 145–149 (2013)

4. Yagi, R. et al. The transcription factor GATA3 is critical for the development of all IL-7Rα-expressing innate lymphoid cells. Immunity40, 378–388 (2014).

5. Sonnenberg, G.F., Mjösberg, J., Spits, H. & Artis, D. Snap Shot: innate lymphoid cells. *Immunity* **39**, 622 (2013).

6.Sonnenberg, G.F. & Artis, D. Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease. *Immunity* **37**, 601–610 (2012).

7. Iwasaki, A. & Medzhitov, R. Regulation of adaptive immunity by the innate immune system. *Science* **327**, 291–295 (2010).

8.Klose, C. S. et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. Cell 157, 340–356 (2014).

9. Xu, W. *et al*. NFIL3 orchestrates the emergence of common helper innate lymphoid cell precursors. *Cell Rep.* **10**, 2043–2054 (2015).

10. Geiger, T.L. *et al*. Nfil3 is crucial for development of innate lymphoid cells and host protection against intestinal pathogens. *J. Exp. Med.* **211**, 1723–1731 (2014).

11. Yu, X. *et al*. The basic leucine zipper transcription factor NFIL3 directs the development of a common innate lymphoid cell precursor. *elife* http://dx.doi.org/10.7554/eLife.0440 (2014).

12. Seillet, C. *et al*. Nfil3 is required for the development of all innate lymphoid cell subsets. *J. Exp. Med.* **211**, 1733–1740 (2014).

13. Kobayashi, T. *et al*. NFIL3-deficient mice develop microbiota-dependent, IL-12/23-driven spontaneous colitis. *J. Immunol.* **192**, 1918–1927 (2014).

14. Seehus, C.R. *et al*. The development of innate lymphoid cells requires TOX-dependent generation of a common innate lymphoid cell progenitor. *Nat. Immunol.* **16**, 599–608 (2015).

15. Aliahmad, P., de la Torre, B. & Kaye, J. Shared dependence on the DNA-binding factor TOX for the development of lymphoid tissue-inducer cell and NK cell lineages. Nat. Immunol. 11, 945–952 (2010).

16. Yang, Q. *et al*. T cell factor 1 is required for group 2 innate lymphoid cell generation. Immunity 38, 694–704 (2013).

17. Mielke, L.A. *et al*. TCF-1 controls ILC2 and NKp46^{+}RORγt^{+} innate lymphocyte differentiation and protection in intestinal inflammation. *J. Immunol.* **191**, 4383–4391 (2013).

18. Serafini N. *et al*. Gata3 drives development of RORγt^{+} group 3 innate lymphoid cells. *J. Exp. Med.* **211**, 199–208 (2014).

19. Hoyler, T. *et al*. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity* **37**, 634–648 (2012).

20. Klein Wolterink, R. G. *et al*. Essential, dose-dependent role for the transcription factor Gata3 in the development of IL-5^{+} and IL-13^{+} type 2 innate lymphoid cells. Proc. Natl Acad. Sci. USA 110, 10240–10245 (2013).

21. Mjosberg J, Bernink J, Golebski K, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. Immunity. 2012;37(4):649-659.

22 114. Spooner, C. J. *et al*. Specification of type 2 innate lymphocytes by the transcriptional determinant Gfi1. *Nat. Immunol.* **14**, 1229–1236 (2013)

23 49. Gordon, S. M. *et al*. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* **36**, 55–67 (2012).

24 50. Szabo, S. J. *et al*. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell***100**, 655–669 (2000).

25 51.Klose, C. S. et al. A T-bet gradient controls the fate and function of CCR6-RORγt+ innate lymphoid cells. Nature494, 261–265 (2013)

26 .52.Rankin, L. C. et al. The transcription factor T-bet is essential for the development of NKp46+ innate lymphocytes via the Notch pathway. Nature Immunol. 14,389–395 (2013)

27 55.Kiss, E. A. et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. Science334, 1561–1565 (2011).

28 56.Lee, J. S. et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. Nature Immunol. 13, 144–151 (2012).

29 57.Veldhoen, M. et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature453, 106–109 (2008).

30.Cherrier, M., Sawa, S. & Eberl, G. Notch, Id2, and RORγt sequentially orchestrate the fetal development of lymphoid tissue inducer cells. J. Exp. Med.209,729–740 (2012).

31.Possot, C. et al. Notch signaling is necessary for adult, but not fetal, development of RORγt+ innate lymphoid cells. Nature Immunol. 12, 949–958 (2011)

32.Wong, S. H. et al. Transcription factor RORα is critical for nuocyte development. Nature Immunol.13, 229–236 (2012)

33. Halim, T. Y. et al. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. Immunity37, 463–474 (2012).

34. Y. Yokota, A. Mansouri, S. Mori, S. Sugawara, S. Adachi, S. Nishikawa, P. Gruss, Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature 397, 702–706 (1999)

35. M. D. Boos, Y. Yokota, G. Eberl, B. L. Kee, Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. J. Exp. Med. 204, 1119–1130 (2007).

36. Male, V. *et al*. The transcription factor E4bp4/Nfil3 controls commitment to the NK lineage and directly regulates Eomes and Id2 expression. J. Exp. Med. 211, 635–642 (2014).

37.M. E. García-Ojeda, R. G. Klein Wolterink, F. Lemaître, O. Richard-Le Goff, M. Hasan, R. W. Hendriks, A. Cumano, J. P. Di Santo, GATA-3 promotes T-cell specification by repressing B-cell potential in pro-T cells in mice. Blood 121, 1749–1759 (2013).

38.N. K. Crellin, S. Trifari, C. D. Kaplan, T. Cupedo, H. Spits, Human NKp44+IL-22+ cells and LTi-like cells constitute a stable RORC+ lineage distinct from conventional natural killer cells. J. Exp. Med. 207, 281–290 (2010).

39. Daussy, C. *et al*. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. *J. Exp. Med.* **211**, 563–577 (2014).

40. Barton, K. *et al*. The Ets-1 transcription factor is required for the development of natural killer cells in mice. Immunity 9, 555–563 (1998).

41. Fuchs A, Vermi W, Lee JS, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-g-producing cells. Immunity. 2013;38(4):769-781

42. Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. Annu Rev Immunol. 2012;30:647-675.

43. Bernink JH, Peters CP, Munneke M, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. Nat Immunol. 2013; 14(3):221-229

44. Moro K, Yamada T, Tanabe M, et al. Innate production of T(H)2 cytokines by adipose tissueassociated c-Kit(1)Sca-1(1) lymphoid cells. Nature. 2010;463(7280):540-544.

45. Fort, M. M. *et al*. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. Immunity 15, 985–995 (2001).

46. Hurst, S. D. *et al*. New IL-17 family members promote Th1 or Th2 responses in the lung: *in vivo* function of the novel cytokine IL-25. J. Immunol. 169, 443–453 (2002).

47. Neill DR, Wong SH, Bellosi A, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature. 2010; 464(7293):1367-1370.

48. Saenz SA, Siracusa MC, Perrigoue JG, et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. Nature. 2010;464(7293):1362-1366.

49. Price AE, Liang H-E, Sullivan BM, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. Proc Natl Acad Sci USA. 2010;107(25):11489-11494

50. Halim, T. Y., Krauss, R. H., Sun, A. C. & Takei, F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. Immunity 36, 451–463 (2012).

51.Monticelli LA, Sonnenberg GF, Abt MC, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. Nat Immunol 2011;12(11):1045-1054.

52. Hendriks RW, et al. Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. Eur J Immunol.1999;29:1912–1918.

53. Robinette, M. L. *et al*. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. Nat. Immunol. 16, 306–317 (2015).

54. J. M. Mjösberg, S. Trifari, N. K. Crellin, et al., "CRTH2 and CD161 define a human IL-25- and IL-33-responsive type 2 innate lymphoid cell type," Nature Immunology, vol. 12, pp. 1055–1062, 2011.

55. Montaldo, E. *et al*. Human RORγt^{+}CD34^{+} cells are lineage-specified progenitors of group 3 RORγt^{+} innate lymphoid cells. Immunity 41, 988–1000 (2014).

56. Cherrier, M., Sawa, S. & Eberl, G. Notch, Id2, and RORγt sequentially orchestrate the fetal development of lymphoid tissue inducer cells. J. Exp. Med. 209, 729–740 (2012).

57. Sciume, G. *et al*. Distinct requirements for T-bet in gut innate lymphoid cells. J. Exp. Med. 209, 2331–2338 (2012).

58. Qiu, J. *et al*. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity 36, 92–104 (2012).

59. Cella M, Fuchs A, Vermi W, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature. 2009;457(7230):722-725.

60. Satoh-Takayama N, Vosshenrich CAJ, Lesjean-Pottier S, et al. Microbial flora drives interleukin-22 production in intestinal NKp46 cells that provide innate mucosal immune defense. Immunity. 2008;29(6):958-970.

61. Cupedo T, Crellin NK, Papazian N, et al. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+CD127+ natural killer-like cells. Nat Immunol. 2009;10(1):66-74

62. Luci C, Reynders A, Ivanov II, et al. Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. Nat Immunol. 2009;10(1):75-82.

63. Hughes T, Becknell B, Freud AG, et al. Interleukin-1beta selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue. Immunity. 2010;32(6):803-814

64. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. Proc Natl Acad Sci USA. 2010;107(24):10961-10966.

65. Sanos, S. L. *et al*. RORγt and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46^{+} cells. Nat. Immunol. 10, 83–91 (2009).

66. Buonocore, S. *et al*. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature 464, 1371–1375 (2010).

67. Vonarbourg, C. *et al*. Regulated expression of nuclear receptor RORγt confers distinct functional fates to NK cell receptor-expressing RORγ^{+} innate lymphocytes. Immunity 33, 736–751 (2010).

68. Yoshida H, Honda K, Shinkura R, Adachi S, Nishikawa S, Maki K, et al. IL-7 receptor alpha+ CD3(−) cells in the embryonic intestine induces the organizing center of Peyer's patches. Int Immunol (1999)

69. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. Nat Immunol (2009)

70. Mebius RE, Miyamoto T, Christensen J, Domen J, Cupedo T, Weissman IL, et al. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3− cells, as well as macrophages. J Immunol (2001)

71. Satoh-Takayama N, Dumoutier L, Lesjean-Pottier S, Ribeiro VS, Mandelboim O, Renauld JC, et al. The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against *Citrobacter rodentium*. J Immunol (2009)

72. Crellin NK, Trifari S, Kaplan CD, Satoh-Takayama N, Di Santo JP, Spits H. Regulation of cytokine secretion in human CD127(+) LTi-like innate lymphoid cells by Toll-like receptor 2. Immunity (2010)

73. Glatzer T, Killig M, Meisig J, Ommert I, Luetke-Eversloh M, Babic M, et al. RORgammat(+) innate lymphoid cells acquire a proinflammatory program upon engagement of the activating receptor NKp44. Immunity (2013)

74. Mjosberg JM, Trifari S, Crellin NK, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. Nat Immunol. 2011;12(11):1055-1062.

75. Dyring-Andersen B, Geisler C, Agerbeck C, et al. Increased number and frequency of group 3 innate lymphoid cells in nonlesional psoriatic skin. Br J Dermatol. 2014;170(3):609-616.

76. Teunissen MBM, Munneke JM, Bernink JH, et al. Composition of Innate lymphoid cell subsets in the human skin: enrichment of NCR(1) ILC3 in lesional skin and blood of psoriasis patients [published online ahead of print March 21, 2014]. J Invest Dermatol.

77. Kim BS, Siracusa MC, Saenz SA, et al. TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. Sci Transl Med. 2013;5(170):170ra16

78. Salimi M, Barlow JL, Saunders SP, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. J Exp Med.2013;210(13):2939-2950.

79. Cortez VS, Fuchs A, Cella M, Gilfillan S, Colonna M.. Cutting edge: salivary gland NK cells develop independently of Nfil3 in steady-state. J Immunol (2014)

80. Schuster IS, Wikstrom ME, Brizard G, Coudert JD, Estcourt MJ, Manzur M, et al. TRAIL+ NK cells control CD4+ T cell responses during chronic viral infection to limit autoimmunity. Immunity (2014)

81. Tessmer MS, Reilly EC, Brossay L.. Salivary gland NK cells are phenotypically and functionally unique. PLoS Pathog (2011)

82. Vivier E, Spits H, Cupedo T. 2009. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nat. Rev. Immunol.* 9:229–34

83. Sawa S, Lochner M, Satoh-Takayama N, Dulauroy S, Berard M, et al. 2011. RORγt+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. Nat. Immunol. 12:320–26

84. Kim S, Han S, Withers DR, Gaspal F, Bae J, et al. 2011. CD117+ CD3− CD56− OX40L high cells express IL-22 and display an LTi phenotype in human secondary lymphoid tissues. *Eur. J. Immunol.* 41:1563–72

85. E. Scandella, B. Bolinger, E. Lattmann, S. Miller, S. Favre, D. R. Littman, D. Finke, S. A. Luther, T. Junt, B. Ludewig, Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. Nat. Immunol. 9, 667–675 (2008)

86.H. Y. Kim, H. J. Lee, Y. J. Chang, M. Pichavant, S. A. Shore, K. A. Fitzgerald, Y. Iwakura, E. Israel, K. Bolger, J. Faul, R. H. DeKruyff, D. T. Umetsu, Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. Nat. Med. 20, 54–61 (2014).

87. Abt MC, Lewis BB, Caballero S, Xiong H, Carter RA, Susac B, et al. Innate immune defenses mediated by two ILC subsets are critical for protection against acute *Clostridium difficile* infection. Cell Host Microbe (2015)

88. Shikotra A, Choy DF, Ohri CM, et al. Increased expression of immunoreactive thymic stromal lymphopoietin in patients with severe asthma. J Allergy Clin Immunol. 2012

89. Chang Y-J, Kim HY, Albacker LA, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. Nat Immunol. 2011;

90. Barlow JL, Bellosi A, Hardman CS, et al. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. J Allergy Clin Immunol. 2012; 129(1):191-8.e1-4.

91. Klein Wolterink RGJ, Kleinjan A, van Nimwegen M, et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. Eur J Immunol. 2012;42(5):1106-1116.

92. Liang H-E, Reinhardt RL, Bando JK, Sullivan BM, Ho IC, Locksley RM. Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. Nat Immunol. 2012;13(1):58-66

93.T. McHedlidze, M. Waldner, S. Zopf, J. Walker, A. L. Rankin, M. Schuchmann, D. Voehringer, A. N. McKenzie, M. F. Neurath, S. Pflanz, S. Wirtz, Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. Immunity 39, 357–371 (2013).

94.E. Hams, M. E. Armstrong, J. L. Barlow, S. P. Saunders, C. Schwartz, G. Cooke, R. J. Fahy, T. B. Crotty, N. Hirani, R. J. Flynn, D. Voehringer, A. N. McKenzie, S. C. Donnelly, P. G. Fallon, IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. Proc. Natl. Acad. Sci. U.S.A. 111, 367–372 (2014).

95. T. A. Doherty, D. Scott, H. H. Walford, N. Khorram, S. Lund, R. Baum, J. Chang, P. Rosenthal, A. Beppu, M. Miller, D. H. Broide, Allergen challenge in allergic rhinitis rapidly induces increased peripheral blood type 2 innate lymphoid cells that express CD84. J. Allergy Clin. Immunol. 133, 1203–1205.e7 (2014).

96. J. Beale, A. Jayaraman, D. J. Jackson, J. D. Macintyre, M. R. Edwards, R. P. Walton, J. Zhu, Y. M. Ching, B. Shamji, M. Edwards, J. Westwick, D. J. Cousins, Y. Y. Hwang, A. McKenzie, S. L. Johnston, N. W. Bartlett, Rhinovirus-induced IL-25 in asthma exacerbation drives type 2 immunity and allergic pulmonary inflammation. Sci. Transl. Med. 6, 256ra134 (2014).

97 115. J. Y. Hong, J. K. Bentley, Y. Chung, J. Lei, J. M. Steenrod, Q. Chen, U. S. Sajjan, M. B. Hershenson, Neonatal rhinovirus induces mucous metaplasia and airways hyperresponsiveness through IL-25 and type 2 innate lymphoid cells. J. Allergy Clin. Immunol. 134, 429–439.e8 (2014).

98. Uhlig HH, McKenzie BS, Hue S, Thompson C, Joyce-Shaikh B, Stepankova R, et al. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. Immunity (2006)

99. Victorino F, Sojka DK, Brodsky KS, McNamee EN, Masterson JC, Homann D, et al. Tissue-resident NK cells mediate ischemic kidney injury and are not depleted by anti-asialo-GM1 antibody. J Immunol (2015)

100. Takayama T., Kamada N., Chinen H., et al. 2010. Imbalance of NKp44(+)NKp46(-) and NKp44(-)NKp46(+) natural killer cells in the intestinal mucosa of patients with Crohn's disease. Gastroenterology 139:882

101. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. J Clin Invest (2008)

102. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kiss EA, Hoyler T, et al. Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. Immunity (2010)

103. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. Immunity (2008)

104. Ciccia F., Accardo-Palumbo A., Alessandro R., et al. 2012. Interleukin-22 and interleukin-22-producing NKp44+ natural killer cells in subclinical gut inflammation in ankylosing spondylitis. Arthritis Rheum. 64:1869.

105. Geremia A., Arancibia-Cárcamo C. V., Fleming M. P., et al. 2011. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. J. Exp. Med. 208:1127.

106 Mette D. Hazenberg and Hergen Spits. Human innate lymphoid cells. Blood 2014 124:700-709; doi: http://dx.doi.org/10.1182/blood-2013-11-42778

107 Ferlazzo G, Pack M, Thomas D, Paludan C, Schmid D, Strowig T, Bougras G, Muller WA, Moretta L, Münz C. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. Proc Natl Acad Sci U S A. 2004 Nov 23; 101(47): 16606–16611.Published online 2004 Nov 9. doi: 10.1073/pnas.0407522101 PMCID: PMC534504 .Immunology

108 Beatriz M Carreno, Michelle Becker-Hapak, and Gerald P Linette CD40 regulates human dendritic cell-derived IL-7 production that, in turn, contributes to CD8+ T-cell antigen-specific expansion. Immunol Cell Biol. 2009 Feb; 87(2): 167–177. Published online 2008 Nov 11. doi: 10.1038/icb.2008.80

109 Lindstedt M1, Lundberg K, Borrebaeck CA. Gene family clustering identifies functionally associated subsets of human in vivo blood and tonsillar dendritic cells. J Immunol. 2005 Oct 15;175(8):4839-46.

110 Roses RE, Xu S, Xu M, Koldovsky U, Koski G, Czerniecki BJ. Differential production of IL-23 and IL-12 by myeloid-derived dendritic cells in response to TLR agonists J Immunol. 2008 Oct 1;181(7):5120-7.

111 Jin JO1, Zhang W2, Du JY2, Yu Q3. BDCA1-positive dendritic cells (DCs) represent a unique human myeloid DC subset that induces innate and adaptive immune responses to Staphylococcus aureus Infection. Infect Immun. 2014 Nov;82(11):4466-76. doi: 10.1128/IAI.01851-14. Epub 2014 Aug 11.

112 Jongbloed SL1, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, Chen CJ, Dunbar PR, Wadley RB, Jeet V, Vulink AJ, Hart DN, Radford KJ. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med. 2010 Jun 7;207(6):1247-60. doi: 10.1084/jem.20092140. Epub 2010 May 17.

113 Morandi B1, Bonaccorsi I, Mesiti M, Conte R, Carrega P, Costa G, Iemmo R, Martini S, Ferrone S, Cantoni C, Mingari MC, Moretta L, Ferlazzo G. Characterization of human

afferent lymph dendritic cells from seroma fluids. J Immunol. 2013 Nov 1;191(9):4858-66. doi: 10.4049/jimmunol.1300760. Epub 2013 Sep 27.

114 Hanif Javanmard Khameneh , Siti Aminah Bte Mohammad Isa ,Lin Min,Fam Wee Nih, Christiane Ruedl . GM-CSF Signalling Boosts Dramatically IL-1Production. Plos One. Published: July 28, 2011. http://dx.doi.org/10.1371/journal.pone.0023025.

115 Oster W1, Brach MA, Gruss HJ, Mertelsmann R, Herrmann F. Interleukin-1 beta (IL-1 beta) expression in human blood mononuclear phagocytes is differentially regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF), M-CSF, and IL-3. Blood. 1992 Mar 1;79(5):1260-5

116 Lenzo JC, Turner AL, Cook AD, Vlahos R, Anderson GP, Reynolds EC, Hamilton JA. 2012. Control of macrophage lineage populations by CSF-1 receptor and GM-CSF in homeostasis and inflammation. Immunology and Cell Biology 90:429–440.

117 Griseri T, McKenzie BS, Schiering C, Powrie F. 2012. Dysregulated hematopoietic stem and progenitor cell activity promotes interleukin-23-driven chronic intestinal inflammation. Immunity 37:1116–1129.