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Invariant NKT Cells Biased for IL-5 Production Act as Crucial Regulators of Inflammation

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Although invariant NKT (iNKT) cells play a regulatory role in the pathogenesis of autoimmune diseases and allergy, an initial trigger for their regulatory responses remains elusive. In this study, we report that a proportion of human CD4+ iNKT cell clones produce enormous amounts of IL-5 and IL-13 when cocultured with CD1d+ APC in the presence of IL-2. Such IL-5 bias was never observed when we stimulated the same clones with α-galactosylceramide or anti-CD3 Ab. Suboptimal TCR stimulation by plate-bound anti-CD3 Ab was found to mimic the effect of CD1d+ APC, indicating the role of TCR signaling for selective induction of IL-5. Interestingly, DNA microarray analysis identified IL-5 and IL-13 as the most highly up-regulated genes, whereas other cytokines produced by iNKT cells, such as IL-4 and IL-10, were not significantly induced. Moreover, iNKT cells from BALB/c mice showed similar IL-5 responses after stimulation with IL-2 ex vivo or in vivo. The iNKT cell subset producing IL-5 and IL-13 could play a major role in the development of allergic disease or asthma and also in the immune regulation of Th1 inflammation. The Journal of Immunology, 2007, 179: 3452–3462.

1Invariant NKT (iNKT)3 cells are a nonconventional population of T cells, expressing a canonical invariant TCR α-chain (Vα14-Jα18 for mice and Vα24-Jα18 for human) and TCR β-chains using limited Vβ segments (Vβ8.2, 2, and 7 in mice and Vβ11 in humans) (1–4). They are selected and restricted by CD1d, a nonclassical MHC class I-like molecule, and proliferate vigorously in response to α-galactosylceramide (αGC), a prototypical iNKT cell ligand, originally isolated from marine sponge (5). Although most iNKT cells express NK cell markers such as CD161, they also contain a small population of cells that are negative for NK cell markers (6). Importantly, CD1d-restricted T cells also contain T cells that neither express the canonical TCR α-chain nor respond to αGC (7, 8). To avoid confusion, it has recently been recommended that iNKT cells should be defined by their reactivity to αGC loaded onto CD1d multimers, instead of expression of NK cell markers (6). iNKT cells comprise CD4+ and CD4+ cells, which show differential expression of regulatory cytokines. In humans, studies have shown that the former produce both Th1 and Th2 cytokines, whereas the latter predominantly produce proinflammatory cytokines such as IFN-γ and TNF-α (9, 10). Accordingly, the CD4+ cells are thought to be the major source of Th2 cytokines for controlling Th1 cell-mediated inflammation or promoting Th2-dependent pathologies.

Although earlier studies have tended to focus on the ability of iNKT cells to down-modulate inflammatory responses, more recent works have shown that they could promote joint inflammation in models of arthritis (11–13) or mediate airway inflammation in bronchial asthma (14, 15). The divergent effects of iNKT cells in inflammatory pathologies are thought to reflect a broad spectrum of their functions in vivo. In fact, iNKT cells explosively produce a number of pro- and anti-inflammatory cytokines after nonphysiological stimulation with αGC (2, 5, 16) or anti-CD3 mAb (17), although stimulation with alternative ligands such as αGC analogues may lead to selective Th1 (18) or Th2 cytokine production (19, 20). Regarding the molecular mechanism for iNKT cell-mediated immune regulation, previous studies have suggested the role of iNKT cell-derived IL-4 or IL-10 in controlling Th1-mediated inflammation (16, 19, 20), whereas the role of IL-13 secreted by iNKT cells has recently been highlighted in the pathogenesis of asthma (14, 15) and ulcerative colitis (21). The published results, however, do not exclude the possible role of other cytokines secreted by iNKT cells. In fact, it is not clear whether iNKT cells could produce specific cytokines that are truly needed to exert regulatory functions or whether they produce cytokines in a redundant way. Another important question is what would trigger the regulatory iNKT cells to promote a cytokine response in vivo during the natural course of disease. Although TCR and/or costimulatory molecule signaling are likely to be the triggers involved, direct evidence for this postulate so far has not been provided.

Based on the observation of neonatal iNKT cells expressing memory-activated phenotype (CD45R0+CD62L+CD25+) (22, 23) and resting adult iNKT cells containing preformed transcripts of IFN-γ and IL-4 (24), it has been suggested that iNKT cells are preactivated by endogenous ligands. If endogenous ligands for iNKT cells are to exist in vivo, we speculate that they transmit a relatively weak signal through TCR (25). Supportive of this idea,

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3Abbreviations used in this paper: iNKT, invariant NKT; αGC, α-galactosylceramide; iGb3, isoglobotrihexosylceramide; HS, healthy subject; MS, multiple sclerosis; DN, double negative; DC, dendritic cell; iDC, immature DC; CBA, cytometric bead array.

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Brigl et al. (26) have recently shown that human iNKT cell clones as well as freshly separated rodent iNKT cells could exert an enormous IFN-γ response, when they react to an endogenous ligand in the presence of costimulatory IL-12 (26). As such, a very weak autoreactive iNKT cell response to CD1d-positive cells could be remarkably augmented by various additional signals such as cytokines and costimulatory molecules. Several candidates for endogenous ligands have been previously reported (27–29). More recent studies have demonstrated that lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) is a possible endogenous ligand naturally presented to iNKT cells in the context of CD1d (30, 31). Notably, Mattner et al. (31) has shown that iNKT cell activation following bacterial infection could be elicited either by stimulation with bacterial glycolipids or by endogenous iGb3 bound to CD1d, depending on the strain of bacteria. This indicates that recognition of endogenous ligand may be critical in triggering at least certain iNKT cell responses to CD1d.

### Materials and Methods

#### Subjects

Venous samples of nine healthy subjects (HS) and 13 multiple sclerosis patients (MS) were used for study (Table I). All the patients had conventional MS, fulfilled standard criteria for the diagnosis of relapsing-remitting MS, and were in remission at examination based on clinical and magnetic resonance imaging assessment. Four patients were on medication for >3 mo: two on low-dose corticosteroids and the other two on IFN-β. HS (33.9 ± 2.2 years old) and MS (37.53 ± 11.8 years old) were age matched. Written informed consent was obtained from all subjects and the Ethics Committee of the National Center of Neurology and Psychiatry approved this study.

#### Abs and reagents

PE-labeled anti-Vα24, FITC-anti-Vβ11, phycoerythrin-Texas Red X-anti-CD4, PC5-anti-CD8, PE-anti-CD206, and anti-mouse IgM were purchased from Immunotech and PE-anti-iNKT cells (specific for invariant Vα24-Jo18 TCR: 6B11) (32), PE-anti-human IL-4, FITC-anti-human IFN-γ, mouse CD1d dimer (dimer X), FITC-anti-mouse TCRβ, PE-anti-mouse NK1.1, and PE-rat anti-mouse IgG1 were purchased from BD Biosciences/BD Pharmingen. All human recombinant cytokines were obtained from PeproTech and microbeads coated with anti-CD14, anti-CD45RO, or anti-PE and the CD4 T cell isolation kit were obtained from Miltenyi Biotec. Flow cytometry was performed on an Epics XL and analyzed with EXPO 32 software (Coulter). Cell sorting was conducted on an Epics Altra (Coulter) or autoMACS cell sorter (Miltenyi Biotec). αGC and OCH (19) were solubilized in DMSO (100 μg/ml). Anti-CD1d mAb (αCD1d 59; IgM) was prepared in the laboratory of S. A. Porcelli.
Human iNKT clones

PBMCs were isolated by density gradient centrifugation and suspended at 1 × 10^6/ml in AIM-V medium (Invitrogen Life Technologies), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS (hereafter referred to as “basic medium”). Cells were stimulated with αGalCer or OCH (100 ng/ml) in the presence of IL-2 (50 IU/ml) and IL-7 (10 ng/ml). After 7 days, half of the medium was changed every 3–5 days with basic medium containing IL-2 (10 IU/ml) and IL-7 (5 ng/ml). Fourteen to 18 days after stimulation, CD4^+ or double-negative (DN) iNKT cells were sorted after staining with the fluorescence-labeled anti-Vβ11, anti-iNKT, anti-CD4, and anti-CD8 Abs. The sorted cells were cultured with fresh allogenic X-irradiated (100 Gy) PBMC at a cell ratio of 1:3, stimulated with 1.0 µg/ml PHA-P (PHA; Sigma-Aldrich), IL-2 (50 IU/ml), and IL-7 (10 ng/ml) for 3 days and then maintained by basic medium supplemented with IL-2 (10 IU/ml) and IL-7 (5 ng/ml). iNKT cell sorting and PHA stimulation was repeated every 4–5 wk. Two to 3 wk after the most recent stimulation, the clones were used for assays, before which they were cultured in cytokine-free medium for at least 4 days.

Coculture experiments

Immature dendritic cells (iDCs) as APCs were derived from CD14^+ monocytes (33). The iDCs were X-irradiated (55 Gy) and seeded at 3 × 10^4 cells/well with or without αGalCer (100 ng/ml) in U-bottom 96-well plates. They were washed and stimulated with iNKT cells at a 1:1 ratio, with or without IL-2 (10 IU/ml). Cytokines in the day 2 supernatant were measured by the Cytomight Beads Array (CBA) kit from BD Biosciences/BD Pharmingen as previously described (34). CD1d-transfected (CD1d-HeLa) and mock-transfected HeLa (mock HeLa) cells were also used for coculture after mitomycin C treatment (50 µg/ml, 30 min). To block the CD1d molecule, anti-CD1d mAb (aCD1d 59) was added to iDC and cultured for an hour. After washing out nonbinding mAb, iNKT cells were added at a 1:1 ratio and incubated with or without IL-2 (1 or 5 IU/ml) for 48 h. IL-5 in the supernatant was measured by CBA.

Microarray analysis

After 24 h of culture with iDCs, iNKT cells were negatively separated from the cell mixture with 95% purity. The iDCs were stained with PE-anti-CD206 and depleted using secondary anti-PE microbeads. mRNA was purified from the iNKT cells and then pooled at ~80°C. The mRNA was labeled with biotin by using the Ovation Biotin System (Nugen Technologies). The targets containing fragmented and biotin-labeled cDNA were hybridized and analyzed on GeneChip Human Genome U133A arrays (Affymetrix). The array probes were scanned and gene transcript levels were determined using algorithms in the GeneChip Analysis Suite software. Gene transcriptions of IL-2-stimulated (IL-2 sample) and vehicle-stimulated iNKT cells (negative control) were separately compared for each clone, and those significantly elevated by IL-2 stimulation were selected by paired t test. All of the genes elevated in any of the clones were analyzed by two-factor ANOVA to investigate a statistical significance.

Intracellular cytokine analysis

We isolated naive CD4^+ T cells from PBMC of HS by positive (CD4 T cell isolation kit) followed by negative selection (CD45RO microbeads). The isolated cells were stimulated by plate-bound anti-CD3 mAb (incubated at 10 µg/ml overnight) with soluble anti-CD25 mAb (2 µg/ml) in AIM-V in the presence of iNKT/DC supernatant, with or without neutralizing anti-IL-5 mAb (10 µg/ml). Three days later, the cells were transferred onto a new plate. Half of the medium was changed every second day. On day 7, the intracellular IFN-γ and IL-4 were stained after restimulating the cells with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 6 h in the presence of monensin (1 µg/ml). Appropriate control Abs were used to define the background immunofluorescence.

Analysis of BALB/c iNKT cells

BALB/c mice in specific pathogen-free conditions were used at 8–13 wk of age. Animal care and use were in accordance with institutional guidelines. Lymphocytes were separated from liver and spleen by gradient centrifugation and stained with FITC-anti-TCRβ and αGc-loaded CD1d mimer (dimer X) with secondary staining by PE-conjugated rat anti-mouse IgGl. Then TCRβ^+ αGc-loaded dimer X^+ cells were sorted by using the Aflra cell sorter. DCs were isolated from splenocytes by using CD11c microbeads and were used after being X-irradiated (30 Gy). The iNKT cells and the DCs were cocultured for 72 h in U-bottom 96-plates at a 1:1 ratio (1.5 × 10^6 cells for each) with or without IL-2. The supernatants were analyzed by CBA. To evaluate in vivo effects of IL-2 on iNKT cells, BALB/c mice were injected i.v. with 5000 IU of IL-2. Two hours later, the mice were sacrificed and their liver lymphocytes were isolated. The isolated cells were carefully stained with αGc-loaded dimer X and TCRβ on ice to avoid direct activation by these reagents. The stained cells were fixed and perforated for staining intracellular IL-5 or IFN-γ according to BD Biosciences protocol, except without any additional in vitro stimulation.

Results

A distinct group of CD4^+ iNKT cell clones produce IL-5 in the presence of IL-2

We have used a total of 26 CD4^+ iNKT cell clones derived from HS or patients with MS to evaluate their self-reactivity. Because we were initially interested in comparing MS with HS regarding the functions of iNKT cells, we used a panel of iNKT cell clones from HS and MS. In the presence of iDCs as APCs, all of the clones vigorously responded to αGc by producing a large amount of IFN-γ (>1000 pg/ml) and variable amounts of IL-4 and IL-5 (500–2500 pg/ml), confirming that they maintained the essential property of iNKT cells to react with αGc. These iNKT cell clones showed very little background response to the iDCs in the simple coculture. However, to our surprise, when we added IL-2 (10 IU/ml),
instead of αGC, to the coculture, 8 of the 26 clones produced an excessive amount of IL-5 (1500–7500 pg/ml; Fig. 1A; Table I). Remarkably, the level of IL-5 induced by IL-2 equaled or exceeded the amount that was induced by αGC (Fig. 1A, right panels). Although αGC induced large quantities of proinflammatory (IFN-γ, TNF-α) and Th2 cytokines from all the clones, exogenous IL-2 induced only a modest amount of the proinflammatory cytokines (20–700 pg/ml) and various amounts of IL-4 (0 pg/ml in Sk.1, 70–230 pg/ml in six other clones) from the eight clones capable of producing IL-5. To obtain deeper insights into this discrepancy, we plotted the ratios for IL-5 to IFN-γ (left upper panel, horizontal axis) (Fig. 1B). Regarding the ability to induce production of IFN-γ, αGC stimulation was much more potent than IL-2 and induced uniformly high responses from all the clones tested (Fig. 1B, lower right panel). A much wider range of IL-5 in quantity was produced after stimulation with IL-2 or αGC (Fig. 1B, upper panels). Interestingly, IL-2 stimulation revealed the presence of a distinct group of clones capable of producing an outstanding amount of IL-5 (1000 pg/ml<), also showing higher IL-5:IFN-γ ratios (Fig. 1B, left upper panel). In contrast, αGC stimulation could not elicit such a clear separation (right upper panel). The addition of a blocking Ab to IL-2R α-chain (anti-CD25 mAb) completely abolished the cytokine production triggered by IL-2 (data not shown). These results suggest that iNKT cells possess a previously unrecognized property to selectively produce an enormous amount of IL-5, which is probably restricted to a subset of CD4+ iNKT cells. In parallel, we have generated three CD4+CD8−DN iNKT cell clones and examined their reactivity to αGC or IL-2 in the same assay. These DN clones produced a large amount of IFN-γ and a lesser amount of TNF-α or Th2 cytokines in response to αGC. Although a large majority of CD4+ iNKT clones produced IL-5 and/or IFN-γ in response to IL-2, none of the DN clones showed a significant response to IL-2 as measured by the production of cytokines (Fig. 1C).

When we evaluated the profile of IL-5 and IFN-γ secretion (Fig. 1B), there was no noticeable difference between iNKT cell clones derived from HS (O) and MS (■). Furthermore, the clones producing a large amount of IL-5 could be generated at a similar frequency from HS and MS: 3 of 11 clones from HS (27.3%) vs 5 of 15 from MS (33.3%) (Table I). We used αGC or its synthetic analog OCH for primary stimulation to generate iNKT cell clones. OCH has a shorter sphinogine chain compared with αGC and has been shown to induce a selective production of Th2 cytokines from iNKT cells (19). To evaluate whether functional differences exist between αGC-derived and OCH-derived clones, we used both αGC and OCH as primary stimulus on PBMCs from all donors, always expanding every sample separately by each of these two glycolipids. A total of 26 iNKT cell clones were derived from 22 donors; pairs of αGC- and OCH-primed clones could be obtained only from 4 of the 22 donors (NkJ, Kai, Kn, and Ok). Although the sample size is not large enough to make any conclusive remarks, it seems that the choice of αGC or OCH is not a key factor in generating the IL-5-producing iNKT clones. Five of 14 clones generated by αGC stimulation (35.7%) produced a large amount of IL-5 in response to IL-2, and similarly 3 of 12 clones stimulated by OCH (25%) were able to do so. Moreover, when we closely examined the four pairs of αGC- and OCH-primed clones generated from the same donors, we still could not find any constant tendency concerning the ability of IL-5 production within these two types of clones (Table I).

The next important task was to evaluate the actual frequency of IL-5-producing iNKT cells within each individual. For this purpose, we freshly isolated PBMCs, stimulated them with IL-2, and examined the frequency of the IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5. However, for unknown reason, we could not reveal the presence of IL-5-producing iNKT cells by this method. Then, we decided to generate a number of CD4+ iNKT cell clones from same donors and examined the frequency of IL-5-producing Vα24+Vβ11+ cell population by flow cytometric demonstration of intracellular IL-5.
donors by the single-cell sorting method, and estimate the frequency of IL-5-producing cells. Although the method is feasible, because it is laborious and time consuming, we selected two donors from whom we could reproducibly generate IL-5-producing clones. We found that two of five single cell-sorted clones derived from one donor efficiently produced IL-5 in response to IL-2. In another donor, the number was one of four (data not shown). This data implies that the frequency of IL-5-producing iNKT cells may reach 25–40% of total CD4+ iNKT cells in individuals who have a higher number of IL-5-producing iNKT cell clones.

**IL-15 could replace IL-2 in mediating the IL-5 production**

To determine whether any cytokines other than IL-2 could also induce IL-5 production, representative IL-5-producing clones Kn.1, Kai.1, and Kai.2 were stimulated with IL-4, IL-7, IL-9, IL-15, IL-12, IL-3, or GM-CSF in the presence of iDCs. Among these cytokines examined, only IL-15 showed an IL-2-like potential to provoke the production of IL-5 from the iNKT cells (Fig. 1D). Of note, receptors for IL-4, IL-7, IL-9, and IL-15 share γ-chain of IL-2R referred to as the common γ-chain, whereas IL-15R also shares β-chain with IL-2R. This implies that the intermediate affinity IL-2R complex composed of the β- and γ-chains would mediate signals needed for IL-5 production. Of further interest, an addition of exogenous IL-5 induced a low but significant amount of IL-4 production. This raises a possibility that, at least in some clones, IL-5 produced by iNKT cells in response to IL-2 may subsequently trigger IL-4 production from the same cells in an autocrine fashion or from other iNKT cells in the close vicinity, thereby augmenting the ability of iNKT cells to polarize the Th cell toward Th2.

**Gene expression profile of iNKT cells responding to IL-2**

To further confirm that our observations represent a previously overlooked property of iNKT cells, we conducted a comprehensive gene expression analysis. An Affymetrix DNA microarray was applied to characterize the mRNA expression of four IL-5-producing clones Kn.1, Kn.2, Kai.2, and Nkj.2. The results showed that 43 genes were significantly up-regulated following IL-2 stimulation in the presence of iDCs (Table II). Most notably, *IL5* was identified as the gene with the highest increase of expression after stimulation (fold increase = 18.86). As the direct consequence of IL-2 stimulation, IL-2Rα (IL2RA) was ranked as the second (fold increase = 13.70) and IL-2Rγ (IL2RG) as the 40th (fold increase = 2.8). Furthermore, *IL13* was ranked as the third (fold increase = 11.4), whereas neither *IL4* nor *IFNG* was among the genes significantly up-regulated in the examined culture condition. The increased expression of *IL13* prompted us to measure the content of the encoded protein in the supernatant by using ELISA. Consistent with the microarray data, the IL-5-producing clones were found to secrete a large amount of IL-13 as well (Fig. 2).

These results indicate that the selective production of IL-5 and IL-13 in response to IL-2 could be a significant property of a subset of CD4+ iNKT cells.
mature DCs appeared to be more potent than iDCs. In contrast, regarding mature DCs may help further promote IL-5 production (Fig. 4).

Comparison of the ability to induce IL-5 between iDC and mature DCs. The ability of iDCs and mature DCs to induce IL-5 from CD4+ iNKT cells in the presence of IL-2 was compared. To obtain mature DCs, iDCs were further stimulated with TNF-α (10 ng/ml) or LPS (2 ng/ml) for 12 h (designated as TNF-α-DC and LPS-DC, respectively).

Representative IL-5 producing clones, Sk.1 and Oz, were stimulated with IL-2 (10 IU/ml) or αGC (100 ng/ml) using these DC populations. Similar results were obtained by both clones. Shown here is the representative data of Sk.1. Supernatants were collected after 48 h and IL-5/IL-4 production was measured by CBA.

Requirement of TCR-CD1d interaction for the selective IL-5 production by iNKT cells

We have further addressed whether the IL-5 production from the iNKT cell clones may be induced in the absence of iDCs. When we stimulated iNKT cells with exogenous IL-2 in the absence of iDCs, only a trace amount of IL-5 was detected (Fig. 3A), elucidating the requirement of iDCs. To determine whether iNKT cells would interact with iDCs via TCR or accessory molecules, we next cultured the iNKT cell clones with CD1d-transfected or mock-transfected HeLa cells and again examined the effect of IL-2. The results showed that CD1d-transfected cells could serve as efficient APCs for the IL-5 production induced by IL-2 (Fig. 3B), whereas mock-treated cells could not. To clarify whether IL-5 production after iNKT cell interaction with iDC also depends on CD1d, we examined the effect of the CD1d-blocking Ab (aCD1d59) on IL-5 production from the iNKT-iDC coculture. As shown in Fig. 3C, addition of the anti-CD1d Ab significantly reduced IL-5 production in response to IL-2. This indicates that TCR-CD1d interaction is critical for iNKT cell clones to produce IL-5, supporting the involvement of TCR signaling. Autoreactive iNKT cells are generally thought to recognize endogenous ligands loaded onto CD1d molecules. Therefore, we tried to stimulate iNKT cells with CD1d dimer (dimer X) loaded with iGb3, a recently identified endogenous ligand for iNKT cells (30, 31). However, loading iGb3 to dimer X was not successful in inducing IL-5 production. When cultured on a plastic plate precoated simply with unloaded dimer X, iNKT cells did not respond to IL-2 (data not shown). We also used TNF-α- or LPS-induced mature DCs as APCs for comparison with iDCs, assuming that up-regulated costimulatory molecules in mature DCs may help further promote IL-5 production (Fig. 4). Regarding αGC-induced IL-5 or IL-4 production, TNF-α-induced mature DCs appeared to be more potent than iDCs. In contrast, iDCs seemed as potent as mature DCs in the induction of IL-5 production by iNKT cells in the presence of IL-2, indicating that the IL-2-induced IL-5 response is not heavily influenced by the maturation state of DCs.

Suboptimal anti-CD3 stimulation mimics the effect of CD1d+ APCs for IL-5 induction from CD4+ iNKT clones in the presence of IL-2.

We have further addressed whether the IL-5 production from the iNKT cell clones may be induced in the absence of iDCs. When we stimulated iNKT cells with exogenous IL-2 in the absence of iDCs, only a trace amount of IL-5 was detected (Fig. 3A), elucidating the requirement of iDCs. To determine whether iNKT cells would interact with iDCs via TCR or accessory molecules, we next cultured the iNKT cell clones with CD1d-transfected or mock-transfected HeLa cells and again examined the effect of IL-2. The results showed that CD1d-transfected cells could serve as efficient APCs for the IL-5 production induced by IL-2 (Fig. 3B), whereas mock-treated cells could not. To clarify whether IL-5 production after iNKT cell interaction with iDC also depends on CD1d, we examined the effect of the CD1d-blocking Ab (aCD1d59) on IL-5 production from the iNKT-iDC coculture. As shown in Fig. 3C, addition of the anti-CD1d Ab significantly reduced IL-5 production in response to IL-2. This indicates that TCR-CD1d interaction is critical for iNKT cell clones to produce IL-5, supporting the involvement of TCR signaling. Autoreactive iNKT cells are generally thought to recognize endogenous ligands loaded onto CD1d molecules. Therefore, we tried to stimulate iNKT cells with CD1d dimer (dimer X) loaded with iGb3, a recently identified endogenous ligand for iNKT cells (30, 31). However, loading iGb3 to dimer X was not successful in inducing IL-5 production. When cultured on a plastic plate precoated simply with unloaded dimer X, iNKT cells did not respond to IL-2 (data not shown). We also used TNF-α- or LPS-induced mature DCs as APCs for comparison with iDCs, assuming that up-regulated costimulatory molecules in mature DCs may help further promote IL-5 production (Fig. 4). Regarding αGC-induced IL-5 or IL-4 production, TNF-α-induced mature DCs appeared to be more potent than iDCs. In contrast, iDCs seemed as potent as mature DCs in the induction of IL-5 production by iNKT cells in the presence of IL-2, indicating that the IL-2-induced IL-5 response is not heavily influenced by the maturation state of DCs.
FIGURE 6. iNKT/iDC coculture supernatant induced Th2 CD4 T cell differentiation in an IL-5-dependent way. Here, we stimulated naive CD4 T cells with plate bound-anti-CD3 mAb (10 µg/ml) and soluble anti-CD28 mAb (2 µg/ml) and evaluated the effect of adding IL-2-induced IL-5-enriched supernatant from iNKT/iDC coculture (Kn.2). The presence of IL-5 in IL-2 sup and its absence in IL-2 sup was confirmed by CBA before the assay. To evaluate the effect of IL-5 in the iNKT/iDC supernatant, assays were also conducted in the presence of anti-IL-5-neutralizing mAb. Seven days after culture, cells were harvested and intracellular cytokines (IL-4 and IFN-γ) were stained after 6 h PMA/ionomycin stimulation. A, Flow cytometry analysis of intracellular IL-4/IFN-γ staining of CD4+ T cells following CD3/CD28 stimulation. The numbers indicate the percentage of cells in the given quadrant. More IL-4-producing cells were generated in the presence of IL-2 sup compared with IL-2 sup. The number of IL-4-producing T cell was reduced when anti-IL-5 mAb was given. In contrast, the number of IFN-γ-producing cells remained unaffected. Shown here is a representative data of two separate experiments with consistent results. B, Effect of anti-IL-5 mAb on the induction of IL-4+ or IFN-γ+ T cells. The frequency of IL-4+ or IFN-γ-producing cells after culture with IL-2 sup was determined as in A. In the presence of anti-IL-5 mAb, IL-4-producing cells were significantly reduced (the mean frequency dropped to 4.3% from 5.76%) (**, p < 0.05, one-factor ANOVA), whereas the frequency IFN-γ-producing cells stayed the same (the mean frequency was 27.0% and 28.2%).

IL-5 from iNKT cells promotes Th2 differentiation of naive CD4+ T cells

Given that some CD4+ iNKT cells selectively produce IL-5, it is important to know whether the iNKT cells may actively modulate an immune response by producing IL-5. To clarify this point further, we have collected the supernatant from iNKT cell clones. Interestingly, the IL-5 response could be elicited by a suboptimal TCR and IL-2 signaling would cause IL-5 production from autoreactive iNKT cells.

Th2 cytokine deviation by IL-2 in BALB/c iNKT cells

The present results demonstrate that predominant production of IL-5, which has not been appreciated as an important property of iNKT cells, would characterize a proportion of autoreactive iNKT cell clones. Interestingly, the IL-5 response could be elicited by a weak TCR stimulus together with IL-2 or IL-15, which might occur during in vivo inflammatory reactions. However, one could argue that we might have seen an artifact arising from use of the iNKT cell clones that were repeatedly stimulated and expanded in vitro. To challenge this criticism, we examined the responsiveness of freshly separated mouse iNKT cells to CD1d+ iDCs in the presence of IL-2. At the beginning, we tried to reproduce the results by using fresh human iNKT cells, but the low frequency of iNKT cells (0.01–0.1% of the PBMC) precluded our attempt. Therefore, freshly isolated iNKT cells and CD11c+ DCs from...
and some TNF-α/H9251 by IL-2 stimulation. In contrast to IL-2, we propose that this selective IL-5 production could represent a physiological iNKT cell response.

BALB/c mice were cocultured in the presence of IL-2 and spleens pooled from six BALB/c mice of the same age by using αGC-loaded CD1d dimer X. iNKT cells were cocultured with magnetically isolated CD11c⁺ splenocytes and stimulated with IL-2 or αGC for 72 h. Cytokines in the supernatant were evaluated by CBA. Data represent mean cytokine concentration from triplicate samples with error bars indicating +SD. Shown here is the representative data from three separate experiments, which gave similar results. A, Cytokine values (picograms per milliliter). B, Cytokine induction evaluated by fold increase from the baseline.

FIGURE 7. Freshly isolated iNKT cells from BALB/c mice produced IL-5 in the presence of IL-2. iNKT cells were isolated from livers and spleens pooled from six BALB/c mice of the same age by using αGC loaded-CD1d dimer X. iNKT cells were cocultured with magnetically isolated CD11c⁺ splenocytes and stimulated with IL-2 or αGC for 72 h. Cytokines in the supernatant were evaluated by CBA. Data represent mean cytokine concentration from triplicate samples with error bars indicating +SD. Shown here is the representative data from three separate experiments, which gave similar results. A, Cytokine values (picograms per milliliter). B, Cytokine induction evaluated by fold increase from the baseline.

FIGURE 8. Increase of IL-5-producing iNKT cell by in vivo administration of IL-2. Liver lymphocyte were isolated after i.v. IL-2 administration and stained for intracellular IL-5 or IFN-γ production. The frequency of αGC-loaded CD1d dimer X⁺ TCRβ⁺ iNKT cells was plotted for IL-5- and IFN-γ-producing cells. Each dot represents data obtained from one mouse. Three mice were used for each condition. IL-5-producing iNKT cells were significantly increased in IL-2 i.v. mice (p < 0.05, Welch’s t test) though no significant difference was observed in IFN-γ-producing iNKT cells.

Discussion

The cardinal property of iNKT cells to produce regulatory cytokines has been well-documented in prior studies. It is, however, of note that the reagents used for inducing cytokines were either αGC, an unnatural ligand for iNKT cells, or anti-CD3 Ab in most cases. These reagents would transmit potent TCR signaling, thereby provoking production of a very wide range of Th1 and Th2 cytokines from iNKT cells. Therefore, it is still unclear as to which cytokines are naturally secreted and which are not involved in physiological immune regulation during the natural course of diseases. In this study, we have reported that some human CD4⁺ iNKT cell clones could selectively produce enormous amounts of IL-5 and IL-13 when cultured with CD1d⁺ APCs in the presence of IL-2 or IL-15. Importantly, the amount of IL-5 was even higher than that induced by αGC. Moreover, DNA microarray analysis identified IL5 and IL13 as the genes that were most highly up-regulated during their response to CD1d⁺ APCs in the presence of IL-2. Subsequent analysis showed that plate-bound anti-CD3 Ab could replace the CD1d⁺ APCs, suggesting that iNKT cells may exhibit the IL-5-producing function after recognizing an endogenous self-ligand expressed by APCs. Also, the IL-5-enriched supernatant from the iNKT cells showed a Th2-biasing effect in vitro, which could be blocked by neutralizing anti-IL-5 mAb. Finally, we showed that freshly isolated iNKT cells could also produce IL-5 in response to CD1d⁺ DCs in the presence of IL-2. These results indicate that a subset of CD4⁺ iNKT cells may use IL-5 and IL-13 as important mediators to regulate Th cell responses in vivo. It is likely that the iNKT cells producing these cytokines may play a decisive role in the control of Th1-mediated pathogenesis or mediating allergic conditions.

It has been well-recognized freshly isolated iNKT cells express activation markers such as CD69 even shortly after birth (23, 35). Together with the recent discovery that iGb3 could serve as an endogenous ligand for iNKT cells (30, 31), it is generally accepted that iNKT cells are autoreactive cells that are being constantly regulated during their response to CD1d⁺ APCs, suggesting that iNKT cells may inhibit the IL-5-producing function after recognizing an endogenous self-ligand expressed by APCs. Also, the IL-5-enriched supernatant from the iNKT cells showed a Th2-biasing effect in vitro, which could be blocked by neutralizing anti-IL-5 mAb. Finally, we showed that freshly isolated iNKT cells could also produce IL-5 in response to CD1d⁺ DCs in the presence of IL-2. These results indicate that a subset of CD4⁺ iNKT cells may use IL-5 and IL-13 as important mediators to regulate Th cell responses in vivo. It is likely that the iNKT cells producing these cytokines may play a decisive role in the control of Th1-mediated pathogenesis or mediating allergic conditions.

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in the presence of IL-2 as a readout. We assume that such a weak self-reactivity of the cells has hampered identifying this potentially important iNKT cell subset in prior studies.

Although we added rIL-2 and IL-15 exogenously to stimulate iNKT cells, these are the cytokines commonly produced in the inflammatory milieu. This allows us to speculate that the IL-5-producing iNKT cells might have a chance to encounter CD1d+ APCs in the presence of either of the cytokines, thereby playing an important role in the local control of inflammation. Interestingly, IL-15 blockade has recently been shown to prevent the induction of allergic airway inflammation (37), implicating indirect evidence for the presence of IL-15 in the site of airway inflammation. Although this study has not identified iNKT cells as a target of IL-15, the critical role of iNKT cells shown in other rodent studies (14, 15) and human asthma (38) supports the idea that local IL-15 may stimulate iNKT cells to produce IL-5 and IL-13, which then leads to augmentation of allergic inflammation involving activation of eosinophils (39, 40). Another point of interest is that the role of IL-2 has been indicated in Th2 polarization processes involving iNKT cells. Although this is most elegantly shown in the case of eradication of certain parasites (41, 42), it is possible that iNKT cells are the target of IL-2 for inducing Th2 polarization. It is also likely that iNKT cells in the inflammatory lesions of MS may produce IL-5 or IL-13 after being triggered by IL-2 or IL-15 in the inflammatory lesions. The IL-5 produced by iNKT cells may directly promote Th2 cell differentiation, thereby deviating Th1/Th2 balance toward Th2. Alternatively, Th2 polarization could be mediated by other cytokines that were triggered by IL-5 in an auto- or paracrine fashion. In fact, we showed that IL-4 was induced by the IL-5-producing iNKT cells in the presence of IL-5 (Fig. 1D) and overproduction of IL-4 from CD4+ iNKT cells could be demonstrated in the remission state of MS (33).

Recent reports have shown that striking Th1 responses against exogenous pathogens could be triggered by iNKT cells after recognizing an endogenous ligand in the presence of IL-12 (26, 31). Given its remarkable homology to the IL-5 response triggered by IL-2 reported here in this article, we speculate that stimulation with an endogenous ligand and locally produced cytokines is a fundamental mechanism that would lead iNKT cells to provoke a decisive response for dealing with infection, allergy, and autoimmunity. In infection models, iNKT cell recognition of iGb3 has been identified as an important trigger for inducing Th1 response by iNKT cells (31). However, iNKT cells may recognize different endogenous Ags (43) and therefore the microenvironment of different tissues or types of inflammation (e.g., Th1 vs Th2) encountered may be instrumental in determining the phenotype of iNKT cytokine production. In this regard, endogenous ligand(s) involved in IL-5 production by IL-2 is an area to be further investigated in the future.

It is important to realize that as much as 8 of the 26 CD4+ iNKT clones (Table I) have demonstrated a bias for IL-5 production following IL-2 stimulation. We speculate that this could arise from the heterogeneity of β-chain CDR3 sequence, although this point is another area that needs to be formally verified. Supportive of this speculation is the recent study showing that individual TCR β-chain may contribute to the variation of Ag recognition among iNKT cells (36). Another possibility is that these IL-5-producing iNKT cells may comprise a distinct lineage with the unique machinery to overproduce IL-5. It may also be due to differences in the frequency of IL-5-biased iNKT cells among each individual. This idea is supported by the observation that IL-5-biased clones tended to be generated from the same individuals (Table I). Furthermore, our preliminary data has demonstrated that IL-2-dependent production from iNKT cells might be subject to mouse strain differences: namely, the production of IL-5 from iNKT cells found in BALB/c could not be demonstrated in C57BL/6 mice (data not shown). This discrepancy between Th1 (C57BL/6) and Th2 (BALB/c) polarized mice may give us an important clue to further analyze this issue.

In summary, the present study has identified presence of human CD4+ iNKT cells that produce IL-5 and IL-13 in response to suboptimal TCR stimulation together with IL-2 or IL-15. Previous studies using αGC or anti-CD3 Ab for stimulation of iNKT cells was unsuccessful in revealing the identity and the unique property of these iNKT cells, reflecting the nonphysiological nature of the methods used for stimulating iNKT cells. Analysis of our data and studies from other groups suggest that this iNKT cell population produce IL-5 and IL-13 in vivo by recognizing an endogenous ligand. In the pathogenesis of allergy, autoimmune diseases or parasite infection, CD4+ iNKT may play a key role in deviating immune responses toward Th2 and thus provide a suitable target for immune intervention. Our results also imply that cytokines could play a major role in instructing the iNKT cell populations to respond differentially in vivo, whether it is beneficial or hazardous. Taking all these into consideration, we propose that sensing the presence of cytokines is probably one of the most fundamental abilities for the iNKT cells that are to be given only a weak TCR signal in vivo.

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Disclosures

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References


