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

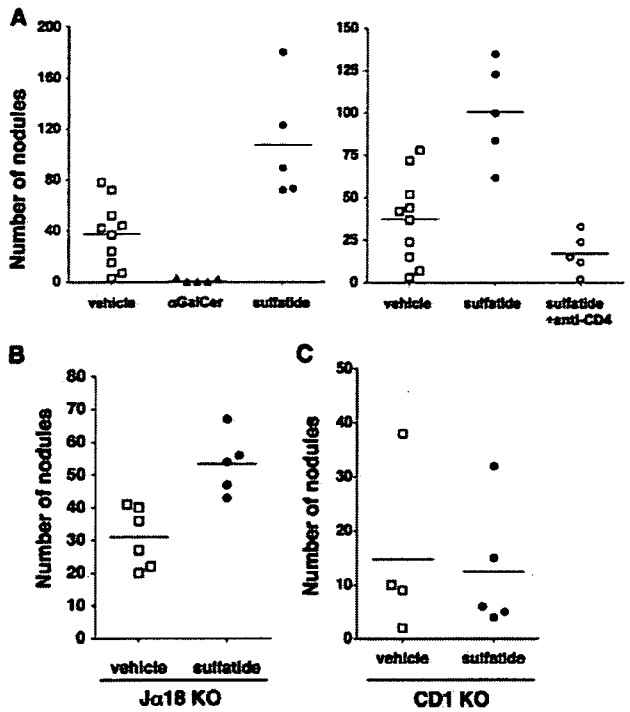

FIGURE 4. The activation of type II NKT cells in vivo enhances tumor development. *A*, BALB/c WT mice were challenged i.v. with 5×10^5 CT26 cells and, the same day, injected i.p. with the vehicle used to dissolve sulfatide (□), 4 μg of αGalCer (▲), or 25 ± 5 μg of sulfatide (●). Some mice that received 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

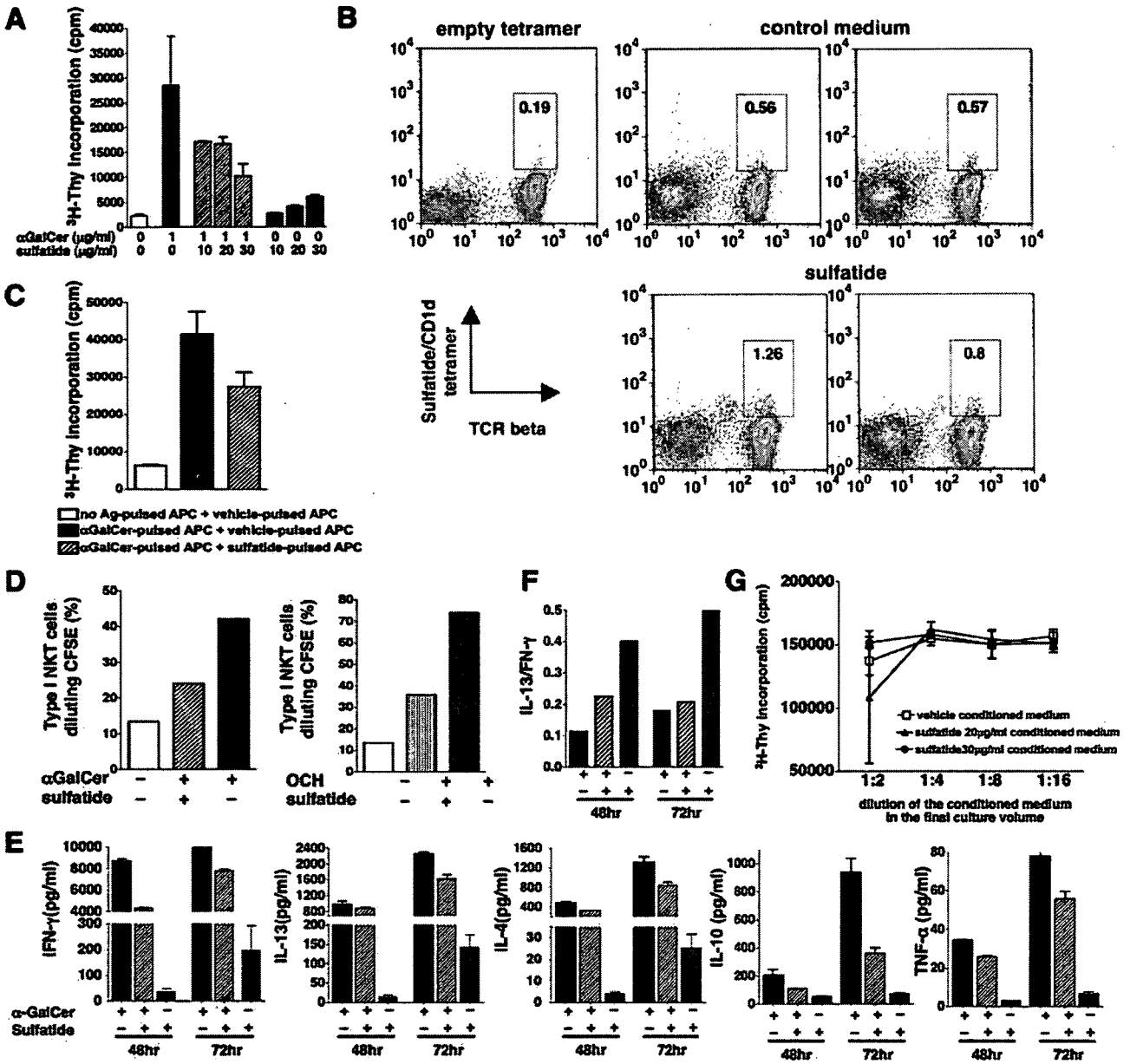

FIGURE 5. Stimulation of type II NKT cells suppresses type I NKT cell proliferation and cytokine secretion. *A*, Naive BALB/c spleen cells were stimulated for 72 h in vitro with αGalCer (1 ng/ml; filled bar), sulfatide (10, 20 or 30 μg/ml; gray bars), sulfatide vehicle (open bar), or both ligands (hatched 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 (0.5×10^6) 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.

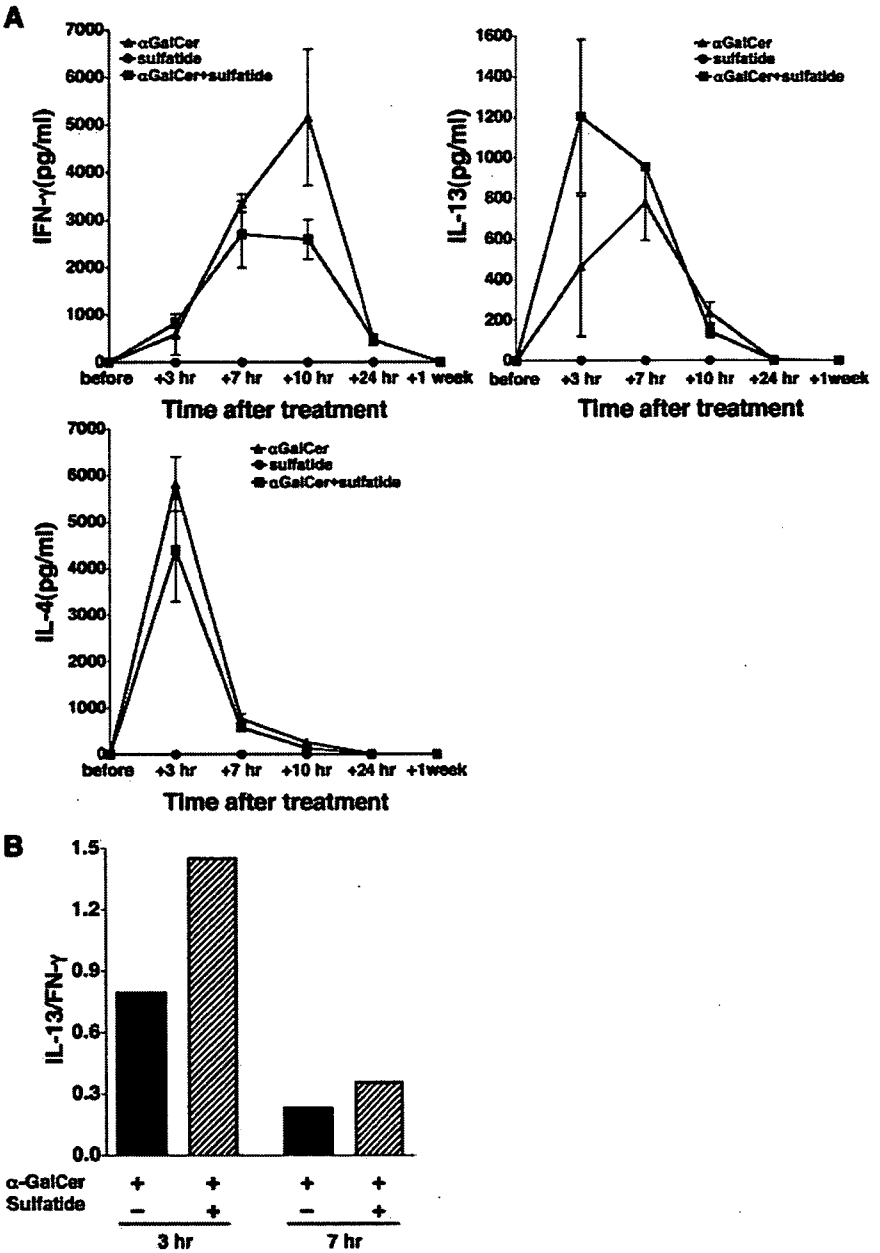

FIGURE 6. In vivo stimulation of type I and type II NKT cells increases IL-13 production and decreases IFN-γ production compared with the stimulation of type I NKT cells alone. *A*, BALB/c WT mice were injected i.p. with αGalCer (▲, 4 μg/mouse), sulfatide (●, 25 ± 5 μg/mouse), or the two NKT cell ligands 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

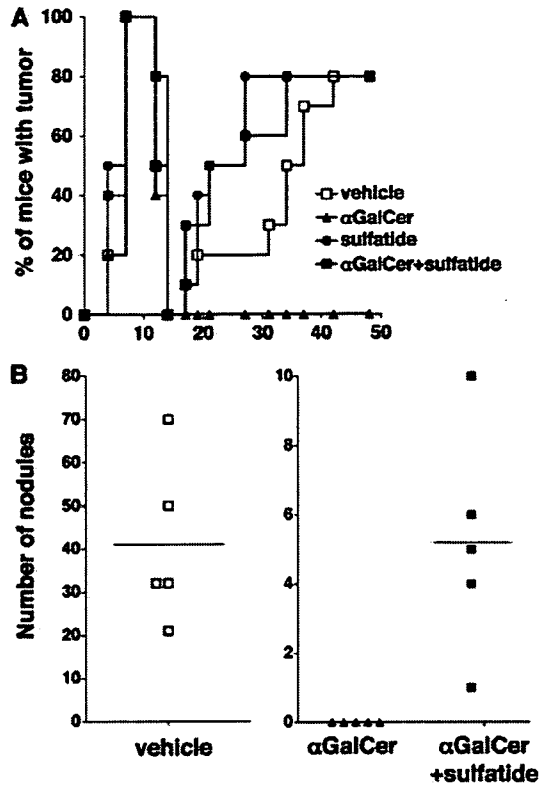

FIGURE 7. Simultaneous in vivo stimulation of type I and type II NKT cells suppresses αGalCer-induced tumor protection. BALB/c WT mice were challenged with 1×10^6 15-12RM cells s.c. (*A*) or 5×10^5 CT26 cells i.v. (*B*). Tumor-challenged WT mice were injected i.p. on the same day of tumor challenge with 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

The authors have no financial conflict of interest.

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Review article

Understanding the behavior of invariant NKT cells in autoimmune diseases

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Abstract

Invariant NKT (iNKT) cells are a unique subset of lymphocytes that recognize glycolipid antigens presented by a monomorphic glycoprotein CD1d. Numerous works have shown that iNKT cells may serve as regulatory cells in autoimmune diseases including multiple sclerosis (MS). However, recent studies have revealed that the presence of iNKT cells accelerates some inflammatory conditions, implying that their protective role against autoimmunity is not predetermined. Here we review recent information concerning the mechanism of how iNKT cells intervene or promote autoimmune inflammation. Although iNKT cells are thought to be specific for a limited set of glycolipids, they may cross-react to self and non-self ligands. Regarding the response to non-self, it is now known that iNKT cells produce enormous amounts of proinflammatory cytokines during the course of infectious diseases, which is triggered by TCR ligation by microbial lipids, cytokines produced from APCs or both. Whereas the strongly activated iNKT cells play a beneficial role in combating environmental pathogens, they could play a deleterious role in autoimmunity by producing disease-promoting cytokines. However, iNKT cells in the steady state would retain an ability to produce anti-inflammatory cytokines, which is needed for terminating the ongoing inflammation. Though an initial trigger for their regulatory responses remains elusive, our recent work indicates that iNKT cells may start regulating inflammation after sensing the presence of IL-2 in addition to recognizing a ubiquitous endogenous ligand. Understanding of how iNKT cells regulate autoimmunity should lead to a more sophisticated strategy for controlling autoimmune diseases.

Keywords: NKT cells; iNKT cells; Multiple sclerosis

Contents

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1. Introduction

Invariant NKT (iNKT) cells are regulatory T lymphocytes reactive to lipid antigen presented by a monomorphic glycoprotein CD1d (Bendelac et al., 2007; Kronenberg, 2005; Taniguchi et al., 2003). Many previous reports have documented that the number or function of iNKT cells is altered in patients with autoimmune diseases such as multiple sclerosis (MS) (Araki et al., 2003; Illés et al., 2000; Kojo et al., 2001; van der Vliet et al., 2001b; Wilson et al., 1998). Studies using transgenic mice that over- or under-express iNKT cells have basically supported the involvement of iNKT cells in the pathogenesis of autoimmune diseases (Miyake and Yamamura, 2007a). Moreover, stimulating iNKT cells with synthetic glycolipids has proven effective for preventing experimental autoimmune encephalomyelitis (EAE) (Miyamoto et al., 2001; Pál et al., 2001) or spontaneous type 1 diabetes (T1D) in NOD mice (Naumov et al., 2001; Sharif et al., 2001), indicating the important role of iNKT cells in controlling pathogenic autoreactivity and maintaining immune homeostasis (Miyake and Yamamura, 2007b). However, more recent studies have shown that iNKT cells may augment inflammatory conditions in models of arthritis (Chiba et al., 2005; Kim et al., 2005; Ohnishi et al., 2005), $CD8^+$ T cell-mediated diabetes (Griseri et al., 2005), experimental colitis (Ronet et al., 2005; Ueno et al., 2005) and airway hypersensitivity reactions (Akbari et al., 2003; Meyer et al., 2007). These results indicate that unlike $CD4^+$ $CD25^+$ regulatory T cells that appear to be a faithful regulator of unwanted immune responses (Sakaguchi and Sakaguchi, 2005), iNKT cells' help is only conditional and would occasionally take part in augmentation of harmful inflammation. How activation of iNKT cells manifests such opposing results and what is an initial trigger for the regulatory iNKT cell responses has remained to be unanswered. Here we review recent advances in the research of iNKT cells that may be relevant for understanding the "Janus-like" behavior of iNKT cells (Wilson and Delovitch, 2003). Our ultimate goal is to seek ways for making iNKT cells serve as a reliable guardian for our health.

2. General properties of iNKT cells

Although iNKT cells express T cell receptor (TCR) α- and β-chains, their TCR diversity is very limited owing to their expression of a single α-chain (Vα14-Jα18 in mice, Vα24-Jα18 in human) coupled with a β-chain rearranged with a limited Vβ gene segments (Vβ8.2, Vβ2 and Vβ7 in mice, Vβ11 in human). Unlike conventional T cells, they constitutively express memory/activated T cell phenotype and are capable of producing enormous amounts of pro- and anti-inflammatory cytokines shortly after TCR engagement (Bendelac et al., 2007; Kronenberg, 2005; Miyake and Yamamura, 2005; Taniguchi et al., 2003). The cytokine burst following iNKT cell activation then triggers a maturation process in downstream cells such as NK cells, dendritic cells (DCs), B cells and T cells, leading to subsequent alteration of a broad range of adaptive immune responses. It is widely accepted that they could behave very much like innate lymphocytes rather than conventional T cells (Mempel et al., 2002), and owing to the rapidity with which they respond to various stimuli, they play an important role in bridging innate and adaptive arms of immune response.

The ability of iNKT cells to produce regulatory cytokines is so outstanding that they could efficiently alter an adaptive immune response. Mouse iNKT cells can produce interferon-γ (IFN- γ), IL-2 (Jiang et al., 2005), -3 (Leite-de-Moraes et al., 2002), -4, -5, -13, -17, -21 (Coquet et al., 2007), GM-CSF (Leite-de-Moraes et al., 2002), and osteopontin (Diao et al., 2004) after an optimal engagement of TCR. However, it does not mean that iNKT cells would purposefully use all the listed cytokines. In fact, it can be assumed that except for extreme conditions (like stimulation with strong agonists), iNKT cells may produce only a set of Th1 or Th2 cytokines in physiological conditions. We support this postulate because the TCR engagement by an endogenous ligand is likely to be modest or suboptimal in most situations (Sakuishi et al., 2007). With regard to their role in balancing immune homeostasis, an organized production of Th1, Th2 or Th17 cytokines is probably required for iNKT cells to conduct meaningful jobs.

3. Exogenous glycolipids stimulatory for iNKT cells

Since a marine sponge-derived glycosphingolipid, α-galactosylceramide (α-GalCer), was discovered as a potent ligand for iNKT cells (Kawano et al., 1997), a synthetic α-GalCer has widely been used for study of iNKT cells as a surrogate ligand (Fig. 1). It is now established that two lipid chains of α-GalCer are inserted to hydrophobic grooves of the CD1d glycoprotein expressed by antigen presenting cells (APCs) (McCarthy et al., 2007), whereas the α-linked sugar moiety is accessible and recognized by the TCR of iNKT cells. Recently, the crystal structure of the invariant TCR and CD1d loaded with α-GalCer has shown a very unique orientation of TCR towards CD1d (Borg et al., 2007), which allows a selective involvement of the invariant α-chain for recognition of the α-linked sugar.

Comparison of α-GalCer with its structurally altered analogues has provided important insights into how iNKT cells may differentially respond to glycosphoingolipids with lipid tail variants (Brutkiewicz, 2006; Miyake and Yamamura, 2007b). As a representative example, we showed previously that an α-GalCer analogue called OCH (Miyamoto et al., 2001; Oki et al., 2004, 2005), with a shorter sphingosine chain (Fig. 1), would selectively stimulate IL-4 production from iNKT cells, whereas α-GalCer stimulation induces both IL-4 and IFN-γ. Accordingly, OCH stimulation of iNKT cells favors a Th2 bias of immune responses *in vivo*, as compared to α-GalCer stimulation.

α-linked sugars such as α-GalCer are not recognized as a product of mammalian cells, implying that α-GalCer is not a physiological ligand for iNKT cells. Currently, it is well recognized that iNKT cells can be activated during infectious diseases (Tupin et al., 2007). Interestingly, it has been reported that α-GalCer-like glycosphingolipids are rather ubiquitously found in the environment, indicating that α-GalCer may be actually derived from bacteria residing with the marine sponge. Whether or not α-GalCer is derived from bacteria, we may ask a number of questions as to whether infectious diseases may influence on autoimmune disease via activation of iNKT cells

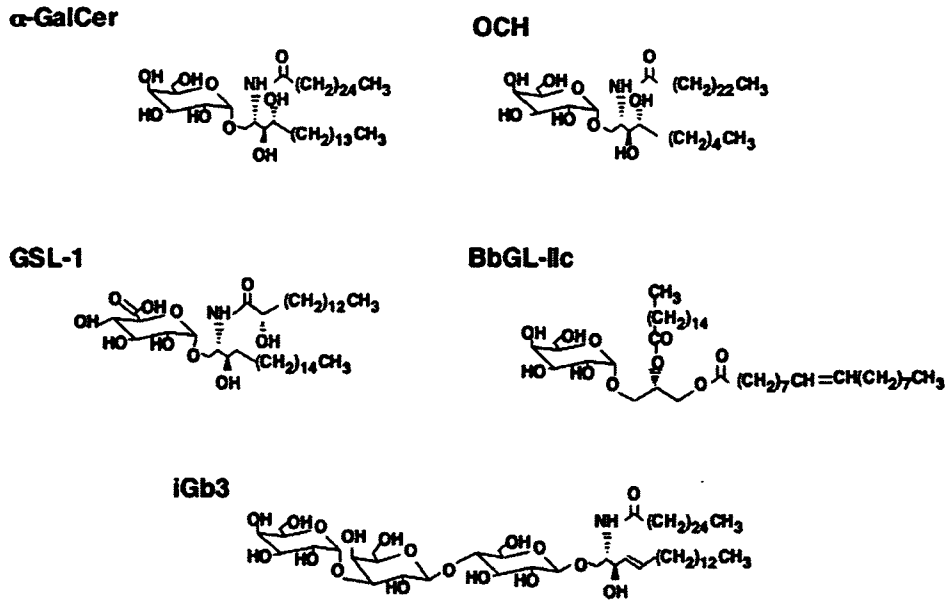

Fig. 1. Structure of glycolipid ligands for iNKT cells. Shown here are the structure of NKT cell agonists: α-galactosylceramide (α-GalCer) (Kawano et al., 1997), an α-GalCer analog called OCH, bearing a shorter sphingosine chain (Miyamoto et al., 2001), *Sphingomonas*-derived glycosphingolipid GSL-1 (Kinjo et al., 2005), *Borrelia burgdorferi*-derived diacylglycerol glycolipid BbGL-IIc (Kinjo et al., 2006), and isoglobotryhexosylceramide (iGb3) (Zhou et al., 2004).

(Godfrey and Berzins, 2006). Although multiple pathways are operative for iNKT cell activation in facing microbial challenge, it has been shown that glycosphingolipids from LPS-negative α-Proteobacteria such as *Sphingomonas* (Fig. 1) could stimulate a proportion of iNKT cells (Kinjo et al., 2005; Mattner et al., 2005). They also found that diacylglycerol glycolipids, extracted from *Borrelia burgdorferi*, stimulate at least 25% of iNKT cells (Kinjo et al., 2006; Kinjo et al., 2005). It is currently thought that arthritis and carditis found in Lyme disease following *B. burgdorferi* infection may be mediated by an autoimmune process. Whether iNKT cells activated by the diacylglycerol lipids may contribute to the pathogenesis of Lyme disease is an interesting question to be addressed. Likewise, an interesting idea is that relapse of MS following infection may be triggered by iNKT cells that are activated in response to microbial stimuli. Of note is that iNKT cells may produce osteopontin, which is reported to trigger relapses of EAE by promoting the survival of activated T cells in the inflammatory site (Hur et al., 2007).

4. Endogenous ligand for iNKT cells: search is not over

Search for an endogenous ligand of iNKT cells has led to the identification of lysosomal glycosphingolipid isoglobotryhexosylcermiade (iGb3) (Fig. 1), a β-linked sugar capable of stimulating iNKT cells as a potential endogenous ligand for mouse and human iNKT cells (Mattner et al., 2005; Zhou et al., 2004). With regard to the role of iGb3 in adaptive immune responses, Mattner et al. reported that Gram-negative, LPS-positive *Salmonella typhimurium* activates NKT cells through the recognition of iGb3, presented by LPS-activated dendritic cells. However, very recent works have cast doubt on the meaning of the iGb3 discovery (Porubsky et al., 2007; Speak et al., 2007). The study by Zhou et al. (2004) indicated that iGb3 presented by CD1d-expressing $CD4^+CD8^+$ thymocytes should be involved in the thymic positive selection of iNKT cells. Porubsky et al. has then generated iGb3 synthetase deficient mice and examined if iNKT cells are really missing in the mice lacking expression of iGb3. They found that the number and function of iNKT cells were as normal as those seen in wild-type mice. Using highly sensitive HPLC assay, Speak et al. sought for the presence of iGb3 in various mouse and human tissues. The only tissue containing iGb3 was the dorsal root ganglion of mice. No iGb3 was detected in any human tissue (Porubsky et al., 2007; Speak et al., 2007). These new findings do not support the idea that iGb3 is central in the selection of iNKT cells and re-opened the search for endogenous ligands for iNKT cells.

With regard to the pathogenesis of MS, it is interesting to know if brain-derived lipids may stimulate iNKT cells. Although such ligands have not been identified yet for iNKT cells, sulfatide derived from the myelin appears to be a ligand for non-invariant NKT cells or type II NKT cells (Godfrey et al., 2004) that bear diverse TCR repertoire although restricted by CD1d glycoprotein (Jahng et al., 2004; Zajonc et al., 2005). This interesting finding leaves room for exploring presence of myelin-derived ligands for iNKT cells that may play a role in the pathogenesis of MS.

5. Human iNKT cells and autoimmune diseases

iNKT cells' recognition of CD1d ligand is well known for its evolutionary conservation across species barriers as indicated by the fact that both mouse and human iNKT cells share a highly homologous CDR3 of TCR α-chain and would cross-recognize α-GalCer (Spada et al., 1998). However, iNKT cells from mouse and human significantly differ in population size in lymphoid organs and peripheral blood (mouse ≫ human). In addition, a clear functional dichotomy for $CD4^+$ and $CD4^-$

populations is found in human (Gumperz et al., 2002; Lee et al., 2002) but not in mouse (Kronenberg and Gapin, 2002). A lower number of the iNKT cells has led to repeated questions about the actual role of iNKT cells in human. However, studies have shown that human iNKT cells show an outstanding ability to proliferate after *in vitro* (van der Vliet et al., 2001a; Yanagisawa et al., 2002) or *in vivo* stimulation with α-GalCer (Chang et al., 2005). Moreover, patients with rare genetic diseases associated with the absence of iNKT cells are reported to suffer from serious viral infections (Levy et al., 2003; Rigaud et al., 2006). These results support a vital role for iNKT cells in maintaining the human health.

The $CD4^+/CD4^-$ dichotomy of human iNKT cells (Gumperz et al., 2002; Lee et al., 2002) is widely appreciated at present. In brief, $CD4^+$ iNKT cells could produce both pro- and anti-inflammatory cytokines after proper stimulation, indicating their ability to balance immune homeostasis. In contrast, $CD4^-$ iNKT cells predominantly produce proinflammatory cytokines such as TNF-α and IFN-γ, but little Th2 cytokines, which is reminiscent of NK cells rather than T cells. A number of studies have addressed the difference between $CD4^+$ and $CD4^-$ iNKT cells in human disease conditions (Araki et al., 2003; Illés et al., 2000; Takahashi et al., 2003). A striking reduction of the total number of iNKT cells in the peripheral blood from remission state MS has been reported from us in previous studies (Araki et al., 2003; Illés et al., 2000). When the $CD4^+$ and $CD4^-$ iNKT cells were analyzed separately, we again noted a remarkable reduction of $CD4^-$ iNKT cells in MS. However, a reduction of $CD4^+$ iNKT cells was only modest. Furthermore, we generated long-term $CD4^+$ iNKT cell lines from MS and healthy subjects and compared their ability to produce IFN-γ and IL-4. We found that the $CD4^+$ NKT cells from subjects with MS produce much more IL-4 than those from healthy subjects, whereas production of IFN-γ was not significantly different. The data collectively support that Th2 biased $CD4^+$ NKT cells may somehow contribute to maintaining the remission state of MS. In contrast, a Th1 bias of iNKT cells has been reported in human type I diabetes (Kent et al., 2005; Wilson et al., 1998). This bias is characterized by the inability to produce IL-4. A similar Th1 bias was also confirmed by using iNKT cell clones derived from draining lymph nodes of affected pancreas from T1D patients (Kent et al., 2005). As such, Th2 bias of iNKT cells during remission of MS seems to be purposeful, whereas the Th1 bias found in T1D could contribute to enhancing pathogenic autoimmunity.

6. iNKT cells regulate autoimmunity in response to exogenous ligands

By using mice lacking CD1d or TCR Jα18 gene that is required for development of iNKT cells, a number of works have proven the role of iNKT cells in self-tolerance and prevention of autoimmunity. Yet, how iNKT cells actually contribute to maintaining self-tolerance remains largely unknown. Earlier works have mainly asked how an exogenous therapeutic ligand such as OCH would modulate autoimmune disease processes. A single injection of OCH protects against development of EAE. However, a simultaneous injection of anti-IL-4 antibody abrogated the preventive effect of OCH. Moreover, disease protective effects of OCH could not be seen in IL-4 knockout mice, indicating that IL-4 produced from iNKT cells is involved in the disease suppression (Miyamoto et al., 2001). Thus, a single NKT cell stimulation with OCH probably inhibits EAE in an Ag-nonspecific mechanism. In contrast, it has been shown by others that repeated injections of α-GalCer would suppress T1D by promoting differentiation and recruitment of tolerogenic DCs in draining lymph nodes (Chen et al., 2005; Gillessen et al., 2003; Naumov et al., 2001). It is possible that presentation of a tissue-specific antigen by tolerogenic DCs may induce Ag-specific regulatory $CD4^+$ T cells secreting IL-10, which accounts for the protection against diabetes.

Without applying an exogenous glycolipid, Lehuen and colleagues have recently shown that iNKT cells could prevent a T cell-transfer model of diabetes by inducing an anergic state of the pathogenic, islet-specific T cells. In contrast to other related works, this suppression did not require Th2 cytokines but was dependent upon direct cell-cell contact (Beaudoin et al., 2002). Subsequent studies showed that the cellular interaction does not involve CD1d recognition by NKT cells (Kent et al., 2005; Novak et al., 2007). Although the mechanism of iNKT cell-mediated regulation in this model remains unclear, it is reminiscent of our work showing that a newly recognized NKT cells (MR1-restricted Vα19 NKT cells) would mediate immune regulation via direct contact with B cells through ICOS-ICOSL interaction independent of TCR recognition (Croxford et al., 2006).

7. Cytokines instruct iNKT cell response towards Th1 or Th2

Although iNKT cells could conduct a tremendous job following stimulation with exogenous ligands or via direct cellular contact, recent studies on the behavior of iNKT cells during *S. typhimurium* infection have highlighted the importance of iNKT cell recognition of an endogenous CD1d ligand in combating against microbial pathogen (Brigl et al., 2003). The work by Brigl et al. showed that iNKT cells would respond to *S. typhimurium* by producing IFN-γ, when co-cultured with DCs. Interestingly, even stimulation with LPS from *S. typhimurium* could similarly induce the IFN-γ production, indicating the involvement of TLRs rather than TCR engagement by bacterial components. Subsequent experiments showed that this IFN-γ production critically required IL-12 that was derived from DCs via TLRs in a MyD88-dependent way. However, IL-12 was not sufficient to cause the iNKT cell production of IFN-γ. It was thought that the production of IFN-γ would require recognition of endogenous CD1d ligand, as anti-CD1d antibodies proved to block the response. Whether or not iGb3 is involved is still not clear, but these results clarified that iNKT cells would exert a decisive effector function (such as a predominant IFN-γ production) when iNKT cells recognize an endogenous ligand in the presence of an exogenous cytokine.

We have recently explored if cytokines other than IL-12 may induce an effector function of iNKT cells. For this aim, human $CD4^+$ iNKT cell clones were stimulated with various cytokines in the presence of DCs. None of the clones co-cultured with

DCs exhibited any noticeable response in the absence of exogenous cytokines. However, 7 out of 27 clones examined produced a large amount of IL-5 and IL-13 when IL-2 was added to the NKT-DC co-cultures. The amount of IL-5 and IL-13 was comparable to that induced with the most potent ligand α-GalCer. However, α-GalCer never induces such a biased response but stimulates production of a broad spectrum of pro- and anti-inflammatory cytokines. Remarkable production of IL-5 and IL-13 but not of other cytokines was also confirmed by conducting DNA microarray analysis. This surprising result raises two points: 1) human $CD4^+$ iNKT cells may comprise functionally distinct populations, including such IL-5/-13 producing clones, and 2) IL-2 may be a critical factor that induces a physiological Th2 response of iNKT cells. Further analysis showed that the production of Th2 cytokines was dependent on the TCR recognition of CD1d ligand. Indeed, addition of anti-CD1d antibody blocked the response, and CD1d lacking APCs could not induce the response. Furthermore, the combination of IL-2 with a weak TCR stimulus by suboptimal concentration of anti-CD3 antibody has reproduced a similar Th2 cytokine production. These results indicate that IL-2 could play a major role in instructing the iNKT cell population to selectively produce Th2 cytokines (Sakuishi et al., 2007). 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*.

IL-12 induced production of IFN-γ (Brigl et al., 2003; Mattner et al., 2005) as well as IL-2 induced production of IL-5 (Sakuishi et al., 2007) depends upon the recognition of endogenous ligand via TCR. However, iNKT cells could also produce a large amount of cytokine in response to cytokine signals independently of TCR signals. It has been shown that iNKT cells can be activated by *Escherichia coli* LPS, and produce IFN-γ, but not IL-4. Nagarajan and Kronenberg have shown that the production of IFN-γ was dependent upon LPS-induced IL-12 and IL-18 from APC, but did not require CD1d-mediated presentation of an endogenous Ag. Furthermore, they showed that exposure to a combination of IL-12 and IL-18 sufficiently activated the iNKT cells (Nagarajan and Kronenberg, 2007). TCR-independent production of Th1 cytokine strongly indicates the innate lymphocyte-like property of iNKT cells.

8. Antigen presenting cells for iNKT cells

To evaluate reactivity of iNKT cells, previous works have mostly used dendritic cells (DCs) or unseparated lymphoid cells as APCs. Recently, two groups have used non-professional APCs for stimulating iNKT cells, and obtained interesting results (Bezbradica et al., 2005; Im et al., 2006). The study by Bezradica et al. has compared the ability of DCs, B cells, hepatocytes, and macrophages to present α-GalCer to mouse NKT cells. Whereas presentation with DCs induced a remarkable production of IFN-γ and IL-4 from NKT cells, α-GalCer-loaded hepatocytes or

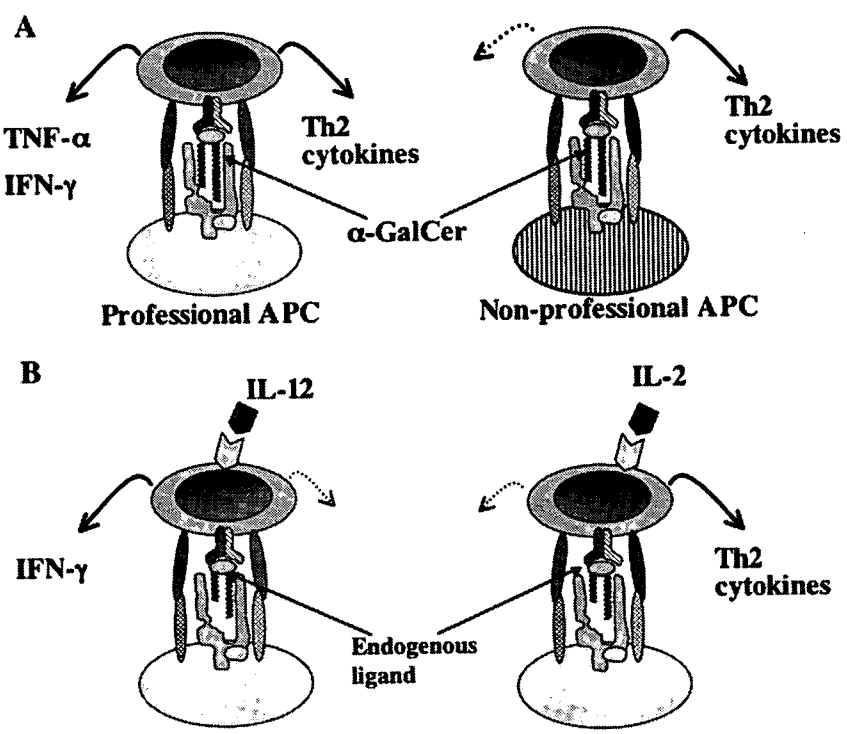

Fig. 2. Activation of iNKT cells by unconventional ways leading to functional bias. A: APC-dependent functional bias of NKT cells. Stimulating NKT cells with α-GalCer presented by professional APCs leads to production of both pro- and anti-inflammatory cytokines (left). However, when non-professional APCs such as Schwann cells (Im et al., 2006) are used, α-GalCer could induce a preferential production of Th2 cytokines from NKT cells. B: Cytokine-dependent functional bias of NKT cells recognizing endogenous ligand/CD1d. NKT cells usually exhibit only a marginal response in response to endogenous ligand bound with CD1d. However, when cytokines are added exogenously, the cells that recognize the endogenous ligand would produce a large amount of selected cytokines. For example, IL-12 induces production of IFN-γ (left) (Brigl et al., 2003), whereas IL-2 provokes IL-5 and IL-13 (right) (Sakuishi et al., 2007).

macrophages did not appear to induce iNKT cells responses. Interestingly, NKT cell stimulation with α-GalCer presented by B cells induced a weak cytokine response characterized by a low production of IL-4. Porcelli and his colleagues have examined the ability of human Schwann cells to present α-GalCer to NKT cells (Im et al., 2006). They showed that iNKT cells produced much lower amounts of proinflammatory cytokines (TNF-α and IFN-γ) but predominantly produced Th2 cytokines (IL-5 and IL-13) when Schwann cells were used as APCs. Although these studies did not examine the NKT cell reactivity to self-CD1d ligand, the results indicate that non-professional APCs tend to provoke production of Th2-associated cytokines from iNKT cells, allowing us to speculate that iNKT cell responses may greatly vary in different organs and tissues resided with different types of APCs.

9. Concluding remarks

Although most previous works have used α-GalCer or anti-CD3 antibody for stimulating iNKT cells to evaluate their functions, recent works have identified various alternative ways by which iNKT cells could be properly and differentially activated (Fig. 2). It is of particular note that iNKT cells exert polarized regulatory functions when exposed to an endogenous CD1d ligand in the presence of cytokines such as IL-12 and IL-2. We speculate that cytokine-triggered activation of iNKT cells should reflect a number of physiological or pathological conditions that could take place in the maintenance of immune homeostasis. Occurrence of Th1 polarization for iNKT cells or robust production of proinflammatory cytokines such as IFN-γ and osteopontin in response to infectious stimuli indicates a new mechanism for exacerbating autoimmune diseases preceded by an infection. Very interestingly, a growing number of potential agonists for iNKT cells have been identified from relatively common pathogens (Tupin et al., 2007). This opens a new possibility that environmental pathogens may play an active role in maintaining the population size and functions of iNKT cells in healthy conditions. Given that the frequency of iNKT cells in the peripheral blood greatly varies among healthy populations, this is an interesting question to be addressed experimentally. This new idea and a prevailing view about the major influence by genetic factors on iNKT cells are not mutually exclusive. Consequently, new approaches exploiting the role of iNKT cells in autoimmunity should probably consider their relation to pathogenic bacteria as well as non-pathogenic microbes.

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Modulation of Vα19 NKT cell immune responses by α-mannosyl ceramide derivatives consisting of a series of modified sphingosines

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We have demonstrated that analogues of α-mannosyl ceramide (α-ManCer) consisting of a series of immunosuppressive 2-aminoalcohol derivatives in place of sphingosine promote a greater immune response from mouse invariant Vα19-Jα26 (AV19-AJ33) TCR-bearing NKT (Vα19 NKT) cells than α-ManCer itself. To further characterize the immune responses of Vα19 NKT cells to the α-ManCer analogues, cytokine production by the cells was examined in detail. We found that certain α-ManCer derivatives individually induced either Th1- or Th2-dominant cytokine production in culture. The Th1- or Th2-biased immune responses of Vα19 NKT cells were dependent on MHC class I-like MR1, since they were induced by coculture with the MR1 transfectants previously loaded with the glycolipids and were inhibited in the presence of anti-MR1 antiserum. Presumably, the recognition of the α-mannosyl residue of the α-ManCer analogues by the invariant TCR is individually modulated, depending on the altered interaction with the groove of the antigen-presenting MR1. Priming of the Vα19 invariant TCR-transgenic mice *in vivo* with these glycolipid derivatives resulted in the induction of the Th1- or Th2-biased immune responses. Thus, these α-ManCer derivatives are likely to be useful in immunotherapy for either Th1 or Th2 excess autoimmune diseases, modulating the function of Vα19 NKT cells.

Key words: Glycolipids · Invariant TCR · NKT cell · Rodents · Th1/Th2

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Introduction

Natural killer T (NKT) cells are defined as lymphocytes bearing both the common NK marker NK1.1, a product of a member of the NKR-P1 gene family, and TCR-CD3 complex [1]. The major component of NKT cells (Vα14 NKT cell) [2, 3] is characterized by the expression of the invariant TCR α chain (mouse Vα14-Jα18, human Vα24-Jα 18), and is positively selected by the no polymorphic MHC class I-like CD1d molecule in association with ß2m [4, 5]. Vα14 NKT cells are responsive to certain glycosphingolipids in the context of CD1d such as α-galactosyl ceramide (α-GalCer, [6]) isolated from marine sponge [7], α-glucuronosyl and α-galacturonosyl

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Abbreviations: α-GalCer: α-galactosyl ceramide · **α-ManCer:** α-mannosyl ceramide · **MNC:** mononuclear cell · **Vα14 NKT cell:** NK1.1$^+$ T cell bearing an invariant Vα14-Jα18 TCR α chain · **Vα19 NKT cell:** NK1.1$^+$ T cell bearing an invariant Vα19-Jα26 TCR α chain

ceramides from α-proteobacteria [8, 9], and intracellular lysosomal isoglobotriaosyl ceramide [10].

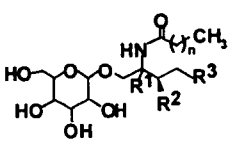
We have recently demonstrated the presence of a novel NK1.1$^+$ T cell repertoire (designated as Vα19 NKT cell in this study) expressing the Vα19-Jα26 invariant TCR α chain [11] that was previously found in human, bovine, and TAP-deficient mouse peripheral blood cells by quantitative PCR analysis [12]. The cells bearing the invariant Vα19-Jα26 TCR are absent in mice lacking the non-classical MHC class I molecule MR1, thus suggesting that they are positively selected by MR1 [13]. It is estimated that Vα19 NKT cells represent 1% of mononuclear cells (MNC) in the liver [11], thus they are a considerably large population as a lymphocyte clone. Localization of the invariant Vα19-Jα26 TCR$^+$ cells in gut lamina propria is also reported [13]. Similar to Vα14 NKT cells [14], Vα19 NKT cells immediately produce large amounts of both Th1- and Th2-promoting immunoregulatory cytokines in response to the engagement of the invariant TCR and thus are considered to have important roles in the regulation of the immune system ([15] and Shimamura, M. *et al.*, Characterization of a novel NKT cell repertoire expressing an invariant Vα19-Jα26 TCRα chain using the invariant TCR transgenic mice, Abstract #4 for the 2nd International Workshop on CD1 antigen presentation and NKT cells, Woods Hole 2002). Therefore, the search for specific antigens for Vα19 NKT cells is quite important in developing new therapies for various immunoregulatory disorders based on the functional modulation of the repertoire.

The self-antigens presented by MR1 have not been identified [16]. The discovery of α-GalCer as a stimulant for Vα14 NKT cells prompted us to investigate artificial glycosphingolipids as agonists for Vα19 NKT cells. We found that α-mannosyl ceramide (α-ManCer) was the best stimulus for Vα19 NKT cells among a series of the synthetic α-glycosyl ceramides with a naturally occurring monosaccharide [15]. This glycolipid was presented by MR1 and caused Vα19 NKT cells to secrete both IL-4 and IFN-γ. Furthermore, we have recently found immunopromotive activity toward Vα19 NKT cells in modified α-ManCer consisting of a series of derivatives of an immunosuppressive antibiotic ISP-I [17] in place of the sphingosine unit [18]; the activity was more intensive than that of the parental α-ManCer. Hence, we have continuously made efforts to characterize the synthetic α-ManCer derivatives for finding stimulants capable of modulating the function of Vα19 NKT cells.

Results

α-ManCer derivatives induce either Th1$^-$ or Th2$^-$ dominant Vα-19 NKT cell responses

The α-ManCer derivatives characterized in this study are listed with conventional abbreviations in Fig. 1. They were tested for potency to induce Th1 or Th2-dominant immune responses from Vα19 NKT cells. Liver mononuclear cells (MNC) isolated from invariant Vα19-Jα26 TCR transgenic (Vα19 Tg) mice with the TCR $\alpha^{-/-}$ background (in which Vα19 NKT cells are the sole component of NKT cells) and C57BL/6 mice as a control (among which Vα14 NKT cells represent the largest proportion) were cultured in the presence of the glycolipids. The amount of IL-4 and IFN-γ secreted into the supernatants was determined (Fig. 2A and B). α-ManCer but not α-GalCer analogues more or less enhanced the production of both IL-4 and IFN-γ by Vα19 Tg$^+$ TCR $\alpha^{-/-}$ but not C57BL/6 cells. These results were in accord with the report [18] that the proliferation and IL-2 production of Vα19 NKT cells were induced in the presence of α-ManCer derivatives in the culture medium. The IL-4 production by Vα19 Tg$^+$ cells predominated on day 1 of culture, whereas the IFN- γ production reached maximum on day 2 of culture (Fig. 2B). This profile of cytokine production is similar to the profile observed in Vα19 Tg$^+$ cells upon TCR engagement with immobilized anti-CD3 antibody. Depletion of the NK1.1$^+$ or TCRαß$^+$ population reduced the responsiveness of the responder cells (Fig. 2C). Thus, it is strongly suggested that the potential to respond to the α-ManCer derivatives was confined to Vα19 NKT cells. Interestingly, the relative intensity of IL-4 to IFN-γ secretion by Vα19 NKT cells was dependent on the chemical structure of the stimulator. α-ManCer



Abbreviations	R^1	R^2	R^3	n	Hexose
Man4PhC16	H	H	4-octylphenyl	14	α-Man
GalC24	H	H	dodecanyl	22	α-Gal
ManC16	H	H	dodecanyl	14	α-Man
Man2HM4PhC16	CH_2OH	H	4-octylphenyl	14	α-Man
Gal2HM4PhC16	CH_2OH	H	4-octylphenyl	14	α-Gal
Man2HMC24	CH_2OH	H	dodecanyl	22	α-Man
Man2HMC16	CH_2OH	H	dodecanyl	14	α-Man
Man3OHC16	H	OH	dodecanyl	14	α-Man

Figure 1. A list of the glycosyl ceramide derivatives characterized in the present study. Glycosphingolipids modified with a 2-hydroxymethyl, 3-hydroxyl, or 4-octylphenyl group are represented as 2HM, 3OH or 4Ph.

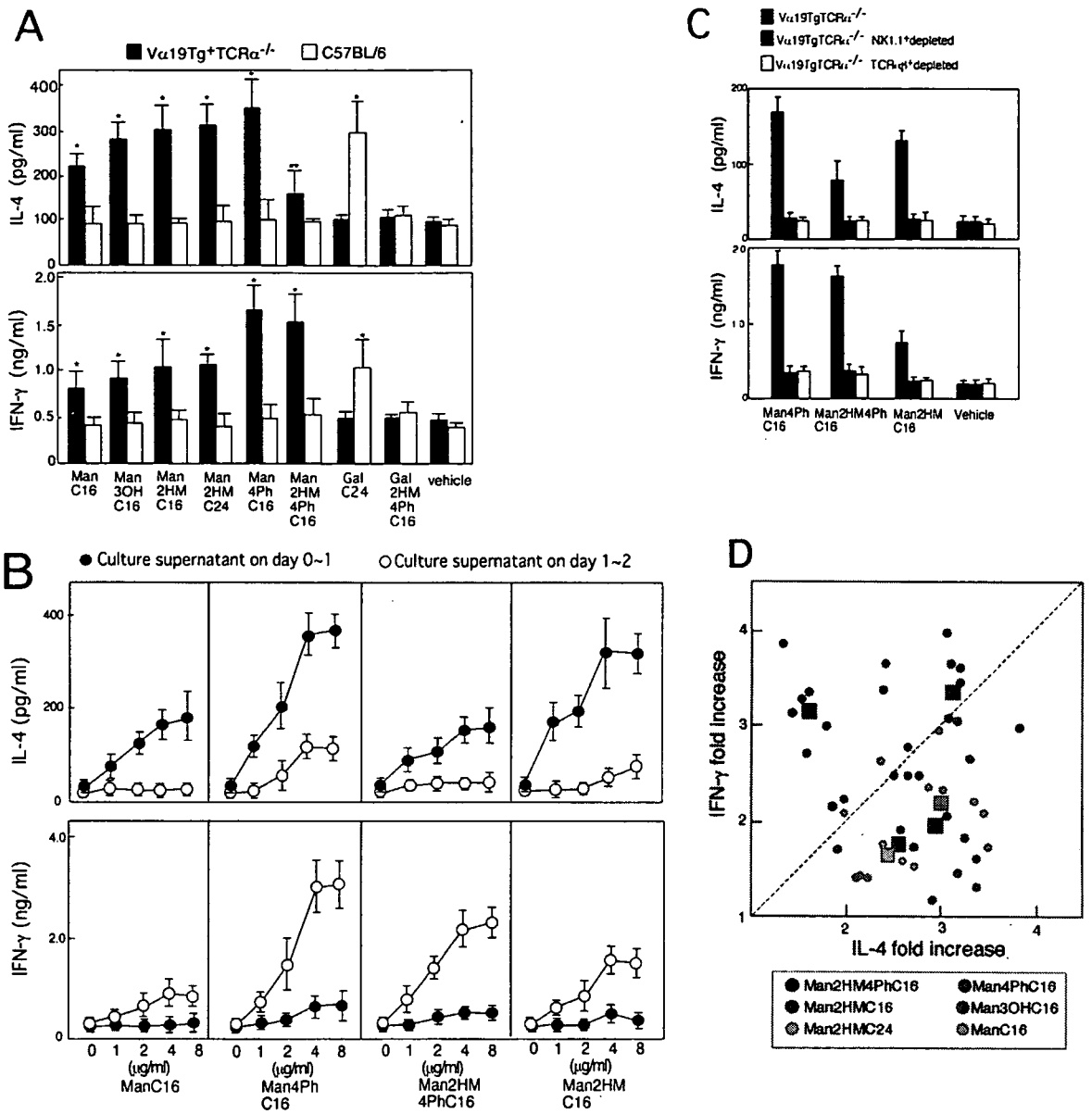


Figure 2. Immune responses of Vα19 NKT cells in culture elicited by α-ManCer derivatives. (A) Liver MNC from Vα19 Tg$^+$ TCRα$^{-/-}$ and C57BL/6 mice were cultured with the addition of glycolipids dissolved in DMSO (2 μg/mL). After 2 days, the immune responses were monitored by measuring the concentrations of IL-4 and IFN-γ in the culture fluid. The filled bars represent the culture of Vα19Tg$^+$TCRα$^{-/-}$ cells, whereas the open bars show the results of C57BL/6 cells. Abbreviations of glycolipids are listed in the legend to Fig. 1. The average of the results obtained from independent eight experiments is indicated. The *p* values in Dunnett's multiple comparison post-test are calculated in comparison with the control. $^*p<0.01$; $^{**}p<0.05$. (B) Dose-dependent activation of Vα19 NKT cells by α-ManCer derivatives in culture. Liver MNC from Vα19 Tg$^+$ TCRα$^{-/-}$ mice were cultured with the indicated dose of glycolipids. After 1 day, the culture fluid was exchanged with the fresh medium with glycolipids. The concentration of cytokines in the culture supernatants (0~1 day, 1~2 day) were determined. One of the three independent experiments giving essentially the same profiles of cytokine production is shown. (C) Determination of the cell populations in the Tg liver responding to the α-ManCer analogues. Liver MNC prepared from Vα19Tg TCRα$^{-/-}$ mice were depleted of NK1.1$^+$ or TCR αß$^+$ cells as described in *Materials and methods*. Cells were cultured with the glycolipids, and the concentration of IL-4 and IFN-γ in the supernatants was determined by ELISA. The average of triplicate cultures in one of the independent three experiments is shown. (D) Modulation of immune responses of Vα19 NKT cells by α-ManCer derivatives. Liver MNC from Vα19 Tg$^+$ TCRα$^{-/-}$ mice were cultured with α-ManCer derivatives as indicated in (A). The concentrations of IL-4 on day 1 of culture and the IFN-γ on day 2 of culture are plotted. Results are shown as the fold-increase relative to the control cultures with the vehicle. Large squares represent the fold-increases in cytokine production on the average.

with either a 2-hydroxymethyl group (Man2HMC16, Man2HMC24), or a 4-phenyl group (Man4PhC16) more intensively induced both IL-4 and IFN-γ production than the α-ManCer without any substitutions in the sphingosine portion (ManC16) or 3-hydroxy α-ManCer (Man3OHC16). On the other hand, the α-ManCer with both 2-hydroxymethyl and 4-phenyl groups (Man2HM4PhC16) induced less IL-4 in Vα19 NKT cells, and the cytokine production was biased to IFN-γ (Fig. 2A and B). To demonstrate more clearly the cytokine profile induced with the individual α-ManCer analogues, the fold-increase in IL-4 production by Vα19Tg$^+$TCRα$^{-/-}$ liver MNC on day 1 *versus* that in IFN-γ production on day 2 in each culture was plotted in Fig. 2D. This profile strongly suggests that manipulation of the sphingosine portion of α-ManCer alters the interaction between invariant Vα19 TCR and the α-mannosyl residue in the glycolipids, resulting in the modulation of the immune responses of Vα19 NKT cells. More detailed cytokine profiles obtained from the culture of hepatic Vα19 NKT cells with representative α-ManCer analogues were examined (Fig. 3). Man4PhC16 induced production of both proinflammatory (IFN-γ, IL-12, IL-17) and Th2-promoting (IL-4, IL-5, IL-10) cytokines. Man2HM4PhC16 promoted proinflammatory whereas Man2HMC16 enhanced Th2-biased cytokine secretion. These results further support that modified α-ManCer are capable of modulating immune responses of Vα19 NKT cells.

Immune responses of Vα19 NKT cells primed *in vivo* with α-ManCer derivatives

The immune responses of Vα19 NKT cells specifically induced by the α-ManCer derivatives were also observed when they were primed *in vivo* with the glycolipids. Spleen cells from Vα19Tg$^+$TCRα$^{-/-}$ and C57BL/6 mice injected 90 min previously with the glycolipids were cultured and cytokines secreted into the supernatants were determined (Fig. 4). Vα19Tg$^+$TCRα$^{-/-}$ splenocytes produced IL-4 and IFN-γ in a similar fashion to those stimulated *in vitro*. They produced increased amount of both IL-4 and IFN-γ with Man4PhC16. Relatively enhanced IL-4 production was observed when the cells were primed with Man2HMC16, whereas IFN-γ production predominated when they were stimulated with Man2HM4PhC16. On the other hand, C57BL/6 cells apparently displayed less responsiveness to these α-ManCer analogues presumably due to the lower frequency of Vα19 NKT cells in the spleen. Thus, α-ManCer derivatives injected into mice possibly target Vα19 NKT cells and promote either Th1- or Th2-dominant immune responses.

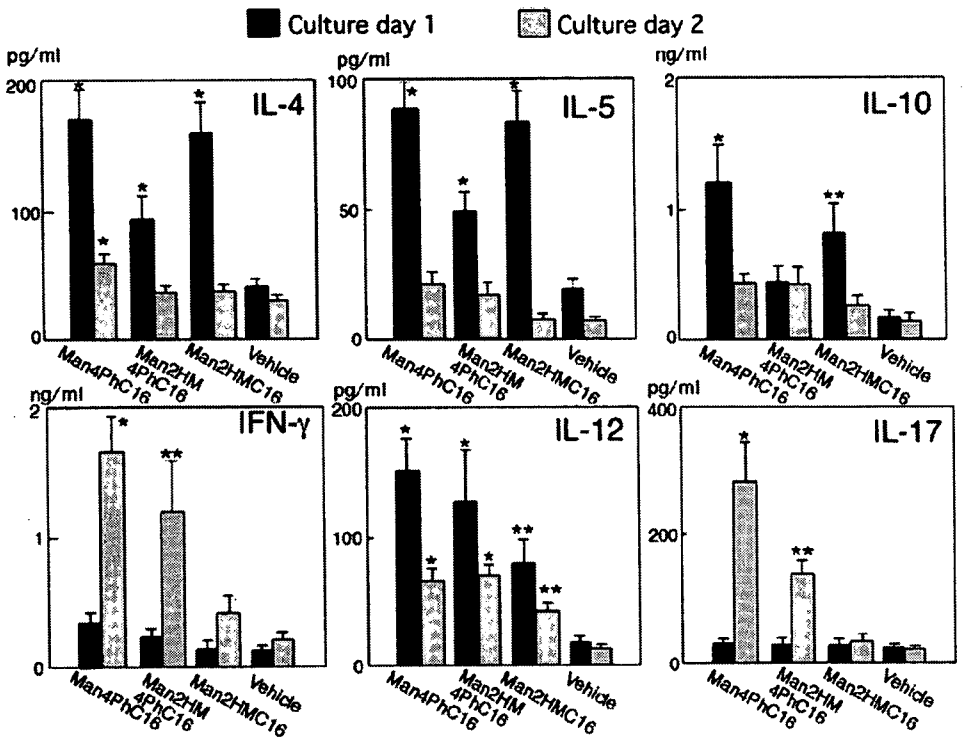


Figure 3. Either Th1- or Th2-dominant cytokine production by Vα19 NKT cells depending on the presence of the α-ManCer derivatives. Liver MNC prepared from Vα19 Tg$^+$ TCRα$^{-/-}$ mice were cultured with the indicated α-ManCer derivative (2 μg/mL). Cytokine production in the culture supernatants on days 1 and 2 was determined by ELISA. One of the three experiments with essentially the similar profiles is shown. The *p* values in Dunnett's multiple comparison post-test are calculated in comparison with the control (cytokine levels in culture with vehicle). * $p<0.01$; ** $p< 0.05$.

