The irregular patch distribution also produces another phenomenon noted in real populations (*7*). The endogenous regular oscillations in our model, together with the exponentially decaying dispersal between patches, generally maintains rather regular cycles. This explains why a wide range of cross-correlation values is observed for pairs of populations. The cycles thus produced are not necessarily persistent. Pronounced and regular population cycles may gradually fade away and subsequently recover (Fig. 1G). Hence, both the amplitude of population fluctuations and the spatial synchrony between pairs of populations changes considerably over time, without any other source of randomness than the spatial location of the interconnected subpopulations. The dispersal is probabilistic because of the assumption of an exponential distribution of dispersal distances, but a regular grid of patches with exactly the same kind of dispersal did not generate the pattern we observed in this model or in the data.

Thus, we conclude that the spatial structure may be of great importance for the dynamics of populations. Most previous studies have concentrated on either the internal dynamics within patches or the rules for dispersal between them (*4*, *5*) in order to explain large-scale spatial dynamics. Here, we used a simple, albeit realistic model for which the assumptions are well substantiated. Delayed density-dependence seems to be the rule rather than exception for a wide range of taxa (*8*, *11*), the exponential distribution of dispersal distances is well established (*12*), and the irregular distribution of patches is true for all natural environments. These are the simple building blocks on which important analyses of spatial population dynamics can rest.

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9. In an arena of 30 units by 30 units, 25 populations were randomly placed (median and interquartile distances were 10.3, 7.4, and 14.2 units). They renewed after

$$X_i(k+1) = (1-m)F[X_i(k), X_i(k-1)] + \sum_{s=1, s\neq i}^{n} M_{si}(k)$$

$i = 1, \ldots, n$, $k = 1, 2, 3, \ldots$,

where $F[X_i(k), X_i(k-1)] = X_i(k)\exp[r + a_1x_i(k) + a_2X_i(k-1)]$, r is an intrinsic rate of increase, and a_1 and a_2 are parameters. M_{si} is the number of immigrants from patch s to patch i after (*3*)

$$M_{si}(k) = mF[X_s(k), X_s(k-1)]\frac{\exp(-cd_{si})}{\sum_{j,j\neq s}\exp(-cd_{sj})}$$

(d_{si} is the distance between patches s and i and $m = 0.1$ and $c = 0.75$ are parameters telling the proportion emigrating and distance emigrated, respectively). The other parameter values used, $r = 0.47$, $a_1 = 0.056$, and $a_2 = -0.1$, yield deterministic cyclic dynamics with a 10.8-year period. The populations were initiated in phase ($X_{i,1} = X_{i,2} = 10$) and left to renew for 1000 generations. From the next 1500 generations, a 68-year sequence was randomly selected for further analyses. Experimentation suggests that the outcome, as we report, is achievable by cyclic dynamics alone or with damped or chaotic dynamics when superimposed with correlated disturbances (*3*, *6*). Thus, the feature we report (Fig. 1, E through G) is not unique for the selected parameters.
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Requirement for $V_\alpha14$ NKT Cells in IL-12–Mediated Rejection of Tumors

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A lymphocyte subpopulation, the $V_\alpha14$ natural killer T (NKT) cells, expresses both NK1.1 and a single invariant T cell receptor encoded by the $V_\alpha14$ and $J_\alpha281$ gene segments. Mice with a deletion of the $J_\alpha281$ gene segment were found to exclusively lack this subpopulation. The $V_\alpha14$ NKT cell–deficient mice could no longer mediate the interleukin-12 (IL-12)–induced rejection of tumors. Although the antitumor effect of IL-12 was thought to be mediated through natural killer cells and T cells, $V_\alpha14$ NKT cells were found to be an essential target of IL-12, and they mediated their cytotoxicity by an NK-like effector mechanism after activation with IL-12.

$V_\alpha14$ NKT cells, originally defined as $NK1.1^+$ T cells (*1*), are a distinct lymphoid lineage expressing several characteristics that demarcate them from T cells, natural killer (NK) cells, and B cells. First, $V_\alpha14$ NKT cells develop outside of the thymus at an early stage of embryogenesis (*2*) and belong to the $CD4^-CD8^-$ or $CD4^+CD8^-$ populations that express heat-stable antigen and the $B220^+$, $Mac\text{-}1^+$, $CD44^+$, $CD45RB^{dim}$, and $Mel\text{-}14^{dim}$ phenotypes (*3*–*5*). Second, $V_\alpha14$ NKT cells express a single invariant T cell receptor α chain (TCRα) encoded by the $V_\alpha14$ and $J_\alpha281$ segments (*4*, *6*, *7*), mostly in association with $V_\beta8$ (*3*, *5*), which is not expressed on conventional T cells (*8*). Because of the unique expression of invariant $V_\alpha14$ TCR, transgenic (tg) mice that only express the invariant $V_\alpha14$ TCR and no endogenous TCRα ($TCR\alpha^{-/-}$ $V_\alpha14^{tg}$) preferentially develop $V_\alpha14$ NKT cells and block conventional $\alpha\beta^+$ T cell development (*8*). Therefore, we speculated that disruption of the genes encoding the invariant $V_\alpha14$ TCR would generate mice lacking $V_\alpha14$ NKT cells while leaving the other lymphoid lineages intact.

IL-12 is known to mediate antitumor effects and was originally hypothesized to interact with NK cells or $CD8^+$ cytotoxic T lymphocytes (CTLs) (*9*). However, recent studies indicate that the systemic administration of IL-12 activates $NK1.1^+$ $TCR^{intermediate}$ cells (which are similar to $V_\alpha14$ NKT cells) and inhibits the hepatic metastasis of tumor (*10*). It is thus unclear whether IL-12 interacts with NK, T, or $V_\alpha14$ NKT cells. It is possible that $V_\alpha14$ NKT cells may be responsible for functions that have been reported for NK cells, because purifications were difficult and the studies were done with a mixture of NK and $V_\alpha14$ NKT cells. We investigated

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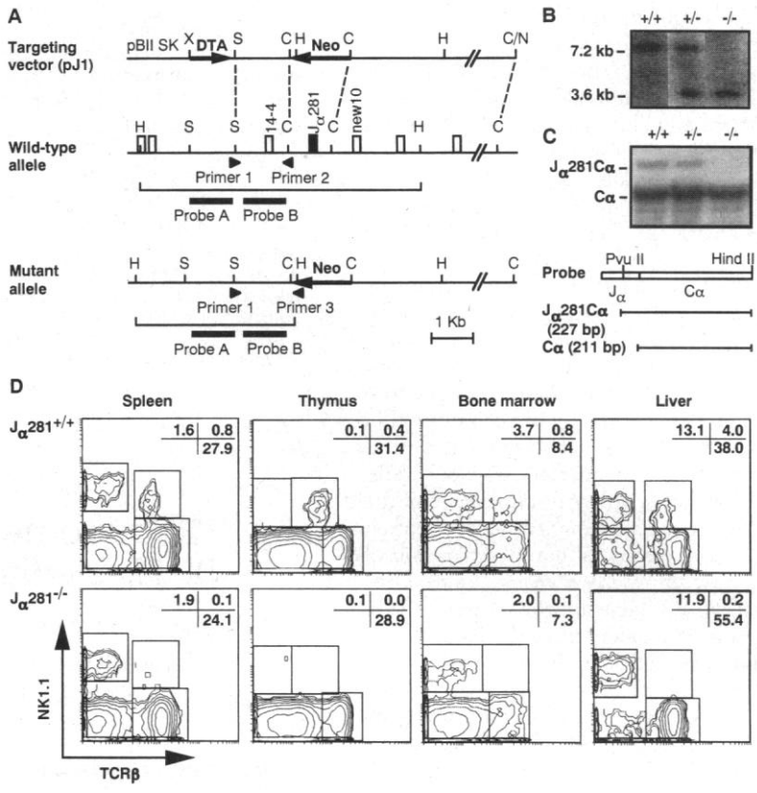

Fig. 1. Generation and analysis of $J_\alpha281^{-/-}$ ($V_\alpha14$ NKT–deficient) mice. (**A**) Targeting construct. Targeting vector pJ1 (top) was designed to replace the Cla I fragment (1.0 kb) containing the $J_\alpha281$ gene segment (middle) with a diphtheria toxin A (DTA) cassette and a neomycin resistant gene cassette (designated Neo) derived from pPGK-*neo*^{r}-poly A (PGK, 3-phosphoglycerate kinase). Sal I–Cla I (1.2 kb) and Cla I (12 kb) genomic fragments of 129Sv/J origin were used for the short and long arms, respectively. The targeting vector was introduced into R1 embryonic stem cells of 129 mice by electroporation. Germline chimeric mice were generated by aggregation methods (*11*). Mutant mice were backcrossed three generations with C57BL/6 mice. Mutant alleles (bottom) were detected by Southern (DNA) blot analysis with probes A and B. X, Xho I; S, Sal I; C, Cla I; H, Hind III; N, Not I. (**B**) Southern blot analysis. Wild-type and mutant alleles were detected by Hind III digestion as 7.2-kb and 3.6-kb bands, respectively. (**C**) Ribonuclease protection assay with the $J_\alpha281C\alpha$ probe (*12*), which detects the $J_\alpha281C\alpha$ (227 bp) and $C\alpha$ (211 bp) bands. (**D**) Fluorescence-activated cell sorter (FACS) profiles. The percent of the gated populations analyzed by FACS (*23*) is indicated. Total numbers of hematopoietic cells in $J_\alpha281^{-/-}$ mice were as follows: 2.2×10^6 NK1.1$^+$, 3.0×10^7 $\alpha\beta^+$ T, 1×10^6 $\gamma\delta^+$ T, 3.0×10^7 IgM$^+$ B, 6.5×10^6 Mac–1$^+$, and 1.5×10^6 Gr-1$^+$ cells.

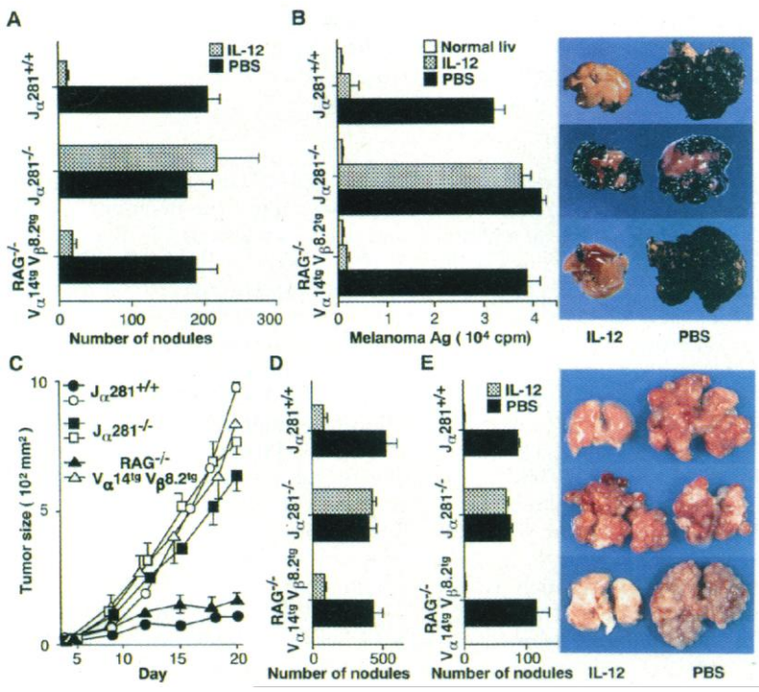

Fig. 2. Inhibition of tumor growth or metastasis in IL-12–treated mice. Protocols are in (*24*). (**A**) Number of metastatic nodules of FBL-3 in the liver. (**B**) Amounts of GM3 melanoma antigens (Ag) in the metastatic liver and their representative photographic views of B16 melanoma. Methods for measurement of melanoma antigens are in (*22*). Each group had five to seven mice; cpm, counts per minute. (**C**) Subcutaneous growth of B16. Symbols for mice treated with PBS (open) or IL-12 (closed) are indicated. (**D**) Number of metastatic nodules of B16 in the lung. (**E**) Number of metastatic nodules of LLC in the lung and their representative photographic views. Each group had three mice.

the antitumor effects of IL-12 to determine if the functional target of the cytokine in vivo is NK cells, CD8$^+$ CTLs, or $V_\alpha14$ NKT cells.

To address this question, we generated mice that lacked invariant $V_\alpha14$ $J_\alpha281$ TCR expression by specific deletion of the $J_\alpha281$ gene segment using homologous recombination and aggregation chimera techniques (*11*) (Fig. 1, A and B). The loss of $J_\alpha281$ gene expression in $J_\alpha281^{-/-}$ mice was confirmed by ribonuclease (RNase) protection assay (*12*) (Fig. 1C). Thus, the invariant $V_\alpha14$ $J_\alpha281$ TCR is not expressed in $J_\alpha281^{-/-}$ mice. The development of the lymphoid organs in $J_\alpha281^{-/-}$ mice was macroscopically normal, and the numbers of lymphocytes were almost the same as in wild-type mice. However, the relative numbers of NK1.1$^+$ TCRβ^+ cells in the thymus, spleen, bone marrow, and liver were reduced in $J_\alpha281^{-/-}$ mice (Fig. 1D). On the other hand, the total number of other hematopoietic cells were not affected (see legend to Fig. 1). Thus, the defects in hematopoietic cell development were subtle, and $V_\alpha14$ NKT cells were exclusively affected. This confirms the requirement of invariant $V_\alpha14$ TCR for the development of $V_\alpha14$ NKT cells (*8*).

To elucidate the functional defects in vivo that arose as a result of the selective loss of the $V_\alpha14$ NKT cell population, we investigated the antitumor activity induced by IL-12. One model was intrasplenic injection

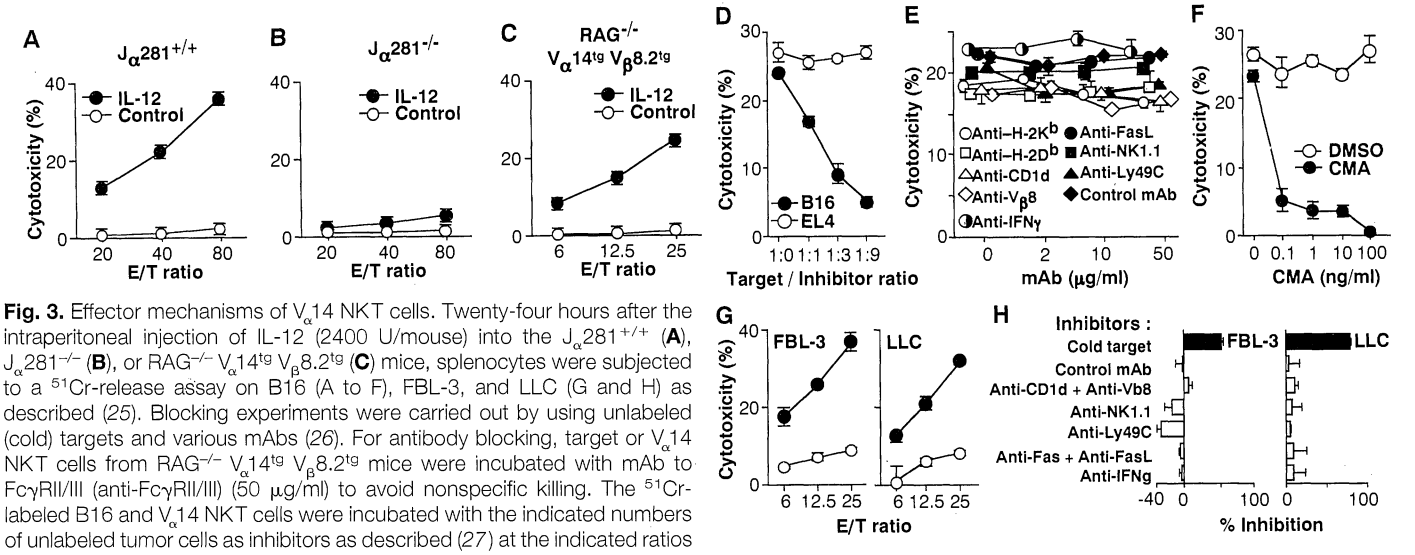

Fig. 3. Effector mechanisms of $V_\alpha14$ NKT cells. Twenty-four hours after the intraperitoneal injection of IL-12 (2400 U/mouse) into the $J_\alpha281^{+/+}$ (**A**), $J_\alpha281^{-/-}$ (**B**), or RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$ (**C**) mice, splenocytes were subjected to a ^{51}Cr-release assay on B16 (A to F), FBL-3, and LLC (G and H) as described (*25*). Blocking experiments were carried out by using unlabeled (cold) targets and various mAbs (*26*). For antibody blocking, target or $V_\alpha14$ NKT cells from RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$ mice were incubated with mAb to FcγRII/III (anti-FcγRII/III) (50 μg/ml) to avoid nonspecific killing. The ^{51}Cr-labeled B16 and $V_\alpha14$ NKT cells were incubated with the indicated numbers of unlabeled tumor cells as inhibitors as described (*27*) at the indicated ratios (**D**) and with mAbs (*26*) at the indicated concentrations (**E**). In some experiments, $V_\alpha14$ NKT cells were incubated with CMA (Wako Pure Chemical Industries, Osaka) or control vehicle (dimethyl sulfoxide; DMSO) (**F**) at the indicated concentrations as described (*19*). IL-12–activated $V_\alpha14$ NKT cells were also tested for their cytotoxicity on FBL-3 and LLC (**G**), whose activities were abrogated by cold targets (**H**) but not by mAbs (*26*). The assays were performed at an effector to target (E/T) ratio of 25:1 in the blocking experiments. The data are expressed as the mean of three cultures ± SD.

of FBL-3 erythroleukemia (Fig. 2A) or B16 melanoma (Fig. 2B), which preferentially metastasize to the liver (*13*). Intraperitoneal administration of IL-12 after the intrasplenic graft of FBL-3 or B16 suppressed metastasis in the liver of $J_\alpha281^{+/+}$ wild-type but not $J_\alpha281^{-/-}$ mice (Fig. 2, A and B). Because the mutant mice exclusively lack $V_\alpha14$ NKT cells, with the other lymphoid populations left intact ($V_\alpha14$ NKT–deficient mice) (Fig. 1D), this implies that $V_\alpha14$ NKT cells are a primary functional target of IL-12 in vivo. To exclude the possibility that a T cell population other than $V_\alpha14$ NKT cells is the primary target of IL-12, we examined antitumor cytotoxicity in transgenic ($V_\alpha14^{tg}$ $V_\beta8.2^{tg}$) mice lacking recombination activating gene (RAG) (RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$), which preferentially generate $V_\alpha14$ NKT cells but block the development of any other lymphocyte lineages, including NK, B, and T cells ($V_\alpha14$ NKT mice) (*14*). In these mice, FBL-3 or B16 tumor cells were rejected as in wild-type mice (Fig. 2, A and B), indicating that NK and T cells are not required for IL-12–mediated tumor rejection. Similar results were also obtained with other tumor systems, such as subcutaneous growth of B16 (Fig. 2C) and pulmonary metastases of B16 (Fig. 2D) or Lewis lung carcinoma (LLC) cells (Fig. 2E). These results confirm the previous observation of the protective effects of IL-12 on those tumors in vivo (*15–17*). In addition, RAG$^{-/-}$ mice having only NK cells could not reject metastasis (*18*), and IL-12 is effective in NK cell–deficient *beige* mice or anti-asialoGM1-treated NK-depleted mice (*17*), indicating that NK cells are not a primary target of IL-12. Thus, conventional T and NK cells seem to be unnecessary for the IL-12–mediated rejection of tumors.

We further examined IL-12–induced antitumor activity in vitro (Fig. 3). The induction of antitumor cytotoxicity was impaired in $J_\alpha281^{-/-}$ mice (Fig. 3B), whereas B16 was killed in RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$ mice to a similar extent as in $J_\alpha281^{+/+}$ mice (Fig. 3, A and C), again confirming that $V_\alpha14$ NKT cells are responsible for IL-12–mediated cytotoxicity. The most important observation on the effector mechanisms is that $V_\alpha14$ NKT cells kill the target through direct contact, because their cytotoxicity was specifically inhibited by unlabeled B16 (Fig. 3D). However, the molecules responsible for the killing interactions have not been identified yet because none of the monoclonal antibodies (mAbs) so far tested successfully blocked $V_\alpha14$ NKT cell–mediated cytotoxicity (Fig. 3E). In addition, the cytotoxicity was abrogated by treatment with concanamycin A (CMA) (Fig. 3F). Because CMA is known to be a specific inhibitor of vacuolar-type H^+-dependent adenosine triphosphatase and it inhibits the activity of perforin (*19*), the effector function is likely to be mediated by NK-like mechanisms. Similar results were also observed with other tumors, including FBL-3 and LLC (Fig. 3, G and H).

Interferon-γ (IFN-γ) is reported to be important in the IL-12–mediated cytotoxicity because it is essential for CTL generation and NK cell activation (*20*). The depletion of CD8$^+$ CTLs and the blocking of IFN-γ

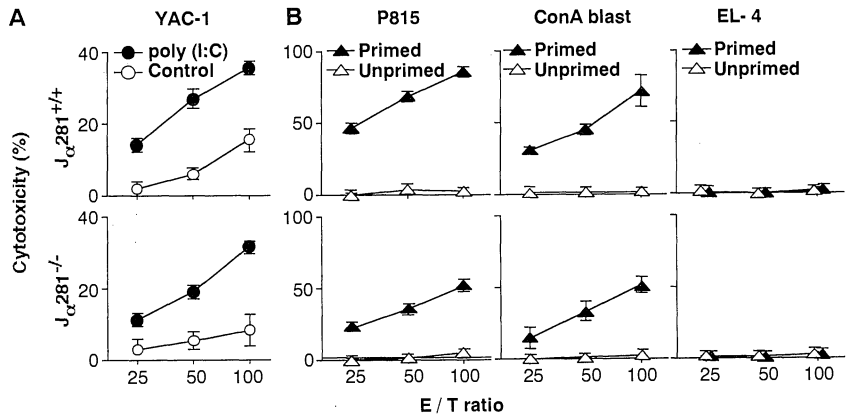

Fig. 4. Potential cytotoxicity of NK and conventional T cells in $J_\alpha281^{-/-}$ mice. (**A**) NK activity. Poly(I:C) (150 μg per mouse; Pharmacia) or PBS (control) was injected intraperitoneally into $J_\alpha281^{+/+}$ and $J_\alpha281^{-/-}$ mice. After 24 hours, spleen cells were tested on YAC-1 cells. (**B**) Allospecific CTL activity. On day 0, P815 mastocytoma (3×10^7; H-2^d) or PBS was injected intraperitoneally (primed and unprimed, respectively) into $J_\alpha281^{+/+}$ and $J_\alpha281^{-/-}$ mice. Spleen cells prepared on day 14 were assayed on P815, ConA-activated BALB/c splenocytes (H-2^d), and EL-4 tumor cells (H-2^b). The data are expressed as the mean of three cultures ± SD.

function with mAb to IFN-γ reduce the efficacy of IL-12 (*16*, *17*). Although $V_\alpha14$ NKT cells are the major source of IFN-γ (*14*), IFN-γ may not be important in the effector phase. This is because high doses of mAbs to IFN-γ do not inhibit $V_\alpha14$ NKT cell–mediated cytolysis (Fig. 3E).

We examined the potential activity of NK and T cells in vitro in the mutant mice; both NK-mediated and T cell–mediated killing functions were potent. The NK activity induced by polyinosinic-polycytidylic acid [poly(I:C)] was as potent in $J_\alpha281^{-/-}$ mice as in wild-type mice (Fig. 4A). Similarly, significant allospecific CTL activity was detected on P815 (H-2^d) and BALB/c (H-2^d) concanavalin A (ConA) blasts, but not on EL-4 (H-2^b), in $J_\alpha281^{-/-}$ mice to the same extent as in wild-type mice (Fig. 4B). Thus, NK and conventional T cells in $J_\alpha281^{-/-}$ mice are functionally active, yet not indispensable for tumor rejection upon IL-12 stimulation.

The primary effect of IL-12 on $V_\alpha14$ NKT cells is also supported by the fact that IL-12 causes an increase in the actual numbers of $V_\alpha14$ NKT cells (about a fourfold increase; 1.5×10^5 to 6×10^5) and in their cell volume (1.3 to 2.5-fold increase) (*21*). It is now clear that a reevaluation of NK and T cell functions in the absence of $V_\alpha14$ NKT cells may alter our understanding of the functions of various subsets of lymphocytes in vivo.

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22. For measurement of the melanoma antigen (GM3 ganglioside), cells or tissues were homogenated. The homogenates of normal or metastatic livers (50 mg) or B16 melanoma were treated with 1 ml of extraction buffer containing 1% NP-40. Varied concentrations of melanoma cell homogenates were used to determine standard curves. Melanoma antigens in the extracts were quantified by radioimmunoassay with ^{125}I-labeled M2590 mAb to melanoma as described [S. Wakabayashi, S. Okamoto, M. Taniguchi, *Jpn. J. Cancer Res.* **75**, 427 (1984); Y. Gunji and M. Taniguchi, *ibid.* **77**, 595 (1986); G. A. Nores, T. Dohi, M. Taniguchi, S. Hakomori, *J. Immunol.* **139**, 3171 (1987)].
23. Cells (1×10^6) were first incubated with mAb to Fc γ receptors II and III (FcγRII/III) (2.4G2) to block nonspecific staining through FcγR, then the cells were incubated with fluorescein isothiocyanate–labeled anti-TCRβ (H57-597) and phycoerythrin–labeled anti-NK1.1 (PK136). Dead cells were excluded by propidium iodide staining, and 10^5 cells were analyzed by EPICS-ELITE (Coulter Electronics, Hialeah, FL) with a logarithmic amplifier as described (*5*).
24. $J_\alpha281^{+/+}$, $J_\alpha281^{-/-}$, and RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$ mice were injected with 2×10^6 B16 or FBL-3 cells in the spleen for liver metastasis, intravenously with 3×10^5 B16 or 2×10^6 LLC cells for pulmonary metastases, or subcutaneously with 2×10^6 B16 cells for subcutaneous tumor growth on day 0. Recombinant murine IL-12 (2400 U/mouse) or phosphate-buffered saline (PBS) was injected on days 3, 5, 7, and 9. On day 14, the mice were killed and either metastatic nodules counted or GM3 melanoma antigens measured by radioimmunoassay in the liver or lung as described (*22*). For subcutaneous tumor growth, injection of IL-12 or PBS was initiated on day 5, and the mice were treated five times per week. The diameters of tumors were measured every day with calipers. The sizes of tumors were expressed as the products of the longest diameter times the shortest diameter (in square millimeters).
25. Target cells were labeled with 100 μCi of sodium chloride (Amersham) per 5×10^6 cells for 1 hour. Effector cells were seeded in 96-well round-bottomed plates at indicated effector/target (E/T) ratios against 1×10^4 target cells. The release of ^{51}Cr from lysed target cells was counted on a γ-counter after a 4-hour incubation at 37°C in 5% CO_2. The percent of specific ^{51}Cr-release was calculated by the following formula: percent of specific lysis = (sample cpm – spontaneous cpm) × 100/(maximum cpm – spontaneous cpm). Spontaneous cpm was calculated from the supernatant of the target cells alone, and the maximum release was obtained by adding 1N HCl to target cells.
26. MAbs to H-2K^b (AF6-885), H-2D^b (KH-95), CD1d (1B1), $V_\beta8$ (MR5-2), NK1.1 (PK136), Ly49C (5E6), IFN-γ (R4-6A2), Fas (Jo2), and Fas ligand (K10) (Pharmingen) were used for blocking experiments.
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CD1d-Restricted and TCR-Mediated Activation of $V_\alpha14$ NKT Cells by Glycosylceramides

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Natural killer T (NKT) lymphocytes express an invariant T cell antigen receptor (TCR) encoded by the $V_\alpha14$ and $J_\alpha281$ gene segments. A glycosylceramide-containing α-anomeric sugar with a longer fatty acyl chain (C_{26}) and sphingosine base (C_{18}) was identified as a ligand for this TCR. Glycosylceramide-mediated proliferative responses of $V_\alpha14$ NKT cells were abrogated by treatment with chloroquine–concanamycin A or by monoclonal antibodies against CD1d/$V_\beta8$, CD40/CD40L, or B7/CTLA-4/CD28, but not by interference with the function of a transporter-associated protein. Thus, this lymphocyte shares distinct recognition systems with either T or NK cells.

An unusual lineage of lymphocytes, $V_\alpha14$ NKT cells, are characterized by their development before thymus formation (*1*), their expression of an invariant TCR encoded by the $V_\alpha14$ and $J_\alpha281$ gene segments (*2*, *3*) mainly associated with $V_\beta8.2$ (*4*), and by the coexpression of the NK1.1 receptor, a marker of NK cells (*5*). The invariant $V_\alpha14$ TCR is essential for the development and function of $V_\alpha14$ NKT cells (*6*–*8*). Contrary to the general rule that the interaction of the TCR with the major histocompatibility complex (MHC) molecules leads to the development of T cells, $V_\alpha14$ NKT cells are selected by CD1d, a nonclassical class Ib molecule (*9*); mutant mice deficient in