

A Critical Role for Natural Killer T Cells in Immunosurveillance of Methylcholanthrene-induced Sarcomas

Nadine Y. Crowe,¹ Mark J. Smyth,² and Dale I. Godfrey¹

¹Department of Pathology and Immunology, Monash University Medical School, Melbourne, Victoria 3181, Australia

²Cancer Immunology, Trescowthick Laboratories, Peter MacCallum Cancer Institute, Melbourne, Victoria 3002, Australia

Abstract

Natural killer (NK) T cells initiate potent antitumor responses when stimulated by exogenous factors such as interleukin (IL)-12 or α -galactosylceramide (α -GalCer), however, it is not clear whether this reflects a physiological role for these cells in tumor immunity. Through adoptive transfer of NK T cells from wild-type to NK T cell-deficient (T cell receptor [TCR] $J\alpha 281^{-/-}$) mice, we demonstrate a critical role for NK T cells in immunosurveillance of methylcholanthrene (MCA)-induced fibrosarcomas, in the absence of exogenous stimulatory factors. Using the same approach with gene-targeted and/or antibody-depleted donor or recipient mice, we have shown that this effect depends on CD1d recognition and requires the additional involvement of both NK and CD8⁺ T cells. Interferon- γ production by both NK T cells and downstream, non-NK T cells, is essential for protection, and perforin production by effector cells, but not NK T cells, is also critical. The protective mechanisms in this more physiologically relevant system are distinct from those associated with α -GalCer-induced, NK T cell-mediated, tumor rejection. This study demonstrates that, in addition to their importance in tumor immunotherapy induced by IL-12 or α -GalCer, NK T cells can play a critical role in tumor immunosurveillance, at least against MCA-induced sarcomas, in the absence of exogenous stimulation.

Key words: rodent • NK T cells • NK cells • tumor immunity • methylcholanthrene

Introduction

NK T cells are a specialized subset of T cells that, in mice, are commonly characterized by the coexpression of NK1.1, and a heavily biased $\alpha\beta$ TCR, with the great majority expressing an invariant V $\alpha 14/J\alpha 281$ TCR α -chain coupled with V $\beta 8.2$, 7 or 2 TCR- β chains (for a review, see references 1 and 2). This TCR specifically recognizes glycolipid-Ag in conjunction with the MHC class I-like molecule, CD1d (for a review, see references 2 and 3). Disruption of either the invariant TCR- α chain, or CD1d, results in a selective deficiency of NK T cells, while all other lymphocyte subsets remain intact (4–7). Upon TCR stimulation, NK T cells rapidly produce both proinflammatory cytokines, (e.g. IFN- γ and TNF- α) and antiinflammatory cytokines (e.g. IL-4, IL-10, IL-13), suggesting an im-

portant role for these cells in immunoregulation. Several studies have shown that NK T cells can suppress cell-mediated immunity and prevent self-tissue destruction. For example, NK T cells prevent pancreatic islet β cell destruction in NOD mice in an IL-4/IL-10-dependent manner (8), mediate systemic suppression associated with anterior chamber-associated immune deviation via IL-10 production (9), induce allograft tolerance (10), and prevent graft-versus-host disease in mice (11). NK T cells have also been shown to suppress tumor antigen-specific, CTL-mediated tumor rejection in an IL-13-dependent manner (12).

In apparent contrast with the above studies, NK T cells promote potent tumor rejection in response to exogenous factors such as IL-12 (4, 13, 14) and α -galactosylceramide (α -GalCer)* (15–17). α -GalCer-induced NK T cell-dependent antitumor activity is dependent on IFN- γ produc-

M.J. Smyth and D.I. Godfrey are co-chief investigators.

Address correspondence to Dale Godfrey, Monash University Medical School, Department of Pathology and Immunology, Commercial Rd., Prahran 3181, Victoria, Australia. Phone: 613-9903-0075; Fax: 613-9903-0018; E-mail: dale.godfrey@med.monash.edu.au

*Abbreviations used in this paper: α -GalCer, α -galactosylceramide; MCA, methylcholanthrene; NMS, normal mouse serum; pfp, perforin; RAG, recombination activation gene; WT, wild-type.

tion, and requires NK cells, (18–20), while the role for CD8⁺ T cells is controversial (19, 20). Despite the clear ability of NK T cells to initiate potent antitumor responses in response to exogenous immunotherapeutic stimuli, whether this represents a physiological role for NK T cells in tumor rejection remains unclear, with two recent studies suggesting an opposing role for NK T cells in tumor immunity. Terabe et al. (12) reported that NK T cells were responsible for incomplete tumor regression by IL-13-mediated inhibition of tumor-specific CTL, suggesting that NK T cells may normally inhibit tumor immunity, perhaps reflecting their immunosuppressive role in autoimmune disease. In contrast, we previously reported that NK T cell-deficient (TCR J α 281^{-/-}) mice were more susceptible to methylcholanthrene (MCA)-induced fibrosarcoma, suggesting an aggressive role for NK T cells in preventing growth of these tumors (21). A disadvantage of the latter study was that it provided little insight into the role of NK T cells and NK T cell-derived factors in this model. It was not clear if NK T cells were directly killing the tumor in a perforin (pfp)-dependent fashion, as suggested by our *in vitro* studies, (21) or if they were acting indirectly via the production of factors which lead to the activation of downstream effectors such as NK cells as has been demonstrated in α -GalCer-induced systems (18–20). It was also unknown whether the NK T cell response, in the absence of α -GalCer, was dependent on an interaction between TCR and CD1d, which might suggest recognition of tumor-derived glycolipid antigen. In this study, using adoptive transfer of NK T cells from wild-type (WT) or various gene-targeted mice, we demonstrate unequivocally that NK T cells can play a critical role in immunosurveillance of MCA-induced fibrosarcomas, and reveal the mechanisms behind this process.

Materials and Methods

Mice. Inbred C57BL/6 WT mice were purchased from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, and Clear Japan, Inc. The following gene-targeted mice were bred at the Peter MacCallum Cancer Institute: C57BL/6-TCRJ α 281-deficient (J α 281^{-/-}) (provided by M. Taniguchi, Chiba University Graduate School of Medicine, Chiba, Japan and backcrossed to C57BL/6 for nine generations) (4); C57BL/6 pfp-deficient (pfp^{-/-}) (from G. Karupiah, John Curtin School of Medical Research, Canberra, Australia and derived from C57BL/6 ES cells) (22); C57BL/6 IFN- γ deficient (IFN- γ ^{-/-}; from Genentech, Inc. and backcrossed to C57BL/6 for 10 generations) (23); C57BL/6 CD1d deficient (CD1d^{-/-}; provided by L. van Kaer, Vanderbilt University School of Medicine, Nashville, TN and backcrossed to C57BL/6 for 10 generations) (6); and C57BL/6 recombination activation gene (RAG)-1 deficient (RAG-1^{-/-}; from L. Corcoran, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia and backcrossed to C57BL/6 for 10 generations). Mice of 6–14 wk of age were used in all experiments that were performed according to animal experimental ethics committee guidelines.

Isolation of Liver Lymphocyte Subsets. Lymphocytes were isolated from the liver as described previously (24). To avoid non-specific binding of antibodies to FcR- γ , cells were preincubated with anti-mouse CD16/32 (2.4G2) mAb (grown in-house) be-

fore staining with FITC-conjugated anti- $\alpha\beta$ TCR (clone H57-597) and PE-conjugated anti-NK1.1 (clone PK-136). All flow cytometry reagents were purchased from BD Pharmingen, unless otherwise indicated. Cells were gated and sorted as described previously (24). After washing twice with PBS, the stained cells were analyzed on a FACStar^{PLUS}™ (Becton Dickinson) and the data processed by the CELLQuest™ program (Becton Dickinson).

Tumor Models *In Vivo*. MCA-1, -3, -4 and CD1.1 were derived from J α 281^{-/-} and CD1d^{-/-} mice, respectively, injected with 100 μ g MCA. B16F10 mouse melanoma and MCA-induced sarcoma cell lines were maintained as described previously (20, 21). For growth of MCA-induced sarcoma lines, groups of WT and gene targeted and/or Ab-depleted mice were injected subcutaneously (right hind leg) with 10⁵ sarcoma cells. On the same day, some mice received liver lymphocytes from WT or gene-targeted mice or FACS[®]-purified liver lymphocyte populations from WT mice, or 2% normal mouse serum (NMS) in PBS (2% NMS.PBS) via intravenous adoptive transfer. In the delayed transfer experiments, some mice received liver lymphocytes 7 d after tumor inoculation. All mice were observed every other day and tumor growth was measured with a caliper square as the product of two diameters. Results were recorded as the mean tumor size (cm²) \pm SEM. Significant difference in the number of mice which remained susceptible to tumor development compared with PBS-treated J α 281^{-/-} groups was determined using a Fisher's exact test (* $P \leq 0.05$; ** $P \leq 0.01$). Significant difference in tumor growth rate, compared with PBS-treated control groups, was determined using a Mann-Whitney U test ($n \geq 4$). In the B16F10 subcutaneous growth model, groups of WT mice were injected subcutaneously (hind leg) with 10⁵ MCA-1 cells, and varying doses of B16F10 were injected subcutaneously within the same region at a distinct site. All mice were observed every other day and B16F10 subcutaneous tumor growth was measured with a caliper square as the product of two diameters. Results were recorded as the mean tumor size (cm²) \pm SEM.

AsialoGM1 and CD8 Depletion. NK cells were specifically depleted in J α 281^{-/-} mice after intraperitoneal injection of 20 μ g rabbit anti-asialoGM1 Ab (Wako Chemicals) on days -1, 0, and 7 (day 0 being day of tumor inoculation) as described previously (25). CD8⁺ cells were specifically depleted using anti-CD8-depleting Ab (clone 53-6.7) (grown in-house) using the same approach.

Results

NK T Cell-deficient Mice Are Susceptible To Sarcoma Growth. We have previously shown that NK T cell-deficient, TCR J α 281^{-/-}, mice (Fig. 1 A) are more susceptible to MCA-induced fibrosarcomas and a fibrosarcoma line (MCA-1) when compared with WT mice, suggesting an important role for NK T cells in preventing the growth of these tumors (21). This phenotype can be demonstrated using several distinct fibrosarcoma lines (Fig. 1 B), including some that were generated from MCA-treated CD1d^{-/-} mice. These data are consistent with previous observations that sarcomas derived from RAG-2^{-/-} mice grow preferentially in RAG-2^{-/-} mice over syngenic WT mice (26).

Adoptive Transfer of NK T Cells into J α 281^{-/-} Mice Restores Protection Against MCA-1 Sarcoma. As the most obvious difference between WT and J α 281^{-/-} mice is the presence of CD1d-restricted NK T cells (Fig. 1 A) (4, 27,

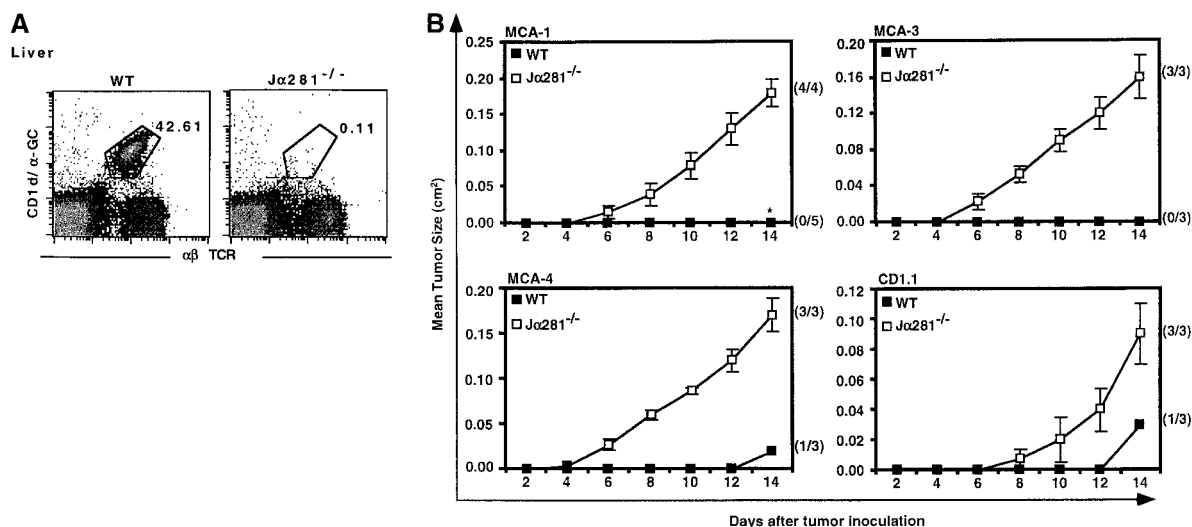


Figure 1. NK T cell-deficient ($J\alpha 281^{-/-}$) mice are more susceptible to growth of MCA-induced sarcoma lines. (A) Liver lymphocytes were isolated from WT and $J\alpha 281^{-/-}$ mice and labeled with CD1d/α-GalCer tetramer and mAb specific for αβ TCR to identify NK T cells. (B) Groups of WT and $J\alpha 281^{-/-}$ mice were injected subcutaneously (hind flank) with 10^5 MCA-1, -3, -4 or CD1.1 sarcoma cell lines. Results were recorded every other day as the mean tumor size (in cm²) ± SEM. Three mice per group were used. Significant difference from the PBS-treated $J\alpha 281^{-/-}$ group was determined using a Mann-Whitney U test when $n \geq 4$. * $P \leq 0.05$.

28), we sought to restore protection in $J\alpha 281^{-/-}$ recipients treated with MCA-1 sarcoma by adoptive transfer of NK T cells. In initial experiments, total liver lymphocytes were transferred, since NK T cells are highly abundant within this population of cells, and we could avoid using mAb to engage cell surface molecules for purification. Over 80% of MCA-1-injected $J\alpha 281^{-/-}$ mice treated with WT liver lymphocytes were able to completely inhibit the growth of MCA-1 (Fig. 2 A), while the rest had significantly smaller tumors (approximately one-fifth the size, measured in cm²; $P \leq 0.01$) than PBS-injected controls (unpublished data). This was similar to the tumor incidence observed in WT mice, the majority of which remained tumor free. An additional control group of MCA-1-injected $J\alpha 281^{-/-}$ mice were treated with $J\alpha 281^{-/-}$ -derived liver lymphocytes, which have all populations except NK T cells. These mice remained susceptible to tumor growth and were indistin-

guishable from PBS-injected controls. Protection was also achieved when transferring WT liver lymphocytes into TCR $J\alpha 281^{-/-}$ mice that had been inoculated with either MCA-3 or MCA-4 tumor cells (unpublished data). This data strongly suggested that inhibition of growth was due to the transfer of CD1d-restricted NK T cells present among the WT liver lymphocytes.

Having established that liver lymphocytes, probably NK T cells, could protect in this system in the absence of cell surface molecule engagement by mAb, we sought to formally define which liver lymphocyte subset was responsible for protection. NK1.1⁺ αβ TCR⁺ (NK T cells), NK1.1⁻ αβ TCR⁺ (T cells), and NK1.1⁺ αβ TCR⁻ (NK cells) were purified by FACS[®] and adoptively transferred intravenously into MCA-1-injected $J\alpha 281^{-/-}$ mice (Fig. 2 B). Mice that received NK T cells (2.5×10^5) were able to inhibit the growth of MCA-1, while those receiving the

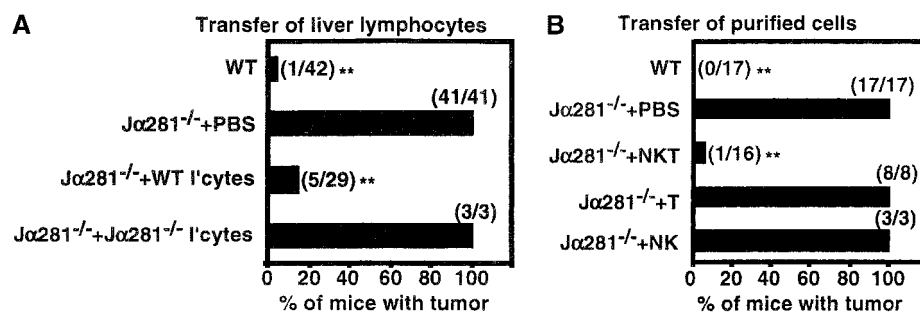


Figure 2. Adoptive transfer of NK T cells protects $J\alpha 281^{-/-}$ mice against MCA-1 tumor growth. Groups of WT and $J\alpha 281^{-/-}$ mice were inoculated subcutaneously (hind flank) with 10^5 MCA-1 cells. (A) Subgroups of $J\alpha 281^{-/-}$ mice then received between 1 and 3×10^6 WT or 3×10^6 $J\alpha 281^{-/-}$ liver lymphocytes, 200 μL 2% NMS.PBS, or (B) 2.5×10^5 purified NK1.1⁺ αβ TCR⁺ (NK T), NK1.1⁻ αβ TCR⁻ (NK), NK1.1⁻ αβ TCR⁺ (T) cells, or 200 μL 2% NMS.PBS, via intravenous adoptive

transfer. NK T cells typically constituted between 30–35% of WT liver lymphocytes, and sorted populations were at least 97, 93, and 94% pure, respectively. Results represent pooled data from 10 (A) or 4 (B) independent experiments (with exception of $J\alpha 281^{-/-}$ liver lymphocytes [A] and purified NK cell [B] transfers from one experiment only, and purified T cell transfers [B] from two experiments). Between three and six mice per group per experiment were used. Total number of mice in each group that developed tumors is shown in parentheses. Significant difference from the PBS-treated $J\alpha 281^{-/-}$ groups was determined using a Fisher's exact test. * $P \leq 0.05$; ** $P \leq 0.01$.

same number of T cells or NK cells could not, specifically defining NK T cells as the population responsible for restoring protection.

Dose Response for NK T Cell-mediated Protection Against MCA-1. To determine the threshold number of NK T cells required to restore protection, several liver lymphocyte doses (3×10^6 , 10^6 , 0.33×10^6 , and 0.11×10^6) (Fig. 3 A), and purified NK T cell doses (2.5×10^5 , 10^5 , and 5×10^4) (Fig. 3 B) were tested. Inhibition of tumor growth was clearly dose-dependent, with 10^6 liver lymphocytes ($\sim 3.0 \times 10^5$ NK T cells) and 2.5×10^5 purified NK T cells required for optimal prevention of tumor growth. As the total number of NK T cells in the protective dose of liver lymphocytes was very similar to that of purified NK T cells, it was unlikely that mAb-binding altered the functional status of transferred NK T cells. Although lower doses of NK T cells failed to completely prevent tumor growth, partial protection was observed with smaller and slower growing tumors corresponding to the number of NK T cells injected.

Factors that Are Critical for NK T Cell-mediated Protection. Having established that transfer of NK T cells effectively protected $J\alpha 281^{-/-}$ mice from tumor growth, we sought to determine which NK T cell molecules were mediating this protection. When liver lymphocytes from $IFN-\gamma^{-/-}$ mice were used as a source of NK T cells, they were unable to prevent tumor growth (Fig. 4 A), despite the

ability of endogenous cells in these $J\alpha 281^{-/-}$ recipients to produce $IFN-\gamma$, indicating an essential role for NK T cell-derived $IFN-\gamma$. In contrast, $pfp^{-/-}$ liver lymphocytes retained the ability to inhibit tumor growth, indicating that direct, pfp -dependent, tumor lysis by NK T cells was not an essential mechanism in this model.

As it was unlikely that NK T cells were acting alone, we further investigated what other cells and/or molecules were important downstream of, or in conjunction with, NK T cell activation. Purified WT NK T cells were transferred into various gene-targeted and/or mAb-depleted mice (Fig. 4 B i–vii). Similar to $J\alpha 281^{-/-}$ mice (Fig. 4 B i), $CD1d^{-/-}$ mice were susceptible to MCA-1 growth (Fig. 4 B ii). However, in contrast to $J\alpha 281^{-/-}$ recipients, NK T cell transfer failed to prevent tumor growth in $CD1d^{-/-}$ mice (Fig. 4 B ii), indicating that $CD1d$ expression by cells other than the tumor, such as APCs, was critical for NK T cell-mediated immunosurveillance. $CD1d$ expression by the tumor itself was not essential; MCA-1 is close to, if not completely, negative for $CD1d$ expression (unpublished data) and, as shown above, a $CD1d^{-/-}$ tumor line grew in $J\alpha 281^{-/-}$ but not WT mice (Fig. 1 B).

$IFN-\gamma^{-/-}$ recipients were also susceptible to MCA-1 growth, and transfer of ($IFN-\gamma$ sufficient) WT NK T cells did not restore complete protection (Fig. 4 B ii), although growth was slower when compared with PBS-injected $IFN-\gamma^{-/-}$ mice. It is possible that transferred WT NK T cells may provide sufficient $IFN-\gamma$ to confer initial protection, however, $IFN-\gamma$ production by downstream non-NK T cells is clearly critical for effective eradication of the tumor. Pfp -deficient recipients were susceptible to MCA-1 growth (Fig. 4 B iv), and WT NK T cells were unable to protect in these mice, although again, the tumor grew at a slower rate when these cells were transferred. This indicated that, although NK T cell-derived pfp was not critical for effective eradication of the tumor, pfp production by other cells, possibly NK or T cells, is clearly important for efficient NK T cell-mediated tumor immunosurveillance (Fig. 4 B iv). It was also noteworthy that both PBS-treated $IFN-\gamma^{-/-}$ and $pfp^{-/-}$ mice had smaller and slower growing tumors when compared with PBS-injected $J\alpha 281^{-/-}$ mice (Fig. 4 B i, iii, and iv; $P \leq 0.01$ and $P \leq 0.05$, respectively). This suggests that NK T cells present within $IFN-\gamma^{-/-}$ and $pfp^{-/-}$ mice may still be able to use other, $IFN-\gamma/pfp$ -independent, pathways/mechanisms to protect against MCA-1 tumor growth, although clearly, NK T cell-derived and non-NK T cell-derived $IFN-\gamma$, and pfp -expression by downstream effector cells appear to be critical in providing optimal protection against this tumor. Transfer of NK T cells into MCA-1-injected, NK or $CD8$ -depleted $J\alpha 281^{-/-}$ mice, or $RAG-1^{-/-}$ mice, revealed that NK, $CD8^+$, and T cells are important for a clear protective response induced by NK T cells (Fig. 4 B v–vii). Tumor growth in PBS-injected, NK, or $CD8$ -depleted $J\alpha 281^{-/-}$, or $RAG-1^{-/-}$ mice (Fig. 4 B v–vii) was not significantly different from that of PBS-injected $J\alpha 281^{-/-}$ mice.

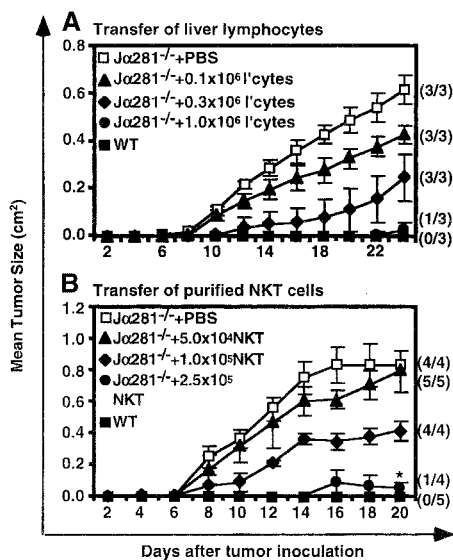


Figure 3. Dose response for NK T cell-mediated protection against MCA-1. Groups of WT and $J\alpha 281^{-/-}$ mice were injected subcutaneously (hind flank) with 10^5 MCA-1 cells. (A) Subgroups of $J\alpha 281^{-/-}$ mice then received either 3×10^6 , 10^6 , 0.33×10^6 , 0.11×10^6 liver lymphocytes or 200 μ L 2% NMS.PBS or (B) 2.5×10^5 , 10^5 , 5×10^4 purified $NK1.1^+ \alpha\beta$ TCR $^+$ (NK T) cells or 200 μ L 2% NMS.PBS, via intravenous adoptive transfer. NK T cells constituted 31% of the liver lymphocyte population and sorted NK T cells were enriched to 98%. Results were recorded as the mean tumor size (cm^2) \pm SEM. Between three and five mice per group were used. Significant difference from the PBS-treated $J\alpha 281^{-/-}$ groups was determined using a Mann-Whitney U test when $n \geq 4$. * $P \leq 0.05$.

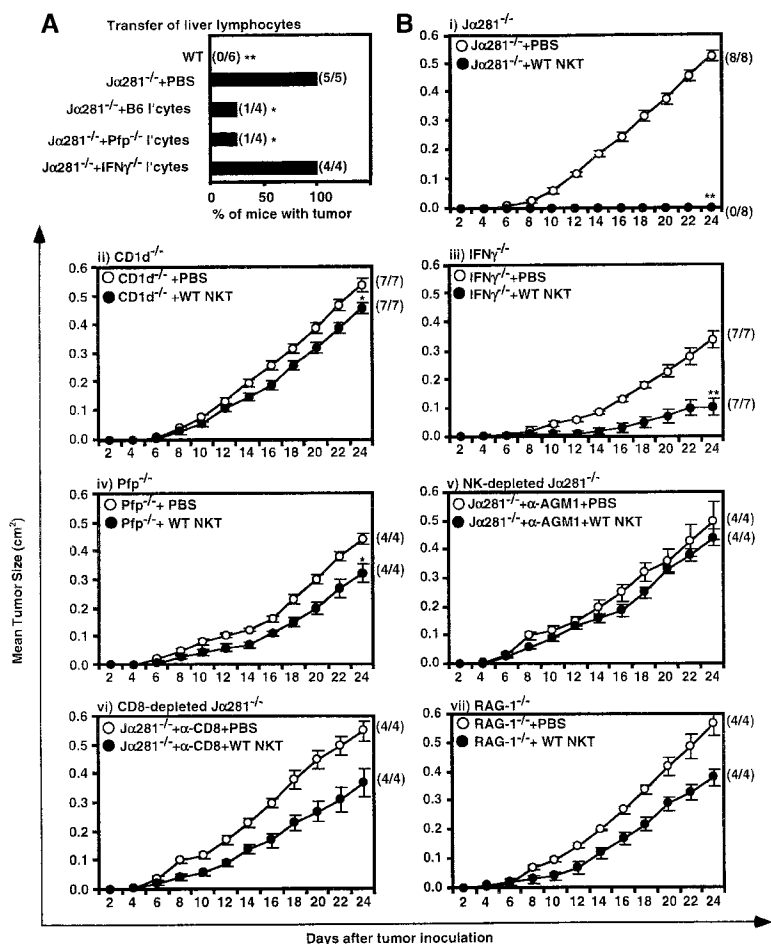


Figure 4. Factors that are critical for NK T cell-mediated protection. (A) WT or J α 281^{-/-} mice were injected subcutaneously (hind flank) with 10⁵ MCA-1 cells. Subgroups of J α 281^{-/-} mice were simultaneously treated with either PBS, or 3 \times 10⁵ NKT cells, as part of a liver lymphocyte population, from either WT, IFN- γ ^{-/-}, or pfp^{-/-} donor mice via intravenous adoptive transfer. Significant difference from the PBS-treated J α 281^{-/-} group was determined by a Fisher's exact test. * $P \leq 0.05$; ** $P \leq 0.01$. (B) Groups of (i) J α 281^{-/-}, (ii) CD1d^{-/-}, (iii) IFN- γ ^{-/-}, (iv) pfp^{-/-}, (v) J α 281^{-/-} mice treated with depleting anti- α -AGM1 Ab, (vi) J α 281^{-/-} mice treated with depleting anti-CD8, and (vii) RAG-1^{-/-} mice, were injected subcutaneously (hind flank) with 10⁵ MCA-1 cells. One-half of each group of mice were simultaneously injected with 2.5 \times 10⁵ sorted WT NK T cells via intravenous adoptive transfer, while the other half were injected intravenously with 2% NMS.PBS. Sorted NK T cells were always at least 97% pure as determined by FACS[®]. (B) i, ii, and iii represent pooled data from two independent experiments, iv represents data from one of these experiments, v, vi, and vii represent data from one independent experiment with three to six mice per group per experiment. Significant difference from the PBS-treated control group of each experiment was determined using a Mann-Whitney U test. * $P \leq 0.05$; ** $P \leq 0.01$.

NK T Cells Are Required Early in the Response Against MCA-1. To investigate whether NK T cells could also protect against established tumors, J α 281^{-/-} mice were treated with WT liver lymphocytes 7 d after MCA-1 injection (Fig. 5). Delayed transfer of WT cells had no effect on tumor growth, indicating that NK T cells are required either earlier in the response, or in significantly greater numbers for effective inhibition of established tumors.

MCA-1 Does Not Induce α -GalCer-like Activation of NK T Cells. We have previously shown that α -GalCer can activate NK T cells that have been adoptively transferred into B16F10-inoculated NK T cell-deficient mice, to initiate potent tumor rejection (20). To investigate whether MCA-1 tumor activates NK T cells in a manner similar to α -GalCer, possibly by providing CD1d-binding glycolipid antigens, WT mice were simultaneously injected with B16F10 and MCA-1. Mice were injected subcutaneously on the hind flank with varying doses of B16F10, and some mice were also injected on the same flank, in an adjacent region, with MCA-1 (Fig. 6). As expected, MCA-1 growth was inhibited, presumably due to the presence of NK T cells, however, this response had no effect on the adjacent growth of B16F10 tumor. Similar results were achieved when B16F10 was injected intravenously into WT mice, some of which were also injected subcutaneous

with MCA-1 (unpublished data). Since B16F10 growth and metastasis is profoundly inhibited after α -GalCer treatment of J α 281^{-/-} mice (20) that have been injected with WT NK T cells, these data suggest that MCA-1 sarcoma does not activate NK T cells to act in a manner similar to that following α -GalCer stimulation.

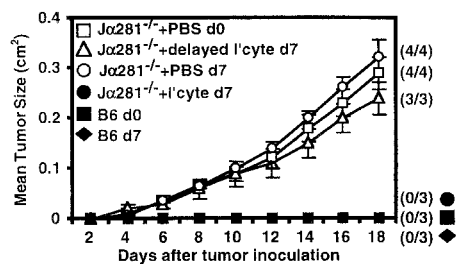


Figure 5. NK T cells are required early in the response against MCA-1. Groups of WT and J α 281^{-/-} mice were inoculated subcutaneously (hind flank) with 10⁵ MCA-1 cells (d0, \square , Δ , \blacksquare). 7 d later, some J α 281^{-/-} mice were injected with 10⁶ WT liver lymphocytes via intravenous adoptive transfer (Δ). As a control to show the WT liver lymphocytes (NK T cells) were protective, other J α 281^{-/-} mice were inoculated with MCA-1 on the same day as lymphocyte transfer (\bullet). NK T cells constituted 27% of the whole liver lymphocyte population as determined by FACS[®]. Results were recorded as the mean tumor size (in cm²) \pm SEM. Four mice per group were used.

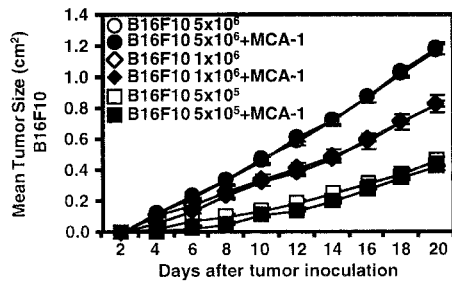


Figure 6. MCA-1 does not induce α -GalCer-like activation of NK T cells. Groups of WT mice were injected subcutaneously (hind flank) with either 5×10^6 , 10^6 , or 5×10^5 B16F10 cells. Some mice were then injected on the same hind flank with 10^5 MCA-1 cells. Results were recorded as the mean tumor size (in cm^2) \pm SEM with five mice per group.

Discussion

It is well established that NK T cells can be induced by either IL-12 or α -GalCer to mediate potent antitumor effects both in vitro and in vivo (4, 15, 16), however, this does not necessarily indicate that NK T cells play a natural/physiological role in tumor recognition and immunity. We recently published that Ja281^{-/-} mice are more susceptible to development of MCA-induced fibrosarcomas when compared with WT mice, suggesting that NK T cells might be important in the prevention of tumor growth in this model (21). Herein, using adoptive transfer techniques and gene-targeted mice, we have clearly demonstrated a critical role for NKT cells in immunosurveillance against MCA-induced fibrosarcomas, and have also identified the key cells and molecules that support this process.

Although NK T cell activation in this system does not require treatment with an exogenous CD1d-binding ligand such as α -GalCer, CD1d expression by recipient cells, possibly APCs, was nonetheless found to be important, indicating that NK T cells were responding to these tumors in a TCR-dependent fashion. What remains to be determined is whether NK T cell activation is caused by the presentation of tumor-derived Ag in the context of CD1d, or rather, whether the presence of CD1d is simply required in addition to other, CD1d-independent, tumor-induced 'danger signals.' It is conceivable that NK T cells were being activated by a CD1d-binding endogenous ligand, mimicking the effects of exogenously added α -GalCer, however, the downstream effector functions after NK T cell activation by MCA-1 were clearly distinct from those following NK T stimulation by α -GalCer. In contrast to α -GalCer-induced tumor rejection (18, 20), NK T cell-mediated tumor immunosurveillance in this model was pfp dependent, involved both NK and CD8⁺ T cells, and most importantly, did not mimic the effect of α -GalCer in its ability to invoke NK T cell-mediated B16F10 rejection.

We previously published that NK T cells can kill MCA-1 in vitro in a pfp-dependent manner when induced by IL-2 and IL-12 (21). This suggested that the critical contribution of NK T cells in vivo was direct tumor cell lysis. However, transferred pfp^{-/-} NK T cells retained their

ability to prevent MCA-1 growth, indicating NK T cell-derived pfp was clearly not critical for protection. WT NK T cells transferred into pfp^{-/-} mice slowed the growth of MCA-1, indicating that, although not essential, NK T cell-derived pfp may make a minor contribution to MCA-1 rejection. On the other hand, pfp-expression by recipient cells was critical, supporting the idea that NK T cell-mediated tumor immunosurveillance relies on downstream pfp-expressing effector cells. These may include NK and CD8⁺ T cells, both of which were required for optimal prevention of sarcoma growth.

NK cells are known to be activated downstream of NK T cells after α -GalCer stimulation (29, 30). This is clearly necessary, although apparently not sufficient, for NK T cell-mediated immunosurveillance in this sarcoma model. These findings are in concert with our previous study showing that NK depletion by anti-asGM1 abrogates protection against primary MCA-induced sarcomas in WT mice (25). The involvement of both NK and CD1d-dependent NK T cells has also been shown to be important in the cyclophosphamide/IL-12-induced regression of a large MCA-induced sarcoma (31). In contrast the role for CD8⁺ T cells in α -GalCer-mediated tumor rejection is controversial (19, 20), although we have now shown that they play a critical role in immunosurveillance of MCA-induced tumor cell lines. Although the susceptibility of CD8-depleted mice to MCA-1 supports previous findings involving other MCA-induced sarcoma lines (32), our earlier findings did not reveal a role for CD8⁺ cells in NK T cell-mediated protection against primary MCA-induction of sarcomas (21). At least two possibilities may explain this. The role of CD8⁺ cells may differ in primary responses to MCA-induced sarcomas when compared with transplanted sarcoma lines; or, as the current experiments involve repopulation of NK T cell-deficient mice with relatively low numbers of NK T cells, it may be possible that in the absence of normal NK T cell numbers, the role for other cells such as CD8⁺ T cells increases.

IFN- γ is a critical factor in α -GalCer-mediated tumor rejection (18–20). This was also the case for the natural tumor surveillance model described herein. Importantly, similar to the α -GalCer model (20), both NK T cell-derived and non-NK T cell-derived IFN- γ was required, suggesting it may function at multiple stages in this process. This cytokine appears to be important for the activation of the downstream effector cells (29, 30, 33), which would explain why it must be initially produced by NK T cells. Additionally, IFN- γ production by recipient effector cells might act to amplify the immune response, or may have antitumor effects of its own (34–36).

The in vivo migratory and homing behavior of NK T cells is very poorly understood, as is the impact that a tumor may have on this process. Using immunohistology, we have failed to detect a significant population of NK T cells at the tumor site in WT mice (unpublished data), nor have we been able to detect these cells, at the doses used for protection, after adoptive transfer into tumor-treated, and nontumor-treated, Ja281^{-/-} mice (unpublished data).

This is probably due to the fact that we are transferring very low numbers of NK T cells, combined with the possibility that they die shortly after being activated in response to the tumor, as has been reported after α -GalCer stimulation (28, 30, 37, 38). The model we favor is that NK T cells are rapidly stimulated by the presence of the tumor cell glycolipids, leading to potent NK T cell-derived IFN- γ production and early activation of effector cells, including NK and CD8⁺ T cells. These effector cells then directly attack the tumor through direct pfp-dependent lysis and IFN- γ secretion. The early recognition of the tumor appears to be key, as delayed transfer of NK T cells provided no detectable protection.

A role for NK T cells in tumor rejection is usually only observed after stimulation with exogenous therapeutic factors such as IL-12 and α -GalCer. In the absence of these factors, some tumors such as B16F10 melanoma and 3LL lung carcinoma grow readily, despite the presence of NK T cells in these mice. Nonetheless, these earlier studies hinted that with the right environment, NK T cells had the potential to mediate tumor immunosurveillance. An obvious question relates to why NK T cells require exogenous stimulation to respond to some tumors, such as B16F10, but not for others, such as the sarcomas used in this study. This may be related to the type of CD1d-binding glycolipid Ag expressed by these tumors, which may be specific for sarcomas, MCA-induced tumors, or other as yet unidentified molecules, that might be differentially expressed by various tumors, such as activating ligands (e.g. NKG2D ligands; reference 39). Another possibility is that resistance of some tumors to NK T cells may relate to the length of time the tumor has been cultured *in vitro*. In support of this, we found that the MCA-1 line became more resistant to NK T cell-mediated tumor immunosurveillance with time in culture (unpublished data).

Clearly, the influence of NK T cells in tumor immunity may vary ranging from suppression (12) to promotion of tumor rejection (this study). Therefore, it will not only be important to determine the signals that activate NK T cells in response to various tumors, but also the signals that regulate the outcome of the NK T cell function (suppressive or aggressive; reference 40). These differential signals may be derived from different APCs (DC versus B cells, macrophages) or tumor cells themselves, in conjunction with distinct microenvironmental factors. These factors may include cytokines such as IL-12 (14, 41, 42) and IL-7 (43–45), which are thought to promote proinflammatory (via increased IFN- γ production), and antiinflammatory (via increased IL-4 and IL-13 production) responses, respectively.

An alternative possibility was that the tumor rejection observed in this model was due to minor transplantation antigens, expressed by the MCA-induced sarcoma lines, that may differ between the J α 281^{-/-} donor mice from which they were originally derived, and the C57BL/6 recipients. We believe this to be highly unlikely for the following reasons. (a) The J α 281^{-/-} mice (originally 129 strain) were backcrossed to C57BL/6 mice nine times before the induction of the sarcomas. Furthermore, we found

a similar pattern of susceptibility in MCA-1-treated CD1d^{-/-} mice, which had also backcrossed to C57BL/6 mice 10 times. Given the number of times each separate line had been backcrossed to C57BL/6 mice, CD1d^{-/-} recipient mice would be unlikely to carry (and therefore be tolerant to) the same minor transplantation antigens that would be present on the J α 281^{-/-} mouse-derived MCA-1 sarcoma line. Therefore, if the response was directed against minor transplantation antigens, we would have predicted that MCA-1 would have been rejected in CD1d^{-/-} mice. (b) MCA-1, MCA-3, and MCA-4 tumor lines were also injected into (129 \times C57BL/6)F1 recipient mice, which would express any of the potential minor transplantation antigens that may be expressed by the tumors. Whereas all these tumors grew as expected in J α 281^{-/-} recipients, they were rejected in F1 mice in an identical fashion to that in C57BL/6 mice (unpublished data). (c) Adoptive transfer of purified, C57BL/6-derived, NK T cells was sufficient to mediate tumor rejection, whereas C57BL/6-derived, conventional NK1.1⁻ T cells had no effect on tumor growth (Fig. 2 B). (d) The difference in tumor growth between C57BL/6 and J α 281^{-/-} recipient mice was already apparent by day 3, which would represent a remarkably rapid response against a minor transplantation antigen. Taken together, these points indicate that this model involves a CD1d-dependent, NK T cell-mediated antitumor response, rather than a more conventional immune response against minor transplantation antigens.

In summary, this study clearly demonstrates a critical role for NK T cells in immunosurveillance of MCA-induced fibrosarcomas, in the absence of exogenous NK T cell stimulation. This is most likely mediated by their acting as an early warning system that initiates an antitumor response subsequently performed by dedicated effectors such as NK cells and/or CTLs. From a clinical viewpoint, this study suggests that maintenance and/or restoration of normal numbers of NK T cells, particularly after immune-depleting conditions, is an important consideration for the prevention of tumor growth and metastasis. Future research should now be aimed at determining whether this role for NK T cells in immunosurveillance extends to other tumor types, as well as gaining a more thorough understanding of the exact molecular mechanisms required for activation and regulation of NK T cell function in the absence of exogenous factors. In particular it will be important to attempt to isolate tumor-derived CD1d-binding ligands that may be key to this response.

The authors wish to thank Dr. Alan Baxter for critically reviewing the manuscript, Prof. Hideo Yagita for helpful discussions, Prof. Mitchell Kronenberg for provision of CD1d/ α -GalCer tetramers, Prof. Masaru Taniguchi for provision of J α 281^{-/-} mice, Prof. Luc Vankaer for provision of CD1d^{-/-} mice, Dr. Phillip Darcy for assistance with tumor measurements, Daniel Pellicci and Konstantinos Kyparissoudis for technical assistance, Dr. Elise Randle-Barrett and Gerard Tarulli for operating the Flow Cytometric Cell Sorter, Christine Hall and the staff at Peter MacCallum Cancer Institute for their maintenance and care of the mice used in these studies, and Shayna Street for providing the J α 281^{-/-}-derived MCA-induced sarcomas.

N.Y. Crowe is supported by an Australian Postgraduate Research Award. M.J. Smyth and D.I. Godfrey are supported by National Health and Medical Research Council of Australia (NHMRC) Research Fellowships and D.I. Godfrey was supported by an ADCORP/Diabetes Australia Fellowship. The project was supported by the Human Frontier Science Program and donations from Rothschild Australia.

Submitted: 17 January 2002

Revised: 18 March 2002

Accepted: 24 April 2002

References

1. Bendelac, A., M.N. Rivera, S.H. Park, and J.H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* 15:535–562.
2. Godfrey, D.I., K.J.L. Hammond, L.D. Poulton, M.J. Smyth, and A.G. Baxter. 2000. NKT cells: facts, functions and fallacies. *Immunol. Today.* 21:573–583.
3. Porcelli, S.A., B.W. Segelke, M. Sugita, I.A. Wilson, and M.B. Brenner. 1998. The CD1 family of lipid antigen-presenting molecules. *Immunol. Today.* 19:362–368.
4. 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 α 14 NKT cells in IL-12-mediated rejection of tumors. *Science.* 278:1623–1626.
5. 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.
6. 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.
7. 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.
8. 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.
9. Sonoda, K.H., M. Exley, S. Snapper, S.P. Balk, and J. Stein-Streilein. 1999. CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune-privileged site. *J. Exp. Med.* 190:1215–1225.
10. Seino, K., K. Yanagisawa, H. Taniguchi, Y. Takada, K. Yuzawa, M. Otsuka, and K. Fukao. 2001. The effect of α -galactosylceramide upon allogeneic rejection. *Transpl. Proc.* 33:437–438.
11. Zeng, D., D. Lewis, S. Dejbakhsh-Jones, F.S. Lan, M. Garcia-Ojeda, R. Sibley, and S. Strober. 1999. Bone marrow NK1.1⁻ and NK1.1⁺ T cells reciprocally regulate acute graft versus host disease. *J. Exp. Med.* 189:1073–1081.
12. Terabe, M., S. Matsui, N. Noben-Trauth, H. Chen, C. Watson, D.D. Donaldson, D.P. Carbone, W.E. Paul, and J.A. Berzofsky. 2000. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat. Immunol.* 1:515–520.
13. Hashimoto, W., K. Takeda, R. Anzai, K. Ogasawara, H. Sakihara, K. Sugiura, S. Seki, and K. Kumagai. 1995. Cytotoxic NK1.1 Ag⁺ $\alpha\beta$ T cells with intermediate TCR induced in the liver of mice by IL-12. *J. Immunol.* 154:4333–4340.
14. Smyth, M.J., M. Taniguchi, and S.E. Street. 2000. The anti-tumor activity of IL-12: mechanisms of innate immunity that are model and dose dependent. *J. Immunol.* 165:2665–2670.
15. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, H. Sato, E. Kondo, M. Harada, H. Koseki, T. Nakayama, et al. 1998. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated V α 14 NKT cells. *Proc. Natl. Acad. Sci. USA.* 95:5690–5693.
16. Nakagawa, R., K. Motoki, H. Ueno, R. Iijima, H. Nakamura, E. Kobayashi, A. Shimozaka, and Y. Koezuka. 1998. Treatment of hepatic metastasis of the Colon26 adenocarcinoma with an α -Galactosylceramide, KRN7000. *Cancer Res.* 58:1202–1207.
17. Toura, I., T. Kawano, Y. Akutsu, T. Nakayama, T. Ochiai, and M. Taniguchi. 1999. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with α -galactosylceramide. *J. Immunol.* 163:2387–2391.
18. Hayakawa, Y., K. Takeda, H. Yagita, S. Kakuta, Y. Iwakura, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Critical contribution of IFN- γ and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of α -galactosylceramide. *Eur. J. Immunol.* 31:1720–1727.
19. Nakagawa, R., I. Nagafune, Y. Tazunoki, H. Ehara, H. Tomura, R. Iijima, K. Motoki, M. Kamishohara, and S. Seki. 2001. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by α -galactosylceramide in mice. *J. Immunol.* 166:6578–6584.
20. Smyth, M.J., N.Y. Crowe, D.G. Pellicci, K. Kyparissoudis, J.M. Kelly, K. Takeda, H. Yagita, and D.I. Godfrey. 2002. Anti-metastatic effect of α -galactosylceramide is mediated by sequential production of IFN- γ by NKT and NK cells. *Blood.* 99:1259–1266.
21. Smyth, M.J., K.Y. Thia, S.E. Street, E. Cretney, J.S. Trapani, M. Taniguchi, T. Kawano, S.B. Pelikan, N.Y. Crowe, and D.I. Godfrey. 2000. Differential tumour surveillance by natural killer (NK) and NK T cells. *J. Exp. Med.* 191:661–668.
22. Kagi, D., B. Lederman, K. Burki, P. Seiler, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature.* 369:31–37.
23. Dalton, D.K., S. Pitts-Meek, S. Keshav, I.S. Figari, A. Bradley, and T.A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon- γ genes. *Science.* 259:1739–1742.
24. 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, et al. 1999. NKT cells are phenotypically and functionally diverse. *Eur. J. Immunol.* 29:3768–3781.
25. Smyth, M.J., N.Y. Crowe, and D.I. Godfrey. 2001. NK and NKT cells collaborate in host protection from MCA-induced fibrosarcoma. *Int. Immunol.* 13:459–463.
26. Shankaran, V., H. Ikeda, A.T. Bruce, J.M. White, P.E. Swanson, L.J. Old, and R.D. Sreiber. 2001. IFN γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature.* 410:1107–1111.
27. Benlagha, K., A. Weiss, A. Beavis, L. Teyton, and A. Bendelac. 2000. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J. Exp. Med.*

- 191:1895–1903.
28. 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.* 192:741–754.
 29. Carnaud, C., D. Lee, O. Donnars, S.H. Park, A. Beavis, Y. Koezuka, and A. Bendelac. 1999. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J. Immunol.* 163:4647–4650.
 30. Eberl, G., and H.R. MacDonald. 2000. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. *Eur. J. Immunol.* 30:985–992.
 31. Karnbach, C., M.R. Daws, E.C. Niemi, and M.C. Nakamura. 2001. Immune rejection of a large sarcoma following cyclophosphamide and IL-12 treatment requires both NK and NK T cells and is associated with the induction of a novel NK T cell population. *J. Immunol.* 167:2569–2576.
 32. Boeson, M., I.M. Svane, A.M. Engel, J. Rygaard, A.R. Thomsen, and O. Werdelin. 2000. CD8⁺ T cells are crucial for the ability of congenic normal mice to reject highly immunogenic sarcomas induced in nude mice with 3-methylcholanthrene. *Clin. Exp. Immunol.* 121:210–215.
 33. Eberl, G., P. Brawand, and H.R. MacDonald. 2000. Selective bystander proliferation of memory CD4⁺ and CD8⁺ T cells upon NK T or T cell activation. *J. Immunol.* 165:4305–4311.
 34. Gee, M.S., C.J. Koch, S.M. Evans, W.T. Jenkins, C.H. Pletcher, J.S. Moore, H.K. Koblish, J. Lee, E.M. Lord, G. Trinchieri, et al. 1999. Hypoxia-mediated apoptosis from angiogenesis inhibition underlies tumor control by recombinant interleukin 12. *Cancer Res.* 59:4882–4889.
 35. Ruegg, C., A. Yilmaz, G. Bieler, J. Bamat, P. Chaubert, and F.J. Lejeune. 1998. Evidence for the involvement of endothelial cell integrin $\alpha V\beta 3$ in the disruption of the tumor vasculature induced by TNF and IFN- γ . *Nat. Med.* 4:408–414.
 36. Yao, L., C. Sgadari, K. Furuke, E.T. Bloom, J. Teruya-Feldstein, and G. Tosato. 1999. Contribution of natural killer cells to inhibition of angiogenesis by interleukin-12. *Blood.* 93:1612–1621.
 37. Leite-De-Moraes, M.C., A. Herbelin, C. Gouarin, Y. Koezuka, E. Schneider, and M. Dy. 2000. Fas/Fas ligand interactions promote activation-induced cell death of NK T lymphocytes. *J. Immunol.* 165:4367–4371.
 38. Osman, Y., T. Kawamura, T. Naito, K. Takeda, L. Van Kaer, K. Okumura, and T. Abo. 2000. Activation of hepatic NKT cells and subsequent liver injury following administration of α -galactosylceramide. *Eur. J. Immunol.* 30:1919–1928.
 39. Wu, J., Y. Song, A.B. Bakker, S. Bauer, T. Spies, L.L. Lanier, and J.H. Phillips. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science.* 285:730–732.
 40. Smyth, M.J., and D.I. Godfrey. 2000. NKT cells and tumor immunity—a double-edged sword. *Nat. Immunol.* 1:459–460.
 41. Kawamura, T., K. Takeda, S.K. Mendiratta, H. Kawamura, L. Van Kaer, H. Yagita, T. Abo, and K. Okumura. 1998. Cutting edge: critical role of NK1⁺ T cells in IL-12-induced immune responses in vivo. *J. Immunol.* 160:16–19.
 42. Leite de Moraes, M.C., G. Moreau, A. Arnould, F. Machavoine, C. Garcia, M. Papiernik, and M. Dy. 1998. IL-4-producing NK T cells are biased towards IFN- γ production by IL-12. Influence of the microenvironment on the functional capacities of NK T cells. *Eur. J. Immunol.* 28:1507–1515.
 43. Gombert, J.M., E. Tancrede-Bohin, A. Hameg, M.C. Leite de Moraes, A.P. Vicari, J.F. Bach, and A. Herbelin. 1996. IL-7 reverses NK1⁺ T cell-defective IL-4 production in the non-obese diabetic mouse. *Int. Immunol.* 8:1751–1758.
 44. Vicari, A.P., A. Herbelin, M.C. Leite-de-Moraes, U. Von Freeden-Jeffry, R. Murray, and A. Zlotnik. 1996. NK1.1⁺ T cells from IL-7-deficient mice have a normal distribution and selection but exhibit impaired cytokine production. *Int. Immunol.* 8:1759–1766.
 45. Hameg, A., C. Gouarin, J.M. Gombert, S. Hong, L. Van Kaer, J.F. Bach, and A. Herbelin. 1999. IL-7 up-regulates IL-4 production by splenic NK1.1⁺ and NK1.1⁻ MHC class I-like/CD1-dependent CD4⁺ T cells. *J. Immunol.* 162:7067–7074.