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Innate IFN-γ–Producing Cells Developing in the Absence of IL-2 Receptor Common γ-Chain

Mariana Resende,*† Marcos S. Cardoso,* Ana R. Ribeiro,* Manuela Flórido,*† Margarida Borges,*‡ António Gil Castro,† Nuno L. Alves,* Andrea M. Cooper,‡,3 and Rui Appelberg*

IFN-γ is known to be predominantly produced by lymphoid cells such as certain subsets of T cells, NK cells, and other group 1 innate lymphoid cells. In this study, we used IFN-γ reporter mouse models to search for additional cells capable of secreting this cytokine. We identified a novel and rare population of nonconventional IFN-γ–producing cells of hematopoietic origin that were characterized by the expression of Thy1.2 and the lack of lymphoid, myeloid, and NK lineage markers. The expression of IFN-γ by this population was higher in the liver and lower in the spleen. Furthermore, these cells were present in mice lacking both the Rag2 and the common γ-chain (γc) genes (Rag2γc mice), indicating their innate nature and their γc cytokine independence. Rag2γc mice are as resistant to Mycobacterium avium as Rag2γc mice, whereas Rag2γc mice lacking IFN-γ are more susceptible than either Rag2γc or Rag2γc γc mice. These lineage-negative CD45+Thy1.2+ cells are found within the mycobacterially induced granulomatous structure in the livers of infected Rag2γcγc animals and are adjacent to macrophages that expressed inducible NO synthase, suggesting a potential protective role for these IFN-γ–producing cells. Accordingly, Thy1.2–specific mAb administration to infected Rag2γcγcγc animals increased M. avium growth in the liver. Overall, our results demonstrate that a population of Thy1.2 non-NK innate-like cells present in the liver expresses IFN-γ and can confer protection against M. avium infection in immunocompromised mice. The Journal of Immunology, 2017, 199: 000–000.

Interferon-γ is a proinflammatory cytokine that plays a major role in protective immune responses against intracellular pathogens. Upon infection, phagocytes will respond by producing cytokines such as IL-12 and IL-18, which will induce NK cells and invariant NK T cells to release IFN-γ during the innate phase of the immune response. At later stages, CD4+ and CD8+ T cells are primed to produce IFN-γ and contribute to pathogen clearance (reviewed in Ref. 1). The major cellular targets of IFN-γ are mononuclear phagocytes that are activated to upregulate Ag processing and presentation pathways, lysosomal activity, and reactive species production, which in turn mediate the microbialidal effect (1, 2). IFN-γ is also responsible for inducing leukocyte recruitment to the infection site and generation of granulomatous lesions, such as the ones induced by mycobacterial infections (1), wherein the newly recruited leukocytes are able to produce more IFN-γ as well as other cytokines that will directly act on the infected cells. Ab-mediated or genetic ablation of IFN-γ signaling severely compromises the in vivo assembly of granulomas in mycobacterial infections (3–5). Complete lack of IFN-γ signaling, such as found in gene-deleted mice, leads to an increased susceptibility to infection relative to that seen when only IFN-γ–producing T cells are lacking (6–9). NK cells are efficient producers of IFN-γ (1, 10) and have been associated with IFN-γ–mediated protection, particularly in the absence of T cells (11, 12). However, mice that lack B and T cells, NK cells, and the other innate lymphoid cells (ILCs), as a result of the deletion of the Rag enzyme required for TCR and BCR recombination and the IL-2R common γ-chain (γc), are more resistant to Chlamydia pneumoniae than Rag1γcγc double-knockout mice (13). In addition, the authors showed that both Rag1γc and Rag1γcγc γc express equivalent levels of Ifng mRNA and protein in infected lungs. These data suggest, therefore, the existence of an additional cellular source of IFN-γ. Indeed, several reports propose the existence of nonconventional IFN-γ–producing cells. Infection with

*IBMC – Instituto de Biologia Molecular e Celular and i3S – Instituto de Investigação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal; †Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho and ICVS/3B’s – PT Government Associate Laboratory, 4170 Braga/Guimarães, Portugal; ‡UCIBIO/REQUIMTE, Departamento de Ciências Biológicas, Laboratório de Biocâmica, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal.

†Current address: Mycobacterial Research Group, Centenary Institute of Cancer Medicine and Cell Biology, Newtown, NSW, Australia.

‡Current address: UCI/BIO/REQUIMTE, Departamento de Ciências Biológicas, Laboratório de Biocâmica, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal.

§Current address: Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, U.K.

ORCID iDs: 0000-0003-4835-3035 (M.R.); 0000-0003-0150-7359 (M.S.C.); 0000-0001-6035-4095 (M.B.); 0000-0002-1567-8389 (N.L.A.); 0000-0001-6050-3863 (A.M.C.); 0000-0002-9447-0665 (R.A.).

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Address correspondence and reprint requests to Mariana Resende, Instituto de Biologia Molecular e Celular, Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal. E-mail address: mrsilva@ibmc.up.pt

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Abbreviations used in this article: γc, γ-chain; dpi, day postinfection; ILC, innate lymphoid cell; ILCγ, ILC precursor; INOS, inducible NO synthase; qPCR, quantitative PCR; Yeti, YFP-enhanced transcript for IFN-γ.

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Esherichia coli or Listeria monocytogenes induces IFN-γ production by splenic innate B cells, which in turn promotes an innate response against these bacteria (14). Human oral epithelial cells have also been shown to produce IFN-γ upon in vitro infection with Candida albicans (15). Expression or secretion of IFN-γ has been detected in peritoneal or splenic macrophages and in bone marrow–derived macrophages or dendritic cells after in vitro stimulation with IL-12/IL-18 or infection with different pathogens (C. pneumoniae, Salmonella typhimurium) (16–21). However, a more detailed study revealed that the lack of purity of the myeloid cell sets cast doubt upon macrophages as the source of the IFN-γ (22, 23). More recently, work from Sturge et al. (24) identified peritoneal neutrophils as an IL-12–independent source of IFN-γ in response to Toxoplasma gondii.

To identify novel cellular sources of IFN-γ, we used reporter mouse models and describe in this article a rare population of IFN-γ–expressing CD45+ Thy1.2+ cells that are of nonlymphoid, nonmyeloid, and non-NK lineage. Moreover, we provide experimental evidence that these cells are functionally relevant, conferring protection against M. avium infection in the liver of lymphopenic Rag2−/−γc−/− mice (lacking both the Rag2 and the common γc genes) mice.

Materials and Methods

Animals

YFP-enhanced transcript for IFN-γ (Yeti) mice were crossed with C57BL/6 Rag2−/− mice at the Trudeau animal house facilities. Yeti mice were generated by the introduction of an IRES-enhanced YFP construct after the stop codon of IFN-γ and is under the control of a bovine growth hormone polyA tail (25). The IFN-γ reporter with endogenous polyA tail (Great) mice (C57BL/6 background) have the IFN-γ−IRES-enhanced YFP reporter cassette under the control of the endogenous ifng 3′ untranslated region and polyA tail (25). All procedures involving live animals were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health, and individual procedures were approved by the Trudeau Institute Animal Care and Use Committee. C57BL/6 IFN-γ−/− mice were purchased from Jackson Laboratories (Bar Harbor, ME), and C57BL/6Rag2−/− mice were obtained from the Centre de Développement des Techniques Avancées for the experimentazione animale (Orleans, France). The two strains were crossed at Instituto de Investigaciones Biomédicas e Celular animal facilities to obtain double-mutant mice. Double-knockout mice were screened by performing PCR with IFN-γ gene-specific primers (according to Jackson Laboratories) from genomic DNA extracted from an ear clipping and by performing flow cytometric analysis of blood samples to detect lymphopenic mice. Mice deficient in both Rag2 and the common γc (B6.Rag2−/−/−γc−/−) were provided by Dr. J. di Santo. Animal care procedures were in accordance with institutional guidelines. This study was previously approved by the Portuguese National Authority for Animal Health–Direcção Geral de Veterinária.

Infection and Ab administration

Mice were infected with Mycobacterium avium strain 2447 by injecting 10⁶ CFUs i.v. at different times of infection, mice were sacrificed and the bacterial burden was determined in the major target organs, the spleen and the liver, by plating serial dilutions of tissue homogenates onto Middlebrook 7H10 medium (Difco, St. Louis, MO). In two experiments, infected Rag2−/−/−γc−/− mice were injected i.p. with 0.4 mg of 30-H12 (anti-Thy1.2) or nonspecific IgG twice a week throughout 60 d of infection.

Abs

The following conjugated mAbs were used in flow cytometry: Brilliant Violet (BV) 421 anti-mouse CD11c, clone N418, Isootype Armenian hamster IgG; Pacific Blue (PB) anti-mouse Ly6G, clone 1A8, Isotype rat IgG2a, k; BV510 anti-mouse CD3, clone 17A2, Isotype rat IgG2b, k; BV510 anti-mouse/human CD11b, clone RM4-5, Isotype rat IgG2b, k; FITC anti-CD90.2 (Thy1.2), clone 30-H12, Isotype rat IgG2b, k; FITC anti-mouse NK1.1, clonePK136, Isotype mouse IgG2a, k; FITC anti-mouse CD115 (Kit), clone 29A14, Isotype rat IgG2a, k; FITC anti-mouse CD49b, clone H9M2, Isotype Armenian hamster IgG; PE anti-mouse CD11c, clone N418, Isotype Armenian hamster IgG; PerCP/Cy5.5 anti-mouse CD19, clone 6D5; Isotype rat IgG2a, k; PerCP/Cy5.5 anti-mouse CD45, clone I3/2,3, Isootype rat IgG2b, k; PerCP/Cy5.5 anti-mouse Ly6A/E (Sca-1), clone D7, Isotype rat IgG2a, k; PerCP/Cy5.5 anti-mouse TCRγδ, clone GL3, Isootype Armenian hamster IgG; PE/Cy7 anti-mouse/human CD11b, clone M1/70, Isotype IgG2b, k; PE/Cy7 anti-mouse DC11c, clone N418, Isotype Armenian hamster IgG; PE/Cy7 anti-mouse DC122 (IL-7Rα), clone TM-B1, Isotype rat IgG2b, k; PE/Cy7 anti-mouse DC127 (IL-7Rα), clone A7/R34, Isotype rat IgG2a, k; PE/Cy7 anti-mouse IFN-γ, clone XMGG1,2, Isootype rat IgG1, k; PE/Cy7 anti-mouse IgL1, Isotype C5l, clone RTK2071, allophycocyanin anti-mouse IA/IE, clone M5/114.15.2, Isotype IgG2b, k; allophycocyanin anti-mouse NKp46, clone 29A14, Isotype rat IgG2a, k; allophycocyanin anti-mouse CD49b, clone D5x, Isotype rat IgM; Alexa Fluor 647 anti-mouse F4/80, clone BM8, Isotype rat IgG2b, k; Alexa Fluor 647 anti-mouse Ly6G, clone BM8, Isotype rat IgG2a, k; Alexa Fluor 700 anti-mouse Ly6C, clone BM8, Isotype rat IgG2a, k; allophycocyanin/Cy7 anti-mouse CD25, clone PC61, Isotype rat IgG1; allophycocyanin/Cy7 anti-mouse F4/80, clone BM8, Isotype rat IgG2a, k (all purchased from BioLegend); eFluor450 anti-mouse CD11c, clone M1/70, Isotype rat IgG2b, k; eFluor450 anti-mouse CD11c, clone N418, Isotype Armenian hamster IgG; PE anti-mouse/human RORγt, clone AFKJS-9, Isotype rat IgG2a, k; PerCP/Cy5.5 anti-mouse TCRγδ, clone H57-957, Isotype Armenian hamster IgG; PE/Texas Red anti-mouse CD45, clone 30-F11, Isotype rat IgG2b, k; PE anti-CD45, clone (from Invitrogen), V450 anti-mouse CD4, clone RM4-5, Isootype rat IgG2a, k; V500 anti-mouse CD8α, clone 53-67, Isotype rat IgG2a, k; V500 anti-mouse CD90.2 (Thy1.2), clone 53-21, Isotype rat IgG2a, k; PE anti-mouse CD90.2 (Thy1.2), clone 53-21, Isotype rat IgG2a, k (from BD Biosciences); purified NOS2 (M-19) rabbit polyclonal IgG (Santa Cruz); Alexa Fluor 488 goat anti-rat IgG (H + L); and Alexa Fluor 647 goat anti-rabbit IgG (H + L) (both from Life Technologies).

Flow cytometry

LIVER and spleen cells were isolated from naive or infected animals. In brief, a single cell suspension was prepared from the spleen or liver by passing the organ through a 70-μm nylon cell strainer, followed by treatment with RBC lysis buffer. Liver cells were further subjected to a 40%-80% Percoll (GE Healthcare) gradient to isolate the mononuclear cells. Cells were washed and the number of viable cells was counted by trypan blue exclusion. The mononuclear cells were then stained with the different combinations of the following Abs: Ly6G-PB, CD90.2-V500, CD11c-PE, CD45-PE-Texas Red, TCRβ-PerCP/Cy5.5, TCRγδ-PerCP/Cy5.5, CD19-PerCP/Cy5.5, CD11b-PE/Cy7, NK1.1-allophycocyanin, NKp46-allophycocyanin, CD49b-allophycocyanin, Ly6C-Alexa Fluor 700, F4/80-allophycocyanin eFluor700, CD11c-eFluor 450, and CD11b-eFluor 450. For the transcription factor analysis, after the surface staining, cells were processed for intracellular staining using the eBioscience “Transcription factor staining buffer set” according to the manufacturer’s instructions and then stained for RORγt-PE, and Tbet-PE/Cy7. Samples were stained using Diva software on an LSR II flow cytometer (BD Biosciences).

Cells from Rag2−/−, Rag2−/−γc−/−, and Rag2−/−IFN-γ−/− mice were stained with the different combinations of the following Abs: CD4-V450, CD45-PE-Texas Red, BV421, CD3-V500, CD8-V500, CD45-PE-Cy7, NK1.1-FTTC, NKp46-FTTC, CD49b-FTTC, CD90.2-PE, CD45-PerCP/Cy5.5, Sca-1-PerCP/Cy5.5, CD122-PE/Cy7, CD127-PE/Cy7, F4/80-PE/Cy7, F4/80-PE/Cy7, Ly6G-A6F47, CD25-aloephycocyanin/Cy7, and F4/80-aloephycocyanin/Cy7. For the intracellular IFN-γ flow cytometry analysis, total spleen cells or total mononuclear liver cells were stimulated with PMA, Ionomycin, and brefeldin A for 5 h at 37°C. After cell surface staining, cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin. Cells were then incubated with IFN-γ–PE/Cy7 or the isotype-matched control for 20 min at room temperature. After two washing steps, cells were acquired by flow cytometry in a FACSCanto II cytometer (BD Biosciences) using a BD FACSDiva software. All data were analyzed using FlowJo software (Tree Star).

Cell sorting

Liver mononuclear cells prepared as described earlier were pooled from three to four naive or infected Rag2−/− mice. For the intracellular IFN-γ– camping, cells were stimulated with PMA, Ionomycin, and Brefeldin A as described above. After two washing steps, cells were acquired by flow cytometry in a FACSCanto II cytometer using a BD FACSDiva software.
expressing CD11b and/or CD11c), and CD45+/Thy1.2+/NK1.1−NKp46+/CD49b−CD11b+CD11c−/F4/80−/Ly6G− (Thy1.2+/non-NK/nonmyeloid cells).

Spleen and liver mononuclear cells pooled from 10 B6.Rag2−/− mice were prepared as described earlier and stained either for surface phenotyping or intracellular IFN-γ detection. Cellular events were acquired using the ImageStream (Amnis; EMD Millipore, Darmstadt, Germany) imaging flow cytometer, and the multiple image analysis was performed using the IDEAS software (v6.0.348, Amnis; EMD Millipore).

RNA extraction and real-time RT-PCR

Total RNA was extracted from sorted populations (RNeasy Micro kit; Qiagen) according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA, using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and Random Hexamers (Fermentas), and real-time PCR (iCycler iQ5; Bio-Rad) was performed using TaqMan Universal PCR Master Mix and primers specific for Hprt, Ifng, Ifi22, and Zbtb16 (Applied Biosystems) according to the manufacturer’s protocols.

Histological and morphometric analysis

Tissues samples were fixed in buffered formalin, embedded, and processed for histology by staining with H&E. Slides were photographed using an Olympus CX31 light microscope equipped with a DP-25 camera (Imaging Software Cell^B; Olympus, Center Valley, PA). One liver section per animal was analyzed, and the ΔΔCt method was used to calculate relative levels of targets compared with Hprt. Data were analyzed using IQ5 Optical System software (Bio-Rad).

Immunofluorescence

Liver tissues were fixed in 4% paraformaldehyde, washed in PBS, incubated for 2 h in a 20% sucrose solution and embedded in optimal cutting temperature compound (OCT), and stored at −80°C until sectioning. Tissue sections (10 μm thickness) were performed in a frozen cryostat and stored at −20°C until use. Slides were saturated in blocking solution (10% FBS, 0.1% Triton X-100 in PBS) for 1 h at room temperature. Rabbit polyclonal IgG specific for inducible NO synthase (iNOS; M-19; Santa Cruz Biotechnology) and rat monoclonal CD90.2 (Thy1.2) (53-21; BD Pharmingen) Abs were diluted in Ab dilution buffer (1% FBS, 0.1% Triton X-100 in PBS) and incubated overnight with tissue sections at 4°C. After washing, sections were incubated with Alexa 647 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-rat IgG Abs (Life Technologies) for 1 h at room temperature. Sections were then counterstained and mounted with DAPI Fluoroshield and imaged in a Leica DMI 6000B inverted microscope using the ORCA-Flash4.0 version 2 (Hamamatsu Photonics) CMOS camera. Images were further analyzed and processed using ImageJ.

Statistical analysis

Results were expressed as means ± 1 SD. Statistical significance was calculated by using the unpaired Student t test or the one-way ANOVA test with a Tukey posttest. A p value <0.05 was considered statistically significant.

Results

IFN-γ is expressed in nonconventional Thy1.2+ cells that lack T, NK, and myeloid lineage markers

The evidence of a T cell– and NK cell–independent IFN-γ–mediated immunity was suggested before by our group (9) and others (6, 13, 26). Hence, to identify a non-T, non-NK cell source of IFN-γ, we used an IFN-γ-YFP reporter mouse (Yeti) backcrossed with Rag1−/− mice (1–4). The YFP+ cells identified by flow cytometry were CD45+, and their frequency was very high in both the livers and the spleens of naive mice (Fig. 1A). To label NK cells, we stained for different NK cell markers, NK1.1, NKp46, and CD49b, using specific mAbs conjugated with a single fluorochrome. Most of the YFP+ cells expressed markers of NK cells, but a minor subset of non-NK, F4/80+/YFP+ cells was additionally detected. Further analysis revealed that these latter cells lacked the myeloid marker CD11b and expressed Thy1.2. To validate these findings, we assessed Ifng gene expression on sorted NK cells (NK1.1−/NKp46−/CD49b−), myeloid cells (CD11b+CD11c+/NK1.1−NKp46+/CD49b+), and the Thy1.2+ non-NK/non-myeloid cells (Thy1.2+/NK1.1−NKp46−CD49b−/CD11b+CD11c−/F4/80−/Ly6G−) from Rag1−/− (nonreporter) animals (Fig. 1B). Although ifng gene expression was detected in NK cells, no Ifng expression was detected in sorted myeloid cells. The non-NK/nonmyeloid Thy1.2+ cells exhibited the same levels of constitutive ifng gene expression as the NK cell subset (Fig. 1C), confirming the flow cytometry data. Despite the negative results for the myeloid cells, and because these cells have been reported as possible sources of IFN-γ (16–21, 24), we decided to further ascertain the capacity of myeloid cells, namely macrophages, to express IFN-γ. To do so, we subjected bone marrow–derived macrophages from C57BL/6 animals to different polarizing stimuli and tested whether the different polarizations could dictate the capacity to express IFN-γ. No Ifng gene expression was detected in any of the macrophage subsets despite their ability to express other cytokines (Supplemental Fig. 1).

Because the IFN-γ-YFP mRNA in Yeti animals is stabilized by a bovine growth hormone polyA tail, it leads to an aberrant expression of IFN-γ (25), as confirmed in this study in the Rag1−/− Yeti mice, which had around 70% YFP+ cells in the liver and 60% in the spleen (Fig. 1A). To ascertain whether our previous results were not an artifact of the Yeti mouse model, we used another reporter mouse where the IFN-γ−/−YFP mRNA is under control of the endogenous IFN-γ-polyA tail (Great mice, IFN-γ reporter with endogenous polyA transcript) in a C57BL/6 background, that is, with no deletion of Rag. The frequency of the YFP+ cells in this mouse model was around 17% in the liver and 2% in the spleen (Supplemental Fig. 2A). To exclude the contribution of the YFP+ cells to expression of T cells, such as CD4+ and CD8+ T cells, NKT cells, and TCRgd+ T cells, we grafted YFP−/CD45−/CD11b−/CD19−/Thy1.2−/Ly5.1−/Ly5.2−/CD4−/CD8−/NKT−/TCRgd−/CD19− cells. An even higher percentage of these cells was found to express YFP (Supplemental Fig. 2B), and a population of Thy1.2+/non-NK/non-T/non-B cells was identified in the liver and spleen of Great mice (Fig. 2A). In the livers, 50% of these Thy1.2+/NK− cells expressed YFP, slightly more than the NK cells (around 40%) (Fig. 2B). In contrast, in the spleen, the frequency of YFP+ cells within the Thy1.2+/non-NK population was low (around 2%). The cell number analysis reflected these differences: despite the higher Thy1.2+/non-NK cell numbers in the spleen (2.3 × 105) when compared with the liver (3.9 × 106), the number of YFP+ cells within this population was higher in the latter (2.2 × 103 and 1.4 × 103, respectively). In contrast, the number of YFP+/NK− cells was higher in the spleen (Fig. 2C). Further, analysis of these Thy1.2−/non-NK cells revealed that, in addition to being F4/80+, they also lacked the expression of CD11b, CD11c, and Ly6G, confirming their nonmyeloid origin, and a small subset of these cells (17.7% in the liver and 33.4% in the spleen) express Ly6C, whereas the rest do not (Fig. 2D). Despite the substantial difference in YFP expression, both the liver and the spleen populations had a similar phenotype. Moreover, the majority of the Thy1.2+/non-NK cells in the liver expressed T-bet (~72%), a
master regulator in IFN-γ-producing cells, and a small fraction of these cells expressed RORγt, in contrast with NK cells, which were all T-bet+ and RORγt2 (Fig. 2E). The spleen presented a similar transcription factor pattern, although the frequency of T-bet+ cells was much lower, paralleling the lower YFP expression.

**CD45+ Thy1.2+ cells with a non-T, non-NK, and nonmyeloid phenotype are found in common γc-deficient mice**

We then asked whether the development of nonconventional IFN-γ-expressing Thy1.2+ cells depended on the common γc component of the IL-2 family of receptors required for the signaling to several ILs such as IL-7 and IL-15 necessary for the development of T cells, B cells, NK cells, and all the other ILCs. To do so, we used IFN-γ nonreporter Rag2+/− γc−/− mice and consistently detected the presence of the Thy1.2+/non-NK/nonmyeloid population in the liver and the spleen (Fig. 3A). The absence of CD68 expression confirmed their nonmyeloid origin (Supplemental Fig. 2C). To ascertain whether these cells express IFN-γ, we assessed gene expression by quantitative PCR (qPCR) on sorted Thy1.2+ nonmyeloid cells and also on myeloid+ (CD11b+ and/or CD11c+) cells from both the liver and the spleen (Fig. 3B). A significant expression of the Ifng gene was detected in sorted Thy1.2+ nonmyeloid cells in the liver, whereas no Ifng expression was detected in the spleen. These findings are in agreement with the very low levels of IFN-γ–YFP detected by cytometry in the spleens of the Great reporter mice (Fig. 2B, 2C) and support the existence of these cells in the absence of reporter gene insertion in the Ifng locus. No Ifng gene expression was detected on myeloid cells (Fig. 3B), but it was found in sorted NK cells from Rag2−/− mice (Supplemental Fig. 3A).

To better characterize the Thy1.2+/non-NK/nonmyeloid population present in both Rag2−/− and Rag2−/− γc−/− mice (see Supplemental Fig. 2D for the gating strategy), we performed a comparative analysis in these cells of markers that are associated with NK cells, lymphoid tissue inducer cells, and other ILCs. The results showed that these cells did not express CD122 (IL-2Rβ), a
marker of NK cell precursors, and a fraction of them expressed MHC class II. Moreover, the majority of these cells express CD127 (IL-7Rα) and Sca-1, with a small fraction expressing low levels of CD25 (Fig. 3C). Further analysis revealed that these cells lacked CD3 and CD8, and in the absence of the γc chain they expressed CD4 (Supplemental Fig. 2E). Interestingly, the expression of both CD127 and CD4 were lower when the γc chain signaling was present (Rag2<sup>−/−</sup>/2 mice). In fact, the CD4 expression was totally abolished in cells of the liver of Rag2<sup>−/−</sup>/2 mice. We next assessed the expression of Id2 and Zbtb16 (encoding for Id2 and PLZF, respectively) in sorted CD45+/Thy1.2+ nonmyeloid cells of Rag2<sup>−/−</sup>/2<sup>γc−/−</sup> mice. As a control, we used NK cells sorted from Rag2<sup>−/−</sup>/2 mice (Supplemental Fig. 2F).

Despite some variability between experiments, we observed that Rag2<sup>−/−</sup>/2<sup>γc−/−</sup>Thy1.2+ cells expressed Id2 in equivalent levels to the Rag2<sup>−/−</sup>/2NK cells. Interestingly, Zbtb16 mRNA was specifically detected in Rag2<sup>−/−</sup>/2<sup>γc−/−</sup>Thy1.2+ liver cells and poorly expressed in the spleen. As expected, NK cells did not express Zbtb16 mRNA (Supplemental Fig. 2F).

**FIGURE 2.** Identification of IFN-γ-expressing Thy1.2+ cells lacking T, NK, or myeloid cell markers in the livers (upper panels) and spleens (lower panels) of Great mice. (A) Expression of Thy1.2 and NK (NK1.1/NKp46/CD49b) markers in cells from YFP-IFN-γ reporter Great mice gated on CD45+/TCRβ<sup>−/−</sup> TCRγδ<sup>−/−</sup> CD19<sup>−/−</sup> cells. (B) YFP expression on gated Thy1.2+NK<sup>−/−</sup> cells and NK<sup>−/−</sup> cells. (C) Number of total Thy1.2+NK<sup>−/−</sup> cells and YFP+/Thy1.2+NK<sup>−/−</sup> cells (upper panel), and total NK cells and YFP+NK<sup>−/−</sup> cells (lower panel). (D) Histograms of CD11b, CD11c, Ly6G, Ly6C expression on Thy1.2+NK<sup>−/−</sup> cells (tinted gray) and NK<sup>−/−</sup> cells (black line). The frequencies of positive cells for each cell marker are indicated in each graph shaded as the corresponding histogram. (E) T-bet and RORγt expression on Thy1.2+NK<sup>−/−</sup> cells and NK<sup>−/−</sup> cells from the liver (upper panel) and the spleen (lower panel). Data are representative from five different experiments with at least three mice each.

The lack of IFN-γ, but not of the common γc, exacerbated the infection and compromised the development of granulomas in a Rag-deficient background

To assess the biological relevance of this rare population of IFN-γ-producing Thy1+ cells, we used a model of *M. avium* infection. We have previously shown that susceptibility of SCID mice to *M. avium* could be exacerbated by the Ab-mediated neutralization of IFN-γ, suggesting an innate source for this cytokine (9). Single-Ab depletions of NK cell subsets (e.g., asialo-GM1<sup>−/−</sup> or Thy1<sup>−/−</sup>) were ineffective at achieving the same exacerbation but were incomplete in their ability to deplete NK cells in SCID mice, suggesting a redundant role for different populations of innate cells in the small but statistically significant protection afforded by IFN-γ in the absence of T cells (9). In this study, we found increased bacterial loads in the livers and spleens of B6.IFN-γ<sup>−/−</sup> mice compared with the C57BL/6 control, as well as in the Rag2<sup>−/−</sup> IFN-γ<sup>−/−</sup> double-knockout animals when compared with the Rag2<sup>−/−</sup> mice (Fig. 4A, Supplemental Fig. 4A). In contrast, Rag2<sup>−/−</sup>/γc<sup>−/−</sup> animals were as able to control the bacterial proliferation...
Thy1.2+/NK cells represent the mean of cells from Rag2 mice as the corresponding histogram. Data are representative of four different experiments with at least four mice each.

Frequencies of positive cells for each cell marker determined according to the fluorescence minus one control (FMO ctrl) are indicated in each graph shaded. Values are normalized to the inner macrophage core. Instead, they just consisted of lymphoid cell accumulations (Fig. 4B). Because both mice lack T cells and Rag2/–γc/– mice are further devoid of NK cells and other ILCs, these data suggest that IFN-γ may be produced by cells other than T cells, NK cells, or other ILC1 subsets as well. The differences in the bacterial loads of both organs between the strains devoid of T cells were evident only at 60 d postinfection (dpi), a time point concomitant with the observation of granulomatous lesions (Fig. 4B, 4C). In contrast, the livers of naive Rag2/–γc/– mice (data not shown).

Next, we measured IFN-γ protein in liver and spleen mononuclear cells stimulated with PMA/Ionomycin and brefeldin A (Fig. 5C). Thy1.2/–non-NK liver cells (upper panel) either from infected Rag2/–γc/– or Rag2/– mice presented equivalent IFN-γ frequencies (around 17%). In contrast, spleen Thy1.2/–non-NK cells (lower panel) did not express significant amounts of IFN-γ. We also used imaging flow cytometry to measure and detect IFN-γ+ cells within the Thy1.2/–non-NK population from both animal models (representative images for IFN-γ+ and IFN-γ– cells are not shown).

As Rag2/– mice (Fig. 4A, Supplemental Fig. 4B). Because both mice lack T cells and Rag2/–γc/– are further devoid of NK cells and other ILCs, these data suggest that IFN-γ may be produced by cells other than T cells, NK cells, or other ILC1 subsets as well. The differences in the bacterial loads of both organs between the strains devoid of T cells were evident only at 60 d postinfection (dpi), a time point concomitant with the observation of granulomatous lesions in these models. At 30 dpi, cell infiltrations were only detected in the C57BL/6 and the B6.INF-γ/– (although the latter with a much lower number) (data not shown). In addition, the lower CFU values observed particularly in the spleens of the different Rag-deficient strains 30 dpi as compared with the Rag-sufficient strains were due to a lower inoculum implantation at the start of the infection because of the very small size of the organ in these animals.

Granuloma assembly is highly dependent on IFN-γ production and is one of the main histopathological features of mycobacterial infections. C57BL/6 mice showed the largest cell infiltration areas, and their granulomas were well developed. The number of lesions in infected IFN-γ−/− or Rag2−/− IFN-γ−/− mouse liver controls was smaller and lacked the prototypical organization of the inner macrophage core. Instead, they just consisted of lymphoid cell accumulations (Fig. 4B, 4C). In contrast, the livers of infected Rag2−/− and Rag2−/−γc−/− mice showed granulomatous structures with a well-developed macrophage core and higher number of lesions (Fig. 4B, 4C).

Despite the extremely low number of the Thy1.2/–non-NK cells in naive Rag2−/−γc−/− mice, their number increased sharply during the course of the infection, with a 10-fold increase in the liver and a 20-fold increase in the spleen at 60 d of infection (Fig. 5A). Strikingly, these cells could be found by immunofluorescence microscopy within the granulomatous lesions in the liver adjacent to macrophages that expressed iNOS, an enzyme known to be induced by IFN-γ (Fig. 5B) and that was also found to be induced in the livers of infected Rag2−/− mice (data not shown).

We used a strategy shown. The frequency of these cells is represented next to the respective gates. (A) CD45+ liver (upper panel) and spleen (lower panel) cells from Rag2−/−γc−/− mice were phenotypically characterized for their expression of CD11b, CD11c, F4/80, Ly6G, and Thy1.2 according to the strategy shown. The frequency of these cells is represented next to the respective gates. (B) Myeloid+ (CD11b+/CD11c+) cells and Thy1.2+ myeloid+ (CD11b+/CD11c+/F4/80+/Ly6G+) cells were sorted from Rag2−/−γc−/− livers and spleens, and the Ifng gene expression was determined by qPCR.

Data represent the mean ± SD from three different experiments. (C) Representative histograms of CD122, MHC-II, CD127, Sca-1, and CD25 expression on Thy1.2+/non-NK liver (upper panel) and spleen (lower panel) cells, and from Rag2−/−γc−/− (tinted gray) and Rag2−/− mice (black line). The frequencies of positive cells for each cell marker determined according to the fluorescence minus one control (FMO ctrl) are indicated in each graph shaded as the corresponding histogram. Data are representative of four different experiments with at least four mice each.

FIGURE 3. Identification of IFN-γ–expressing Thy1.2+ cells in mice lacking Rag-2 and IL-2Rγc. (A) CD45+ liver (upper panel) and spleen (lower panel) cells from Rag2−/−γc−/− mice were phenotypically characterized for their expression of CD11b, CD11c, F4/80, Ly6G, and Thy1.2 according to the strategy shown. The frequency of these cells is represented next to the respective gates. (B) Myeloid+ (CD11b+/CD11c+) cells and Thy1.2+ myeloid+ (CD11b+/CD11c+/F4/80+/Ly6G+) cells were sorted from Rag2−/−γc−/− livers and spleens, and the Ifng gene expression was determined by qPCR. Values are normalized to Hprt expression. Reactions were run in triplicate. Each point represents the analysis of mRNA pooled from 10 animals. Data are representative of four different experiments with at least four mice each.
shown in Fig. 5D). As control, IFN-γ was also detected in NK cells from Rag2²⁻/⁻ mice infected i.v. for 30 or 60 d. Data represent the mean CFUs ± SD from at least five mice per group of one of three experiments. (B) Representative histology of liver sections stained with H&E from mice infected for 60 d. Photos were taken with a light microscope Olympus CX31 with a DP-25 camera using the Imaging Software Cell^B. Pictures are shown with original magnification ×10 and ×40 in the upper and lower panels, respectively. (C) Percentage of infiltrated area in the livers of the indicated mice 60 dpi. Data represent the mean ± SD from six to eight mice per group. Each point represents one liver section. The statistical analysis of the differences between mutant mice and the C57BL/6 control group is shown above each column. Additional comparisons between other groups are indicated by horizontal bars. The p value was determined by a one-way ANOVA test with a Tukey multiple comparisons posttest (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

The lack of IFN-γ, but not of the common γc, exacerbates the infection in a Rag-knockout background. (A) M. avium 2447 burden in the spleen and liver of C57BL/6, IFN-γ²⁻/⁻, Rag2²⁻/⁻ IFN-γ²⁻/⁻, Rag2²⁻/⁻, and Rag2²⁻/⁻ γc²⁻/⁻ mice infected i.v. for 30 or 60 d. Data represent the mean CFUs ± SD from at least five mice per group of one of three experiments. (B) Representative histology of liver sections stained with H&E from mice infected for 60 d. Photos were taken with a light microscope Olympus CX31 with a DP-25 camera using the Imaging Software Cell^B. Pictures are shown with original magnification ×10 and ×40 in the upper and lower panels, respectively. (C) Percentage of infiltrated area in the livers of the indicated mice 60 dpi. Data represent the mean ± SD from six to eight mice per group. Each point represents one liver section. The statistical analysis of the differences between mutant mice and the C57BL/6 control group is shown above each column. Additional comparisons between other groups are indicated by horizontal bars. The p value was determined by a one-way ANOVA test with a Tukey multiple comparisons posttest (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Thy1.2+ cells of Rag2²⁻/⁻ γc²⁻/⁻ mice have a protective role during M. avium infection in the liver

To assess whether Thy1.2⁺/non-NK cells displayed a protective role in lymphopenic mice infected with M. avium, we depleted the Thy1.2 population in infected Rag2²⁻/⁻ γc²⁻/⁻ mice using mAbs (clone 30-H12). The depletion was conducted over 60 d of infection and confirmed by flow cytometry (Fig. 6A). Although no differences in the bacterial proliferation were observed in the spleens of anti-Thy1.2⁺-treated versus control animals, the Ab depletion reverted the 2-fold decrease in liver mycobacterial loads observed in Rag2²⁻/⁻ γc²⁻/⁻ mice when compared with an IFN-γ⁺/⁺ deficient host (Fig. 6B). These results suggest that the Thy1.2⁺ IFN-γ⁺-producing cells can functionally participate in the immune response against M. avium.

Discussion

The most important producers of IFN-γ in the mammalian organism are T lymphocytes and NK cells. B cells, epithelial cells, but more consistently myeloid cells, such as macrophages, DCs, and neutrophils, have also been reported as capable of producing this cytokine (14–21, 24). Recently, ILCs distinct from NK cells...
FIGURE 5. IFN-γ-expressing Thy1.2+ cells and iNOS expression in granuloma macrophages from Rag2+/-γc+/-infected mice. (A) Number of Thy1.2+ myeloid cells in uninfected (ui) or M. avium–infected livers and spleens from Rag2−/−γc−/− mice 60 dpi. Data represent the mean ± SD from at least five mice per group. (B) Thy1.2 and iNOS expression in granuloma-like structures of the liver at 60 dpi. Photos were taken with an inverted microscope LEICA DMI 6000B with the ORCA-Flash4.0 version 2 (Hamamatsu Photonics) CMOS camera. (C) Intracellular IFN-γ expression in CD45+ Thy1.2+/NK−/myeloid− liver (upper panel) and spleen (lower panel) cells from both Rag2−/−γc−/− and Rag2−/− mice and in CD45+NK+ cells from Rag2−/− mice after 30 d of infection. Isotype control for IFN-γ is shown in spleen CD45+Thy1.2+/NK−/myeloid− cells from (Figure legend continues)
and CD45+NK+ cells from Rag2

Flow Imager camera. The selected images are representative of the CD45+/Thy1.2+/NK
(upper panel) and spleen cells (lower panel) from

Using flow cytometry, we found a small population of CD45+

M. avium
during the course of infection. A different clone of Thy1.2 was used to identify the frequency of depleted Thy1.2 cells (clone 53-21). (0.01, ****

c control, the bacterial loads of infected Rag2

Tukey multiple comparisons posttest (**

from two experiments. Both experiments were performed with four to five mice per group. The

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Because the latter is required for the generation of NK cells and

both the expression of Rag and the common γc of the IL-2R. Further studies should address whether this new population may represent a distinct or precursor lineage of innate-like cells. In contrast, the fact that these cells are CD127γc (IL-7Rαc) and are present in very small numbers in the absence of γc suggests that the signaling via the IL-7Rαγc heterodimer may

be important for their development. Supporting this hypothesis are the increased numbers of these cells in immune-competent mice (C57BL/6.Great), where the presence of γc allows the signaling via IL-7Rα. Alternatively, the IL-7Rα could form a heterodimer complex with the thymic stromal lymphopoietin receptor for thymic stromal lymphopoietin signaling, as shown during the induction of Th2 responses (30, 31). A further possibility is that the cells found in Rag2γc−/− mice are heterogeneous and represent additional populations, which may include ILC subsets requiring γc signaling and therefore are absent in the Rag2γc−/−mutant mice. Finally, it is possible that the population of Thy1.2γc cells found in the double-mutant mice may represent the precursors of γc-dependent cells blocked in their development because of the lack of γc signaling. This last hypothesis is supported by the fact that Rag2γc−/−Thy1.2γc− mice express Id2 mRNA. Moreover, these cells express Zbtb16 (encoding for PLZF), particularly in the liver. PLZF was described as being expressed in a precursor stage of ILC, an ILC progenitor (ILCp) that gives rise to all ILCs, except lymphoid tissue inducer cells and NK cells, and described as Lin−Id2−IL-7Rα−CD25−αβγ− cells (27, 32, 33). Klose and colleagues

and named type 1 and type 3 ILCs were shown to express IFN-γ and to participate in the response to infection and inflammation (27–29). Based on our previous work, where we found T cell– and NK cell–dependent IFN-γ–mediated immunity (9), we used IFN-γ reporter mice to look for additional IFN-γ–producing cells. Using flow cytometry, we found a small population of CD45γc Thy1.2γc non-T, non-B, non-NK, and nonmyeloid cells that expressed YFP in two distinct reporter strains. The IFN-γ–YFP expression by these cells was particularly evident in the liver, whereas in the spleen the expression was rather low. A cellular population with a similar phenotype was found in nonreporter Rag-deficient mice. These latter cells expressed IFN-γ as confirmed by qPCR. These cells were also found in mice that lack both the expression of Rag and the common γc of the IL-2R. Because the latter is required for the generation of NK cells and all the other ILCs, further studies should address whether this new population may represent a distinct or precursor lineage of innate-like cells. In contrast, the fact that these cells are CD127γc (IL-7Rαγc) and are present in very small numbers in the absence of γc suggests that the signaling via the IL-7Rαγc heterodimer may

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Rag2γc−/−γc−/− animals. Data are from one representative independent experiment out of three. (D) Images of IFN-γ+ or IFN-γ− liver cells taken using a Flow Imager camera. The selected images are representative of the CD45γcThy1.2γcNK−/myeloid− cells from Rag2γc−/−γc−/− (I) and Rag2γc−/−γc−/− (II) animals, and CD45γcNK− cells from Rag2γc−/− mice (III). The p value was determined by a one-way ANOVA test with a Tukey multiple comparisons posttest (**p < 0.01, ****p < 0.0001).
(27) have shown that although some ILC lineages are dependent on IL-7R signaling, common lymphoid progenitor and ILCp bone marrow numbers were only slightly reduced in IL-7R−/− animals. Importantly, the ILCp were mainly found in mucosal organs such as the small intestine and the liver, and were sparse in the spleen.

It is evident that the Rag2−/−γc−/− Thy1.2+ cells identified in this article are phenotypically different in the liver and the spleen. Whether they belong to the same lineage is also unclear. It is not unusual for a certain cell type to differ phenotypically according to the microenvironment where it resides. Significant variations of the same ILC subset within different organs have been reported. For instance, Cortez and colleagues (34) reported a distinct subset of ILC1 in the salivary glands that shares features between ILC1 and NK cells. Their work also highlights how the particular microenvironment of the salivary glands can impact the ILC development. Robinette et al. (35) show that the discrimination between ILC1 and NK cells can be trickier in the spleen than the liver, because of the fact that these cells express different surface markers in these organs.

To understand the physiological relevance of the IFN-γ-producing Thy1.2+non-NK cells, we explored their role in an experimental M. avium infection model because the control of this opportunistic pathogen is strictly dependent on IFN-γ (3–5, 36, 37). In the absence of T cells, the major producers of IFN-γ, Rag-deficient mice were still more resistant than mice lacking both Rag and IFN-γ, suggesting a T cell–independent source of this cytokine. During M. avium infection, we observed an increase in the numbers of a novel CD45+Thy1.2+ non-NK and nonmyeloid IFN-γ-producing population of cells. The ability of these infected cells to produce IFN-γ protein was also confirmed by intracellular staining after PMA/ ionomycin stimulation. Importantly, these cells were found to localize within granulomatous lesions in the liver in the vicinity of cells staining positive for iNOS, which is most frequently induced by IFN-γ. Further, Thy1.2-specific mAb-mediated cell depletion increased the pathogen growth in the liver of Rag2−/−γc−/− mice to the levels of Rag2−/−γc−/−IFN-γ−/− mice while reducing the cell infiltration area. Together, our data demonstrate that this non-conventional population of IFN-γ-producing cells accumulates in the liver, within the granuloma, and mediates improved control of bacterial growth. These cells were not frequent in the spleen, and their depletion did not affect the bacterial burden in this organ, supporting a liver-specific action of Thy1.2+ cells in response to mycobacterial infection. We cannot exclude the possibility that the control of bacterial proliferation observed in Rag2−/−γc−/− mice might also rely on IFN-γ–independent compensatory mechanisms such as TNF production. In the spleen, Thy1.2+non-NK cells produce minute amounts of IFN-γ, suggesting the involvement of another protective mechanism. The fact that Thy1.2 depletion in the infected Rag2−/−γc−/− animals did not affect the bacterial proliferation control in the spleens also supports this hypothesis. It is still possible that other cells may secrete IFN-γ and compensate for the removal of these CD45+Thy1.2−non-NK, nonmyeloid cells in the spleen. Macrophages, dendritic cells, or PMNs may be responsible for the splenic production of IFN-γ as described in other infection models (18–21). However, we failed to detect such cytokine production in these myeloid cells by either the use of reporter mice or by qPCR of sorted populations. The regulation of IFN-γ expression in these cells is likely different and not constitutive, as is found in ILCs. Thus, the lack of detection in myeloid cells by qPCR may relate to inadequate triggering stimuli or kinetic constraints.

Taken together, our study reveals a unique subset of innate IFN-γ–producing cells that develops in the absence of IL-2R common γc. The lower amounts of IFN-γ expressed by cells other than T cells, NK cells, or other ILCs in the spleen compared with the liver may explain why these cells were never detected before. In addition, the majority of the studies exploring in vivo responses to infection and the specific role of different cell populations have been done with spleen cells. In fact, there are very few studies exploring the different liver cell populations using flow cytometry and, to our knowledge, this is the first work to unveil the cellular expression of IFN-γ by nonconventional cells in the liver. Thus, our work has identified a new cell subset capable of producing IFN-γ constitutively and with a protective role toward M. avium infection in an immune-compromised mouse model.

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Disclosures

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