

# IL-2/CD40-driven NK cells install and maintain potency in the anti-mesothelioma effector/memory phase

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## Abstract

Murine and human mesothelioma tumors are susceptible to immunotherapy, particularly when immune adjuvants are delivered locally. We have shown that direct injection of IL-2 plus agonist anti-CD40 antibody induces regression of large mesothelioma tumors. These studies aimed to determine if NK cells contribute to IL-2/CD40 antibody-driven tumor eradication. We show that NK cells infiltrate developing mesothelioma tumors; however, their absence (in beige mice or in asialo GM<sub>1</sub> antibody-depleted C57BL/6J mice) does not alter tumor growth rates suggesting that they cannot function as effector cells in this microenvironment. Anti-CD40 antibody treatment did not alter the percent of NK cells in treated tumors or in draining lymph nodes (dLNs), and tumor resolution occurred in the absence of NK cells. However, a two-tumor model showed that NK cells contributed to CD40-driven systemic immunity leading to resolution of untreated distal tumors. IL-2 treatment led to increased proportions of NK cells in tumors and dLNs, and in the absence of NK cells, IL-2 lost its therapeutic effect. In contrast, the absence of NK cells did not reduce the anti-tumor activity of the IL-2/anti-CD40 antibody combination yet tumors recurred in NK-deficient mice and > 37% of tumor cell re-challenged mice were unable to provide protection, implying insufficient memory. Furthermore, untreated distal tumors in NK-depleted mice were less readily cured than in immunologically intact mice. These data show that NK cells infiltrate mesothelioma tumors, which, after local IL-2 and/or anti-CD40 antibody treatment, provide help for the acquisition and/or maintenance of systemic immunity and long-term effector/memory responses.

**Keywords:** innate immune response, local therapy, tumor

## Introduction

NK cells play an important role in innate immunity (1–3). NK cells rapidly secrete pro-inflammatory factors and are equipped with activating or inhibiting receptors that define various NK cell subsets (4). NK cells can respond to infection in minutes to days, and this early innate response may coordinate T- and B-cell activation (5). NK cells can be important mediators of anti-tumor immunity as they are able to lyse tumor cells *in vivo* and *in vitro* without prior sensitization. This includes eliminating metastatic cells in the circulation (6–8 and reviewed by 9, 10). Furthermore, protection from the development of carcinogen-induced tumors may be NK cell dependent (7, 11). Thus, NK cells represent targets for anti-cancer immunotherapy (12–16).

Malignant mesothelioma is an aggressive cancer and, despite improved treatment regimens, patient prognosis post diagnosis remains dismal (17–19). Thus, new treatment options are desperately needed. There is evidence in animal and human studies that the immune system can restrain mesothelioma tumor development (20–24, reviewed by 19, 25). The occasional reports of spontaneous remissions in mesothelioma patients support these observations (26–28). Furthermore, asbestos, which is the main carcinogen for mesothelioma, can directly affect NK function (29). These observations make it important to understand the role of NK cells in mesothelioma.

Human clinical trials have reproducibly shown that IL-2 can exert anti-cancer activity (reviewed by 25). While systemic

IL-2 administration is associated with significant toxicity, locally applied IL-2 has been shown to be a rational, safe and effective anti-cancer treatment option in many different human cancers, including mesothelioma (30–35) and reviewed by (25). We have previously shown that IL-2 or an agonist anti-CD40 antibody (FGK45) cure murine mesothelioma tumors if administered intratumorally (i.t.) as single agents into small tumors (36, 37). When combined, IL-2 and anti-CD40 antibody resolve larger mesothelioma tumor burdens (36–38). The cells responsible for this effect are yet to be fully defined and may include NK cells.

NK cells respond rapidly to IL-2 (39–41) and asbestos-driven suppression of NK activity can be restored by IL-2 (29). Other studies have shown that agonist anti-CD40 antibody may also target NK cells (16, 42). Early studies suggested that while mesothelioma tumor cells are resistant to NK killing, they are sensitive to IL-2-driven lymphokine-activated killer (LAK) cells (43). While the identity of LAK cells remains unclear, IL-2-activated NK cells have been implicated (44, 45).

It has been hypothesized that the microenvironment of tumors likely to respond to local therapies must already contain relevant immune cells (25). Thus, these studies aimed firstly to identify mesothelioma tumor-infiltrating NK cells and assess their role in developing mesothelioma tumors; secondly to determine if NK cells contribute to local or distal tumor regression after local IL-2 and/or agonist anti-CD40 antibody treatment and finally to determine if NK cells contribute to the installation of IL-2/CD40-driven memory. The AE17 murine model of mesothelioma used in these studies was induced in mice with asbestos fibers and inoculation of cloned tumor cells into mice results in progressing tumors histologically similar to human mesothelioma (36). This study shows that NK cells infiltrate developing mesothelioma tumors, which, after local IL-2 and agonist anti-CD40 antibody treatment, provide help for the acquisition and/or maintenance of systemic immunity and long-term effector/memory responses.

## Methods

### Mice

Female C57BL/6J (H-2<sup>b</sup>) and beige mice aged 6–8 weeks were obtained from the Animal Resources Centre (Perth, Australia). All mice were used in accordance with institutional guidelines and approval of the University of Western Australia and Curtin University's Animal Ethics Committees (AEC). Mice were injected subcutaneously (s.c.) with  $5 \times 10^5$  tumor cells per site and tumor growth monitored. Animals were sacrificed when tumors reached 100 mm<sup>2</sup> as per AEC conditions.

### Murine tumor model

AE17 is a malignant mesothelioma cell line derived from C57BL/6J mice injected with asbestos fibers as previously described (36).

### Anti-asialo GM<sub>1</sub> depletion

Anti-asialo GM<sub>1</sub> (Wako Chemicals, Neuss, Germany) was used according to the manufacturer's instructions to deplete

NK cells. Animals were given two doses intraperitoneally (i.p.) before treatment (20 µl per dose) and then a dose every 4–5 days, throughout treatment. NK cell depletion was checked at the start and end of the experiment by flow cytometric analysis of samples stained for NK1.1 and αβ TCR (NK cells: NK1.1<sup>+</sup>αβTCR<sup>neg</sup>) and was 80–90% effective.

### Antibodies

Four monoclonal antibodies, NK1.1, DX5, 4D11 (all from Becton Dickinson, Mountain View, CA, USA) and AGM-1 (from Wako Chemicals) directed against molecules expressed on murine NK cells were used. Antibodies targeting the NK1.1 surface antigen, encoded by the gene *NKR-P1C*, are the most widely used NK cell marker for flow cytometric analysis on selected strains of mice (46), including the C57BL/6J strain used in this study. The 4D11 antibody reacts with an inhibitory receptor, Ly49G2 (also known as LGL-1), on the surface of NK cells and mediates negative regulation of NK cytolytic activity (47). DX5 targets CD49b (also known as α2 Integrin or Very Late Antigen-2; (48)). Anti-asialo GM<sub>1</sub> targets the glycosphingolipid asialo GM<sub>1</sub> antigen. Anti-αβTCR or anti-CD3 (Becton Dickinson) were used to discriminate NKT cells (NK1.1<sup>+</sup>αβTCR<sup>+</sup> or NK1.1<sup>+</sup>CD3<sup>+</sup>) from NK cells (NK1.1<sup>+</sup>αβTCR<sup>neg</sup> or NK1.1<sup>+</sup>CD3<sup>neg</sup>).

### IL-2 and agonist anti-CD40 antibody

Proleukin (rhIL-2; Cetus Corporation, Emeryville, CA, USA) and anti-CD40 antibody (FGK45; WEHI, Melbourne, Victoria, Australia) were diluted in PBS to the required concentration as previously described (38). Mice bearing small tumors (<20 mm<sup>2</sup>) were treated with the IL-2 and anti-CD40 antibody monotherapies, while mice with large tumors (25–60 mm<sup>2</sup>) were treated with the IL-2/anti-CD40 antibody combination therapy as previously described (49). Briefly, intratumoral (i.t.) treatment was given three times per week for 2 weeks consisting of PBS, IL-2 (20 µg per dose) anti-CD40 antibody (40 µg per dose) or IL-2/anti-CD40 antibody. The definition of small versus large tumors is relative to the maximum allowable size, 100 mm<sup>2</sup> as per AEC conditions.

### Flow cytometry

Samples were prepared as a single-cell suspension and stained for flow cytometric analysis using the antibodies described above. Analysis was performed on a FACScan (Becton Dickinson) using Cell Quest software or FlowJo software.

### Immunohistochemistry

Frozen sections (10 µm) of OCT-embedded draining lymph node (dLN) and tumors were fixed in cold acetone and washed, and endogenous peroxidase was blocked using 1% hydrogen peroxide. Endogenous biotin was blocked using the avidin blocking kit (Vector Laboratories, Burlingame, CA, USA). Primary antibody directed against Ly49G2 (4D11; Becton Dickinson) or isotype control (Rat IgG2a; Becton Dickinson) were applied for 45 min at room temperature. This was followed by sequential incubations with secondary biotinylated antibodies (rat anti-mouse Ig; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and streptavidin HRP (Dako, Glostrup, Denmark), washing with PBS

between steps. Staining was visualized by precipitating 3,3-diaminobenzidine (Sigma-Aldrich, Sydney, Australia) and counterstaining with hematoxylin. Sections were rinsed in ethanol, cleared in xylene and mounted using Depex. Slides were visualized on a Zeiss Axioskop 2 plus microscope with attached Zeiss AxioCam digital camera and Axiovision 3.1 software (Carl Zeiss, Oberkochen, Germany).

### Statistical analysis

Statistical significance was calculated using GraphPad PRISM 4.0. Student's *t*-test was used to determine differences between two populations. One-way analysis of variance was used to determine differences between more than two populations.

## Results

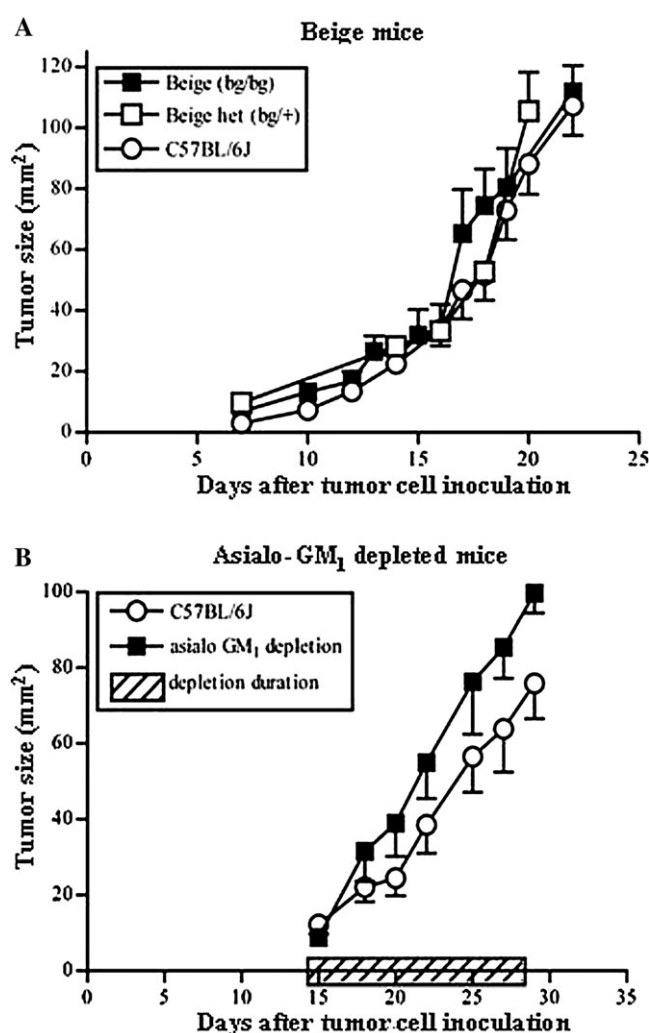
### NK cells infiltrate mesothelioma tumors early in tumor development

Recent studies of human mesothelioma tumor tissue showed the presence of infiltrating CD16<sup>+</sup> or CD56<sup>+</sup> NK cells (50, 51). However, to date, no study has systematically investigated NK cells in murine mesothelioma models during tumor progression. Thus, flow cytometric analysis of tumor samples stained with anti-NK antibodies (NK1.1, Ly49G2, CD49b and asialo GM<sub>1</sub>) was performed at different stages of AE17 tumor growth. The data revealed putative NK cells in the mesothelioma tumor microenvironment by day 7 of tumor progression with decreasing proportions of NK cells in larger mesothelioma tumors (Supplementary Figure 1, available at *International Immunology* Online).

### NK cells do not hinder mesothelioma tumor progression

DX5<sup>+</sup> or CD49b<sup>+</sup> NK cells are reported to represent fully functional NK cells (48) and their presence in the mesothelioma microenvironment imply an effector function that could slow tumor growth. Thus, the next series of experiments assessed the role of NK cells during mesothelioma tumor development using NK cell impaired beige mice (Fig. 1A). The beige mutation blocks the normal process of NK cell degranulation, leading to impaired antibody-dependent and antibody-independent cytotoxicity of tumor cells (52). However, the function of NKT cells in mice with the beige mutation appears to be intact (53). No differences in tumor growth were seen in C57BL/6J, beige (C57BL/6J-*Lyst*<sup>bg/bg</sup>) and heterozygous (C57BL/6J-*Lyst*<sup>bg/+</sup>) mice inoculated with AE17 cells (Fig. 1A).

Since beige mice have impaired NK activity but are not NK cell-free (and NK cells are still capable of producing cytokines in beige mice, (54, 55)), the role of NK cells was also tested in C57BL/6J mice depleted of NK cells by treatment with the anti-asialo GM<sub>1</sub> antibody. The anti-asialo GM<sub>1</sub> antibody is reported to deplete NK, but not NKT, cells (11). Depletions were commenced when tumors were palpable (3 × 3 mm<sup>2</sup>) and continued until tumors reached 100 mm<sup>2</sup>. There was a slight increase in tumor growth in mice depleted of NK cells; however, the difference did not reach statistical significance (Fig. 1B). Taken together, these data imply that NK cells do not impact on mesothelioma tumor growth rate.



**Fig. 1.** NK cells do not hinder mesothelioma tumor progression. C57BL/6J, beige (C57BL/6J-*Lyst*<sup>bg/bg</sup>) and beige heterozygous (C57BL/6J-*Lyst*<sup>bg/+</sup>) mice were inoculated with  $5 \times 10^5$  AE17 cells and tumor growth monitored (A). In a separate experiment, C57BL/6J mice were inoculated with  $5 \times 10^5$  AE17 cells and tumors allowed to develop to a palpable size ( $3 \times 3$  mm<sup>2</sup>). Anti-asialo GM<sub>1</sub> depletion was commenced and continued until tumors reached 100 mm<sup>2</sup> (B). Data are pooled from two experiments ( $n = 8$ –10 mice per group) and shown as mean  $\pm$  SEM.

### NK cell proportions increase in tumors responding to IL-2 +/- anti-CD40 antibody treatment

We have previously shown that intratumoral (i.t.) treatment with IL-2 induces mesothelioma complete tumor regression mediated by CD4<sup>+</sup> and CD8<sup>+</sup> T cells (36). We have also shown that i.t. agonist anti-CD40 antibody leads to complete tumor regression mediated by CD8<sup>+</sup> T cells and follicular B cells (37). Unexpectedly, the combination of IL-2 with agonist anti-CD40 antibody required CD8<sup>+</sup> T cells and granulocytes to resolve mesothelioma tumors (38). However, the role of NK cells was not examined in those studies. Thus, the next series of experiments were designed to determine if NK cells contributed to treatment-induced tumor regression.

Immunohistochemistry showed that Ly49G2<sup>+</sup> NK cells expanded in response to IL-2 +/- anti-CD40 antibody (Fig. 2A).

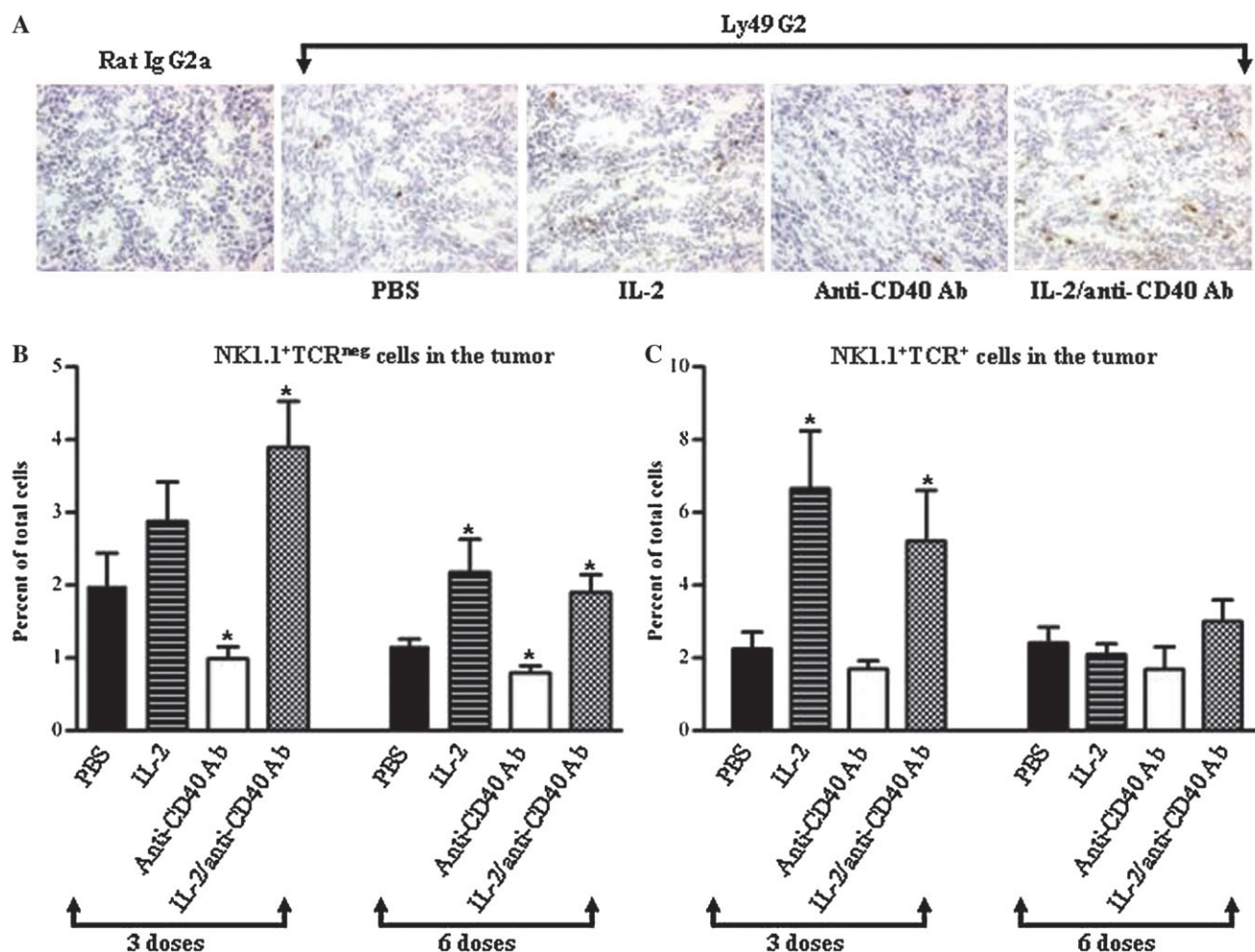
Dual staining showed that 4D11<sup>+</sup> cells did not co-express CD3 within tumors and dLN from IL-2/anti-CD40 antibody-treated mice (Supplementary Figure 2, available at *International Immunology* Online) and therefore did not represent NKT cells or a T-cell subset. Flow cytometric analysis of dual stained samples (NK1.1 and  $\alpha\beta$ TCR) was used to determine the percentage of NK cells (NK1.1<sup>+</sup> $\alpha\beta$ TCR<sup>neg</sup>) and NKT cells (NK1.1<sup>+</sup> $\alpha\beta$ TCR<sup>+</sup>) infiltrating tumors. Only the IL-2/anti-CD40 antibody combination induced a significant albeit small increase in the percent of tumor-associated NK cells midway through therapy (dose 3). By the final sixth dose tumors regressing in response to both the IL-2 monotherapy and the combination protocol contained significantly higher percents of NK cells than diluent (PBS)-treated progressing tumors (Fig. 2B). This data is in agreement with others who have shown that IL-2 administration results in a significant expansion of a Ly49G2<sup>high</sup> NK cell subset (41). The anti-CD40 antibody monotherapy negatively affected NK cell proportions at

both time points; i.e. the NK cell proportions decreased. Taken together, these data suggest that NK cells are positioned to play a role in IL-2 +/- anti-CD40 antibody induced anti-tumor immunity but not in the anti-CD40 antibody monotherapy.

Interestingly, during IL-2 +/- anti-CD40 antibody treatment, there was a transient, but significant, increase of NKT cells proportions midway through therapy (Fig. 2C). By the final dose, their proportions had decreased to those seen in progressing PBS-treated tumors. The anti-CD40 antibody monotherapy did not induce any significant changes in NKT cell proportions. These data suggest that activated NKT cells may play a transient role in IL-2-induced anti-tumor immunity; however, further studies are required to confirm this.

*NK and NKT cells increase in dLNs during IL-2 +/- anti-CD40 antibody therapy*

The percent of NK cells within dLNs peaked after three doses of IL-2 +/- anti-CD40 antibody and remained significantly

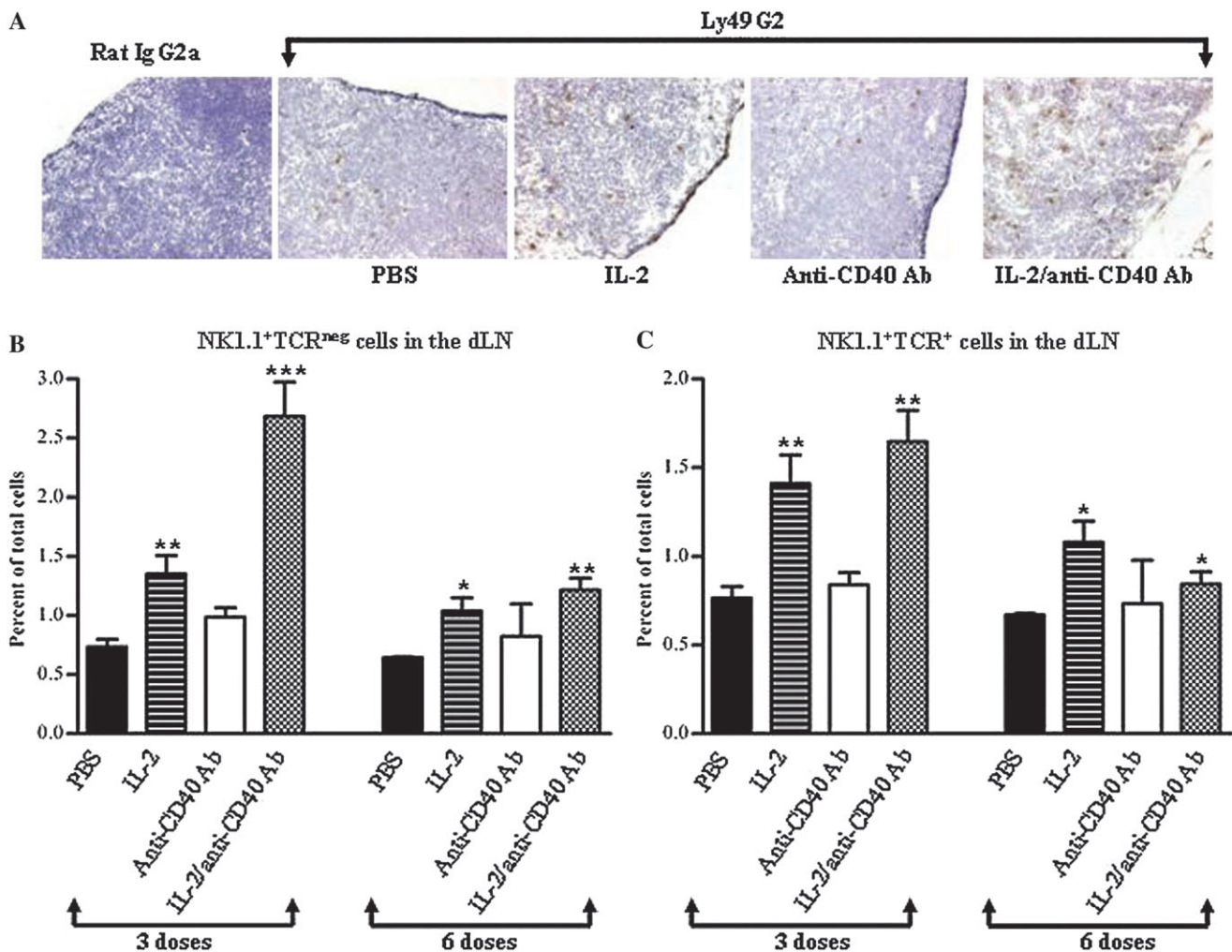


**Fig. 2.** Ly49G2<sup>+</sup> tumor-infiltrating cells increase in IL-2 +/- anti-CD40 antibody-treated mice. AE17 tumors collected from mice treated with three doses of PBS, IL-2, anti-CD40 antibody or IL-2/anti-CD40 antibody were cryosectioned and immunostained using an anti-Ly49G2 antibody (4D11) or rat IgG2a isotype on 10  $\mu$ m sections (A). Representative photos are shown (magnification  $\times 200$ ) from two experiments ( $n = 6$  mice per group). Tumors collected from mice bearing small AE17 tumors treated with three or six doses of PBS, IL-2, anti-CD40 antibody and mice bearing large AE17 tumors treated with IL-2/anti-CD40 antibody were disaggregated and cells dual stained with NK1.1-PE and  $\alpha\beta$ TCR-FITC antibody for NK cells (NK1.1<sup>+</sup>TCR<sup>neg</sup>; B) and NKT cells (NK1.1<sup>+</sup>TCR<sup>+</sup>; C). Data is shown as mean  $\pm$  SEM of the percent of total cells are pooled from two experiments ( $n = 8$  mice per group). \* =  $P < 0.05$  comparing treated groups to PBS-treated mice.

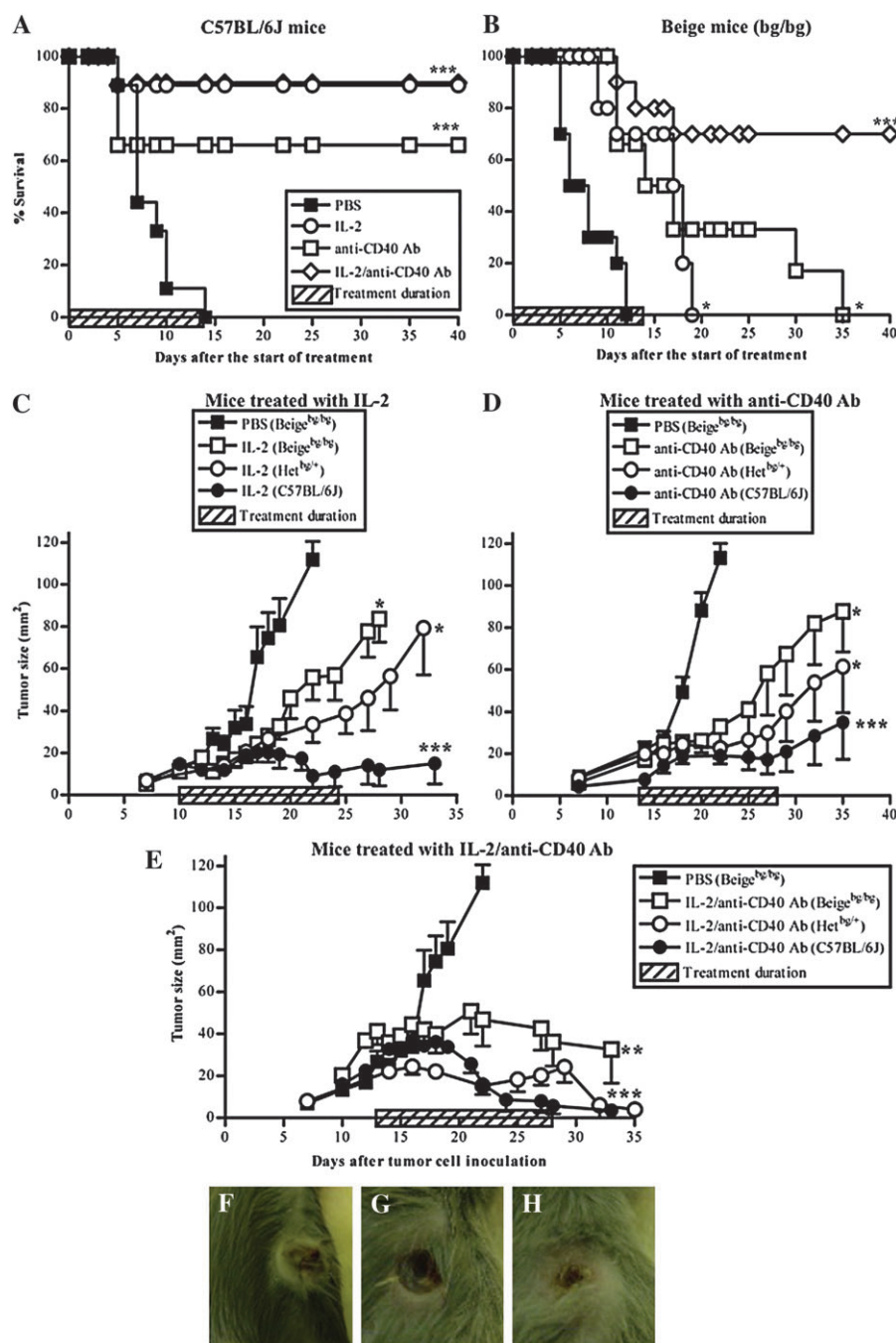
higher than PBS controls after six doses (Fig. 3B). Similarly, NKT cells were significantly increased after three and six doses with IL-2 +/- anti-CD40 antibody (Fig. 3C). No changes in the percentage of either NK or NKT cells were seen in the dLN after anti-CD40 antibody treatment. These results were confirmed by immunohistochemistry using anti-Ly49G2 antibody (Fig. 3A).

*NK cells are effectors for the IL-2 or anti-CD40 antibody monotherapies but are redundant for the combination therapy*  
C57BL/6J and beige (C57BL/6J-*Lyst<sup>bg/bg</sup>*) AE17-bearing mice were treated with i.t. PBS, IL-2, anti-CD40 antibody or both as described above. Treatment of beige mice with the IL-2 or anti-CD40 antibody monotherapies significantly improved survival (Fig. 4B) relative to the PBS controls, although unlike C57BL/6J mice (Fig. 4A), no cures were seen as the tumors eventually grew (Fig. 4B). Interestingly, the

combination IL-2 and anti-CD40 antibody treatment significantly enhanced survival in 71% of beige mice (Fig. 4B), similar to C57BL/6J mice (92%; Fig. 4A). Beige mice may have defective granulocytes (56), cytotoxic T-cell and antibody responses, while beige heterozygous mice have intact granulocyte (56), cytotoxic T-cell (57) and antibody responses (58). However, studies have suggested that beige heterozygous mice may have slightly impaired NK status (59–61). While the effect of the beige mutation on the IL-2 or anti-CD40 antibody effect was more pronounced in beige mice, it was still seen in heterozygous animals (Fig. 4C and D). These data suggest that NK cells function as effectors in these therapies; note the tumor burden is small. In contrast, the combination IL-2 and anti-CD40 antibody treatment induced complete regression of large tumors in the majority of beige and heterozygous animals (Fig. 4E). NK cells in beige mice lack cytotoxicity are still capable of



**Fig. 3.** NK and NKT cells increase in dLNs of IL-2 +/- anti-CD40 antibody-treated mice. dLNs collected from mice treated with three doses of PBS, IL-2, anti-CD40 antibody or IL-2/anti-CD40 antibody were cryosectioned and immunostained using an anti-Ly49G2 antibody (4D11) or rat IgG2a isotype on 10 µm sections (A). dLNs were collected from mice bearing small AE17 tumors treated with three or six doses of PBS, IL-2, anti-CD40 antibody or mice bearing large AE17 tumors treated with IL-2/anti-CD40 antibody. Cells were dual stained with NK1.1-PE and αβTCR-FITC antibody for NK cells (NK1.1<sup>+</sup>TCR<sup>neg</sup>; B) and NKT cells (NK1.1<sup>+</sup>TCR<sup>+</sup>; C) and the percent of total cells calculated relative to the isotype controls. Pooled data from two experiments (*n* = 8 mice per group) are represented as mean ± SEM. \* = *P* < 0.05; \*\* = *P* < 0.01, \*\*\* = *P* < 0.001 comparing treated groups to PBS-treated mice.



**Fig. 4.** NK cells are required for the IL-2 or anti-CD40 antibody monotherapies but are redundant for the combination therapy. C57BL/6J (A, C–E), beige (C57BL/6J-*Lyst*<sup>bg/bg</sup>; B and C–E) or beige heterozygous mice (C57BL/6J-*Lyst*<sup>bg/+</sup>; C–E) were inoculated with  $5 \times 10^5$  AE17 cells and tumors allowed to develop. I.t. treatment was given three times per week for 2 weeks consisting of PBS, IL-2 (small tumors, A–C) anti-CD40 antibody (small tumors, A, B and D) or IL-2/anti-CD40 antibody (large tumors, A, B and E). Data are shown as survival (A and B) or tumor growth (C–E) and are pooled from two experiments ( $n = 5$ –10 mice per group) and represented as mean  $\pm$  SEM. \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$  comparing treatment groups to PBS-treated controls. Representative photos show necrosis in C57BL/6J mice (F), beige mice (G) or beige heterozygous mice (H) after four doses.

producing cytokines (54, 55), which could lead to the effects observed with the IL-2/anti-CD40 antibody treatment. However, similar results were seen when NK cells were depleted using the anti-asialo GM<sub>1</sub> antibody during IL-2 +/- anti-CD40 antibody therapy (38), these data suggest that NK cells are not important in this setting.

Necrosis was observed with IL-2/anti-CD40 antibody treatment in beige (Fig. 4G), heterozygous mice (Fig. 4H) and anti-asialo GM<sub>1</sub>-depleted mice (data not shown), similar to C57BL/6J mice (Fig. 4F). Thus, NK cells are not responsible for tumor necrosis induced by this treatment. Overall, the data imply that NK cells may be required for the IL-2 or

anti-CD40 antibody single agents to be effective against small tumors but that NK cells are not required for the eradication of large tumors when IL-2 and anti-CD40 antibody are combined.

#### *NK cells contribute to the eradication of distal untreated tumors*

The next series of experiments addressed the role NK cells play in eradicating distal-untreated tumors using a two-tumor model. To do this, mice were inoculated with AE17 cells s.c. into both the left and right flanks on day 0. Two days before the start of anti-CD40 antibody with or without co-administration of IL-2 treatment, animals were depleted of NK cells using anti-asialo GM<sub>1</sub> antibody. Depletions continued throughout the treatment period; depleted NK cells returned 5 days after the last dose. IL-2 was not shown here as we have previously reported that IL-2 does not impact on untreated distal tumors (38).

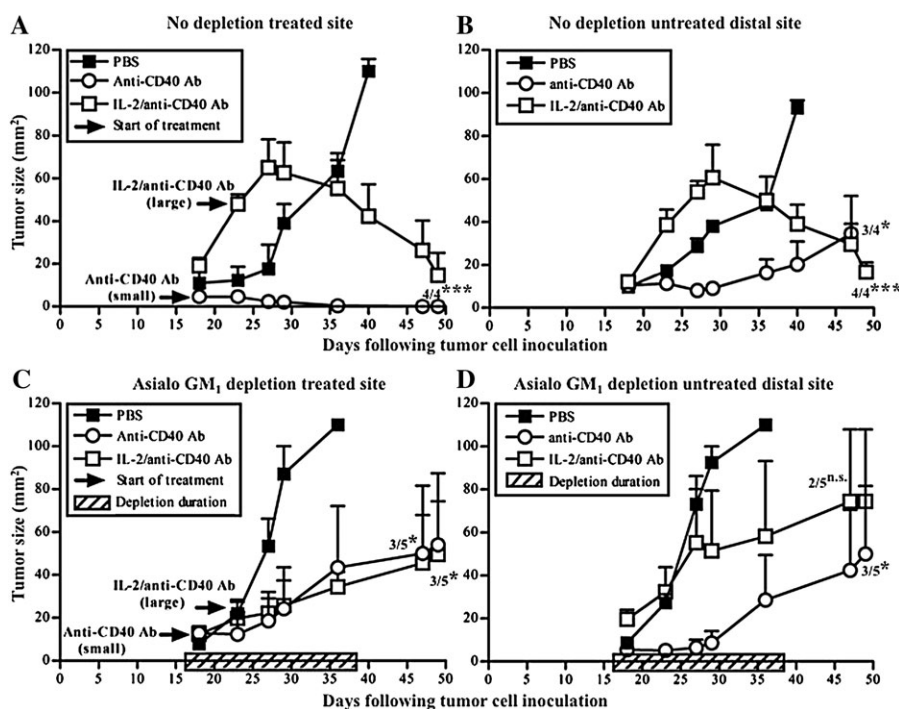
Immunologically intact (i.e. no depletion) mice responded to the anti-CD40 antibody alone or the combination treatment of IL-2/anti-CD40 antibody leading to complete regression of treated site tumors in 8/8 mice (Fig. 5A). Untreated distal tumors regressed in 3/4 mice treated with anti-CD40 antibody monotherapy and 4/4 mice receiving IL-2/anti-CD40 antibody (Fig. 5B). Mice depleted of NK cells maintained a partial anti-tumor response after anti-CD40 antibody or IL-2/anti-CD40 antibody with 3/5 mice in each group exhibiting complete

regression of the treated-site tumors (Fig. 5C). Similarly, a partial effect was seen in untreated contralateral tumors with anti-CD40 antibody alone (3/5 mice with complete tumor regression) or IL-2/anti-CD40 antibody (2/5 mice with complete tumor regression; Fig. 5D). These data imply that CD40 +/- IL-2-driven NK cells contribute to the induction and/or maintenance of systemic responses that lead to eradication of distal tumors.

#### *NK cells play a role in the acquisition of long-term effector/memory responses*

Mice bearing single AE17 tumors that had completely regressed after the anti-CD40 antibody monotherapy or the IL-2/anti-CD40 antibody combination, with or without asialo GM<sub>1</sub> depletion, were left for 3 months and then re-challenged with  $5 \times 10^5$  AE17 cells at the contralateral site (Table 1). In some mice depleted of NK cells with asialo GM<sub>1</sub> depletion, tumors recurred within 3 months (4/14 mice). Tumor recurrence did not occur in mice that were immunologically intact (no depletion) during treatment (0/12 mice) and these mice were completely protected from re-challenge (9/9 mice). Interestingly, mice in which NK cells were depleted with asialo GM<sub>1</sub> depletion during treatment were only partially protected from rechallenge (5/8 mice; Table 1).

As discussed above, the anti-CD40 antibody monotherapy and IL-2/anti-CD40 antibody treatment induced complete tumor regression in some beige mice, beige heterozygous



**Fig. 5.** IL-2/CD40-driven NK cells promote regression of untreated distal tumors. C57BL/6J mice were inoculated with  $5 \times 10^5$  AE17 cells s.c. into the left and right flanks and tumors allowed to develop. Two days before the start of i.t. treatment (i.e. anti-CD40 antibody into small tumors and IL-2/anti-CD40 antibody into large tumors, indicated by arrows), animals were left immunologically intact (A and B) or depleted of NK cells using anti-asialo GM<sub>1</sub> antibody (C and D). Depletions were continued for 14–16 days. I.t. treatment was given three times per week for 2 weeks with PBS, anti-CD40 antibody or IL-2/anti-CD40 antibody. Data are from one experiment ( $n = 4–5$  mice per group) and shown as mean  $\pm$  SEM. The numbers, 4/4, 3/4, 3/5 and 2/5, represent the number of mice with complete tumor regression (at the local-treated site or the distal-untreated site) out of the total mice treated per group. \* =  $P < 0.05$ , \*\*\* =  $P < 0.001$  comparing treatment groups to PBS-treated controls. ns = not significant.

**Table 1.** NK cells are required for development of effective memory

Number of mice	Anti-CD40 antibody	IL-2/anti-CD40 antibody
No depletion		
Tumor regression at end of treatment	6/6	12/12
Tumor recurrence post-treatment	0/6	0/12
Protected from rechallenge at contralateral site	3/3	9/9
Asialo GM <sub>1</sub> depletion		
Tumor regression at end of treatment	6/9	14/18
Tumor recurrence post-treatment	2/6	4/14
Protected from rechallenge at contralateral site	1/2	5/8
Beige heterozygous (C57BL/bg) mice		
Tumor regression at end of treatment	2/5	8/10
Tumor recurrence post-treatment	2/2	6/8
Beige (bg/bg) mice		
Tumor regression at end of treatment	0/6	10/14
Tumor recurrence post-treatment	NA	8/10

C57BL/6J mice inoculated with  $5 \times 10^5$  AE17 tumor cells s.c. on day 0 were left to develop into tumors ranging from 16 to 40 mm<sup>2</sup> before therapy commenced. Mice were depleted of NK cells using the anti-asialo GM<sub>1</sub> antibody 1–2 days before the start of treatment consisting of 3 i.t. injections per week for 2 weeks with the anti-CD40 antibody (40 µg per dose) monotherapy into small tumors, or the IL-2 (20 µg per dose)/anti-CD40 antibody (40 µg per dose) combination into large tumors. Mice demonstrating complete tumor regression after treatment were left for 3 months and then re-challenged with  $5 \times 10^5$  AE17 at the contralateral site. Data shown are the number of mice with complete tumor regression, tumor recurrence (after regression with treatment) and the number protected after rechallenge in the different treatment groups. In a separate experiment, beige homozygous (C57BL/6J-*Lyst*<sup>bg/bg</sup>) and beige heterozygous (C57BL/6J-*Lyst*<sup>bg/+</sup>) mice were inoculated with  $5 \times 10^5$  AE17 cells and tumors allowed to develop. I.t. treatment was given as described above. Mice with complete tumor regression after treatment were monitored for tumor recurrence. Data shown are the number of mice with complete tumor regression and then tumor recurrence in each of the different treatment groups.

mice and asialo GM<sub>1</sub>-depleted mice (Table 1). These mice were monitored for tumor recurrence. Recurrences at the original tumor site occurred in a number of animals (Table 1). Tumors reappeared in beige mice (Fig. 6B) sooner than in heterozygous animals (Fig. 6C). Tumors never recurred in normal C57BL/6J mice (Fig. 6A); these mice were monitored for the remainder of their natural lives (>500 days).

These results suggest that even though NK cells are not critically required for tumor eradication induced during and immediately after IL-2/anti-CD40 antibody therapy, they are required for the induction and/or maintenance of potent long-term effector/memory responses. These data suggest that IL-2/CD40-driven NK cells are involved in the acquisition and maintenance of effector/memory T-cell populations.

## Discussion

Several studies, including our own, have shown that murine and human mesothelioma tumors are susceptible to local IL-2 therapy (25,30–36). Our recent studies have also shown that local agonist anti-CD40 antibody mediates complete regression of mesothelioma tumors (37). We have shown that local IL-2 combined with anti-CD40 antibody is particularly effective at inducing the complete regression of monotherapy-resistant large mesotheliomas (38). NK cells are highly responsive to IL-2 (39–41) and may also respond to agonist anti-CD40 antibody (16). Thus, while CD8<sup>+</sup> T cells are key effectors in IL-2 with or without anti-CD40 antibody-driven anti-tumor immunity (49), it is possible that tumor-infiltrating NK cells contribute to tumor regression. This contribution may be through direct lysis of tumor cells or indirectly via interactions with antigen-presenting cells and CD8<sup>+</sup> T cells (42,62–64).

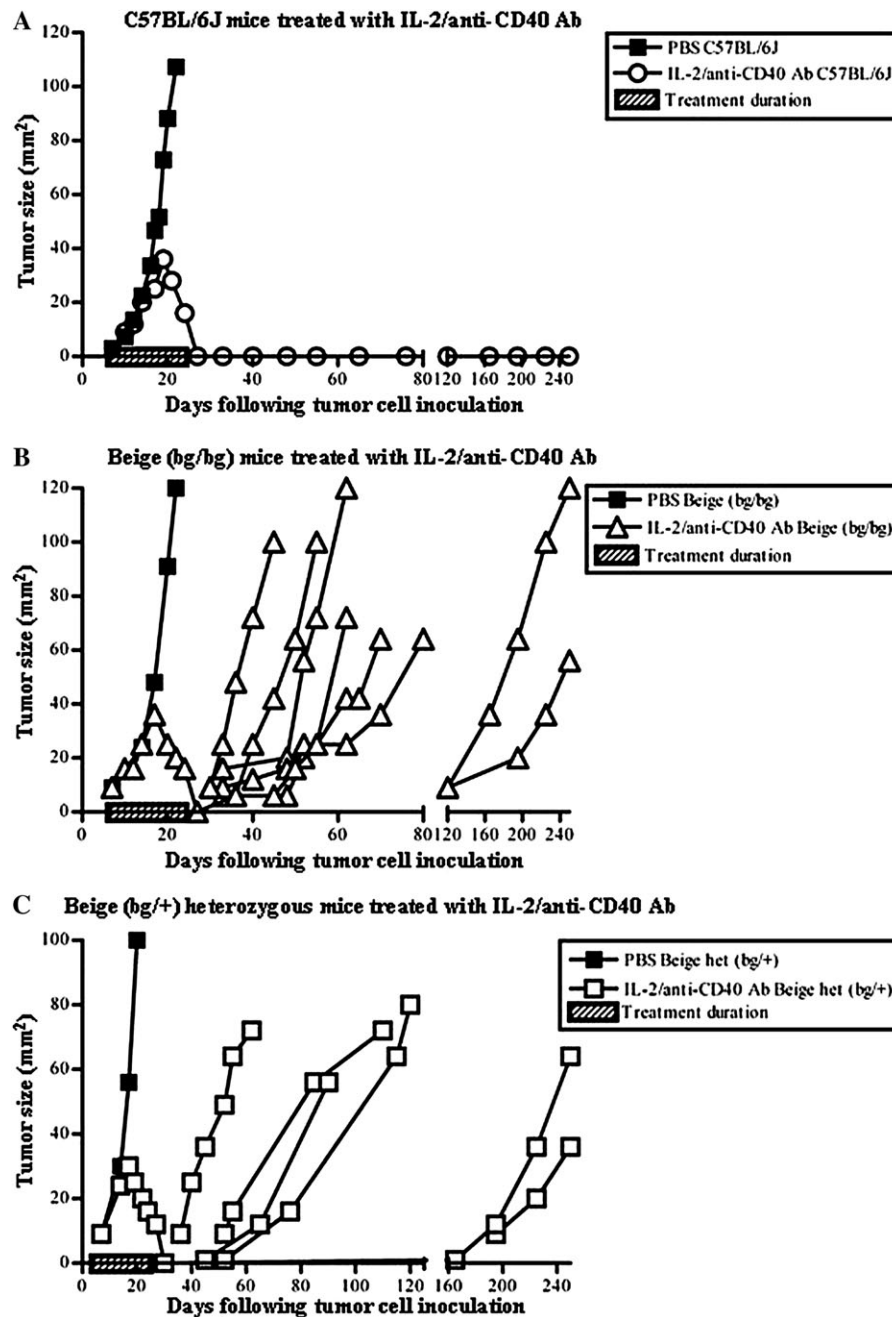
These studies initially aimed to determine if NK cells infiltrate murine mesothelioma tumors. Varying numbers of puta-

tive NK cells were seen in developing mesothelioma tumors. NK cells were located at the periphery and scattered throughout the tumors. These data suggest the murine mesothelioma tumor microenvironment is similar to human mesothelioma in which a CD16<sup>+</sup> or CD56<sup>+</sup> NK cell infiltrate is seen (50, 51).

Increasing exposure to asbestos is reported to be associated with decreasing NK cell effector function despite a concomitant increase in the number of circulating NK cells (65). Furthermore, mesothelioma patients are reported to exhibit a profound deficiency in NK cell function (66). These data imply a role for NK cells during the early stages of mesothelioma tumor development. In contrast, a recent study showed that while mesothelioma patients with a high density of CD8<sup>+</sup> cells had a significantly longer overall survival post surgery than those with a low density of CD8<sup>+</sup> cells, there was no survival benefit according to the density of CD56<sup>+</sup> NK cells (51). In our studies, use of beige mice with impaired NK cell function and NK-depleted C57BL/6J mice failed to reveal an effector function for NK cells during mesothelioma development. Taken together, the human and mouse data show that NK cells infiltrating mesothelioma tumors do not exert significant effector function, particularly at the late stages of tumor development, possibly on account of rapid impairment of NK cell function by mesothelioma-associated factors.

Tumor-infiltrating NK cells represent potential targets for local therapies. Thus, we examined the role of NK cells in murine mesothelioma tumors responding to local IL-2 and/or anti-CD40 antibody. In our hands, the locally applied monotherapies using IL-2 or anti-CD40 antibody were only effective in small tumor burdens, whereas their combination proved effective in much larger tumors (38).

There are reports that stimulatory anti-CD40 antibodies indirectly activate NK cells to mediate anti-tumor immunity



**Fig. 6.** NK cells are required for the prevention of tumor recurrence. C57BL/6J (A), beige (B, C57BL/6J-*Lyst*<sup>bg/bg</sup>) and beige heterozygous mice (C, C57BL/6J-*Lyst*<sup>bg/+</sup>) that exhibited complete tumor regression after IL-2/anti-CD40 antibody treatment were monitored for tumor recurrence. Tumor growth is shown for individual mice with tumor recurrence for beige mice ( $n = 8$ ) and beige heterozygous mice ( $n = 6$ ).

(16). Here, we show that while local delivery of anti-CD40 antibody alone mediated the complete regression of small tumors, it did not significantly alter the percent of NK cells or NKT cells in mesothelioma tumors or in dLNs. Similarly, CD40-driven anti-tumor immunity was only slightly impaired in the absence of NK cells. Thus, NK cells do not play a major role in i.t. anti-CD40 antibody-driven eradication of treated site tumors. In contrast, the two-tumor model showed that NK cells were significant contributors to CD40-driven systemic immunity such that untreated distal tumors could

be eradicated. The mechanism of this contribution is unclear. A possible scenario is enhanced cross talk and activation between dendritic cells, NK cells and T cells or between NK cells and B cells and requires further investigation.

The percent of both NK and NKT cells increased within mesothelioma tumors and dLNs after local treatment with either IL-2 alone or the IL-2/anti-CD40 antibody combination. Furthermore, in the absence of functional NK cells (i.e. in beige mice or in NK-depleted C57BL/6J mice), the IL-2 monotherapy lost its therapeutic effect. Note that although

beige mice and mice depleted with the anti-asialo GM<sub>1</sub> antibody lack functional NK cells, they are reported to contain functional NKT cells (11, 53). Therefore, NKT cells alone do not appear to exert effector function, although further studies are required to confirm this. Importantly, IL-2-driven NK cells appear to exert effector function. Our previous studies suggest that this IL-2-driven NK function is likely to involve cross talk with dendritic cells and CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as has been suggested by others (42,62–64), because depletion of either T-cell population completely ablates IL-2-driven tumor regression in this model (36).

The absence of NK cells only slightly reduced the anti-tumor activity of the IL-2/anti-CD40 antibody combination. One possible explanation is that both agents activate a number of different immune cell types, and the removal of NK cells leaves behind other effector cells that can eradicate tumors. Our previous studies found that CD8<sup>+</sup> T cells and granulocytes were key anti-tumor effectors activated via the IL-2/anti-CD40 antibody combination (38). Interestingly, however, tumors recurred in beige homozygous and heterozygous mice and asialo GM<sub>1</sub>-depleted mice thought to be cured of mesothelioma tumors following treatment with IL-2 and anti-CD40 antibody. These data suggest that in the absence of NK cells, effector CD8<sup>+</sup> T cells and granulocytes restrain rather than completely eradicate tumor cells. Furthermore, untreated distal tumors in mice depleted of NK cells with asialo GM<sub>1</sub> were less readily cured than in immunologically intact mice. We have recently shown that IL-2/anti-CD40-induced resolution of distal tumors is dependent on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (49). Thus, NK cells may play a key role in licensing other effector cells, most likely T cells, to levels that ensure complete tumor cell eradication throughout the body.

Our data further imply a role for NK cells in the acquisition and/or maintenance of memory responses. We have shown that CD4<sup>+</sup> and CD8<sup>+</sup> T cells play a critical role in the development of IL-2/anti-CD40 driven memory responses (49), thus our data implies a key role for NK cells in regulating T-cell memory responses. Taken together, these data are in agreement with studies demonstrating that NK cells regulate adaptive immune responses (67–71) and are essential for T-cell expansion and development of immunologic memory (70). It is possible that NK cells function as memory cells (72). It is also possible that B-cell activation and memory-induction driven by CD40-CD40L interactions between NK cells and B cells becomes important (73); future studies are required to address these issues. Beige homozygous and heterozygous mice have impaired NK cell lytic activity (52, 55,59–61); however, their NK cells are still capable of producing cytokines (54). Therefore, our studies suggest that memory induced by NK cells is not mediated by NK-derived cytokines. Other possible mechanisms include CD40/CD40L interactions and/or DC cross talk. Thus, NK-derived signals received by T and/or B cells may affect their capacity to become memory cells.

In summary, our data show that endogenous NK cells do not impact on mesothelioma tumor development. However, local application of IL-2 and/or agonist anti-CD40 antibody recruits NK cell help for the acquisition and maintenance of systemic immunity and long-term effector/memory responses. These data suggest that IL-2/CD40-driven NK cells can play

a significant role in determining the breadth and duration of anti-mesothelioma immune responses.

## Supplementary data

Supplementary data are available at *International Immunology* Online.

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