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A ‘natural’ way to provide innate mucosal immunity

James P. Di Santo^{1,2}, Christian A.J. Vosshenrich^{1,2} and Naoko Satoh-Takayama^{1,2}

¹Innate Immunity Unit, Institut Pasteur, Paris, France

²Inserm U668, Institut Pasteur, Paris, France

Correspondence:

James P. Di Santo
Institut Pasteur
25 rue du Docteur Roux
75724 Paris France
Tel. : 33145688696
Fax : 33140613510
james.di-santo@pasteur.fr

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Summary

The mucosal barrier comprises a layered defense system including physio-chemical and immunological strategies to contain commensal microflora while protecting the host against potential pathogens. In contrast to the clearly established and well-characterized role for the adaptive immune system in intestinal defense, our knowledge on innate immune mechanisms that operate in the gut is much less defined. The recent identification of novel innate lymphoid cells (ILC), including 'NK-like' cells that naturally produce IL-22 and appear to play a role in intestinal defense, demonstrate an unexpected and increasing complexity in mucosal innate immunity.

Introduction

Homeostasis between the intestinal microbiota and the immune system is required for efficient energy and metabolite extraction from food, protection from pathogenic microbes, degradation of xenobiotics and maintenance of a competent epithelial barrier [1,2]. This equilibrium necessitates a constant dialogue mediated by pattern recognition receptors (PRRs) on epithelial and immune cells and pathogen-associated molecular patterns expressed by a complex ($\sim 10^{14}$ bacteria and 10^3 species) microbiota [3,4]. The intestinal immune system is a dynamic structure that comprises of a vast network of lymphoid tissues, lymphoid cells and dendritic cells (DCs) within the epithelium and lamina propria (LP). Lymphocytes reinforce the mucosal barrier via IgA production and epithelial cell activation resulting in production of mucus and anti-bacterial peptides. In the LP, DCs capture microbial antigens and drive pro-inflammatory Th17 cells and regulatory T cell responses [5,6]. Distinct bacterial taxa induce the development of lymphoid tissues [7], while Th17 and Treg cell differentiation shape the bacterial community demonstrating the reciprocal impact of the intestinal immune system on symbiotic microbiota [8,9,10].

In addition to the aforementioned adaptive immune component, innate lymphocytes including natural killer (NK) cells [11,12] and mucosal-associated invariant T (MAIT) cells [13] are also found in the intestine. While the function of MAIT cells has not yet been fully defined [14], it is known that NK cells can rapidly detect and then destroy stressed, transformed or infected target cells [15]. As NK cells can be found in lymphoid and non-lymphoid tissues and circulate in the blood and lymph [16], they may have a role in systemic immunosurveillance against various pathogens and tumors. NK cells are also capable of prompt secretion of several types of cytokines (including IFN- γ and TNF- α) that play an important role in the activation of DC and macrophages, thereby increasing their antigen-processing and anti-microbial activity, respectively. Chemokine secretion by NK cells can

influence inflammatory responses and immune defense through recruitment of hematopoietic effector cells [17].

The aforementioned biological functions of NK cells fit squarely within the realm of ‘T-helper 1-like’ (pro-inflammatory) activities of the immune response, supporting the long-held view that NK cells represent a homogeneous cohort of Th1-polarized innate lymphocytes. Still, evidence has accumulated suggesting that NK cells may exhibit phenotypic and functional ‘diversity’ [18,19] that operates at the level of cell activation (attributes of resting, primed and chronically stimulated NK cells differ; [20,21,22-24] as well as at the level of different tissue environments. NK cells that are found in the thymus, lymph node, liver, pancreas and uterus have markedly different cell surface phenotypes and biological functions [25-27,28,29]. Thus tissue microenvironments may condition the ultimate biological effector program of NK cell subsets.

A family of natural cytotoxicity receptors (NCR) has been characterized that appear to play important roles in NK cell target recognition [30]. NKp46 (encoded at the *Ncr1* locus in mice) is highly conserved NCR in mammals and has been proposed as an NK cell-specific marker [31]. The analysis of NKp46⁺ cells in the intestinal mucosa demonstrated that NKp46 also delineates a subset of innate lymphoid cells (ILC) that differ from classical NK cells. This review will update our current knowledge on IL-22-producing NKp46⁺ cells as well as other novel ‘natural’ immune defense pathways that operate at mucosal surfaces.

NKp46 identifies a novel subset of ‘non-NK’ cells in the murine intestine: NCR22 cells

Whereas NK cells have been documented in the intestinal mucosa [11,12], their developmental pathways and biological roles are not fully understood. NK cells, by virtue of their rapid cytokine response, might play an important role in intestinal immunity by interfacing with intestinal DC to regulate immune responses. Alternatively, NK cells may

eliminate stressed or infected cells within the intestinal lamina propria (LP) or epithelium. In this way, intestinal NK cells could contribute to the maintenance of epithelial homeostasis.

Recently, several independent groups have characterized human and murine mucosal lymphocytes expressing natural cytotoxicity receptor family members [32,33,34,35,36]. In the mouse, NKp46 had been previously shown to be highly and specifically expressed in immature and mature NK cells in the mouse [31,37] and ‘knock-in’ mice bearing a GFP reporter at the *Ncr1* locus [38] were used in one study to identify these cells in the gut [32]. Unlike splenic NK cells that were relatively homogeneous in NK1.1 expression, intestinal NKp46⁺ cells clearly comprised distinct subsets, including a large population of CD127⁺NK1.1⁻ cells that lacked many of the markers of mature NK cells (Ly49 family members, CD11b, CD27, NKG2D). Moreover, this subset of NKp46⁺ cells in the intestine lacked perforin, natural cytotoxicity and did not transcribe IFN- γ , and thus bore little functional resemblance to classical NK cells found in the bone marrow or spleen [32,35,36]. In contrast, these unusual gut NKp46⁺ cells expressed the nuclear hormone receptor retinoic acid receptor-related orphan receptor gamma t (ROR γ t, encoded at the *Rorc* locus) that plays an essential role in the development of Lymphoid Tissue-inducer (LTi) cells [39] and in the differentiation of Th17 cells [5]. Further studies demonstrated that intestinal NKp46⁺ cells produced substantial amounts of interleukin (IL)-22, but little IL-17A [32,33,35], thereby distinguishing them from Th17 T cell subsets [5,6,40,41]. Nevertheless, IL-22⁺NKp46⁺ cells required *Rorc* for their development [32,35,36] and their homeostasis was dependent on the presence of microbial flora, as germ-free mice showed a strong decrease in the absolute numbers of NKp46⁺ cells that express *Rorc* and *Il22* [32,35]. While commensal microbe segmented filamentous bacterium (SFB) induces intestinal Th17 T cells [8,9], its role in regulating innate IL-22 production in the gut is unknown. In contrast, development of NK1.1⁺ NKp46⁺ intestinal cells was unaffected by

Rorc deficiency and these cells developed normally in the absence of microbial flora. Taken together, these observations identify a novel subset of intestinal mucosal NKp46⁺ cells that can be clearly distinguished from ‘classical’ NK cells in terms of phenotype, function, transcription factor dependency and interactions with microbial flora (Figure 1).

Studies by the Colonna [●●33] and Spits [●●34] laboratories using human fetal and adult lymphoid tissue identified novel innate lymphocyte subsets characterized by CD127 (IL-7R α), CD56 and NKp44 expression with strong IL-22 production in response to IL-23. ‘NK22’ cells in humans [●●33], like their murine counterparts, express *Rorc* [●●33,●●34], and the aryl hydrocarbon receptor (*Ahr*) shown to be critical in the regulation of IL-22 production [42]. As the mouse intestinal cells express NKp46 and show robust production of IL-22 following IL-23 stimulation [43], we have designated these as ‘NCR22’ cells [●●44], a term that could also apply to the human counterparts. While a standard nomenclature for IL-22-producing innate cells has yet to be defined, we would caution the use of ‘NK’ for these cells that thus far have not been shown to exert natural killing.

Soon after their discovery, the question whether IL-22-producing NKp46⁺ cells were more related (developmentally and functionally) to NK cells or to LTi cells was raised [45,46]. In the next sections, we will examine recent data that impacts on this debate as well as outstanding issues in NCR22 biology that are the subject of ongoing investigation.

Relationship of NCR22 cells to classical NK cells

In addition to the phenotypic and functional differences between NK cells and NCR22 cells, several aspects of the developmental process suggested that these two cell types were distinct (Figure 1). Unlike intestinal NK1.1⁺ cells, NCR22 cells were unaffected by the absence of IL-15 signaling [●●32,●●35]. Still, since immature NK cell precursors are also IL-15-independent [47] and NCR22 have attributes of immature NK cells, it was possible that

NCR22 cells expressing *Rorc* might represent precursors to more mature classical NK cells. Moreover, previous studies demonstrated that fetal *Rorc*⁺ LTi cells could develop into lytic NK1.1⁺ NK cells following culture *in vitro* [48], while immature NK cells in the secondary lymphoid tissue of humans and mice were shown to express *Rorc* [49,50].

A recent series of papers addressed the developmental relationship of human and mouse *Rorc*-expressing NKp46⁺ cells to classical NK cells [●●44,●●51]. Our group showed that NCR22 cells shared a developmental relationship with NK cells and LTi cells by expressing and requiring the transcriptional repressor Id2 for normal development and homeostasis [●●44,52]. We further demonstrated that IL-7 was critically required for NCR22 development. Finally, we used a fate-mapping approach to follow the destiny of *Rorc*-expressing cells and found that the vast majority of classical NK cells (in the bone marrow, liver, lymph node, spleen and intestine) were not descendants of *Rorc*⁺ precursors [●●44]. In an independent study from the Spits' laboratory [●●51], human lineage-negative *RORC*⁺ precursors in fetal and adult lymphoid tissues were shown to acquire CD56 and NCR expression, but failed to develop functional characteristics of mature NK cells (these cells remained IL-22⁺ and lacked perforin and granzyme B expression). Collectively, these studies clearly exclude a major role for *Rorc*-expressing hematopoietic precursors as developmental intermediates in the differentiation of classical NK cells, and reinforce the notion that NCR22 and NK cells derive from distinct developmental pathways. Along these lines, it has been recently shown that the transcription factor E4bp4/Nfil3 is a critical determinant of NK cell development [●●53,●●54] and might provide a 'signature' to dissect classical NK cell differentiation [55]. While E4bp4/Nfil3-deficient mice do not appear to have defects in formation of secondary lymphoid tissue (suggesting normal LTi function), it is not yet reported whether NCR22 differentiation in these mice is affected.

Relationship of NCR22 cells to Lymphoid Tissue inducer (LTi) cells

LTi cells are classically defined as CD3⁻CD4⁺ hematopoietic cells that promote the formation of lymphoid tissues (including LN, PP and intestinal isolated lymphoid follicle, ILF) via a cross-talk with stromal cells that results in the recruitment of B and T lymphocytes to functionally distinct zones [56]. Several signals have been identified that are critical to this process. LTi cells from *Rorc*-deficient mice are not generated and stromal cells in lymphoid tissue anlagen from *Rorc*^{-/-} mice fail to express VCAM and ICAM resulting in abortive lymphoid tissue genesis [39]. LTi cells express CD127 and IL-7 is required for the activation of LTi cells to express membrane-bound lymphotoxin LTα1β2 heterotrimer that activates stromal cells through the LTβR [57]. Chemokine/chemokine receptor interactions (operating through CCR7 and CXCR5) also play a critical role in LTi localization and subsequent lymphoid tissue development [57,58].

While LTi and intestinal NCR22 cells share *Rorc* and *Il7* dependency for their development (Figure 1), the chemokine/chemokine receptor interactions that dictate tissue localization of NCR22 cells are not yet identified. Moreover, there has been some debate about where NCR22 cells localize as two groups identified these cells in cryptopatches (CP) using NKp46 antibodies [●●35,●●36], while another group found Ncr1-GFP expressing cells in the LP and in ILFs, but not in CP [●●32]. One possible explanation might involve the distinction between CP and ILF (that represent a continuum) [59]. However, their localization in the LP might be more consistent with their role in IL-22 production to stimulate epithelial function. Nevertheless, LTβR signals appear dispensable for the development of NCR22 cells (Satoh-Takayama, unpublished), whereas these signals are critical for lymphoid tissue organogenesis suggesting that CP/ILF structures are not obligatory for NCR22 differentiation.

Do LTi and NCR22 cells subserve similar biological functions? Like NCR22 cells, both human and mouse LTi cells have been shown to express IL-22 and also IL-17

[●●34,58,●60]. Nevertheless, IL-17 and IL-22 are not required for the development of lymphoid tissues, suggesting other roles for IL-17/IL-22-producing LTi and NCR22 cells, including inflammation and immunity to infection (see below). The analysis of lymphoid tissue induction *in vivo* is technically challenging, although elegant studies have shown *bona fide* lymphoid tissue organogenesis following transfer of purified LTi cells [61]. *In vitro* surrogates of this process include assays that measure induction of VCAM and ICAM expression on stromal cells using co-culture systems. While human CD56⁺IL-22⁺ cells show activity in this assay [●●34,●●51], it remains to be shown that murine NCR22 cells have LTi-like activity *in vitro* or *in vivo*. In the absence of demonstrable LTi activity, the use of the term ‘NKR-LTi’ [●●35] could be cautioned. Furthermore, since only a fraction of LTi or NCR22 cells produce IL-22 after stimulation, it is not clear whether IL-22 production and LTi-like activity are exerted by the same cell or by functionally distinct subsets within LTi and NCR22 cell populations being analyzed. Defining the functional relationship of IL-22 producing NKp46⁺ cells to classical LTi cells remains an area of further investigation.

Roles for NCR22 cells in intestinal immune defense and tissue homeostasis

IL-22 was initially identified as a member of the IL-10 family and is expressed by Th17 and Th22 T cells, NK cells, NKT cells and $\gamma\delta$ T cells subsets [62]. IL-22 exhibits both anti-inflammatory as well as pro-inflammatory properties, depending on the tissue context [63]. A protective role for IL-22 in immune defense has been demonstrated at mucosal surfaces using lung and intestinal infection models [●●64,●●65]. While the cellular source of mucosal IL-22 remains a matter of debate, IL-22 production is retained in Rag-deficient mice consistent with an innate immune cell origin [●●64]. Considering the prompt production of IL-22 by NCR22 cells, we hypothesized that intestinal NCR22 cells were involved in immune defense against the pathogen *Citrobacter rodentium*. Using Rag-deficient mice lacking the γ_c

chain, we found that the complete absence of NCR22 cells was correlated with accentuated susceptibility to *C. rodentium* [●●32]. In contrast, mice lacking classical NK cells showed partial susceptibility [●●33,43] consistent with a minor role for NK1.1⁺ cells in defense against this pathogen. Surprisingly, Ncr1-deficient mice showed no increased susceptibility to *C. rodentium* [43] suggesting that Ncr1 ligand-driven signals (if present) are not essential for the anti-microbial response. In a separate set of studies, the Flavell lab demonstrated that intestinal IL-22 production from innate cells also was required for immune protection in the dextran sulfate-induced (DSS) colitis model [●●66]. In both *C. rodentium* and DSS models, increased IL-23 production (likely via PRR-triggered DCs) was upstream of the enhanced IL-22 production. The likely cellular target of IL-22 was the intestinal epithelium, since IL-22 was shown to stimulate epithelial cells to promote secretion of anti-microbial proteins (β -defensins, RegIII family members and lipocalin 2) that reinforce mucosal barrier function [●●64,●●65]. Accordingly, RegIII β and RegIII γ transcripts are strongly reduced in epithelial cells from mice lacking intestinal NKp46⁺ cells [●●32,●●35], and while these mice still maintain the capacity to restrict entry of commensal microflora, their susceptibility to pathogenic micro-organisms is accentuated. In this way, intestinal NKp46⁺ cells provide a form of ‘pre-emptive’ immune defense that operates to strengthen the epithelial barrier.

Since IL-22 has also been shown to have pro-inflammatory properties [63], it is possible that NCR22 cells may be involved in intestinal pathologies under certain conditions. Infection by *Toxoplasma gondii* in mice causes an ileitis that is associated with increased local IL-22 production [●67]. While CD4⁺ T cells appear to be the major IL-22 producers in this context, the residual IL-22 production in Rag-deficient mice infected with *T. gondii* suggests additional potential pathological roles for NCR22 cells or LTi cells. Recently, innate IL-23-responsive colitis-promoting cells that appear hardwired for IL-17 production have been identified [●●68]. These *Rorc*-dependent cells appear functionally distinct from NCR22

cells, but share several phenotypic (CD127, CD90) and developmental characteristics (Figure 1). Whether these innate cells represent unique lineages or differentiation states within a single innate cell lineage is unknown.

Roles for NCR22 or NCR22-like cells at other tissue sites

These observations suggest that an intestinal ‘niche’ conditions the differentiation of diverse NKp46⁺ cell subsets that are important for mucosal immunity. An obvious next question is whether NKp46⁺ cells are present at other mucosal sites or in other tissues under steady-state conditions (where they might be involved in tissue homeostasis) or recruited to these sites following infection or inflammation. Sites where IL-22 has been shown to play an important role in immune defense or in inflammation (such as liver, skin, and lung) could be considered. Increased epithelial turnover that is a hallmark of certain skin disorders, including psoriasis [69] may be driven by local hyper-secretion of IL-22. While NCR22 cells are poorly represented in normal skin [●●36], inflammation may result in recruitment of NCR22 cells. Subsequent IL-23 stimulation could then lead to increased IL-22 production and exacerbation of epithelial pathology. As IL-22 polymorphisms have been associated with susceptibility to certain types of intestinal cancer [70], it is interesting to consider the role for NCR22 cells in the initiation and progression of intestinal tumors.

Conclusions

The identification of NCR22 cells that are ‘naturally’ programmed for IL-22 production identifies a novel cellular immune defense mechanism in the gut. In addition to classical NK cells (IFN- γ ⁺), LTi cells (IL-17⁺/IL-22⁺) and Thy1⁺ cells (IL-17⁺/IFN- γ ⁺) [●●68], NCR22 constitute a novel emerging family of innate lymphoid cells (ILC) that have prompt cytokine production capacity and unique roles in immune defense, especially at mucosal

surfaces. Very recently, two additional ILCs were identified in mice: IL-13-producing cells in ‘fat associated lymphoid clusters’ (FALC), and IL-13 and IL-5-producing ‘nuocytes’ (both of these cell types are implicated in immune protection against *Nippostrongylus brasiliensis*) [71,72]. Collectively, these novel ILC demonstrate a remarkable range of functional capacities (Figure 1) that resemble that of differentiated T cell subsets. Understanding the mechanisms governing innate diversity may provide evidence for an evolutionary relationship to those operating in T cells.

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See annotation ••71.

Figure 1. **Diversity of Innate Lymphoid Cells.** Several distinct innate lymphoid cells (ILC) have been recently identified that have diverse roles in innate immunity. NK cells were the first described ILC, followed by IL-22-producing NKp46+ cells (NCR22) and lymphoid tissue inducer (LTi) cells. ILC development relies on distinct cytokine and transcription factors as indicated. NCR22 cells require interactions with commensal flora for their homeostasis. See main text for further details.

Diversity of Innate Lymphoid Cells

