Intestinal Microbes Affect Phenotypes and Functions of Invariant Natural Killer T Cells in Mice

GERHARD WINGENDER,[*,‡] DARIUSZ STEPNIAK,[*] PHILIPPE KREBS,[§,‖] LIN LIN,[¶] SARA MCBRIDE,[#] BO WEI,[¶] JONATHAN BRAUN,[¶] SARKIS K. MAZMANIAN,[#] and MITCHELL KRONENBERG[*]

[*]Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, California; [‡]Institute for Molecular Medicine, University of Bonn, Bonn, Germany; [§]Department of Genetics, The Scripps Research Institute, La Jolla, California; [‖]Division of Experimental Pathology, Institute of Pathology, University of Bern, Bern, Switzerland; [¶]Department of Pathology and Laboratory Medicine, University of California Los Angeles, Los Angeles, California; and [#]Division of Biology, California Institute of Technology, Pasadena, California

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BACKGROUND & AIMS: Invariant natural killer T (*i*NKT) cells undergo canonical, Vα14–Jα18 rearrangement of the T-cell receptor (TCR) in mice; this form of the TCR recognizes glycolipids presented by CD1d. *i*NKT cells mediate many different immune reactions. Their constitutive activated and memory phenotype and rapid initiation of effector functions after stimulation indicate previous antigen-specific stimulation. However, little is known about this process. We investigated whether symbiotic microbes can determine the activated phenotype and function of *i*NKT cells. **METHODS:** We analyzed the numbers, phenotypes, and functions of *i*NKT cells in germ-free mice, germ-free mice reconstituted with specified bacteria, and mice housed in specific pathogen-free environments. **RESULTS:** Specific pathogen-free mice, obtained from different vendors, have different intestinal microbiota. *i*NKT cells isolated from these mice differed in TCR Vβ7 frequency and cytokine response to antigen, which depended on the environment. *i*NKT cells isolated from germ-free mice had a less mature phenotype and were hyporesponsive to activation with the antigen α-galactosylceramide. Intragastric exposure of germ-free mice to *Sphingomonas* bacteria, which carry *i*NKT cell antigens, fully established phenotypic maturity of *i*NKT cells. In contrast, reconstitution with *Escherichia coli*, which lack specific antigens for *i*NKT cells, did not affect the phenotype of *i*NKT cells. The effects of intestinal microbes on *i*NKT cell responsiveness did not require Toll-like receptor signals, which can activate *i*NKT cells independently of TCR stimulation. **CONCLUSIONS: Intestinal microbes can affect *i*NKT cell phenotypes and functions in mice.**

Keywords: αGalCer; T-Cell Activation; Mucosa; TLR.

BASIC AND TRANSLATIONAL AT

Invariant natural killer T (*i*NKT) cells are a unique subset of T lymphocytes characterized by the expression of an invariant T-cell antigen receptor (TCR) rearrangement, Vα14-Jα18 in mice (Vα14*i* NKT cells) and an orthologous Vα24-Jα18 (Vα24*i*) in human beings, and the recognition of antigens presented by CD1d, a nonpolymorphic major histocompatibility complex class I–like antigen-presenting molecule.[1–4] CD1d binds lipid structures, and one of the best-studied *i*NKT cell antigens is α-galactosylceramide (αGalCer), a synthetic version of a glycolipid originally isolated from a marine sponge.[1]

*i*NKT cells express surface molecules characteristic of antigen-experienced lymphocytes, and antigenic stimulation leads to the rapid induction of effector functions by *i*NKT cells such as the production of T-helper type (T_h)1 and T_h2 cytokines and potent cytotoxicity.[1–4] As a consequence of their vigorous early response, *i*NKT cells have been implicated in diverse immune reactions, including the pathogenesis of inflammatory diseases of the liver, pancreas, and intestine. Similar data in human patients are relatively sparse, still they suggest comparable roles for *i*NKT cells in different contexts. In the case of inflammatory bowel disease, most of the findings are consistent with a protective role for *i*NKT cells during T_h1-mediated diseases and a deleterious one in T_h2 diseases.[5,6] The fact that *i*NKT cells can cause either beneficial or detrimental effects in different disease models illustrates their dichotomous function and their ability to polarize the ensuing immune response in either a T_h1 or T_h2 direction.[7] In contrast to this diversity in the functional outcome, a protective role of *i*NKT cells almost uniformly has been reported both in animal models and in human patients with type I diabetes.[8,9]

In addition to αGalCer, glycolipid antigens known to stimulate the majority of *i*NKT cells have been reported in a few types of bacteria. One is *Sphingomonas/Sphingobium* species, which have glycosphingolipids similar to the original sponge antigen.[10,11] Another includes *Borrelia burgdorferi*, the causative agent of Lyme disease,[12] and *Streptococcus*

Abbreviations used in this paper: αGalCer, α-galactosylceramide; APC, antigen presenting cell; CCR9, C-C chemokine receptor type 9; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; GF, germ-free; *i*NKT, invariant natural killer T; IEL, intraepithelial lymphocytes; IL, interleukin; Jax, Jackson Laboratory; LI, large intestine; LPL, lamina propria lymphocytes; MyD88, Myeloid differentiation primary response gene (88); RF, restricted flora; SI, small intestine; SPF, specific pathogen free; Tac, Taconic Farms; TCR, T-cell antigen receptor; T_h, T-helper type; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRIF, TIR-domain-containing adapter-inducing interferon-β; Vα14*i*, invariant Vα14 to Jα18 TCR rearrangement.

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pneumoniae. Several additional pathogens have been reported to have glycolipid antigens that activate *i*NKT cells, including *Leishmania donovani* and *Helicobacter pylori*,[13–15] but in such cases it may be only a subset of the cells that are stimulated. More generally, the distribution and prevalence of *i*NKT cell antigens in the microbiota and in the wider environment, as well as their role in *i*NKT cell function under noninflammatory conditions, remain to be determined.

The constitutively activated phenotype of *i*NKT cells has been attributed to the presence of self-agonist glycolipid ligands that drive the selection of these cells and stimulate them continually in the periphery. Although there is some evidence for this, we set out to determine the role of intestinal bacteria in shaping the phenotype and function of *i*NKT cells. Our hypothesis was substantiated by the previous finding that ribosomal DNA sequences from *Sphingobium yanoikuyae* and related species are found in the mouse intestine,[16,17] suggesting these could be commensal organisms. Furthermore, sequences from the related bacteria *Novosphingobium aromaticivorans* have been found in the human intestine.[18] In addition, we showed previously that intragastric challenge with *S yanoikuyae* stimulated peripheral *i*NKT cells.[16] This indicated that gut-derived *i*NKT cell antigens are capable of activating peripheral *i*NKT cells. Here, we show that intestinal bacteria can modulate the phenotype, TCR Vβ-use, and the immune responses of *i*NKT cells, and that antigens from commensals that engage the semi-invariant TCR are likely a contributing factor.

Material and Methods

Mice and Cell Lines

Mice were housed under specific pathogen-free (SPF) conditions at the animal facilities of the La Jolla Institute for Allergy and Immunology (La Jolla, CA), the Scripps Research Institute (La Jolla, CA), and the Department of Pathology and Laboratory Medicine (Los Angeles, CA), or housed under germ-free conditions at the California Institute of Technology (Pasadena, CA) in accordance with the Institutional Animal Care Committee guidelines. C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or from Taconic Farms (Hudson, NY), Swiss Webster germ-free and SPF-housed animals were purchased from Taconic Farms, and B6.129S1-*Il12b*^{tm1Jm}/J (*interleukin* [*IL*]-*12p40*$^{-/-}$) were purchased from the Jackson Laboratory. Myeloid differentiation primary response gene 88 (MyD88) and TRIF (*Lps2*) double-deficient mice[19] and restricted flora (RF) mice have been described previously.[20,21] *S yanoikuyae* and *Escherichia coli* were purchased from the American Type Culture Collection (Manassas, VA). The T-cell lymphoma RMA was transfected virally to stably express CD1d as previously described,[22] resulting in the line RMA-CD1d.

Reagents and Monoclonal Antibodies

αGalCer was obtained from the Kirin Pharmaceutical Research Corporation (Gunma, Japan). Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was obtained from Invitrogen (Carlsbad, CA). Monoclonal antibodies against the following mouse antigens were used in this study: β_7-integrin (M293), C-C chemokine receptor type 9 (CCR9) (9B1, eBioCW-1.2), CD1d (1B1), CD3ε (145.2C11, 17A2), CD4 (GK1.5, RM4-5), CD5 (53-7.3), CD8α (53−6.7, 5H10), CD19 (1D3, 6D5), CD25 (PC61.5), CD44 (IM7), CD45R (B220, RA3-6B2), CD69 (H1.2F3), CD103 (2E7), CD122 (TM-b1), CD127 (A7R34), TCRβ (H57-597), NK1.1 (PK136), Vβ2 (B20.6), Vβ7 (TR310), granulocyte macrophage colony-stimulating factor (MP1-22E9), IL-2 (JES6-5H4), IL-4 (11B11), IL-13 (eBio13A), interferon-γ (XMG1.2), and tumor necrosis factor (TNF)-α (MP6-XT22). Antibodies were purchased from BD Biosciences (San Diego, CA), BioLegend (San Diego, CA), eBioscience (San Diego, CA), or Invitrogen. αGalCer-loaded CD1d tetramers were produced as described.[23]

Cell Preparation, In Vivo Challenge, and Flow Cytometry

Single-cell suspensions from liver, spleen, thymus, and intestine were prepared as described.[24,25] In vivo cytotoxicity assays and cell staining for flow cytometry were performed as reported previously.[24] *i*NKT cells were activated in vivo by intravenous injection of 1 μg αGalCer and analyzed 90 minutes later. Bacterial suspensions were gavaged using a 20G × 1.5 feeding needle.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Comparisons were drawn using a 2-tailed Student *t* test or an analysis of variance test. *P* values $<.05$ were considered significant and are indicated as follows: $^*P < .05$, $^{**}P \leq .01$, and $^{***}P \leq .001$. Each experiment was repeated at least twice, and background values were subtracted.

Results

Distribution and Phenotype of Intestinal iNKT Cells

We examined the frequency of *i*NKT cells in different sites in the intestine because there have been conflicting data on the frequency and distribution of these cells in the gut mucosa.[5] We analyzed *i*NKT cells in the lamina propria lymphocyte (LPL) and intraepithelial lymphocyte (IEL) compartments of the small intestine (SI) and large intestine (LI). *i*NKT cells readily could be detected in LPLs and IELs from both small and large intestines of SPF C57BL/6J mice (Figure 1*A*). The signal was specific for *i*NKT cells, as indicated by the low background when using unloaded CD1d tetramers (Figure 1*A*). We consistently observed a higher frequency of *i*NKT cells in the small intestine than in the LI and a higher frequency in LPLs than in IELs (Figure 1*B*). It is notable that the frequency of *i*NKT cells in the SI-LPL was comparable with the value in the spleen. The majority of intestinal *i*NKT cells were CD4$^+$ and NK1.1$^+$, with the exception of decreased NK1.1 expression by LI-IEL *i*NKT cells (Figure 1*C* and Supplementary Figure 1*A*). Most intestinal *i*NKT cells were CD69$^+$, CD44$^+$, and CD122$^+$, similar to their splenic counterparts (data not shown). Furthermore, only a small fraction of intestinal *i*NKT cells expressed CD103 (Supplementary Figure 1*B* and *C*), and intestinal *i*NKT cells also were mostly negative for the β_7-integrin (data not shown). In contrast, we

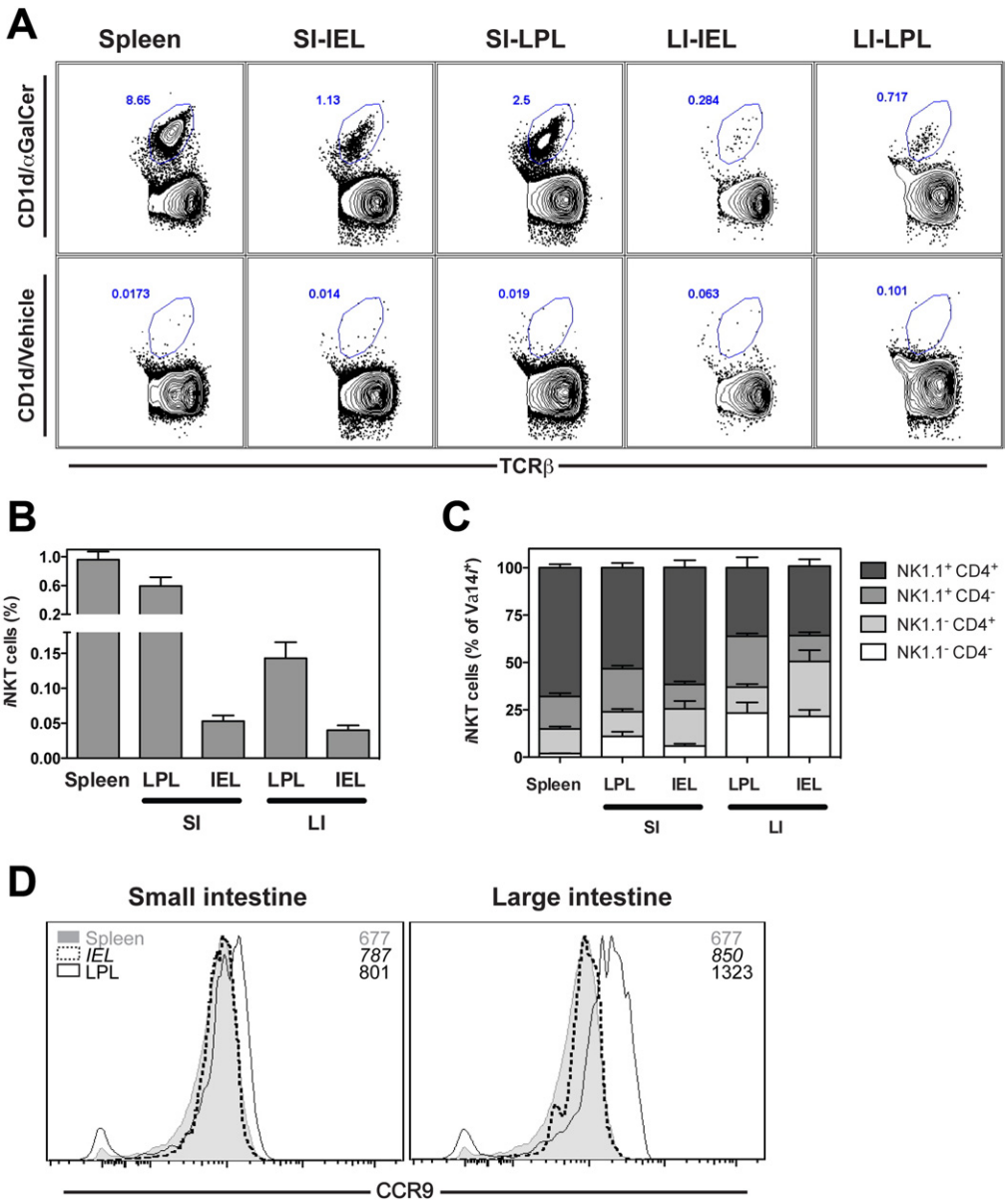

Figure 1. Distribution and phenotype of intestinal *i*NKT cells. (*A*) Lymphocytes from the indicated sites were incubated either with αGalCer loaded or unloaded CD1d-tetramers, analyzed by flow cytometry, and the frequencies of tetramer-positive cells within live TCRβ$^+$CD44$^+$CD8α$^-$CD19$^-$ cells are shown. (*B* and *C*) Relative percentage of *i*NKT cells within (*B*) total live lymphocytes and (*C*) their expression of CD4 and NK1.1, from indicated sites. The graphs summarize data from 3–5 independent experiments, with 6–9 samples per group. (*D*) Representative expression of CCR9 on *i*NKT cells derived from the spleen (*tinted in both panels*), IEL (*dashed*), or LPL (*black line*) from the small or large intestine. The *numbers in histograms* denote the geometric mean values for CCR9 on *i*NKT cells.

detected expression of CCR9 on *i*NKT cells derived from the LPL (Figure 1*D*).

Environmental Influences on the Responsiveness and Vβ-Use of iNKT Cells

It has been reported that the housing conditions provided by the commercial vendors at Taconic Farms (Tac) and the Jackson Laboratory (Jax), and the consequent difference in the intestinal microbiota, can impact the composition and function of conventional CD4$^+$ T lymphocytes in the intestine.[17] To test if such differences could influence the responsiveness of peripheral *i*NKT cells, we directly compared their phenotype and function in SPF C57BL/6 animals from both vendors. The percentage of *i*NKT cells in the thymus, spleen, and liver of Tac mice tended to be lower than in Jax mice, a difference that was statistically significant in all experiments, however, only in the spleen (Figure 2*A*). Primary Vα14*i* NKT cells commonly use 3 Vβ chains paired with the invariant TCR α-chain. Vβ8.1/2 is most abundant, comprising approximately 55% of the total, with the other principal ones being Vβ7 (14%) and Vβ2 (7%).[1,26] The analysis of the Vβ-use of the *i*NKT cells from Tac and Jax C57BL/6 mice revealed a significantly higher frequency of Vβ7$^+$ *i*NKT cells in the thymus, spleen, and liver of Tac mice (Figure 2*B*). No difference, however, was observed for Vβ7 use of *i*NKT cells in either SI-IELs or SI-LPLs (Supplementary Figure 2*A*), or for the frequency of Vβ2$^+$ *i*NKT cells in any of the organs analyzed (Supplementary Figure 2*A* and *B*). The decrease of Vβ7$^+$ *i*NKT cells in Jax mice was balanced by a correlative increase of *i*NKT cells expressing Vβ8 (data not shown). Furthermore, we observed no significant differences between Tac and Jax *i*NKT cells in the surface expression of NK1.1, CD4, CD25, CD44, CD69, and CD122 in any tissue analyzed (data not shown).

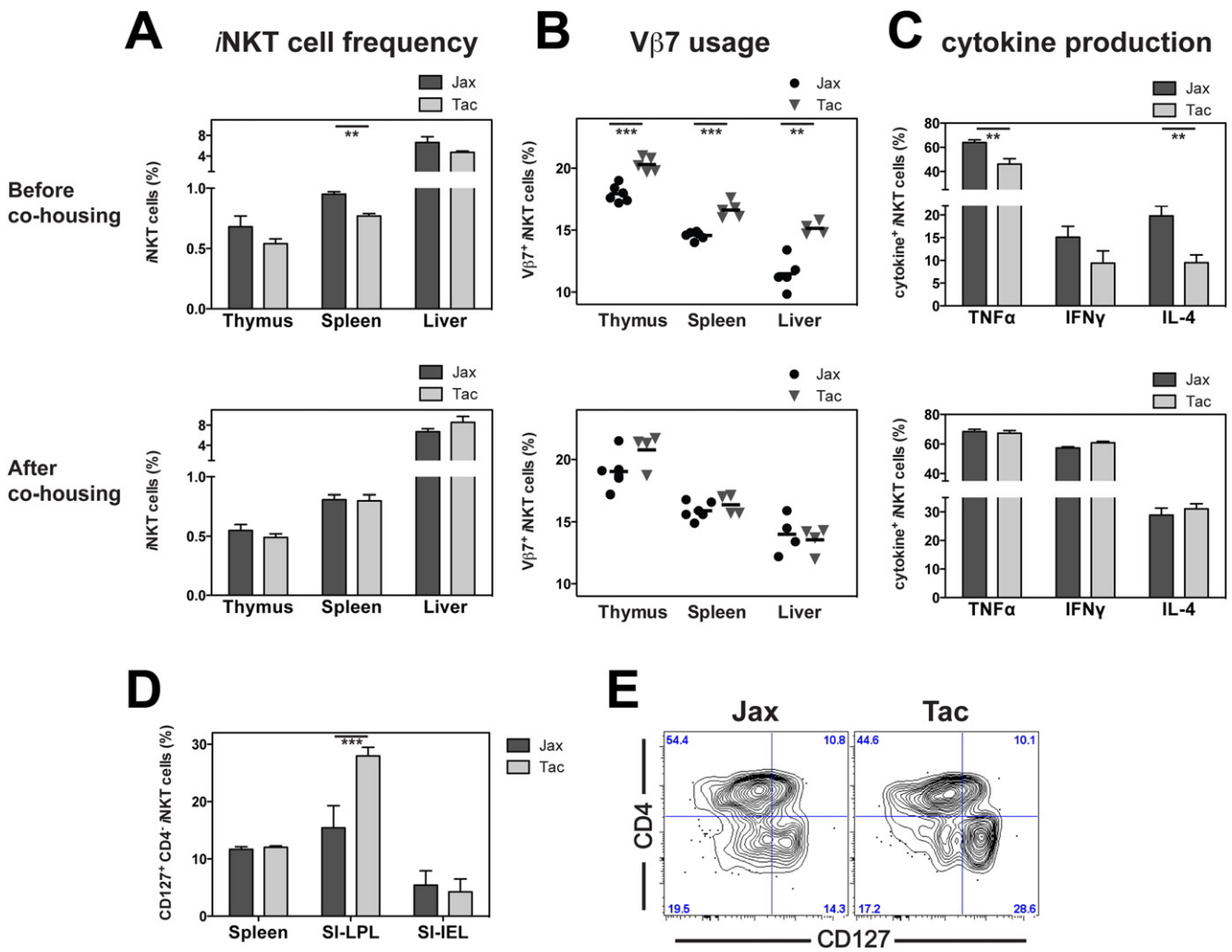

Figure 2. Environmental influences on the responsiveness and Vβ-use of *i*NKT cells. (*A–C*) C57BL/6 animals, purchased from either Taconic Farms (Tac) or Jackson Laboratory (Jax), were analyzed either within 1 week after delivery (*top panels*), or, alternatively, newborn offspring from Tac or Jax mice were co-housed from 2–5 days after birth until analysis 8–10 weeks later (*lower panels*). Relative frequency of (*A*) *i*NKT cells and (*B*) their Vβ7-use in indicated organs is shown. (*C*) Production of indicated cytokines by splenic *i*NKT cells 90 minutes after intravenous injection of αGalCer was analyzed by intracellular staining. Representative data from 4 (*top panels*) or 3 (*lower panels*) independent experiments are shown. (*D* and *E*) Frequency of CD127$^+$CD4$^-$ *i*NKT cells in (*D*) indicated organs or from (*E*) SI-LPL from indicated mice. Representative data from 3 independent experiments are shown. $^{**}P \leq .01$, $^{***}P \leq .001$.

However, we noted in the SI-LPL, but not in any other organ analyzed, a significantly increased frequency of CD127$^+$CD4$^-$ *i*NKT cells in the Tac- compared with the Jax-derived mice (Figure 2*D* and *E*, and data not shown). We also assessed the effector functions of the *i*NKT cells after activation with αGalCer. The frequency of cytokine-producing *i*NKT cells tended to be lower in Tac mice (Figure 2*C*). This difference, however, was statistically significant only for TNF-α in all 4 experiments, whereas significance for differences in IL-4 and interferon-γ was not observed consistently. The lower TNF-α production of Tac *i*NKT cells could not be explained by the difference in the Vβ-use because the cytokine production in the Tac *i*NKT cells was reduced irrespective of the Vβ-chain expressed (Supplementary Figure 2*C*).

To determine if the observed differences were acquired and did not stem from minor variations caused by genetic drift, we co-housed newborn offspring of Tac and Jax mice to allow the environmental factors, including the intestinal microbiota, to equalize. When analyzed side-by-side 8 to 10 weeks later, we did not find a difference in *i*NKT cell frequency or in Vβ-use and function (Figure 2*A–C*). Therefore, these data clearly show that differences in the environment of Tac- and Jax-derived animals can modulate the frequency, Vβ-use, and cytokine production of *i*NKT cells.

iNKT Cells From Germ-Free Mice Are Hyporesponsive

To determine more directly if the normal gut microbiota affects the development and function of peripheral *i*NKT cells, we compared *i*NKT cells derived from Swiss Webster animals raised in germ-free (GF) conditions with those from mice raised in SPF conditions. Although relative *i*NKT cell numbers recovered from GF and SPF animals did not differ significantly (data not shown), we

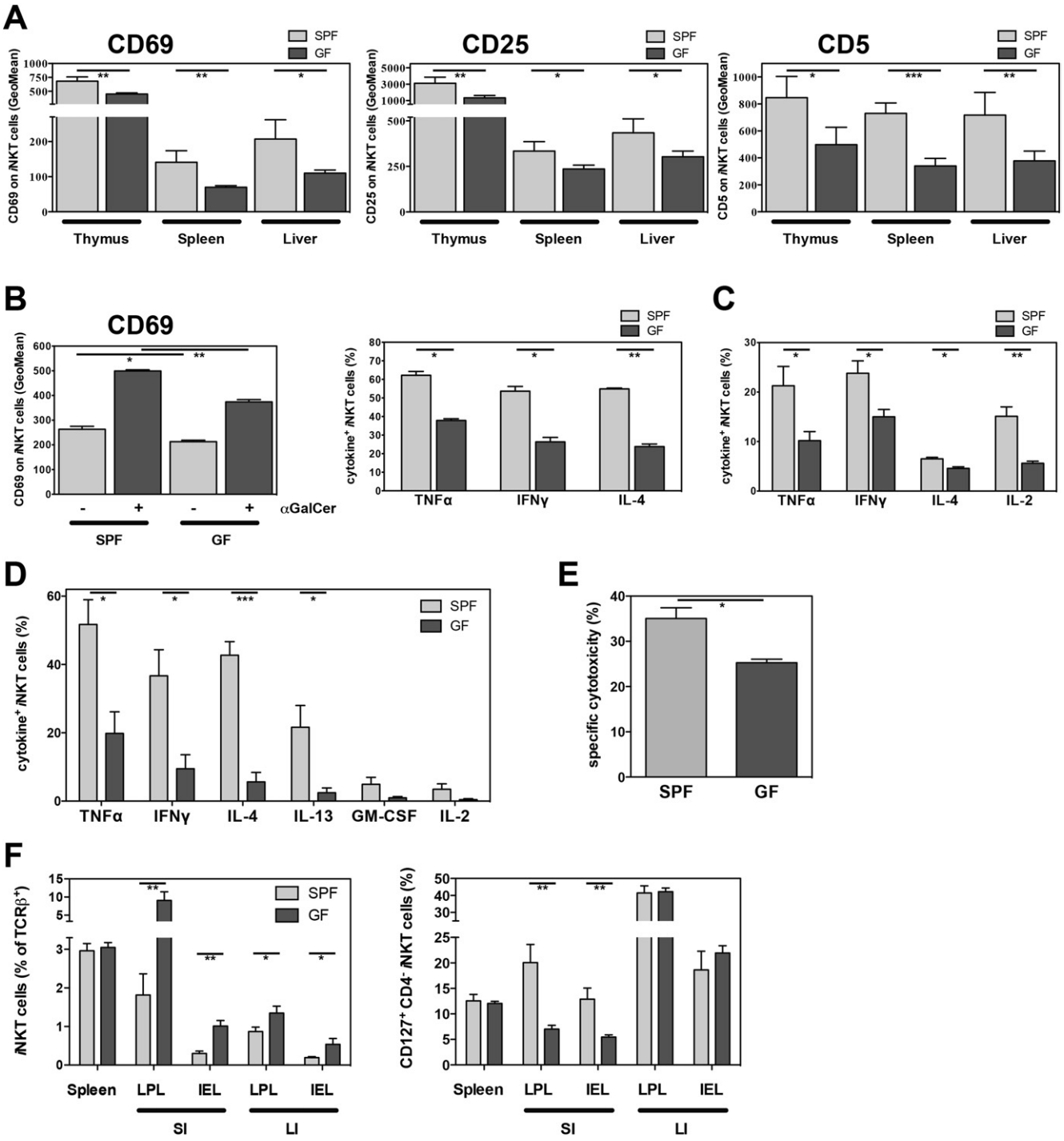

Figure 3. *i*NKT cells from germ-free animals are hyporesponsive. (*A*) Expression of CD69, CD25, and CD5 by *i*NKT cells from indicated organs derived from GF- or SPF-housed Swiss Webster mice. (*B*) Expression of CD69 (*left panel*) and indicated cytokines (*right panel*) by splenic *i*NKT cells from GF- or SPF-housed Swiss Webster mice with or without αGalCer challenge in vivo (90 min). The expression of CD69 after αGalCer increased on SPF-derived *i*NKT cells 1.9-fold (GeoMean), whereas the increase on GF-derived *i*NKT cells was lower at 1.75-fold ($P_{(SPF \pm \alpha GalCer \text{ vs } GF \pm \alpha GalCer)} = .004$). (*C*) Splenocytes from GF and SPF Swiss Webster mice were co-cultured with αGalCer-loaded RMA-CD1d cells for 4 hours, and cytokine production by *i*NKT cells was analyzed by intracellular staining. (*D*) GF- or SPF-housed animals on the C57BL/6 background were injected with αGalCer, and the cytokine production by splenic *i*NKT cells was analyzed 90 minutes later. The graph summarizes data from 2 independent experiments, with 4–5 mice per group. (*E*) αGalCer-specific in vivo cytotoxicity in spleen 4 hours after injection of B-cell targets into GF or SPF housed Swiss Webster mice. Representative data from 2 independent experiments are shown. (*F*) Relative percentage of *i*NKT cells within TCRβ+ live lymphocytes (*left*) and of $CD127^+CD4^-$ *i*NKT cells (*right*) from indicated organs of GF- or SPF-housed Swiss Webster animals. The graphs summarize data from 3 independent experiments, with 5–8 mice per group. $^*P < .05$, $^{**}P \leq .01$, $^{***}P \leq .001$.

observed that unstimulated *i*NKT cells from the spleen, liver, and thymus of GF mice uniformly expressed lower levels of the activation markers CD69, CD25, and CD5 (Figure 3*A* and Supplementary Figure 3*A*). When GF and SPF animals were challenged with the potent *i*NKT cell antigen αGalCer, the difference in CD69 expression between GF and SPF mice was even more pronounced (Figure 3*B*), suggesting that *i*NKT cells from GF animals respond less vigorously. Importantly, cytokine production by *i*NKT cells from GF animals, as measured by intracellular cytokine staining, was significantly lower compared with their SPF counterparts (Figure 3*B*). We also observed a similar difference after stimulating splenocytes from GF and SPF mice with αGalCer in vitro (Supplementary Figure 3*B*). *i*NKT cells are not highly dependent on costimulation for activation,[27] and the expression level of CD1d on antigen-presenting cells (APCs) was comparable in SPF and GF animals (data not shown). Nonetheless, it was possible that differences in the maturation state of APCs caused the reduced responses of *i*NKT cells from GF mice. To avoid the influence of endogenous APCs, we stimulated splenocytes from GF- and SPF-raised animals with αGalCer-loaded, CD1d-transfected RMA lymphoma cells in vitro. In this experimental set-up and similar to previous results, *i*NKT cells derived from GF animals produced significantly less cytokines than cells from SPF animals (Figure 3*C*). These data show that, independently of any putative effect on APCs, *i*NKT cells from GF mice respond less vigorously to antigen stimulation than *i*NKT cells from SPF animals.

Because Swiss Webster mice are not fully inbred, we aimed to confirm that *i*NKT cells from GF mice are hyporesponsive by testing GF animals on the C57BL/6 background. Similar to their Swiss Webster counterparts, splenic *i*NKT cells from C57BL/6 GF animals showed a significant impairment in antigen-stimulated cytokine production (Figure 3*D*) and up-regulation of CD69 expression (Supplementary Figure 3*C*).

Apart from cytokine production, activated *i*NKT cells display potent cytotoxic activity. To test if the presence of the intestinal microbiota affects the cytotoxic potential of *i*NKT cells, we injected GF- and SPF-housed Swiss Webster animals with CFDA-SE-labeled B cells loaded in vitro with αGalCer, and measured cytotoxicity in vivo 4 hours later.[24] The αGalCer-specific in vivo cytotoxicity in GF mice was significantly lower than that observed in SPF animals (Figure 3*E*), indicating that the microbiota also is important for the development and/or maintenance of the cytotoxic capability of *i*NKT cells. Altogether these data show that *i*NKT cells from GF animals are hyporesponsive to antigen stimulation in a cell-intrinsic fashion.

Furthermore, we found a significantly higher frequency of intestinal *i*NKT cells in GF than in SPF Swiss Webster mice in all 4 intestinal compartments (Figure 3*F*), suggesting that the homing/expansion of *i*NKT cells to the intestine does not require the gut microbiota to the same extent as for αβT cells.[28] The analysis of the Vβ-use of the *i*NKT cells from GF and SPF C57BL/6 mice revealed a significantly lower frequency of $V\beta7^+$ *i*NKT cells in the thymus and spleen of GF mice (Supplementary Figure 3*D*). Similar to other organs analyzed (Figure 3*A*), in GF mice the expression of CD69 was lower on intestinal *i*NKT cells than in SPF mice (Supplementary Figure 3*E* and data not shown). Furthermore, although no differences for the expression of CD103, β_7-integrin, and CCR9 on intestinal *i*NKT cells from GF compared with SPF mice were observed (Supplementary Figure 3*F* and data not shown), the frequency of $CD127^+CD4^-$ *i*NKT cells in the small intestine was lower in the GF animals (Figure 3*F*).

Bacterial Products Promote iNKT Cell Responsiveness in a Toll-Like Receptor–Independent Fashion

Bacterial products, via Toll-like receptor (TLR) signaling and induction of IL-12 and other cytokines by APCs, can activate *i*NKT cells even in the absence of a microbial antigen that engages their TCR. To establish if this alternative route of stimulation plays a role in shaping the *i*NKT cell antigen responsiveness to intestinal microbiota, we used MyD88 and TRIF double-deficient animals, which cannot respond to TLR ligands.[29] We did not detect any phenotypic differences between C57BL/6 control and $MyD88^{-/-}Trif^{Lps2/Lps2}$ mice (Figure 4*A*). Activation of *i*NKT cells from $MyD88^{-/-}Trif^{Lps2/Lps2}$ animals with αGalCer caused phenotypic changes that also were indistinguishable from the controls (Figure 4*A*). Furthermore, we did not observe differences in αGalCer-induced cytokine production by *i*NKT cells (Figure 4*B*). Similarly, analysis of $IL\text{-}12^{-/-}$ animals showed no phenotypic or functional differences with *i*NKT cells from wild-type animals (Figure 4*C* and *D*). Consistent with these data, the frequency and phenotype of intestinal *i*NKT cells in the $IL\text{-}12^{-/-}$ animals were similar to C57BL/6 control mice (Supplementary Figure 4). These data suggest that the pathways of *i*NKT cell stimulation that depend on TLR stimulation of APCs cannot account for the hyporesponsive phenotype and function of *i*NKT cells in GF mice.

Bacterial Reconstitution Corrects the Hyporesponsive Phenotype of iNKT Cells

We then tested if the hyporesponsive phenotype of *i*NKT cells in GF animals could be reversed. To this end, we co-housed GF with SPF animals for 4 weeks under SPF conditions. After this time, we found that the phenotype of *i*NKT cells from SPF mice was indistinguishable from the ones of previously GF mice (Figure 5*A*).

Next, we reconstituted GF animals by gavage with live bacteria, either with the *Sphingomonas/Sphingobium* species *S yanoikuyae*, which have *i*NKT cell antigens,[10] or with *E coli*, which are devoid of such antigens (data not shown). Analysis of CD69 expression on *i*NKT cells showed that reconstitution with *S yanoikuyae* was sufficient to normalize the hyporesponsive phenotype of *i*NKT cells from GF mice (Figure 5*B* and *C*). In contrast, reconstitution of the GF animals with *E coli* bacteria did not cause such a change in the *i*NKT cell phenotype (Figure 5*B* and *C*).

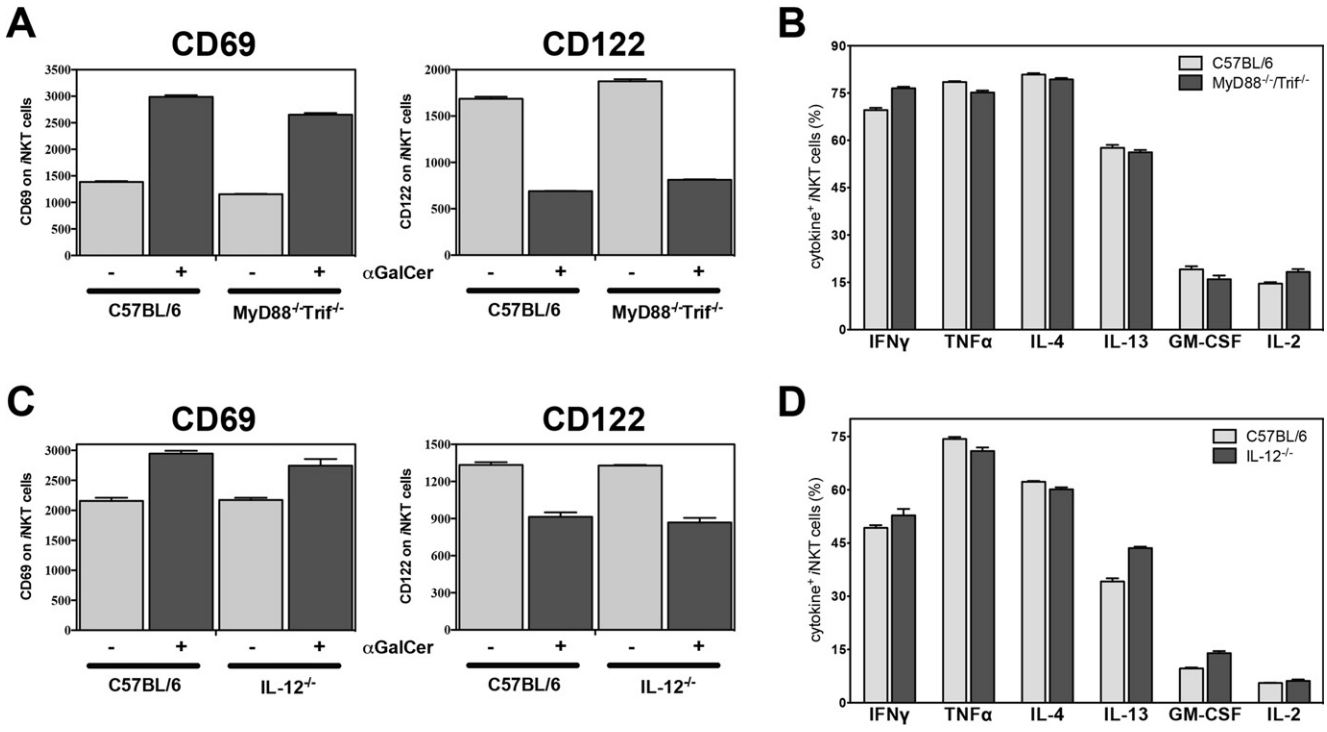

Figure 4. Bacterial products promote *i*NKT cell responsiveness in a TLR-independent fashion. (*A* and *B*) C57BL/6J wild-type and MyD88$^{-/-}$Trif$^{Lps2/Lps2}$ animals were either mock treated or injected with αGalCer and 90 minutes later the expression of indicated (*A*) surface markers and (*B*) cytokines by splenic *i*NKT cells was analyzed. (*C* and *D*) C57BL/6J wild-type and IL-12$^{-/-}$ animals were either mock treated or injected with αGalCer, and 90 minutes later the expression of indicated (*C*) surface markers and (*D*) cytokines by splenic *i*NKT cells was analyzed. Representative data from 2 independent experiments are shown. GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon.

These data suggest that intestinal-derived *i*NKT cell–specific antigens from microbes are necessary to render peripheral *i*NKT cells fully mature and ready to respond. Similar to C57BL/6 GF (Supplementary Figure 3*D*), *i*NKT cells from SW-GF animals displayed a lower frequency of Vβ7$^+$ cells and this frequency normalized after reconstitution with *S yanoikuyae*, but not with *E coli* (Figure 5*D*), suggesting antigen-driven proliferation of *i*NKT cells.

To analyze the effect of a limited set of intestinal organisms on the responsiveness of *i*NKT cells, we also tested mice bearing an RF.[16] RF mice carry an altered and reduced microbiota, including different fungal and bacterial species, as compared with SPF mice.[20,21] The bacterial microbiota of RF mice is enriched for *Firmicutes* species and devoid of *Sphingomonas/Sphingobium* species.[16] Although *i*NKT cell numbers are reduced in RF mice,[16] their response to αGalCer can be measured. *i*NKT cells derived from RF mice displayed a higher frequency of Vβ7$^+$ *i*NKT cells in the spleen (Supplementary Figure 5). Under resting conditions, splenic *i*NKT cells from RF mice expressed lower CD69 levels and displayed a lower up-regulation of this marker after αGalCer stimulation (Figure 5*E*). Furthermore, fewer *i*NKT cells produced cytokines in the RF mice compared with the SPF controls (Figure 5*F*), recapitulating the data we obtained from the GF animals.

Discussion

Here, we report a detailed record of the distribution and phenotype of *i*NKT cells in the intestine. Furthermore, we show that bacterial products from the intestinal microbiota contribute to the full responsiveness of peripheral Vα14*i* NKT cells and can modulate their phenotype and TCR Vβ-use. *i*NKT cells from SPF mice derived from different vendors differed in the frequency of *i*NKT cells, the proportion that expressed Vβ7, and in their cytokine response after antigen stimulation. In addition, *i*NKT cells derived from GF animals displayed a less mature phenotype and were hyporesponsive to antigen-specific activation, as measured by up-regulation of activation markers and the production of cytokines. These effects on the acute, antigen-specific response of *i*NKT cells in GF mice could be reversed days after oral exposure to bacteria expressing *i*NKT cell antigens. Furthermore, full *i*NKT cell maturation and the constitutive activation state of these cells did not require TLR-mediated signals. Together, these findings suggest that antigens from the microbiota that engage the semi-invariant TCR likely are responsible for the effects observed.

In light of these findings, we were surprised that *i*NKT cells were increased in GF mice in the lamina propria and epithelium of the small and large intestines, although similar to their counterparts in the spleen and liver, the intestinal *i*NKT cells from GF mice expressed lower amounts of the activation antigen CD69. These data are consistent with a recent report showing that *i*NKT cells are increased in the colon of GF mice owing to increased production of the chemokine CXCL16.[30] Interestingly, colonization of neonatal mice with intestinal flora pre-

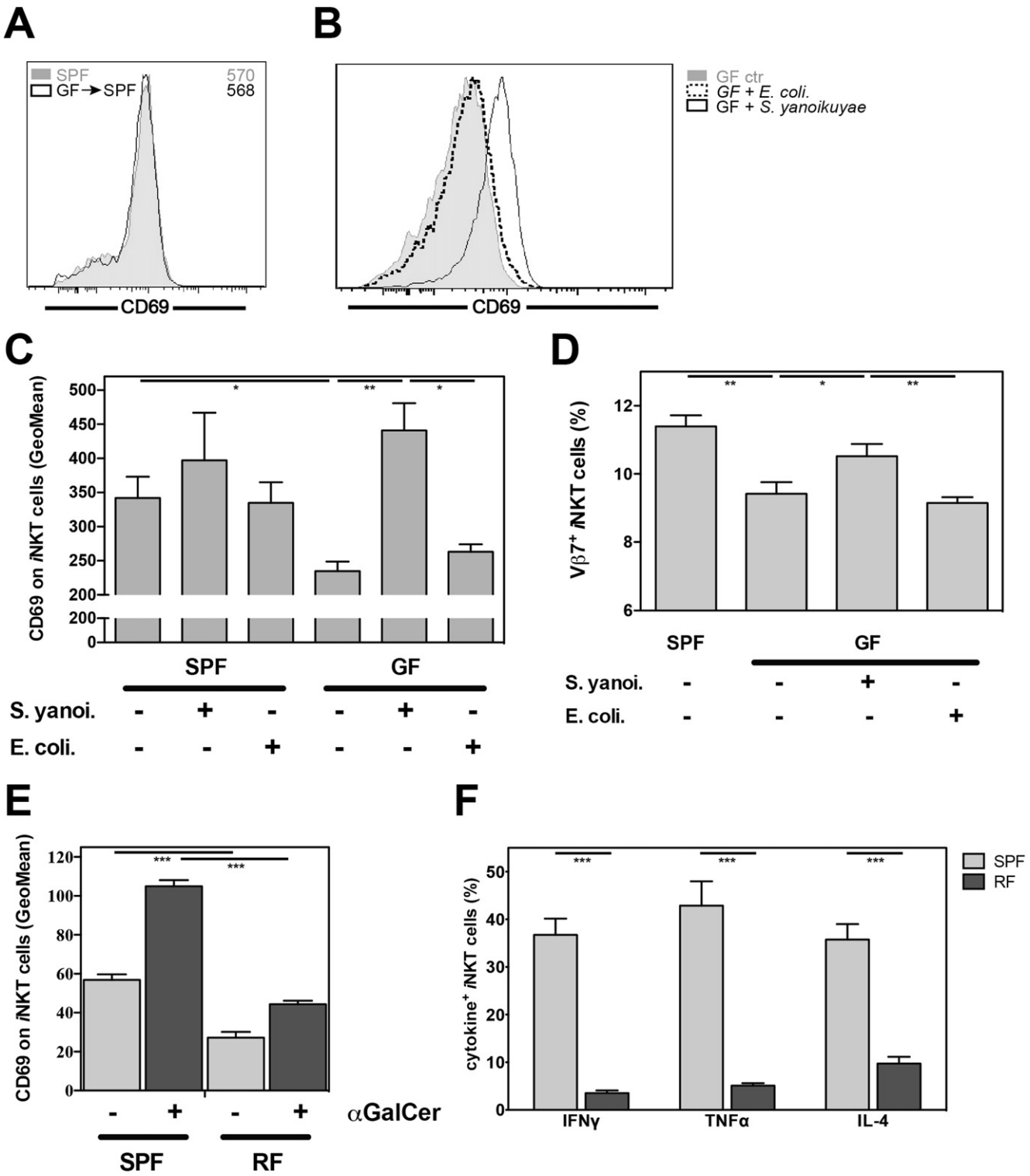

Figure 5. Bacterial exposure corrects the hyporesponsive phenotype of *i*NKT cells. (*A*) GF- and SPF-housed Swiss Webster mice were co-housed for 4 weeks, and expression of CD69 in splenic *i*NKT cells was analyzed. The *numbers in histograms* denote the geometric mean values for CD69 on *i*NKT cells. (*B–D*) GF- and SPF-housed Swiss Webster mice were mock treated or intragastrically challenged with either *S yanoikuyae* or *E coli* bacteria as indicated. Four to 5 days later, the expression of CD69 in splenic *i*NKT cells was analyzed and is represented as an example histogram (*B*) or as a summary (*C*). Furthermore, the relative frequency of V$\beta7^+$ *i*NKT cells is shown (*D*). Expression of CD69 (*E*) and indicated cytokines (*F*) by splenic *i*NKT cells from RF and SPF-housed C57BL/6 mice with or without αGalCer challenge in vivo (90 min). Representative data from (*A*, *E*, and *F*) 2, (*D*) 3, or (*B* and *C*) 4 independent experiments are shown. IFN, interferon. $^*P < .05$, $^{**}P \leq .01$, $^{***}P \leq .001$.

vented both the increased accumulation of *i*NKT cells in the intestine and the contribution of these cells to inflammation in the intestine and the lung,[30] providing additional evidence for the modulation of *i*NKT cell function as well as the number of *i*NKT cells by intestinal microbes.

The frequency and distribution of *i*NKT cells in intestinal tissue has not been fully characterized, despite the role of *i*NKT cells in several models of inflammatory bowel disease and intestinal infections.[5,6] Many of the studies reporting the presence of NKT cells in the intestine relied on the co-expression of the TCR/CD3ε complex and NK cell receptors,[5,6] which does not allow for the unequivocal identification of *i*NKT cells. By using CD1d tetramers loaded with αGalCer, however, *i*NKT cells have been reported in LPLs[31,32] and in SI-IELs, where 80% of them were NK1.1^{neg}[26]. A later report, however, did not detect *i*NKT cells in SI-IELs.[31] Here, we report on the presence of *i*NKT cells in IELs and LPLs of both small and large intestines. We detected a higher relative percentage of *i*NKT cells in the small rather than the LI, and also generally more in the LPL than in the IEL compartments. The frequencies in LI-LPLs were comparable with those in the lymph node, and for the SI-LPLs were comparable with those in the spleen. These data show that *i*NKT cells constitute a significant lymphocyte population within the lamina propria.

It has been reported that *i*NKT cells can influence the microbial colonization and the composition of intestinal bacteria.[33] Here, we provide evidence that this influence is mutual. *i*NKT cells derived from SPF animals from 2 different vendors, Taconic Farms and Jackson Laboratory, showed differences in the frequency of *i*NKT cells, Vβ7-use, and cytokine production. These differences were dependent on the environment because CD1d expression was not different between the 2 strains, and co-housing of the offspring diminished them. Interestingly, although *i*NKT cells expressing Vβ7 have a lower avidity for αGalCer,[34] it has been inferred that they have a higher avidity for the endogenous selecting antigen(s).[35] The environment-dependent increase in Vβ7$^+$ *i*NKT cells in the Tac C57BL/6 mice therefore could be owing to differences in intestinal *i*NKT cell antigens. However, in preliminary experiments, we could not recover detectable antigenic *i*NKT cell activity in the intestinal contents from SPF mice (data not shown). Although a difference in the intestinal microbiota likely is responsible, especially considering the known differences between Jax and Tac mice,[17] further experiments are required to determine the parameters in the environment that are responsible for the differences in *i*NKT cells between mice from the 2 vendors.

The finding that *i*NKT cells from GF Swiss Webster mice were hyporesponsive is in contrast to that by Park et al,[36] in which no impairment of the *i*NKT cell response of GF animals was detected. Several technical differences, however, set our study apart from the previous one, including the following: (1) the use of CD1d/αGalCer-tetramers to unequivocally detect Vα14*i* NKT cells, in contrast to measuring NK1.1$^+$TCRβ$^+$ cells, the only tools available at that time; (2) the quantitative analysis of activation marker expression levels by determining the mean fluorescence intensity, rather than expression by NKT cells per se; and, finally, (3) the analysis of the *i*NKT cell cytokine response on a single-cell level, rather than analysis of cytokine messenger RNA from total splenocytes. However, despite the marked differences we observed in *i*NKT cells from GF mice, we should not overlook the significant phenotypic and functional overlap they have with *i*NKT cells from SPF mice, including an expanded population, increased activation marker expression compared with naive T lymphocytes, and the ability of some of the cells, albeit a reduced percentage, to produce effector cytokines rapidly.

Our data obtained from MyD88$^{-/-}$Trif$^{Lps2/Lps2}$ and IL-12$^{-/-}$ mice indicated that TLR ligands from the intestinal contents are not required for the full maturation of peripheral *i*NKT cells. These data do not exclude a potential role for other sensing molecules, such as RIG-I–like receptors and NOD-like receptors,[37] but their role in *i*NKT cell activation currently are unknown. Therefore, the indirect or cytokine-mediated pathway for *i*NKT cell activation is likely not responsible for the homeostatic maintenance of the highly activated and responsive state of these cells in SPF mice. Furthermore, reconstitution of GF mice by oral administration of *S yanoikuyae*, which contain relatively high-affinity glycosphingolipid antigens for the *i*NKT cell TCR, could recover the full phenotypic maturity of these cells. In the absence of an isogenic *S yanoikuyae* strain, we performed reconstitution with *E coli*, a bacterium believed to lack antigens for the *i*NKT cell TCR. Administration of *E coli* did not normalize the phenotype of *i*NKT cells. These data show the importance of intestinal bacterial products for facilitating the full degree of *i*NKT cell responsiveness, and they suggest that antigens for the semi-invariant TCR are responsible.

Because *i*NKT cell antigens to date have been identified only from a few bacterial sources,[3,4] the distribution of *i*NKT cell antigens in the microbiota, and more generally in the environment, remains incompletely characterized. We previously showed specific *i*NKT cell antigens in *S yanoikuyae*.[10] Such *Sphingomonas/Sphingobium* species are ubiquitously present in water and soil[15] and are commensal species in the gut.[16,17] Therefore, we cannot exclude that a similar bacteria is a source of the intestinal *i*NKT cell antigens. Still, the *S yanoikuyae* species were not reported to differ substantially between Tac and Jax C57BL/6 animals.[17] In this context the observation is of interest that mice bearing an RF were not able to support full reactivity of *i*NKT cells. RF mice lack *Sphingomonas/Sphingobium* species,[16] but also numerous other bacteria species normally present in SPF mice.[20,21] We expect, however, that additional bacteria, many of them noninfectious, contain *i*NKT cell antigens. For example, patients with primary biliary cirrhosis expressed antibodies against enzymes from the *Sphingomonas/Sphingobium* species *N aro-*

maticivorans.[18] *N aromaticivorans* was detected in the gut of primary biliary cirrhosis patients,[18] and the activation of *i*NKT cells by *N aromaticivorans*–derived antigens was linked to disease progression.[38,39] These data showed that commensal bacteria expressing *i*NKT cell antigens can contribute to *i*NKT cell-mediated inflammation. Together with our data, these findings suggest that the composition of the intestinal microbiota may be an important exacerbating or causative factor in autoimmune diseases, with a possible contribution of *i*NKT cells.

The body exchanges substances with the environment via the mucosal surfaces of the lung and the intestine. We recently showed that *i*NKT cell antigens are present in house dust and that the adjuvant effect they exerted during airway inflammation is dependent on *i*NKT cells.[19] Here, we show that materials from the intestinal microbiota, likely *i*NKT cell antigens, modulate the phenotype and function of peripheral *i*NKT cells. Together, these reports show that *i*NKT cells are sensitive in responding to the environment and that antigens recognized by these cells are far more prevalent than previously anticipated.

Importantly, our findings indicate that the composition of the intestinal microbiota influences the cytokine responsiveness of *i*NKT cells. It is thus conceivable that such modulation not only could pertain to the magnitude of the antigen-induced cytokine response, but also its polarization. Given the important role *i*NKT cells play in numerous infectious and autoimmune diseases, our findings imply that the intestinal microbiota-mediated modulation of *i*NKT cells significantly could affect the outcome of these diseases.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.04.017.

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Reprint requests

Address requests for reprints to: Mitchell Kronenberg, PhD, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, San Diego, California 92037. e-mail: mitch@liai.org; fax: (858) 752-6990.

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Conflicts of interest

The authors disclose no conflicts.

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