Skin and Peripheral Lymph Node Invariant NKT Cells Are Mainly Retinoic Acid Receptor-Related Orphan Receptor γt^+ and Respond Preferentially under Inflammatory Conditions[1]

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Lymph nodes (LNs) have been long considered as comprising few invariant NKT (iNKT) cells, and these cells have not been studied extensively. In this study, we unravel the existence of stable rather than transitional LN-resident NK1.1$^-$ iNKT cell populations. We found the one resident in peripheral LNs (PLNs) to comprise a major IL-17-producing population and to express the retinoic acid receptor-related orphan receptor γt (RORγt). These cells respond to their ligand α-galactosylceramide (α-GalCer) in vivo by expanding dramatically in the presence of LPS, providing insight into how this rare population could have an impact in immune responses to infection. PLN-resident RORγt^+ NK1.1$^-$ iNKT cells express concomitantly CCR6, the integrin α-chain α_E (CD103), and IL-1R type I (CD121a), indicating that they might play a role in inflamed epithelia. Accordingly, skin epithelia comprise a major RORγt^+ CCR6$^+$CD103$^+$CD121a$^+$ NK1.1$^-$ cell population, reflecting iNKT cell composition in PLNs. Importantly, both skin and draining PLN RORγt^+ iNKT cells respond preferentially to inflammatory signals and independently of IL-6, indicating that they could play a nonredundant role during inflammation. Overall, our study indicates that RORγt^+ iNKT cells could play a major role in the skin during immune responses to infection and autoimmunity. ***The Journal of Immunology,*** **2009, 183: 2142–2149.**

Mouse Vα14 NKT (invariant NKT (iNKT))[3] cells are a small subset of CD4$^+$ or CD4$^-$CD8$^-$ double-negative (DN) $\alpha\beta$ T cell population expressing a semi-invariant TCR composed of an invariant Vα14Jα18 chain combined with one of three Vβ segments (Vβ8, 7, or 2) (1). They are capable of recognizing CD1d-presenting self-glycolipids, such as isoglobotrihexosylceramide or exogenous glycolipids found in certain bacteria, as well as the synthetic α-galactosylceramide (α-GalCer), originally isolated from marine sponges.

Mouse iNKT cells can be identified using CD1d tetramers loaded with α-GalCer regardless of their expression of differentiation markers such as NK1.1, the molecule most commonly associated with the NK lineage (2, 3). The usage of CD1d tetramers allows for the identification of iNKT cells that are devoid of most NK-associated markers, including NK1.1 (4, 5). Because NK1.1$^-$ appear before NK1.1$^+$ iNKT cells during ontogeny, in the thymus, spleen, and liver, and because NK1.1$^+$ iNKT cells accumulate progressively and dominate the iNKT cell population in these organs in older mice, it has been accepted that the NK1.1$^-$ iNKT cells are immature and that their ultimate fate is to become NK1.1$^+$ iNKT cells. Therefore, studies aimed at understanding iNKT cell functions have focused mostly on the NK1.1$^+$ iNKT cell population.

In response to TCR ligation, iNKT cells promptly produce large amounts of both IFN-γ and IL-4. They have been implicated in the regulation of immune responses associated with a broad range of diseases and have been shown to promote inflammatory Th1 and immunomodulatory Th2 responses (6). Several hypotheses have been proposed to reconcile such a diversity of functions. iNKT cell subsets differing in respect to their ability to secrete Th1- vs Th2-type cytokines was suggested, among other possibilities.

Numerous studies aimed at identifying distinct functional subsets among iNKT cells have been performed. In humans, these studies showed that DN iNKT cells produce predominantly Th1 cytokines, whereas CD4$^+$ iNKT cells are the exclusive producers of Th2-type cytokines upon primary stimulation (7). In mice, there is no evidence for a disparity in cytokine secretion between CD4$^+$ and DN iNKT cells, although one study did show distinct functional properties between liver CD4$^+$ and DN iNKT, the latter being more efficient at rejecting tumors (8). Other studies pointed out phenotypic distinctions among iNKT cells based on their expression of chemokine receptors and adhesion molecules, depending on the organ where they reside: CXCR5 in the spleen (9), and CXCR6 and LFA-1 in the liver (10, 11).

Several lines of research have converged to the recent discovery of iNKT-expressing retinoic acid receptor-related orphan receptor γt (RORγt) and secreting IL-17. However, neither of these studies shows a phenotype that can distinguish between IL-17$^+$ and IL-17$^-$ NK1.1$^-$ iNKT cells (12–15). Furthermore, whether these

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[3] Abbreviations used in this paper: iNKT, invariant NKT; 7-AAD, 7-aminoactinomycin D; α-GalCer, α-galactosylceramide; BM-DC, bone marrow-derived dendritic cell; DC, dendritic cell; DN, double negative; LN, lymph node; PLN, peripheral LN; RORγt, retinoic acid receptor-related orphan receptor γt; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; WT, wild type.

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cells, representing minor iNKT cell populations in the liver and spleen, secrete exclusively IL-17 was not reported. Finally, McNab et al. (16) suggested the existence of mature, stable NK1.1$^-$ iNKT cells in the spleen. However, a detailed functional and phenotypical characterization of this subset is still lacking.

There are few reports concerning lymph node (LN)-resident iNKT cells because of their rarity. Early reports using CD1d tetramers indicated that they have a high frequency of NK1.1$^-$ iNKT cells (3). Other studies indicated phenotypic and functional differences between iNKT cells colonizing mediastinal and mesenteric (splanchnic) and peripheral LNs (PLNs) (17); however, these studies focused on NK1.1$^+$ iNKT in Vα14 transgenic animals.

In this study, we uncovered LN NK1.1$^-$ iNKT cell populations representing mature iNKT cells at a final stage of their development. Contrary to previously described organ-resident iNKT cells, which are mostly NK1.1$^+$, NK1.1$^-$ iNKT cells represent the majority of CD4$^+$ and DN iNKT cells in LNs. The latter, mainly located in PLNs, produce IL-17, but not IFN-γ or IL-4, and express the nuclear receptor and the transcription factor RORγt. We also found that most of skin-resident iNKT, like PLN iNKT cells, express CCR6, CD103, and CD121a in addition to producing IL-17, and that both populations respond in vivo preferentially to inflammatory signals and could be involved in immune responses to infection and autoimmunity.

Materials and Methods

Mice

C57BL/6 mice were purchased from Janvier. IL-6$^{-/-}$ mice were purchased from The Jackson Laboratory. *Rorc(γt)-Gfp^{TG}* mice were generated, as previously described (18), and backcrossed nine times to C57BL/6. All mice were maintained under specific pathogen-free conditions at our animal facility, and experimental studies were in accordance with the Institutional Animal Care and Use Guidelines.

In vitro stimulation

Cell suspensions were prepared from thymus, liver, spleen, and LNs and enriched in iNKT cells by depletion of CD8$^+$ and CD19$^+$ cells using mouse depletion dynabeads (Invitrogen) and following the manufacturer's instructions. Ear skin was directly incubated in RPMI 1640 medium containing collagenase type VIII (Sigma-Aldrich) and DNase I (Sigma-Aldrich), and cell suspensions were obtained, as described previously (18). Cells were incubated with 0.1 ng/ml α-GalCer-preloaded bone marrow-derived dendritic cells (BM-DCs), obtained after culture with GM-CSF (PeproTech) at 2 ng/ml for 6 days, or PMA (5 ng/ml)/ionomycin (500 ng/ml), for 4 h in the presence of 5 μg/ml brefeldin A (all purchased from Sigma-Aldrich). IL-17 content in culture supernatants of sorted LN subsets, stimulated with BM-DCs in the presence of 100 ng/ml α-GalCer, was measured by ELISA using the DuoSet ELISA kit from R&D Systems and following the manufacturer's instructions.

Abs and flow cytometry

PE-Texas Red- and PerCP-Cy5.5-conjugated mAbs against B220 clone RA3-6B2; PerCP-Cy5.5- and Alexa Fluor 700-conjugated mAbs against CD4 clone RM4-5; PerCP-Cy5.5-conjugated mAb against NK-1.1 clone PK136; FITC- and PE- conjugated mAb against Ki67 clone B56; PE-conjugated mAb against CD121a clone 35F5; Alexa Fluor 647-conjugated mAb against CCR6 clone 140706; nonconjugated mAbs against CD8α clone 53-6.7, and CD19 clone 1D3; and 7-aminoactinomycin D (7-AAD) were obtained from BD Biosciences. Alexa Fluor 488-conjugated mAb against IL-17 clone eBio17B7; PE-conjugated mAbs against TCRβ clone H57-597, IL-4 clone 11B11, IFN-γ clone XMG1.2, and RORγt clone AFKJS-9; PE-Cy5-conjugated mAb against heat-stable Ag clone M1/39; PE-Cy7-conjugated mAb against NK-1.1 clone PK136; FITC- and PE-conjugated mAbs against CD103 clone 2E7; and Pacific Blue-conjugated mAb against CD4 clone RM4-5 and CD45 clone 30-F11 were purchased from eBiosciences. PE-Cy7-conjugated mAb against heat-stable Ag clone M1/69 and Alexa Fluor 700-conjugated mAb against TCRβ clone H57-597 were purchased from Biolegend. FITC- and PE-conjugated mAbs against CCR6 clone 140706 were from R&D Systems. Pacific Orange-conjugated mAb against CD4 clone RM4-5 was obtained from Invitrogen. CD1d-α-GalCer or -PBS57 tetramers were produced with streptavidin-allophycocyanin (BD Biosciences) or -PE-Cy7 (eBiosciences) and used for staining, as described previously (2). BD Fixation/Permeabilization Kit (cytokine detection) and Foxp3 Staining Buffer Set from eBiosciences (transcription factors and Ki67 detection) were used for intracellular staining, according to manufacturer's instructions. Flow cytometry was performed with FACSAria (BD Biosciences), and data were analyzed with FACSDiva software v6.1.2 (BD Biosciences) and FlowJo 7.2.5 (Tree Star).

In vivo stimulation

Four-month-old C57BL/6 mice were injected with 100 μl of PBS containing LPS (25 μg), or LPS and α-GalCer (0.05 μg), or one million BM-DCs, activated or not with LPS (Sigma-Aldrich) at 5 μg/ml, and preloaded or not with 10 ng/ml α-GalCer. Contralateral footpads were injected with 100 μl of PBS.

Induction of skin inflammation

Induction of inflammation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich) started at day 0 with application of 20 μl of 0.01% TPA suspended in acetone (10 μl in each side of the ear) and repeated every 2 days, as described previously (19, 20). A control group treated with acetone was included in each experiment.

Results

Identification of LN-resident NK1.1$^-$ iNKT cells

Previous studies showed that most iNKT cells exported from the thymus are NK1.1$^-$ and that they acquire NK1.1 in the periphery (4, 5). Thus, it has been believed that peripheral NK1.1$^-$ iNKT cells in normal mice represent recent thymic emigrants at an immature transitional stage of their development and that NK1.1 expression is a marker of maturation. We monitored the kinetics of the natural NK1.1$^-$ to NK1.1$^+$ transition during iNKT cell maturation, by tracking iNKT cells with CD1d tetramers loaded with α-GalCer, and found that this maturation takes place in the thymus, liver, and spleen, leading to an accumulation of NK1.1$^+$ iNKT cells as mice age (Fig. 1). However, whereas NK1.1$^+$ iNKT cell proportions plateau at ~2 mo of age in the thymus and liver, accumulation of these cells occurs with slower kinetics in the spleen, where 4 mo are needed. Moreover, the frequency of steady-state splenic NK1.1$^-$ iNKT cell populations is higher than those found in the thymus or liver. Analysis of pooled cells from maxillary, axillary, inguinal, and mesenteric LNs (pooled LNs) revealed that few NK1.1$^-$ iNKT cells progress to the NK1.1$^+$ stage, regardless of mouse age, with equilibrium established as early as 1 mo of age (Fig. 1 and Table S1).[4] The absence of NK1.1$^+$ iNKT cells in LNs might reflect a suboptimal environment that does not permit immature NK1.1$^-$ iNKT cells to reach the NK1.1$^+$ stage and/or favor NK1.1$^-$ iNKT cell maturation and maintenance. Accordingly, it was reported that the stimulating capabilities of APCs in mesenteric LNs are distinct from those of splenic APCs (17). The frequency of NK1.1$^+$ iNKT cells in LNs might also reflect the number of immature vs mature NK1.1$^-$ iNKT cells that reach distinct LNs. Regardless, these results indicate that the NK1.1$^-$ to NK1.1$^+$ transition does not occur in the LN, revealing a stable, nontransitional NK1.1$^-$ iNKT cell subset, possibly representing a mature iNKT cell population. Accordingly, IL-2Rβ (CD122), moderately and highly expressed on liver and spleen NK1.1$^-$ and NK1.1$^+$ iNKT cells, respectively, is not expressed on LN NK1.1$^-$ iNKT cells (Fig. 2). This supports the probability that they represent a distinct iNKT cell population that is or becomes unresponsive to IL-15, which is described to be crucial for NK1.1$^-$ to NK1.1$^+$ transition (21).

Phenotypic characterization of LN-resident NK1.1$^-$ iNKT cells

Based on our previous finding showing that equilibrium between NK1.1$^-$ and NK1.1$^+$ iNKT cells is established in all organs tested

[4] The online version of this article contains supplemental material.

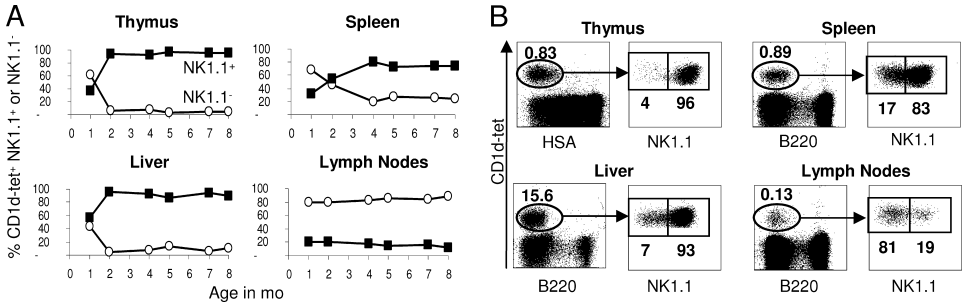

FIGURE 1. Kinetics of iNKT cell maturation and identification of mature LN NK.1.1$^-$ iNKT cells. *A*, Thymus, liver, spleen, and LN iNKT cells from C57BL/6 mice (age 1–8 mo) were stained with CD1d tetramers and anti-NK1.1 mAb. The percentages of NK1.1$^+$ (■) or NK1.1$^-$ (○) tetramer$^+$ (Tet$^+$) iNKT cells are shown. LN cell analyses were performed on pooled maxillary, axillary, inguinal, and mesenteric LNs. *B*, Shown are representative dot plots of NK1.1 expression on iNKT cells in 4-mo-old mice. Data are representative of at least three individual experiments, with three mice pooled in each experiment.

between 2 and 4 mo of age, we decided to characterize phenotypically NK1.1$^-$ iNKT cells using 4-mo-old mice to allow unambiguous distinction between transitional and resident NK1.1$^-$ iNKT cells. Separate phenotypic analysis of peripheral (axillary, maxillary, popliteal, and inguinal) and mesenteric LNs showed, as previously observed with pooled LNs, that the majority of LN iNKT cells draining different areas are NK1.1$^-$ (Fig. 3 and Table S1). We also found a higher frequency of DN than CD4 NK1.1$^-$ iNKT cells in PLNs, whereas the opposite picture was observed in mesenteric LNs. In thymic, splenic, and liver NK1.1$^+$ iNKT cells, the frequency of CD4$^+$ NK1.1$^+$ iNKT cells was found to be higher in all of these organs (data not shown). In LNs, it is possible that some subsets expand, home, or are retained preferentially in some LNs, but not others. Other factors or receptors, yet to be defined, might explain these differences in subset composition. More importantly, we found that ~70–80% of NK1.1$^-$ PLN iNKT cells coexpress CCR6, CD103, and CD121a and ~10% of CCR6$^+$CD103$^+$CD121a$^+$ cells express CD4 (Fig. 3*A* and Table S1). This population is minor in mesenteric LNs (Fig. 3*B* and Table S1). These results indicate that NK1.1$^-$ iNKT cells are composed of two populations, as follows: the first expresses CD4 and is essentially located in mesenteric LNs; the second is DN and mainly located in PLNs, expressing simultaneously CCR6, CD103, and CD121a.

Functional characterization of LN-resident NK1.1$^-$ iNKT cells

NK1.1$^-$ iNKT cells were shown to produce IL-17 in the thymus, liver, spleen, and lung (12–15). To determine whether those in the LNs produce IL-17, we stimulated LN iNKT cells in vitro, with α-GalCer-loaded BM-DCs. The concentration was optimized to avoid NK1.1 and TCR down-modulation (Fig. S2), which is reported to occur after strong TCR engagement. We found that IL-17 was produced by NK1.1$^-$ iNKT cells in all LNs tested, with the highest frequency of IL-17$^+$ cells in PLNs (Fig. 4*A*). We also found that liver, spleen, and thymic NK1.1$^-$ iNKT cells produce IL-17, thus confirming previous studies (Fig. 4*A*). However, the frequencies of these cells do not exceed 1% of total iNKT cells. Moreover, we found in PLNs that all IL-17$^+$ iNKT cells express concomitantly CCR6, CD103, and CD121a, and ~10% of these cells express CD4 (Fig. 4*B* and Table S1). In mesenteric LNs, containing low frequencies of CCR6$^+$CD103$^+$CD121a$^+$ cells, IL-17$^+$ iNKT cells are not clearly defined by their coexpression of the three surface markers, indicating a phenotypic heterogeneity within IL-17$^+$ iNKT cells (Fig. 4*B* and Table S1). This heterogeneity is also seen in liver, spleen, and thymus IL-17$^+$ iNKT cells (data not shown). We confirmed the secretion of IL-17 by quantifying this cytokine in the culture supernatant of sorted PLN CCR6$^+$CD103$^+$CD121a$^+$ NK1.1$^-$ iNKT cells stimulated with α-GalCer-loaded BM-DCs (Fig. 4*C*).

In mice, Th17 cells were shown to produce IL-17, but not IFN-γ and IL-4, and express the transcription factor RORγt, important for IL-17 production, but not T-bet and GATA-3, respectively, important for IFN-γ and IL-4 production (22). To check to what extent LN IL-17$^+$ iNKT cells are related to Th17 cells, we analyzed PLN iNKT cells in *Rorc(γt)-Gfp^{TG}* mice, in which GFP$^+$ cells are RORγt$^+$. We found that GFP$^+$, but not GFP$^-$ iNKT cells produce IL-17 after stimulation (Fig. 4*D*). In addition, we found that GFP$^+$ iNKT cells in PLNs are CCR6$^+$CD103$^+$CD121a$^+$ NK1.1$^-$ and are mainly DN, a phenotype identical with PLN IL-17$^+$ iNKT cells from wild-type (WT) mice, confirming that IL-17$^+$ and RORγt$^+$ iNKT cells represent the same population (Fig. S3*A*). We also found that LN GFP$^+$ NK1.1$^-$ iNKT cells from *Rorc(γt)-Gfp^{TG}* mice (Fig. S3*B*) and LN IL-17$^+$ NK1.1$^-$ iNKT cells from WT mice (Fig. 4*D*) do not produce IFN-γ, whereas a minute fraction of cells has the potential to secrete IL-4. We confirmed the presence of IL-4, but not IFN-γ, in the culture supernatant of sorted PLN CCR6$^+$CD103$^+$CD121a$^+$ NK1.1$^-$ iNKT cells after stimulation with α-GalCer-loaded BM-DCs (data not shown). We found that LN IL-17$^+$ iNKT cells do not express the transcription factor T-bet (Fig. S4), and that these cells are not related to T regulatory cells, because they do not express Foxp3 (Fig. S4), GITR, or CTLA-4, all associated with this subset (data not shown).

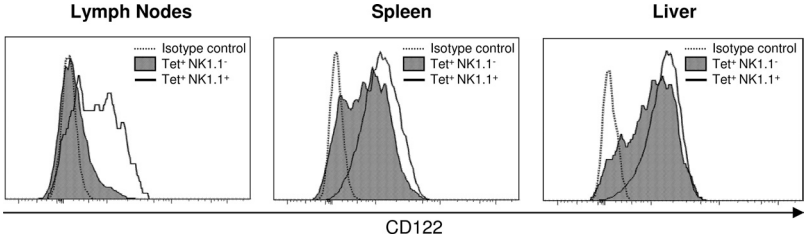

FIGURE 2. IL-2Rβ expression among iNKT cells. Liver, spleen, and LN cells were stained with CD1d tetramers and mAbs directed against NK1.1 and CD122, and shown is IL-2Rβ expression, as mentioned. Data are representative of three experiments.

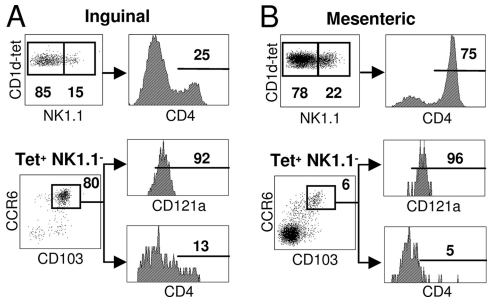

FIGURE 3. CCR6$^+$CD103$^+$CD121a$^+$ NK.1.1$^-$ iNKT cells represent a major iNKT cell population in PLNs. Inguinal (*A*; representative of PLNs) and mesenteric (*B*) LNs from 4-mo-old C57BL/6 mice were stained with CD1d tetramers and mAbs directed against NK1.1, CCR6, CD103, CD121a, and CD4. Note that Tet$^+$ iNKT are mainly NK1.1$^-$ (*upper left*, *A* and *B*), with DN and CD4$^+$ being the main populations in peripheral and mesenteric LNs, respectively (*upper right*, *A* and *B*), and that CCR6$^+$CD103$^+$ cells are the major population among peripheral, but not mesenteric Tet$^+$ NK1.1$^-$ iNKT cells (*lower left dot*, *A* and *B*), with CCR6$^+$CD103$^+$, but not CCR6$^-$CD103$^-$ cells expressing CD121a and being mainly CD4$^+$ (*lower right*, *A* and *B*). Data are representative of at least 10 individual experiments.

Altogether, these results, supported by *Rorc(γt)-Gfp^{TG}* mice, show that the concomitant expression of CCR6, CD103, and CD121a in PLNs is strongly associated with IL-17 production by iNKT cells.

IL-17$^+$ CCR6$^+$CD103$^+$CD121a$^+$ NK1.1$^-$ iNKT cells respond and expand preferentially in vivo to α-GalCer in the presence of LPS

Studies performed by Parekh et al. (23) have shown that iNKT cells are capable of substantial in vivo expansion in response to α-GalCer treatment. iNKT cell expansion was observed in many organs and was maximal ~3 days after injection. This expansion was accompanied by secretion of IL-4 and IFN-γ as early as 2 h after α-GalCer treatment. Two hours after i.p. injection of α-GalCer, we did not detect IL-17 production by PLN iNKT cells, whereas more than half of splenic iNKT cells produce IFN-γ and IL-4 and less than 1% produce IL-17 (Fig. S5). Also, popliteal LN iNKT cells did not produce IL-17 or expand 2 h after α-GalCer injection in the footpad (data not shown). We thus decided to analyze iNKT cell response at different time points after α-GalCer or α-GalCer-loaded BM-DC injection in the footpad. We observed an optimal amplification of popliteal LN iNKT cells 3 days postinjection and found that expanded cells do not produce IL-17 ex vivo at this time point or any other time tested between 1 and 5 days (Fig. 5*A* and data not shown). In vitro stimulation of expanded popliteal LN iNKT cells showed that these cells keep their potential to secrete IL-17 and indicated that popliteal LN IL-17$^+$ iNKT cells did not expand preferentially because their frequency remained unchanged 3 days postinjection (Fig. 5*A*). To mimic what

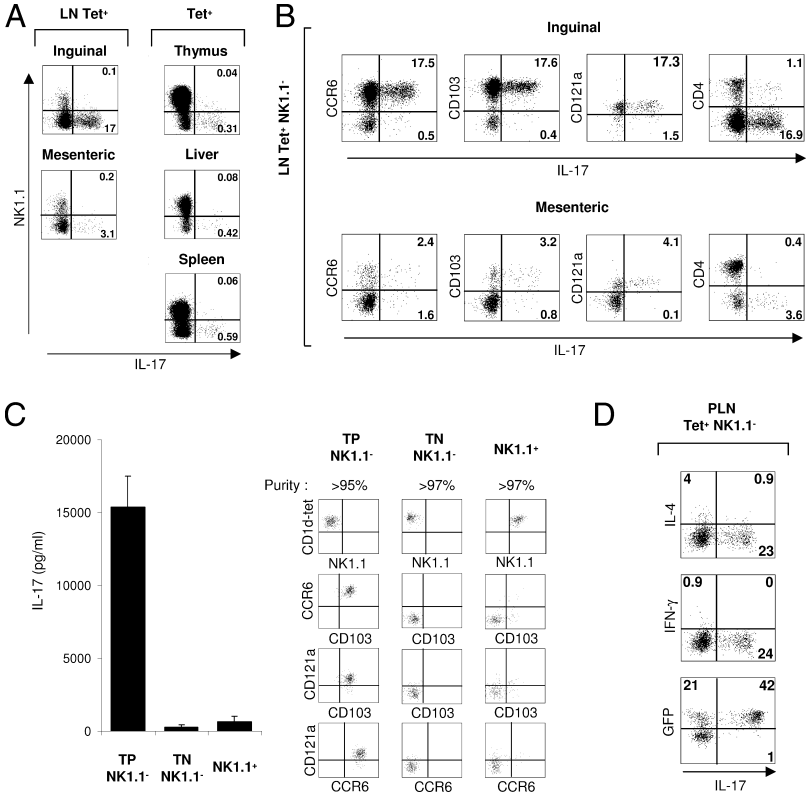

FIGURE 4. CCR6, CD103, and CD121a define IL-17$^+$ iNKT cells in PLNs and express RORγt. *A*, Inguinal (representative of PLNs) and mesenteric LNs, thymus, liver, and spleen were enriched in iNKT cells and stimulated with α-GalCer-loaded BM-DCs. Tet$^+$ iNKT cells were then analyzed for NK1.1 and IL-17 expression. Note that Tet $^+$ IL-17$^+$ iNKT cells are NK1.1$^-$, with the highest frequency of IL-17$^+$ cells being in PLNs. Data are representative of at least 10 individual experiments, with 3 mice pooled in each experiment. Numbers represent percentages. *B*, LN cells were stimulated as in *A*, and shown is CCR6, CD103, CD121a, and CD4 expression on Tet$^+$ IL-17$^+$ NK1.1$^-$ iNKT cells. Data are representative of at least four individual experiments, with pooled PLN cells from 20 mice in each experiment. *C*, Sorted Tet$^+$ CCR6$^+$CD103$^+$CD121a$^+$ NK1.1$^-$ (TP), CCR6$^-$CD103$^-$CD121a$^-$ NK1.1$^-$ (TN), and NK1.1$^+$ LN iNKT cells, as shown in histograms for postsort check (purity superior to 95%), were stimulated for 48 h with BM-DCs in the presence of 100 ng/ml α-GalCer, and IL-17 content in the culture supernatant was measured by ELISA. Data are representative of at least three individual experiments, with pooled PLN cells from at least 20 mice in each experiment. *D*, PLN iNKT cells from C57BL/6 (*upper* and *middle dot plots*) or *Rorc(γt)-Gfp^{TG}* (*lower dot plots*) mice were stimulated with PMA/ionomycin, and Tet$^+$ NK1.1$^-$ iNKT cells were checked for IL-17, IL-4, and IFN-γ production. Data are representative of at least two individual experiments, with 3 pooled mice in each experiment.

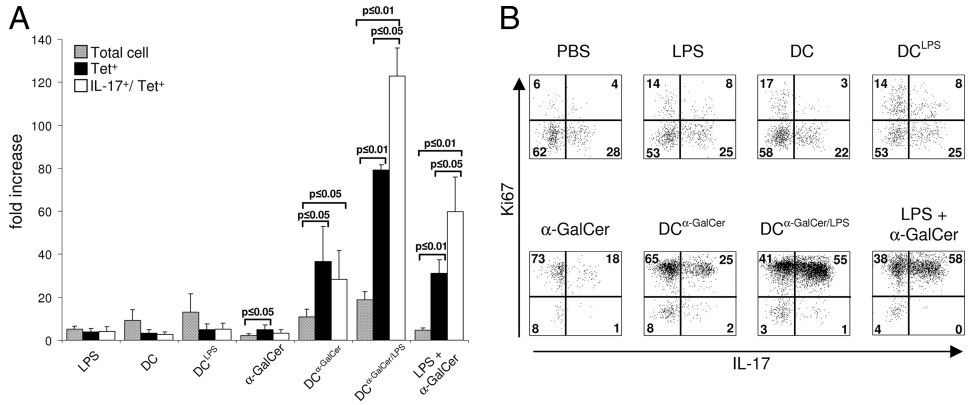

FIGURE 5. In vivo expansion of PLN iNKT cells. Mice were injected in the footpad with 100 μl of PBS containing one of the following: LPS, LPS and α-GalCer, one million BM-DCs (DC), DCs preloaded with α-GalCer (DC$^{\alpha\text{-GalCer}}$), DCs stimulated with LPS (DC$^{\text{LPS}}$), and DCs activated with LPS and preloaded with α-GalCer (DC$^{\alpha\text{-GalCer/LPS}}$). Contralateral footpads were injected with 100 μl of PBS. Three days after injection, cells from popliteal LNs were counted, assessed for their frequency of Tet$^+$ iNKT cells, and, in parallel, stimulated with PMA/ionomycin. Four hours later, cells were subjected to intracellular staining to assess IL-17 production and Ki67 expression. *A*, Shown are the fold increase in total cell numbers, Tet$^+$, and IL-17$^+$/Tet$^+$ iNKT cells in popliteal LNs after different condition injections as compared with contralateral LNs after PBS injection. *B*, Shown are representative dot plots of IL-17 production and Ki67 expression by Tet$^+$ iNKT cells. Numbers represent percentages. Data are representative of five experiments, with three mice pooled in each experiment. Values of p by unpaired Student's t test are shown.

would occur in physiopathological conditions after migration of activated dendritic cells (DCs) or Langerhans cells to LNs under inflammatory conditions, we injected LPS-activated BM-DCs loaded with α-GalCer. Three days later, the overall popliteal LN iNKT cells expanded, but importantly, we found an increased frequency of popliteal LN IL-17$^+$ iNKT cells, indicating that they expanded preferentially under these stimulation conditions (Fig. 5*A*). The increase in popliteal LN IL-17$^+$ iNKT cell number is due to cell proliferation of LN-resident cells rather than to enhanced iNKT cell recruitment because they highly express Ki67, a nuclear cell proliferation-associated Ag expressed in all active stages of the cell cycle (Fig. 5*B*). We also observed a preferential expansion of popliteal LN IL-17$^+$ iNKT cells after coinjection of free α-GalCer and LPS, indicating that LPS-responsive CD1d-expressing cells present at the site of injection or in the draining LN could recapitulate the preferential amplification of popliteal LN IL-17$^+$ iNKT cells observed in response to α-GalCer presented by LPS-activated BM-DCs (Fig. 5*A*). Overall, our results indicate that resting NK1.1$^-$ iNKT cells respond to antigenic challenge in vivo by dramatically expanding while keeping their potential to produce IL-17. Importantly, IL-17$^+$ iNKT cells respond preferentially under inflammatory conditions, providing insight into how this rare population could have an impact in immune responses to infection.

Skin-resident iNKT cells are mainly RORγt$^+$ CCR6$^+$CD103$^+$CD121a$^+$ NK1.1$^-$ iNKT and respond preferentially under inflammatory conditions

We analyzed iNKT cell composition in the skin and gut, organs connected to PLN and mesenteric LNs, respectively. We found that skin, but not gut (intraepithelial lymphocytes, lamina propria lymphocytes, and Peyer's patches), contains a dominant PLN-like NK1.1$^-$ CD4$^-$ iNKT cell population (Fig. 6*A*). Moreover, we found a high frequency of iNKT cells expressing CCR6, CD103, and CD121a, and most iNKT cells express CD103 (Fig. 6*A*). Skin-resident CCR6$^+$CD103$^+$CD121a$^+$ NK1.1$^-$ iNKT cells produce IL-17 after stimulation with PMA/ionomycin (Fig. 6*B*). The frequency of IL-17$^+$-producing cells is, however, lower than that observed in draining maxillary LNs (Fig. 6*B* and Table S1). This is probably not due to a differential expression of RORγt, important for IL-17 production, because we found that skin and PLN iNKT cells from *Rorc(γt)-Gfp^{TG}* express the same level of RORγt (data not shown). Importantly, the frequency of IL-17-producing iNKT cells increased after induction of ear skin inflammation with topical application of TPA (Fig. 6*B*). TPA application is also accompanied by an increase in ear thickness and in number of iNKT cells, with a peak at day 8, preceded by increased iNKT cell numbers in the draining maxillary LNs (Fig. S6). Skin and maxillary LN iNKT cells proliferate after TPA application, as assessed with Ki67 staining (Fig. 6*C*). Importantly, we observed a preferential expansion of RORγt$^+$ among iNKT cells (Fig. 6*C*).

Overall, our results indicate that skin and PLN iNKT cells have similar phenotypical and functional features, and that both populations respond preferentially under inflammatory conditions.

IL-6-independent generation and response of RORγt$^+$ CCR6$^+$CD103$^+$CD121a$^+$ NK1.1$^-$ iNKT cells

It has been shown previously that IL-6 is not required for IL-17 production from spleen iNKT cells (15). To address the question of whether PLN and skin iNKT cell generation and function are altered in the absence of IL-6, we analyzed the phenotype and the function of both populations in IL-6$^{-/-}$ mice. We found that iNKT cells are present in the skin and PLN of IL-6$^{-/-}$ mice with similar composition and absolute numbers to what we observed in WT animals, although iNKT cell frequencies in IL-6$^{-/-}$ mice were higher (Fig. 7 and data not shown). Moreover, the frequency of IL-17$^+$ iNKT cells, detected after stimulation with PMA/ionomycin, is unaltered in the mutant mice (data not shown). This indicates that skin and PLN IL-17$^+$ iNKT cells are normally generated in the absence of IL-6 and with no intrinsic defect in their capability to produce IL-17. To address whether their function could be altered in vivo in the absence of IL-6, we analyzed both populations after ear inflammation with TPA. We found that skin and PLN iNKT cells expanded, as assessed by Ki67 staining (data not shown), and their absolute numbers exceeded what we observed in WT animals (Fig. 7). This increase in iNKT cell numbers in the absence of IL-6 might be due to the lower frequency of cell types that compete with iNKT cells for growth and differentiating factors. In addition, skin and PLN iNKT cells produce IL-17 in the absence of IL-6, with a frequency of IL-17$^+$-producing cells similar to the one observed in WT animals (Fig. 7). Overall, our

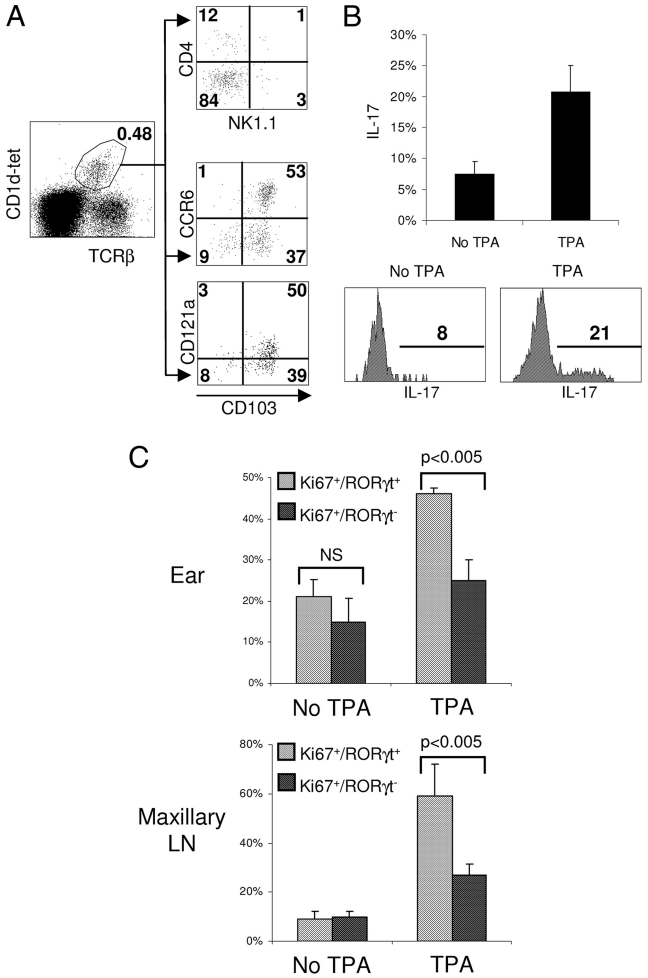

FIGURE 6. IL-17$^+$ CCR6$^+$CD103$^+$CD121a$^+$ iNKT cells are skin resident and respond preferentially under inflammation conditions. *A*, Ear cell suspensions from 4-mo-old C57BL/6 mice were stained with CD1d tetramers and mAbs directed against NK1.1, CCR6, CD103, CD121a, and CD4. Dot plots are gated on live (7-AAD$^-$) CD45$^+$. Data are representative of four individual experiments, with pooled ear cells from at least 20 mice in each experiment. *B*, Histograms (*upper*) show frequency of IL-17-producing cells among iNKT cells after PMA/ionomycin stimulation in normal and TPA-inflamed skin. *Lower* are representative histograms. Data are representative of four individual experiments, with pooled ear cell suspensions from at least 10 mice in each experiment. *C*, Histograms show frequency of Ki67$^+$ iNKT cells among RORγt$^+$ or RORγt$^-$ subsets in normal or TPA-inflamed skin ear (*upper*) and in maxillary LNs (*lower*). Data are representative of four individual experiments, with pooled ear cells from at least 10 mice in each experiment. Values of p by unpaired Student's t test are shown.

results indicate that neither the generation nor the function of PLN and skin iNKT cells is affected in the absence of IL-6, and that iNKT cells could play crucial and nonredundant roles during inflammation.

Discussion

We show the existence of a stable rather than transitional NK1.1$^-$ iNKT cell population resident in LN. The absence of IL-2Rβ expression at the surface of these cells, crucial for iNKT cell maturation (24), indicates that they are not equipped to transit to the NK1.1$^+$ stage like previously described for conventional iNKT cells, and thus represent a distinct iNKT cell population. Importantly, we found that PLNs comprise a major DN RORγt$^+$ IL-17-secreting subset defined by the expression of CCR6, CD103, and CD121a. We also unravel the existence of a phenotypic and functionally equivalent population in the skin, and found that both PLN and skin iNKT cell populations respond preferentially to inflammatory signals, independently of IL-6. CD103 (associated with β_7) was initially described as a marker for intraepithelial T cells residing in the gut wall and skin, and important for their retention (25). We found CD103 to be expressed on most skin-resident iNKT cells, and thus might play a role in their retention. CD103 expressed on PLN IL-17$^+$ NK1.1$^-$ iNKT cells is most likely not related to LN homing, especially because E-cadherin, recognized by CD103 (26), is not expressed on endothelium or LNs. Our results indicate the lack of CD62L and CCR7 expression (data not shown), which are key homing receptors mediating lymphocyte entry into LNs, and suggest that other receptors, yet to be determined, are involved in this process or that they are highly mobile in lymph. The presence of CD103 on IL-17$^+$ iNKT cells might indicate their previous maturation in a TGF-β-rich milieu. Accordingly, TGF-β has been proposed as a key factor in the development of iNKT cells (27). Early data showed that CD103 enhanced CD3-induced activation (28); its costimulatory function could point toward a role in the development of this subset. CCR6 was mainly shown to be expressed on subsets of T cells, DCs, and Langerhans cells, and required for the trafficking of these cells via CCL20 expressed by epithelial cells (29). The expression of CCR6 on Th17 cells was reported on human and mouse T cells (30, 31), and has been shown to be involved in the recruitment of pathogenic T cells in different autoimmune diseases (29). Our in vitro chemotaxis experiments show that PLN iNKT cells are attracted by CCL20 (data not shown). We could hypothesize that during inflammatory conditions, CCR6$^+$IL-17$^+$ NK1.1$^-$ iNKT cells could be attracted by CCL20 produced by inflamed epithelial cells. The presence of CD103 on IL-17$^+$ NK1.1$^-$ iNKT cells predisposes them for retention within epithelial sites, allowing them to participate in local responses. Accordingly, CD103 was shown to be expressed on highly potent regulatory CD25$^+$ and CD25$^-$ T cell subsets in mice (32), and data from CD103-deficient animals suggest that this molecule might indeed be involved in the control of autoimmunity in the skin (33). Thus, the chemokine receptor CCR6 and the integrin CD103 can be regarded as novel markers for an IL-17$^+$ NK1.1$^-$ iNKT cell subset specialized in exerting function within the skin. The functions of CCR6 and CD103 and their ligands in controlling in vivo migration and function of IL-17$^+$ NK1.1$^-$ iNKT cells remain to be established by analysis of knockout animals. In addition to the migratory potential of PLN iNKT cells to inflamed skin, our results indicate that iNKT cells are already present in this site. These cells are less potent in producing IL-17 than their PLN counterpart in normal conditions, but this potential increases after inflammation. We also show that PLN iNKT cells respond to their ligand α-GalCer preferentially in the presence of LPS, indicating that they might be involved in the response to inflammation upon bacterial infection. The mechanisms underlying the preferential response of IL-17$^+$ iNKT cells in the presence of inflammatory signals are under investigation. We could speculate that, once activated, IL-17$^+$ iNKT cells in general will expand and respond specifically to cytokines such as IL-21 or IL-23. Accordingly, splenic IL-17$^+$ iNKT cells have been shown to constitutively express IL-23R and to respond to IL-23 (15), and iNKT cells were also shown to respond to IL-21 (34). The specific expression of CD121a in iNKT cells could play a crucial role in this preferential response. Accordingly, an in vitro study showed that the presence of IL-1 expanded selectively Foxp3$^-$NK1.1$^-$ CD1d tetramer-positive cells enriched in IL-17 producers in a CD4$^+$CD25$^+$ T cell culture (35). Also, IL-1, which is a major cytokine connected to inflammation, was shown recently to be

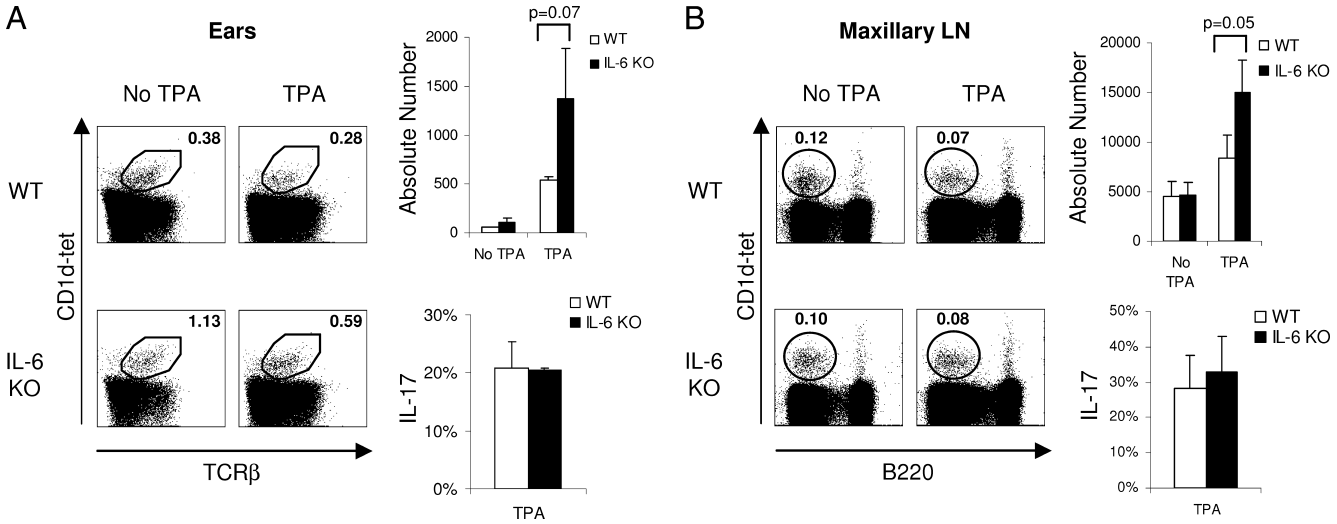

FIGURE 7. Generation and function in vivo of iNKT cells from PLNs and skin are IL-6 independent. *A*, Ear cell suspensions from 4-mo-old C57BL/6 mice and IL-6$^{-/-}$ mice were stained with CD1d tetramers and mAb directed against TCRβ in normal or TPA-inflamed skin. Representative dot plots are gated on live (7-AAD$^-$) CD45$^+$ cells. Histograms show absolute number (*upper*) and percentage of IL-17 (*lower*) among iNKT cells from C57BL/6 mice and IL-6$^{-/-}$ mice after PMA/ionomycin stimulation. Data are representative of three individual experiments, with pooled ear cell suspensions from at least 10 mice in each experiment. Values of *p* by unpaired Student's *t* test are shown. *B*, Shown are representative dot plots of maxillary LN cells from 4-mo-old C57BL/6 mice and IL-6$^{-/-}$ mice stained with CD1d tetramers, and mAb directed against B220 in normal or TPA-inflamed skin. Histograms show absolute number (*upper*) and percentage of IL-17 (*lower*) among iNKT cells from C57BL/6 mice and IL-6$^{-/-}$ mice after PMA/ionomycin stimulation. Data are representative of three individual experiments, with pooled maxillary LN cells from at least 10 mice in each experiment. Values of *p* by unpaired Student's *t* test are shown.

required to prime human CCR6$^+$ Th17 cells to enable IL-23-induced cytokine release (36). Our own results show that ear inflammation after TPA application is accompanied by 5-fold increase in IL-1 transcript (data not shown). Interestingly, although IL-6 was shown to be a major factor for Th17 cell differentiation, our results indicate that IL-17$^+$ iNKT cell generation and function occur in the absence of this proinflammatory cytokine. Thus, IL-1 and other factors yet to be determined are likely to play key roles in iNKT cell responses in the absence of IL-6 and indicate that iNKT cells could play a nonredundant role during inflammation.

IL-17$^+$ iNKT cells have been described in other studies in the thymus, liver, spleen, and lung, but not in LNs or skin (13, 15). In these studies, it was suggested that all NK1.1$^-$ iNKT cells have the potential to secrete IL-17. Our study shows heterogeneity among NK1.1$^-$ iNKT cells, as exemplified by the few IL-17-secreting cells found in mesenteric LNs. Also, in these studies, tissue-resident IL-17$^+$ NK1.1$^-$ iNKT cells represented a minor subset compared with IL-4- and IFN-γ-producing NK1.1$^+$ iNKT cells. We found this composition inverted in PLNs and skin, because NK1.1$^-$ IL-17$^+$ cells represent the major population among iNKT cells. This composition heterogeneity could explain the diversity of iNKT cell function depending on the anatomic location. This physical separation of iNKT cells with antagonistic functions could solve part of the paradox of how iNKT cells with different potentials can respond to similar ligands. Finally, previous studies did not connect IL-17$^+$ iNKT cells with specific phenotypic characteristics, such as CCR6, CD103, and CD121a, which, in the case of PLN and skin IL-17$^+$ iNKT cells, are most likely important for exerting their function. More work must be done to show in vivo evidence for a role of IL-17$^+$ iNKT cells and other NK1.1$^-$ iNKT subsets in relevant pathological situations.

In conclusion, our results represent a functional and phenotypical characterization of mature, stable NK1.1$^-$ iNKT cells, mainly located in LNs. Our study shows that the ones located in PLN mainly secrete the proinflammatory cytokine Th17 and are armed to exert their function within epithelia, such as the skin, where an equivalent population of iNKT cells was also detected. Importantly, both populations respond preferentially to inflammation independently of Th17 differentiating signals. Our study opens a new area of investigation regarding the role of these cells in immune responses to infection and autoimmunity.

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Disclosures

The authors have no financial conflict of interest.

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