Identification of an IL-17–producing NK1.1\textsuperscript{neg} iNKT cell population involved in airway neutrophilia

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Invariant natural killer T (iNKT) cells are an important source of both T helper type 1 (Th1) and Th2 cytokines, through which they can exert beneficial, as well as deleterious, effects in a variety of inflammatory diseases. This functional heterogeneity raises the question of how far phenotypically distinct subpopulations are responsible for such contrasting activities. In this study, we identify a particular set of iNKT cells that lack the NK1.1 marker (NK1.1\textsuperscript{neg}) and secrete high amounts of interleukin (IL)-17 and low levels of interferon (IFN)–γ and IL-4. NK1.1\textsuperscript{neg} iNKT cells produce IL-17 upon synthetic (α–galactosylceramide [α–GalCer]) or PBS-57), as well as natural (lipopolysaccharides or glycolipids derived from \textit{Sphingomonas wittichii} and \textit{Borrelia burgdorferi}), ligand stimulation. NK1.1\textsuperscript{neg} iNKT cells are more frequent in the lung, which is consistent with a role in the natural immunity to inhaled antigens. Indeed, airway neutrophilia induced by α–GalCer or lipopolysaccharide instillation was significantly reduced in iNKT-cell–deficient Jα18\textsuperscript{−/−} mice, which produced significantly less IL-17 in their bronchoalveolar lavage fluid than wild-type controls. Furthermore, airway neutrophilia was abolished by a single treatment with neutralizing monoclonal antibody against IL-17 before α–GalCer administration. Collectively, our findings reveal that NK1.1\textsuperscript{neg} iNKT lymphocytes represent a new population of IL-17–producing cells that can contribute to neutrophil recruitment through preferential IL-17 secretion.
of myeloid progenitors (CFU cells) and neutrophils from the bone marrow to the periphery (8). Yet, it is still not clear how iNKT cells promote neutrophil recruitment to inflammatory sites and what mediators are involved.

The newly described cytokine IL-17 is a likely candidate for this task because it has already been implicated in airway neutrophilia induced by endotoxin exposure (17, 18). Furthermore, it has been documented that in IL-17 receptor–deficient mice, the host defense against lung bacterial infection is impaired (19).

Based in these data, we set out to examine whether stimulated iNKT cells were able to produce IL-17, and whether this cytokine mediated the neutrophil recruitment. We found that a small subset of iNKT cells lacking the NK1.1 marker generated high amounts of IL-17, together with low IFN-γ levels, in response to several iNKT cell ligands, namely, α-GalCer or its analogue PBS-57, as well as glycolipids derived from S. wittichii and Borrelia burgdorferi. This NK1.1neg iNKT cell subset was more frequent among lung iNKT cells, which is in accordance with a potential contribution to the airway neutrophilia elicited by intranasal (i.n.) exposure to α-GalCer, PBS-57, or LPS.

RESULTS AND DISCUSSION

α-GalCer stimulation induces IL-17 production

iNKT cells are plausible candidates for IL-17 production (20–24), considering that their biological activities overlap with most of those ascribed to this proinflammatory mediator. We tested this hypothesis using mononuclear cells (MNCs) isolated from the liver, where iNKT cells are more abundant than in other organs, and compared IL-17 production by total hepatic MNCs from wild-type C57BL/6 and iNKT cell-deficient (Jα18−/−) mice in response to the iNKT cell–specific antigen ligand α-GalCer. As shown in Fig. 1 A, IL-17 was easily detected in cell supernatants from wild-type mice and accumulated during the 72-h incubation period. In contrast, it failed to be produced by MNCs from Jα18−/− or from CD1d−/− mice (Fig. 1 A), which are both iNKT-cell deficient.

We further addressed the question of whether the capacity to induce IL-17 production was shared by more physiological ligands of iNKT cells, such as glycosphingolipids from Sphingomonas sp and diacylglycerol antigens from B. burgdorferi, which causes Lyme disease (11, 12). We found that liver cells from wild-type, but not from Jα18−/−, mice produced IL-17 in response to the galacturonic acid–containing S. wittichii glycosphingolipid (GalA-GSL) and, to a lesser extent, to some synthetic variants of BbGLII from B. burgdorferi (Fig. 1 C). Our results concord with previous studies identifying BbGLIIc as the best BbGLII variant for iNKT cell activation (12) and prove that ligands with more physiological relevance than α-GalCer can also induce IL-17 production.

It has been widely documented that iNKT cells produce large amounts of both IFN-γ and IL-4 in response to α-GalCer (1–4). Knowing that both cytokines are potent inhibitors of IL-17 production (22, 23), we examined how this activity was affected when endogenous IFN-γ and/or IL-4 production was abolished in genetically modified IFN-γ−/− or IL-4−/− mice and/or in the presence of neutralizing anti–IL-4 mAbs. The lack of either cytokine resulted in a clear increase of IL-17 secretion after α-GalCer activation (Fig. 1 B), which was further enhanced in the absence of both, indicating that IL-4 and IFN-γ are produced endogenously and contribute similarly to the inhibition.

The iNKT NK1.1neg subset is the major source of IL-17 after α-GalCer stimulation

It is well established that α-GalCer acts specifically on iNKT cells (5). However, other cells could be secondarily stimulated and potentially produce IL-17 in our experimental model. To confirm the direct involvement of iNKT cells in IL-17 production, we gated the tetramer CD1d/α-GalCer+ population...
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from hepatic MNCs and sorted them into two subsets according to their NK1.1 expression (Fig. 2 A). Upon stimulation with α-GalCer, IL-17 was only detected in supernatants of NK1.1neg iNKT cells (Fig. 2 B), along with very low amounts of IL-4 and IFN-γ (Fig. 2, C and D). In contrast, the NK1.1pos subset produced high levels of the latter two cytokines (Fig. 2, C and D), but little IL-17 (Fig. 2 B), proving that it responded normally to α-GalCer stimulation. GalA-GSL and BbGLIIc ligands also induced IL-17 production by sorted NK1.1neg, but not NK1.1pos, iNKT cells (Fig. 2 E). NK1.1pos iNKT cells were activated by these ligands because they produced IL-4 (Fig. 2 F). No detectable IL-17 production was observed when sorted T cells from Jα18−/− mice were stimulated with α-GalCer (Fig. 2 G). The conclusion that the NK1.1neg subset is the main source of IL-17 among iNKT cells was confirmed by intracellular cytokine staining, as shown in Fig. 2 H.

With the exception of IL-17, which is produced by NK1.1neg iNKT cells, and IL-4, IFN-γ, and IL-3, which are produced more efficiently by NK1.1pos than NK1.1neg iNKT cells, the cytokine profile generated by the two subsets in response to α-GalCer was essentially the same, as assessed by a protein array detecting 32 different cytokines (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20061551/DC1). Moreover, NK1.1neg and NK1.1pos iNKT cells were undistinguishable by the expression of major iNKT cell markers, such as CD4, CD44, CD62L, CD69, Ly49A, and Ly49C, which occurred at similar levels (Fig. S2). Furthermore, both populations shared the Vβ bias that is typical for NK1.1pos iNKT cells (Fig. 2 I).

NK1.1neg and NK1.1pos iNKT cell subsets are functionally distinct

The preferential production of IL-17 by NK1.1neg iNKT cells raised the question of whether their NK1.1pos counterparts were unable to produce the same amount because of the inhibition exerted by endogenous IL-4 and IFN-γ. To address this issue, we blocked both cytokines by the corresponding neutralizing mAbs before stimulation with α-GalCer. Even though approximately fourfold more IL-17 was produced by NK1.1pos cells in these conditions (Fig. 3 A), the concentrations remained eight times lower than those generated by the NK1.1neg subset (Fig. 3 B), indicating that the two populations are functionally distinct. Nonetheless, the IL-17 production by the NK1.1neg population remained sensitive to down-regulation by IL-4 and IFN-γ, as assessed by the strong inhibitory effect of exogenous cytokines (Fig. 3 B).

α-GalCer (not depicted). Data represent the mean ± the SD of two to three individual experiments. *P < 0.05. (H) Intracellular IL-17 staining was performed after in vitro stimulation of liver MNCs and analyzed among gated CD1dα-GalCer tetramers + NK1.1pos iNKT and CD1dα-GalCer tetramers + NK1.1neg iNKT liver MNCs were stimulated with α-GalCer or synthetic B. burgdorferi glycolipids (BbGL-II [IIc] or GalA-GSL [GSL]; E and F) plus irradiated liver MNCs from Jα18−/− mice as APCs. Sorted CD4−CD62L− T cells from Jα18−/− mice were stimulated with α-GalCer plus irradiated liver MNCs from Jα18−/− mice as APCs (G), 3 d later, IL-17 (B, E, and G), IL-4 (C and F), and IFN-γ (D) were measured in the supernatants. No cytokine was detected in the absence of α-GalCer stimulation, in the absence of APCs or when APCs alone were stimulated with from hepatic MNCs and sorted them into two subsets according to their NK1.1 expression (Fig. 2 A). Upon stimulation with α-GalCer, IL-17 was only detected in supernatants of NK1.1neg iNKT cells (Fig. 2 B), along with very low amounts of IL-4 and IFN-γ (Fig. 2, C and D). In contrast, the NK1.1pos subset produced high levels of the latter two cytokines (Fig. 2, C and D), but little IL-17 (Fig. 2 B), proving that it responded normally to α-GalCer stimulation. GalA-GSL and BbGLIIc ligands also induced IL-17 production by sorted NK1.1neg, but not NK1.1pos, iNKT cells (Fig. 2 E). NK1.1pos iNKT cells were activated by these ligands because they produced IL-4 (Fig. 2 F). No detectable IL-17 production was observed when sorted T cells from Jα18−/− mice were stimulated with α-GalCer (Fig. 2 G). The conclusion that the NK1.1neg subset is the main source of IL-17 among iNKT cells was confirmed by intracellular cytokine staining, as shown in Fig. 2 H.

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Recent studies reported that TGF-β and IL-6 are required for driving the differentiation of naive CD4 T cells into Th17 cells (25), thus prompting us to verify whether NK1.1<sup>+</sup> iNKT cells become more efficient IL-17 producers in these conditions. Fig. 3 C clearly shows that this is true for naive conventional T cells, but not for NK1.1<sup>+</sup> iNKT cells, even though they retained their ability to produce both IL-4 and IFN-γ (Fig. 3, D and E), which proves responsiveness to stimulation. In addition, we tested the effect of IL-23 on NK1.1<sup>+</sup> iNKT cells, knowing that it enhances IL-17 production by conventional T cells (26). Yet, once again, this treatment did not increase IL-17 secretion by NK1.1<sup>+</sup> iNKT cells (Fig. 3 F), suggesting that NK1.1<sup>−</sup> and NK1.1<sup>+</sup> cells are, indeed, functionally distinct iNKT cell subsets.

**Physiological relevance of NK1.1<sup>−</sup> iNKT and IL-17 in early host defense to airborne antigens**

Because of their constant exposure to foreign antigens, airways and lungs depend on a competent immune response to avoid deleterious inflammatory responses caused by inefficient clearance of pathogens. Having established that iNKT cells are potent IL-17 producers, we addressed the question of their participation in airway neutrophilia resulting from exposure to α-GalCer, PBS-57, or LPS, which is another iNKT cell ligand (27), or LPS. We first verified that pulmonary iNKT cells could produce IL-17 upon activation, which was actually the case for MNCs from wild-type (A–D) and Jα<sup>18<sup>−/−</sup> mice (A–C) after exposure to these ligands (Fig. 4, A–C). We next sorted NK cells from pulmonary MNCs and found once again that only the NK1.1<sup>−</sup> subset responded to α-GalCer stimulation in terms of IL-17 production (Fig. 4 D). Remarkably, this subpopulation turned out to be much more frequent in the lung than in the liver (Fig. 4 E) or in the spleen (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20061551/DC1) because it comprises up to 40% of pulmonary iNKT cells in naive mice (Fig. 4 E).
from their NK1.1<sup>pos</sup> counterpart because of their high production of IL-17 and low secretion of IFN-γ and IL-4. The existence of distinctive subpopulations provides a possible explanation for the contrasting effects exerted by iNKT cells. In support of this idea, a recent report demonstrates that liver CD4<sup>pos</sup> NK cells are unique in their capacity to confer an antitumor response (28). Our findings provide the first evidence for a particular NK1.1<sup>pos</sup> iNKT subset endowed with a preferential IL-17–producing profile induced by various antigens that we propose to name iNKT17 cells. The fact that this phenotype is more abundant in the lung than in liver or spleen (Fig. S3) supports the notion that specialized iNKT cell subsets may reside in different organs.

**MATERIALS AND METHODS**

**Animals.** 7–9-wk-old C57BL/6 mice were purchased from Janvier. Jx<sup>18<sup>−/−</sup></sup>, CD1d<sup>−/−</sup>, and IFN-γ<sup>−/−</sup>–deficient mice (29, 30) were bred in our own facilities. All mice were kept in well-controlled animal housing facilities and had free access to tap water and pellet food. Animal experiments were performed according to the French Institutional Committee.

**Cell preparation.** Lymphocytes were isolated from the liver, spleen, or lung, as previously described (15, 30).

**FACS analysis and sorting of iNKT cells and conventional, naive T cells.** MNCs were stained with anti-V<sub>β</sub>8.1/8.2 (clone MR5-2–, anti-Vβ8.3 (clone 1B3.3), anti-Vβ7 (clone TR310; mAb provided by S. Latour [Institut National de la Santé et de la Recherche Médicale, Paris France] and J.C. Bories [EA3963, Paris, France]), or anti-NK1.1 (clone PK136) mAb (Institut National de la Santé et de la Recherche Médicale, Paris France) and V<sub>β</sub>8.1/8.2 (clone MR5-2), anti-CD1d<sup>−</sup>GalCer tetramers (plasmids containing CD1d and b2m genes were provided by M. Kronenberg, La Jolla Institute for Allergy and Immunology, San Diego, CA). NK1.1<sup>pos</sup> iNKT (tetramers<sup>pos</sup>) and NK1.1<sup>neg</sup> iNKT (tetramers<sup>neg</sup>) cells were then sorted. In parallel, splenocytes were stained with anti-CD4 (clone RM4-5) and anti-CD62L (clone Mel14) antibodies before sorting of conventional naive CD4<sup>+</sup>CD62L<sup>+</sup> T cells. All cells were sorted using a FACSVantage cell sorter (Becton Dickinson).

**Cell culture.** A final concentration of 10<sup>5</sup> liver or lung MNCs or 2.5 × 10<sup>5</sup> sorted iNKT cells per milliliter were cultured with or without irradiated liver MNCs (5 Gy) from Jx<sup>18<sup>−/−</sup></sup> or CD1d<sup>−/−</sup> mice as APCs, at a ratio of 1:2. Cells were cultured in RPMI 1640 medium containing antibiotics, 10% FCS, 4 mg/ml β-mercaptoethanol, and 200 mM glutamine (all from Invitrogen) incubated at 37°C with 100 ng/ml α-GalCer solution, 100 ng/ml PBS-57, 10 μg/ml αGalCer tetramers (plasmids containing CD1d and b2m genes were provided by M. Kronenberg, La Jolla Institute for Allergy and Immunology, San Diego, CA), NK1.1<sup>pos</sup> iNKT (tetramers<sup>pos</sup>) and NK1.1<sup>neg</sup> iNKT (tetramers<sup>neg</sup>) cells were then sorted. In parallel, splenocytes were stained with anti-CD4 (clone RM4-5) and anti-CD62L (clone Mel14) antibodies before sorting of conventional naive CD4<sup>+</sup>CD62L<sup>+</sup> T cells. All cells were sorted using a FACSVantage cell sorter (Becton Dickinson).

**Determination of cytokines.** The levels of IL-17A (R&D Systems), IL-4, and IFN-γ were assessed by ELISA, as previously described (28, 29). Cytokine protein array II was purchased from Ray Biotech and used for analyzing supernatants from α-GalCer–stimulated sorted NK1.1<sup>pos</sup> and NK1.1<sup>neg</sup> iNKT cells according to the manufacturer’s instructions.

**Intracellular cytokine staining.** Liver MNCs were stimulated for 4 h with 10<sup>−8</sup> M PMA (Sigma-Aldrich), 10<sup>−7</sup> M ionomycin, and 10 μg/ml brefeldin A. Cells were then washed and incubated with C1dα-GalCer tetramer–APC, anti-NK1.1 PerCP-Cy-5.5, and anti-TCRβ-FITC. For intracellular staining, cells were fixed with 4% PFA, washed, and permeabilized.
with 0.5% saponin (Sigma-Aldrich), and then further incubated with anti-IL-17-PE or isotype control (BD Biosciences). The cells were washed and analyzed on a FACSCalibur (Becton Dickinson) using CellQuest software (BD Biosciences).

In vivo treatment. Mice received a single i.n. administration of 2 μg α-GalCer (Kirin Brewery Co., Ltd.), 2 μg PBS-S7 (Sigma-Aldrich), or 10 μg LPS (Sigma-Aldrich) 24 h before sacrifice. In some experiments, mice received 100 μg of anti-IL-17 mAb (R&D Systems) or control Ig (Sigma-Aldrich) i.p. 24 h before ligand administration. Differential cell counts were determined in BALF 24 h after ligand instillation, as previously described (30).

Statistical analysis. A nonparametric Mann-Whitney test was used to calculate significance levels for all measurements. P values <0.05 were considered statistically significant.

Online supplemental material. Fig. S1 shows cytokine profile of NK1.1<sup>−</sup> and NK1.1<sup>+</sup> liver iNKT cells stimulated with α-GalCer for 3 d. Different cytokines were analyzed using mouse cytokine array II membranes. Fig. S2 shows that NK1.1<sup>−</sup> and NK1.1<sup>+</sup> iNKT cells express similar levels of CD4, CD69, CD44, CD62L, Ly49A, and Ly49C markers. All antibodies used were obtained from Becton Dickinson. Fig. S3 shows the percentage of NK1.1<sup>−</sup> iNKT and NK1.1<sup>+</sup> iNKT cells among gated CD1d/α-GalCer tetramers “TCRB+” iNKT splenocytes.

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