Immunology and Cell Biology (2010), 1–13

www.nature.com/icb

ORIGINAL ARTICLE

CD40-activated B cells contribute to mesothelioma tumor regression

Connie Jackaman[1,5], Scott Cornwall[2,3,4,5], Peter Thomas Graham[2,3,4] and Delia Jane Nelson[1,2,3,4]

Targeting CD40, a member of the tumor necrosis factor superfamily, using agonist antibodies (Abs) produces dramatic antitumor effects. Indeed, high-dose intravenous anti-CD40 Ab 'licenses' dendritic cells (DCs) that instruct activated CD8$^+$ cytotoxic T cells to leave lymph nodes (LNs) and penetrate the mesothelioma tumor microenvironment. However, toxic side effects and the potential of an 'overwhelmed' immune response warrant an alternative approach. In this study, we show that injecting lower doses of anti-CD40 Ab directly into the tumor bed avoided toxic side effects and prolonged survival in 60% of mice, with most cured. Unexpectedly, DCs in tumors and LNs 'disappeared', CD8$^+$ tumor-specific T-cell numbers and function were not enhanced, and T cells did not infiltrate regressing tumors. CD4$^+$ or CD8$^+$ depletion only marginally hindered anti-CD40 Ab efficacy implying another effector mechanism. B-cell numbers significantly increased in tumors, draining LNs and spleens during intratumoral anti-CD40 Ab treatment. CD40 targeting had no effect on splenic B-1 cells, obliterated marginal zone B cells and promoted follicular (FO) B-cell activity. Adoptive transfer of tumor antigen-experienced, CD40-activated B cells, or their immunoglobulin products, which recognized autoantigens on mesothelioma cells, protected against tumor challenge. Finally, studies using B-cell knockout mice showed that successful treatment of established tumors required the presence of B cells. Thus, these data suggest that CD40-activated FO B cells can become an important component of an effective antitumor immune response.
Immunology and Cell Biology advance online publication, 13 July 2010; doi:10.1038/icb.2010.88

Keywords: tumor immunity; B cells; cytotoxic T cells; agonist anti-CD40 antibody; tumor targeting

A number of tumors showed promising sensitivity to agonist anti-CD40 antibody (Ab) therapy.[1–4] Intravenous (i.v.) anti-CD40 Ab therapy is often used, yet it could lead to serious side effects and risks 'exhausting' the immune system.[2,5–7] Intratumoral (i.t.) administration of agonist anti-CD40 Ab is an alternative strategy that directly targets the tumor microenvironment and may avoid unwanted side effects.[2,6,8] In this study, we used a murine model of malignant mesothelioma (MM) because (1) it has already shown sensitivity to systemically administered anti-CD40 Ab;[3,4] (2) other immunotherapeutic agents have been effective when administered directly into MM tumors;[9–12] (3) there are MM patients with accessible tumors making this approach clinically applicable[9,13,14] and (4) the search for alternative therapies for MM is crucial as long-term survivors after standard therapies such as chemotherapy, surgery and/or irradiation remain rare.[15]

Most studies using anti-CD40 Ab as an anticancer therapy recognize tumor-specific cytotoxic lymphocytes (CTLs) driven by CD40-licensed dendritic cells (DCs) as the main effector cell involved,[2,3,16] particularly when high doses of anti-CD40 Ab are delivered systemically. Yet, CD40 is a 50 kDa type I membrane glycoprotein member of the tumor necrosis factor superfamily that was initially identified as a surface marker on B cells.[17] In B cells, CD40/CD40L (CD154) interactions promote clonal expansion, differentiation, isotype switching and maturation, and lead to germinal center formation in spleen and lymph nodes (LNs).[17,18] Moreover, recent studies have reported a positive role for CD40-activated B cells in tumor immunity.[19–22] CD40-activated B cells have been also used to identify tumor antigens.[23] These studies are particularly promising as B cells are considered to have a minor role in several cancers (reviewed by Canevari *et al.*[24]). In human mesothelioma, Abs to private and public tumor antigens can be detected as tumors progress, suggesting a deleterious role for B cells.[25]

The debilitating features of a persistent humoral immune response in association with Th-2-like cytokines include exacerbating local chronic inflammation and generating a protumorigenic microenvironment.[26–29] Furthermore, tumor antigen-specific Abs bound to tumor cells may protect from killer cells.[30] Similarly, B cells have been shown to disable CD4$^+$ T-cell help[31] and depleting B cells can improve protective T-cell immunity[31–35] implying a suppressive role. However, other studies have shown that loss of B cells impairs T-cell immunity.[36–38] Vaccination

[1]School of Medicine and Pharmacology, Queen Elizabeth II Medical Centre, University of Western Australia, Perth, Western Australia, Australia; [2]Immunology and Cancer Group, School of Biomedical Sciences, Curtin University of Technology, Perth, Western Australia, Australia; [3]Western Australia Biomedical Research Institute, Curtin University of Technology, Perth, Western Australia, Australia and [4]Curtin Health Innovation Research Institute, Curtin University of Technology, Perth, Western Australia, Australia
[5]These authors contributed equally to this work.
Correspondence: Dr DJ Nelson, School of Biomedical Sciences, Curtin University of Technology, Kent street, Bentley, Perth, Western Australia 6102, Australia.
E-mail: Delia.Nelson@curtin.edu.au
Received 7 February 2010; revised 7 June 2010; accepted 8 June 2010

studies also imply a beneficial role for Abs. For example, a significant association between the development of an antitumor antigen Ab response and prolonged survival was observed after vaccination of melanoma patients (reviewed by Canevari *et al.*[24]). The results may be cancer model dependent and/or reflect B-cell depletion methodologies, nonetheless the potential for CD40-activated B cells to improve anti-tumor immunity in mesothelioma warrants closer scrutiny. Hence, this study systematically examined the effect i.t. administration of anti-CD40 Ab into established MM tumors had on tumor growth and on lymphoid and tumor-infiltrating DCs, T cells and B cells.

RESULTS

Targeting anti-CD40 Ab into the tumor microenvironment induces tumor regression

The first series of experiments investigated whether i.t. agonist anti-CD40 Ab therapy had an effect on two genetically different murine MM tumor models *in vivo*. To do this, we injected AE17 cells derived in C57BL/6 mice[12] and AB1 cells derived in BALB/c mice[39] into C57BL/6 (Figures 1a and b) and BALB/c (Figures 1c and d) mice, respectively, on day 0 and allowed them to develop into established tumors that measured 16–25 mm^2. Agonist anti-CD40 Ab was then administered i.t. at 40 (Figures 1a–d), 80 and 100 μg per dose every second day over 2 weeks. Diluent (phosphate-buffered saline (PBS)-injected) control mice were included. Tumor regression in response to the 40 μg anti-CD40 Ab dose was observed in both models (Figures 1b and d) in at least 60% of mice (Figures 1a and c), and these mice remained tumor free for the duration of their natural lives (that is, >18 months). Responses to the higher doses were identical to those seen for the lower 40 μg dose (data not shown); thus, dose escalation did not offer additional benefit. A further group included AE17-bearing mice treated with the isotype (IgG_{2a}) control Ab (data not shown). In comparison to PBS-treated mice, rat IgG_{2a}-treated mice showed no significant decrease in tumor growth rate. These results confirm that rat IgG_{2a} treatment does not reduce tumor growth rate or prolong survival.

The efficacy of i.t. anti-CD40 Ab was also tested using the Lewis lung carcinoma tumor cell line. The same regimen used for MM had little effect (data not shown); however, daily i.t. injections of anti-CD40 Ab delayed tumor growth (Supplementary Figure 1B) and extended survival (Supplementary Figure 1A).

In vitro cultured AE17 and AB1 MM tumor cells are $CD40^-$ (Supplementary Figure 2). In contrast, the positive controls (*ex vivo* B cells prepared from LN of normal C57BL/6 mice) were clearly $CD40^+$. Hence, the effects of anti-CD40 Ab could not be exerted directly through CD40-expressing tumor cells. Thus, we turned our attention to the role of the immune system.

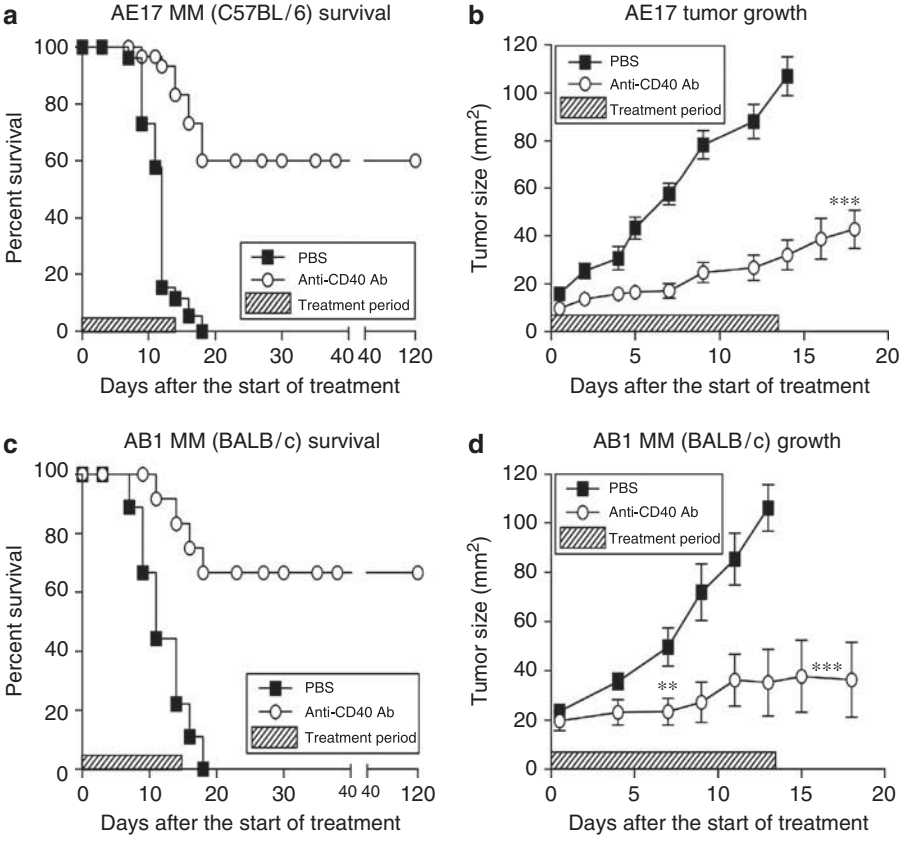

Figure 1 Mesothelioma tumors regress in response to i.t. agonist anti-CD40 Ab therapy. C57BL/6J (**a**, **b**) and BALB/c (**c**, **d**) mice were injected subcutaneously with 5×10^5 AE17 and 1×10^6 AB1 MM tumor cells, respectively, on day 0 and left to develop. When tumors were 16–25 mm^2 i.t. agonist anti-CD40 Ab (40 μg per dose) or PBS (the diluent control) commenced and continued every second day. Tumor growth (**b**, **d**; mean ± s.e.m.) and percent survival (Kaplan–Meier plots; **a**, **c**) of tumor-bearing mice are shown; for AE17-bearing mice pooled data are from 10 experiments with 3–5 mice per group, the total number of mice for each treatment group was 40; for AB1-bearing mice pooled data are from five experiments with 2–5 mice per group, the total number of mice for each treatment group was 16. Differences in tumor growth at each time point comparing PBS to anti-CD40 Ab treatment were analyzed using an unpaired *t*-test. For AE17-tumor-bearing mice, $P<0.0001$ (***) at days 7, 9, 12, 14, 16 and 18. For AB1-tumor-bearing mice, $P<0.001$ (**) at days 7 and 9 and $P<0.0001$ (***) at days 11, 13 and 15.

Tumor antigen presentation is not improved after i.t. anti-CD40 Ab

Previous studies in mesothelioma used agonist anti-CD40 Ab to promote T-cell immunity by DC activation.[3,4] Thus, we first assessed the impact of i.t. anti-CD40 Ab treatment on tumor antigen presentation using the AE17-sOVA model that expresses a 'marker' tumor antigen (ovalbumin, OVA) and adoptive transfer of 5,6-carboxyfluorescein-succinimidyl-ester (CFSE)-labeled OT-1T cells, which are specific to OVA. OVA tumor antigen presentation levels were not improved in the draining LN (dLN) at the third dose (Figures 2a–c) or sixth dose (data not shown).

DCs 'disappear' from i.t. anti-D40 Ab-treated tumors and dLNs

To investigate possible reasons for the lack of improved tumor antigen presentation, we tracked CD11c$^+$ DCs in dLNs and in AE17 tumors during anti-CD40 Ab treatment. Both the percent (Figure 2d) and total numbers (Figure 2e) of DCs transiently increased in dLN midway through treatment (third dose), but then had diminished by the sixth dose. DCs also appeared to reduce in tumors shrinking in response to anti-CD40 Ab (Figure 2f).

T cells are not critically required for i.t. anti-CD40 Ab-mediated tumor regression

The data above suggest that DCs were not activated by anti-CD40 Ab. However, the antigen presentation assay may not be sensitive enough to reveal the consequences of activated DCs, which could be improved CTL function, and increased numbers of CD8$^+$ T cells, including tumor-specific CTL, infiltrating the tumor microenvironment. Thus, we conducted a series of experiments assessing the role of T cells in mediating the antitumor activity observed in Figure 1 in response to i.t. anti-CD40 Ab therapy. The first two experiments used the AE17-sOVA model enabling monitoring of tumor-specific immune responses.[12] The lytic capacity of tumor-specific CD8$^+$ T cells was assessed using an *in vivo* CTL assay. Differentially fluorescently (CFSE) labeled target cells (spleen cells from naive mice incubated with or without the SIINFEKL peptide) were co-injected i.v. into AE17-sOVA tumor-bearing mice. No SIINFEKL-specific lytic activity was seen in mice bearing the AE17 parental tumor in the dLN (Figure 3a). In contrast, mice bearing AE17-sOVA tumors showed some endogenous lytic activity in the dLN without anti-CD40 Ab treatment. Anti-CD40 Ab did not significantly increase this tumor-specific CTL activity after three or six doses; the latter being a time point when treated tumors are clearly regressing.

Tumor-infiltrating, tumor-specific CD8$^+$ T cells were identified using major histocompatibility complex (MHC) class I-restricted OVA (SIINFEKL)-specific tetramers. Anti-CD40 Ab treatment led to a decrease in the percent of detectable tumor-specific CD8$^+$ cells in AE17-sOVA tumors after both three and six doses (Figure 3b).

In the final set of experiments addressing the role of T cells, we returned to the parental AE17 model. AE17 tumor-bearing mice were

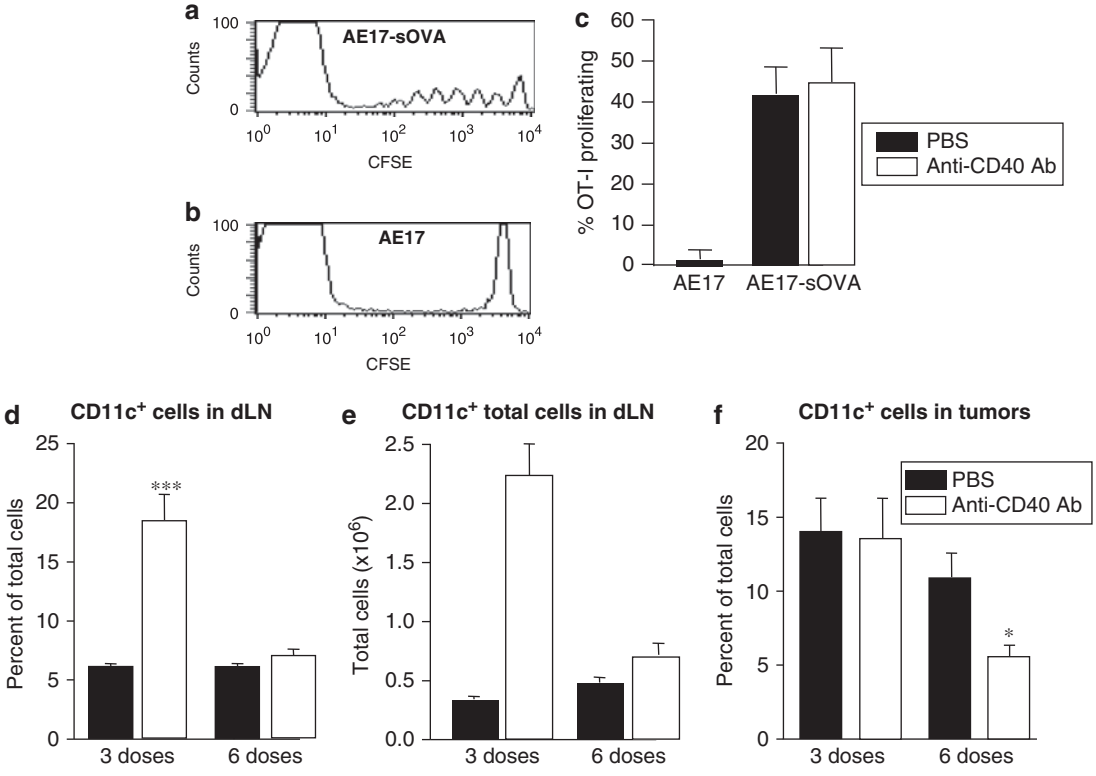

Figure 2 Low doses of i.t. anti-CD40 Ab does not license DCs. To assess changes in antigen presentation, C57BL/6J mice bearing AE17 or AE17-sOVA tumors were given three i.t. doses of PBS or anti-CD40 Ab. Three days before analysis these mice were the recipients of 1×10^7 adoptively transferred, CFSE-labeled, tumor antigen-specific, CD8$^+$ T cells prepared from OT-1 mice. The dLNs were harvested from recipient mice, stained for CD8 and FACS analysis performed by gating on CD8$^+$ CFSE$^+$ cells. Representative FACS histograms are shown from AE17-sOVA (**a**) and AE17 (**b**) tumor-bearing mice that were not treated with anti-CD40 Ab. Treatment with anti-CD40 Ab did not increase the percent of proliferating OT-1 T cells (**c**); pooled data from two experiments (six mice per group; shown as mean ± s.e.m). dLNs (**d**, **e**) and tumors (**f**) taken from AE17 or AE17-sOVA tumor-bearing mice after three or six i.t. doses of PBS or anti-CD40 Ab were prepared as single suspensions, and stained for CD11c$^+$ DCs and data recorded as the percent of total cells (**d**, **f**) or as total cell numbers (**e**). Pooled data (**d**–**f**) are from two experiments (8–10 mice per group) and represented as mean ± s.e.m.; $^*P<0.05$, $^{***}P<0.0001$.

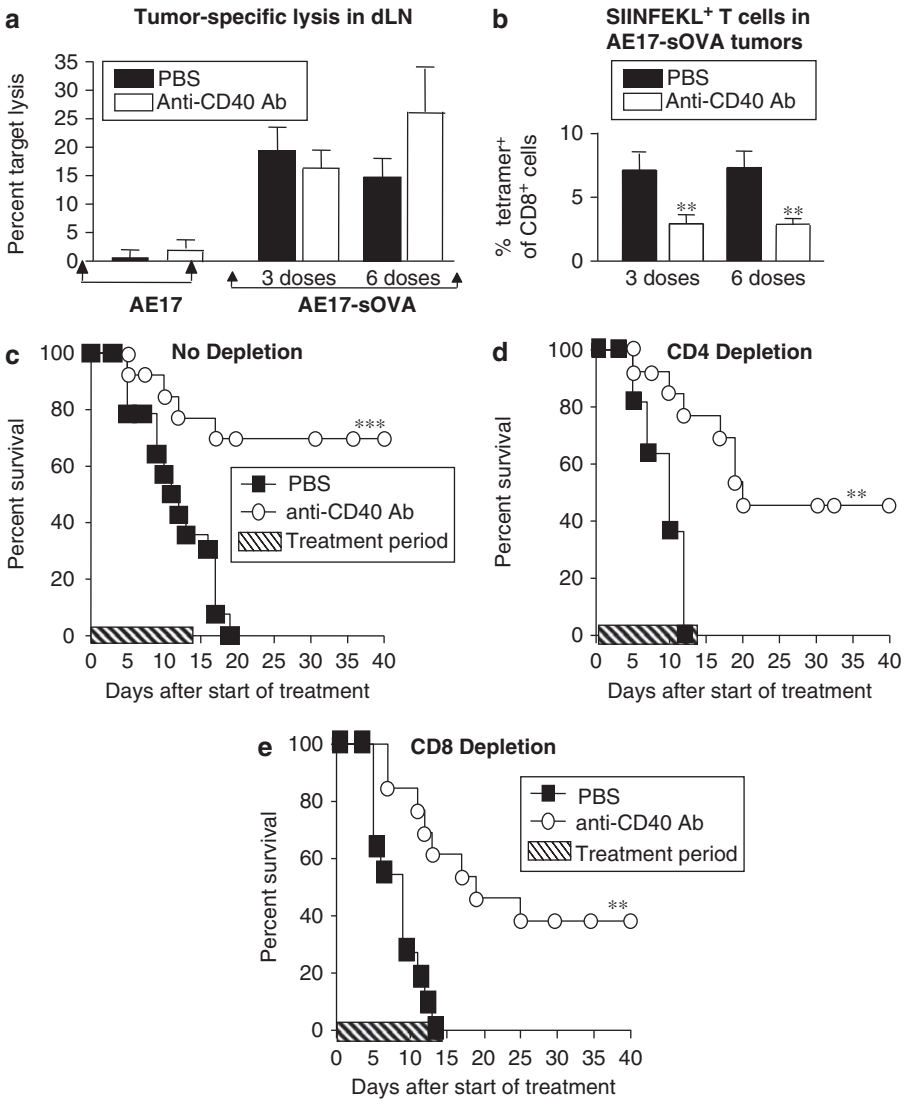

Figure 3 T cells represent one effector arm after i.t. anti-CD40 Ab. An *in vivo* CTL assay involving adoptively transferred, fluorescently labeled, target cells expressing MHC class I bound SIINFEKL was used to determine the lytic ability of tumor-specific CTL in the dLN of animals bearing AE17 or AE17-sOVA tumors after three or six i.t. doses of PBS or anti-CD40 Ab; pooled data shown as mean ± s.e.m. are from three experiments with nine mice per group (**a**). The SIINFEKL tetramer was used to identify tumor-specific $CD8^+$ T cells that had infiltrated AE17-sOVA tumors after three or six i.t. doses of PBS or anti-CD40 Ab. Single-cell suspensions were co-stained for the SIINFEKL tetramer and CD8 and analyzed by FACS (**b**); pooled data shown as mean ± s.e.m. are from three experiments with 10–12 mice per group. $^{**}P<0.01$ comparing treated groups to PBS-treated controls. The role of CD4 and CD8 T cells was addressed by depleting mice AE17 tumor-bearing mice of CD4 (**d**) or CD8 (**e**) cells using specific mAbs 1 day before commencing anti-CD40 Ab therapy, and maintaining depletion throughout the treatment regimen and the data compared with the no-depleted controls (**c**). Pooled data are shown as survival (Kaplan–Meier plots) from three experiments with a total of 10–15 mice per group. $^{***}P<0.0001$.

depleted of $CD4^+$ or $CD8^+$ T cells (using monoclonal Ab) during the anti-CD40 Ab treatment period. In these experiments, 70% of control (that is, no depletion) mice responded to anti-CD40 Ab treatment (Figure 3c). In contrast, 40 and 50% of mice experienced anti-CD40 Ab-induced tumor regression and long-term survival after CD4 and CD8 depletion, respectively (Figures 3d and e). Thus, a significant number of mice still experienced anti-CD40 Ab-driven survival in the absence of $CD8^+$ or $CD4^+$ T cells, albeit in slightly lower numbers. These data suggest that although T cells may have a role in locally administered anti-CD40 Ab-induced antitumor responses, their role is not as critical as expected and other mechanisms must be operating.

Only B cells increase within the tumor microenvironment during i.t. anti-CD40 Ab treatment

Fluorescent-activated cell sorting (FACS) analyses of *ex vivo* tumors prepared as single-cell suspensions showed that by the third dose of anti-CD40 Ab there was a significant increase in the percent of B cells in MM tumors that persisted to the sixth dose (Figure 4c). In contrast, $CD4^+$ T cell numbers appeared to decrease, whereas $CD8^+$ T cell numbers remained low (Figures 4a and b). Immunohistochemical staining of AE17 tumors collected after the third dose of PBS or anti-CD40 Ab supported these observations (data not shown). Note that we also examined natural killer (NK) cells ($NK1.1^+CD3^-$), NKT cells ($NK1.1^+CD3^+$), macrophages ($F4/80^+$) and granulocytes ($GR\text{-}1^+$),

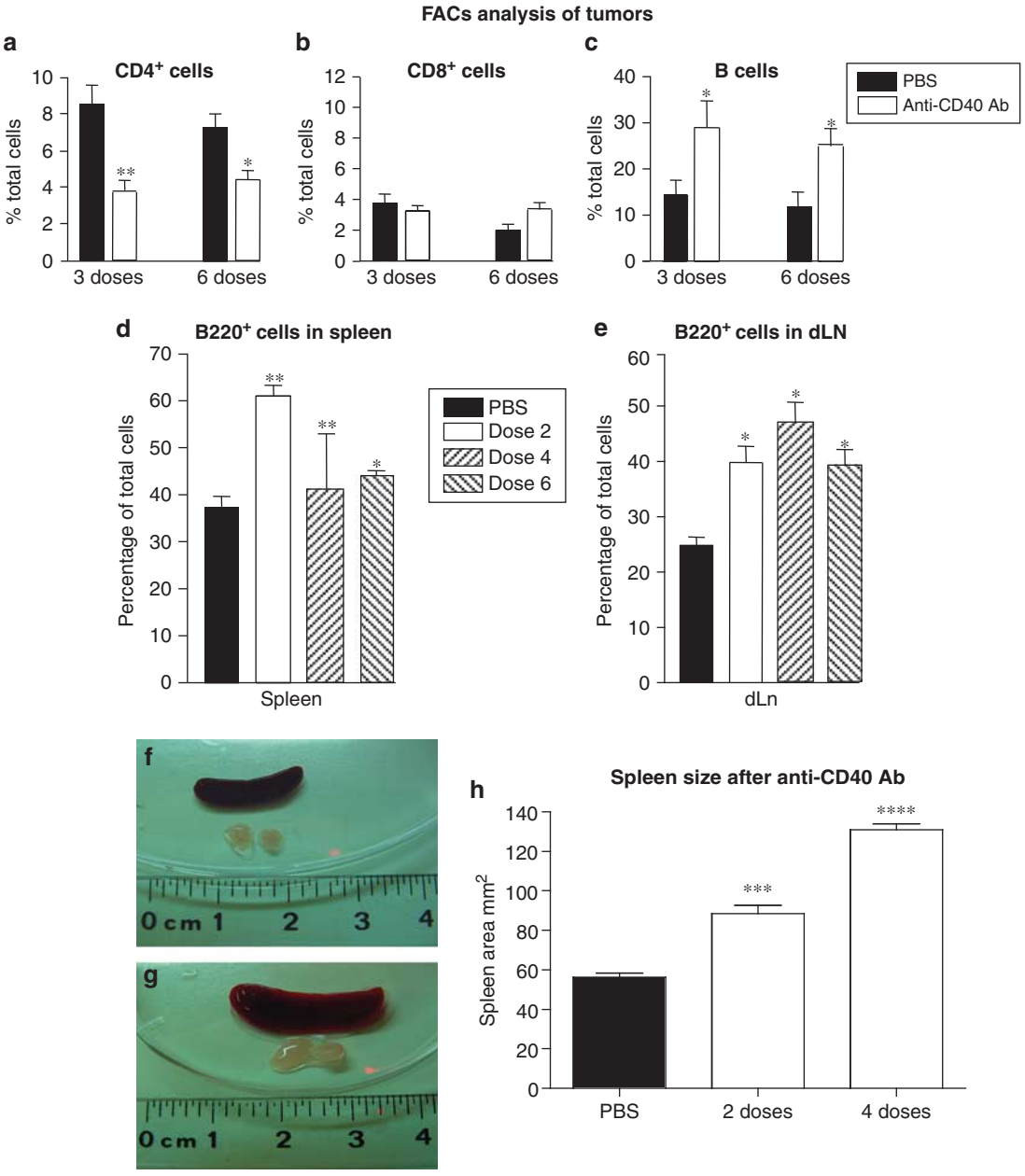

Figure 4 Only B cells increase in anti-CD40 Ab-treated tumors. C57BL/6J mice bearing AE17 tumors were given three or six i.t. doses of PBS or anti-CD40 Ab. Tumors were prepared as a single-cell suspension. FACS analysis of $CD4^+$ T cells (**a**), $CD8^+$ T cells (**b**) or $B220^+$ B cells (**c**) was used to calculate the percentage of total cells. Pooled data from two experiments (8–9 mice per group) are represented as mean ± s.e.m. $^*P<0.05$; $^{**}P<0.01$ comparing anti-CD40 Ab treated to PBS-treated groups. The spleens (**d**) and dLNs (**e**) were also harvested after two, four and six doses of anti-CD40 Ab, prepared as single cells and stained for B220. The data from two experiments with three mice per group are shown as mean percent of total cells (± s.e.m. with $^*P<0.05$; $^{**}P<0.01$ and $^{***}P<0.005$). Representative photographs for PBS-treated (**f**) and anti-CD40 Ab-treated (**g**) spleen and dLN are shown after three doses. Spleens were taken from five mice per group after two and four doses with i.t. anti-CD40 Ab therapy and two dimensions measured using microcallipers (**h**). Pooled data are shown as mean spleen area (± s.e.m. with $^{***}P<0.005$ and $^{****}P<0.005$).

all of which were not increased during or after anti-CD40 Ab treatment (data not shown).

B cells increase in secondary lymphoid organs during agonist anti-CD40 Ab therapy

FACS analyses of spleens (Figure 4d) and dLNs (Figure 4e) showed that B-cell proportions had significantly increased by the second dose of anti-CD40 Ab. Their proportions remained significantly elevated in the dLNs throughout the treatment period. By the fourth i.t. dose of anti-CD40 Ab, tumor dLNs (data not shown) and spleens (Figures 4f–h) had more than doubled in size relative to dLNs from PBS-treated mice. As a result, total cell numbers also increase thus the total number of splenic B cells would increase on account of spleen size.

Tumor antigen-experienced, CD40-stimulated B cells can protect against tumor growth

To generate tumor antigen-experienced, CD40-stimulated B cells, we treated *in vivo* AE17 MM tumor-bearing C57BL/6J mice with

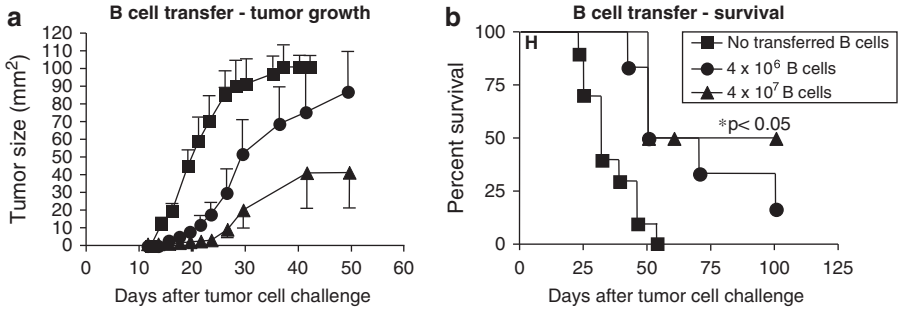

Figure 5 CD40-activated B cells protect against tumor challenge. Naive C57BL mice were the recipients of adoptively transferred, splenic $CD19^+$ B cells (**a**, **b**) prepared from mice with AE17 tumors regressing in response to anti-CD40 Ab treatment. After 3 weeks all mice, as well as unmanipulated tumor growth controls (no transferred B cells), were challenged with AE17 tumor cells; data are from one of the two representative experiments (each with six mice per group) shown as mean tumor growth rate ± s.e.m. (**a**) or survival (**b**).

anti-CD40 Ab and splenocytes collected from only those mice that experienced tumor regression. Positively selected $CD19^+$ B cells (>95% purity) were transferred into naive C57BL/6J mice. After 3 weeks mice were challenged with AE17 tumor cells. Recipients of $CD19^+$ B cells from anti-CD40 Ab-treated tumor-bearing mice showed a significant level of protection relative to control mice (that is, those that did not receive any transferred cells). In mice given 4×10^6 B cells, 5 out of 6 mice (Figures 5a and b) developed tumors 24 days later; 1 protected mouse remained tumor free for >100 days. Mice given 10-fold more (4×10^7) B cells were better protected with three mice remaining tumor free for 3 months, and the one mouse that was tested was completely protected against a second tumor rechallenge (data not shown). B cells transferred from anti-CD40 Ab-treated healthy mice did not provide any form of protection (data not shown).

Agonist anti-CD40 Ab therapy increases IgM and IgG Ab levels in tumor-bearing mice

Enzyme-linked immunosorbent assays (ELISAs) using pooled serum from three experiments, with five mice per group, showed an increase in immunoglobulin M (IgM) levels (relative to PBS and rat IgG_{2a}-treated controls) at the fourth and sixth doses of anti-CD40 Ab (data not shown). By the sixth dose, anti-CD40 Ab-treated mice produced significantly higher serum IgG levels than the two control groups (Figure 6a), suggesting that agonist anti-CD40 Ab promotes Ig class switching and maturation to memory B cells and plasma cells.

Sera collected from tumor-bearing mice that (1) had been cured by anti-CD40 Ab therapy or (2) were not treated and had progressing tumors, as well as sera from (3) normal healthy mice and (4) healthy mice given six doses of anti-CD40 Ab, were adjusted to contain 25 μg IgG and transferred into naive mice. The recipient mice were challenged with tumor cells 24 h later, and survival (Figure 6b) and tumor growth (Figure 6c) were monitored. Unmanipulated control mice, as well as mice that received sera from untreated tumor-bearing mice or received sera taken from healthy mice died quickly because of tumor progression. Interestingly, there was a slight delay in tumor growth in mice that were given sera taken from healthy mice given anti-CD40 Ab (Figures 6b and c); however, all mice subsequently developed tumors. Tumor growth was significantly retarded in mice given sera from mice that had been cured of their tumors by i.t. anti-CD40 Ab; that is, there was delayed tumor progression in two out of five mice whereas the remainder were completely protected and survived beyond 100 days (Figure 6b).

NK cells are not involved in CD40-mediated tumor rejection

NK cells were depleted just before and throughout the anti-CD40 Ab treatment period. The absence of NK cells did not alter tumor growth (data not shown) and survival (Figure 6d). These data suggest that NK cells do not function as Ab-dependent cell-mediated cytotoxicity (ADCC) effector cells in this system.

Anti-CD40 Ab treatment amplifies Ab responses that target autoantigens

FACS analysis was used to assess the presence of tumor-specific Ab in sera from healthy and AE17-bearing mice that had, or had not, been treated with anti-CD40 Ab. To do this we incubated AE17 tumor cells or a syngeneic mouse fibroblast cell line (3T3 cells) with the sera, and then linked it to anti-mouse IgM or IgG. Preliminary experiments were conducted to optimize the staining protocol. This was achieved by titrating the IgM and IgG Abs to identify a concentration that generated a detectable signal without background staining. The data shown were generated from flow cytometric analysis after gating on viable cells. Purified sera from healthy and AE17 tumor-bearing mice contained low levels of IgM (Figures 7a, b and e) and IgG (Figures 7c, d and f) that reacted with surface antigens expressed by both AE17 and 3T3 cells. When healthy mice were treated with anti-CD40 Ab, these levels increased on the surface of AE17 cells, but not on 3T3 cells, suggesting that healthy sera contain Abs that recognize autoantigens overexpressed by the AE17 tumor cell line (Figures 7e and f). Similar results were seen with sera from anti-CD40 Ab-treated AE17 tumor-bearing mice (Figures 7e and f). Similar results, albeit at higher levels, were seen when the cells were permeabilized for examination of intracellular IgG antigens (Figure 7g). Despite the increased presence of auto-Abs, we did not see macroscopic signs of autoimmunity such as lethargy, diarrhea or vitiligo in mice treated with anti-CD40 Ab.

Anti-CD40 Ab treatment targets follicular B cells

Healthy and tumor-bearing mice were examined for alterations in B-cell subpopulations in secondary lymphoid organs and tumors during anti-CD40 Ab treatment. $CD3^-CD5^-B220^+$ B cells were divided into marginal zone (MZ) and follicular (FO) B cells. MZ cells express high levels of CD21 and low levels of CD23, whereas FO B cells express high levels of both[40] (Figure 7h). The most striking observation was the complete disappearance of splenic MZ B cells in healthy and tumor-bearing mice treated with anti-CD40 Ab (Figure 7i). In contrast, FO B-cell proportions increased by 11.02 ± 0.87%, which was statistically significant (Figure 7j). Note

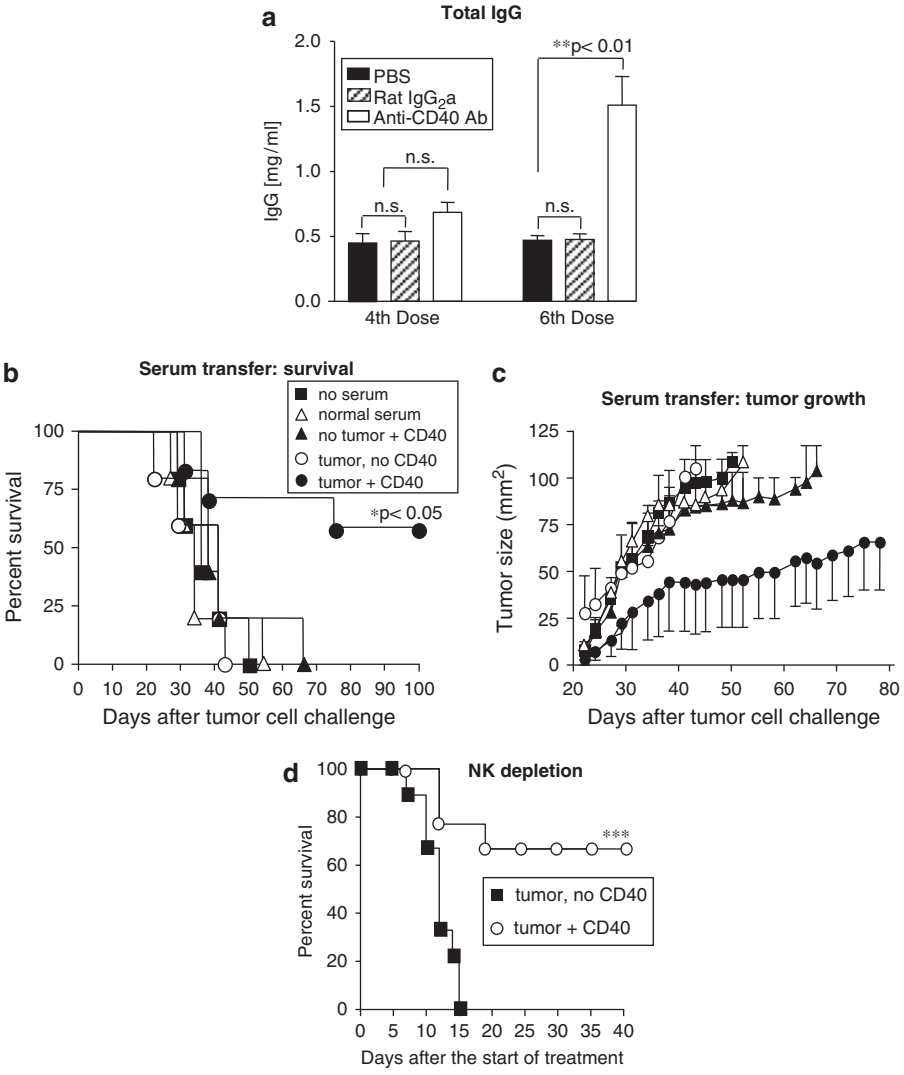

Figure 6 CD40-driven sera protects against tumor challenge. Individual sera from one experiment with five mice per group treated with four or six doses of anti-CD40 Ab, rat IgG_2a or PBS was used to measure total IgG in each mouse (**a**); data are shown as mean ± s.e.m. Sera were also collected from unmanipulated (normal serum) and anti-CD40 Ab-treated healthy mice (no tumor+CD40), as well as from mice with AE17 untreated, progressing tumors (tumor, no CD40) and from mice with AE17 tumors regressing in response to anti-CD40 Ab treatment (tumor+CD40). All sera were adjusted to contain 25 μg of IgG and transferred into naive mice and challenged 24 h later with AE17 tumor cells (**b**, **c**). Tumor growth control mice did not have transferred sera (no serum). Data are from three representative experiments with five recipient mice per group and shown as mean ± s.e.m. The role of NK cells was addressed by depleting AE17-bearing mice of NK cells using a specific mAb 1 day before commencing anti-CD40 Ab therapy, and maintaining depletion throughout the treatment regimen (**d**); pooled data from two experiments (6–10 mice per group) are shown as survival and $***P<0.001$ comparing treated groups to no-depletion controls (shown in Figure 3c).

that as the spleens in anti-CD40 Ab-treated mice swell, this amounts to a large increase in total FO B-cell numbers. Very few (<0.8% of total B cells) B-1 cells, identified by co-expression of $B220^+CD5^+CD21^+$,[41] were seen in any of the tissues examined and anti-CD40 Ab treatment had little effect on this subset (data not shown).

The B-cell subsets were also examined for CD40 expression. Before therapy, B-1 cells expressed low levels of surface CD40, FO cells showed intermediate levels, whereas MZ cells were high expressors; CD40 expression levels in B-1 and FO cells remained unchanged after anti-CD40 Ab treatment (data not shown). Thus, FO and MZ B cells have the capacity to directly respond to agonist anti-CD40 Ab treatment.

B cells are required for successful treatment of established tumors with anti-CD40 Ab

The data above suggest that B cells may be involved in the antitumor activity shown in Figure 1. Thus, the next series of experiments were designed to assess the antitumor role of B cells during i.t. anti-CD40 Ab treatment of established tumors. In these experiments, T-and B-cell-deficient severe combined immunodeficiency (SCID) mice (on the BALB/c background) were used to create B-cell 'empty' and B-cell 'full' mice. To do this, spleen cells taken from normal, healthy BALB/c mice were either (1) unfractionated, (2) positively immuno-selected for B cells (to create B-cell full mice) or (3) immunodepleted of B cells (to create B-cell empty mice) and transferred through the tail vein into SCID mice. Hence, in the unfractionated group, recipient

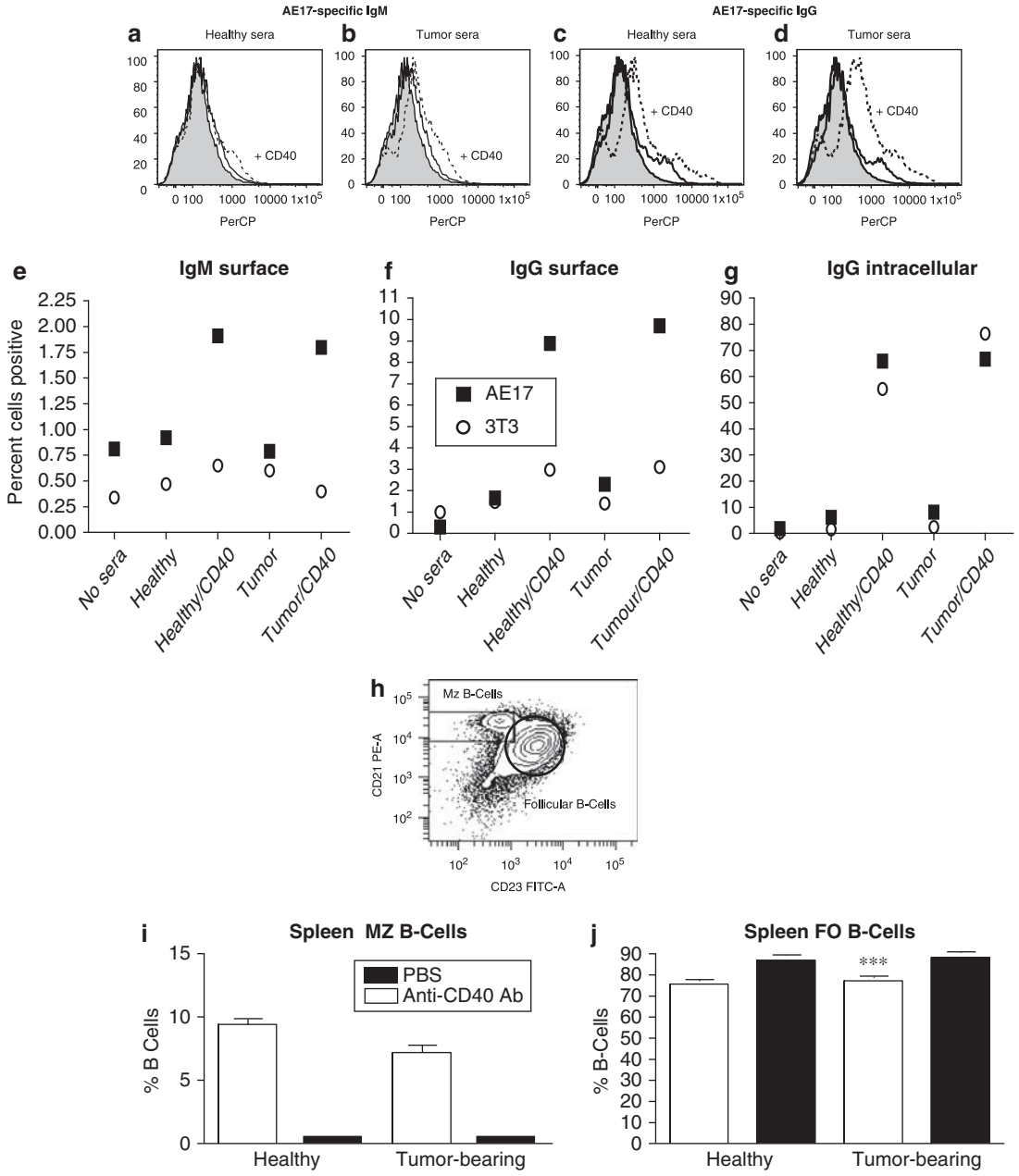

Figure 7 CD40-activated FO B cells secrete auto and tumor-specific Abs. Purified pooled sera (used in Figure 6) from healthy versus tumor-bearing mice treated with PBS or anti-CD40 Ab were incubated *in vitro* with AE17 tumor cells or 3T3 mouse fibroblast cells and linked to PerCP-conjugated Streptavidin by biotinylated anti-mouse IgG or anti-mouse IgM for FACS analysis. Representative histograms showing IgM$^+$ (**a**, **b**) or IgG$^+$ (**c**, **d**) cells incubated with pooled sera from healthy (**a**, **c**) or AE17 tumor-bearing (**b**, **d**) mice; filled line represents AE17 cells incubated with fetal calf serum, solid line represents cells incubated sera from PBS animals and the dashed line represents tumor cells incubated sera from anti-CD40 Ab-treated animals. Data determined by gating on PerCP$^+$ cells show the percent of total AE17 (filled square symbols) or 3T3 cells (open round symbols) that were linked to purified surface IgM (**e**) and IgG serum Abs (**f**) or to intracellular IgG (**g**). Healthy and tumor-bearing mice treated with anti-CD40 Ab were examined midway through treatment (after the third injection) for alterations in splenic B-cell subpopulations. B cells were initially gated for B220$^+$ and CD3$^-$ expression. B220$^+$ CD5$^-$ B cells were divided into MZ cells, which express high levels of CD21 and low levels of CD23, and FO B cells, which express high levels of CD21 and CD23 (**h**). Graphs of splenic MZ (**i**) and FO (**j**) B cells show pooled data from one experiment with five mice per group $^{***}P<0.001$.

SCID mice were reconstituted with both T and B cells (shown as total spleen recipients in Figure 8a) and were used as intact immune controls. To ensure successful engraftment, we left all groups of SCID mice for 7 weeks before (1×10^6) AB1 tumor-cell inoculation. Once the tumors were established either PBS or agonist anti-CD40 Ab (40 μg per dose) were administered i.t. every second day.

In the first series of experiments, SCID mice were the recipients of unfractionated spleen cells (total spleen recipient) or B220$^-$ splenoctyes (B220-cell-depleted mice). The total spleen SCID recipients clearly responded to the anti-CD40 Ab treatment relative to their PBS controls (Figure 8a); although this response was not as robust as seen in BALB/c mice (Figures 1c and d). In contrast, the B-cell-

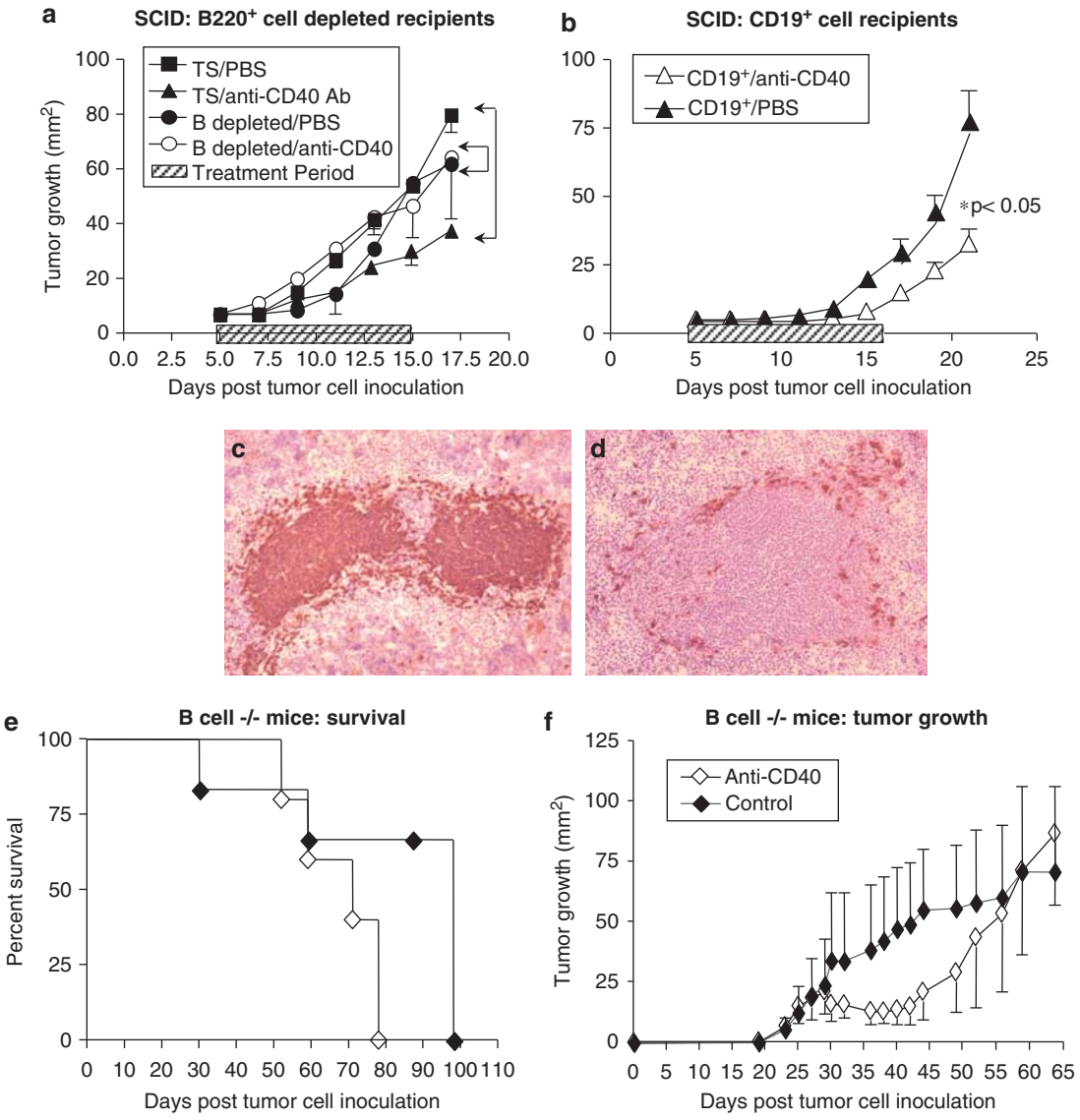

Figure 8 Successful anti-CD40 Ab treatment requires B cells. SCID mice were reconstituted with different cell populations for 7 weeks before subcutaneous inoculation with 1×10^6 AB1 tumor cells. Once tumors were palpable, i.t. anti-CD40 Ab or PBS treatment was commenced and continued every second day. In one experiment with three mice per group, SCID mice received either unfractionated (total spleen, TS) spleen cells or B220-depleted cells (**a**); pooled data are shown as mean tumor growth rate ± s.e.m. In another series of experiments SCID mice received $CD19^+$ B cells (**b**). Representative photographs show that successful engraftment of $CD19^+$ B cells in SCID spleens was seen at the end of the experiment, when the mice were killed (**c**); a low level of $CD8^+$ staining was seen in the same spleens (**d**). The data are from one of the two experiments with three mice per group and shown as mean tumor growth rate ± s.e.m. B $cell^{-/-}$ mice (**e**, **f**) were injected subcutaneously with 5×10^5 AE17 MM tumor cells on day 0. When tumors reached 16–25 mm^2 i.t. agonist anti-CD40 Ab or PBS treatment commenced; pooled data from one experiment with five mice per group are shown as survival (**e**) and mean tumor growth rate ± s.e.m. (**f**).

depleted group was completely incapable of responding to anti-CD40 Ab therapy (relative to their PBS-treated controls; Figure 8a). These data suggest that B cells are required for anti-CD40 Ab-mediated antitumor activity.

As B220 can be expressed on other cell types, another series of experiments using the B-cell-specific marker, CD19, to positively select B cells (purities of >90% were determined by FACS analysis) was conducted. Figure 8c shows that the spleens of SCID mice were successfully reconstituted with $CD19^+$ B cells. Note that we also looked for $CD8^+$ cells in spleens of the $CD19^+$ B cells SCID recipients at the end of the experiment. Low numbers of $CD8^+$ cells were seen in MZs that, on account of their location and size, may be $CD8^+$ DCs or macrophages (Figure 8d). These mice were inoculated with AB1 tumor cells and given i.t. PBS or agonist anti-CD40 Ab. Anti-CD40 Ab treatment significantly improved the survival of mice given $CD19^+$ B cells (Figure 8b). Finally, B $cell^{-/-}$ mice were inoculated with AE17 tumor cells, and once the tumors reached 16–25 mm^2 anti-CD40 Ab treatment commenced. The anti-CD40 Ab-treated mice initially appeared to benefit from treatment for a period of 25 days; however, this difference did not reach a statistically significant difference and all developed tumors (Figures 8e and 8f). These data suggest that i.t. anti-CD40 Ab treatment is only fully effective when B cells are present.

DISCUSSION

Systemic administration of 100 μg activating anti-CD40 Ab into MM-bearing mice has been shown to generate transient tumor reduction mediated by 'cross-armed' tumor-specific CTL.[3] However, cessation of treatment was associated with rapid tumor reemergence. In this study, we show that a short-term regimen consisting of six lower (40 μg) doses of agonist anti-CD40 Ab administered directly into MM tumors provoked tumor regression, prolonged survival and permanently cured at least 60% of mice for the remainder of their natural lives. This is particularly promising for MM as these tumors can be accessible for i.t. administration, a feature that has already capitalized upon in the clinic.[9,13,14]

On the basis of previous studies using agonist anti-CD40 Ab, we expected that our i.t. approach would also be mediated by tumor-specific T cells driven by highly activated DCs.[2,42,43] In this study, DCs seemed to leave the tumor microenvironment and emigrate into the dLN midway through i.t. anti-CD40 Ab therapy. However, this DC emigration was short-lived and dLN DC levels rapidly diminished toward the end of treatment. Furthermore, tumor antigen presentation levels in the dLNs were not altered during anti-CD40 Ab treatment. These DCs may have 'prematurely' apoptosed in a manner similar to recent studies examining CD40-activated memory $CD4^+$ T cells.[7] Nonetheless, it was still possible that these DCs had been licensed such that they evoked potent tumor-specific effector CTL. Unexpectedly, tumor-specific CTL function was not enhanced in regional LNs, and there was no obvious $CD4^+$, $CD8^+$ or tumor-specific $CD8^+$ T-cell infiltration into tumors. Finally, depletion of $CD4^+$ or $CD8^+$ T cells only marginally reduced anti-CD40 Ab efficacy. Thus, although T cells may be involved in locally targeted CD40-mediated antitumor activity, their role was not as critical as expected. In fact, the only tumor-infiltrating immune cell type that increased in number was the B cell. These observations prompted us to more closely examine the effect anti-CD40 Ab had on B cells in tumor-bearing animals. Note that the role of B cells after systemic administration of anti-CD40 Ab in mesothelioma has not yet been thoroughly scrutinized, possibly on account of the above-mentioned crucial role of cross-armed $CD8^+$ T cells.[3] It is possible that CD40-activated B cells function as antigen-presenting cells in that system, as has been shown by others.[19]

In this study, we showed that by the end of the 2-week anti-CD40 Ab treatment period mice had developed significantly increased serum IgM and IgG levels; this was a time point when tumors were clearly regressing. These mice also showed increased proportions of splenic and LN B cells. These results suggest that B cells were rapidly stimulated by agonist anti-CD40 Ab. This was particularly true in the spleen, wherein B cells proliferated after the second dose contributing to a substantial increase in spleen size. CD40/CD40L interactions are known to stimulate B-cell proliferation within secondary lymphoid organs. In germinal centres, CD40-stimulated B cells can develop into Ab-secreting plasmablasts,[44] memory B cells or IgG-secreting cells (isotype switching). CD40-activated B cells can be rescued from apoptosis[45,46] and may even present tumor antigens to $CD4^+$ T cells,[21] thereby offering help to T cells. However, others have shown a detrimental side to CD40 engagement on B cells including ablation of germinal center formation, premature termination of the humoral immune response, prevention of B-cell memory and interference with B-cell migration.[47,48] We found that anti-CD40 Ab treatment obliterated splenic MZ B cells. This may have been because of their higher endogenous CD40 expression levels and apoptosis as a consequence of 'over'-activation. Nonetheless, these data suggest that MZ B cells do not provide antitumor activity. B-1 cells were found in very low numbers, expressed little or no CD40 and did not respond to i.t. anti-CD40 Ab. In contrast, FO cells increased in numbers in the spleens and tumors of anti-CD40 Ab-treated mice. This may be because FO cells expressed intermediate CD40 levels and survived CD40 activation. Thus, our data suggest that CD40-activated FO B cells proliferate in spleen, where isotype switching and plasmablast generation occurs.

The role of B cells in tumor immunity remains unresolved. In a virus-induced tumor model, mice depleted of B cells failed to develop tumor immunity.[31] In contrast, a tumor transfected to express allogeneic MHC class I antigens was rejected more frequently in SCID mice reconstituted with T cells compared with those reconstituted with T and B cells.[31] In this study, SCID mice were used to create B-cell empty, B-cell full and immune intact mice before tumor establishment and anti-CD40 Ab treatment. Mice devoid of $B220^+$ or $CD19^+$ B cells did not respond to i.t. anti-CD40 Ab therapy. In contrast, SCID mice reconstituted with both T and B cells (that is, the total spleen recipients) responded well to therapy, and mice filled with only $CD19^+$ B cells showed an improved capacity to respond to anti-CD40 Ab. Similarly, the use of B-cell knockout mice showed that the loss of B-cells prevented CD40-driven cures. These studies suggest that although T cells have a role in locally driven antitumor immunity, they require a significant level of help from CD40-driven B cells.

Abs in animal models and in cancer patients have been shown to recognize surface and intracellular tumor antigens (reviewed by Canevari *et al.*[24]). Furthermore, some are capable of inducing complement-dependent and ADCC tumor cell lysis.[49] In human mesothelioma, Abs to tumor antigens are detected as tumors progress,[25] and we noted that AE17 mesothelioma tumor cells grew slower in B $cell^{-/-}$ mice than in C57Bl/6 wild type (data not shown), suggesting a deleterious role for B cells. We show that transferred sera containing IgM and IgG generated by *in vivo* CD40-activated B cells provided significant antitumor protection. Our studies suggest that local anti-CD40 Ab treatment amplifies the generation of auto-Abs directed against antigens overexpressed by MM tumors. Depletion of NK cells did not reduce anti-CD40 Ab antitumor efficacy showing that NK cells are not involved in ADCC in this system, although the role of monocytes and eosinophils is unknown and we have not yet confirmed that complement is involved. Thus, it is possible that CD40-activation turns IL-10 secreting suppressor B cells into immune enhancing B cells by as yet unidentified means. CD40-activated B cells have been shown to function as antigen-presenting cells,[19] and this might occur after systemic administration to induce effector T cells. However, in our hands i.t. administration did not induce an overt $CD8^+$ effector T-cell response. Nonetheless, one possibility is that they might have a key role in the generation of memory T cells, as we found that the i.t. anti-CD40 Ab induced a potent long-term protective memory response (data not shown).

Although others have shown that cross-armed $CD8^+$ T cells are key effectors after systemic anti-CD40 Ab administration in mesothelioma[3] and other cancers,[1,2,8] our data imply an important role for B cells after i.t. anti-CD40 Ab. The reasons for these different protective mechanisms are not yet clear. Note that the route of delivery appears to be irrelevant, as we have shown that antitumor responses induced by i.t. IL-2 are completely T-cell dependent.[12] Thus, the i.t. route does not skew immune response away from T cells. Our data suggest that the antitumor response induced by i.t. anti-CD40 Ab relied on FO B cells, although the mechanism remains unclear. It is possible that CD40-activated splenic FO B cells proliferate and mature into plasma cells, which secrete significant amounts of Abs directed at autoantigens overexpressed by tumor cells. These auto-Abs may

induce ADCC involving macrophages. This theory is attractive as there are a large number of macrophages in the tumor microenvironment in this model.[50] Future studies will attempt to elucidate the role of macrophages in response to i.t. anti-CD40 Ab.

In summary, these studies clearly show that low-dose i.t. agonist anti-CD40 Ab therapy provides greater and longer lasting antitumor activity than higher doses that are systemically administered. Our data suggest that the antitumor response induced by i.t. anti-CD40 Ab relies on FO B cells and not on $CD8^+$ T cells. It is possible that CD40-activated splenic FO B cells mature into plasma cells that secrete Abs directed at autoantigens overexpressed by mesothelioma tumor cells. These Abs may induce ADCC, although NK cells are not involved. This hypothesis is supported by adoptive transfer studies using tumor antigen-experienced, CD40-stimulated B cells or their IgG products to provide protection in the recipients. Moreover, SCID reconstitution and B-cell knockout studies confirmed that B cells are essential for tumor-targeted agonist anti-CD40 Ab to be effective.

METHODS

Mice

Female C57BL/6J, BALB/c mice aged 6–8 weeks and SCID mice (which are deficient in B and T cells) were obtained from the Animal Resources Center (Perth, Western Australia) and maintained under standard animal housing conditions at University of Western Australia animal holding facility. The OT-1 ($H\text{-}2^b$) T-cell receptor transgenic mouse line, expressing a T-cell receptor recognizing the dominant $H\text{-}2^b$-restricted OVA epitope (SIINFEKL) was kindly supplied by Dr F Carbone and Dr W Heath (University of Melbourne, Australia) and bred in house at the University of Western Australia animal holding facility. The μMT mice that contain no B cells because of a targeted mutation in the transmembrane region of the IgM heavy chain[51] were kindly supplied by Dr D Tarlington (Walter Eliza Hall Institute, 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.

Murine cell lines

AE17 and AB1 are MM cell lines derived from the peritoneal cavity of C57BL/6J or BALB/c mice, respectively, which were injected with asbestos (Crocidolite) fibers. Injection of AE17 and AB1 cells into naive mice results in MM tumors histologically similar to the human MM, as previously described.[39] AE17s-OVA was developed by transfecting the AE17 parental cell line with cDNA coding for sOVA, as previously described.[12] The Lewis lung carcinoma cell line developed in C57BL/6J mice was obtained from the American Tissue Culture Collection (Manassas, VA, USA). 3T3 cells, a standard murine fibroblast cell line,[52] were kindly provided by Professor Erik Helmerhorst (Curtin University, Perth, Western Australia). All cell lines were maintained in media that consisted of RPMI 1640 (Invitrogen, Auckland, New Zealand) supplemented with 10% turbo bovine serum (Invitrogen), 50 $\mu g\,ml^{-1}$ gentamicin (Pharmacia, Bentley, Western Australia, Australia), 60 $\mu g\,ml^{-1}$ benzylpenicillin (CSL, Melbourne, Victoria, Australia) and 0.05 mM 2-mercaptoethanol (Merck, West Point, PA, USA). Transfected tumor cell lines were maintained in the same medium supplemented with 400 $\mu g\,l^{-1}$ neomycin analog G418 (geneticin; Invitrogen). Cells were cultured at 37 °C in a 5% CO_2-humidified atmosphere.

Agonist anti-CD40 monoclonal Ab injections

Agonist rat anti-murine CD40 Ab (FGK45) and a rat IgG2a control Ab (both from the Walter and Elisa Hall Institute, Melbourne, or the Monoclonal Antibody Facility, Western Australian Institute for Medical Research, Perth) were stored at −20 °C (at 1 $mg\,ml^{-1}$) and diluted in PBS to 0.4 $mg\,ml^{-1}$ so that 40 μg per 100 μl per injection was delivered i.t. by a 26-gauge needle. The endotoxin levels in these Abs are $<0.1\,EU\,ml^{-1}$ (measured by each supplier using an endotoxin detection kit, documentation supplied). In one series of experiments the same dose of anti-CD40 Ab was administered i.v. The PBS diluent (100 μl per dose) was included as another control. Six injections per mouse were given three times per week for 2 weeks.

In vivo tumor growth

Confluent AE17 and AB1 tumor cells (80%) were harvested using trypsin and prepared for injection by washing twice in PBS. Viability was always >90%. Mice were injected subcutaneously in the left flank with 5×10^5 AE17 tumor cells and 1×10^6 AB1 tumor cells, per mouse in 100 μl of PBS. Tumor growth was monitored regularly and tumors were measured using microcallipers. Mice were sacrificed using methoxyflurane (Medical Developments International, Springvale, Victoria, Australia) when tumors were 100 mm^2 in accordance with the requirements of institutional Animal Ethics Committees.

Harvesting tumors and lymphoid organs for FACS staining and immunohistochemistry

Mice were killed using methoxyflurane, and tumors, tumor-dLNs, non-dLN and spleens were removed. For FACS analyses, single-cell suspensions were obtained by gently mashing tumors and lymphoid organs between two frosted glass slides, removing debris by filtration through a 30-μm-thick nylon mesh (BD Falcon, Bedford, MA, USA), centrifuging cells at 1200 r.p.m. for 6 min and resuspending the cell pellet in PBS or 2% turbo bovine serum. Where relevant, lysis buffer (1 M Tris-HCl, pH 7.2) was used to lyse red blood cells. For immunohistochemistry, tumors, dLNs, non-dLNs and spleens were embedded into O.C.T. freezing medium and stored at −80 °C. Sections of 10 μm were cut using a Leica Jung CM cryostat 1800 (Leica, Wetzlar, Germany) and placed onto poly-L-lysine (Sigma, St Louis, MO, USA) coated slides.

FACS staining and analysis

Cells were washed in PBS or 2% turbo bovine serum, adjusted to 10^6 cells per ml and 200 μl per well transferred into 96-well plates (BD Falcon) and stained for FACS analysis using Abs and their isotype control. Abs directed against mouse CD11c (clone HL3), CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD21 (clone 7G6), CD5 (clone 53-7.3), CD40 (clone 3/23) and CD23 (clone B3B4) were obtained from Pharmingen (San Diego, CA, USA). Anti-CD40 (clone 1C10) was from Caltag (Burlingame, CA, USA). Abs directed against mouse CD19 (clone MB19-1), and CD45R, an isoform of CD45 (B220; clone RA3-6B2) were from eBioscience (San Diego, CA, USA) B220 (clone RA3-6B2) was also purchased from BioLegend (San Diego, CA, USA). In one series of experiments, biotinylated anti-mouse IgM (R6-60.2) or IgG (A85-1) were linked to streptavidin-conjugated PerCP (all from Pharmingen). Cells were incubated for 30 min to 1 h at 4 °C, in the dark, washed in PBS and fixed in 2% paraformaldehyde for 15 min at 4 °C in dark. After fixation, cells were washed in PBS/2% turbo bovine serum and analyzed on a FACSCalibur (BD Biosciences, CA, USA) using CellQuest or FlowJo software or on a FACSCanto II (BD Biosciences) using Diva software.

Tetramer staining

Single-cell suspensions were resuspended in 100 μl of Fc block (CD16/32; Pharmingen) and incubated for 30 min on ice in the dark. Washed samples (in PBS/0.1%bovine serum albumin (BSA)/0.02% azide) were resuspended in 50 μl SIINFEKL MHC K^b-PE tetramer (diluted 1:200; kindly donated by Dr Andrew Brooks, University of Melbourne, Victoria, Australia) and incubated for 1 h at room temperature (RT) in the dark. Samples were washed, resuspended in 100 μl anti-CD8 tricolor (Caltag), incubated for 30 min and prepared for FACS analysis.

Immunohistochemistry

Air-dried sections were fixed in cold absolute ethanol for 15 min, washed in PBS and endogenous peroxidases blocked by a 10 min incubation at RT with 1% H_2O_2 solution (6% H_2O_2 stock; Orion Laboratories, Perth, Western Australia, USA). After a repeat wash, sections were incubated using an Avidin/Biotin blocking kit (Vector Laboratories Burlingame, CA, USA) as per manufacturer's instructions to prevent endogenous nonspecific avidin or biotin activity. After washing, the appropriate isotype controls and primary Abs directed against murine $CD4^+$ and $CD8^+$ T cells (clones RM4-5 and 53-6.7, respectively; Pharmingen) and B cells (B220) were incubated for 60 min at RT,

washed in PBS and sequentially linked to the secondary Ab (anti-rat biotin; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and streptavidin–horseradish peroxidase (Dako, Glostrup, Denmark) during serial 30-min incubation periods between extensive washing. After another wash in PBS, one Sigma FAST (D-4168) 3,3-diaminobenzidine tablet and one urea hydrogen tablet (Sigma) were added to 1 ml of ddH_2O to serve as a peroxidase substrate (125 µl per section). After a 5–20 min incubation, sections were washed in PBS, counterstained in Gill's Hematoxylin for 10 s, washed in tap water and blued in Scott's water (7 g $NaHCO_3$, 40 g $MgSO_4$ in 2 l H_2O) for 20 s. The sections were then dehydrated in increasing concentrations of ethanol, cleared in xylene (Merke, Kilsyth, Victoria, Australia) and mounted with DPX (Ultramount; Scot Scientific, Perth, Western Australia, Australia) and a coverslip (Esco cover glass; Biolab Scientific, Melbourne, Victoria, Australia).

In vivo depletion studies

For depletion of $CD4^+$ or $CD8^+$ cells, two doses (150 µg per dose) of either the anti-CD4 monoclonal Ab (YTS-191) or the anti-CD8 monoclonal Ab (YTS-169) (both hybridoma cell lines were obtained from the European Collection of Animal Cell Cultures, Salisbury, UK) were injected intraperitonially one day before treatment and continued (three doses per week; 100–150 µg per dose) until the end of anti-CD40 Ab treatment. Mice were killed and spleens tested (by FACS analysis) at the start and end of the experiment for staining of $CD4^+$ or $CD8^+$ cells. This was determined as 95–99% effective for $CD8^+$ depletions and 90–95% effective for $CD4^+$ depletions. Anti-asialo GM_1 (Wako Chemicals, Neuss, Germany) was used according to the manufacturer's instructions to deplete NK cells. Animals were given two doses intraperitonially before treatment (20 µl per dose) and then a dose every 4 days throughout treatment. Depletion of NK cells was shown by double staining for NK1.1 and αβ T-cell receptor (both from Pharmingen) to be 80–90% effective.

In vivo analysis of CTL function (*in vivo* CTL assay)

Target cells for *in vivo* evaluation of cytotoxic activity were prepared as previously described.[12] Briefly, C57BL/6 spleen and LN cell suspensions were RBC-lysed, the cells washed and divided into two populations. One population was pulsed with 10^{-6} M SIINFEKL for 90 min at 37 °C, washed in PBS and labeled with a high concentration (5 µM) of CFSE (Molecular Probes, Eugene, OR, USA). Control, uncoated target cells were labeled with a low concentration of CFSE (0.5 µM). 10^7 cells of each population were mixed in 200 µl PBS and i.v. injected into each recipient mouse. Specific *in vivo* cytotoxicity was determined by collecting the dLNs, non-dLNs, spleens and tumors from recipient mice 18 h after injection and the number of cells in each target cell population determined by flow cytometry. The ratio between the percentages of uncoated versus SIINFEKL coated ($CFSE^{lo}/CFSE^{high}$) was calculated to obtain a numerical value of cytotoxicity. Further controls included naive and PBS-treated recipient mice. To normalize data allowing inter-experimental comparisons, we calculated ratios between the percentages of peptide coated in control versus tumor-bearing mice.

Quantifying murine IgG and IgM using ELISA

Total IgG levels were determined by coating ELISA plates (Nunc Maxisorp, Roskilde, Denmark) with chrompure mouse IgG (Jackson ImmunoResearch Laboratories) or serum samples overnight at 4 °C in carbonate coating buffer (0.15 M Na_2CO_3, 0.35 M $NaHCO_3$ (pH 9.6)). The wells were then washed three times in washing buffer (PBS, 0.05% Tween-20; Sigma) and blocked in PBS, 2% BSA for 60 min at RT. After a repeat wash, biotin-conjugated goat anti-mouse IgG Ab (clone poly4053; diluted in PBS, 0.05% Tween-20, 0.1% BSA; BioLegend) was added and incubated for 60 min at RT. The wells were again washed three times in washing buffer before addition of streptavidin–horseradish peroxidase (p0397; Dako). After a final wash, 100 µl of the substrate K-Blue (ELISA Systems, Windsor, Queensland, Australia) was added to each well and incubated at RT until sufficient color change occurred before stopping with 100 µl of 1 M HCl. Optical densities were read at 410 nm.

Total IgM was determined using a sandwich IgM ELISA. ELISA plates were coated with rat anti-mouse IgM (Pharmingen) at 0.25 µg ml^{-1} in carbonate coating buffer, incubated overnight at 4 °C, washed and blocked in PBS, 2% BSA. Samples and standards (mouse IgM; BioLegend) were added and incubated overnight at 4 °C. After three washes, biotin-conjugated anti-mouse IgM (clone R6-60.2; Pharmingen) was added to each well, and after 60 min incubation at RT, linked to Streptavidin–horseradish peroxidase and the substrate K-Blue as per the IgG ELISA.

Purifying serum

Blood from cardiac bleeds was allowed to clot for 1 h at RT, centrifuged at 2000 *g* for 20 min and serum was collected. Sera from three separate experiments were pooled, diluted in running buffer (20 mM Na_2HPO_4, pH 7.3) and loaded onto a Protein G column (HiTrap Protein G HP; Amersham Biosciences AB, Uppsala, Sweden) at 1.5 ml min^{-1}, and this was repeated three times. After washing the column with running buffer, bound IgG was eluted with 1 ml aliquots of 100 mM glycine (pH 2.7). Eluted fractions were neutralized with 20 µl 1 M Tris (pH 9), pooled and dialyzed in a dialysis cassette 10 000 MWCO (Pierce, Rockford, IL, USA) against purified water for at least 24 h and concentrated using a Vivaspin20 Centrifuge Concentrator 10 000 MWCO cutoff at 3000 *g* (VivaScience, Hanover, Germany). The unbound, IgG-depleted eluate was collected and used for the IgM studies after dialysis and concentration. The IgG and IgM content was measured by ELISA.

B-cell isolation

Spleens were dissociated between sterile frosted glass slides in PBS/1% BSA (as described above) and the resulting single-cell suspension prepared to the required concentration and incubated with the appropriate Ab. To isolate $B220^+$ B cells, we incubated four Dynabeads conjugated to the anti-mouse B-cell Ab (B220; Dynal Biotech, Oslo, Norway) per estimated target B cells with splenocytes for 20 min at 4 °C with gentle rotation. Bead-captured cells were collected by magnetic separation. To isolate $CD19^+$ B cells, we incubated 10 µl of CD19 Microbeads (Miltenyi Biotec Australia, North Ryde, NSW, Australia) per 10^7 total cells for 15 min at 4 °C. Washed cells were resuspended in 500 µl of buffer per 10^8 total cells before magnetic separation with MS column and miniMACS separator (Miltenyi Biotech Australia).

Lyons Parish assay: CFSE-labeling and T- and B-cell proliferation

The Lyons Parish assay was used to assess *in vivo* changes in tumor antigen presentation levels using adoptively transferred tumor-specific T cells. To do this, we labeled cells with CFSE (Molecular Probes, Eugene, OR, USA) as previously described.[53] The cells were resuspended in 20 ml RPMI at 10^7 cells ml^{-1} and incubated with 1 µl of CFSE stock solution (5 mM in dimethyl sulfoxide) for 10 min at room temperature. Cells were washed through fetal calf serum four times and PBS twice, and 10^7 cells i.v. injected into each recipient mouse. CFSE-labeled cells were recovered from secondary lymphoid organs 3 days after adoptive transfer and analyzed for B220, CD19, CD4 and CD8 expression by FACS analysis.

Statistical analysis

Statistical significance comparing each time point or dose was determined by an unpaired *t*-test; *P*-values of <0.05 were considered significant. Statistical analysis was conducted using GraphPad Prism v3.00 (San Diego, CA, USA).

ACKNOWLEDGEMENTS

We thank the Mesothelioma Applied Research foundation (MARF), the Australian Lung Foundation (ALF) and the Western Australian Cancer Council for funding this project. We especially thank the Canberra-based Asbestos Research Group who raised funds in memory of their relatives, Gerald Willey and Peter Thurbon, both of whom died from mesothelioma.

1 Toes RE, Schoenberger SP, van der Voort EI, Offringa R, Melief CJ. CD40-CD40Ligand interactions and their role in cytotoxic T lymphocyte priming and anti-tumor immunity. *Semin Immunol* 1998; **10**: 443–448.
2 van Mierlo GJ, den Boer AT, Medema JP, van der Voort EI, Fransen MF, Offringa R *et al.* CD40 stimulation leads to effective therapy of CD40(-) tumors through induction of strong systemic cytotoxic T lymphocyte immunity. *Proc Natl Acad Sci USA* 2002; **99**: 5561–5566.
3 Stumbles PA, Himbeck R, Frelinger JA, Collins EJ, Lake RA, Robinson BW. Cutting edge: tumor-specific CTL are constitutively cross-armed in draining lymph nodes and

transiently disseminate to mediate tumor regression following systemic CD40 activation. *J Immunol* 2004; **173**: 5923–5928.
4 Nowak AK, Robinson BW, Lake RA. Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. *Cancer Res* 2003; **63**: 4490–4496.
5 Hamzah J, Nelson D, Moldenhauer G, Arnold B, Hammerling GJ, Ganss R. Vascular targeting of anti-CD40 antibodies and IL-2 into autochthonous tumors enhances immunotherapy in mice. *J Clin Invest* 2008; **118**: 1691–1699.
6 Jackaman C, Lew AM, Zhan Y, Allan JE, Koloska B, Graham PT *et al.* Deliberately provoking local inflammation drives tumors to become their own protective vaccine site. *Int Immunol* 2008; **20**: 1467–1479.
7 Berner V, Liu H, Zhou Q, Alderson KL, Sun K, Weiss JM *et al.* IFN-gamma mediates CD4+ T-cell loss and impairs secondary antitumor responses after successful initial immunotherapy. *Nat Med* 2007; **13**: 354–360.
8 Todryk SM, Tutt AL, Green MH, Smallwood JA, Halanek N, Dalgleish AG *et al.* CD40 ligation for immunotherapy of solid tumours. *J Immunol Methods* 2001; **248**: 139–147.
9 Davidson JA, Musk AW, Wood BR, Morey S, Ilton M, Yu LL *et al.* Intralesional cytokine therapy in cancer: a pilot study of GM-CSF infusion in mesothelioma. *J Immunother* 1998; **21**: 389–398.
10 Caminschi I, Venetsanakos E, Leong CC, Garlepp MJ, Robinson BW, Scott B. Cytokine gene therapy of mesothelioma. Immune and antitumor effects of transfected interleukin-12. *Am J Respir Cell Mol Biol* 1999; **21**: 347–356.
11 van Bruggen I, Nelson DJ, Currie AJ, Jackaman C, Robinson BW. Intratumoral poly-N-acetyl glucosamine-based polymer matrix provokes a prolonged local inflammatory response that, when combined with IL-2, induces regression of malignant mesothelioma in a murine model. *J Immunother* 2005; **28**: 359–367.
12 Jackaman C, Bundell CS, Kinnear BF, Smith AM, Filion P, van Hagen D *et al.* IL-2 intratumoral immunotherapy enhances CD8+ T cells that mediate destruction of tumor cells and tumor-associated vasculature: a novel mechanism for IL-2. *J Immunol* 2003; **171**: 5051–5063.
13 Mukherjee S, Haenel T, Himbeck R, Scott B, Ramshaw I, Lake RA *et al.* Replication-restricted vaccinia as a cytokine gene therapy vector in cancer: persistent transgene expression despite antibody generation. *Cancer Gene Ther* 2000; **7**: 663–670.
14 Robinson BW, Mukherjee SA, Davidson A, Morey S, Musk AW, Ramshaw I *et al.* Cytokine gene therapy or infusion as treatment for solid human cancer. *J Immunother* 1998; **21**: 211–217.
15 Nelson DJ, Robinson BW, Allan J, van der Most R. Gene therapy of mesothelioma. *Expert Opin Biol Ther* 2005; **5**: 1039–1049.
16 Staveley-O'Carroll K, Schell TD, Jimenez M, Mylin LM, Tevethia MJ, Schoenberger SP *et al. In vivo* ligation of CD40 enhances priming against the endogenous tumor antigen and promotes CD8+ T cell effector function in SV40T antigen transgenic mice. *J Immunol* 2003; **171**: 697–707.
17 Banchereau J, Briere F, Liu YJ, Rousset F. Molecular control of B lymphocyte growth and differentiation. *Stem Cells* 1994; **12**: 278–288.
18 Lee BO, Moyron-Quiroz J, Rangel-Moreno J, Kusser KL, Hartson L, Sprague F *et al.* CD40, but not CD154, expression on B cells is necessary for optimal primary B cell responses. *J Immunol* 2003; **171**: 5707–5717.
19 Coughlin CM, Vance BA, Grupp SA, Vonderheide RH. RNA-transfected CD40-activated B cells induce functional T-cell responses against viral and tumor antigen targets: implications for pediatric immunotherapy. *Blood* 2004; **103**: 2046–2054.
20 Kim SK, Nguyen Pham TN, Nguyen Hoang TM, Kang HK, Jin CJ, Nam JH *et al.* Induction of myeloma-specific cytotoxic T lymphocytes *ex vivo* by CD40-activated B cells loaded with myeloma tumor antigens. *Ann Hematol* 2009; **88**: 1113–1123.
21 Lapointe R, Bellemare-Pelletier A, Housseau F, Thibodeau J, Hwu P. CD40-stimulated B lymphocytes pulsed with tumor antigens are effective antigen-presenting cells that can generate specific T cells. *Cancer Res* 2003; **63**: 2836–2843.
22 Buhmann R, Nolte A, Westhaus D, Emmerich B, Hallek M. CD40-activated B-cell chronic lymphocytic leukemia cells for tumor immunotherapy: stimulation of allogeneic versus autologous T cells generates different types of effector cells. *Blood* 1999; **93**: 1992–2002.
23 Kondo E, Gryschok L, Schultze JL, von Bergwelt-Baildon MS. Using CD40-activated B cells to efficiently identify epitopes of tumor antigens. *J Immunother* 2009; **32**: 157–160.
24 Canevari S, Pupa SM, Menard S. 1975-1995 revised anti-cancer serological response: biological significance and clinical implications. *Ann Oncol* 1996; **7**: 227–232.
25 Robinson C, Callow M, Stevenson S, Scott B, Robinson BW, Lake RA. Serologic responses in patients with malignant mesothelioma: evidence for both public and private specificities. *Am J Respir Cell Mol Biol* 2000; **22**: 550–556.
26 Houghton AN, Uchi H, Wolchok JD. The role of the immune system in early epithelial carcinogenesis: B-ware the double-edged sword. *Cancer Cell* 2005; **7**: 403–405.
27 Tan TT, Coussens LM. Humoral immunity, inflammation and cancer. *Curr Opin Immunol* 2007; **19**: 209–216.
28 de Visser KE, Korets LV, Coussens LM. *De novo* carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* 2005; **7**: 411–423.
29 Schreiber H, Wu TH, Nachman J, Rowley DA. Immunological enhancement of primary tumor development and its prevention. *Semin Cancer Biol* 2000; **10**: 351–357.
30 Manson LA. Anti-tumor immune responses of the tumor-bearing host: the case for antibody-mediated immunologic enhancement. *Clin Immunol Immunopathol* 1994; **72**: 1–8.
31 Qin Z, Richter G, Schuler T, Ibe S, Cao X, Blankenstein T. B cells inhibit induction of T cell-dependent tumor immunity. *Nat Med* 1998; **4**: 627–630.
32 Brodt P, Gordon J. Anti-tumor immunity in B lymphocyte-deprived mice. I. Immunity to a chemically induced tumor. *J Immunol* 1978; **121**: 359–362.
33 Barbera-Guillem E, Nelson MB, Barr B, Nyhus JK, May Jr KF, Feng L *et al.* B lymphocyte pathology in human colorectal cancer. Experimental and clinical therapeutic effects of partial B cell depletion. *Cancer Immunol Immunother* 2000; **48**: 541–549.
34 Monach PA, Schreiber H, Rowley DA. CD4+ and B lymphocytes in transplantation immunity. II. Augmented rejection of tumor allografts by mice lacking B cells. *Transplantation* 1993; **55**: 1356–1361.
35 Shah S, Divekar AA, Hilchey SP, Cho HM, Newman CL, Shin SU *et al.* Increased rejection of primary tumors in mice lacking B cells: inhibition of anti-tumor CTL and TH1 cytokine responses by B cells. *Int J Cancer* 2005; **117**: 574–586.
36 Gordon J, Holden HT, Segal S, Feldman M. Anti-tumor immunity in B-lymphocyte-deprived mice. III. Immunity to primary Moloney sarcoma virus-induced tumors. *Int J Cancer* 1982; **29**: 351–357.
37 Schultz KR, Klarnet JP, Gieni RS, HayGlass KT, Greenberg PD. The role of B cells for *in vivo* T cell responses to a Friend virus-induced leukemia. *Science* 1990; **249**: 921–923.
38 DiLillo DJ, Yanaba K, Tedder TF. B cells are required for optimal CD4+ and CD8+ T cell tumor immunity: therapeutic B cell depletion enhances B16 melanoma growth in mice. *J Immunol* 2010; **184**: 4006–4016.
39 Marzo AL, Lake RA, Lo D, Sherman L, McWilliam A, Nelson D *et al.* Tumor antigens are constitutively presented in the draining lymph nodes. *J Immunol* 1999; **162**: 5838–5845.
40 Zhang XK, Moussa O, LaRue A, Bradshaw S, Molano I, Spyropoulos DD *et al.* The transcription factor Fli-1 modulates marginal zone and follicular B cell development in mice. *J Immunol* 2008; **181**: 1644–1654.
41 Hardy RR. B-1 B cell development. *J Immunol* 2006; **177**: 2749–2754.
42 Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 1998; **393**: 480–483.
43 Diehl L, den Boer AT, Schoenberger SP, van der Voort EI, Schumacher TN, Melief CJ *et al.* CD40 activation *in vivo* overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat Med* 1999; **5**: 774–779.
44 Calame KL. Plasma cells: finding new light at the end of B cell development. *Nat Immunol* 2001; **2**: 1103–1108.
45 Fang W, Nath KA, Mackey MF, Noelle RJ, Mueller DL, Behrens TW. CD40 inhibits B cell apoptosis by upregulating bcl-xL expression and blocking oxidant accumulation. *Am J Physiol* 1997; **272**: C950–C956.
46 Mackus WJ, Lens SM, Medema RH, Kwakkenbos MJ, Evers LM, Oers MH *et al.* Prevention of B cell antigen receptor-induced apoptosis by ligation of CD40 occurs downstream of cell cycle regulation. *Int Immunol* 2002; **14**: 973–982.
47 Erickson LD, Vogel LA, Cascalho M, Wong J, Wabl M, Durell BG *et al.* B cell immunopoiesis: visualizing the impact of CD40 engagement on the course of T cell-independent immune responses in an Ig transgenic system. *Eur J Immunol* 2000; **30**: 3121–3131.
48 Erickson LD, Durell BG, Vogel LA, O'Connor BP, Cascalho M, Yasui T *et al.* Short-circuiting long-lived humoral immunity by the heightened engagement of CD40. *J Clin Invest* 2002; **109**: 613–620.
49 Jasinska J, Wagner S, Radauer C, Sedivy R, Brodowicz T, Wiltschke C *et al.* Inhibition of tumor cell growth by antibodies induced after vaccination with peptides derived from the extracellular domain of Her-2/neu. *Int J Cancer* 2003; **107**: 976–983.
50 Jackaman C, Cornwall S, Lew AM, Zhan Y, Robinson BW, Nelson DJ. Local effector failure in mesothelioma is not mediated by CD4+ CD25+ T-regulator cells. *Eur Respir J* 2009; **34**: 162–175.
51 Kitamura D, Roes J, Kuhn R, Rajewsky K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 1991; **350**: 423–426.
52 Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J Cell Biol* 1963; **17**: 299–313.
53 Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J Immunol Methods* 1994; **171**: 131–137.

Supplementary Information accompanies the paper on Immunology and Cell Biology website (http://www.nature.com/icb)