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

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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.  


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, 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 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
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 × 10⁵ 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 × 10⁵ 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.

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\(^\circ\))\(^\circ\)) 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 (Matrey) as described earlier (9), dissolved in vehicle (0.5% polysorbate 20), and injected i.p. with PBS. OCH was prepared as described by Oki et al. (34). Purified rat anti-mouse CD4 (clone GK1.5) was obtained from Harlan Bio-products 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\(^\circ\) (35), was synthesized by NeoMPS.

In vivo tumor assay

A single cell suspension of 5 × 10⁵ CT26 cells or 1 × 10⁶ 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 metastasis 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 at 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 × 10⁵ 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

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1 Abbreviations used in this paper: αGalCer, α-galactosylceramide; WT, wild type.
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 with 10% FCS and then washed twice. 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 × 10⁻³ 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

![FIGURE 3](http://www.jimmunol.org/)

Stimulation of type I 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 I 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 I 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 respond 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.
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 αGal-Cer-pulsed APC. To examine spleen cell or CD4+ cell proliferation in vitro, 2.5 μg/μl [3H]thymidine was added during the final 8 h of a 72-h culture. At the end of the culture the [3H]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), 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 MHCM Tetramer Core Facility, Atlanta, GA). For the visualization of type II NKT cells, spleen cells were incubated in a 96-well plate (0.8 × 10^4/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 FACScalibur 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 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. 1A, 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 1A, left panel).

Now we tested whether the immunosuppressive type II NKT cell is CD4+ (Fig. 1B). 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 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.
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 [3H]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-TCR 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-TCR, 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. 1A, 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. 2A). 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. 2B). 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 α-GalCer, 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 α-GalCer or OCH. When spleen cells of tumor-challenged mice were cultured in vitro with either α-GalCer 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 α-GalCer 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 α-GalCer 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 α-GalCer or OCH protected WT mice from CT26 tumor growth (Fig. 3C). As expected, αGalCer (Fig. 3D) 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. 3E). 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. 4A) and Jα18KO mice (Fig. 4B), whereas in CD1dKO mice (Fig. 4C) 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. 4A, 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.
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. 5A) 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 μg/ml sulfatide for 48 h. The average number of sulfatide-specific cells increased 2-fold in 48 h (after subtracting background) (Fig. 5B). Interestingly, when both stimuli were given concurrently the strong in vitro proliferation induced by αGalCer (Fig. 5A) 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. 5D). 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. 5C). 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. 5E). Moreover, the higher IL-13/IFN-γ ratio produced by sulfatide-stimulated cells compared with αGalCer-stimulated cells (Fig. 5F) 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 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. 5G). 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. 6A). 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. 6B). 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, type II NKT cell 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 abrogated the protection induced by type I NKT cell stimulation (Fig. 7A). 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. 7B, 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-oGalCer-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 Jo18KO 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 oGalCer 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 as 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 Jo18KO 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 (Jo18KO mice) at an early stage of tumor growth. At a very early time point, Jo18KO 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 Jo18KO mice was made at the same early tumor stage in which a higher susceptibility to tumor growth was observed in Jo18KO 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 studied this population in vivo. In accordance with previous observations (20, 44), the in vivo activation of type I NKT cells with the strong agonist oGalCer completely protected the mice from tumor growth. Further, we found that the OCH analog of oGalCer, 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 oGalCer. 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 oGalCer 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 oGalCer-induced (Fig. 5, A, C, and D) or OCH-induced (Fig. 5D) proliferation was reduced in vitro and in vivo (E. Ambrosino, M. Terabe and J. A. Berzofsky unpublished observations). Moreover the oGalCer-induced cytokine production was reduced and skewed toward a higher IL-13/IFN-γ (Fig. 5F) ratio in vitro, and a similar trend was observed in vivo (Fig. 6B). 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 oGalCer or sulfatide and then mixed (Fig. 5C), ruling out a possible competition by sulfatide for oGalCer binding to CD1d molecules or a direct antagonistic effect of sulfatide on the same cell as oGalCer 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 oGalCer 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 oGalCer (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 oGalCer treatment was either reversed or reduced when sulfatide was coadministered 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 oGalCer 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. 4C) or in mice depleted of CD4+ T cells (Fig. 4A) 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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