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Naoko Satoh-Takayama, Sarah Lesjean-Pottier, Shinichiro Sawa, Christian A. J. Vosschenrich, Gérard Eberl, et al.. Lymphotoxin- β receptor-independent development of intestinal IL-22-producing NKp46+ innate lymphoid cells.. European Journal of Immunology, 2011, 41 (3), pp.780-6. 10.1002/eji.201040851 . pasteur-01402741

HAL Id: pasteur-01402741

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Submitted on 4 May 2017

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LT β R-independent development of intestinal IL-22-producing NKp46⁺ innate lymphoid cells

Naoko Satoh-Takayama^{1,2}, Sarah Lesjean-Pottier^{1,2}, Shinichiro Sawa³, Christian A.J. Vosshenrich^{1,2}, Gérard Eberl³ and James P. Di Santo^{1,2}

¹Innate Immunity Unit, Institut Pasteur, Paris, France

²Inserm U668, Paris, France

³Lymphoid Tissue Development Unit, Institut Pasteur, Paris, France

Key words: innate / lymphotoxin / cryptopatch / NK cell

Address correspondence to:

James P. Di Santo
Innate Immunity Unit
Department of Immunology
Institut Pasteur
25 rue du Docteur Roux
Paris, France
F-75724
Tel : +331 45 68 86 96
Fax: +331 40 61 35 10
Email : disanto@pasteur.fr

Abbreviations : CP, cryptopatch; ILC, innate lymphoid cell; ILF, isolated lymphoid follicle; LT, lymphotoxin; LT β R, LT β receptor; NCR22, IL-22-producing NKp46⁺ cell; ROR γ t, nuclear hormone receptor retinoic acid receptor-related orphan receptor gamma t.

Abstract

The natural cytotoxicity receptor NKp46 (encoded by *Ncr1*) is an activating receptor expressed by innate lymphoid cells (ILC), including NK cells, a subset of $\gamma\delta$ T cells and intestinal ROR γ t⁺ IL-22⁺ cells (NCR22 cells). Previous studies have proposed a critical role for NCR22 cells in mucosal barrier function through IL-22-mediated production of anti-bacterial peptides from intestinal epithelial cells. Lymphoid tissue inducer (LTi) cells are ROR γ t⁺ ILCs that orchestrate programmed and inducible lymphoid tissue development (including lymph nodes (LN), Peyer's patches (PP) and intestinal isolated lymphoid follicles from cryptopatches (CP)) through cell-surface expressed lymphotoxin (LT) $\alpha_1\beta_2$ heterotrimers. While LTi and NCR22 cells share phenotypic and functional characteristics, their relationship remains unclear, and a model of CP-dependent development of NCR22 cells from LTi cells has been proposed. Here we test this model using LT β R-deficient *Ncr1*^{GFP/+} mice (that lack all LN, PP and CP). We find that NCR22 cells develop normally in the absence of LT β R, achieving functional competence and a normal tissue localization in the lamina propria. These results indicate that organized lymphoid tissue structures are not requisite for the generation of the intestinal NCR22 cell compartment and suggest a dichotomy in the biological roles of LTi and NCR22 cells.

Introduction

Innate lymphoid cells (ILC) represent a diversified, rapid, first-line defense system that comprises circulating cells that patrol tissues and resident cells that provide immuno-surveillance at mucosal surfaces [1]. Natural Killer (NK) cells are one well-characterized ILC subset that detects infected or transformed target cells through a panoply of activating and inhibitory receptors reacting with stress-induced and MHC class I ligands, respectively [2]. Natural cytotoxicity receptors (NCR, including NKp30, NKp44 and NKp46 in man and NKp46 in mice, encoded at the *Ncr1* locus) represent one class of activating receptors that are selectively expressed by NK cells [3]. Ligand recognition by NCRs is implicated in diverse types of immune responses (anti-viral, anti-tumor and autoimmune responses; [4-7], although the precise structure of the specific ligand moieties recognized in some of these contexts remains to be defined.

Recently, several groups have reported novel innate NKp46⁺ cell populations present in the murine intestine having the capacity to selectively produce high levels of IL-22 [8-10]. Using *Ncr1*^{GFP/+} reporter mice [11], we were able to characterize several distinct CD3⁻NKp46⁺ cell subsets in intestinal lamina propria that differed in CD127 (IL-7R α) and NK1.1 expression [8, 12]. IL-22 production appeared restricted to the CD127⁺NK1.1⁻ cell subset that highly expressed the nuclear hormone receptor retinoic acid receptor-related orphan receptor gamma t (ROR γ t). Moreover, these ROR γ t⁺ NKp46⁺CD127⁺ cells lacked cytotoxic potential [8-10]. Recently, we showed that IL-22-producing NKp46⁺ cells (that we will refer to as “NCR22” cells) represent a distinct lineage from conventional NK cells in mice [13]; similar findings were obtained

for the analogous populations in humans [14]. The biological roles for NCR22 cells remain to be fully defined, but based on their distinct cytokine profile, these cells have been proposed to act as amplifiers of mucosal barrier function through IL-22-mediated stimulation of anti-bacterial peptides from intestinal epithelial cells [1, 15, 16].

Lymphoid tissue inducer (LTi) cells are ROR γ t-expressing innate cells involved in lymphoid tissue development [17]. NCR22 cells share some phenotypic (CD127) and functional (ROR γ t, IL-22) characteristics of LTi cells [8-10, 18-20], although there are several notable differences (expression of CD4, NKp46, CD117 and IL-17). Innate and adaptive lymphocytes that express ROR γ t can be found in several anatomically distinct regions of the gut, clustering in Peyer's patches (PP), cryptopatches (CP) and intestinal lymphoid follicles (ILF), while being dispersed throughout the lamina propria [17]. ROR γ t⁺ LTi cells are present in lymph nodes (LN), PP, CP and ILF, and LTi function appears critical for the formation of these distinct lymphoid structures [17, 21]. Whether NCR22 cells participate in lymphoid tissue development is unclear. Previous studies proposed a CP localization and origin for NCR22 cells based on immunohistological analysis using polyclonal anti-NKp46 antibodies [9, 10]. In this model, LTi cells would be a precursor for NCR22 cells that would further differentiate within CP structures under the influence of microbial stimulation by commensal bacteria [9, 22]. How CP (or other lymphoid tissue structures) condition NCR22 differentiation is not known.

To test this CP-dependent model, we studied NCR22 cells in mice deficient for lymphotoxin- β receptor (LT β R). Membrane-bound lymphotoxin (LT) hetetotrimers

(LT $\alpha_1\beta_2$) are expressed on the surface of LTi cells and deliver critical signals to LT β R⁺ stromal cells that initiate and structure lymphoid tissue development [23, 24]. Accordingly, *Ltbr*^{-/-} mice fail to develop LN, PP, CP and ILF [25] and therefore represent a useful model to test whether CP (or other organized lymphoid tissues) are essential for NCR22 homeostasis and function.

Results and Discussion

Absence of NKp46⁺RORγt⁺ cells in intestinal cryptopatches

Several papers have reported that intestinal aggregates, such as cryptopatches (CP) and isolated lymphoid follicles (ILF), harbor various subsets of RORγt⁺ cells, including LTi cells and NKp46⁺ cells [9, 10, 17, 26]. While the role for LTi cells in the maturation of CP to ILF has been clearly demonstrated [21, 27], the functional role for CP-resident NKp46⁺ cells has been less clear. We previously showed that several commercially available polyclonal goat-anti-mouse NKp46 antibodies failed to show sufficient staining specificity, since these reagents were unable to discriminate between NKp46⁺ cells in *Ncr1*^{GFP/+} reporter mice [11] and NKp46⁻ cells in *Ncr1*^{GFP/GFP} mice and mostly reacted with GFP⁻ cells in either of these two strains [12]. Since previous studies localizing NKp46⁺ cells to CP in C57BL/6 mice used these same reagents [9, 10], we first re-assessed this issue using a different set of mouse models (*Ncr1*^{GFP/+} mice) and reagents (monoclonal anti-NKp46 antibodies).

Sections of small intestine from *Ncr1*^{GFP/+} mice were stained with GFP- and RORγt-specific antibodies. We found abundant RORγt⁺ cells within CP structures (Figure 1A). These lymphoid aggregates were clearly identified as CP and were not more mature ILF since they lacked B220⁺ B cells (data not shown). We failed to co-localize NKp46⁺RORγt⁺ cells to these CP structures and moreover, the few NKp46⁺ cells that were detected, appeared RORγt⁻. Similar results were obtained in *Rorc*^{GFP/+} mice [28] stained with anti-GFP antibodies and a mouse-specific NKp46 mAb (Figure

1B). In contrast, in immature ILF (harboring CD3⁺ T cells), we found more frequent NKp46⁺ cells, both ROR γ T⁺ and ROR γ t⁻ (Supplemental Figure 1). These results indicate that NKp46⁺ cells are rare in intestinal CP and contrast with previous results suggesting that up to 30% of ROR γ t⁺ cells in CP were NKp46⁺ [9, 10]. It remains possible that these previous studies showing NKp46⁺ cells within lymphoid clusters were visualizing NKp46⁺ cells in more mature ILF.

Normal development of intestinal NKp46⁺CD127⁺ cells in the absence of LT β R

The lineage relationship between LT_i and IL-22-producing NKp46⁺ cells (here denoted NCR22 cells) is supported by the important role for cytokines (IL-7) and transcription factors (Id2, Rorc) in the development of these two ILC subsets [8-10, 13, 19, 26, 29]. One model proposed that local environmental stimuli within the CP could promote maturation of resident LT_i cells to acquire NK cell markers [9, 22], thereby generating NCR22 cells. If true, then disruption of organized lymphoid structures (through genetic means that block lymphoid tissue development) might impact on the generation, localization and / or function of NCR22 cells. LT β R signaling in lymphoid tissue organizer stromal cells is essential for development of CP, PP, LN and ILF in the gut [20]. We therefore created a model whereby the localization and function of intestinal CD3⁻NKp46⁺ cells could be traced in mice lacking LT β R. *Ncr1*^{GFP/+} reporter mice were placed on the LT β R-proficient (*Ltbr*^{+/+}) or -deficient (*Ltbr*^{-/-}) background. We then examined the phenotype and absolute numbers of CD3⁻NKp46⁺ (GFP⁺) cell subsets that differentially express NK1.1 and/or CD127 as previously described [8].

Previous studies have described the critical role for LT β R signaling in the development of cytolytic NK1.1⁺ NK cells in the bone marrow and spleen [30]. Accordingly, we found that intestinal NK1.1⁺ cells were also selectively ablated in the absence of LT β R (Figure 2A). In contrast, the proportions and absolute numbers of the CD127⁺NK1.1⁻ subset of NKp46⁺ cells were unaltered in *Ltbr*^{-/-} mice (Figure 2A; data not shown). Further phenotypic analysis of the intestinal CD127⁺NK1.1⁻ subset revealed that these cells normally expressed CD244 (2B4), but lacked expression of CD122 and CD4 (Figure 2B). These results suggest that LT β R signaling differentially influences development of classical NK cells and NCR22 cells, being essential for the former but not the latter.

LT β R signals are dispensable for IL-22-production by NCR22 cells

The vast majority of intestinal NKp46⁺ cells that have the CD127⁺NK1.1⁻ phenotype are NCR22 cells that express the transcription factor ROR γ t and promptly produce IL-22 following short-term (4 hr) stimulation with IL-23 [12]. We next assessed whether these characteristics were maintained the intestinal CD127⁺NK1.1⁻ subset in the absence of LT β R. Total small intestinal LPL were cultured *in vitro* with or without IL-23 stimulation, and IL-22 expression was determined using intracellular staining. We observed that steady-state levels of IL-22 were not altered in the absence of LT β R, with IL-22 production being restricted to CD127⁺ cells (Figure 2C). Moreover, IL-23-induced IL-22 production appeared normal in *Ltbr*^{-/-} mice with a similar fraction of IL-22⁺ cells that showed a normal IL-22 staining intensity. Absolute numbers of

NCR22 cells in the intestinal LPL were unchanged in the absence of $LT\beta R$ (Supplemental Figure 2).

We next analyzed whether the phenotype of NCR22 cells was altered in $Ltbr^{-/-}Ncr1^{GFP/+}$ mice. Small intestine LPLs were isolated and stimulated with IL-23, and the phenotype of $CD3^{-}GFP^{+}IL-22^{+}$ cells was analyzed. We found that $Ltbr^{-/-}$ NCR22 cells normally expressed $ROR\gamma t$ and failed to express IL-17 (Figure 2D). Interestingly, NCR22 cells expressed CD90.2 (Thy1.2), a marker that was recently described on an inflammatory IL-17-producing innate lymphoid cell that can be recruited to the large intestine and is implicated in colitis exacerbation [31]. These results demonstrate that CD90 (and Sca-1; data not shown) are expressed by several intestinal ILCs, including NCR22 cells, NK cells and the novel colonic IL-17⁺ ILC [31].

NCR22 cells normally localize to the lamina propria in the absence of $LT\beta R$

Lastly, we used immunohistology to analyze the localization of NCR22 cells in $Ltbr^{-/-}Ncr1^{GFP/+}$ mice. We failed to detect CP or ILFs in the absence of $LT\beta R$, and LN and PP were absent in these mice (data not shown) in agreement with previous reports [25, 27]. We localized NCR22 cells using anti-GFP antibodies in $Ltbr^{-/-}Ncr1^{GFP/+}$ mice. We found dispersed $GFP^{+}ROR\gamma t^{+}$ cells in the lamina propria that lacked CD3 (Figure 3). In addition, $ROR\gamma t^{+}$ cells as well as classical NK cells ($GFP^{+}ROR\gamma t^{-}$) and other undefined $ROR\gamma t^{+}$ ILCs ($CD3^{-}GFP^{-}$) were observed. No obvious differences in the distribution or frequency of these different subsets were detected in mice invalidated for $LT\beta R$ compared to control mice (data not shown). These results rule out an obligatory

role for $\text{LT}\beta\text{R}$ signaling and for organized lymphoid tissues structures for the development, localization and function of NCR22 cells.

Concluding remarks

A subset of intestinal innate lymphoid cells (ILC), including NCR22 cells, IL-17⁺ innate cells and LTi cells, demonstrate a shared dependence on the transcription factor $\text{ROR}\gamma\text{t}$ [8, 19, 31]. Still, these distinct ILCs are involved in different biological processes, such as lymphoid tissue organogenesis (LTi), maintenance of epithelial barrier function (NCR22 cells) and pro-inflammatory pathologies (IL-17⁺ colonic ILCs). The developmental relationships of these different $\text{ROR}\gamma\text{t}^+$ ILCs remains unclear as are the molecular mechanisms that generate and regulate their effector functions. We provide evidence that cryptopatches, (where one ILC - LTi cells - reside) are not required for the differentiation of an apparently distinct ILC subset (NCR22 cells). The fact that NCR22 cell differentiation unfolds normally in the absence of $\text{LT}\beta\text{R}$ demonstrates that lymphoid tissues are not essential for NCR22 function and do not support a model of CP-dependent NCR22 differentiation from LTi cells [9]. Nevertheless, the important role for $\text{LT}\beta\text{R}$ signaling in intestinal epithelial defense has been recently demonstrated [32]. In this report, $\text{LT}\beta\text{R}$ -deficient mice were shown to be highly susceptible to infection by *Citrobacter rodentium*, an attaching and effacing gram⁻ bacterium that requires ILC-produced IL-22 for immune defense [33]. Interestingly, epithelial cells were shown to be the $\text{LT}\beta\text{R}^+$ targets in this context, suggesting that NCR22 cells could provide two critical signals (lymphotoxin $\text{LT}\alpha_1\beta_2$

and IL-22) that collaborate to activate epithelial cells. Identification of the molecular cues that allow selective localization of NCR22 cells in the lamina propria will be important to better understand the regulation of this critical ILC-epithelial cell crosstalk that promotes intestinal immune defense.

Materials and Methods

Mice

Lymphotoxin- β receptor-deficient (*Ltbr*^{-/-}) mice [34] on C57BL/6 background were kindly provided by Dr. Bernhard Ryffel (CDTA, Orleans) and were crossed with *Ncr1*^{GFP/+} mice [11] (kindly provided by Dr. Ofer Mandelboim (Hebrew University, Jerusalem) to generate *Ltbr*^{+/+}*Ncr1*^{GFP/+} or *Ltbr*^{-/-}*Ncr1*^{GFP/+} mice. Mice harboring a BAC with GFP under the control of the *Rorc* promoter (*Rorc*^{GFP/+} mice) have been described previously [28]. All mice were housed under specific pathogen-free condition at the Institut Pasteur. Mice (6-12 weeks old) were used for experiments that followed the guidelines provided by the Animal Care Use Committee of the Institut Pasteur and were performed in accordance with French law (authorizations A7515-06, A7515-07 and 75-579).

Cell preparation, flow cytometric analysis and intracellular cytokine staining

Isolation and flow cytometric analysis of lamina propria lymphocytes (LPL) was performed as previously described [8, 12]. Fluorochrome-coupled antibodies included anti-CD3 (eBioscience), anti-CD127 (eBioscience), anti-NK1.1 (BioLegend), anti-2B4 (eBioscience), anti-CD90.2 (Thy1.2, BD Pharmingen), anti-CD122 (eBioscience), and anti-CD4 mAb (eBioscience). Intracellular staining was performed as described [8, 12].

Immunohistology

Immunohistological analysis of 6 μm sections of small intestine was performed as previously described [8, 12]. Antibodies included anti-GFP (Invitrogen), mAb anti-ROR γ t (PE; eBioscience), anti-NKp46 (29A1.4; eBioscience), hamster anti-CD3 (Invitrogen), Alexa488-conjugated anti-rabbit IgG and anti-hamster Alexa647 conjugated IgG (Invitrogen).

Statistical analysis

Statistical differences between groups were determined using the unpaired Student *t* test. P values <0.05 were considered significant.

Acknowledgments

We would like to thank Ofer Mandelboim (Hebrew University, Jerusalem) and Bernhard Ryffel (CDTA, Orleans) for *Ncr1*^{GFP/+} mice and LTβR-deficient mice, respectively. N.S.-T. was the recipient of fellowship from Japan Society for the Promotion of Science (JSPS), Japan. The Innate Immunity Unit at the Institut Pasteur receives support from the Institut Pasteur, Inserm, and the ANR.

Conflict of Interest

The authors declare no conflict of interest with respect to this work.

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Figure Legends

Figure 1. **Analysis of NKp46⁺ cells in intestinal cryptopatches.** A) Sections from the small intestine of *Ncr1*^{GFP/+} mice were stained with anti-GFP (green) and anti-RORγt (red). Right panels are showing each single staining. B) Sections from the small intestine of *RORγt*^{GFP/+} mice stained with anti-GFP (green) and anti-NKp46 (red). CP structures are shown. Magnification: 40X.

Figure 2. **Analysis of intestinal NKp46⁺ cell subsets from *Ltbr*^{+/+}*Ncr1*^{GFP/+} and *Ltbr*^{-/-}*Ncr1*^{GFP/+} mice.** A) Comparison of NK1.1 versus CD127 (IL-7Rα) expression on gated CD3⁻GFP⁺ lamina propria lymphocytes. Representative results of five independent experiments are shown. B) Expression levels of CD224 (2B4), CD122 (IL-2Rβ) or CD4 on the CD127⁺NK1.1⁻ subset of intestinal CD3⁻NKp46⁺ cells. Staining using *Ltbr*^{+/+}*Ncr1*^{GFP/+} mice (shaded), *Ltbr*^{-/-}*Ncr1*^{GFP/+} mice (solid line) mouse) and isotype control staining (dashed line). Data are representative of 3 independent experiments. C) Comparison of IL-22-production from gated CD3⁻GFP⁺ cells in *Ltbr*^{+/+}*Ncr1*^{GFP/+} and *Ltbr*^{-/-}*Ncr1*^{GFP/+} mice. Total LPL were stimulated with IL-23 for 4 hours. Control un-stimulated LPL samples are shown for comparison. Data are representative of four independent experiments. D) Phenotype of NCR22 cells in *Ltbr*^{+/+}*Ncr1*^{GFP/+} and *Ltbr*^{-/-}*Ncr1*^{GFP/+} mice. Data are representative of three independent experiments.

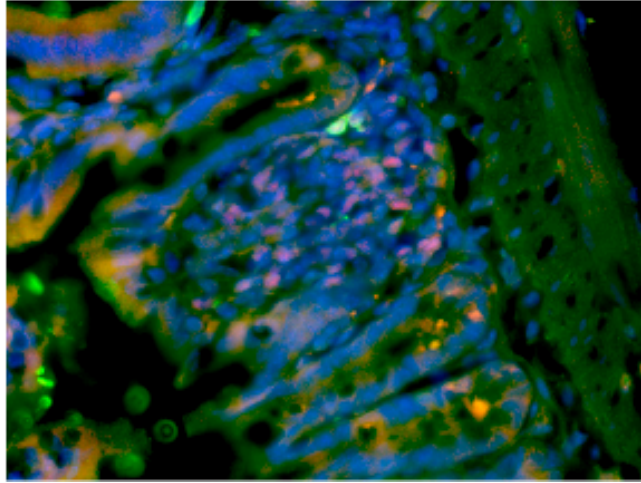
Figure 3. **Immunohistological analysis of *Ltbr*^{-/-}*Ncr1*^{GFP/+} mice.** Sections from the small intestine of *Ltbr*^{-/-}*Ncr1*^{GFP/+} mice were stained with DAPI (blue), anti-GFP (green), anti-RORγt (red) and anti-CD3 (white). The left-top panel show merged image. Bottom panels show individual fluorescence images. Magnification: 20X.

Supporting Information

Supplemental Figure 1. Serial sections from small intestine of *ROR γ ^{GFP/+}* mice were stained as follows: A) anti-GFP (green), anti-NKp46 (red) and anti-CD3 (blue). Merged and individual images are shown. B) anti-GFP (green), anti-CD11c (red) and anti-B220 (blue). Merged image is shown. Magnification: 40X.

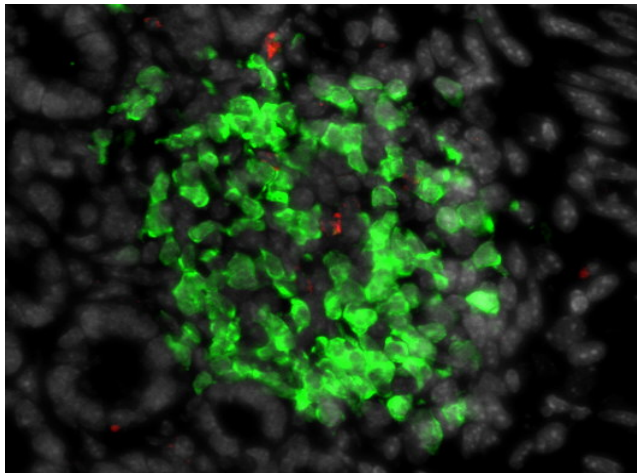
Supplemental Figure 2. Comparison of absolute numbers of IL-22⁺CD127⁺ innate lymphoid cells (CD3⁻GFP⁺) in isolated LPL cells from *Ltbr^{+/+}Ncr1^{GFP/+}* mice and *Ltbr^{-/-}Ncr1^{GFP/+}* mice. Results are from four mice of each genotype analyzed.

A



DAPI/GFP (Ncr1) /ROR γ t

B



GFP (ROR γ t) /NKp46

