Differential antitumor immunity mediated by NKT cell subsets in vivo

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We showed previously that NKT cell–deficient TCR Jα18$^{-/-}$ mice are more susceptible to methylcholanthrene (MCA)-induced sarcomas, and that normal tumor surveillance can be restored by adoptive transfer of WT liver-derived NKT cells. Liver-derived NKT cells were used in these studies because of their relative abundance in this organ, and it was assumed that they were representative of NKT cells from other sites. We compared NKT cells from liver, thymus, and spleen for their ability to mediate rejection of the sarcoma cell line (MCA-1) in vivo, and found that this was a specialized function of liver-derived NKT cells. Furthermore, when CD4$^+$ and CD4$^-$ liver-derived NKT cells were administered separately, MCA-1 rejection was mediated primarily by the CD4$^-$ fraction. Very similar results were achieved using the B16F10 melanoma metastasis model, which requires NKT cell stimulation with α-galactosylceramide. The impaired ability of thymus-derived NKT cells was due, in part, to their production of IL-4, because tumor immunity was clearly enhanced after transfer of IL-4–deficient thymus-derived NKT cells. This is the first study to demonstrate the existence of functionally distinct NKT cell subsets in vivo and may shed light on the long-appreciated paradox that NKT cells function as immunosuppressive cells in some disease models, whereas they promote cell-mediated immunity in others.

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Abbreviations used: α-GalCer, α-galactosylceramide; CFSE, carboxyfluorescein diacetate succinimidyl ester; MCA, methylcholanthrene, NMS, normal mouse serum.

NKT cells are a unique population of T cells that mediate potent immunoregulatory roles in vivo. Found in mice and humans, NKT cells express a heavily biased TCR repertoire; most cells express the TCR Vα14-Jα18 chain coupled to TCR Vβ8.2, Vβ7, or Vβ2 in mice, and TCR Vα24-Jα18 coupled to TCR Vβ11 in humans (for review see reference 1). This TCR specifically recognizes glycolipid antigens that are presented in the context of CD1d. The best known example of such an antigen is α-galactosylceramide (α-GalCer) (reviewed in reference 2); however, recent studies revealed the identify of several other glycolipid antigens that are recognized by the NKT cell antigen receptor, including the endogenous lysosomal glycosphingolipid, isoglobotrihexosylceramide (3, 4); glycolipids that are derived from some gram-negative, LPS-negative bacteria, including *Ehrlichia* and *Sphingomonas* (4–7); mycobacterial phosphatidylinositol mannoside (8); and phosphoethanolamine (9). The interaction between this TCR and the CD1d molecule is so critical during NKT cell development, that mice lacking either factor are selectively deficient in NKT cells (10–13). NKT cells are detected wherever conventional T cells are found, although their frequency relative to other T cells is tissue dependent; the highest NKT cell/T cell ratio is found in the liver (14–17).

A striking characteristic of NKT cells is their ability to produce Th1 (e.g., IFN-γ and TNF) and Th2 type (e.g., IL-4 and -13) cytokines rapidly upon primary stimulation. This suggests an important and diverse role for these cells in immunoregulation, although how one cell type that produces Th1 and Th2 type cytokines can direct an immune response clearly is a major paradox in the NKT cell field. Many studies in mouse models have demonstrated that NKT cells can influence a broad range of diseases, including autoimmune diseases such as type 1 diabetes, graft-versus-host disease, graft rejection, systemic suppression of cell-mediated immunity after introduction of antigen into the anterior chamber of the

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eye. They also contribute to airway hypersensitivity and contact sensitivity in mice. These activities are generally thought to be due to the production of type 2 cytokines, including IL-4, -10, and -13, by NKT cells (for reviews see references 18–21). In contrast to the studies that showed that NKT cells can suppress cell-mediated immunity, many other studies demonstrated that NKT cells also can promote potent cell-mediated antitumor responses (e.g., rejection of B16F10 melanoma) when stimulated with exogenous IL-12 or α-GalCer (for review see reference 22). Even in the absence of exogenous stimulation, NKT cells are essential for preventing the development and growth of MCA-induced sarcomas (23). We demonstrated, with both tumor models, that the defective tumor immunity that was observed in NKT cell–deficient Jα18$^{-/-}$ mice was restored by adoptive transfer of NKT cells that were isolated from the liver of WT donor mice, and that this protection was dependent upon IFN-γ production by the transferred NKT cells (24, 25).

In studies in which we used adoptive transfer to restore NKT cell–mediated tumor rejection in Jα18$^{-/-}$ mice (24, 25), we used liver-derived NKT cells because of their relative abundance, and it was assumed that these were representative of all NKT cell populations in vivo. At least two phenotypically distinct subsets of NKT cells exist in the liver and other tissues of mice, defined as CD4$^+$CD8$^-$ (CD4$^+$) and CD4$^-$CD8$^-$ double negative, and an additional CD4$^-$CD8$^+$ subset exists in humans (1). In vitro culture of CD4$^+$ and CD4$^-$ NKT cells from mice or humans, and NKT cells from various tissues in mice, revealed that NKT cell subsets can behave differently, at least in terms of their cytokine production in vitro (15, 26–30). Together, these studies raised the intriguing possibility that NKT cell subsets may be differentially responsible for the diverse functions that are attributed to these cells in vivo. We sought to test this hypothesis by using adoptive transfer of various NKT cell subsets into two distinct models of tumor immunity. Our results provide the first evidence to directly demonstrate the existence of functionally distinct NKT cell subsets in vivo. Whereas liver CD4$^-$ NKT cells are capable of promoting tumor rejection, other NKT cell subsets, including thymus and spleen-derived NKT cells and liver-derived CD4$^+$ NKT cells, are far less potent in this capacity. Our results have far-reaching implications for all studies that examine NKT cell function, and indicate that care must be taken when NKT cell subsets are being examined and manipulated.

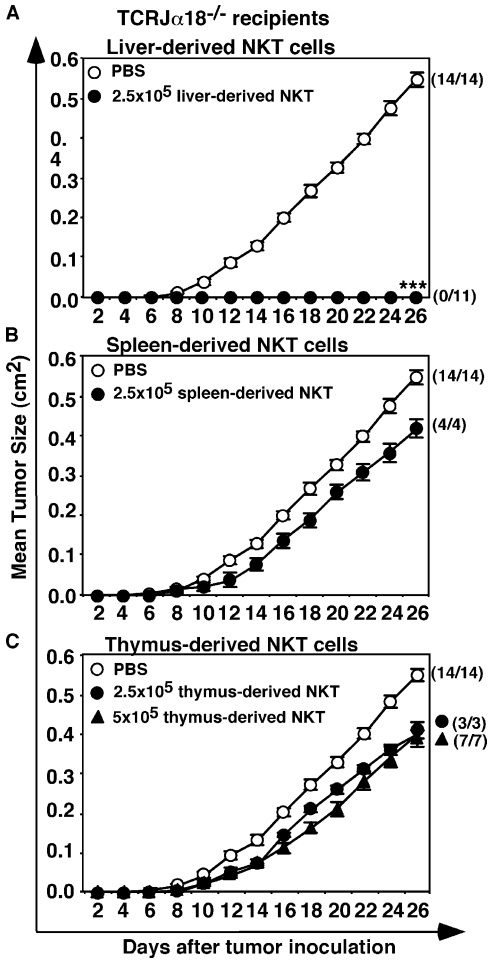

Figure 1. Liver-derived NKT cells are optimal mediators of MCA-1 tumor immunosurveillance. TCR Jα18$^{-/-}$ mice were inoculated s.c. in the right hind flank with 10^5 MCA-1 cells. Groups of TCR Jα18$^{-/-}$ mice then received sorted liver- (A), spleen- (B), or thymus-derived (C) NKT cells or, 2% NMS in PBS by way of i.v. injection. Sorted populations were always ≥94%, ≥91%, and ≥97% pure, respectively. Results were recorded every other day as the mean tumor size (cm^2) ± SEM. Data are pooled from three (A), one (B), and two (C) experiment(s) with three to five mice/group/experiment. The number of mice in each group is indicated in parentheses. PBS-treated TCR Jα18$^{-/-}$ control data are the same for A, B, and C and is shown for comparison. Significant difference in tumor growth rate was determined between the PBS-treated TCR Jα18$^{-/-}$ control group and the mice that received liver, spleen, or thymus-derived NKT cells using a Kruskal-Wallis statistical test, followed by a Dunn's post test. ***$P \leq 0.001$.

RESULTS

Liver-derived NKT cells are unique in their ability to prevent MCA-1 sarcoma growth

We reported that NKT cell–deficient TCR Jα18$^{-/-}$ mice were more susceptible to the development and growth of MCA-induced sarcomas than WT mice (23). We also demonstrated that protection against sarcoma growth was restored to TCR Jα18$^{-/-}$ mice upon adoptive transfer of liver-derived WT NKT cells (24) (Fig. 1 A). Liver-derived NKT cells were used in these studies because of their relative abundance, and it was assumed that these cells were functionally representative of all NKT cell populations in vivo. To test this hypothesis directly, NKT cells were isolated from the liver, spleen, and thymus of WT mice and adoptively transferred into MCA-1–injected TCR Jα18$^{-/-}$ recipients. Consistent with our earlier report (24), transfer of 2.5×10^5 liver-derived NKT cells into MCA-1–inoculated TCR Jα18$^{-/-}$ mice induced complete inhibition of tumor growth (Fig. 1 A). In contrast, the equivalent number of spleen- or thymus-derived NKT cells from the same donor mice failed

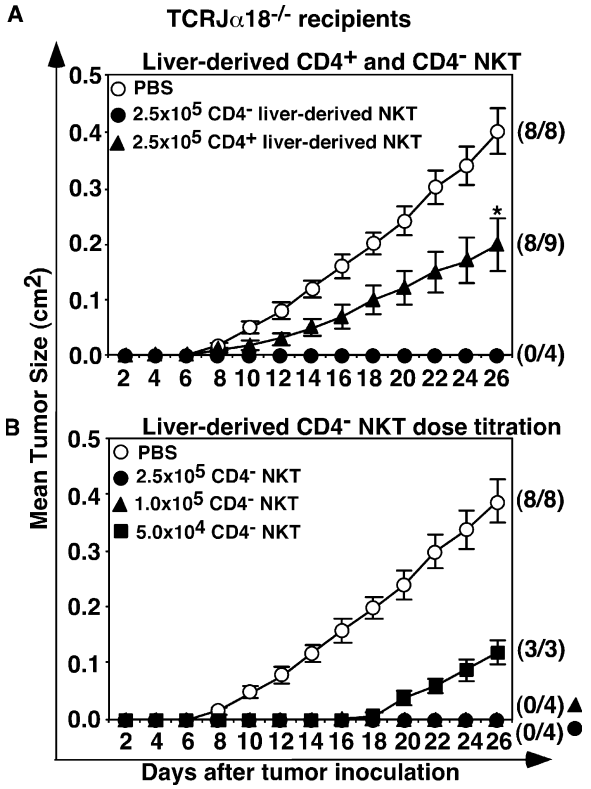

Figure 2. Liver-derived CD4$^-$ NKT cells are the most potent mediators of MCA-1 tumor immunosurveillance. TCR Jα18$^{-/-}$ mice were inoculated s.c. in the right hind flank with 10^5 MCA-1 cells. Groups of TCR Jα18$^{-/-}$ mice then received sorted CD4$^+$ or CD4$^-$ liver-derived NKT cells, or 2% NMS in PBS (A), or a range of liver-derived CD4$^-$ NKT cell doses (2.5×10^5, 10^5 or 5×10^4 cells), by way of i.v. injection (B). Results were recorded every other day as the mean tumor size (cm^2) $\pm$ SEM. Data are pooled from two independent experiments (A and B). We used three to five mice/group/experiment. The number of mice with measurable tumors over total mice per group is indicated in parentheses. PBS-treated TCR Jα18$^{-/-}$ control data are the same for A and B, and are shown for comparison. Significant difference in tumor growth rate was determined between the PBS-treated control group and mice receiving CD4$^+$ NKT cells, using a Mann-Whitney U test. *$P < 0.05$.

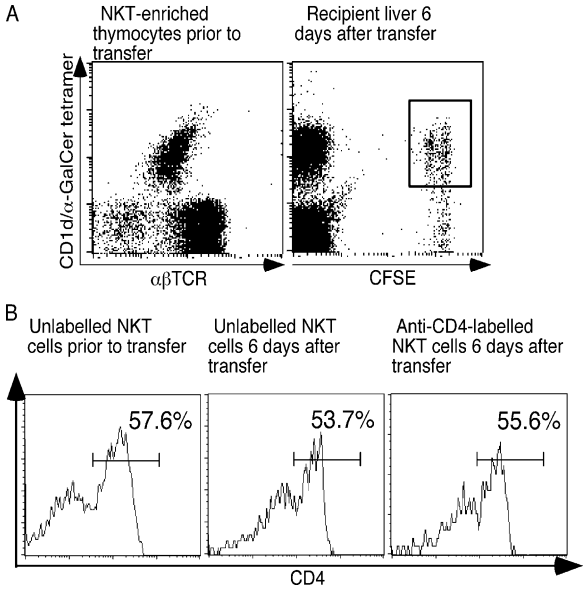

Figure 3. Thymus-derived NKT cells detected several days after transfer. NKT cell–enriched thymocytes were CFSE-labeled and transferred into WT recipients by way of i.v. injection (A, left). 6 d after transfer, organs were harvested and screened for the presence of CFSE$^+$CD1d/α-GalCer$^+$ cells (A, right). These cells were gated on, and analyzed for, CD4 expression (B, middle and right). Some mice received CFSE-labeled cells that were labeled with anti-CD4 before transfer (B, right). Data are representative of six (A) or two (B) experiments with one or two mice/group.

to inhibit tumor growth (Fig. 1, B and C). In fact, even twice as many (5×10^5) thymus-derived NKT cells were unable to restore protection (Fig. 1 C), which suggests that liver NKT cells have unique functional characteristics that are necessary for efficient rejection of the MCA-1 sarcoma.

Liver-derived CD4$^-$, but not liver CD4$^+$, NKT cells mediate tumor immune surveillance

Given that liver-derived NKT cells are unique in their ability to mediate MCA-1 rejection, we sought to determine whether CD4$^+$ and CD4$^-$ subsets of liver-derived NKT cells possessed this capacity. Each subset was sorted and adoptively transferred into MCA-1–inoculated TCR Jα18$^{-/-}$ recipients. As shown in Fig. 2 A, CD4$^-$ NKT cells completely restored protection against MCA-1, whereas the equivalent number of CD4$^+$ NKT cells did not. At best, CD4$^+$ NKT cells slowed tumor growth when compared with PBS-injected controls. Considering that CD4$^-$ cells make up ~30% of the liver-derived NKT cell population, and that the antitumor effects resided primarily within this compartment, it was hypothesized that a lower dose of these cells than that established for unfractionated liver-derived NKT cells (2.5×10^5) (24), would still restore complete protection. MCA-1–inoculated TCR Jα18$^{-/-}$ recipients were injected with a range of CD4$^-$ doses (2.5×10^5, 10^5, 5×10^4 cells), and were monitored for tumor development (Fig. 2 B). Restoration of tumor rejection was dose dependent; 2.5×10^5 and 10^5 CD4$^-$ cells completely inhibited tumor growth, while 5×10^4 cells considerably delayed tumor onset and progression. Given that CD4$^-$ NKT cells represent ~30% of total NKT cells in liver (unpublished data), the number of CD4$^-$ cells required for complete tumor inhibition (10^5) was similar to the number of these cells ($>0.8 \times 10^5$) present in the minimum dose of unfractionated liver-derived NKT cells (2.5×10^5) necessary for complete protection (24). This supports the concept that the CD4$^-$ subset is mostly, if not completely, responsible for the antitumor effects of liver-derived NKT cells.

Survival of NKT cell subsets after adoptive transfer

One explanation for the lack of protection mediated by thymus-derived NKT cells is that they might not survive after transfer into the periphery. To address this possibility, NKT cell–enriched thymocytes were carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled and transferred

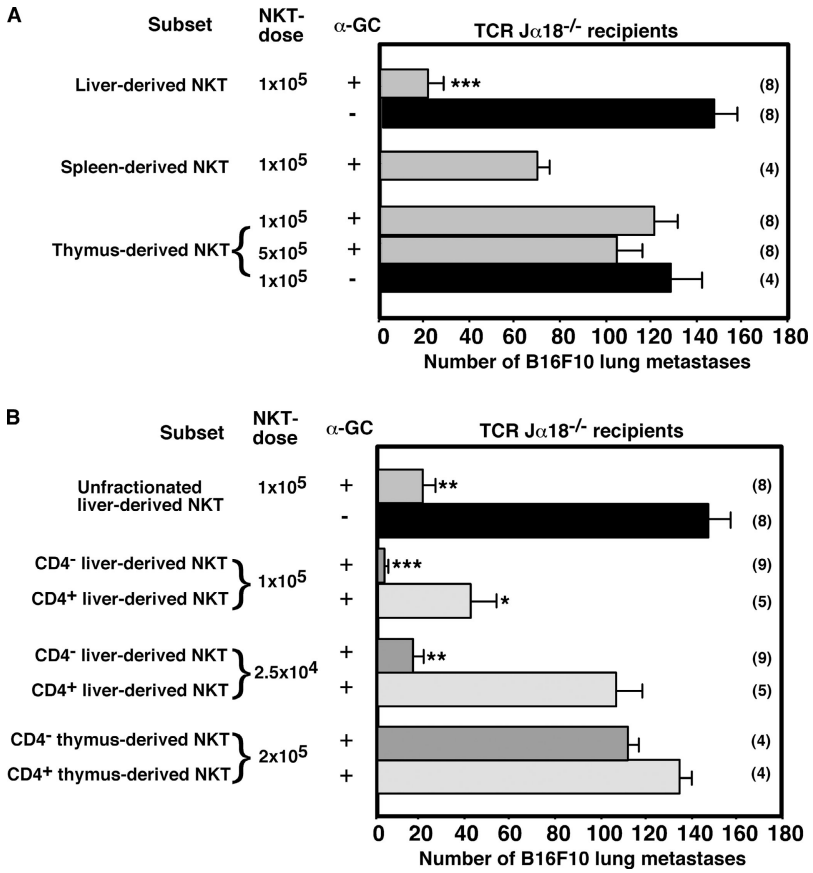

Figure 4. Liver-derived NKT cells are most efficient mediators of α-GalCer–induced antitumor immunity. TCR Jα18$^{-/-}$ mice were inoculated with 5 × 10^5 B16F10 cells by way of i.v. injection. 3 h later, some mice received unfractionated liver-derived lymphocytes (A and B), NKT cell–enriched thymocytes or splenocytes, sorted thymus-derived NKT cells (A), or sorted liver- or thymus-derived CD4$^+$ or CD4$^-$ NKT cell subsets (B) by way of i.v. injection. On the same day as lymphocyte transfer, and again on days 4 and 8, some mice also received 2 μg α-GalCer or vehicle by way of i.p. injection. 14 d after tumor inoculation, lungs were harvested, and B16F10 colonies were counted and recorded as the mean number ± SEM. Data are pooled from two independent experiments and the total number of mice in each group is indicated in parentheses. Significant difference in tumor growth rate was determined between mice receiving NKT cells and vehicle, and mice receiving NKT cells and α-GalCer, using a Kruskal-Wallis test, followed by a Dunn's post test. *P < 0.05; **P < 0.01; ***P ≤ 0.001.

into WT recipients. 6 d later, organs were harvested and analyzed for the presence of CFSE$^+$ CD1d/α-GalCer tetramer$^+$ cells. These cells were clearly detected in liver (Fig. 3 A), and to a lesser extent in spleen, PBLs, and BM (not depicted) for at least a week after transfer. In other experiments, we could detect clear populations of thymus-derived NKT cells 8 weeks after adoptive transfer (unpublished data). Transferred liver- and spleen-derived NKT cells were also detectable in these tissues (albeit at a lower rate relative to the number of NKT cells injected, which was consistent with a previous report [31]) with a similar tissue distribution (unpublished data). Another explanation for the inability of CD4$^+$ NKT cells to confer complete tumor inhibition is that CD4 labeling for FACS sorting may induce the preferential depletion of this subset in vivo. To address this possibility, mice received CFSE-labeled NKT cells that were, or were not, labeled with anti-CD4 before transfer. As shown in Fig. 3 B, prelabeled CD4$^+$ NKT cells were detected at the same frequency as nonprelabeled CD4$^+$ NKT cells 6 d after transfer, indicating that CD4 labeling did not affect survival of these cells.

Liver-derived NKT cells are predominant mediators of α-GalCer–induced antitumor immunity

One possible explanation for the differential responsiveness of NKT cell subsets in these studies is that liver CD4$^-$ NKT cells are activated selectively in response to the MCA-1 tumor. Therefore, we used a model in which all NKT cells are stimulated to test whether this distinction existed. We previously reported that adoptive transfer of liver-derived NKT cells into α-GalCer–treated, B16F10-inoculated TCR Jα18$^{-/-}$ recipients inhibited B16F10 metastases in the lungs (25). To determine whether spleen- and thymus-derived NKT cells promoted similar antitumor immunity, unfractionated liver-derived lymphocytes, NKT cell–enriched thymocytes, and splenocytes were adoptively transferred into α-GalCer–treated, B16F10-inoculated, TCR Jα18$^{-/-}$ mice. Each transferred population contained 10^5 NKT cells, which

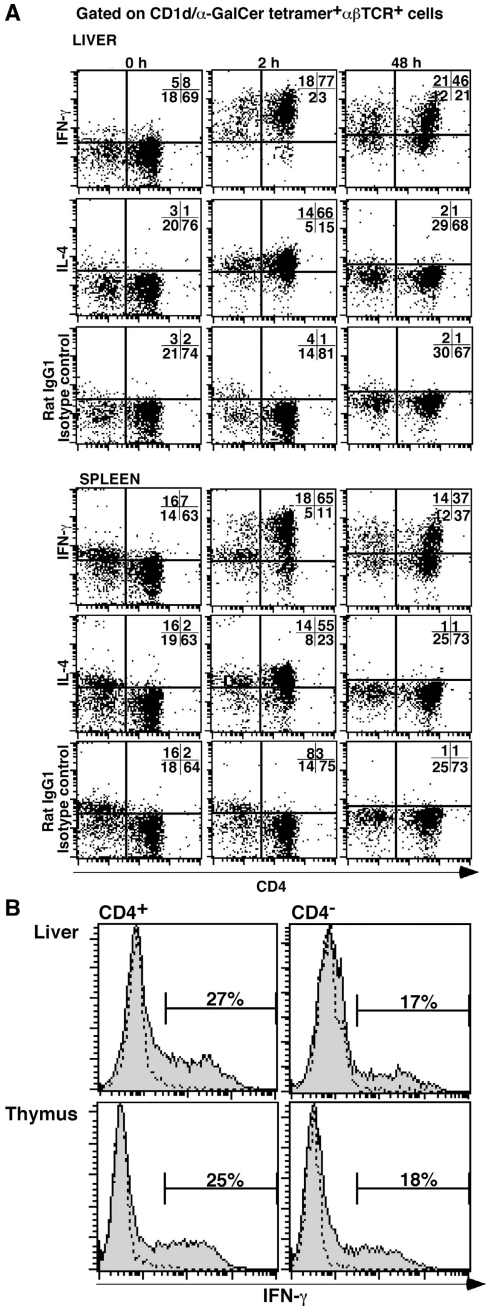

Figure 5. Cytokine production by NKT cell subsets after α-GalCer stimulation. WT mice received 2 μg α-GalCer or vehicle by way of i.p. injection and were killed 2 or 48 h later (A). Liver- and spleen-derived lymphocytes were cultured without further stimulation for 2 h in Brefeldin A. After culture, cells were labeled with surface mAb, fixed, and permeabilized for intracellular cytokine staining. Cells were analyzed by flow cytometry, and CD1d/α-GalCer$^+$αβTCR$^+$ cells were gated on and CD4$^+$ and CD4$^-$ subsets screened for intracellular IFN-γ production. Gates were set based on the isotype control at each time point. (B) Liver lymphocytes and NKT cell–enriched thymocytes (with sorted spleen-derived DCs) were cultured for 12 h with 100 ng/ml α-GalCer. Brefeldin A was added for the final 2 h of culture. After culture, cells were prepared for intracellular cytokine staining (as for A). NK1.1$^+$αβTCR$^+$ cells from the liver and NK1.1$^+$ cells from the thymus were electronically gated and CD4$^+$ and CD4$^-$ subsets were analyzed for intracellular IFN-γ production (filled line graph: DCs with α-GalCer; dotted line: nonstimulated control).

we determined previously to be a protective dose (25). As expected, liver-derived NKT cells effectively inhibited B16F10 metastases in the lungs (Fig. 4 A). However, consistent with earlier findings from the MCA-1 model, spleen-derived NKT cells were less effective than were liver-derived NKT cells at reducing the number of metastases. Even more striking was the inability of NKT cell–enriched thymocytes or sorted thymus-derived NKT cells to inhibit tumor metastases, even when a fivefold increase in NKT cell dosage (5×10^5) was used (Fig. 4 A). In some experiments, NKT cells were purified by FACS sorting based on NK1.1 versus αβTCR expression from liver, spleen, and thymus, with similar results.

Liver-derived CD4$^-$ NKT cells are the most potent mediators of α-GalCer–induced antitumor immunity

To determine whether CD4$^+$ and CD4$^-$ subsets of liver-derived NKT cells were capable of mediating α-GalCer–induced antitumor immunity, each subset was sorted and adoptively transferred into α-GalCer–treated, B16F10-inoculated, TCR Jα18$^{-/-}$ recipients (Fig. 4 B). Whereas both subsets promoted α-GalCer–induced antitumor immunity, this was mediated more potently by the CD4$^-$ subset. Significant ($P < 0.01$) antitumor activity was observed after transfer of 2.5×10^4 CD4$^-$ NKT cells, whereas equivalent numbers of CD4$^+$ NKT cells had little effect. For comparison, we also injected some mice with thymic NKT cells that had been subdivided into CD4$^+$ and CD4$^-$ subsets (Fig. 4 B). As for unfractionated thymic NKT cells, neither CD4$^+$ nor CD4$^-$ subsets showed enhanced protection.

We demonstrated previously that NKT cell–derived IFN-γ is critical for tumor immunosurveillance against MCA-1 and α-GalCer–induced antitumor immunity against B16F10 (24, 25). To investigate whether differences in IFN-γ production were responsible for the functional differences that were observed, WT mice were injected with α-GalCer, and CD4$^+$ and CD4$^-$ NKT cell subsets from the liver and spleen were analyzed for their production of intracellular cytokines. As shown in Fig. 5 A, no clear differences were observed; all populations produced similar levels of IFN-γ and IL-4 after α-GalCer stimulation. Because thymic NKT cells are not stimulated by α-GalCer in situ (16, 32), thymus- and liver-derived NKT cells were isolated from naive mice and stimulated in vitro with sorted spleen-derived DCs and 100 ng/ml α-GalCer for 12 h (Fig. 5 B). Intracellular cytokine staining of these cells demonstrated that IFN-γ and IL-4 production was similar between CD4$^+$ and CD4$^-$, subsets from the thymus and liver.

NKT cell–derived IL-4 production impairs antitumor function of thymus-derived NKT cells

To investigate whether IL-4 and/or IL-10 production antagonizes the potential of some NKT cell subsets to confer protection, NKT cells were enriched or purified from the thymus and liver of WT and IL-4$^{-/-}$ mice and adoptively transferred into α-GalCer–treated, B16F10-inoculated,

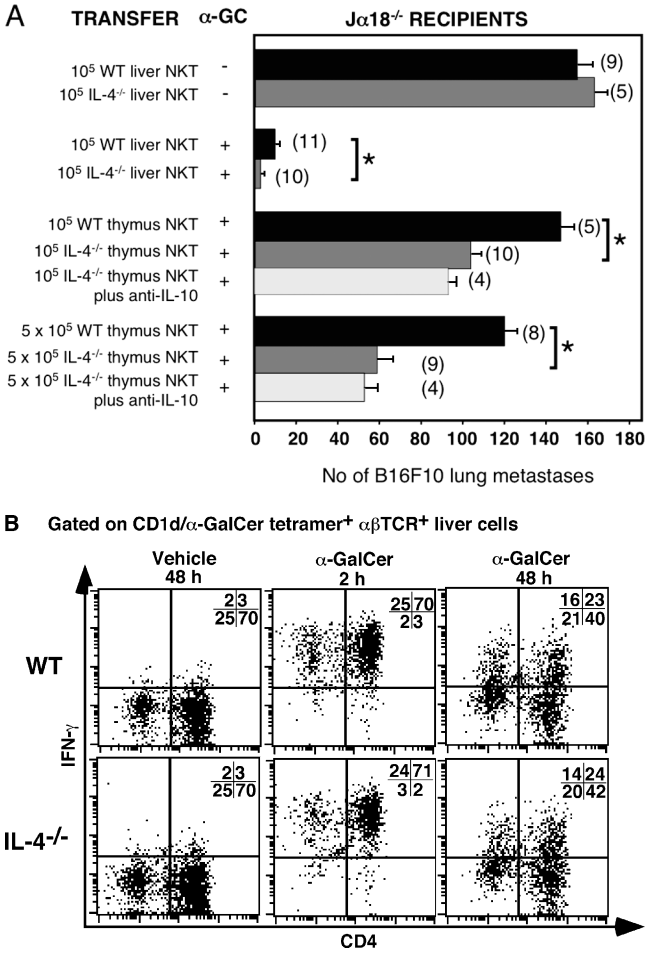

Figure 6. NKT cell–derived IL-4 production impairs antitumor function of thymus-derived NKT cells. (A) TCR Jα18$^{-/-}$ mice were inoculated with 5 × 10^5 B16F10 cells by way of i.v. injection. 3 h later, some mice received unfractionated liver-derived lymphocytes, NKT cell–enriched thymocytes, or sorted liver- and thymus-derived NKT cells from WT and IL-4$^{-/-}$ donors by way of i.v. injection. For IL-10 neutralization, some mice received 400 μg anti–IL-10 on the same day as lymphocyte transfer (day 0), and again on days 4 and 8 by way of i.p. injection. Some mice also received 2 μg α-GalCer or vehicle by way of i.p. injection on days 0, 4, and 8. 14 d after tumor inoculation, lungs were harvested, and B16F10 colonies were counted and recorded as the mean number ± SEM. The number of mice in each group is indicated in parentheses. Results from mice receiving WT or IL-4$^{-/-}$ NKT cells from thymus or liver were compared using a Mann-Whitney rank sum test. *P < 0.05. (B) WT and IL-4$^{-/-}$ mice received 2 μg α-GalCer or vehicle by way of i.p. injection and were killed 2 or 48 h later. Liver-derived lymphocytes were cultured without further stimulation for 2 h in Brefeldin A, before being labeled with surface mAb, fixed, and permeabilized for intracellular cytokine staining. Cells were analyzed by flow cytometry and CD1d/α-GalCer$^+$αβTCR$^+$ cells were electronically gated and analyzed for intracellular IFN-γ production and CD4 expression.

TCR Jα18$^{-/-}$ mice, some of which also were treated with a neutralizing anti–IL-10 mAb. The activity of this batch of mAb was confirmed by using an inhibition ELISA (unpublished data) at two to four times the amount (400 μg/mouse on days 0, 4, and 8) known to effectively block IL-10 signaling in other in vivo models (33–35). As shown in Fig. 6 A, IL-4$^{-/-}$ liver-derived NKT cells were significantly ($P < 0.05$) more protective than were the same number of WT liver-derived NKT cells. Furthermore, IL-4$^{-/-}$ thymus-derived NKT cells also were able to protect in this model, in sharp contrast to WT thymus-derived NKT cells. Neutralizing anti–IL-10 had little additional effect. One possible explanation for the enhanced tumor immunity after transfer of IL-4$^{-/-}$ cells was that in the absence of IL-4 production, NKT cell–derived IFN-γ production was enhanced. We tested this by comparing IFN-γ production by NKT cells in IL-4$^{-/-}$ and WT mice after α-GalCer administration in vivo. As shown in Fig. 6 B, IFN-γ production was comparable between CD4$^+$ and CD4$^-$ NKT cell subsets from each strain 2 and 48 h after stimulation. Taken together, this suggests that thymus-derived NKT cells are capable of responding to the tumor, but are unable to do so because IL-4 production antagonizes their ability to mediate IFN-γ–dependent tumor rejection. How liver-derived NKT cells inhibit tumor growth—despite similar levels of IL-4 production to thymus-derived NKT cells—is unclear but might relate to other (undefined) cytokines that are expressed differentially between these NKT cell subsets, or by differences in their ability to transactivate NK cells and DCs. This latter possibility was investigated using in vitro cultures in which NKT cells from thymus or liver of WT mice were cocultured for 12 h, in the presence of α-GalCer, with liver mononuclear cells from Jα18$^{-/-}$ mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050953/DC1). This induced IFN-γ production by NK cells, enhanced CD69 expression by DCs and NK cells, and increased the percentage of CD86$^+$ DCs. However, we found no obvious difference in the ability of thymus- and liver-derived NKT cells in this assay that would explain the differential antitumor immunity observed.

DISCUSSION

We previously reported that adoptive transfer of liver-derived NKT cells restores antitumor immunity to TCR Jα18$^{-/-}$ mice (24, 25). These and other studies presumed that liver-derived NKT cells were a functionally homogenous population that was representative of all NKT cells. However, our current study demonstrates that liver NKT cells, and primarily the CD4$^-$ subset, are unique in their ability to confer protective antitumor responses. It is not clear why spleen- and thymus-derived NKT cells do not confer similar protective responses, but there are several plausible explanations. We considered the possibility that thymus-derived NKT cells are functionally less mature than NKT cell populations that are found in the periphery. Yet our data, and that of other investigators, clearly demonstrate that thymus-derived NKT cells are potent cytokine producers after in vitro stimulation (15, 17, 36–39), and that these cells survive in the periphery after adoptive transfer (31). Furthermore, adoptive transfer of thymus- (40) or spleen-derived (41) NKT cells into NOD mice conferred IL-4–

and/or IL-10–dependent suppression of type 1 diabetes, which demonstrated that these cells can mediate effector functions in vivo. Spleen-derived NKT cells are also clearly functional because they can mediate anterior chamber-associated immune deviation in an IL-10–dependent manner (42), and can promote airway hyperresponsiveness by way of IL-4 and -13 production (43); this further supports the idea that they are not functionally immature. Clearly, thymus- and spleen-derived NKT cells can influence immune responses upon adoptive transfer in vivo. This raises the possibility that these cells are specifically less efficient at mediating tumor rejection compared with liver-derived NKT cells, and by definition, should be regarded as a functionally distinct subset. This has interesting implications for other studies that involve the adoptive transfer of liver-derived NKT cells (e.g., references 44, 45), and raises several important questions, such as whether the effects that are mediated by NKT cells in these studies are also influenced by the NKT cell tissue of origin. Considering that these responses appeared to be dependent on NKT cell–derived IL-4 (i.e., the opposite of our current study), this raises the question of whether these functional roles also are unique to liver NKT cells, and whether $CD4^+$ and $CD4^-$ subsets of liver NKT cells contribute equally to the various diseases in which NKT cells are known to play a role.

The ability of IL-$4^{-/-}$ thymus-derived NKT cells to restore α-GalCer–induced antitumor immunity supports the idea that these cells are functionally active upon adoptive transfer, and that they can recognize and respond to the tumor, but that the production of IL-4 antagonizes their ability to promote effective tumor rejection. These findings are reminiscent of observations in a model of experimental autoimmune encephalomyelitis, in which the Th1-type disease was heightened in IL-$4^{-/-}$ mice and was inhibited in IFN-$\gamma^{-/-}$ mice after α-GalCer treatment (46).

This study is the first in vivo demonstration of functionally distinct NKT cell subsets in two disease models. In vitro studies have reported differences in cytokine production between $CD4^+$ and $CD4^-$ NKT cell subsets from mice (15) and humans (28, 30, 47, 48); however, the physiological significance of these results is unclear and does not easily explain the differences in our study. We were unable to determine any clear differences in cytokine production that could explain the functional disparity between $CD4^+$ and $CD4^-$ liver NKT subsets. Both subsets produced similar levels of IFN-γ and IL-4 by cytokine staining, and were detected in similar locations after adoptive transfer, including at the tumor site (albeit in very low numbers) (unpublished data). Further study of factors that are expressed differentially between NKT cell subpopulations is warranted.

Our results demonstrate the importance of studying distinct NKT cell subsets in the various models in which these cells are involved. Specifically, the tissue of origin (e.g., liver, thymus, or spleen) and the subset within that tissue (e.g., $CD4^+$ or $CD4^-$) needs to be considered because the use of different subsets may influence results dramatically. It should be emphasized that our study may not prove to be the most complete breakdown of NKT cell subsets, and that additional ways to subdivide NKT cell populations, based on their functional distinction, may become apparent. An attractive hypothesis is that different NKT cell subsets have different functional roles in vivo. Thus, $CD4^-$ liver-derived NKT cells may be very efficient at promoting Th1-type cell-mediated immunity, whereas other NKT cell subpopulations, such as thymus-, spleen-, and liver-derived $CD4^+$ NKT cells, might be more efficient at conferring immunosuppressive effects. This needs to be tested by comparing the activity of these subsets in other models of NKT cell–dependent immune responses that require a cell-mediated Th1-like or immunosuppressive Th2-like effect. The ability to separate potentially suppressive and/or aggressive NKT cell subsets will be an important step forward for NKT cell research, especially now that these cells are being targeted in α-GalCer–based clinical trials. Global activation of NKT cells by administration of soluble α-GalCer and/or α-GalCer–pulsed DCs could be detrimental because they may induce opposing effects that reduce the efficiency of treatment and/or cause undesirable effects. These could be eliminated by targeting individual NKT cell subsets, which might be achieved by in vitro isolation and stimulation of the desired subset before transfer, or potentially by the targeted suppression of particular subsets in vivo. Future studies should also aim to determine whether NKT cell subsets are preconditioned in their relative environments before transfer, or whether distinct functional capabilities are intrinsic to each subset upon export from the thymus. Thus, the novel findings that are reported in this study are likely to have major implications for future studies that investigate the potential of NKT cells in immunotherapy.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 WT mice were bred in-house at the Department of Microbiology and Immunology Animal House, Melbourne University, or the Peter MacCallum Cancer Institute Animal House. TCR J$\alpha18^{-/-}$ mice (provided by M. Taniguchi, Chiba University Graduate School of Medicine, Chiba, Japan and backcrossed to C57BL/6 for 12 generations) and C57BL/6 IL-$4^{-/-}$ mice (The Jackson Laboratory) were bred at the Peter MacCallum Cancer Institute. Mice of 6–14 wk of age were used in all experiments that were performed according to animal experimental committee guidelines.

Isolation of NKT cell subsets. Lymphocytes were isolated from the liver by cutting it into small pieces and gently pressing it through 200-μm mesh sieves into PBS containing 2% FCS (Commonwealth Serum Laboratories). Lymphocytes were separated from hepatocytes and cellular debris by way of a 33% isotonic Percoll density gradient (GE Healthcare) performed at room temperature. Exposed DNA was removed using DNase (35 μg/ml final concentration) (Roche Diagnostics). Lymphocytes were isolated from the thymus, spleen, and LNs by cutting the organs and gently grinding them between two frosted glass slides into PBS containing 2% FCS. BM was harvested by flushing femurs with PBS containing 2% FCS. Blood was collected, by way of cardiac puncture, into 10 ml of PBS to prevent clotting. Liver, spleen, BM, and PBLs were depleted of RBCs using red cell lysis buffer (Sigma-Aldrich).

NKT cell enrichment. For thymus-derived NKT cell enrichment, thymocytes were labeled with anti-CD8 (clone 3.155) and anti-CD24 (J11D) (both grown in-house), and bound cells were depleted using rabbit comple-

ment (C-six Diagnostics). Clumping of dead cells was avoided by using DNase, and viable cells were collected using a histopaque (1.083 g/ml) gradient (Sigma-Aldrich) performed at room temperature. Cells were washed twice before being surface labeled for flow cytometric sorting. Spleen-derived NKT cells were enriched by B cell depletion. This was achieved in one of two ways in different experiments, either by incubating spleen cells on 90-mm petri dishes that were coated with purified goat anti–mouse Ig (15 μg/ml; Caltag), or anti-B220 labeling followed by magnetic depletion with anti-rat Ig–coated Dynabeads (Dynal). In an alternative enrichment procedure, splenocytes were labeled with α-GalCer–loaded CD1d tetramers (CD1d/α-GalCer) followed by anti-PE microbeads (Miltenyi Biotec). Cells were passed through an automated magnetic cell sorter (autoMACS) enrichment column (Miltenyi Biotec), and collected into PBS containing 2% FCS and 2 mM EDTA (Sigma-Aldrich).

Antibodies and flow cytometric cell sorting. For sorting of NKT cell populations, unfractionated or NKT cell–enriched populations were labeled with combinations of antibodies, including FITC-conjugated anti-αβTCR (clone H57-597), FITC-conjugated anti-CD4 (clone RM4-5), PE-conjugated anti-NK1.1 (clone PK-136), biotinylated anti-CD4 (clone RM4-5), or allophycocyanin-conjugated anti-αβTCR (clone H57-597). For DCs, splenocytes were labeled with PE-conjugated anti-MHC class II (clone M5/114.15.2) and biotinylated anti-CD11c (clone HL3). Biotinylated antibodies were revealed using streptavidin-CyChrome. To avoid nonspecific binding of antibodies to FcR-γ, cells were incubated with anti–mouse CD16/32 (clone 2.4G2) (grown in-house). All flow cytometry reagents were purchased from BD Biosciences unless otherwise indicated. After cell surface labeling, cells were sorted using a FACStar^{PLUS} (Becton Dickinson) or MoFlow (DakoCytomation). Desired populations were collected into 2% normal mouse serum (NMS) in PBS. A sample of sorted cells was always analyzed to assess the purity of these populations. Sorted NK1.1$^+$αβTCR$^+$ (NKT cell) populations were always ≥95% pure, and CD4^{high} (CD4$^+$) and CD4$^{-/low}$ (CD4$^-$) NKT cell subsets were always ≥96% pure, unless otherwise indicated.

Culture media. Culture media contained RPMI 1640 (GIBCO BRL) supplemented with 5% FCS (JRH), 100 U/ml penicillin (GIBCO BRL), 100 μg/ml streptomycin (GIBCO BRL), and 2 mM Glutamax (GIBCO BRL).

Intracellular cytokine staining after in vivo and in vitro α-GalCer stimulation. For in vivo stimulation, WT or IL-4$^{-/-}$ mice received 2 μg α-GalCer or vehicle by way of i.p. injection, and were killed 2 or 48 h later. Lymphocytes were isolated from the liver and spleen and cultured for 2 h, without further stimulation, in 5 μg/ml Brefeldin A (Sigma-Aldrich). For in vitro stimulation, NKT cell–enriched thymocytes or unfractionated liver-derived lymphocytes were cultured for 12 h with sorted spleen-derived DCs and 100 ng/ml α-GalCer in culture media (Life Technologies). Brefeldin A was added for the last 2 h of culture. After culture, in vivo and in vitro stimulated cells were labeled with combinations of cell surface antibodies, including FITC-conjugated anti-CD4 (clone RM4-5), CyChrome-conjugated CD1d/α-GalCer tetramer (produced in-house), PerCP-conjugated anti-CD4 (clone RM4-5), and allophycocyanin-conjugated anti-αβTCR (clone H57-597). Following cell surface labeling, cells were washed once in PBS, and fixed in 0.5% paraformaldehyde in PBS (BDH Chemicals) for 30–45 min in the dark at room temperature. Cells were washed twice in PBS and incubated for 1 h in the dark at room temperature with PE-conjugated anti–IFN-γ (clone XMG1.2), anti-IL-4 (clone 11B11), or rat IgG1 (clone R3-34 as an isotype control) in 0.05% saponin (Sigma-Aldrich) in 5% FCS in PBS.

MCA-1 subcutaneous sarcoma model. The MCA-1 sarcoma cell line was derived from a TCR Jα18$^{-/-}$ mouse injected with 100 μg MCA and maintained as described previously (23). For growth of MCA-1, WT and TCR Jα18$^{-/-}$ mice were injected s.c. in the right hind flank with 10^5 sarcoma cells. From 1–2 h later, subgroups of TCR Jα18$^{-/-}$ recipient mice received one of the following populations of cells from WT donors by way of i.v. injection: unfractionated liver-derived lymphocytes; sorted liver-, spleen-, or thymus-derived NK1.1$^+$αβTCR$^+$ cells; sorted liver-derived CD4$^+$ or CD4$^-$ NKT cell subsets; or 2% NMS in PBS. Tumor growth was measured every second day with a caliper square as the product of two diameters. Results were recorded as the mean tumor size (cm^2) ± SEM.

B16F10 lung metastases model. B16F10 melanoma cells were maintained as described previously (25). WT or TCR Jα18$^{-/-}$ mice received 5×10^5 B16F10 cells by way of i.v. injection. 3 h later, some TCR Jα18$^{-/-}$ recipient mice received one of the following populations from WT donors by way of i.v. injection, unless otherwise indicated: unfractionated liver-derived lymphocytes, NKT cell–enriched thymocytes or splenocytes, sorted thymus-derived NK1.1$^+$αβTCR$^+$ cells, sorted liver- and thymus-derived CD4$^+$ or CD4$^-$ NKT cell subsets, or 2% NMS in PBS. 1–2 h after lymphocyte transfer, and again on days 4 and 8, mice also received 2 μg α-GalCer (provided by Kirin Breweries Pharmaceutical Research Laboratories, Gunma, Japan, that was prepared in saline supplemented with 0.5% polysorbate-20). For IL-10 neutralization, some mice also were treated with 400 μg anti–IL-10 (clone JES-2A5) or control IgG on days 0, 4, and 8. Mice were killed on day 14, and surface lung metastases were counted with the aid of a dissecting microscope as described previously (49). Data were recorded as the mean number of metastases ± SEM.

In vivo detection of adoptively transferred NKT cells. NKT cell–enriched thymocytes were washed once in 0.1% BSA in PBS, before being labeled in 1 ml 0.1% BSA in PBS with 4 μl of 1 mM 5-(and-6)-CFSE (Invitrogen) for 10 min at 37°C in the dark. The reaction was quenched with PBS containing 20% FCS before cells were washed twice in RPMI 1640 and once in PBS, and transferred into recipient mice by way of i.v. injection. In some experiments, cells were labeled with anti-CD4 before transfer. Organs (liver, spleen, PBLs, BM, LNs, and thymus) were harvested 6 d after transfer, and screened by flow cytometry for the presence of CFSE$^+$ cells. CFSE$^+$ cells were analyzed for CD1d/α-GalCer tetramer-binding, αβTCR, and CD4 expression.

Statistical tests. Significant differences in tumor growth rate between PBS/vehicle-injected TCR Jα18$^{-/-}$ control groups and multiple test groups were determined using a Kruskal-Wallis test, followed by a Dunn's post test. Significant differences between vehicle-injected TCR Jα18$^{-/-}$ control groups and a single test group were determined using a Mann-Whitney U test.

Online supplemental material. Fig. S1 shows that thymus, spleen, and liver NKT cells induce similar amounts of bystander cell activation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050953/DC1.

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REFERENCES

1. Godfrey, D.I., H.R. MacDonald, M. Kronenberg, M.J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? *Nat. Rev. Immunol.* 4:231–237.
2. Hayakawa, Y., D.I. Godfrey, and M.J. Smyth. 2004. Alpha-galactosylceramide: potential immunomodulatory activity and future application. *Curr. Med. Chem.* 11:241–252.
3. Zhou, D., J. Mattner, C. Cantu III, N. Schrantz, N. Yin, Y. Gao, Y. Sagiv, K. Hudspeth, Y.P. Wu, T. Yamashita, et al. 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science.* 306:1786–1789.
4. Mattner, J., K.L. Debord, N. Ismail, R.D. Goff, C. Cantu III, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, et al. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature.* 434:525–529.
5. Kinjo, Y., D. Wu, G. Kim, G.W. Xing, M.A. Poles, D.D. Ho, M. Tsuji, K. Kawahara, C.H. Wong, and M. Kronenberg. 2005. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature.* 434:520–525.
6. Wu, D., G.W. Xing, M.A. Poles, A. Horowitz, Y. Kinjo, B. Sullivan, V. Bodmer-Narkevitch, O. Plettenburg, M. Kronenberg, M. Tsuji, et al. 2005. Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells. *Proc. Natl. Acad. Sci. USA.* 102:1351–1356.
7. Sriram, V., W. Du, J. Gervay-Hague, and R.R. Brutkiewicz. 2005. Cell wall glycosphingolipids of *Sphingomonas paucimobilis* are CD1d-specific ligands for NKT cells. *Eur. J. Immunol.* 35:1692–1701.
8. Fischer, K., E. Scotet, M. Niemeyer, H. Koebernick, J. Zerrahn, S. Maillet, R. Hurwitz, M. Kursar, M. Bonneville, S.H. Kaufmann, and U.E. Schaible. 2004. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc Natl Acad Sci USA.*101:10685–10690.
9. Rauch, J., J. Gumperz, C. Robinson, M. Skold, C. Roy, D.C. Young, M. Lafleur, D.B. Moody, M.B. Brenner, C.E. Costello, and S.M. Behar. 2003. Structural features of the acyl chain determine self-phospholipid antigen recognition by a CD1d-restricted invariant NKT (iNKT) cell. *J Biol Chem.* 278:47508–47515.
10. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for V(Alpha)14 Nkt Cells in Il-12-Mediated Rejection of Tumors. *Science.* 278:1623–1626.
11. Chen, Y.H., N.M. Chiu, M. Mandal, N. Wang, and C.R. Wang. 1997. Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. *Immunity.* 6:459–467.
12. Mendiratta, S.K., W.D. Martin, S. Hong, A. Boesteanu, S. Joyce, and L. Van Kaer. 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity.* 6:469–477.
13. Smiley, S.T., M.H. Kaplan, and M.J. Grusby. 1997. Immunoglobulin E production in the absence of interleukin-4–secreting Cd1-dependent cells. *Science.* 275:977–979.
14. Eberl, G., R. Lees, S.T. Smiley, M. Taniguchi, M.J. Grusby, and H.R. MacDonald. 1999. Tissue-specific segregation of CD1d-dependent and CD1d-independent NK T cells. *J. Immunol.* 162:6410–6419.
15. Hammond, K.J.L., S.B. Pelikan, N.Y. Crowe, E. Randle-Barrett, T. Nakayama, M. Taniguchi, M.J. Smyth, I.R. van Driel, R. Scollay, A.G. Baxter, and D.I. Godfrey. 1999. NKT cells are phenotypically and functionally diverse. *Eur. J. Immunol.* 29:3768–3781.
16. Matsuda, J.L., O.V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C.R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 192:741–753.
17. Hammond, K.J.L., D.G. Pellicci, L.D. Poulton, O.V. Naidenko, A.A. Scalzo, A.G. Baxter, and D.I. Godfrey. 2001. CD1d-restricted NKT cells: an interstrain comparison. *J. Immunol.* 167:1164–1173.
18. Godfrey, D.I., and M. Kronenberg. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. *J. Clin. Invest.* 114:1379–1388.
19. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 23:877–900.
20. Brigl, M., and M.B. Brenner. 2004. CD1: antigen presentation and T cell function. *Annu. Rev. Immunol.* 22:817–890.
21. Wilson, S.B., and T.L. Delovitch. 2003. Janus-like role of regulatory iNKT cells in autoimmune disease and tumour immunity. *Nat. Rev. Immunol.* 3:211–222.
22. Smyth, M.J., N.Y. Crowe, Y. Hayakawa, K. Takeda, H. Yagita, and D.I. Godfrey. 2002. NKT cells—conductors of tumor immunity? *Curr. Opin. Immunol.* 14:165–171.
23. Smyth, M.J., K.Y.T. Thia, S.E.A. Street, E. Cretney, J.A. Trapani, M. Taniguchi, T. Kawano, S.B. Pelikan, N.Y. Crowe, and D.I. Godfrey. 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med.* 191:661–668.
24. Crowe, N.Y., M.J. Smyth, and D.I. Godfrey. 2002. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J. Exp. Med.* 196:119–127.
25. Smyth, M.J., N.Y. Crowe, D.G. Pellicci, K. Kyparissoudis, J.M. Kelly, K. Takeda, H. Yagita, and D.I. Godfrey. 2002. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood.* 99:1259–1266.
26. Yang, Y., A. Ueno, M. Bao, Z. Wang, J.S. Im, S. Porcelli, and J.W. Yoon. 2003. Control of NKT cell differentiation by tissue-specific microenvironments. *J. Immunol.* 171:5913–5920.
27. Lee, P.T., K. Benlagha, L. Teyton, and A. Bendelac. 2002. Distinct functional lineages of human V alpha-24 natural killer T cells. *J. Exp. Med.* 195:637–641.
28. Gumperz, J.E., S. Miyake, T. Yamamura, and M.B. Brenner. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J. Exp. Med.* 195:625–636.
29. Bollyky, P.L., and S.B. Wilson. 2004. CD1d-restricted T-cell subsets and dendritic cell function in autoimmunity. *Immunol. Cell Biol.* 82: 307–314.
30. Rogers, P.R., A. Matsumoto, O. Naidenko, M. Kronenberg, T. Mikayama, and S. Kato. 2004. Expansion of human Valpha24(+) NKT cells by repeated stimulation with KRN7000. *J. Immunol. Methods.* 285:197–214.
31. Matsuda, J.L., L. Gapin, S. Sidobre, W.C. Kieper, J.Y.T. Tan, R. Ceredig, C.D. Surh, and M. Kronenberg. 2002. Homeostasis of V(alpha)14i NKT cells. *Nat. Immunol.* 3:966–974.
32. Pellicci, D.G., A.P. Uldrich, K. Kyparissoudis, N.Y. Crowe, A.G. Brooks, K.J. Hammond, S. Sidobre, M. Kronenberg, M.J. Smyth, and D.I. Godfrey. 2003. Intrathymic NKT cell development is blocked by the presence of alpha-galactosylceramide. *Eur. J. Immunol.* 33:1816–1823.
33. Nishigori, C., D. Yarosh, A. O'Connor, V. Shreedhar, S.E. Ullrich, P. Cox, and M. Kripke. 1998. HindIII liposomes suppress DTH responses in vivo and induce epidermal IL-10 in vitro. *J. Immunol.* 161:2684–2691.
34. Hunter, M., A. Wang, C. Hirota, and D. McKay. 2005. Neutralizing anti-IL10 antibody blocks the protective effect of tapeworm infection in a murine model of chemically induced colitis. *J. Immunol.* 174:7368–7375.
35. Gorczynski, R., Z. Chen, H. Zeng, and X. Ming Fu. 1997. Specificity for in vivo graft prolongation in gd TCR+ hybridomas derived from mice given portal vein donor specific preimmunization and skin allografts. *J. Immunol.* 159:3698–3706.
36. Zlotnik, A., D.I. Godfrey, M. Fischer, and T. Suda. 1992. Cytokine production by mature and immature $CD4^{-}CD8^{-}$ T cells. Alpha beta-T cell $receptor^{+}$ $CD4^{-}CD8^{-}$ T cells produce IL-4. *J. Immunol.* 149:1211–1215.
37. Arase, H., N. Arase, K. Nakagawa, R.A. Good, and K. Onoe. 1993. NK1.1+ CD4+ CD8− thymocytes with specific lymphokine secretion. *Eur. J. Immunol.* 23:307–310.
38. Pellicci, D.G., K.J.L. Hammond, A.P. Uldrich, A.G. Baxter, M.J. Smyth, and D.I. Godfrey. 2002. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1(−) CD4(+) CD1d-dependent precursor stage. *J. Exp. Med.* 195:835–844.
39. Benlagha, K., T. Kyin, A. Beavis, L. Teyton, and A. Bendelac. 2002. A thymic precursor to the NK T cell lineage. *Science.* 296:553–555.
40. Hammond, K.J.L., L.D. Poulton, L.J. Palmisano, P.A. Silveira, D.I. Godfrey, and A.G. Baxter. 1998. Alpha/beta-T cell receptor (Tcr)(+)Cd4(−)Cd8(−) (Nkt) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (Nod)/Lt mice by the influence

of interleukin (Il)-4 and/or Il-10. *J. Exp. Med.* 187:1047–1056.
41. Lehuen, A., O. Lantz, L. Beaudoin, V. Laloux, C. Carnaud, A. Bendelac, J.F. Bach, and R.C. Monteiro. 1998. Overexpression of natural killer T cells protects V-alpha-14-J-alpha-281 transgenic nonobese diabetic mice against diabetes. *J. Exp. Med.* 188:1831–1839.
42. Sonoda, K.H., D.E. Faunce, M. Taniguchi, M. Exley, S. Balk, and J. Stein-Streilein. 2001. NK T cell–derived IL-10 is essential for the differentiation of antigen-specific T regulatory cells in systemic tolerance. *J. Immunol.* 166:42–50.
43. Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, T. Nakayama, M. Taniguchi, M.J. Grusby, R.H. DeKruyff, and D.T. Umetsu. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* 9:582–588.
44. Campos, R.A., M. Szczepanik, A. Itakura, M. Akahira-Azuma, S. Sidobre, M. Kronenberg, and P.W. Askenase. 2003. Cutaneous immunization rapidly activates liver invariant Valpha14 NKT cells stimulating B-1 B cells to initiate T cell recruitment for elicitation of contact sensitivity. *J. Exp. Med.* 198:1785–1796.
45. Kim, H.Y., H.J. Kim, H.S. Min, S. Kim, W.S. Park, S.H. Park, and D.H. Chung. 2005. NKT cells promote antibody-induced joint inflammation by suppressing transforming growth factor {beta}1 production. *J. Exp. Med.* 201:41–47.
46. Pal, E., T. Tabira, T. Kawano, M. Taniguchi, S. Miyake, and T. Yamamura. 2001. Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of V alpha 14 NK T cells. *J. Immunol.* 166:662–668.
47. Lee, P.T., K. Benlagha, L. Teyton, and A. Bendelac. 2002. Distinct functional lineages of human V(alpha)24 natural killer T cells. *J. Exp. Med.* 195:637–641.
48. Lin, H., M. Nieda, and A.J. Nicol. 2004. Differential proliferative response of NKT cell subpopulations to in vitro stimulation in presence of different cytokines. *Eur. J. Immunol.* 34:2664–2671.
49. Smyth, M.J., K.Y.T. Thia, S.E.A. Street, D. MacGregor, D.I. Godfrey, and J.A. Trapani. 2000. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *J. Exp. Med.* 192:755–760.