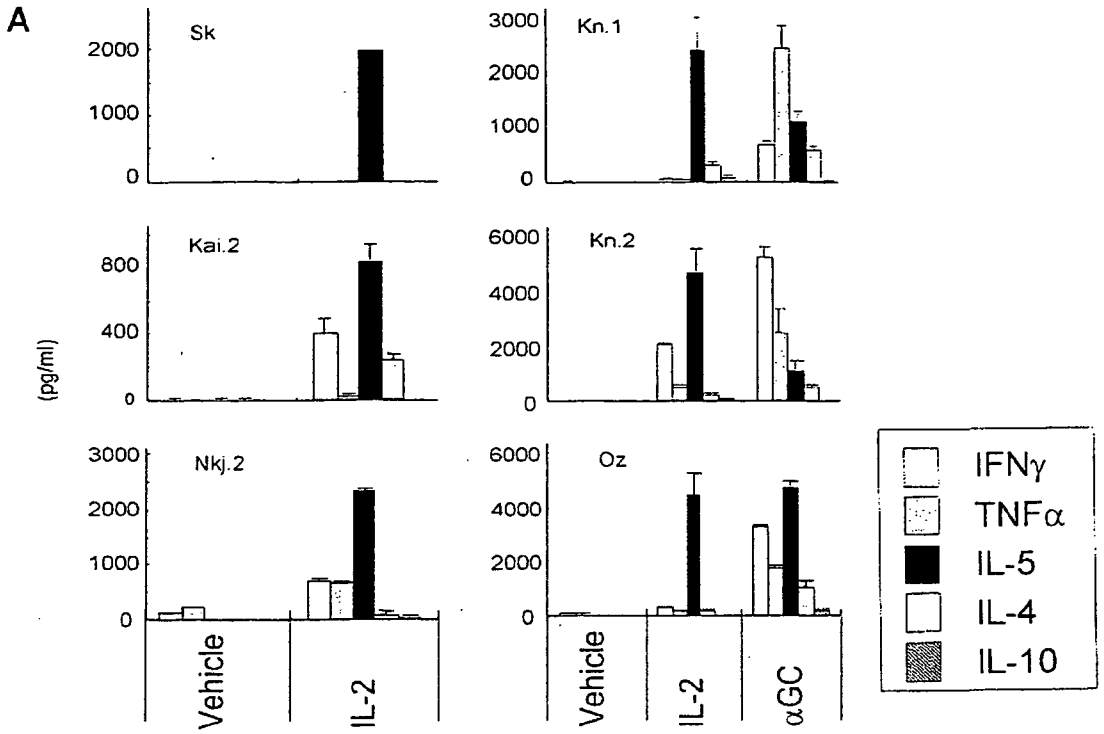
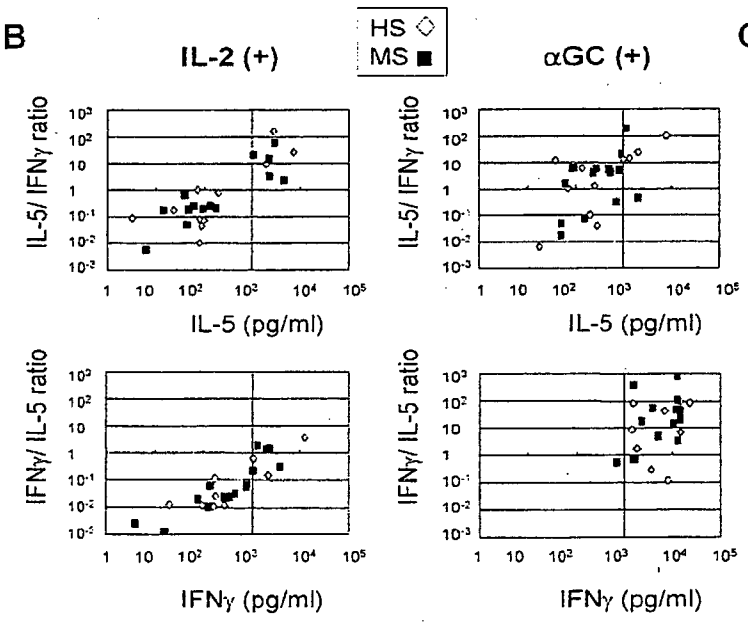
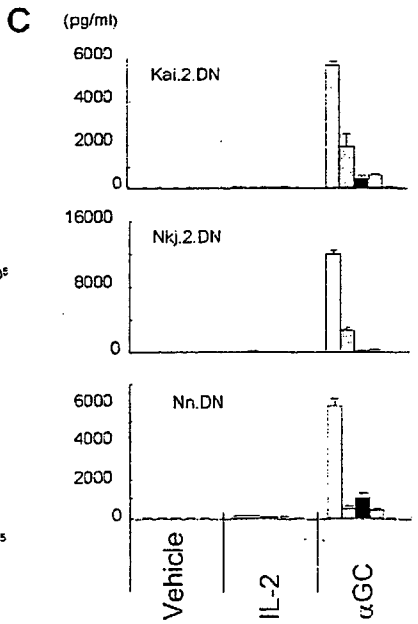
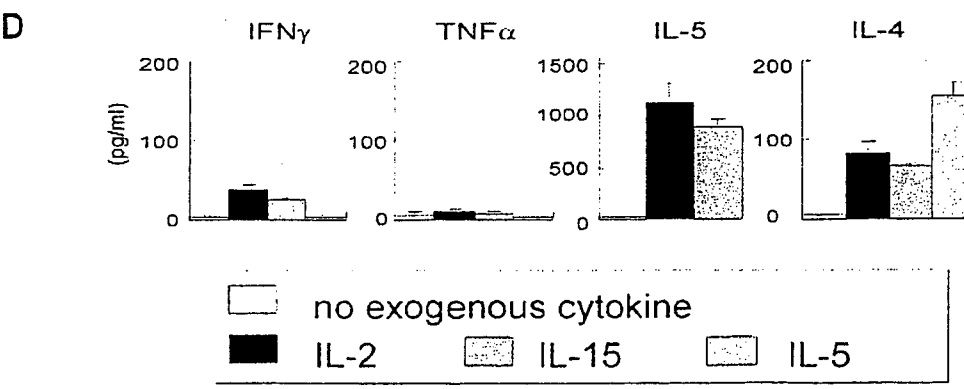

A
Sk
Kn.1
Kai.2
Kn.2
Nkj.2
Oz
(pg/ml)
Vehicle
IL-2
αGC
IFNγ
TNFα
IL-5
IL-4
IL-10


B
IL-2 (+)
αGC (+)
HS ◇
MS ■
IL-5/ IFNγ ratio
IFNγ/ IL-5 ratio
IL-5 (pg/ml)
IFNγ (pg/ml)


C
(pg/ml)
Kai.2.DN
Nkj.2.DN
Nn.DN
Vehicle
IL-2
αGC


D
IFNγ
TNFα
IL-5
IL-4
(pg/ml)
no exogenous cytokine
IL-2
IL-15
IL-5

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 αGC 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 αGC (100 ng/ml) in U-bottom 96-multiwell plates. Six hours later, they were washed and added 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 Cytometric 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) was 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-CD28 mAb (2 μg/ml) in AIM-V in the presence of iNKT/iDC 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 dimer (dimer X) with secondary staining by PE-conjugated rat anti-mouse IgG1. Then $TCR\beta^-$ αGC-loaded dimer X^+ cells were sorted by using the Altra 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 (1.5×10^4 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),

FIGURE 1. Production of IL-5 by human iNKT cell clones in the presence of exogenous IL-2. iNKT cell clones were cultured with the same number of allogenic iDCs (3×10^4 of each/well) in the presence or absence of exogenous IL-2 (10 IU/ml). The iDCs were preincubated for 6 h with αGC (100 ng/ml) or DMSO (vehicle) then washed before adding iNKT clones. After 48 h, concentrations of cytokines (IFN-γ, TNF-α, IL-5, IL-4, and IL-10) in the supernatant were determined by CBA. All data represent mean cytokine concentration from triplicate samples with error bars indicating −SD. *A*. Exogenous IL-2 induces IL-5 production from some iNKT cell clones. Shown are the representative experiments using two clones from HS (Sk and Kai.2) and four clones from MS (Kn.1, Kn.2, Oz, and Nkj.2). Clones in the *left panels* were stimulated with IL-2 alone, whereas those in the *right panels* were stimulated with IL-2 or αGC for comparison. *B*. IL-2 or αGC responses of each iNKT clone evaluated by production of IL-5 and IFN-γ. All $CD4^+$ iNKT cell clones from HS (○) or MS (■) were stimulated with IL-2 (*left panels*) or αGC (*right*), and the content of IL-5 and IFN-γ in the supernatant was measured by CBA. In each panel, results of individual clones are plotted according to the production of IL-5 (picograms per milliliter) vs IL-5-IFN-γ ratio (*upper panels*) or IFN-γ production vs IFN-γ-IL-5 ratio (*lower panels*). By conducting this analysis, we could identify a distinctive group of clones that produced high IL-5 in response to IL-2 and presented with a high IL-5-IFN-γ ratio (*left, upper panel*). *C*. DN iNKT cell clones respond to αGC but not to IL-2. $CD4^-CD8^-$ DN iNKT cells were derived from HS and MS in parallel with $CD4^-$ iNKT clones, and the assay was conducted exactly the same as $CD4^+$ iNKT cell clones. Their cytokine responses to IL-2 and αGC were compared. Shown are the data of two clones from HS (Kai.2, DN, and Nn.DN) and one from MS (Nkj.2.DN). The counterpart $CD4^-$ clones of Kai2.DN and Nkj2.DN produced IL-5 in response to IL-2 (*A*), whereas the Nn.DN counterpart did not (data not shown). Data represent mean cytokine concentration from triplicate samples and error bars indicate +SD. The same legend for cytokine is used as in *A*. *D*. IL-15 also stimulates IL-5 production from the clones responsive to IL-2. iNKT cell clones producing IL-5 in response to IL-2 were cultured with iDCs for 48 h in presence of IL-3, -4, -5, -7, -9, -12, -15, GM-CSF (10 ng/ml), or IL-2 (10 IU/ml). Cytokines in the coculture supernatant were measured by CBA. Experiments using three clones (Kn.1, Kai.1, and Kai.2) gave similar results. Shown here is the cytokine production induced by exogenous IL-2 (10 IU/ml) and IL-15 and IL-5 (10 ng/ml) by clone Kn.1. Note that IL-5 data for exogenous IL-5 (1716.4 pg/ml) is eliminated from the graph. Data represent mean cytokine concentration from triplicate samples with error bars indicating +SD.

instead of αGC to the coculture. 8 of the 26 clones produced an excessive amount of IL-5 (1500–7500 pg/ml; Fig. 1*A*; Table I). Remarkably, the level of IL-5 induced by IL-2 equaled or exceeded the amount that was induced by αGC (Fig. 1*A*, *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 or IFN-γ to IL-5 (vertical axis) vs quantities of IL-5 or IFN-γ in the supernatant (horizontal axis) (Fig. 1*B*). 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. 1*B*, *lower right panel*). A much wider range of IL-5 in quantity was produced after stimulation with IL-2 or αGC (Fig. 1*B*, *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. 1*B*, *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. 1*C*).

When we evaluated the profile of IL-5 and IFN-γ secretion (Fig. 1*B*), there was no noticeable difference between iNKT cell clones derived from HS (◇) 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 sphingosine 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 $V\alpha24^+V\beta11^+$ 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

Table II. *List of all genes significantly up-regulated after iDC and IL-2 stimulation*[a]

No.	Fold	Gene Name
1	18.86	**IL-5 (colony-stimulating factor, eosinophil)**
2	13.70	**IL-2R, α**
3	11.40	**IL-13**
4	9.40	**IL-17R B**
5	7.93	**Chemokine (CC motif) ligand 4**
6	6.79	**Granzyme A (CTL-associated serine esterase 3)**
7	6.76	**Matrix metalloproteinase 12 (macrophage elastase)**
8	6.13	HEG homolog
9	6.05	Pim-1 oncogene
10	5.57	**NK cell transcript 4**
11	5.47	Hypothetical protein MAC30
12	5.46	Protein tyrosine phosphatase, receptor type, K
13	5.31	Arginine-rich, mutated in early stage tumors
14	5.00	A disintegrin and metalloproteinase domain 19 (meltrin β)
15	4.91	Cyclin D2
16	4.88	Hepatoma-derived growth factor, related protein 3
17	4.61	β5-tubulin
18	4.47	**Chemokine (CC motif) receptor 2**
19	4.12	Suppressor of var1, 3-like 1 (*Saccharomyces cerevisiae*)
20	3.91	Sideroflexin 1
21	3.89	**CD48 Ag (B cell membrane protein)**
22	3.70	Phosphoglycerate kinase 1
23	3.63	Bromodomain adjacent to zinc finger domain, 1B
24	3.52	Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)
25	3.52	Karyopherin (importin) β1
26	3.46	Chromosome 4 open reading frame 9
27	3.45	Emopamil-binding protein (sterol isomerase)
28	3.37	Nucleoporin 50 kDa
29	3.35	Lactate dehydrogenase B
30	3.29	UDP-Gal:βGlcNAc β 1,4-galactosyltransferase, polypeptide 5
31	3.28	Proteasome (prosome, macropain) subunit, α type, 1
32	3.26	RAS guanyl-releasing protein 1 (calcium and DAG regulated)
33	3.20	Proteasome (prosome, macropain) subunit, α type, 1
34	3.17	Proteasome (prosome, macropain) activator subunit 2 (PA28 β)
35	3.17	Heat shock 60-kDa protein 1 (chaperonin)
36	3.00	Proteasome (prosome, macropain) activator subunit 1 (PA28 α)
37	2.94	Chaperonin containing TCP1, subunit 4 (δ)
38	2.86	Synaptotagmin XI
39	2.84	Proteasome (prosome, macropain) subunit, α type, 1
40	2.80	**IL-2R, γ (severe combined immunodeficiency)**
41	2.61	Polypyrimidine tract-binding protein 1
42	2.55	Polypyrimidine tract-binding protein 1
43	2.52	Ribosomal protein S4, X-linked

[a] $CD4^-$ iNKT cell clones were cocultured with allogenic iDCs in the presence or absence of exogenous IL-2. The iNKT cells were separated and examined by using DNA microarray. Listed are all the genes that were significantly up-regulated (paired *t* test, $p < 0.05$) in the iNKT clone cells by the presence of exogenous IL-2. The genes are listed in order by fold increase of control. Immune-related genes are highlighted in bold.

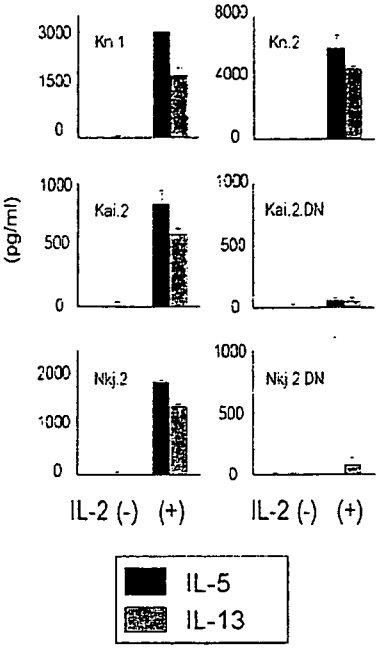


FIGURE 2. Exogenous IL-2 also triggered IL-13 production. $CD4^+$ iNKT cell clones Kn.1, Kai.2, Nkj.2, and Kn.2 as well as DN clones Kai.2. DN and Nkj.2. DN were stimulated with iDCs in the presence of IL-2. Supernatants were collected 48 h later and used for measurement of IL-5 (CBA) and -13 (ELISA). All of the data represent mean cytokine concentration from triplicate samples with error bars indicating + SD.

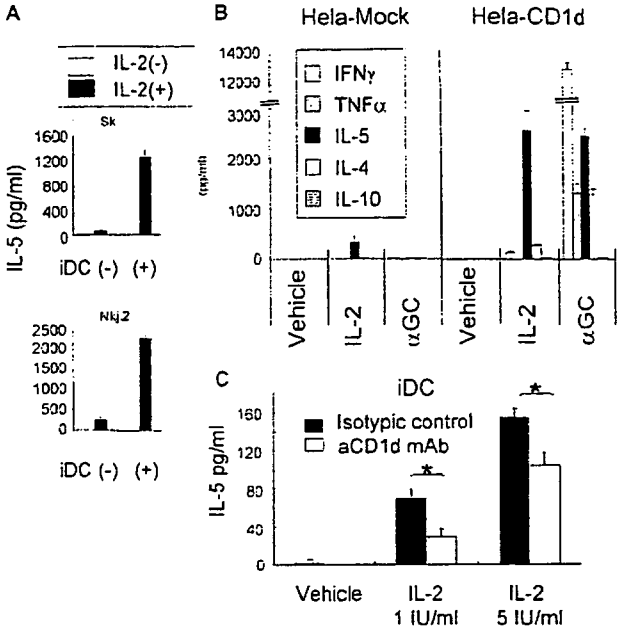


FIGURE 3. TCR-CD1d interaction was required for IL-2-mediated IL-5 production from iNKT clones. *A*. Presence of iDCs is critical for IL-5 production. $CD4^+$ iNKT clone cells Sk and Nkj.2 were cultured with or without iDCs in the presence or absence of IL-2 (10 IU/ml). Cytokines in the supernatant were measured by CBA after 48 h coculture. *B*. Comparison of CD1d- and Mock-transfected HeLa cells for IL-5 induction. The cytokine production of $CD4^+$ iNKT clone cells (Kn.1 and Kn.2) in response to IL-2 (10 IU/ml) and αGC (100 ng/ml) was evaluated by culturing them with CD1d-transfected or mock-transfected HeLa cells. For αGC stimulation, HeLa cells were loaded with αGC or DMSO (vehicle) for 12 h before incubation with iNKT cells. *C*. Suppression of IL-5 production by anti-CD1d blocking Ab. IL-5 production of $CD4^+$ iNKT clone cells (Kai.1 and Kk) in response to IL-2 stimuli (1 IU/ml, 5 IU/ml) was evaluated in the coculture with iDCs. To evaluate the involvement of CD1d, we added anti-CD1d mAb (aCD1d 59; 10 μg/ml) or isotype control Ab from the start of culture. Data represent mean cytokine concentration from triplicate samples with error bars indicating +SD. Although not indicated here, this blocking Ab significantly suppressed IFN-γ production induced by αGC stimulation by the same clones (*, $p < 0.01$, one-factor ANOVA).

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 cell clones may reach 25–40% of total $CD4^+$ iNKT cells in individuals who have a higher number of IL-5-producing iNKT cells.

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. 1*D*). 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 simulation (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.

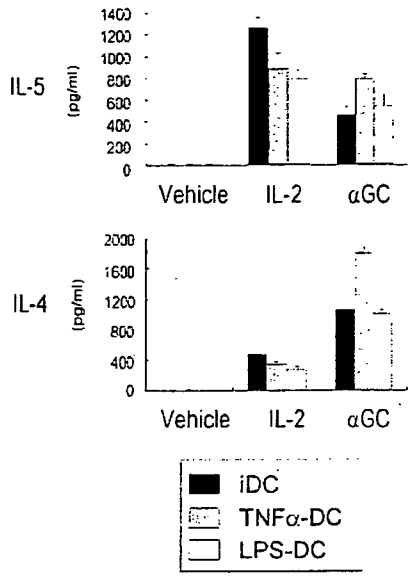


FIGURE 4. Comparison of the ability to induce IL-5 between iDC and mature DCs. The ability of iDCs and mature DCs to induce IL-5 from $CD4^+$ iNKT cells in the presence of IL-2 was compared. To obtain mature DCs, iDCs were further stimulated with TNF-α (10 ng/ml) or LPS (2 ng/ml) for 12 h (designated 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.

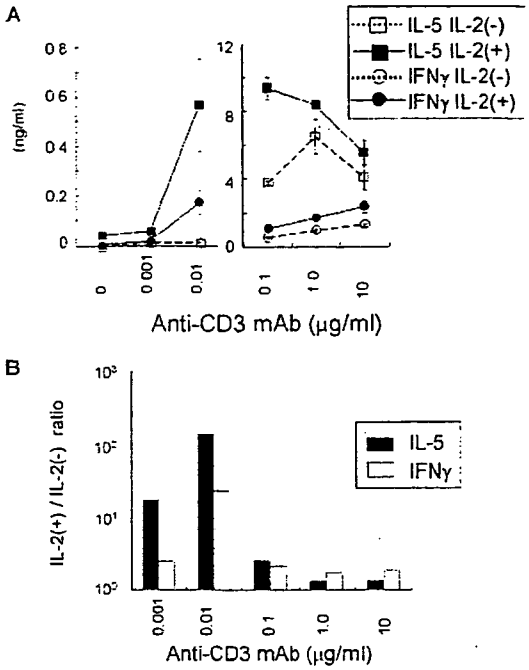


FIGURE 5. Suboptimal anti-CD3 stimulation mimics the effect of $CD1d^+$ APCs for IL-5 induction from $CD4^+$ iNKT clones in the presence of IL-2. $CD4^+$ iNKT cell clones Kn.2 and Kai.2 were stimulated by plate-bound anti-CD3 mAb (0.001–10 μg/ml) in the presence or absence of IL-2 (10 IU/ml). IL-5 and IFN-γ in the supernatant were measured by using ELISA. Clones Kn.2 and Kai.2 gave similar results. Shown are representative data obtained from Kn.2. Data represent mean cytokine concentration from triplicate samples and error bars indicate +SD. *A*. Production of IL-5 and IFN-γ when stimulated with various concentrations of plate bound anti-CD3 Ab. Horizontal axis indicates concentration of anti-CD3 mAb used (micrograms per milliliter), whereas amount of the cytokines (nanograms per milliliter) are shown in vertical axis. *B*. The effect of exogenous IL-2 on the cytokine values. To evaluate the augmenting effect of exogenous IL-2 on cytokine production, the amounts of cytokine produced at the presence of IL-2 were divided by those obtained at the absence of IL-2. This $IL\text{-}2^+\text{-}IL\text{-}2^-$ ratio was obtained using the data shown in *A*. Exogenous IL-2 induced IL-5 most efficiently and selectively when iNKT clones were stimulated at a suboptimal dose of anti-CD3 (0.01 μg/ml).

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. 3*A*), 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. 3*B*), 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. 3*C*, 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 response to 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 with exogenous IL-2 promotes IL-5 production

As demonstrated above, CD1d expression on APCs (Fig. 3*B*) as well as the presence of exogenous IL-2 is critically required for IL-5 induction from iNKT cells. Our speculation is that IL-5-producing iNKT cells are autoreactive to CD1d ligand, but they cannot mount the detectable IL-5 response unless accessory IL-2 signaling is provided. This is based on the premise that the endogenous ligand expressed by DCs could not provide sufficiently strong TCR signals able to provoke IL-5 production. To test this hypothesis, we explored whether suboptimal cross-linking of TCR in the presence of IL-2 could induce selective IL-5 production. We stimulated iNKT cells with plate-bound anti-CD3 mAb in the presence of IL-2. Using this APC-free system, we found that even without adding IL-2, the iNKT clone cells produce a large amount of IL-5 and somewhat a lesser amount of IFN-γ at higher concentrations of anti-CD3 mAb (0.1, 1.0, and 10 μg/ml)

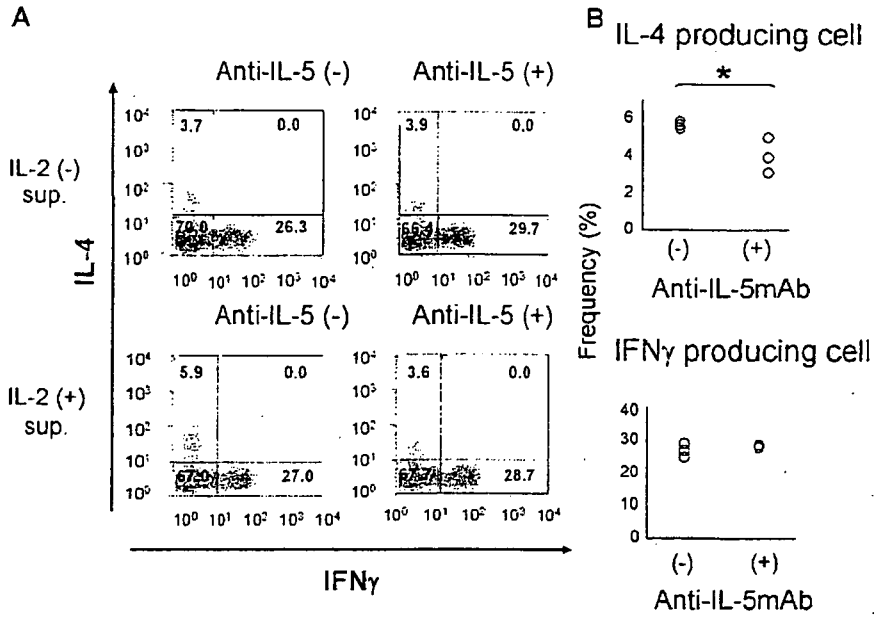


FIGURE 6. iNKT/iDC coculture supernatant induced Th2 CD4 T cell differentiation in an IL-5-dependent way. Here, we stimulated naive CD4 T cells with plate bound-anti-CD3 mAb (10 μg/ml) and soluble anti-CD28 mAb (2 μg/ml) and evaluated the effect of adding IL-2-induced IL-5-enriched supernatant from iNKT/iDC 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%).

(Fig. 5*A*). In this range of high Ab concentration, production of both IL-5 and IFN-γ was almost equally augmented by adding exogenous IL-2 (Fig. 5*B*), as judged by IL-2$^+$-IL-2$^-$ ratios. As expected, the lower concentration of anti-CD3 (0.001 and 0.01 μg/ml) without IL-2 induced only trace amounts of IL-5 or IFN-γ from iNKT cell clones. However, when exogenous IL-2 was added, the production of the cytokines was greatly augmented (Fig. 5*A*). In particular, IL-5 production at 0.01 μg/ml anti-CD3 was greatly and selectively induced. The IL-2$^+$-IL-2$^-$ ratio for IL-5 was 140.2, whereas that for IFN-γ was ~23.0 (Fig. 5*B*). As such, a suboptimal TCR stimulation with anti-CD3 could mimic the stimulation with CD1d$^+$ APCs, supporting our hypothesis that the suboptimal TCR and IL-2 signaling would cause IL-5 production from autoreactive iNKT cells.

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 cells that were stimulated with CD1d$^+$ iDCs and IL-2 (IL-2$^+$ sup), or from those stimulated with the iDCs alone (IL-2$^-$ sup). Then, we examined the effect of adding these supernatants on in vitro differentiation of naive T cells induced with plate-bound anti-CD3 mAb stimulation. After 7 days of culture with the supernatant, the percentage of IL-4- or IFN-γ-producing CD4$^+$ T cells was enumerated by intracellular cytokine staining. The results showed that the addition of IL-2$^+$ sup induced higher frequencies of IL-4$^+$CD4$^+$ T cells, as compared with IL-2$^-$ sup (5.9 vs 3.7% in Fig. 6*A*, *left*). In contrast, IFN-γ$^+$CD4$^+$ T cells did not differ significantly (27.0 vs 26.3%). Similar results were obtained in repeated experiments, indicating that IL-2$^+$ sup possess the biological activity to induce significant Th2 polarization of Th cells. To assess the role of IL-5 in the supernatant, we added neutralizing IL-5 Ab to the IL-2$^+$ sup and then evaluated its effect in the same culture system. The results showed that presence of anti-IL-5 significantly lowered the frequency of the IL-4$^+$CD4$^+$ T cells on day 7 (Fig. 6*B*, *upper*), whereas it did not change the percentage of IFN-γ$^+$ T cells (Fig. 6*B*, *upper*). Collectively, these results suggest that the IL-5 secreted by iNKT cells could contribute to mediating Th2 bias, although other factors such as IL-13 may also play some role.

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

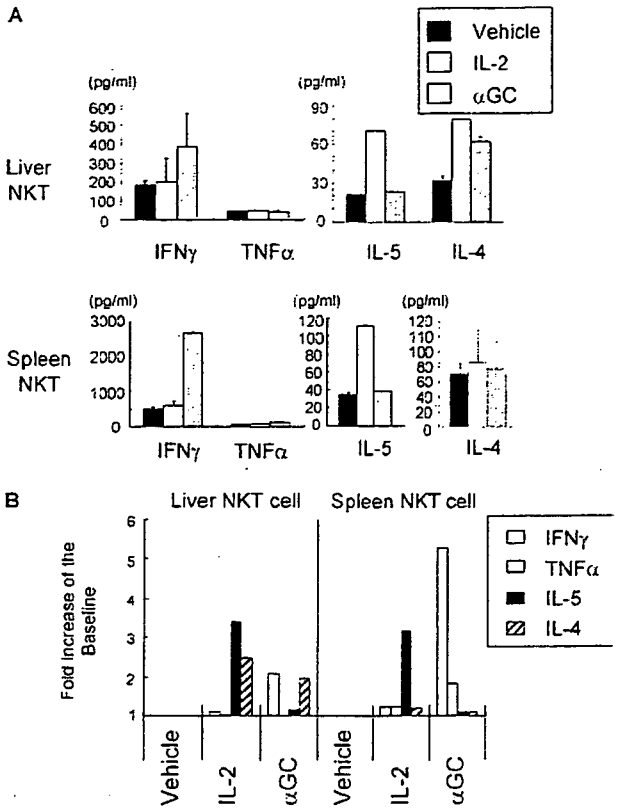


FIGURE 7. Freshly isolated iNKT cells from BALB/c mice produced IL-5 in the presence of IL-2. iNKT cells were isolated from livers and spleens pooled from six BALB/c mice of the same age by using αGC loaded-CD1d dimer X. iNKT cells were cocultured with magnetically isolated CD11c$^-$ splenocytes and 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.

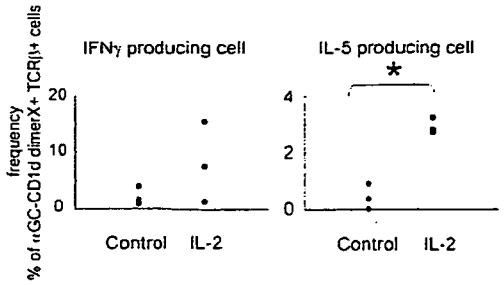


FIGURE 8. Increase of IL-5-producing iNKT cell by in vivo administration of IL-2. Liver lymphocyte were isolated after i.v. IL-2 administration and stained for intracellular IL-5 or IFN-γ production. The frequency of αGC-loaded CD1d dimer X$^+$ TCRβ$^+$ iNKT cells was plotted for IL-5- and IFN-γ-producing 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.

BALB/c mice were cocultured in the presence of IL-2 or αGC. Compared with human iNKT clone-iDC cocultures, the mouse cocultures showed a relatively high background response in any cytokine measured. However, as seen in humans, exogenous IL-2 apparently augmented the production of IL-5 from liver and spleen iNKT cells (Fig. 7), whereas neither IFN-γ nor TNF-α was altered by IL-2 stimulation. In contrast to IL-2, αGC stimulation induced a massive production of IFN-γ from liver and spleen iNKT cells and some TNF-α from spleen iNKT cells. However, IL-5 triggered by αGC was negligible.

To further convince ourselves of the significance of the physiological effect of IL-2 on iNKT cells, we investigated the effect of IL-2 on iNKT cells in vivo. We injected the cytokine i.v. to BALB/c mice. Two hours later, we separated liver lymphocytes and analyzed the intracellular expression of IL-5 by flow cytometry analysis. Consistent with the ex vivo IL-2 stimulation data, a significant increase in the number of IL-5-producing iNKT cells was observed in the BALB/c mice injected with IL-2, while a similar increase in the number of IFN-γ-producing iNKT cells could not be found (Fig. 8). These data imply that rodent iNKT cells are able to produce IL-5 ex vivo as well as in vivo in response to IL-2 as demonstrated in human iNKT clones. Taken together, we propose that this selective IL-5 production could represent a physiological iNKT cell response.

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 upregulated 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 stimulated by self-ligand(s). Consistent with this concept, studies have shown that a proportion of iNKT cell clones exhibit various degrees of self-reactivity as measured by proliferative responses against CD1d$^+$ APCs (29, 36). However, it has remained unclear what percentage of iNKT cells may exhibit such an autoreactive response as demonstrated by simple proliferation assays. In this regard, it is noteworthy that the self-reactivity of our iNKT cell clones could be demonstrated by using their ability to produce IL-5

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 autocrine 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. 1*D*) 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

The authors have no financial conflict of interest.

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Cross-Regulation between Type I and Type II NKT Cells in Regulating Tumor Immunity: A New Immunoregulatory Axis[1]

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Negative immunoregulation is a major barrier to successful cancer immunotherapy. The NKT cell is known to be one such regulator. In this study we explored the roles of and interaction between the classical type I NKT cell and the poorly understood type II NKT cell in the regulation of tumor immunity. Selective stimulation of type II NKT cells suppressed immunosurveillance, whereas stimulation of type I NKT cells protected against tumor growth even when responses were relatively skewed toward Th2 cytokines. When both were stimulated simultaneously, type II NKT cells appeared to suppress the activation in vitro and protective effect in vivo of type I NKT cells. In the absence of type I, suppression by type II NKT cells increased, suggesting that type I cells reduce the suppressive effect of type II NKT cells. Thus, in tumor immunity type I and type II NKT cells have opposite and counteractive roles and define a new immunoregulatory axis. Alteration of the balance between the protective type I and the suppressive type II NKT cell may be exploited for therapeutic intervention in cancer. ***The Journal of Immunology*****, 2007, 179: 5126–5136.**

To prevent diseases induced by autoimmune attack or to control collateral damage during an immune response, the immune system has developed many mechanisms of negative regulation. In the context of tumor immunity, the strict regulation of immune responses to maintain self-tolerance and prevent autoimmunity can represent a barrier to successful anti-tumor therapy. Because cancer vaccines to date have been able to induce robust T cell responses but only little clinical benefit (1), a greater understanding of these barriers and the discovery of ways to circumvent them may be necessary to finally realize the promise of cancer vaccines and immunotherapy. Recently, several cell types with suppressive function have been described such as the widely studied $CD4^+CD25^+$ T regulatory cell (2), M2 or tumor-associated macrophages (3–5), myeloid-derived suppressor cells (6–8), and the CD1d-restricted NKT cell (9–16). However, there is only limited information on the interaction between different regulatory cells (12, 17–19).

In this report we will propose a novel axis between regulatory subsets of NKT cells with opposite and counter-regulatory functions. In earlier studies, NKT cells appeared to have a paradoxical role in tumor immunity because they were shown to promote (20–23) as well as suppress (10–13, 16) tumor immunosurveillance. NKT cells are specialized T cells that recognize lipid Ags presented by CD1d molecules and also express NK cell receptors. Although the classical (or type I) NKT cell carrying an invariant TCR using the Vα14Jα18 TCR α-chain is the most prevalent and extensively studied, NKT cells are a heterogeneous population composed of different subsets (24). A less well-studied type of NKT cell, the so-called type II NKT cell with a more diverse TCR repertoire, has been identified in both humans and mice (25–29). Little is yet known about type II NKT cell physiologic function. Although a role for type II NKT cells has been documented in a transgenic model of hepatitis B virus infection (30), *Trypanosoma cruzi* infection (31), and experimental autoimmune encephalomyelitis (9) as well as in humans in the immunopathogenesis of ulcerative colitis (32), their role in tumor immunity is not known. Further, no interaction between the two types of NKT cells has been reported.

Although the range of actions attributed to date to NKT cells is extremely diverse and in some case conflicting, it is important to consider that not all of the studies discriminated between type I and type II NKT cells. We recently found that type II NKT cells were sufficient to suppress tumor immunosurveillance (12), but it was not clear whether type I NKT cells could also suppress as well as protect. In this study, to define the relative roles and potential interactions of these two classes of NKT cells, for the first time we compared their roles in the immune response against the same tumors by selectively stimulating them with specific ligands. We directly showed a role for type II NKT cells in suppressing tumor immunosurveillance, defining an important physiological function for the little-studied type II NKT cell. In contrast, type I NKT cells were directly involved in protection, indicating opposite roles for the two NKT cell subsets. Moreover when the two NKT cell populations were simultaneously stimulated, the effect of type I NKT cell activation was suppressed by type II NKT cell activation both in vitro and in vivo in two different tumor models, suggesting a counter-regulation between the two NKT cell subsets and identifying a potential new immunoregulatory axis.

Materials and Methods

Mice

Female BALB/c mice were purchased from Animal Production Colonies, Frederick Cancer Research Facility (National Institutes of Health, Frederick, MD). BALB/c CD1d-deficient mice (CD1dKO mice; provided by M. Grusby, Harvard University, Boston MA) and BALB/c Jα18-deficient

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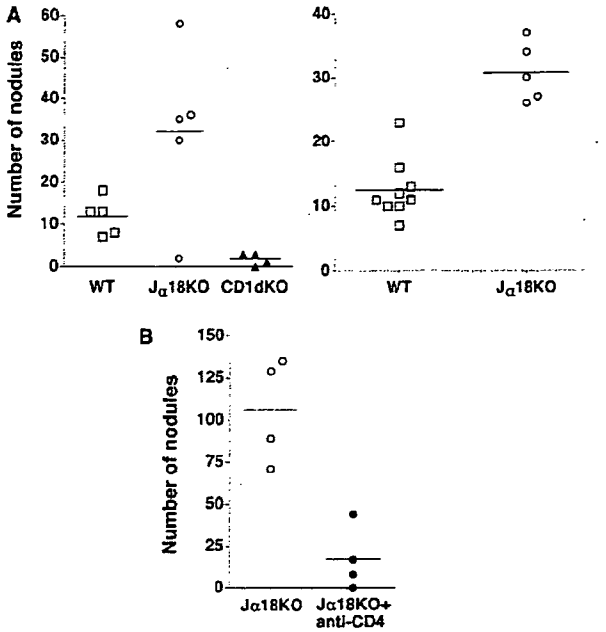

FIGURE 1. The presence of CD4$^-$ CD1d-restricted type II NKT cells down-regulates tumor immunosurveillance and the absence of type I NKT cells correlates with a higher susceptibility to tumor growth. *A*, On day 0 in two different experiments, 5×10^5 CT26 tumor cells were injected i.v. into BALB/c WT, Jα18KO, and CD1dKO mice. Eight days after tumor challenge the mice were sacrificed and the number of lung nodules was counted. At an early stage of tumor growth, Jα18KO (○) were more susceptible (*left panel*, $p = 0.04$; *right panel*, $p = 0.001$; Mann-Whitney test) and CD1dKO mice (▲) were less susceptible ($p = 0.02$; Mann-Whitney test) than WT mice (□). The experiment was repeated five times with similar results, and two representative experiments are shown. The horizontal bars indicate the means. *B*, On day 0, 5×10^5 CT26 tumor cells were injected i.v. into BALB/c Jα18KO mice. Anti-CD4 (1.5 mg; clone GK1.5) was injected i.p. on days −2, −1, 0, and 7. Mice were sacrificed 11 days after tumor challenge. CD4$^+$ T cell depletion (●) significantly decreased the number of tumor nodules in BALB/c Jα18KO mice ($p = 0.03$ against untreated WT mice; Mann-Whitney test). The Ab treatment was confirmed to result in >99% depletion of CD4$^+$ T cells by flow cytometry using a staining Ab that is not blocked by GK1.5. The results shown are representative of two similar experiments. The horizontal bars indicate the means.

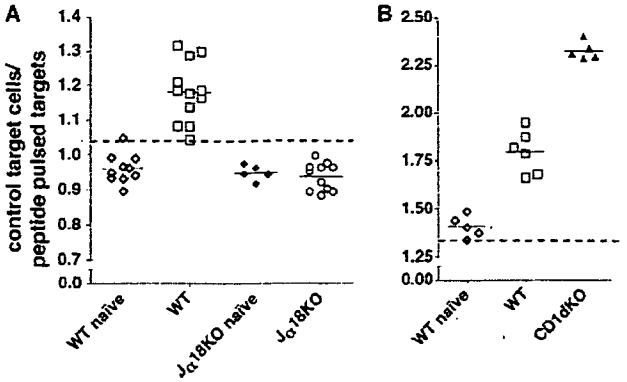

FIGURE 2. The presence of type II NKT cells but the absence of type I NKT cells correlates with a lower tumor-specific CTL response in vivo at an early stage of tumor growth. To examine the anti-tumor CTL response in BALB/c WT and Jα18KO mice at the same tumor stage as in Fig. 1, the in vivo CTL assay was performed. *A*, At this stage of tumor growth, tumor-challenged WT mice (□) were able to weakly but significantly respond against tumor-Ag pulsed target cells ($p = 0.0001$ against naive WT mice; Mann-Whitney test). In contrast, tumor-challenged Jα18KO mice (○), like naive WT (◇) and naive Jα18KO (◆) mice, showed no detectable specific CTL response against the same target. *B*, In a different experiment from that in *A*, to examine the anti-tumor CTL response in BALB/c CD1dKO mice at similar early time point, BALB/c CD1dKO and WT mice were compared by in vivo CTL assay. Tumor-challenged WT mice showed, again, a tumor-specific CTL response ($p = 0.004$ against naive WT mice; Mann-Whitney test) and BALB/c CD1dKO showed a strong tumor-Ag specific response ($p < 0.0001$ against naive and $p = 0.004$ against challenged WT mice; Mann-Whitney test). The horizontal bars indicate the means. The horizontal dashed line indicates the ratio of control target cells to specific target cells in the mixture injected into the mice. The experiments in *A* and *B* were repeated twice.

mice (Jα18KO mice; provided by M. Taniguchi, RIKEN Institute, Yokohama, Japan and by D. Umetsu, Harvard Medical School, Boston, MA) were bred at the National Cancer Institute (Bethesda, MD) under pathogen-free conditions. Female mice >6 wk of age were used for experiments. All experiments were approved by the National Cancer Institute's institutional animal care and use committee.

Tumor cell lines

The colon carcinoma CT26 cell line was maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, nonessential amino acids, sodium pyruvate, streptomycin and penicillin, and 2-ME (5×10^{-5} M). The fibrosarcoma line 15-12RM (33) was maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, nonessential amino acids, sodium pyruvate, streptomycin and penicillin, 2-ME (5×10^{-5} M), and 200 μg/ml G418.

Reagents

Synthetic α-galactosylceramide (αGalCer)[3] was provided by Kirin Brewery (Tokyo, Japan). For study, the stock solution was further diluted with PBS. Myelin-derived 3′-sulfogalactosylceramide (sulfatide) was purified (>90%) from a >98% pure sulfatide preparation from bovine brain (Matreya) as described earlier (9), dissolved in vehicle (0.5% polysorbate 20), and diluted with PBS. OCH was prepared as described by Oki et al. (34). Purified rat anti-mouse CD4 (clone GK1.5) was obtained from Harlan Bioproducts for Science. The AH1 peptide (SPSYVYHQF), a CTL epitope from murine leukemia virus gp70 expressed in CT26 tumor cell line and presented by H-2L^d (35), was synthesized by NeoMPS.

In vivo tumor assay

A single cell suspension of 5×10^5 CT26 cells or 1×10^6 15-12RM cells in 200 μl of PBS was injected i.v. or s.c., respectively, in mice. In the case of the CT26 lung metastases model, the determination of pulmonary metastasis was performed as previously described (13). In the 15-12RM tumor model, tumor size was measured periodically by caliper gauge.

In vivo Ab or glycolipid treatment

Purified rat anti-mouse CD4 was diluted to 1.5 mg in 200 μl of PBS and injected i.p. 2 consecutive days before tumor challenge, the day of challenge, and then 1 wk after the last injection. The glycolipids were diluted from the stock solutions at the desired concentration in PBS and administered i.p. αGalCer was administered at the concentration of 4 μg/mouse, and OCH was administered at the concentration of 2 μg/mouse (because this same concentration was reported to be effective in vivo in an autoimmune disease model (36)). When the sulfatide was titrated in vitro, we observed a very reproducible biphasic dose-response curve, typical for most T cell responses, with the maximum effect at 20–30 μg of sulfatide. In all of our experiments sulfatide showed an effect at a dose range of 20–30 μg/mouse in vivo or 20–30 μg/ml in vitro, so the amount of sulfatide used is therefore indicated as 25 ± 5 μg or μg/ml.

In vivo CTL assay

A single cell suspension of 5×10^7 naive spleen cells/ml was left unpulsed (control target cells) or pulsed with AH1 peptide (specific target cells) for 2 h at 37°C. After washing, spleen cells were then incubated with the fluorescent dye CFSE (Molecular Probes) at either 5 μM (specific target

[3] Abbreviations used in this paper: αGalCer, α-galactosylceramide; WT, wild type.

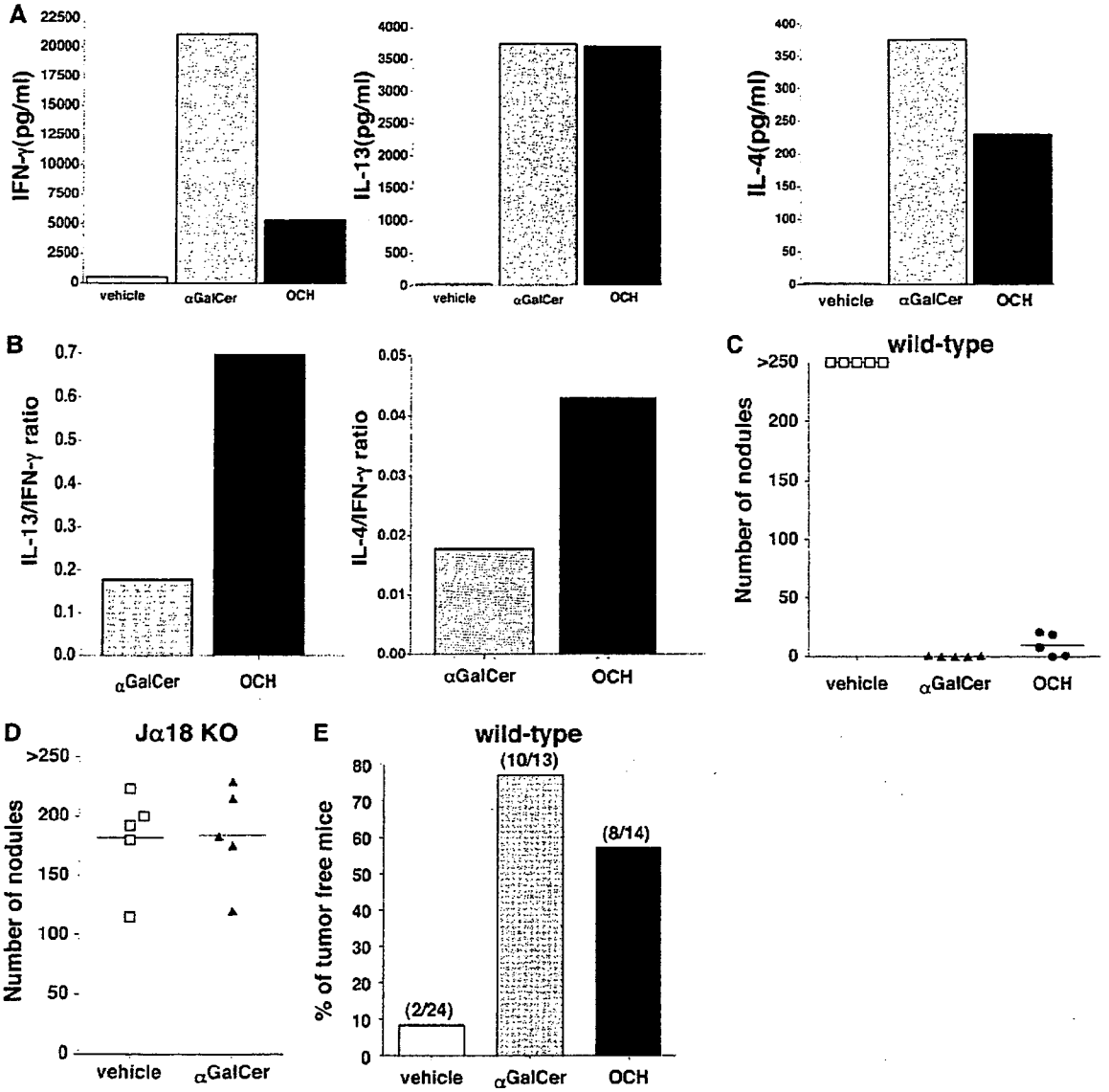

FIGURE 3. Stimulation of type 1 NKT cells protects mice from tumor growth. *A*. BALB/c spleen cells were stimulated in vitro with either 100 ng/ml αGalCer (gray bar) or 100 ng/ml OCH (filled bar), or vehicle (open bar). *A*. The amount of IFN-γ, IL-13, and IL-4 produced in vitro after 48 h of culture was examined by Luminex assay. *B*. The weak type 1 NKT cell agonist OCH induced a higher ratio of IL-13/IFN-γ and IL-4/IFN-γ secretion compared with αGalCer. *C* and *D*. The effect of type 1 NKT cell stimulation by either vehicle (□), αGalCer (▲) or OCH (●) on the CT26 in vivo tumor growth was investigated. In different experiments, BALB/c WT (*C*) and Jα18KO (*D*) mice were challenged with 5×10^5 CT26 cells i.v. and were injected i.p. with 4 μg per mouse of αGalCer or 2 μg per mouse of OCH on the same day. *C*. Two weeks after tumor challenge all of the mice were sacrificed and the number of lung nodules was counted. In WT mice, αGalCer treatment prevented CT26 growth ($p = 0.008$ against vehicle-treated WT mice; Mann-Whitney test), and OCH-treated mice were protected from CT26 tumor growth ($p = 0.008$ against vehicle-treated WT mice; Mann-Whitney test). *D*. CT26-challenged Jα18KO mice did not responded to αGalCer and were not protected from tumor growth. *E*. Protection by either αGalCer or OCH in the 15-12RM s.c. fibrosarcoma model. BALB/c WT mice were challenged s.c. with 1×10^6 15-12RM cells and, on the same day, were injected i.p. with vehicle (open bar), 4 μg per mouse of αGalCer (gray bar), or 2 μg per mouse of OCH (filled bar). The presence of a recurrent tumor was measured on day 50. WT mice were protected against recurrence of the 15-12RM fibrosarcoma by either αGalCer ($p < 0.0001$ against vehicle-treated WT mice; log rank test) or OCH ($p = 0.0006$ against vehicle-treated WT mice; log rank test). The numbers indicate the number of tumor-free mice in each group. The data presented are in a pool from three independent experiments.

cells) or 0.5 μM (control target cells) for 15 min at room temperature. Spleen cells were washed and then incubated for 30 min at room temperature in RPMI 1640 with10% FCS and then washed twice more. The two spleen cell populations were finally mixed together at a 1:1 ratio and injected i.v. into experimental mice. After ~18 h the mice were sacrificed and single-cell suspensions from their spleens were processed individually to evaluate the presence of 5 μM or 0.5 μM CFSE-labeled cells by flow cytometry (FACScalibur, BD Biosciences). The results are reported as ratio of the proportion of control target cells over the proportion of specific target cells left in each mouse (control target cells/peptide-pulsed targets).

In vitro cell activation and proliferation

A single-cell suspension of splenocytes was prepared from naive BALB/c mice. The cells were cultured at a density of 8×10^5/well in a 96-well plate in 200 μl of RPMI 1640 supplemented with 10% FCS, L-glutamine, non-essential amino acids, sodium pyruvate, streptomycin and penicillin and 2-ME (5×10^{-5} M). In some wells OCH (100 ng/ml), αGalCer (1–100 ng/ml), sulfatide (25 ± 5 μg/ml), sulfatide vehicle, or both αGalCer and sulfatide were added to the culture. Twenty-four, 48, and 72 h after the stimulation 100 μl of culture medium was collected from the wells and

stored at −70°C until cytokine measurement. In other experiments, $CD4^-$ T responder cells were purified by positive selection using autoMACS (Miltenyi Biotec) after staining with mouse anti-CD4 magnetic beads (Miltenyi Biotec). APC were obtained by the depletion of T cells using mouse anti-CD90 beads (non-T APC), incubated with αGalCer or sulfatide at 37°C for 2 h, and washed. $CD4^+$ cells (0.5×10^6) were incubated with 0.125×10^6 Ag-pulsed or vehicle-pulsed APC. Each APC population was pulsed with a single Ag. The final number of APC was maintained equal among the groups. In some experiments we tested the effect of medium from type II NKT cell-activated cultures. $CD4^-$ cells (0.5×10^6) were stimulated with 0.25×10^6 APC pulsed with different concentrations of sulfatide. Seventy-two hours later the supernatant was harvested and added at different final dilutions in cultures of $CD4^-$ cells stimulated with αGalCer-pulsed APC. To examine spleen cell or $CD4^-$ cell proliferation in vitro, 2.5 μCi/ml [^{3}H]thymidine was added during the final 8 h of a 72-h culture. At the end of the culture the [^{3}H]thymidine incorporation was evaluated with a MicroBeta counter (Wallac, PerkinElmer).

Cytokine assay

The concentration of IFN-γ, IL-4, IL-13, IL-10, or TNF-α in the culture supernatant (48 and 72 h long) or in the plasma samples was determined by a LINCOplex kit (Linco Research) using a Bio-Plex System (Bio-Rad) according to the manufacturer's instructions. The samples were analyzed in duplicate or triplicate, depending on the experiment.

Flow cytometry

Purified non-T APC were blocked with anti-CD16/CD32 (clone 2.4G2; BD Biosciences) and then stained with anti-mouse B220 (clone RA3-6B2), CD11c (clone N418), and CD11b (clone M1/70) Abs (all from eBioscience). For analysis of cell proliferation, total spleen cells were labeled with CFSE and cultured as indicated. At the end of the culture, the cells were blocked with anti-CD16/CD32 (clone 2.4G2; BD Biosciences) and then stained with Abs anti-mouse TCRβ, CD4, and CD1d-tetramer loaded with PBS57 (an αGalCer analog) (National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility, Atlanta, GA). For the visualization of type II NKT cells, spleen cells were incubated in a 96-well plate (0.8×10^6/well) with medium alone or sulfatide (25 ± 5 μg/ml) for 48 h. At the end of the incubation the cells were harvested, blocked with anti-CD16/CD32, and then stained with anti-mouse TCRβ and a sulfatide-loaded CD1d tetramer (9). All of the samples incubated with Abs were then washed and analyzed on a FACSCaliber flow cytometer by using CellQuest software (BD Biosciences) Flowjo (Tree Star).

Statistical analysis

The data were analyzed using the nonparametric Mann-Whitney or log rank test for in vivo data and Student's *t* test for in vitro data as indicated by using GraphPad Prism 4 software (version 4.0b; GraphPad Software). The data were considered significant at $p < 0.05$.

Results

The presence of $CD4^+$ CD1d-restricted non-Vα14Jα18$^-$ (type II) NKT cells down-regulates tumor immunosurveillance and the absence of type I NKT cells correlates with a higher susceptibility to tumor growth and a lower tumor Ag-specific cytotoxic response in vivo

We have previously reported that a $CD4^-$ CD1d-restricted NKT cell suppresses tumor immunosurveillance (10, 13) and that a CD1d-restricted type II NKT cell is sufficient for the down-regulation of tumor immunosurveillance (12). In contrast, several groups have reported that type I NKT cells can enhance tumor immunosurveillance (21–23). Therefore, we asked whether type I NKT cell-deficient Jα18KO mice, which retain type II NKT cells, have higher susceptibility in the CT26 lung metastasis model in which we observed a suppressive function of type II NKT cells. We previously showed no difference in the tumor growth between wild-type (WT) and Jα18KO mice, as both strains of mice developed >250 tumor nodules/mouse at a late stage of tumor growth in contrast to CD1dKO mice, which lack both subsets of NKT cells and were partially protected (12). Because the lung metastasis model loses sensitivity once the number of tumor nodules reaches the saturation level (>250 tumor nodules/mouse), we compared

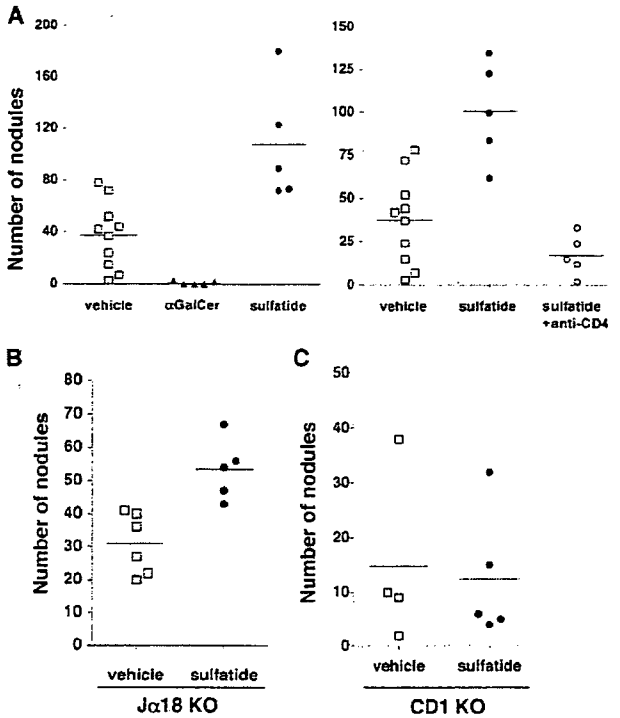

FIGURE 4. The activation of type II NKT cells in vivo enhances tumor development. *A*, BALB/c WT mice were challenged i.v. with 5×10^5 CT26 cells and, the same day, injected i.p. with the vehicle used to dissolve sulfatide (□), 4 μg of αGalCer (▲), or 25 ± 5 μg of sulfatide (●). Some mice that received sulfatide were also injected with 1.5 mg of anti-CD4 (clone GK1.5) two consecutive days before tumor challenge, the same day as tumor challenge, and 1 wk later (○). The number of tumor nodules in the lungs was monitored and when it reached ~50 in control WT mice all of the experimental mice were sacrificed and the number of nodules was determined. Sulfatide significantly increased the number of lung metastases compared with vehicle treated mice in two independent experiments shown of four with similar results (in both panels, $p = 0.03$ against vehicle-treated WT mice; Mann-Whitney test). αGalCer again protected the mice from tumor growth ($p = 0.0007$ against vehicle-treated WT mice; Mann-Whitney test). *B* and *C*, Jα18KO and CD1KO mice were challenged i.v. with 5×10^5 CT26 cells and, the same day, injected i.p. with the amount of vehicle used to dissolve sulfatide (□) or 25 ± 5 μg of sulfatide (●). Sulfatide significantly increased the number of lung metastases compared with vehicle-treated mice in Jα18KO mice ($p = 0.004$ against vehicle-treated mice; Mann-Whitney test). Sulfatide was not effective in CD1dKO mice.

the tumor growth in WT and Jα18KO mice at earlier stages of tumor growth. When the number of nodules reached ~15 in WT mice, we observed a greater number of CT26 lung nodules in Jα18KO mice than in WT mice (Fig. 1*A*, *right* and *left panels*). Thus, at a very early stage the absence of type I NKT cells but presence of type II NKT cells makes the mice more susceptible to tumor growth. Even at this early stage of tumor growth, NKT cell-deficient CD1dKO mouse were protected from tumor growth because they lack the suppressive type II NKT cell (Fig 1*A*, *left panel*).

Now we tested whether the immunosuppressive type II NKT cell is $CD4^+$ (Fig. 1*B*). Syngeneic BALB/c Jα18KO mice were challenged i.v. with CT26 tumor cells. The mice were depleted of $CD4^+$ T cells with an anti-CD4 mAb two consecutive days before, the same day and 1 wk after tumor challenge. The depletion of $CD4^+$ T cells protected Jα18KO mice from tumor growth. Because we previously reported that $CD4^+CD25^+$ T regulatory cells

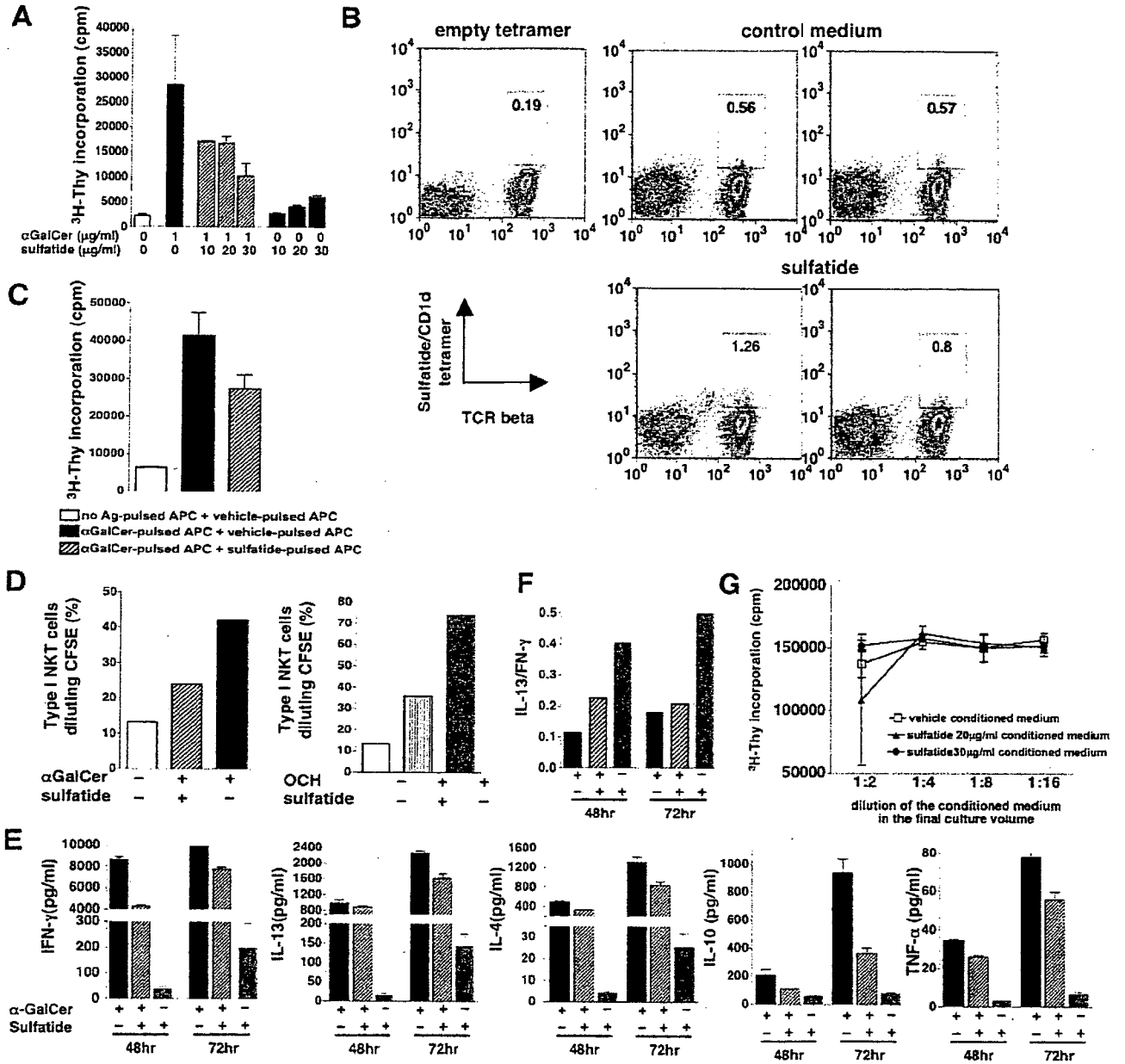

FIGURE 5. Stimulation of type II NKT cells suppresses type I NKT cell proliferation and cytokine secretion. *A*. Naive BALB/c spleen cells were stimulated for 72 h in vitro with αGalCer (1 ng/ml; filled bar), sulfatide (10, 20 or 30 μg/ml; gray bars), sulfatide vehicle (open bar), or both ligands (hatched bars; sulfatide was added 30 min after αGalCer, to avoid possible competition). The NKT cell agonist-induced proliferation was examined by [^{3}H]thymidine incorporation. Sulfatide induced a small but significant in vitro proliferation (with 30 μg/ml sulfatide, $p < 0.0001$ against unstimulated cells; Student's t test). The proliferative response induced by αGalCer stimulation was greatly suppressed when type II NKT cells were stimulated in the same culture (with 30 μg/ml sulfatide, $p = 0.03$ against αGalCer-stimulated cells; Student's t test). The experiment was repeated three times. *B*. Naive BALB/c spleen cells were stimulated for 48 h in vitro with sulfatide (25 ± 5 μg) or medium and then stained with a sulfatide-loaded CD1d tetramer to visualize sulfatide-reactive cells. In cultures stimulated with sulfatide (*lower panels*) the proportion of sulfatide-reactive type II NKT cells expanded ~2-fold (after subtracting background). (Note that in contrast to observations with type I NKT cells, staining of sulfatide-CD1d tetramer binding type II NKT cells with anti-TCRβ does not show intermediate level fluorescence, consistent with previous observations of TCR staining of such type II NKT cells (40, 47).) *C*. CD4$^+$ cells (0.5×10^6) were stimulated with 0.125×10^6 T cell-depleted APC. The T cell-depleted APC were mixtures of an equal number of vehicle-pulsed and unpulsed cells (open bar), an equal number of vehicle-pulsed and αGalCer-pulsed cells (50 ng/ml; filled bar), or an equal number of αGalCer-pulsed and sulfatide-pulsed cells (25 ± 5 μg/ml; hatched bar). When the cells were stimulated with both αGalCer and sulfatide-pulsed APC the proliferation was significantly lower than that of CD4$^+$ cells stimulated with only αGalCer-pulsed APC ($p = 0.03$ against αGalCer-stimulated cells; Student's t test). The experiment was repeated four times with similar results. *D*. Stimulation of type II NKT cells with sulfatide decreases the proportion of type I NKT cells undergoing proliferation after stimulation with αGalCer or OCH. Total spleen cells (0.8×10^6) were labeled with CFSE and then stimulated in vitro with 10 ng/ml αGalCer (filled bar, *left panel*) or 100 ng/ml OCH (gray bar, *right panel*), vehicle (open bars), or 25 ± 5 μg of sulfatide plus αGalCer (hatched bar, *left panel*) or OCH (straight lined bar, *right panel*). After 72 h, the cells were stained with αGalCer analog-CD1d tetramer and anti-TCRβ, and the gated population positive for both parameters was evaluated for the dilution of CFSE as a measure of proliferation. The combination of sulfatide and αGalCer or OCH reduced the proportion of αGalCer analog-CD1d tetramer-positive (type I NKT) cells diluting CFSE. The graphs are representative of two independent experiments. *E*. The pattern of cytokines secreted in culture supernatants under different conditions of NKT cell stimulation was examined

do not play a major role in the suppression of tumor immunosurveillance in this model (12), this result directly demonstrates that the CD1d-restricted type II NKT cell (present in Jα18KO mice and absent in CD1d KO mice: see Fig. 1*A*, *left panel*) responsible for the negative regulation of tumor immunosurveillance against the CT26 tumor is $CD4^-$.

At the same early tumor stage in which we observed a greater susceptibility of Jα18KO mice compared with WT mice to tumor growth, WT mice showed a weak but significant specific CTL response against tumor Ag-pulsed cells in contrast to Jα18KO mice, which did not show any tumor-specific killing (Fig. 2*A*). Although the weak cytotoxic response we observed in WT mice was not sufficient to protect them, nevertheless it likely accounts for their slightly lower susceptibility to tumor growth compared with Jα18KO mice, which completely lacked such a response. At this same time point CD1dKO mice, which were protected from tumor growth, showed a strong tumor-Ag specific cytotoxic response compared with both naive and challenged WT mice (Fig. 2*B*). These results suggest that type I NKT cells counteract the immunosuppressive function of type II NKT cells in tumor immunosurveillance and the inhibition of CTL activity and that the presence of type II NKT cells is sufficient for the negative regulation of tumor immunosurveillance by suppressing the $CD8^+$ T cell-dependent tumor rejection.

Stimulation of type I NKT cells protects from tumor growth

To better examine the function of type I NKT cells, we stimulated this cell population using type-specific agonists. Type I NKT cells can release large amounts of both Th1 and Th2 cytokines upon stimulation. It has been shown that the cytokine profile of activated type I NKT cells is different when stimulated with different Ags. α-GalCer (KRN7000), a strong agonist of type I NKT cells, has been reported to induce high IFN-γ and IL-4 (37) with a preferential release of Th1 cytokines. OCH, a weaker agonist of type I NKT cells, has been reported to induce a higher ratio of IL-4/IFN-γ than αGalCer, leading to a suppression of a Th1-mediated autoimmune disease, experimental autoimmune encephalomyelitis (34, 36, 38). Because we have reported that a Th2 cytokine, IL-13, plays a critical role in the down-regulation of tumor immunosurveillance by NKT cells (10, 13), to determine whether the final effect of type I NKT cell stimulation was due to preferential Th1 or Th2 cytokine induction, we stimulated type I NKT cells with either αGalCer or OCH. When spleen cells of tumor-challenged mice were cultured in vitro with either αGalCer or OCH, the latter induced a lower level of IFN-γ production and a higher ratio of IL-13/IFN-γ and IL-4/IFN-γ (Fig. 3, *A* and *B*) released in the supernatant than the former. To compare these two different stimulations of type I NKT cells in vivo, we treated mice with either αGalCer or OCH by using two different tumor models in which type II NKT cells suppress CTL-mediated tumor immunosurveillance (Fig. 3, *C–E*). WT mice received either 4 μg/mouse of αGalCer or 2 μg/mouse of OCH and were challenged the same day with either CT26 cells i.v. or 15-12RM cells s.c. Either αGalCer or OCH protected WT mice from CT26 tumor growth (Fig. 3*C*). As expected, αGalCer (Fig. 3*D*) and OCH (data not shown) did not show any effect in Jα18KO mice, which lack type I NKT cells. Also, both protected mice against recurrence of the 15-12RM fibrosarcoma (Fig. 3*E*). Thus, the in vivo stimulation of type I NKT cells protects in both of these tumor models, confirming a protective role of type I NKT cells in tumor immunosurveillance. The higher ratio of IL-13/IFN-γ released after OCH vs αGalCer stimulation of type I NKT cells may account for the slightly lower protection, although the levels of protection observed after OCH or αGalCer treatment were not statistically different. Nevertheless these findings suggest that, within the range of the cytokine profiles we could test, type I NKT cell stimulation protects against tumor development.

Type II NKT cell stimulation enhances tumor growth

Having demonstrated a protective role of type I NKT cells in tumor immunosurveillance, we examined the role of type II NKT cells. Because, at the moment, no marker is known to be specific for type II NKT cells and no mice selectively lacking type II NKT cells are available, we examined the role of this NKT subpopulation by selectively stimulating them in vivo. Only a few lipids specific for noninvariant NKT cells have been characterized. Among these, the myelin-derived glycolipid sulfatide (or 3′-sulfogalactosylceramide) has been reported to selectively stimulate a non-αGalCer-reactive CD1d-restricted NKT cell (9). It should be noted that the use of sulfatide as a selective stimulant for type II NKT cells, just like the widespread use of αGalCer as a selective stimulant for type I NKT cells, does not imply that these are the physiologic ligands for CD1d, as the latter is clearly not even a mammalian product. Thus, the doses and concentrations used were based on titrations to determine optimal dose (see *Materials and Methods*) and not on any evidence about physiologic concentrations, which do not apply. WT, Jα18KO, and CD1dKO mice were treated with sulfatide or with vehicle and were challenged with CT26 cells i.v. (Fig. 4). When WT mice had ~30–50 lung nodules, by which time it is too late to observe the difference between WT and Jα18KO mice, all of the experimental animals were sacrificed. Sulfatide significantly increased the number of tumor nodules in both WT (Fig. 4*A*) and Jα18KO mice (Fig. 4*B*), whereas in CD1dKO mice (Fig. 4*C*) sulfatide did not show any effect. Because Jα18KO mice lack type I NKT cells and CD1dKO mice lack both type I and type II NKT cells, type II NKT cells are necessary for the suppression of tumor immunosurveillance induced by sulfatide whereas type I NKT cells are not. Moreover, treatment with anti-CD4 mAb canceled the effect of sulfatide (Fig. 4*A*, *right panel*). These observations comparing WT, CD1dKO, and Jα18KO mice as well as CD4 depletion taken together directly showed that the activity of sulfatide is mediated by $CD4^+$ type II NKT cells and excluded nonspecific or toxic effects of sulfatide.

after 48 (*left three bars*) and 72 (*right three bars*) hours of culture. αGalCer was used at 1 ng/ml and sulfatide was used at 25 ± 5 μg/ml. The experiment was repeated twice with similar results. The simultaneous stimulation of type I and type II NKT cells (hatched bars) significantly suppressed the αGalCer-induced cytokine production (filled bars) except for IL-13 and IL-4 at 48 h ($p < 0.04$ against αGalCer-stimulated cells: Student's *t* test). *F*. The results from the first two panels in Fig. 5*E* are plotted as a ratio between IL-13 and IFN-γ induced by stimulation with αGalCer (filled bars), sulfatide (gray bars), or both αGalCer and sulfatide (hatched bars). *G*. $CD4^+$ cells (0.5×10^n) were stimulated with 0.25×10^6 T-depleted APC pulsed with αGalCer (50 ng/ml). In the same cultures, supernatants from 0.5×10^6 $CD4^+$ cells stimulated with vehicle (—) or sulfatide pulsed-APC (▲, 20 μg/ml; ●, 30 μg/ml) were added at different final dilutions (from 1/2 to 1/16 dilution). No difference in cell proliferation was significant. The experiment was repeated twice with similar results.

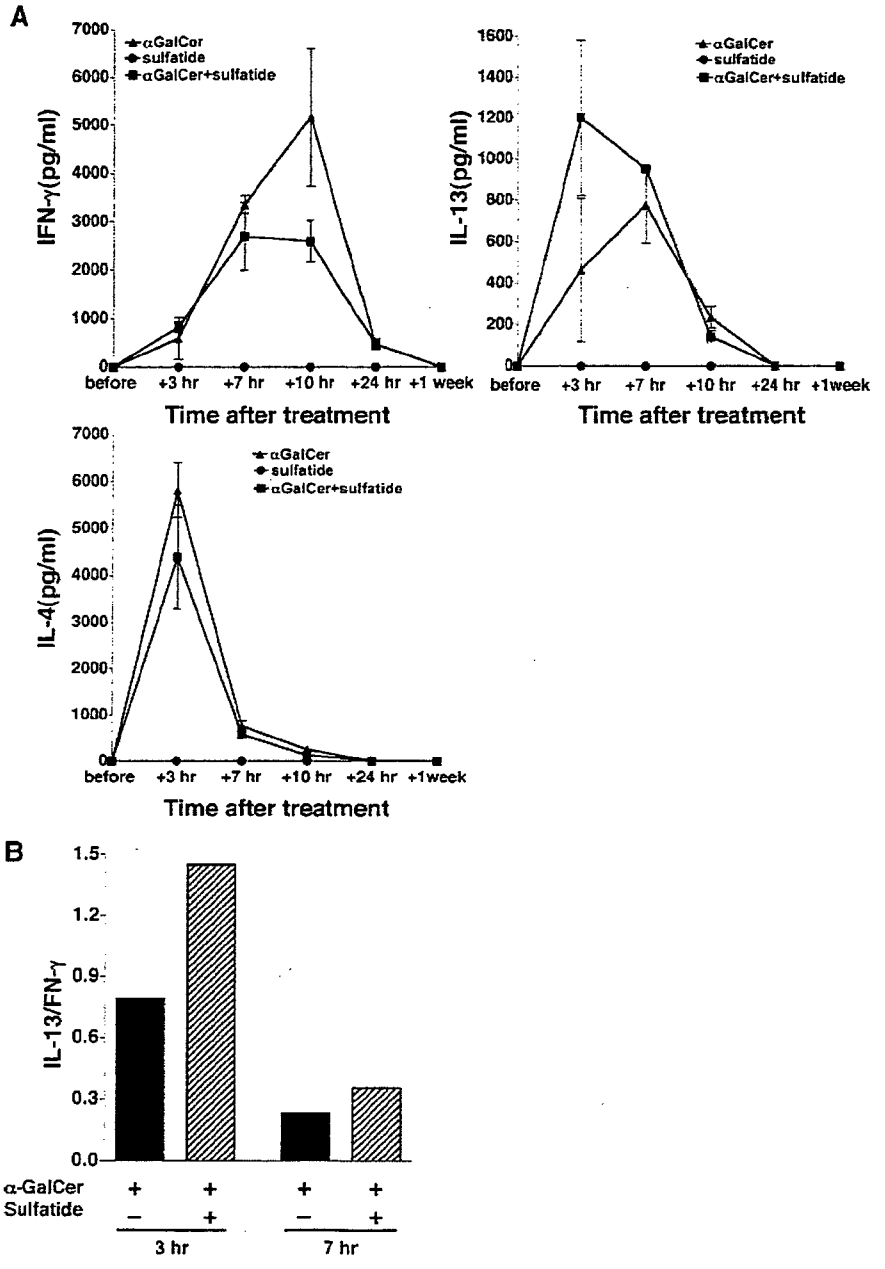

FIGURE 6. In vivo stimulation of type I and type II NKT cells increases IL-13 production and decreases IFN-γ production compared with the stimulation of type I NKT cells alone. *A*. BALB/c WT mice were injected i.p. with αGalCer (▲, 4 μg/mouse), sulfatide (●, 25 ± 5 μg/mouse), or the two NKT cell ligands simultaneously (■, sulfatide was injected 15–30 min later). The mice (three per group) were bled before treatment and 3, 7, 10, and 24 h and 1 wk after treatment. Plasma samples were collected and the presence of IFN-γ, IL-13, and IL-4 in the circulation was analyzed. αGalCer stimulated high levels of all three cytokines (▲). Sulfatide treatment (●) did not induce any detectable cytokine production, nor did vehicle (data not shown). Treatment with both αGalCer and sulfatide (■) reduced IFN-γ (*upper left panel*) and increased IL-13 (*upper right panel*) compared with αGalCer alone. IL-4 (*bottom panel*) was only weakly decreased 3 h after treatment by the combination. The results were not statistically significant but showed a trend between αGalCer alone and αGalCer plus sulfatide (for IFN-γ, $p = 0.19$ at 7 h and $p = 0.05$ at 10 h; for IL-13, $p = 0.11$ at 3 h and $p = 0.29$ at 7 h; for IL-4, $p = 0.25$ at 3 h; Student's t test), and the same trend of IFN-γ decrease, IL-13 increase, and weak change in IL-4 after αGalCer plus sulfatide treatment compared with αGalCer treatment alone was reproducible in two independent experiments. *B*. The results from the first two panels in Fig. 6*A* are plotted as a ratio between IL-13 and IFN-γ induced by stimulation with αGalCer (filled bars) or both αGalCer and sulfatide (hatched bars).

Stimulation of type II NKT cells suppresses the proliferation and cytokine production induced by type I NKT cell stimulation

The previous observations suggest opposite roles of type I and type II NKT cells. To examine whether the two NKT cell subpopulations can cross-talk and regulate each other, we stimulated them at the same time, first in vitro (Fig. 5). Total spleen cells from WT mice were stimulated in vitro with vehicle, type I NKT cell agonists (50 ng/ml αGalCer or 50 ng/ml OCH), the type II NKT cell agonist sulfatide (10, 20, 30 μg/ml), or each single type I NKT cell agonist and the sulfatide simultaneously (sulfatide was added 30 min later to avoid possible competition for CD1d molecules). αGalCer (Fig. 5*A*) and OCH (data not shown) stimulation induced a strong proliferative response, whereas the stimulation induced by sulfatide was weaker but significant and dose dependent. The weaker response to sulfatide can be explained by the weaker signal induced and by the 5-fold lower frequency of sulfatide-reactive cells vs αGalCer-reactive cells in the spleen (9). This also accounts for the need to use a higher concentration of sulfatide compared with αGalCer, to obtain an effect. To confirm that the type II NKT cells themselves are proliferating in response to sulfatide, we stained cells with a sulfatide-CD1d tetramer to enumerate type II NKT cells stimulated with medium alone or with 25 ± 5 μg/ml sulfatide for 48 h. The average number of sulfatide-specific cells increased ~2-fold in 48 h (after subtracting background) (Fig. 5*B*). Interestingly, when both stimuli were given concurrently the strong in vitro proliferation induced by αGalCer (Fig. 5*A*) or OCH (data not shown) was significantly suppressed. Similarly, by examining the proportion of αGalCer analog-CD1d tetramer-positive cells diluting the fluorescent dye CFSE, we observed significantly fewer type I NKT cells undergoing proliferation when sulfatide was added in cultures stimulated with αGalCer or OCH (Fig. 5*D*). This result confirms by direct staining that it is the type I NKT cell itself whose proliferation is

being inhibited rather than some bystander cells. To completely rule out any possible competition between αGalCer and sulfatide for CD1d molecules, purified $CD4^+$ responders were cultured with Ag-pulsed T-depleted APC, pulsed separately with either 50 ng/ml αGalCer alone or 25 ± 5 μg/ml sulfatide alone, and then mixed (Fig. 5*C*). Again, simultaneous stimulation of type II NKT cells suppressed type I NKT cell proliferation by αGalCer, although the degree of suppression was less than when soluble Ags were added and left in the culture. This may be due to the low affinity of sulfatide for CD1d (9) and some loss of sulfatide upon washing the pulsed cells. In vivo as well, the αGalCer- and OCH-induced expansion of type I NKT cells is suppressed when type II NKT cells are simultaneously activated by sulfatide (data not shown). Likewise, the strong cytokine induction by αGalCer was suppressed when sulfatide was added to the same spleen cell culture (Fig. 5*E*). Moreover, the higher IL-13/IFN-γ ratio produced by sulfatide-stimulated cells compared with αGalCer-stimulated cells (Fig. 5*F*) indicates a skewed cytokine profile of type II NKT cells compared with type I NKT cells.

To examine whether the down-regulation of type I NKT cell activation by type II NKT cells is mediated by soluble factors, $CD4^+$ responders cells were incubated with αGalCer-pulsed APC and different dilutions of conditioned medium obtained by stimulating $CD4^-$ responder cells with sulfatide-pulsed APC. At any of the dilutions tested, the conditioned medium from activated type II NKT cells did not suppress the proliferation of activated type I NKT cells (Fig. 5*G*). This result may suggest that the suppression of type I NKT cell activation by type II NKT cells is not or not only mediated by soluble factors.

Consistent with in vitro observations, simultaneous stimulation in vivo of both types of NKT cells with αGalCer (4 μg/mouse) and sulfatide (25 ± 5 μg/mouse) resulted in a trend (not quite statistically significant, $p = 0.05$ for IFN-γ and $p = 0.11$ for IL-13 at the peak time of difference for each; see Fig. 6 legend) toward lower IFN-γ and IL-4 levels in the plasma compared with the levels induced by αGalCer (Fig. 6*A*). Interestingly, the level of IL-13 was increased. Although the changes in the cytokine profiles were not statistically significant, a tendency toward a higher IL-13/IFN-γ ratio was observed in vivo after the simultaneous stimulation of type I and type II NKT cells (Fig. 6*B*). Thus, these in vivo cytokine data are consistent with the more compelling in vitro data discussed above and the in vivo tumor protection discussed below showing that type I and type II NKT cells have mutually opposing effects. The stimulation of type II NKT cells down-regulates type I NKT cell proliferation and cytokine production (especially IFN-γ) and increases IL-13 production, which is critical for NKT cell-mediated suppression (10, 13).

Simultaneous activation in vivo of type I and type II NKT cells suppresses type I NKT cell-mediated protection against tumors

Finally, we examined whether the counter-regulation of type I and type II NKT cells we showed by proliferation and cytokine production in vitro and in vivo had a clinical impact on the tumor growth in vivo (Fig. 7). Mice challenged with either 15-12RM cells s.c. or CT26 cells i.v., were treated with αGalCer (4 μg/mouse), sulfatide (25 ± 5 μg/mouse), both ligands simultaneously (sulfatide 30 min later than αGalCer to avoid any possible competition of sulfatide for the CD1d molecules, although neither is likely to saturate in vivo), or vehicle. Similar to the CT26 tumor model, in the 15-12RM tumor model (Fig. 7*A*) type II NKT stimulation made recurrence more rapid, although the difference was not statistically significant. In the 15-12RM tumor model, stimulation of both type I and type II NKT cells in vivo completely

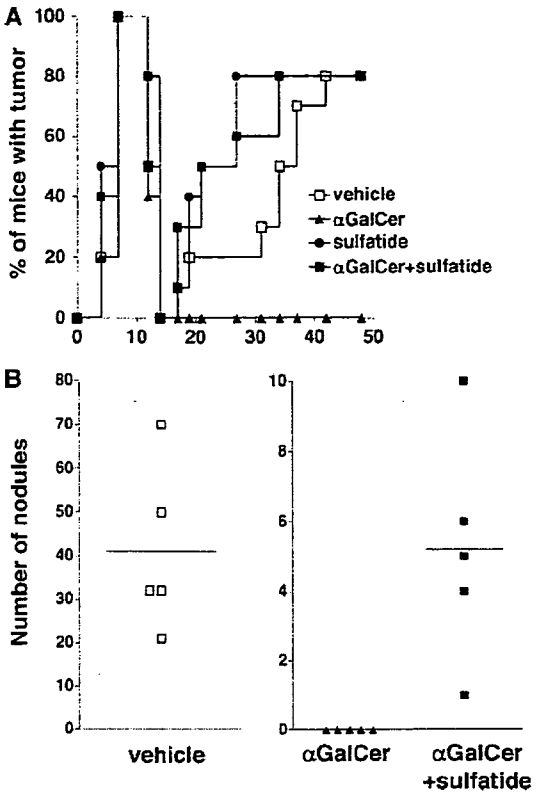

FIGURE 7. Simultaneous in vivo stimulation of type I and type II NKT cells suppresses αGalCer-induced tumor protection. BALB/c WT mice were challenged with 1×10^6 15-12RM cells s.c. (*A*) or 5×10^5 CT26 cells i.v. (*B*). Tumor-challenged WT mice were injected i.p. on the same day of tumor challenge with αGalCer (▲, 4 μg/mouse), sulfatide (●, 25 ± 5 μg/mouse), the vehicle used to dissolve the lipids (□), or the two NKT cell ligands simultaneously (■, sulfatide was injected 15–30 min later). *A*, In the 15-12RM tumor model the stimulation of type II NKT cells enhanced the tumor growth, although not significantly. Simultaneous stimulation of type I and type II NKT cells completely eliminated the protection induced by type I NKT cell stimulation alone ($p = 0.001$ against αGalCer-treated mice; log rank test). *B*, In the CT26 tumor model the costimulation of type I and type II NKT cells reduced the protection by type I NKT cell stimulation alone ($p = 0.008$ against αGalCer-treated mice; Mann-Whitney test).

abrogated the protection induced by type I NKT cell stimulation (Fig. 7*A*). Using the CT26 tumor model, a similar effect was seen. Although the number of tumor nodules in the mice treated with both αGalCer and sulfatide was still significantly lower than that of mice treated with vehicle, it was significantly higher than that of mice treated with αGalCer alone (Fig. 7*B*, *right panel*). Thus, the treatment with sulfatide significantly reduced the protection induced by αGalCer and, conversely, the αGalCer counteracted the suppression by sulfatide. These final results confirm the biological significance in vivo of the counter-regulation between type I and type II NKT cells observed in vitro and indicate that cross-regulation between the two cell populations in vivo can determine the clinical outcome in two different tumor models.

Discussion

In this study we have discovered the first evidence for cross-regulation between type I and type II NKT cells and have shown that this interaction can determine the clinical outcome in two murine malignancies. First, we directly showed that a $CD4^+$ CD1d-restricted type II NKT cell suppresses CTL-mediated tumor immunosurveillance, leading to tumor development. This supports our

previous reports showing that $CD4^-$ NKT cells are necessary for, or that type II NKT cells are sufficient for, the suppression of tumor immunosurveillance (10–13, 16). Type I NKT cells, in contrast, have been described in different models as enhancing tumor immunosurveillance (20–23). Consistent with those reports, we also showed that the lack of type I NKT cells accelerates tumor growth and the selective stimulation of type I NKT cells induces protection. In contrast, here for the first time we found that the stimulation of type II NKT cells (by sulfatide, which appears to act selectively through this NKT cell subset) enhances tumor growth. Furthermore, we found that the simultaneous activation of both type I and type II NKT cells results unexpectedly in a clear suppressive effect of type II NKT cells over type I NKT cell stimulation, with the down-regulation of type I NKT cell activation in vitro and diminished protection against tumors in vivo in two different tumor models. These findings identify a novel immunoregulatory axis between the two subsets of NKT cells with opposite functions.

Although little is still known about the physiological roles of type II NKT cells, recently several groups have succeeded in characterizing a role for this NKT cell subset in different immune responses, including infectious and autoimmune diseases (9, 30–32, 39). These studies raised attention to a little-studied NKT cell subpopulation, which can potentially play a role in a wider range of immune responses. However, in the context of tumor immunity, beyond our initial observation (12) there are no reports on the potential roles of type II NKT cells. In this study, for the first time, we directly investigated the activity of the little-studied type II NKT cell in the regulation of tumor immunosurveillance. Recently, Jahng et al. (9) characterized the myelin-derived lipid Ag sulfatide as a selective ligand for a proportion of the type II NKT cells. The in vivo activation of a non-αGalCer-reactive type II NKT cell with sulfatide suppressed pathological autoimmune responses in a murine model (9, 40). In our study, we took advantage of the activation of a proportion of type II NKT cells by sulfatide to study them in our tumor models. In both NKT cell-intact WT mice and type I NKT cell-deficient Jα18KO mice, treatment with sulfatide increased the number of lung nodules. Tumor growth in CD1dKO mice was not affected by sulfatide treatment, confirming that sulfatide is specifically activating type II NKT cells and is not exerting nonspecific effects or toxicity. Although we have not yet identified the specific type II NKT cell Ag in our tumor models, these findings suggest that the suppressive cells are sulfatide-reactive and their activation by sulfatide directly demonstrates their ability to down-regulate tumor immunosurveillance, although it does not imply that sulfatide is the physiologic ligand. Indeed, similarly αGalCer itself is not a physiologic type I NKT cell ligand and is not expressed in humans or other mammals at all, but it has been widely used a tool to study type I NKT cell activity. Sulfatide is the simplest member of a class of acidic glycolipids containing sulfate esters that are found in many tissues as well as many tumors (41, 42). In humans, different classes of CD1 molecules have been shown to present members of the sulfatide family (43). We plan to investigate whether any of these tumor-derived lipids are involved in type II NKT cell activation in our tumor models.

Over the past years many studies have investigated the role of NKT cells in tumor immunosurveillance, mainly focusing on type I NKT cells, and have implicated this cell population primarily in the promotion of tumor immunosurveillance (21–23). We previously reported that type I NKT cell-deficient Jα18KO mice would eventually develop lung nodules as well as WT mice (12). To examine the role of type I NKT cells over the course of tumor growth, we compared the tumor growth in their presence (WT mice) or absence (Jα18KO mice) at an early stage of tumor growth. At a very early time point, Jα18KO mice are more susceptible to tumor growth and have no CTL immune response to tumor Ag-pulsed cells in contrast to WT mice, indicating, in accordance with a previous study (21), that type I NKT cells contribute to the natural tumor immunosurveillance during early tumor growth. The observation of a weak anti-tumor CTL response in WT mice but not in Jα18KO mice was made at the same early tumor stage in which a higher susceptibility to tumor growth was observed in Jα18KO mice compared with WT mice. The two observations seems reasonably correlated; we therefore reason that such a weak immune response in WT mice; although too weak to mediate significant protection against tumor growth, is the result of less suppression of the immune system by type II NKT cells in the presence of type I NKT cells. To further examine the protective role of the type I NKT cell, we stimulated this population in vivo. In accordance with previous observations (20, 44), the in vivo activation of type I NKT cells with the strong agonist αGalCer completely protected the mice from tumor growth. Further, we found that the OCH analog of αGalCer, shown to preferentially induce Th2 cytokines in type I NKT cells and to suppress Th1-induced autoimmune disease (36, 45), strongly suppressed tumor growth as well. This suggests a protective role for type I NKT cells within the range of the cytokine profiles we could test. Although this result makes less likely a role of type I NKT cell-secreted Th2 cytokines in the suppression of tumor immunosurveillance, the OCH ligand does not completely skew the immune response toward Th2 and induces a reasonable level of IFN-γ production, although at lower levels compared with αGalCer. It would be of interest to further investigate the clinical effect of a complete Th2 skewing of type I NKT cell activation.

Finally, we investigated whether type I and type II NKT cells could potentially cross-talk when both cell populations were stimulated simultaneously. Although the stimulation with sulfatide is much weaker than the stimulation with αGalCer in terms of the induction of proliferation and cytokine release in vitro, surprisingly, when both types of NKT cells were stimulated at the same time the αGalCer-induced (Fig. 5, *A*, *C*, and *D*) or OCH-induced (Fig. 5*D*) proliferation was reduced in vitro and in vivo (E. Ambrosino, M. Terabe and J. A. Berzofsky unpublished observations). Moreover the αGalCer-induced cytokine production was reduced and skewed toward a higher IL-13/IFN-γ (Fig. 5*F*) ratio in vitro, and a similar trend was observed in vivo (Fig. 6*B*). The same counteractive effect was observed even when type II NKT cells were stimulated 15–30 min later than type I NKT cells or when APC were independently pulsed with αGalCer or sulfatide and then mixed (Fig. 5*C*), ruling out a possible competition by sulfatide for αGalCer binding to CD1d molecules or a direct antagonistic effect of sulfatide on the same cell as αGalCer upon the stimulation of type I NKT cells independently of type II NKT cells. The lesser degree of suppression in the culture in which APC were pulsed with αGalCer or sulfatide and then mixed to stimulate $CD4^+$ cells compared with that in the culture in which soluble Ags were added may be due to the lower affinity of sulfatide for CD1d molecules compared with αGalCer (9). Thus, it is unlikely that the suppression of type I NKT activation by type II NKT cells is a result of competition for CD1d binding. Most importantly, the clinical protective effect of αGalCer treatment was either reversed or reduced when sulfatide was coadministrated in vivo, in that the protection induced by type I NKT cell stimulation was partially or completely lost, depending on the tumor model (Fig. 7). In vivo, the expression of CD1d is so widespread that these molecules could not be anywhere near saturation by αGalCer or sulfatide at the doses administrated, again ruling out direct competition of

these ligands for CD1d. Also, we found no evidence of a nonspecific cytotoxic effect of sulfatide on APCs, type I NKT cells, or conventional T cells either in vitro or in vivo (data not shown) by evaluating cell numbers and proportions of the different populations (T cell subsets, B cells, myeloid dendritic cells, and plasmacytoid dendritic cells) remaining, their surface markers, and their propidium iodide staining after culture in sulfatide or vehicle. Also, the lack of effect of sulfatide in CD1dKO mice (Fig. 4*C*) or in mice depleted of $CD4^-$ T cells (Fig. 4*A*) excludes a nonspecific or toxic effect as the mechanism of tumor growth enhancement. For all of these reasons taken together we believe that sulfatide most likely acts directly on type II NKT cells, which recognize the sulfatide presented by CD1d, and, therefore, that it is the type II NKT cells that mediate the downstream effects; however, we cannot absolutely exclude more complex mechanisms involving other cells not tested in these studies.

In view of the central role of IL-13 in mediating the suppressive activity of NKT cells in tumor immunosurveillance (10, 11, 13, 46) in the tumor models used in this study, the tendency toward an increase in IL-13 secretion in vivo when type II NKT cells were simultaneously stimulated with type I NKT cells may contribute to the suppression of protection in mice treated with both αGalCer and sulfatide. The difference between the complete reversal of protection in the 15-12RM model and the partial reversal of protection in the CT26 model may relate to the greater sensitivity of the CT26 lung metastasis model to IFN-γ and NK cells activated by IFN-γ. Because the suppression of αGalCer-induced IFN-γ production by sulfatide was incomplete, the residual cytokine may be sufficient to partially protected in the lung metastasis model, but not in the s.c. 15-12RM tumor model. Nevertheless, the results demonstrate in two different models a novel suppressive effect of type II NKT cells on the ability of type I NKT cells to protect against cancer.

In conclusion, in this study we have defined a complex regulatory pathway of tumor immunosurveillance in which both subsets of NKT cells are involved and play opposite roles, forming a novel immunoregulatory axis. Furthermore, our data suggest a cross-talk between them, resulting in a counter-regulation of functions. Because we could not directly examine whether the interaction between type I and type II NKT cells occurs naturally in vivo, as for most studies, we had to stimulate the different cell populations to examine their activity. Nevertheless, our results clearly show that the described interaction has biologic significance in vivo in two different tumor models. At the moment we do not have detailed information about the mechanism through which type II NKT cells inhibit type I NKT cell activation. Also, we cannot distinguish a direct suppressive effect from one mediated through an intermediate cell such as a dendritic cell (47). The evidence that medium from type II NKT cell-activated cultures, when added to type I NKT cell-activated cultures, was not sufficient to inhibit their proliferation and that blocking soluble factors (such as IL-13 and TGFβ; E. Ambrosino, M. Terabe and J. A. Berzofsky unpublished observations) did not inhibit the suppressive activity of a type II NKT cell suggests that the mechanism of suppression could be by cell-to-cell contact rather than by soluble factors. Further studies will be performed to test either hypothesis. The final result is a balance between the type I and type II NKT cell activities regulating tumor immunosurveillance.

Because one of the mechanisms that may limit the effectiveness of immunotherapy of cancer is the active suppression of immune responses by lymphocytes, the blockade or elimination of these regulatory cells may represent a strategy for improving antitumor vaccines (48, 49). In this context, our studies suggest that the alteration of the balance between the protective type I and the suppressive type II NKT cell may be exploited for therapeutic intervention in cancer.

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Disclosures

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