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Commensal Microbiota and CD8⁺ T Cells Shape the Formation of Invariant NKT Cells

Bo Wei,* Gerhard Wingender,† Daisuke Fujiwara,* Diana YuHui Chen,* Michael McPherson,‡ Sarah Brewer,* James Borneman,§ Mitchell Kronenberg,† and Jonathan Braun*‡

Commensal bacteria play an important role in the formation of the immune system, but the mechanisms involved are incompletely understood. In this study, we analyze CD1d-restricted invariant NKT (iNKT) cells in germfree mice and in two colonies of C57BL/6 mice termed conventional flora and restricted flora (RF), stably bearing commensal microbial communities of diverse but distinct composition. In germfree mice, iNKT cells were moderately reduced, suggesting that commensal microbiota were partially required for the antigenic drive in maintaining systemic iNKT cells. Surprisingly, even greater depletion of iNKT cell population occurred in RF mice. This was in part attributable to reduced RF levels of intestinal microbial taxa (Sphingomonas spp.) known to express antigenic glycosphingolipid products. However, memory and activated CD8⁺ T cells were also expanded in RF mice, prompting us to test whether CD8⁺ T cell activity might be further depleting iNKT cells. Indeed, iNKT cell numbers were restored in RF mice bearing the CD8α⁻/⁻ genotype or in adult wild-type RF mice acutely depleted with anti-CD8 Ab. Moreover, iNKT cells were restored in RF mice bearing the Prf1⁻/⁻ phenotype, a key component of cytolytic function. These findings indicate that commensal microbiota, through positive (antigenic drive) and negative (cytolytic depletion by CD8⁺ T cells) mechanisms, profoundly shape the iNKT cell compartment. Because individuals greatly vary in the composition of their microbial communities, enteric microbiota may play an important epigenetic role in the striking differences in iNKT cell abundance in humans and therefore in their potential contribution to host immune status.

The composition of the enteric microbial community is diverse (1, 2) and represents a unique and possibly stable epigenetic trait distinguishing individuals throughout their life (3, 4). Family studies indicate that microbial composition is an important epigenetic trait whose biologic functionalities may be preserved at the biochemical rather than phylogenetic level (5–7). Accordingly, enteric microbiota composition represents a potentially important factor in individual physiology and disease susceptibility. Enteric bacteria influence the abundance of immune constitution of intestinal lymphoid follicles and Peyers’s patches, as revealed by antibiotic treatment and genetic manipulations such as mice deficient in activation-induced cytidine deaminase, which disables somatic diversification and attendant immune microbial control (8–12). Local and systemic immune cell populations are numerically and functionally attenuated in germfree (GF) mice (13), and this context has permitted the identification of some microbial products that shape normal immune cell formation (6, 14, 15).

Modeling the diverse microbial composition of the human, our laboratory has developed mice stably bearing distinct microbial communities of complex composition, termed conventional flora (CF) and restricted flora (RF). As characterized by conventional microbiologic and molecular phylotyping, both microbial flora include hundreds of phylotypes spanning the major bacterial and fungal taxa (16, 17). However, the RF and CF microbial communities display broad-based compositional differences at the species to family levels: preferentially Bacteroidetes and Firmicutes phylotypes in CF and RF mice, respectively, features that also distinguish enteric microbial communities among humans with obese and lean dietary habits (18). RF mice are strikingly deficient in innate-like immune cell populations, including marginal zone (MZ) B cells and plasmacytoid dendritic cells (pDCs) (16, 19). Moreover, this phenotype is mediated by a CD8⁺ T cell population, expanded and activated by RF microbiota, that cytolytically targets these innate immune cell types (16, 19). Although the mechanisms linking RF microbiota and this cytolytic T cell population are uncertain, they may involve targeting of a Qa-1–restricted Ag shared by the depleted innate immune cell types (19).

Invariant NKT (iNKT) cells are an important innate-like lymphocyte population programmed for CD1d-restricted recognition of glycolipid Ags of host or microbial origin (20–23). Systemically distributed and notably localized in extra lymphoid sites, such as the liver (24), they play diverse roles in effector and regulatory responses to microbial infection (25–27), chronic inflammatory diseases (28–30), and tumor immunity (31, 32). In this study, we analyzed the effects of commensal microbiota on the iNKT population. First, we found that GF mice were partially decreased in...

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Abbreviations used in this paper: αGalCer, α-galactosylceramide; CF, conventional flora; DC, dendritic cell; GF, germfree; iNKT, invariant NKT lymphocyte; Int, intestine; MZ, marginal zone; NIH, National Institutes of Health; pDC, plasmacytoid dendritic cell; RF, restricted flora; UCLA, University of California at Los Angeles; WT, wild-type.

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nNKT cell numbers, suggesting that microbiota is a significant source of antigenic glycosphingolipid required for their full systemic expansion. Second, nNKT cells were depleted in mice bearing RF microbiota, a process mediated by cytolytic CD8\(^+\) T cells. Thus, the commensal microbial community systemically shaped nNKT cell populations by two distinct mechanisms, one that stimulated, presumably by Ag-driven expansion of nNKT cells, and another that induced a CD8\(^+\) T cell population targeting nNKT cells for cytolytic depletion. The implication is that distinct members of the microbial community differentially shape the nNKT cell population, which opens new strategies to identify microbiota epigenetically modulating specific host immune traits.

**Materials and Methods**

**Mice**

C57BL/6 mice bearing CF were obtained from The Jackson Laboratory (Bar Harbor, ME) and kept in specific pathogen-free conditions monitored for a panel of pathogenic microorganisms based on serologic and microbiologic screening by the University of California at Los Angeles (UCLA), Division of Laboratory Animal Medicine. C57BL/6 mice bearing RF were kept in a separate facility maintained by the UCLA Department of Radiation Oncology. RF mice were periodically tested for RF status by aerobics and anaerobic culture of cecal contents and index molecular phylotypes (16). With respect to specific pathogen-free status, both CF and RF mice were monitored for the absence by serology or culture of a panel of viral, fungal, and bacterial pathogenic taxa, including Helicobacter spp.

**CD8\(^+\) Abs used for CD8\(^+\) T cell depletion were purchased from BioXCell (West Lebanon, NH).** Cells were recovered by centrifugation at 2000 rpm for 5 min, washed twice with PBS-57, and re-suspended in 100 μl of PBS-57. CD1d tetramers loaded with α-GaCer were used. Mouse CD1d tetramers loaded with αGalCer were recently developed by Dr. P. Savage and associates (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD11c (HL3), CD44, CD62L (MEL-14), CD69, CD122, NK1.1, DX-5, and Ly-6C were purchased from BioLegend (San Diego, CA). Anti-GaCer-α (α-GaCer) loaded CD1d tetramers were as described previously (35). Mouse CD1d tetramers loaded with α-GalCer were purchased from BioXCell (West Lebanon, NH). Cells were re-suspended at 10,000 cells/μl in PBS-57, and aliquots of 5, 10, or 20 μl were added to 95% purity, were i.v. injected into CF mice at 4 × 10\(^5\) cells/mouse. Mice were sacrificed 3 d after CD8\(^+\) T cell transfer, and splenic DNA was extracted from mucosal samples

Intestinal epithelium-associated tissue samples were isolated from small and large intestines of age- and gender-matched CF and RF mice as reported previously (16). DNA was extracted by the CLS-Y buffer and a 30-s bead-beating step with the FastDNA Spin Kit (MP Biomedicals, Solon, OH) and a FastPrep instrument setting of 5.5, as described by the manufacturer. Purified DNA samples were size fractionated by electrophoresis in 1% agarose gels. DNA > 3 kb was excised without exposure to UV or ethidium bromide and recovered using the QiAquick Gel Extraction Kit (Qiagen, Valencia, CA), following the manufacturer’s instructions, except that the gel pieces were not exposed to heat.

**Sphingomonas-selective PCR**

Partial Sphingomonas small-subunit rRNA genes were amplified in 20-μl PCRs with the following conditions: 50 mM Tris (pH 8.3), 500 μg/ml BSA, 2.5 mM MgCl\(_2\), 250 μM each deoxynucleotide triphosphate, 400 nM of each primer, 1 μl of mucosal DNA, and 1 U of TaqDNA polymerase. The Sphingomonas-selective primers were as follows: Sphingo 108F (5’-GGCTAAGCCTGTGAACTG-3’) and Sphingo 420R (5’-TTACACCACTTAAAGGCTTC-3’) (37). All reactions were combined and heated at 94°C for 5 min. Forty cycles of PCR were then performed at 94°C for 20s, 58°C for 30s, and 72°C for 30s, followed by 72°C for 5 min. PCRs were run on a 1% agarose gel and ethidium bromide stained. The Sphingomonas spp. were detected by ethidium bromide-stained bands at 370 and 420 bp.

**DNA extraction from mucosal samples**

DNA was extracted from mucosal samples of CF and RF mice using the CLS-Y buffer and a 30-s bead-beating step with the FastDNA Spin Kit (MP Biomedicals, Solon, OH) and a FastPrep instrument setting of 5.5, as described by the manufacturer. Purified DNA samples were size fractionated by electrophoresis in 1% agarose gels. DNA > 3 kb was excised without exposure to UV or ethidium bromide and recovered using the QiAquick Gel Extraction Kit (Qiagen, Valencia, CA), following the manufacturer’s instructions, except that the gel pieces were not exposed to heat.

**Sphingobium yanoikuyae challenge**

*Sphingobium yanoikuyae* bacterium was cultured in anaerobic conditions in anaerobic culture of cecal contents and index molecular phylotypes (16). All the mice examined in the study were age and gender matched. The animal procedures were carried out in accordance with the animal research protocols approved by UCLA and La Jolla Institute of Allergy and Immunology institutional animal research committees.

**Reagents**

Fluorochrome Ab conjugates reactive to mouse CD3e (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD11c (HL3), CD44, CD62L (MEL-14), CD69, CD122, NK1.1, DX-5, and Ly-6C were purchased from BioXCell (West Lebanon, NH). Anti-MPDCA-1 was obtained from Millenyi Biotec (Auburn, CA). The α-galactosylceramide (α-GaCer) loaded CD1d tetramers were as described previously (35). Mouse CD1d tetramers loaded with α-GalCer, an analog of α-GaCer recently developed by Dr. P. Savage and colleagues (36) were obtained from the NIH Tetramer Core Facility located at the Emory Vaccine Center at Yerkes (Atlanta, GA). Monoclonal anti-CD8α and CD8β Abs used for CD8\(^+\) T cell depletion were purchased from BD Biosciences and Biolegend (San Diego, CA), respectively.

**Cell isolation and flow cytometric analysis**

Single-cell suspensions were prepared from spleen, thymus, and liver of experimental mice. For spleen and thymus, single-cell suspensions from spleen and thymus were made RBC-free by ammonium chloride lysis. For liver lymphocyte isolation, liver tissue from individual mice was homogenized gently in a 100-μm cell strainer in a petri dish containing 10 to 20 ml of DMEM with antibiotics and 10% FCS (Life Technologies, Long Island, NY). Cells were recovered by centrifugation at 2000 rpm for 5 min, resuspended with 7 ml of 40% Percoll, and layered on 5 ml of 80% Percoll in 15 ml of Corning tubes. Liver lymphocytes were then isolated by Percoll gradient centrifugation at 2000 rpm for 30 min, and cells in the lymphocyte layer were collected for flow cytometric analysis.

Identification of nNKT cells was performed in accord with standard criteria (24), using multiparameter staining for CD1d tetramers loaded with α-GaCer (or PBS-57), anti-CD3ε, anti-TCRβ, and in some cases anti-NK1.1 Abs. Except when specified as PBS-57, CD1d tetramers loaded with α-GaCer were used. Data were collected on a FACSCalibur or LSRII flow cytometer (BD Biosciences) and analyzed using CellQuest (BD Biosciences) or FlowJo (Tree Star, Ashland, OR) software.

**Statistical analysis**

Statistical analysis was performed by using Prism software (www.graphpad.com). The percentages and the absolute number of nNKT cells in different samples were analyzed by using an unpaired two-tailed Student t test for two groups, or one-way ANOVA for three or more data sets, with a 95% confidence interval. Values of p < 0.05 were considered to be significantly different.

**Results**

RF mice are deficient for nNKT cells

We recently studied the changes in lymphocyte and dendritic cell (DC) populations and function programmed by divergent resident
intestinal microbial communities. Compared with CF mice, RF mice had a profound numerical and functional depletion of pDCs and innate-like (MZ and B-1 B cells) (16, 19). These changes were associated with an expanded CD8\(^+\) T cell population (38), whose cytolytic activity was causally involved in the depletion of pDCs and innate-like B cells in RF mice (16, 19). Although conventional (CD4\(^+\) and CD8\(^+\)) T cells were only modestly altered in RF mice (38), we wanted to assess the n\(\text{NKT}\) cell compartment, another innate-like lymphocyte population whose immune functions have been under extensive study. To determine the status of n\(\text{NKT}\) cells in RF mice, their abundance was examined by flow cytometry, identifying n\(\text{NKT}\) cells by conventional multiparameter criteria including binding to glycolipid (\(\alpha\)-GalCer) or PBS-57–loaded CD1d tetramers, along with anti-TCR\(\beta\) and anti-NK1.1 Abs.

As exemplified in representative individuals, we initially observed that n\(\text{NKT}\) cells were significantly reduced in RF mice in all evaluated compartments, including spleen, liver, and thymus (Fig. 1). We further tested whether the absence of intestinal bacteria in GF mice might also affect n\(\text{NKT}\) cells. As shown in Fig. 1, the amount of n\(\text{NKT}\) cells in GF mice were significantly decreased as compared with age- and gender-matched CF control mice. However, much more profound deficiency of n\(\text{NKT}\) cells was observed in RF mice (Fig. 1). Analysis of NK1.1\(^+\) lymphocytes of the liver in CF, RF, and GF mice confirmed the selective depletion of n\(\text{NKT}\) cells (NK1.1 intermediate and TCR\(\beta\)^+ ) versus conventional NK cells (NK1.1 high and TCR\(\beta\)-neg) in GF and RF mice and the much more profound depletion in RF mice (Fig. 1B). Quantitatively, n\(\text{NKT}\) cell numbers in GF mice were only moderately reduced, at 50–75% of CF levels for both percentage and absolute numbers in spleen, liver and thymus (Fig. 1D). Similar results were obtained from >24 mice belong to two different GF colonies (Materials and Methods). In contrast, n\(\text{NKT}\) cells in RF mice were reduced >2-fold in the spleen and >5-fold in the liver and thymus. Thus, RF mice displayed a systemic decrease of n\(\text{NKT}\) cell number, most strikingly in the liver where they are most prevalent.

These findings indicated that commensal intestinal microbiota were not strictly required for n\(\text{NKT}\) cell formation but that they contributed to the higher systemic levels of n\(\text{NKT}\) cells achieved in mice bearing CF microflora. However, the modest deficiency of n\(\text{NKT}\) cells in GF mice was notable for two reasons. First, it indicated that the intestinal microbial products were not absolutely required, some host autonomous processes were sufficient for substantial generation of the n\(\text{NKT}\) cell subset. Second, it suggested that the profound depletion of n\(\text{NKT}\) cells in RF mice involved a microbial-induced process distinct from microbial Ag drive.

The role of enteric bacteria in n\(\text{NKT}\) cells formation

Antigenic drive is an important factor in n\(\text{NKT}\) cell expansion and the constitutive activation state of these cells. Although an important role for the still undefined cellular Ag(s) is likely, the pertinent glycolipid Ags also may be of microbial origin (20–24). Accordingly, a simple explanation for the deficiency of n\(\text{NKT}\) cells in RF mice is the absence of microbial species producing relevant microbial glycolipid Ags.

Previously, we observed extensive differences in the composition of intestinal enteric microbiota between CF and RF mice (16, 17). Glycosylceramides from the cell wall of Sphingomonas have been identified as the CD1d ligands stimulating n\(\text{NKT}\) cells (20, 23). Therefore, we wondered whether the reduced n\(\text{NKT}\) cell levels in RF mice might be associated with reduced levels of Sphingomonas in the RF versus CF intestinal microbial community. When Sphingomonas was detected in the epithelium-associated samples by specific 16S rRNA PCR, levels of Sphingomonas spp were significantly decreased in the small and large intestines of RF mice as compared with CF mice (Fig. 2A). This result suggests that the absence of stimulating microbial Ags in the intestinal microbiota might partially attribute to the n\(\text{NKT}\) cell deficiency in RF mice.

We further tested whether the intestinal colonization of certain species of bacteria, bearing known n\(\text{NKT}\) cell Ags, could affect n\(\text{NKT}\) cells in the periphery. For this purpose, S. yanoikuyae bacteria (23) were gastrically inoculated in GF mice to observe their effects on n\(\text{NKT}\) cells. Indeed, intragastric application of S. yanoikuyae to C57BL/6 GF mice led to a significant increase in relative n\(\text{NKT}\) cell numbers in liver and spleen within a day (Fig. 2B). Furthermore, the activation markers CD69 and CD25 were upregulated on n\(\text{NKT}\) cells in liver (Fig. 2C) and spleen (data not shown), indicating that enteric bacteria bearing n\(\text{NKT}\) cell Ags systemically affected n\(\text{NKT}\) cells.

Expanded CD8\(^+\) T cells in RF mice display the memory and cytolytic phenotypes

RF mice are distinguished by an expansion of CD8\(^+\) T cells (38), which play a direct role in depleting innate or innate-like immune cell populations including pDCs and MZ B cells (16, 19). We therefore asked whether the distinctive reduction of n\(\text{NKT}\) cells in RF mice are also involved in this expanded CD8\(^+\) T cell population. To further define the phenotype of the expanded CD8\(^+\) T cell population, we evaluated several markers of T cell activation and memory in CF and RF mice (Fig. 3). In contrast to CF mice, most RF CD8\(^+\) T cells expressed a high level of CD44, as reported previously (23) (Fig. 3A). This suggested that the expanded CD8\(^+\) T cells in RF mice were part of a memory cell population. Multiparameter analysis of CD8\(^+\)CD44\(^+\) T cells was therefore performed for expression of Ly-6C and CD122, useful in distinguishing effector and central memory T cells (39, 40). As shown in Fig. 3B, CD8\(^+\)CD44\(^+\) T cells in RF mice were increased for the Ly-6C^+CD122^- subpopulation. Accordingly, the CD8\(^+\) T cell expansion in RF mice primarily represented expansion of a central memory population. Because Ly-6C^+ and CD122 expression were correlated with IFN-\(\gamma\) production and cytolytic activity, these findings were in accord with the increase in these effector functions, such as the increased IFN-\(\gamma\) production by CD8\(^+\) T cells in RF mice (16).

Effect of CD8\(^+\) T cells on the depletion of n\(\text{NKT}\) cells in RF mice

To determine whether CD8\(^+\) T cells in RF mice contributed to the decrease in n\(\text{NKT}\) cell numbers, we rederived CD8\(\alpha^-\) CF mice with RF microbiota and examined whether the genetically determined absence of CD8\(^+\) T cells would reverse the RF-associated deficit of n\(\text{NKT}\) cells. The CD8\(^-\)/CF mice indeed showed an increase in n\(\text{NKT}\) cell numbers in RF mice, including the spleen, liver, and thymus (Fig. 4A). In contrast, under normal CF condition, CD8\(^-\)/CF mice showed normal n\(\text{NKT}\) cells that were similar with wild-type (WT) CF mice. Comparing groups of individuals, the mean percentages and absolute numbers of n\(\text{NKT}\) cells in spleen, liver, and thymus of the RF CD8\(\alpha^-\) mice (Fig. 4B) were even higher than those in WT CF mice.

CD8\(\alpha\) is expressed on a variety of cell types in addition to conventional CD8\(\alpha\beta\) T cells, and the absence of these populations during ontogeny may impact n\(\text{NKT}\) cell differentiation. To assess the effect of CD8\(\alpha\) cells in the adult mouse on n\(\text{NKT}\) cells in the absence of a developmental defect, RF mice were acutely depleted of these cells by anti-CD8\(\alpha\) or anti-CD8\(\beta\) Ab treatment and analyzed 3 d after the last treatment. As expected, CD8\(^+\) T cells were profoundly decreased after anti-CD8 treatment (Fig. 5A). When n\(\text{NKT}\) cells were analyzed, a striking restoration of n\(\text{NKT}\) cells was
observed after 3 wk of continuous CD8+ T cell depletion (Fig. 5B). It should be noted that after anti-CD8 treatment, the residual iNKT T cell population may have been altered with respect to the levels of its CD4 and CD8 single- and double-positive components. To determine the timing of iNKT cell recovery in relation to the CD8+ T cell depletion, a time course was performed in mice receiving anti-CD8 Ab treatment (Fig. 5C). This revealed that iNKT cell levels began to recover 2 wk after CD8+ T cell depletion. However, thereafter iNKT cells again declined. The reduction was not surprising, because CD8+ T cell numbers begin to recover after 2 wk because of clearance of the Ab to subtherapeutic levels (bar graph in Fig. 5C). Taken together, these observations indicated that CD8+ T cells actively mediate depletion of iNKT cells in adult RF mice.

Perforin is a membrane-disruptive protein required for cytolysis by CD8+ T cells and NK cells. To determine whether CD8+ T cells in RF mice suppress iNKT cells through CTL-mediated cytotoxicity, we established a colony of RF Prf1−/− mice by cesarean rederivation. As shown in Fig. 6A, the Prf1−/− genotype restored iNKT cells in RF mice to the normal amount in CF mice. Compared with age- and gender-matched Prf1+/+ RF mice, CF Prf1−/− and RF Prf1−/− mice displayed normal or even increased iNKT cells (Fig. 6B), considered both as percentage and absolute numbers. These results demonstrated that perforin-mediated cytotoxicity plays a role in the depletion of iNKT cells in RF mice. However, we note that these data leave open the possibility that NK cells may also be involved in iNKT cell deficiency in RF mice.

**RF CD8+ T cells do not directly deplete iNKT cells in CF mice**

Transfer of RF CD8+ T cells into CF mice acutely depleted pDCs and MZ B cells, providing evidence of in vivo cytolysis by RF CD8+ T cells of these target cell types (16, 19). Using these same conditions, we therefore tested whether RF CD8+ T cell transfer also depleted iNKT cells. Accordingly, RF CD8+ T cells were transferred i.v. into CF mice and, at the same time, stimulated with RF enteric microbial Ags i.p., which is required for optimal CD8+ T cell activity in the CF recipient mice (16, 19). However, RF CD8+ T recipients showed no change in splenic or liver iNKT cell abundance (Fig. 7A). In contrast, as a positive control, splenic iNKT cells were successfully depleted under these conditions (Fig. 7B).

Thus, the predominant population of peripheral iNKT cells is not acutely susceptible to in vivo cytolysis by RF CD8+ T cells.

**Discussion**

The present study uncovered two distinct effects of the intestinal microbial community on iNKT cells. The iNKT cell population was moderately reduced in GF mice, suggesting that the commensal microbiota positively modulates the iNKT cell population. Conversely, in mice bearing RF microbiota, iNKT cells were greatly reduced, a process dependent on active depletion by CD8+ T cells and cytolytic function mediated by perforin. This discussion addresses the potential mechanisms underlying these different modes of interaction between the iNKT cell population and the host commensal microbial community.
The expansion of the iNKT cell population is highly dependent on interaction with cognate glycolipid/CD1d complexes in the thymus (24, 41). Some CD1d ligands are presumed to be of self-origin as exemplified by the discovery of an endogenous lysosomal glycosphingolipid, iGb3, as a potential CD1d ligand (20, 21). Although the characterization of the full scope of endogenous ligands remains an active research area, peripheral encounter with self- or foreign Ags presented by CD1d in principle could modulate or fine-tune the iNKT cell population, in terms of number, activation state, or expression of maturation markers such as NK1.1 (24, 31, 42–45).

Although an earlier study did not find any difference in iNKT cells in GF as compared with conventional mice (46), in this study, we report a significant decrease in the frequency of iNKT cells. The divergent results can be attributed to differences in the current study, but not the study of Park et al. (46), which provides evidence that host production of glycosphingolipid may represent an endogenous mammalian source of antigenic glycolipid. One possibility is that dietary Ags derived from food or its microbial contaminants affect the homeostasis of iNKT cells. In addition, mice bearing null mutations of glycolipid biosynthesis (51 and processing, including microsomal triglyceride transfer protein (52) and adaptor protein AP-3 (53), are deficient in iNKT cells. These observations suggest that host production of glycosphingolipid may represent an endogenous mammalian source of antigenic glycolipids. However, these mutations also may affect the processing and intracellular trafficking of exogenous glycolipids (54, 55) including those of commensal microbial origin (56). Thus, the current study can most simply be interpreted as evidence that the much more extensive depletion of iNKT cells in GF mice. This suggests that other sources may provide an important, alternate source of antigenic glycolipid.

A second phenotype related to the commensal microbiota was the much more extensive depletion of iNKT cells observed in mice bearing the RF microbiota enriched for Firmicutes spp. Several lines of evidence indicated that this depletion was mediated by CD8α T cells. First, RF mice bear an expanded memory CD8α T cell population, and RF mice genetically lacking CD8α displayed normal and even increased levels of iNKT cells. This finding potentially implicated a variety of CD8α∗ cell types (e.g., conventional CD8αβ T cells and CD8αα T cells or lymphoid DCs) (57). However, treatment of RF mice with anti-CD8α or CD8β Ab substantially restored iNKT cell levels. This rapid replenishment suggested that iNKT cell numbers were suppressed by an active species is significantly decreased in the small and large intestines of RF mice as assayed by phylotype-specific 16S rRNA PCR (Fig. 2A). The decreased colonization of Sphingomonas spp. in RF mice may at least partially contribute to the deficiency of iNKT cells, because Sphingomonas spp. were not detected in GF mice (Fig. 2B, 2C).

It is notable that substantial levels of iNKT cells were preserved in GF mice. This suggests that other sources may provide an important, alternate source of antigenic glycolipid. One possibility is that dietary Ags derived from food or its microbial contaminants affect the homeostasis of iNKT cells. In addition, mice bearing null mutations of glycolipid biosynthesis (51 and processing, including microsomal triglyceride transfer protein (52) and adaptor protein AP-3 (53), are deficient in iNKT cells. These observations suggest that host production of glycosphingolipid may represent an endogenous mammalian source of antigenic glycolipids. However, these mutations also may affect the processing and intracellular trafficking of exogenous glycolipids (54, 55) including those of commensal microbial origin (56). Thus, the current study can most simply be interpreted as evidence that commensal microbiota are at least partially required for iNKT cell homeostasis.
A defect in the \textit{i}NKT cell precursor, by contrast, might be less reversible or at least might require a longer time for the restoration of the population. The action of CD8$^+$ cells in this setting could have been indirect, for example, through competition for IL-15 or production of inhibitory products affecting T cell activation and expansion (58, 59). However, RF mice bearing a null mutation for perforin, a critical component of cell-mediated cytolysis, were also restored for \textit{i}NKT cells. Taken together, these findings strongly suggest the \textit{i}NKT cell deficiency in RF mice was mediated by the cytolytic action of CD8$^+$ T cells.

Certain other immune cell types, including pDCs and MZ B cells, are also selectively depleted by the cytotoxic action of CD8$^+$ T cells in RF mice (16, 19). In contrast, many other cell types, including conventional myeloid DCs, follicular B cells, and memory CD4$^+$ T cells, are preserved in RF mice. The reason why only a few cell types, including \textit{i}NKT cells, pDCs, and MZ B cells, are coordinately depleted is uncertain. One possibility is that these cell types may share a common target Ag recognized by the relevant CD8$^+$ T cell population. In the case of MZ B cells, there is direct evidence that they are targeted via the MHC class 1b molecule Qa-1 (19). Moreover, the molecular stress response associated with activation via the NF-kB pathway, or the metabolic stress of cellular proliferation (60, 61), includes up-regulation of Qa-1 and a shift in Qa-1 peptide loading to Hsp60-derived peptides (62) targeted by Qa-1-restricted CD8$^+$ T cells (63–65). The role of such cytolytic T cells in targeting \textit{i}NKT cells provides a simple explanation for the systemic depletion of \textit{i}NKT cells, including those residents in sites remote from the intestine such as in the thymus.

In this regard, it was notable that during the short-term action of in vivo-transferred RF CD8$^+$ T cells, a significant depletion was detectable for pDCs and MZ B cells (16, 19) but not of \textit{i}NKT cells (this study). Although the reason for this difference is uncertain, it suggests two explanations. First, peripheral pDCs and MZ B cells bear higher amounts of the putative shared target Ag (such as Qa-1/Hsp60 peptide), permitting their more efficient targeting. Alternatively, the target Ag may be predominantly expressed by precursor rather mature \textit{i}NKT cells. Because the mature \textit{i}NKT cell population turns over slowly, such precursor cell depletion would not be manifested in the mature \textit{i}NKT cell population over the short term. These ideas predict that quantitative levels of Qa-1 and Hsp60 expression, or Qa-1/Hsp60 peptide loading, are physiologically elevated in these target populations, which may be experimentally testable predictions.

The mechanism linking commensal RF microbiota with induction of this cytolytic CD8$^+$ T cell population is unknown. Speculatively, we note that both murine Qa-1/Hsp60 and human HLA-E/Hsp60 CD8$^+$ T cell responses are elicited by certain microbiota, including \textit{Listeria} and \textit{Salmonella} (63, 66). This cross-reactivity has been linked to microbial GroEL (192–200) and mammalian Hsp60 (216–224), which encode a Qa-1/HLA-E binding nonapeptide with identical residues for TCR contact (–FD–Y). Thus, we speculate that RF microbiota may be distinguished from CF microbiota by either microbial community members responsible for greater...
bioavailability of such Hsp60 mimics or by traits that elicit or provide adjuvant activity for the local enteric mucosal stress response, leading to immunogenicity and expansion of an Hsp60/Qa-1 CD8+ T cell population.

The mechanism(s) by which RF microbiota induce the striking RF immunologic phenotype is a complex issue. The data from GF mice indicate that enteric organisms only contribute incrementally to iNKT levels, because the population is largely preserved in GF mice. Depletion of CD8+ T cells in RF mice increases iNKT cell numbers. A and B, RF mice were treated with anti-CD8α (or saline, the vehicle control) for 3 wk and analyzed by FACS at 3 d after the last treatment for levels of CD4+ and CD8+ T cells (A) or iNKT cells (B). C, Dynamic change of absolute number of iNKT cells (curves) and percentage of CD8+ T cells (bar graphs) in liver of anti-CD8α Ab-treated RF mice and RF control at indicated time points. Similar results were obtained using an anti-CD8β Ab (data not shown). Absolute numbers of iNKT cells in RF and control RF mice were compared at week 2 and week 3 after anti-CD8α treatment; p values were analyzed by Student t test. Data are representative of three individual experiments and >24 mice per group.

FIGURE 5. Depletion of CD8+ T cells in RF mice increases iNKT cell numbers. A and B, RF mice were treated with anti-CD8α (or saline, the vehicle control) for 3 wk and analyzed by FACS at 3 d after the last treatment for levels of CD4+ and CD8+ T cells (A) or iNKT cells (B). C, Dynamic change of absolute number of iNKT cells (curves) and percentage of CD8+ T cells (bar graphs) in liver of anti-CD8α Ab-treated RF mice and RF control at indicated time points. Similar results were obtained using an anti-CD8β Ab (data not shown). Absolute numbers of iNKT cells in RF and control RF mice were compared at week 2 and week 3 after anti-CD8α treatment; p values were analyzed by Student t test. Data are representative of three individual experiments and >24 mice per group.

FIGURE 6. Perforin (Prf1)−/− mice bearing RF microflora have normal iNKT cell numbers. Re-derived Prf1−/− mice bearing RF microflora were examined for the presence of iNKT cells. A, The percentage of PBS-57–loaded CD1d tetramer+ iNKT cells within lymphocytes from spleen and liver of CF, CF Prf1−/−, RF, and RF Prf1−/− mice are indicated. B, Percentages and absolute number (C) of iNKT cells in CF, CF Prf1−/−, RF, and RF Prf1−/− mice in lymphocytes of indicated organs. Statistical analysis of p values using Student t test compared WT CF versus CF Prf1−/− mice, CF Prf1−/− versus RF WT, and RF WT versus RF Prf1−/− mice were indicated. The data are representative of at least three individual experiments with >12 mice in each group.
mouse. Conversely, broad-spectrum antibiotic treatment on RF mice produced only modest and inconsistent reversal of the immune phenotypes in RF mice (B. Wei and J. Borneman, unpublished observations). The interpretation of this finding is uncertain, because restoration of depleted immune cell types in RF mice was reliably converted (to CF composition) only when the microbiota were manipulated in the immediate postnatal period (19). Finally, and conversely, RF CD8+ T cells modify the composition of the enteric microbiotal community (67). Taken together, the interplay of microbiotal composition for such immunologic traits are complex, and its delineation will require fastidious experimental design with respect to host ontology, and both selective manipulation and careful longitudinal analysis of microbial and immune effector composition.

The composition of commensal microbial communities is a distinguishing human trait, which is profoundly affected by diet and other lifestyle factors. Because the modern urban lifestyle is associated with striking increases in certain chronic inflammatory and autoimmune diseases, the mechanisms linking lifestyle, commensal microbiota, and such disease susceptibility is of considerable interest. The present study provides evidence for two mechanisms linking the commensal microbiota with the mature iNKT population. Our findings may provide an explanation for the striking difference in iNKT cells number found in the peripheral blood of different individuals (68). Furthermore, considering the pleiotropic roles of NKT cells in host defense and immunoregulation, these findings suggest potentially relevant processes contributing to the link between the commensal microbiota and the associated profile of disease susceptibilities.

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Disclosures

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References


