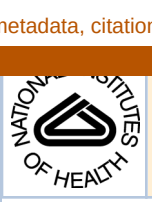

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Intestinal Microbes Affect Phenotypes and Functions of Invariant Natural Killer T cells in Mice

Gerhard Wingender[*,†], **Dariusz Stepniak**[*], **Philippe Krebs**[‡,Π], **Lin Lin**[¶], **Sara McBride**[§], **Bo Wei**[¶], **Jonathan Braun**[¶], **Sarkis K. Mazmanian**[§], and **Mitchell Kronenberg**[*,1]

[*]Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037, USA [†]Institute for Molecular Medicine, University of Bonn, Bonn, Germany [‡]Department of Genetics, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA [Π]Division of Experimental Pathology, Institute of Pathology, University of Bern, Bern, Switzerland [¶]Department of Pathology and Laboratory Medicine, University of California Los Angeles, 10833 Le Conte Ave; CHS 13-222, Los Angeles, CA 90095 [§]Division of Biology, California Institute of Technology, 1200 E. California Bl., Pasadena, CA 91125, USA

Abstract

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 (SPF) environments.

Results—SPF 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 hypo-responsive to activation with the antigen α-galactosylceramide. Intra-gastric 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

[1]Author for correspondence: La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, San Diego, CA, 92037, Tel: (858) 752- 6540, Fax: (858) 752-6990, mitch@liai.org, URL: http://www.liai.org.

Competing Interest

The authors have no competing interests regarding this work.

Author contributions

G.W. and M.K. designed research, G.W., D.S., P.K., L.L. B.W. and S.M. conducted experiments and acquired data, G.W. and M.K wrote the manuscript, S.K.M., J.B. and M.K. obtained funding. All authors approved the manuscript.

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

Introduction

Invariant natural killer T (*i*NKT) cells are a unique subset of T lymphocytes characterized by the expression of an invariant TCR rearrangement, Vα14-Jα18 in mice (Vα14*i* NKT cells) and an orthologous Vα24-Jα18 (Vα24*i*) in humans, and the recognition of antigens presented by CD1d, a non-polymorphic MHC 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_h1- 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 has almost uniformly 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 cell have been reported in two types of bacteria. One type is *Sphingomonas/Sphingobium* species, which have glycosphingolipids similar to the original sponge antigen[10,11]. The second type, *Borrelia burgdorferi*, is the causative agent of Lyme disease[12]. Several additional pathogens have been reported to have glycolipid antigens that activate *i*NKT cells, include *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 non-inflammatory 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. While 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-usage and the immune responses of *i*NKT cells, and that antigens from commensals that engage the semi-invariant TCR are a likely contributing factor.

Material and Methods

Mice and cell lines

Mice were housed under SPF conditions at the animal facilities of the La Jolla Institute for Allergy and Immunology (La Jolla, CA), the 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 from Taconic Farms; and B6.129S1-*Il12b*^{tm1Jm}/J (*IL-12p40*$^{-/-}$) from Jackson Laboratory. MyD88 and TRIF (*Lps2*) double deficient mice[19] and restricted flora (RF) mice have been described previously[20,21]. *Sphingobium yanoikuyae* and *Escherichia coli* were purchased from American Type Culture Collection (Manassas, VA). The T cell lymphoma RMA was virally transfected to stably express CD1d as previously described[22], resulting in the line RMA-CD1d.

Reagents and monoclonal antibodies

α-galactosylceramide (αGalCer) was obtained from the Kirin Pharmaceutical Research Corporation (Gunma, Japan). CFDA-SE was obtained from Invitrogen (Carlsbad, CA). Monoclonal antibodies (mAbs) against the following mouse antigens were used in this study: β_7-integrin (M293), 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), GM-CSF (MP1-22E9), IL-2 (JES6-5H4), IL-4 (11B11), IL-13 (eBio13A), IFNγ (XMG1.2) and TNFα (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 i.v. injection of 1μg αGalCer and analyzed 90min later. Bacterial suspensions were gavaged using a 20Gx1.5 feeding needles.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Comparisons were drawn using a two-tailed Student t-test or ANOVA test. p-values <0.05 were considered significant and are indicated with *p<0.05, **p≤0.01 and ***p≤0.001. 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 LPL and IEL compartments of the small (SI) and large intestines (LI). *i*NKT cells could readily be detected in LPL and IEL from both, small and large intestine of specific-pathogen-free (SPF) C57BL/6J mice (Figure 1A). The signal was specific for *i*NKT cells, as indicated by the low background when utilizing unloaded CD1d tetramers (Figure 1A). We consistently observed a higher frequency of *i*NKT cells in the small intestine than in the large intestine; and a higher frequency in the LPL than in IEL (Figure 1B). It is notable that the frequency of *i*NKT cells in the SI-LPL was comparable to 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 1C and Supplemental figure 1A). 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 (Supplemental figure 1B, 1C), and intestinal *i*NKT cells also were mostly negative for the β_7-integrin (data not shown). In contrast, we detected expression of CCR9 on *i*NKT cells derived from the LPL (Figure 1D).

Environmental influences on the responsiveness and Vβ-usage 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 which was statistically significant in all experiments however only in the spleen (Figure 2A). Primary Vα14*i* NKT cells use commonly three 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-usage of the *i*NKT cells from Tac and Jax C57BL/6 mice revealed a significantly higher frequency of $V\beta7^+$ *i*NKT cells in the thymus, spleen and liver of Tac mice (Figure 2B). No difference, however, was observed for Vβ7 usage of *i*NKT cells in either SI-IEL or SI-LPL (Supplemental figure 2A), or for the frequency of $V\beta2^+$ *i*NKT cells in any of the organs analyzed (Supplemental figure 2A, 2B). The decrease of $V\beta7^+$ *i*NKT cells in Jax mice was balanced out 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). 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 to the Jax derived mice (Figure 2D, 2E and data not shown). We also assessed the effector functions of the *i*NKT cells following activation with αGalCer. The frequency of cytokine-producing *i*NKT cells tended to be lower in Tac mice (Figure 2C). This difference, however, was statistically significant only for TNFα in all four experiments, while significance for differences in IL-4 and IFNγ was not consistently observed. The lower TNFα production of Tac *i*NKT cells could not be explained by the difference in the Vβ-usage, as the cytokine production in the Tac *i*NKT cells was reduced irrespective of the Vβ expressed (Supplemental figure 2C).

To determine if the observed differences were acquired and did not stem from minor variations due to genetic drift, we co-housed newborn offsprings of Tac and Jax mice, to allow the environmental factors, including the intestinal microbiota, to equalize. When analyzed side-by-side after eight to ten weeks later, we did not find difference in *i*NKT cell frequency, nor in Vβ-usage and function (Figure 2A–C). Therefore, these data clearly demonstrate that differences in the environment of Tac- and Jax-derived animals can modulate the frequency, Vβ-usage 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 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 3A and supplemental figure 3A). 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 3B), 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 to their SPF counterparts (Figure 3B). We also observed a similar difference after stimulating splenocytes from GF and SPF mice with αGalCer in vitro (Supplemental figure 3B). *i*NKT cells are not highly dependent on co-stimulation 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 APC 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 the previous results, *i*NKT cells derived from GF animals produced significantly less cytokines than cells from SPF animals (Figure 3C). These data demonstrate 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.

As 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 3D) and up-regulation of CD69 expression (Supplemental figure 3C).

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 CFSE-labeled B cells loaded *in vitro* with αGalCer, and measured cytotoxicity *in vivo* four hours later[24]. The αGalCer specific *in vivo* cytotoxicity in GF mice was significantly lower than that observed in SPF animals (Figure 3E), indicating that the microbiota is also important for the development and/or maintenance of the cytotoxic capability of *i*NKT cells. Altogether these data demonstrate that *i*NKT cells from GF animals are hypo-responsive 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 four intestinal compartments (Figure 3F), 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-usage 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 (Supplemental figure 3D). Similar to other organs analyzed (Figure 3A), in GF mice the expression of CD69 was lower on intestinal *i*NKT cells than in SPF mice (Supplemental figure 3E and data not shown). Furthermore, while no differences for the expression of CD103, β_7-integrin and CCR9 on intestinal *i*NKT cells from GF compared to SPF mice were observed (Supplemental figure 3F and data not shown), the

frequency of $CD127^{+}CD4^{-}$ *i*NKT cells in the small intestine was lower in the GF animals (Figure 3F).

Bacterial products promote iNKT cell responsiveness in a TLR independent fashion

Bacterial products, via 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. In order to establish if this alternative route of stimulation plays a role in shaping the *i*NKT cell antigen responsiveness to intestinal microbiota, we utilized 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 4A). Activation of *i*NKT cells from $MyD88^{-/-}Trif^{Lps2/Lps2}$ animals with αGalCer caused phenotypic changes that were also indistinguishable from the controls (Figure 4A). Furthermore, we did not observe differences in αGalCer-induced cytokine production by *i*NKT cells (Figure 4B). Similarly, analysis of $IL\text{-}12^{-/-}$ animals showed no phenotypic or functional differences with *i*NKT cells from wild-type animals (Figure 4C, 4D). 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 (Supplemental 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 four 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 5A).

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 hypo-responsive phenotype of *i*NKT cells from GF mice (Figure 5B, 5C). In contrast, reconstitution of the GF animals with *E. coli* bacteria did not cause such a change in the *i*NKT cell phenotype (Figure 5B, 5C). 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 (Supplemental figure 3D) *i*NKT cells from SW-GF animals displayed a lower frequency of $V\beta7^{+}$ cells and this frequency normalized following reconstitution with *S. yanoikuyae*, but not with *E. coli* (Figure 5D), 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 a restricted flora (RF)[16]. RF mice carry an altered and reduced microbiota, including different fungal and bacterial species as compared to SPF mice[20,21]. The bacterial microbioata of RF mice is enriched for *Firmicutes spp* and devoid of *Sphingomonas/Sphingobium spp*[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 $V\beta7^{+}$ *i*NKT cells in the spleen (Supplemental 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 following αGalCer stimulation (Figure 5E). Furthermore, fewer *i*NKT cells produced cytokines in the RF mice compared to the SPF controls (Figure 5F), recapitulating the data we obtained in 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-usage. *i*NKT cells from specific-pathogen-free (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. Additionally, *i*NKT cells derived from GF animals displayed a less mature phenotype and were hypo-responsive 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 like their counterparts in the spleen and liver, the intestinal *iN*KT 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 due to increased production of the chemokine CXCL16 (Olszak et al., *Science* in press, *DOI: 10.1126/science.1219328*). Interestingly, colonization of neonatal mice with intestinal flora prevented 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 (Olszak et al., *Science* in press, *DOI: 10.1126/science.1219328*), providing additional evidence for the modulation of *i*NKT cell function as well as number 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. Using CD1d tetramers loaded with αGalCer, however, *i*NKT cells have been reported in LPL[30,31] and in SI-IEL, where 80% of them were NK1.1^{neg}[26]. A later report, however, did not detect *i*NKT cells in SI-IEL[30]. Here we report on the presence of *i*NKT cells in IEL and LPL of both small and large intestine. We detected a higher relative percentage of *i*NKT cells in the small rather than the large intestine, and also generally more in the LPL than in the IEL compartments. The frequencies in LI-LPL were comparable to those in the lymph node, and for the SI-LPL to those in the spleen. These data demonstrate 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[32]. Here we provide evidence that this influence is mutual. *i*NKT cells derived from SPF animals from two different vendors, Taconic Farms and Jackson Laboratory, showed differences in the frequency of *i*NKT cells, Vβ7-usage and cytokine production. These differences were dependent on the environment, as CD1d expression was not different between the two strains and co-housing of the offspring diminished them. Interestingly, although *i*NKT cells expressing Vβ7 have a lower avidity for αGalCer[33], it has been inferred that they have a higher avidity for the endogenous selecting antigen(s)[34]. The environment-dependent increase in Vβ7^{+} *i*NKT cells in the Tac C57BL/6 mice could therefore be due 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). While a difference in the intestinal microbiota is likely 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 two vendors.

The finding that *i*NKT cells from GF Swiss Webster mice were hypo-responsive are in contrast to those by Park et al.[35], where 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: (a) the use of CD1d/αGalCer-tetramers to unequivocally detect Vα14*i* NKT cells, in contrast to measuring $NK1.1^{+}TCR\beta^{+}$ cells, the only tools available at that time; (b) the quantitative analysis of activation marker expression levels by determining the mean fluorescence intensity, rather than expression by NKT cells *per se*; and finally (c) the analysis of the *i*NKT cell cytokine response on a single cell level, rather than analysis of cytokine mRNA 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 to naïve 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 $IL\text{-}12^{-/-}$ 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, like RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs)[36], but their role in *i*NKT cell activation are currently 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 carried out 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 demonstrate 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.

As *i*NKT cell antigens have so far only been identified 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 demonstrated specific *i*NKT cell antigens in *Sphingobium 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 likely source of the intestinal *i*NKT cell antigens. Still, the *Sphingomonas yanoikuyae* species were not reported to substantially differ between Tac and Jax C57BL/6 animals[17]. In this context the observation is of interest that mice bearing a restricted flora (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 non-infectious, contain *i*NKT cell antigens. For example, patients with primary biliary cirrhosis (PBC) expressed antibodies against enzymes from the *Sphingomonas/Sphingobium* species *Novosphingobium aromaticivorans*[18]. *N. aromaticivorans* was detected in the gut of PBC patients[18] and the activation of *i*NKT cells

by *N. aromaticivorans*-derived antigens was linked to disease progression[37,38]. These data demonstrated 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 other 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 demonstrated 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 demonstrate 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 could significantly affect the outcome of these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

αGalCer	α-galactosylceramide
APC	antigen presenting cell
GF	germ-free
i	invariant
IEL	intraepithelial lymphocytes
LI	large intestine
LPL	lamina propria lymphocytes
mAb	monoclonal antibody
NKT	Natural Killer T
RF	restricted flora
SI	small intestine
SPF	specific pathogen free
T_h1	T helper type 1

T_h2	T helper type 2
TLR	toll like receptor
Vα14*i*	invariant Vα14 to Jα18 TCR rearrangement

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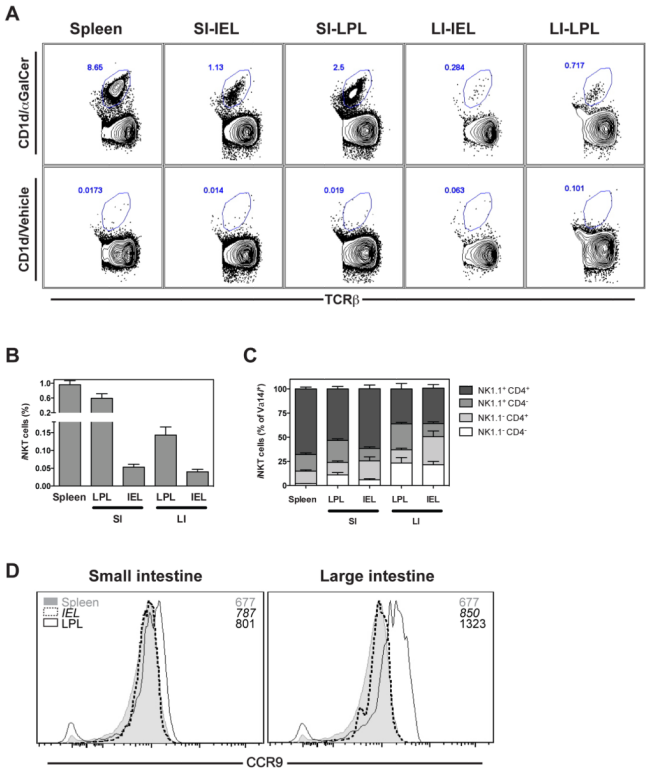

Figure 1. Distribution and phenotype of intestinal *i*NKT cells

(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, C)** Relative percentage of *i*NKT cells within total live lymphocytes (B) and their expression of CD4 and NK1.1 (C), 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.

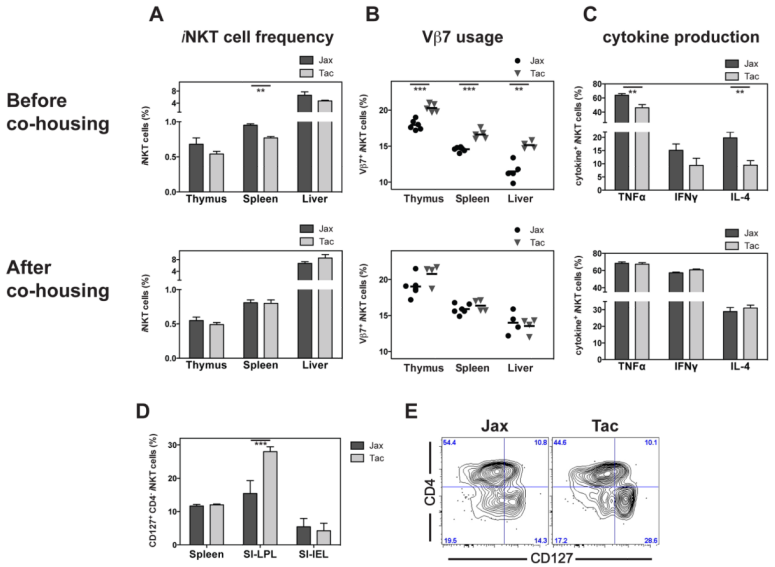

Figure 2. Environmental influences on the responsiveness and Vβ-usage of *i*NKT cells

(A-C) C57BL/6 animals, purchased from either Taconic Farms (Tac) or Jackson Laboratory (Jax), were either analyzed within one week after delivery (top panels). Or alternatively, new-born 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 *i*NKT cells (A) and their Vβ7-usage (B) in indicated organs is shown. Production of indicated cytokines by splenic *i*NKT cells 90min after i.v. injection of αGalCer was analyzed by intracellular staining (C). Representative data from four (top panels) or three (lower panels) independent experiments are shown. **(D, E)** Frequency of $CD127^{+}CD4^{-}$ *i*NKT cells in indicated organs (D) or from SI-LPL (E) from indicated mice. Representative data from three independent experiments are shown.

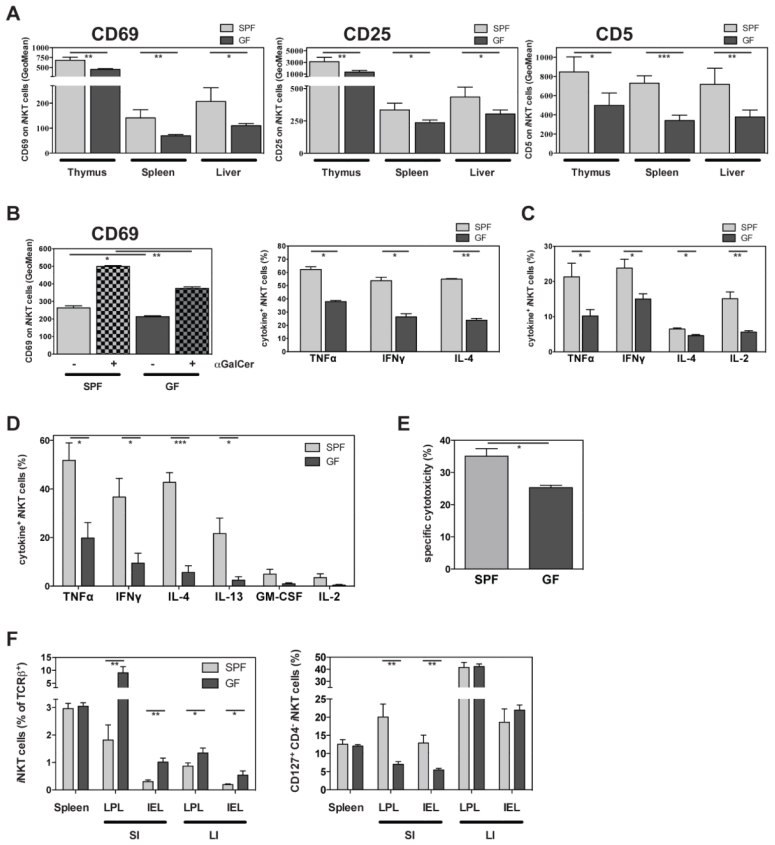

Figure 3. *i*NKT cells from germ-free animals are hyporesponsive

(A) Expression of CD69, CD25 and CD5 by *i*NKT cells from indicated organs derived from germ-free (GF) or specific-pathogen-free (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 (90min). The expression of CD69 following αGalCer increased on SPF derived *i*NKT cells 1.9fold (MFI), whereas the increase on GF derived *i*NKT cells was lower at 1.75fold ($p_{(SPF +/- \alpha GalCer \text{ vs } GF +/- \alpha GalCer)} = 0.004$). **(C)** Splenocytes from GF and SPF Swiss Webster mice were co-cultured with αGalCer loaded RMA-CD1d cells for 4h 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 90min later. The graph summarizes data from two independent experiments, with 4–5 mice per group. **(E)** αGalCer-specific *in vivo* cytotoxicity in spleen 4h after injection of B cell targets into GF or SPF housed Swiss Webster mice. Representative data from two independent experiments are shown. **(F)** Relative percentage of *i*NKT cells within $TCR\beta^+$ 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 three independent experiments, with 5–8 mice per group.

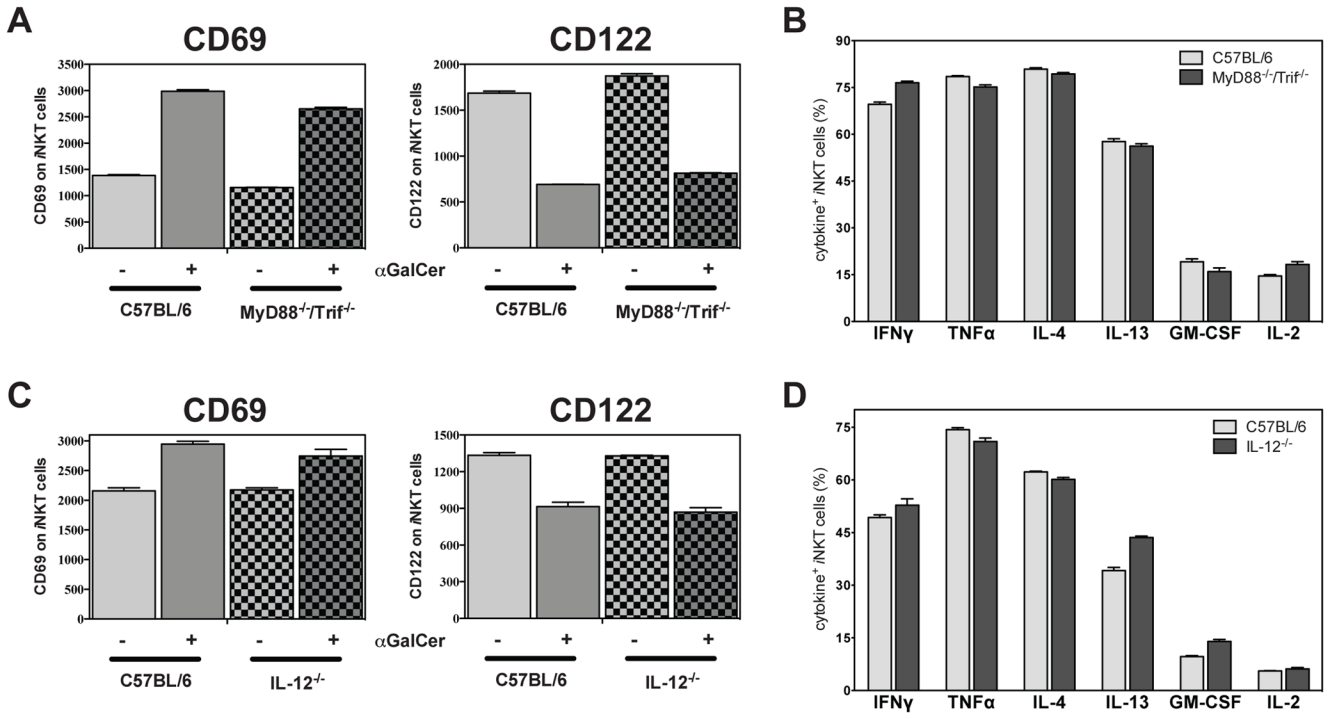

Figure 4. Bacterial products promote *i*NKT cell responsiveness in a TLR independent fashion

(A, B) C57BL/6J wild-type and MyD88$^{-/-}$Trif$^{Lps2/Lps2}$ animals were either mock treated or injected with αGalCer and 90min later the expression of indicated surface markers (A) and cytokines (B) by splenic *i*NKT cells was analyzed. **(C, D)** C57BL/6J wild-type and IL-12$^{-/-}$ animals were either mock treated or injected with αGalCer and 90min later the expression of indicated surface markers (C) and cytokines (D) by splenic *i*NKT cells was analyzed. Representative data from two independent experiments are shown.

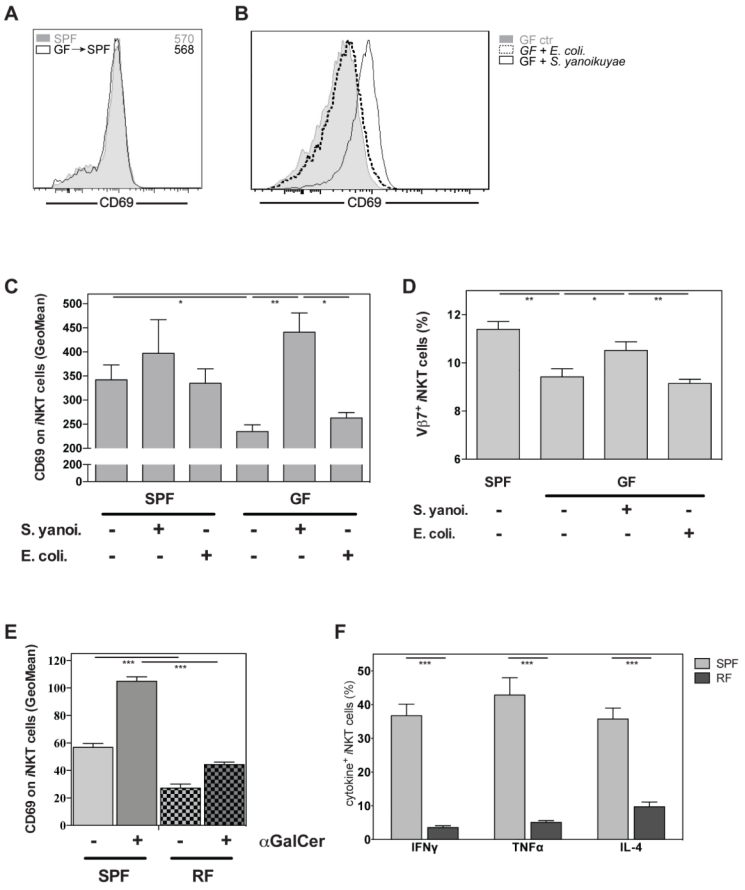

Figure 5. Bacterial exposure corrects the hyporesponsive phenotype of *i*NKT cells

(A) GF and SPF housed Swiss Webster mice were co-housed for four 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 intra-gastrically challenged with either *S. yanoikuyae* or *E. coli* bacteria as indicated. Four to five days later the expression of CD69 in splenic *i*NKT cells was analyzed and is represented as example histogram (B) or as summary (C). Furthermore, the relative frequency of $V\beta7^+$ *i*NKT cells is shown (D). **(E, F)** Expression of CD69 (E) and indicated cytokines (F) by splenic *i*NKT cells from restricted-flora (RF) and SPF housed C57BL/6 mice with or without αGalCer challenge in vivo (90min). Representative data from two (A, E, F), three (D) or four (B, C) independent experiments are shown.