

# Inhibitory Effects of B Cells on Antitumor Immunity

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## Abstract

**B-cell functions in antitumor immunity are not well understood. In this study, we evaluated the role of B cells in the development of antitumor immunity using Friend murine leukemia virus gag-expressing mouse EL-4 (EL-4 gag), D5 mouse melanoma, or MCA304 mouse sarcoma cells. To screen tumors for susceptibility to B-cell-deficient immune environments, spleen cells from naive C57BL/6 [wild-type (WT)] and B-cell knockout (BKO) mice were cultured with irradiated tumor cells *in vitro*. When cells were stimulated with EL-4 gag or D5 (but not MCA304 tumors), IFN- $\gamma$  production from CD8 T cells and natural killer cells was markedly decreased in WT compared with BKO cultures. IFN- $\gamma$  production was correlated with CD40 ligand expression on the tumor and inversely with interleukin-10 (IL-10) production by B cells. Sorted WT B cells produced more IL-10 than CD40 knockout (CD40KO) B cells when cocultured with EL-4 gag or D5 (but not MCA304). IFN- $\gamma$  production by BKO cells was reduced by the addition of sorted naive WT B cells (partially by CD40KO B cells) or recombinant mouse IL-10. *In vivo* tumor progression mirrored *in vitro* studies in that WT mice were unable to control tumor growth whereas EL-4 gag and D5 tumors (but not MCA304) were eliminated in BKO mice. Robust *in vivo* antitumor CTLs developed only in BKO tumor-challenged mice. Our studies provide the first mechanistic basis for the concept that B-cell depletion could therapeutically enhance antitumor immune responses to certain tumors by decreasing IL-10 production from B cells. (Cancer Res 2006; 66(15): 7741-7)**

## Introduction

The aim of cancer therapy is long-term elimination of tumor cells without damage to healthy cells (1, 2). Surgery and radiotherapy are limited to treatment of local tumors, and chemotherapy is limited by toxicity due to the low level of tumor specificity (1). There is considerable evidence indicating that the immune system can recognize and destroy tumor cells (2–5). For example, certain immunocompromised animals and humans are susceptible to spontaneous tumor growth (3), and immunosuppressive therapies after transplantation can be associated with an increased incidence of cancer (6). Natural occurrence of adaptive cellular and humoral immune responses to tumor-associated antigens has been observed in cancer patients (7, 8), although host antitumor immune responses may become ineffective due to tumor escape mechanisms, such as changes in tumor antigenicity or induction of regulatory immune cells (9–12). To

date, the possibility of broadly effective anticancer immunotherapy has not been realized.

In B-cell-deficient mice, enhanced antitumor immunity was associated with increased T-cell or natural killer (NK) cell activities, although the direct role of the B-cell deficiency in the tumor immunity has not been determined thus far (13, 14). To our knowledge, ours is the first report that addresses the mechanisms by which B cells inhibit immune responses against tumors. Our data show that antitumor immunity is highly enhanced in the absence of B cells due to up-regulation of both innate and adaptive immunity, in cases where CD40 ligand (CD40L) is expressed on tumor cells. After *in vitro* tumor challenge, increased IFN- $\gamma$  production from CD8 T cells and NK cells was observed in the absence of B cells, whereas in wild-type (WT) spleen cells, high amounts of interleukin (IL)-10 were produced by B cells, resulting in decreased IFN- $\gamma$  production. CD40-CD40L interactions between B cells and tumor cells were associated with B-cell IL-10 production. In *in vivo* experiments, tumor growth was suppressed in B-cell knockout (BKO) mice. Robust CD8 T-cell responses against tumor cell MHC class I peptide were induced in BKO mice after tumor cell challenge. After tumor cell elimination, memory CD8 T cells were detected.

Taken together, our data provide a mechanistic explanation for why B cells can limit effective immune responses against certain tumor cells and suggest that induction of temporary B-cell deficiency has a potential role in cancer immunotherapy.

## Materials and Methods

**Mice.** C57BL/6, BKO (C57BL/6 background and homozygous for a targeted mutation in the gene for immunoglobulin heavy chain 6), IL-10 knockout (IL-10KO; C57BL/6 background and homozygous for a targeted mutation in the gene for IL10), and CD40 knockout (CD40KO; C57BL/6 background and homozygous for a targeted mutation in the gene for Cd40) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were used under a protocol approved by the Center for Biologics Evaluation and Research (CBER) Animal Care and Use Committee and in accordance with NIH guidelines for animal use and care.

**Cell lines.** Mouse EL-4 cells [carrying entire Friend murine leukemia virus (MuLV) and expressing gag (EL-4 gag)] were kindly provided by Dr. Antonio Rosato (University of Padua, Padua, Italy). The gag contains the immunodominant CD8 T-cell epitope (gag<sub>85-93</sub>) that is responsible for rejection of EL-4 gag cells. D5 mouse melanoma cells and MCA304 mouse sarcoma cells were kindly provided by Dr. Raj Puri [Food and Drug Administration (FDA), Bethesda, MD]. A hybridoma producing anti-CD8 antibody 2.43 clone (rat IgG2b) was a gift of Dr. John Morris (National Cancer Institute, Bethesda, MD), and a hybridoma producing anti-NK-1.1 PK136 clone (mouse IgG2a) was a gift of Dr. Ronald Schwartz (National Institute of Allergy and Infectious Diseases, Bethesda, MD). A hybridoma producing anti-CD40-stimulating antibody FGK45 (rat IgG2a) was a kind gift of Dr. Antonius Rolink (University of Basel, Basel, Switzerland). EL-4 gag cells were maintained in complete Iscove's modified Dulbecco's medium (Quality Biological, Inc., Gaithersburg, MD) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT). D5 melanoma cells and MCA304 sarcoma cells were maintained in complete RPMI 1640 (Quality Biological)

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containing 10% FBS. 2.43 hybridoma cells and PK136 hybridoma cells were maintained in complete DMEM (Quality Biological) containing 10% FBS. FGK45 hybridoma cells were maintained in complete RPMI 1640 containing 10% FBS and recombinant IL-6 (2 ng/mL; PeproTech, Rocky Hill, NJ).

**Cell irradiation.** For *in vitro* experiments, tumor cells were irradiated: EL-4 gag, 1.5  $K_{rad}$ ; D5, 0.5  $K_{rad}$ ; and MCA304, 0.5 or 1.5  $K_{rad}$ , using a cesium-137 radiation device, Gammacell 1000 Elite (Nordion International, Inc., Vancouver, British Columbia, Canada).

**Antibodies and reagents.** Anti-NK-1.1-FITC, anti-CD8 $\alpha$ -CyChrome, anti-IFN- $\gamma$ -APC, anti-IL-10-FITC, anti-B220-CyChrome, anti-CD40L-biotin, streptavidin-FITC, anti-CD127-PE, purified anti-CD16/CD32, no azide/low endotoxin (NA/LE) anti-CD40L, NA/LE hamster IgG isotype control, and NA/LE rat IgG2a isotype control were purchased from BD Pharmingen (San Diego, CA). Anti-rat IgG2a-FITC was purchased from BioLegend (San Diego, CA). 5-(and-6)-Carboxylfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR). Brefeldin A (GolgiPlug), Cytofix/Cytoperm solution, and Perm/Wash solution were purchased from BD Pharmingen. Recombinant mouse IL-10 (rIL-10) was purchased from PeproTech. The MuLV gag<sub>85-93</sub> peptide CCLCLTVFL was synthesized in the CBER Core Facility (FDA). Heat-killed *Brucella abortus* (HKBA) strain 1119.1 was kindly provided by Dr. Barbara Martin (U.S. Department of Agriculture, Ames, IA).

**Tumor/spleen cell cocultures and intracellular cytokine staining.** Unless otherwise indicated, spleen cells or B cells were cultured at  $1 \times 10^6$  per well with EL-4 gag, MCA304, or D5 cells at  $2 \times 10^5$  per well in 48-well plates for 1 to 4 days. Tumor cells were irradiated before coculture.

For intracellular cytokine staining, tumor-cocultured spleen cells were restimulated with nonirradiated tumor cells before, staining with the addition of brefeldin A (1.0  $\mu$ g/mL) for 4 hours. Cells were permeabilized and washed using Cytofix/Cytoperm solution and Perm/Wash solution followed by staining.

**IL-10 ELISA.** IL-10 levels in a cell culture supernatant were measured by ELISA (BioLegend). IL-10 bound to the immobilized capture antibody was developed using TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). rIL-10 (BioLegend) was used for the standard curve.

**Cytokine beads array assay.** Several different cytokines in cell culture supernatant were measured using the Cytometric Bead Array (CBA) kit (BD Biosciences, San Diego, CA). Cytokines were stained according to the manufacturer's protocol. Briefly, capture beads and phycoerythrin (PE) detection reagent were incubated with standard samples or test samples for 2 hours, washed in wash buffer, and then analyzed by BD CBA software and the FACSCalibur flow cytometer.

**Isolation of B cells.** A mouse CD19 positive selection kit (StemCell Technologies, Vancouver, British Columbia, Canada) was used to select B cells from mouse spleens. Briefly, mouse spleen cells were incubated with CD19PE labeling reagent (PE-labeled anti-CD19 antibody) for 15 minutes. After washing in complete RPMI 1640 with 10% FBS, cells were incubated with a tetrameric antibody complex (anti-PE  $\times$  anti-dextran) for 15 minutes and then magnetic nanoparticles (dextran-coated magnetic beads) were added. Ten minutes later, cells were washed and CD19<sup>+</sup> B cells were positively selected using EasySep magnet (StemCell Technologies). Preparations were typically >95% CD19<sup>+</sup> by flow cytometric analysis.

**In vivo experiments.** For the *in vivo* experiments, cell doses and injection sites were as follows: EL-4 gag,  $2 \times 10^7$  per mouse, i.d.; D5,  $1 \times 10^6$  per mouse, i.m.; and MCA304,  $1 \times 10^6$  per mouse, i.m. I.m. injections were given in the left hind leg. HKBA was given at a dose of  $10^8$  per mouse i.p. for tumor challenge experiments and at  $10^6$  per mouse i.v. for *in vivo* CTL experiments. MuLV gag peptide was injected at a dose of 100  $\mu$ g/mouse. Anti-NK-1.1 and anti-CD8 antibodies for depletion experiments were injected i.p. daily for 3 days on a weekly basis at 100  $\mu$ g/mouse per injection.

**Tumor measurement.** Tumor size was assessed using calipers. Mice bearing large tumors of >20-mm diameter were euthanized in accordance with the approved animal study protocol.

**Antibody preparation from hybridomas.** Anti-CD40-stimulating antibody for *in vitro* studies, anti-CD8 antibody, and anti-NK-1.1 antibody for *in vivo* tumor studies were purified from cell culture supernatants using

protein G chromatography. Briefly, after loading of a mixture of cell culture supernatant and binding buffer [0.1 mol/L sodium acetate (pH 5.2); 1:1] to HiTrap protein G columns (Amersham Biosciences, Uppsala, Sweden), bound IgG was eluted with 0.1 mol/L glycine followed by immediate neutralization with 1 mol/L Tris base. Antibody solution was dialyzed with a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) against PBS and stored at 4°C until use.

**In vivo CTL assay.** To assess *in vivo* CTL activity after tumor challenge, an *in vivo* CTL assay was done as described (15). A mixture of MuLV gag<sub>85-93</sub>-coated, CFSE<sup>high</sup> (labeled with 5  $\mu$ mol/L CFSE) target mouse spleen cells and noncoated CFSE<sup>low</sup> (labeled with 0.5  $\mu$ mol/L CFSE) control mouse spleen cells (in equal volume, containing  $1 \times 10^8$  cell/mL each) were injected into recipient mice (i.v., total  $2 \times 10^7$  cells per mouse). Recipient mice were previously immunized with HKBA ( $10^8$  organisms per mouse) mixed with MuLV gag<sub>85-93</sub> (100  $\mu$ g) or had received EL-4 gag cells ( $2 \times 10^7$  per mouse) in the left hind leg 6 days before target cell injection. Eighteen hours after transfer of targets, cells in spleens were analyzed by flow cytometry. The percentage of specific lysis was calculated using the following formula to determine a value for cytotoxicity: M1/M2 ratio = % total of noncoated CFSE<sup>low</sup> cells / % total of MuLV gag<sub>85-93</sub>-coated CFSE<sup>high</sup> cells; percentage specific lysis =  $1 - [M1/M2 \text{ (PBS group)} / M1/M2 \text{ (immunized or tumor group)}]$ .

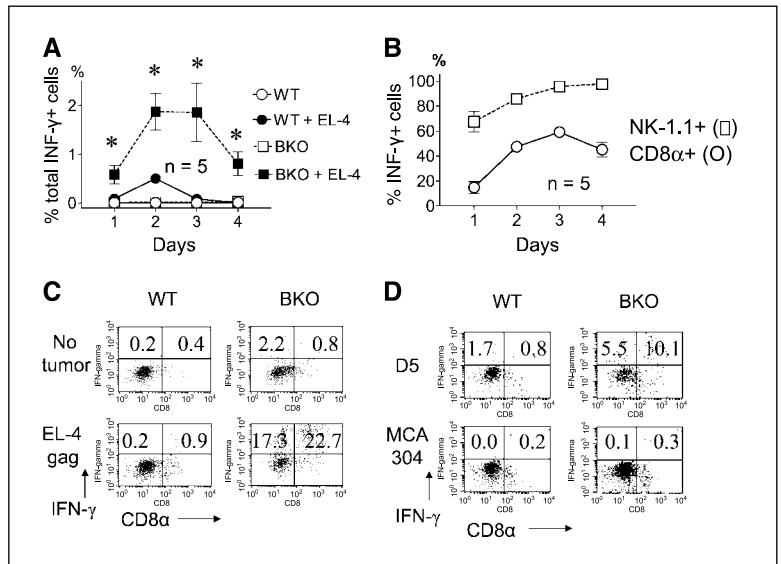
## Results

**Enhanced IFN- $\gamma$  production from spleen cells in the absence of B cells.** We developed an *in vitro* coculture system to determine the effects of B-cell deficiency on cytokine production after tumor cell challenge. Spleen cells from naive WT mice or naive BKO mice were cultured with irradiated EL-4 gag cells for 1 to 4 days and then restimulated with nonirradiated EL-4 gag cells before flow cytometry. IFN- $\gamma$ , a cytokine that is often associated with antitumor immunity (3), was analyzed. The proportion of IFN- $\gamma$ -expressing spleen cells from BKO mice was higher than that seen in naive WT mice (Fig. 1A). Most IFN- $\gamma$ -producing cells from BKO spleen cells at day 3 or 4 were NK-1.1 positive (Fig. 1B). These IFN- $\gamma^+$  NK-1.1<sup>+</sup> cells were either CD8 $\alpha$  positive or negative (Fig. 1C). The CD8 $\alpha$  cells are probably T cells because they were also CD3e<sup>+</sup> (data not shown). The NK-1.1 marker can be expressed both on NK cells and activated CD8 T cells (16). These results indicate that substantially enhanced IFN- $\gamma$  responses by CD8 T cells and NK cells are induced by EL-4 gag cells or D5 tumor cells but not MCA304 sarcoma cells (Fig. 1D).

IFN- $\gamma$  production can be modified by other cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-12, or IL-10 (17). To determine correlates of IFN- $\gamma$  up-regulation in BKO mice, supernatants from EL-4 gag tumor/spleen cell cocultures were analyzed. Higher levels of proinflammatory cytokines, such as IFN- $\gamma$ , TNF- $\alpha$ , and MCP-1, were released from cocultures of BKO cells and irradiated EL-4 gag cells compared with WT cultures (Fig. 2A-C). BKO cell cultures produced less IL-6 and IL-10 compared with WT cell cultures (Fig. 2D and E). No significant differences were observed between WT and BKO cultures with respect to IL-4, IL-5, and IL-12 p70 (data not shown).

IL-10 can inhibit IFN- $\gamma$  production by T cells in other systems (18). We hypothesized that IL-10 inhibits IFN- $\gamma$  production in the tumor/spleen cell cocultures. To determine the cellular source of IL-10, spleen cells from WT or BKO mice were cocultured with irradiated EL-4 gag cells. In the WT cocultures, IL-10<sup>+</sup> cells were predominantly B220<sup>+</sup> (Fig. 3A). There were significant differences in CD8 T-cell proportions between naive WT and BKO mouse spleens [percentage CD8 T cells:  $11.0 \pm 0.9$  (WT),  $20.1 \pm 2.1$  (BKO),  $P < 0.01$ ; NK cells:  $3.9 \pm 0.5$  (WT),  $5.0 \pm 0.6$ , not significant,  $n = 3$ ].

**Figure 1.** IFN- $\gamma$  expression by spleen cells after coculture with EL-4 gag cells *in vitro*. Naive spleen cells from WT or BKO mice were cocultured with irradiated EL-4 gag cells. Cells were restimulated with nonirradiated EL-4 gag cells just before intracellular cytokine staining and flow cytometric analysis. In the control group, naive spleen cells were cultured for 4 days without any challenges. **A**, percentage of IFN- $\gamma$ <sup>+</sup> spleen cells. \*,  $P < 0.01$ , WT (EL-4 gag) versus BKO (EL-4 gag) at days 1 to 4. **B**, proportion of IFN- $\gamma$ <sup>+</sup> BKO spleen cells that express CD8 $\alpha$  (○) or NK-1.1 (□) at days 1 to 4. **C**, CD8 $\alpha$  and IFN- $\gamma$  expression in NK-1.1<sup>+</sup> cells 3 days after EL-4 gag cell challenge *in vitro*. Numbers, percentage of IFN- $\gamma$ <sup>+</sup> cells. **D**, CD8 $\alpha$  and IFN- $\gamma$  expression in NK-1.1<sup>+</sup> cells 3 days after coculture with D5 or MCA304 tumor cells. **C** and **D**, results are representative of four experiments.



To control for differences in BKO versus WT spleens, sorted WT B cells were added to the BKO cultures. The same level of IL-10 production as in WT cell cultures was observed in the coculture of sorted WT B cells and BKO spleen cells. These data indicate that EL-4 gag cells stimulate B cells to produce IL-10.

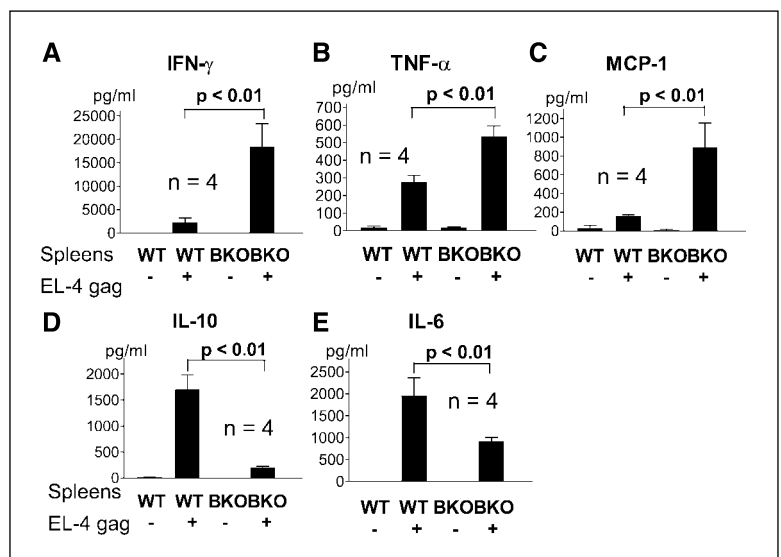
The ability of B cells to secrete IL-10 after tumor cell exposure was studied using three different tumor cell lines: EL-4 gag cells, D5 melanoma cells, and MCA304 sarcoma cells. Sorted WT B cells produced IL-10 when cocultured with irradiated EL-4 gag or D5 cells but not MCA304 cells (Fig. 3B). By flow cytometry, sorted B cells stimulated with EL-4 gag or D5 cells expressed more IL-10 than nonstimulated control B cells (Fig. 3C). MCA304 cells failed to induce IL-10 from B cells, consistent with the data obtained by ELISA (Fig. 3B). These results suggest that (a) tumor cells can induce B cells to secrete IL-10 and (b) tumors vary in ability to stimulate B cells to secrete IL-10.

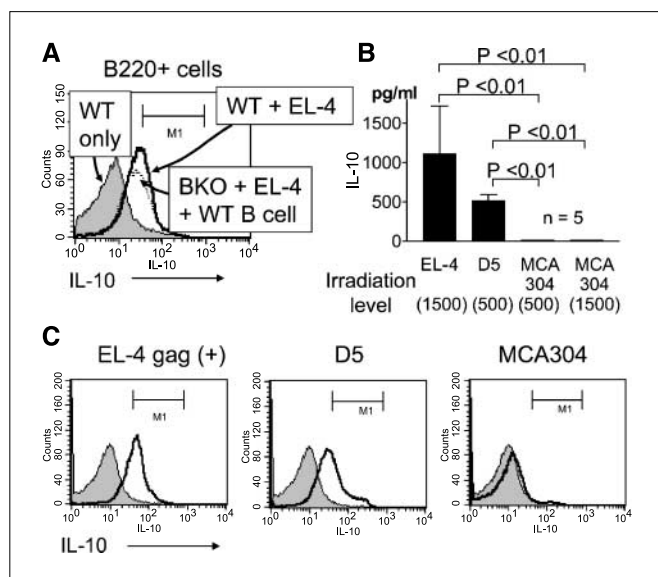
**CD40-CD40L is associated with inhibitory effects of IL-10 B cells on antitumor immunity and is necessary for IFN- $\gamma$  production.** B cells can be activated by CD40L expressed on EL-4

cells to proliferate or secrete immunoglobulins (19, 20). CD40L was expressed on tumor cells that induced IL-10 from B cells (Fig. 4A) but not on MCA304 cells. To study whether CD40-CD40L interactions are important for IL-10 production, different types of sorted B cells were cocultured with irradiated EL-4 gag cells. IL-10 production was significantly attenuated when tumor cells were cultured with CD40KO B cells compared with WT B cells, whereas differences in IL-10 levels were also observed between IL-10KO and CD40KO B cells (Fig. 4B). These results indicate that B-cell IL-10 production is partially dependent on CD40-CD40L interactions. Furthermore, IL-10 production by B cells was attenuated by anti-CD40L blocking antibody (Fig. 4C).

To confirm the effects of IL-10 on IFN- $\gamma$  production in this model, mouse rIL-10 was added to cocultures of BKO or IL-10KO spleen cells with EL-4 gag cells. IFN- $\gamma$  production was diminished in BKO spleen cells by rIL-10 (Fig. 4D). Tumor-stimulated IFN- $\gamma$  production by BKO cells was higher than from IL-10KO cells, but both types of spleen cells produced more IFN- $\gamma$  than WT spleen cells (Figs. 4D and 1C). Because IL-10-deficient spleen cells did

**Figure 2.** Cytokine production from mouse spleen cells after coculture with irradiated EL-4 gag cells. Naive WT or BKO spleen cells were cocultured with irradiated EL-4 gag cells for 3 days. Cytokine production in cell culture supernatants was measured by cytokine beads array assay. Control spleen cells were unstimulated. **A**, IFN- $\gamma$ . **B**, TNF- $\alpha$ . **C**, MCP-1. **D**, IL-10. **E**, IL-6.





**Figure 3.** IL-10 production from B cells after a tumor challenge. *A*, irradiated EL-4 gag cells were cocultured with naive WT spleen cells (solid line) or with naive BKO spleen cells plus sorted naive WT B cells ( $5 \times 10^5$  per well; dotted line) for 3 days. Cells were then restimulated with nonirradiated EL-4 gag cells before flow cytometry. Gray shaded area, unstimulated WT spleen cells. Percentage of B220<sup>+</sup> cells expressing IL-10: WT, only 4.3%; WT + EL-4 gag, 27.6%; BKO + EL-4 gag + sorted WT B cells, 27.1%. Results are representative of five experiments. *B*, sorted WT B cells were cultured with either irradiated EL-4 gag, D5, or MCA304 cells for 3 days followed by measurement of IL-10 in culture supernatants. *C*, cells cultured in (*B*) were restimulated with nonirradiated tumor cells at 3 days and stained for IL-10. Shaded areas, control B cells were not restimulated. Cells are gated for B220. Percentage of IL-10<sup>+</sup>, B220<sup>+</sup> cells: control, 1.4%; EL-4 gag, 51.7%; D5, 32.6%; MCA304, 3.6%. Results are representative of two independent experiments.

not produce as much IFN- $\gamma$  as BKO cells, it seemed possible that low levels of IL-10 could have a priming or enhancing effect on IFN- $\gamma$  production. However, a low dose of rIL-10 did not increase IFN- $\gamma$  production from IL-10KO spleen cells (Fig. 4D). These results indicate that B cells may reduce IFN- $\gamma$  production by producing IL-10. To confirm the results, sorted B cells from WT, IL-10KO, or CD40KO mice were added to cocultures of BKO spleen and EL-4 gag cells. The proportion of IFN- $\gamma$ -expressing NK-1.1<sup>+</sup> cells was reduced significantly by WT B cells (but not by IL-10KO B cells; Fig. 4E). IFN- $\gamma$  suppression was partially reversed by CD40KO B cells relative to WT B cells (Fig. 4E). These results suggest that (a) IL-10 produced from B cells lowers IFN- $\gamma$  production and (b) this effect is partially CD40-CD40L dependent.

MCA304 cells induced neither IL-10 from WT B cells nor IFN- $\gamma$  from BKO-derived NK and CD8 T cells. To study whether IFN- $\gamma$  production can be induced by providing a signal via CD40-CD40L interactions, MCA304 cells were incubated with anti-CD40-stimulating antibody (clone FGK45), which mimics the action of CD40L (21). MCA304 cells were CD40 negative, Fc receptor positive, and FGK45 bound to MCA304 in a dose-dependent manner at 3 to 30  $\mu$ g/mL presumably through Fc receptors (Fig. 4F). After washing, these "CD40-stimulating antibody-expressing" MCA304 cells induced IFN- $\gamma$  from NK (Fig. 4G) and CD8 T cells (Fig. 4H), indicating that CD40-CD40L interactions can trigger IFN- $\gamma$  production.

**Tumor growth inhibition in BKO mice is dependent on CD8 $\alpha^+$  or NK $^+$  cells.** To test whether antitumor immunity *in vivo* is enhanced in the absence of B cells, we used a model of antitumor

vaccination developed in our laboratory. Previously, we showed partial tumor inhibition in WT mice immunized with an adjuvant (HKBA) plus tumor antigen (22). In the present study, all WT control mice were euthanized due to exponential EL-4 gag tumor growth, but in contrast, immunized mice were partially protected and 4 of 5 mice were tumor-free for >30 days (Fig. 5A). Antitumor immunity was enhanced in BKO mice, in that EL-4 gag tumor growth was completely inhibited in immunized mice and even unimmunized BKO mice eliminated tumors by day 30 (Fig. 5B). *In vivo* depletion of NK-1.1<sup>+</sup> or CD8<sup>+</sup> cells reversed antitumor immunity in BKO mice (Fig. 5C). These results show that NK-1.1- or CD8-expressing cells are crucial for elimination of tumor cells in naive BKO mice, consistent with results of the *in vitro* experiments. D5 melanoma cell growth was also inhibited in BKO mice (Fig. 5D), whereas MCA304 sarcoma cells grew continuously (Fig. 5E). Importantly, BKO mice showed enhanced *in vivo* antitumor immunity only when challenged with tumor cells that induced opposite patterns of *in vitro* IFN- $\gamma$  and IL-10 production in WT compared with BKO cultures. These results indicate that spontaneous tumor elimination in the absence of B cells depends on up-regulated CD8 T-cell/NK cell responses and correlates with increased IFN- $\gamma$  and decreased IL-10 production.

**Increased *in vivo* CD8 T-cell responses against a tumor MHC class I antigen in BKO mice.** To further characterize the function of CD8 T cells in tumor immunity, CTL responses were assessed *in vivo*. CD8 T-cell responses induced by a mixture of HKBA and MuLV gag peptide or by EL-4 gag cells were higher in BKO mice than in WT mice (Fig. 6A), consistent with tumor suppression results and further supporting the idea that antitumor CD8 T-cell responses are up-regulated in BKO mice.

The strength of primary effector CD8 T-cell responses does not always correlate with memory cell development (23). To determine whether tumor challenge resulted in memory CD8 T-cell responses, antigen-specific IFN- $\gamma$  recall responses were measured in spleen cells from BKO mice 8 weeks after tumor injection. None of the mice had clinical signs of residual tumor ("tumor resistant"). Tumor-naive mice were used in control cultures. In tumor-resistant BKO cultures, high levels of IFN- $\gamma^+$  CD8 T cells were observed up to 4 days after class I peptide stimulation. The majority of IFN- $\gamma^+$  CD8 T cells were memory cell marker IL-7 receptor  $\alpha$  positive (CD127<sup>+</sup>; Fig. 6C; ref. 24). Increased levels of IFN- $\gamma$  production from CD8 T cells were also observed in D5-resistant BKO mice (Fig. 6D).

## Discussion

In mice, early progressive tumor growth results in T-cell-mediated "concomitant immunity" against an established tumor. Loss of this immunity has been attributed to development of regulatory T cells after the tumor reaches a certain size (25). This observation leads to the question of whether eliminating CD25<sup>+</sup>CD4<sup>+</sup> regulatory T-cell functions may be useful in cancer immune therapies (26). B cells may also have a regulatory role relevant to antitumor T-cell responses. Examples of B-cell-mediated T-cell suppression include murine lipopolysaccharide-stimulated B cells that induce T-cell anergy by producing transforming growth factor- $\beta$  (27) and limitation of autoreactive CD4 T-cell responses in the presence of B cells (28). Human CD40-stimulated B-cell leukemia cells induced allogenic T-cell anergy by producing IL-10 and were therefore judged to be unlikely to induce a protective antileukemia immune response (29). In contrast, B-cell depletion did not boost IL-2-induced immune responses against renal cell

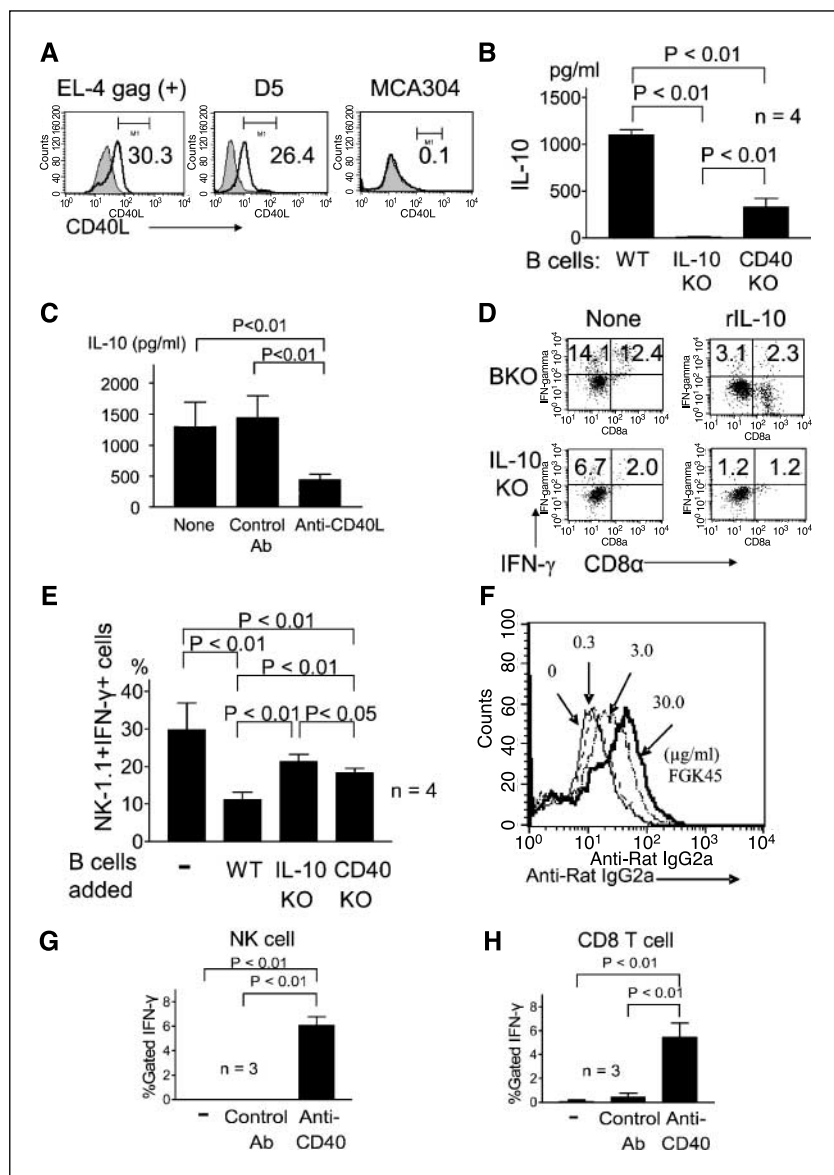
carcinoma and melanoma, but tumor CD40L expression was not assessed in this study (30). Our study shows that B cells can function as regulatory cells early after challenge with certain tumors. Enhanced tumor immunity in BKO mice is correlated with IFN- $\gamma$  production from NK and CD8 T cells, which is partially dependent on CD40L expression on tumor cells. CD40L is expressed in several human cancer cells with both hematopoietic and nonhematopoietic origins, such as T-cell lymphoma (31), renal carcinoma (32), melanoma (33), breast cancer (34), and bladder cancer (35), although the effects of B cells on CD40L-expressing tumors remain unclear. Our data support a model whereby B-cell stimulation via CD40 results in IL-10 secretion (36), which in turn diminishes CD8 and NK cell IFN- $\gamma$  secretion, antitumor activity, and CD8 T-cell memory development.

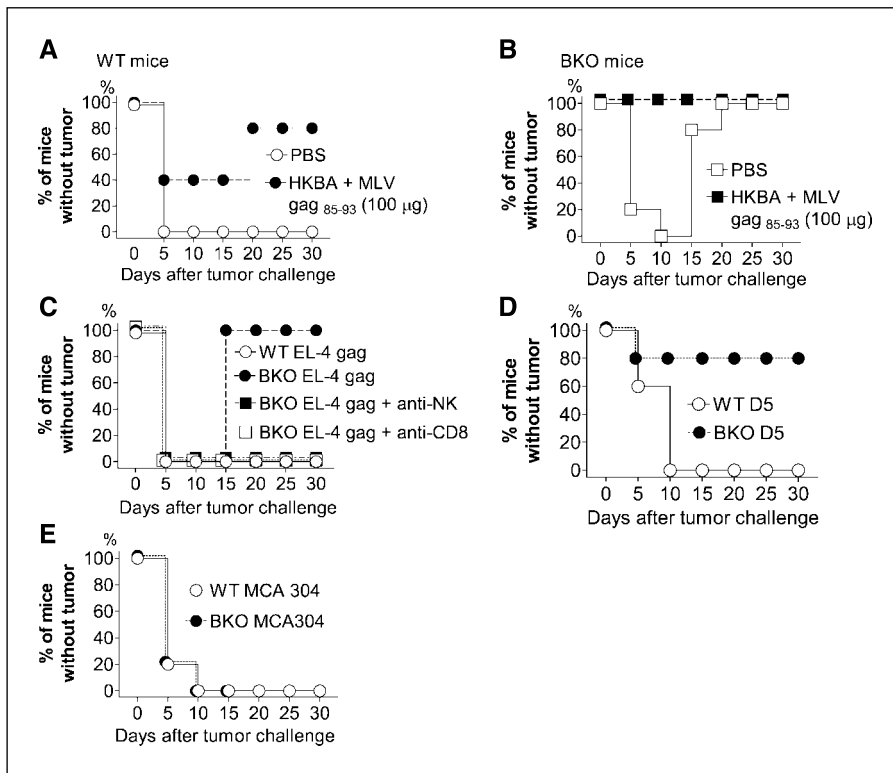
There are many conflicting observations about the role of IL-10 in antitumor responses (18, 37). IL-10 can down-regulate dendritic cell activation, resulting in decreased antitumor activity (27). IL-10 can also decrease MHC class I expression on tumor cells (38). On the other hand, rIL-10 injection at certain times promotes effector

function of antitumor CD8 T cells (39), and cytotoxicity of NK cells against tumor cells can be increased by rIL-10 (40). Clinical trials have shown both proinflammatory and anti-inflammatory effects of rIL-10 (37). Those studies were done using exogenously administered IL-10, whereas our studies focus on the effects of IL-10 physiologically provided by B cells. In our model, IFN- $\gamma$  production was attenuated by rIL-10 or by adding IL-10-expressing WT B cells. IL-10KO cells, however, did not produce as much IFN- $\gamma$  as BKO cells when cocultured with tumors. Of note, low amounts (0.2 ng/mL) of IL-10 are present in BKO and tumor cocultures (Fig. 2D). Interestingly, rIL-10 at a dose of 0.2 ng/mL did not increase IFN- $\gamma$  in IL-10KO and tumor cocultures. These observations suggest that IL-10 may be necessary for higher IFN- $\gamma$  production only if present in the appropriate amount and produced at a specific time.

The overall effects of modified IFN- $\gamma$  and IL-10 production due to B-cell deficiency on antitumor CD8 T-cell function were confirmed by an *in vivo* CTL assay. We showed that antitumor CD8 T-cell responses were up-regulated in BKO mice after a tumor

**Figure 4.** The effect of CD40-CD40L interactions on IL-10 production from B cells and on IFN- $\gamma$  production from NK and CD8 T cells. **A**, CD40L expression on tumor cells. Nonstimulated tumor cells (EL-4 gag, D5, or MCA304) were stained with control antibody (gray shaded area) or anti-mouse CD40L monoclonal antibody (solid line). Numbers, percentage of gated CD40L<sup>high</sup> cells. **B**, reduced IL-10 production in the absence of CD40-CD40L interaction. Sorted B cells were cocultured with irradiated EL-4 gag cells for 3 days, and supernatants were assayed for IL-10. **C**, inhibitory effect of anti-CD40L blocking antibody on IL-10 production. Sorted WT B cells and irradiated EL-4 gag cells were cocultured with anti-CD40L blocking antibody or control antibody (Ab) for 3 days. Supernatant IL-10 was measured by ELISA. **D**, rIL-10 decreases IFN- $\gamma$  production by BKO cells, although IL-10KO cells produce less IFN- $\gamma$  than BKO cells. BKO or IL-10KO spleen cells were cocultured with irradiated EL-4 gag cells for 3 days with or without rIL-10 (2 ng/mL to BKO and 0.2 ng/mL to IL-10KO). After restimulation with nonirradiated EL-4 gag cells, IFN- $\gamma$  production was measured by flow cytometry. Cells are gated as NK-1.1<sup>+</sup>. Numbers, percentage of gated IFN- $\gamma$ <sup>+</sup>, CD8 $\alpha$ <sup>+</sup>, or CD8 $\alpha$ <sup>-</sup> cells. **E**, IFN- $\gamma$  inhibition in BKO spleens is partially dependent on CD40-CD40L. BKO spleen cells and irradiated EL-4 gag cells were cocultured with or without sorted B cells for 3 days and then restimulated with nonirradiated EL-4 gag cells before flow cytometry. **F**, anti-CD40 antibody binds to MCA304 cells through Fc receptor in a dose-dependent manner. Irradiated MCA304 cells were cultured with FGK45 at various doses, and binding was detected at 30 minutes using anti-rat IgG2a as a second antibody for flow cytometry. **G** and **H**, anti-CD40-stimulating antibody bound to MCA304 cells induces IFN- $\gamma$  from BKO spleen cells. Irradiated MCA304 cells were cocultured with FGK45 or control antibody (30  $\mu$ g/mL) for 30 minutes. After washing with PBS, MCA304 cells were cocultured with BKO spleen cells for 3 days. After restimulation with MCA304-bound FGK45, IFN- $\gamma$  production from NK (**G**) and CD8 T cells (**H**) was evaluated by flow cytometry.



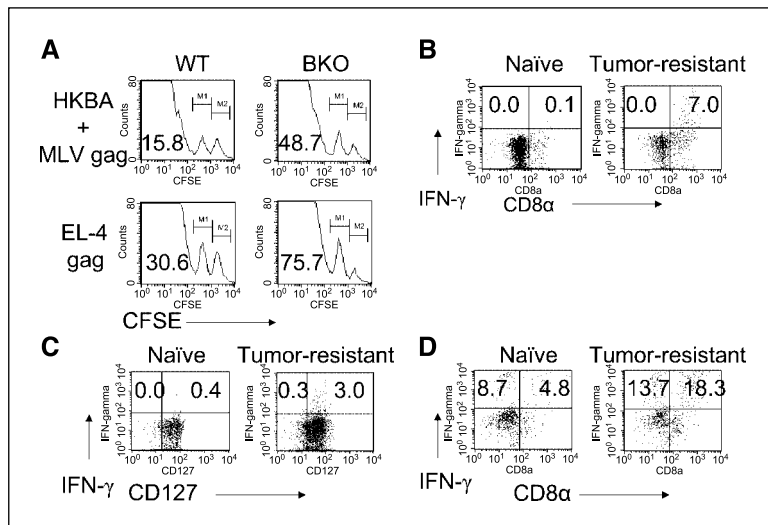


**Figure 5.** Tumor remission in the absence of B cells. WT (A) or BKO (B) mice were immunized with PBS or a mixture of HKBA and MuLV gag peptide and challenged with EL-4 gag cells 7 days later. C, naive BKO mice were injected i.p. with depleting antibodies against NK-1.1 or CD8 for 3 consecutive days per week. One week after antibody injections, mice were challenged with EL-4 gag cells. D and E, naive WT or BKO mice were challenged with D5 (D) or MCA304 (E). Percentage of mice without visible or palpable tumor. At least five individual mice were used per group.

cell challenge. In tumor-resistant BKO mice, memory CD8 cells were detected, whereas naive IL-10KO mice did not inhibit initial EL-4 gag cell growth (data not shown). These data suggest that (a) in BKO mice, spontaneous effective tumor cell elimination is induced due to the development of vigorous effector and memory CD8 T cells and (b) a certain balance of proinflammatory and anti-inflammatory cytokines, such as IFN- $\gamma$  and IL-10, may be needed for optimal antitumor immunity.

Our data show that CD40-CD40L interactions are important for B-cell IL-10 production. Recent publications suggest that B-cell-derived IL-10 or proinflammatory cytokine secretion can be affected by duration of CD40 stimulation. *In vitro*, tumor lysate-loaded CD40-stimulated B cells are effective antigen-presenting

cells that promote antitumor T-cell responses (41). These experiments differ from ours with regards to prolonged anti-CD40 stimulation, which may be less physiologically relevant. Briefer B-cell CD40 stimulation seems to result in anti-inflammatory effects (42). Thus, duration of CD40 stimulation could be critical. Presence or absence of B-cell receptor stimulation can also affect CD40-mediated cytokine production. Duddy et al. (36) showed that stimulation through CD40 alone resulted in IL-10 production from B cells and, consequently, reduced levels of proinflammatory cytokine production by the same B cells. The effects of CD40 ligation on B-cell IL-10 production are reversed by B-cell receptor stimulation (36). Based on these observations, we speculate that, in our model, CD40 ligation in the relative absence of B-cell receptor



**Figure 6.** A, *in vivo* CTL after an immunization with HKBA + MuLV gag peptide or EL-4 gag cells in WT or BKO mice. Numbers, calculated % specific lysis. B to D, memory CTL development in BKO mice after a tumor cell challenge. Naive BKO mice were challenged with EL-4 gag or D5 cells, resulting in tumor clearance by 4 weeks. Eight weeks after the tumor challenge, spleen cells were harvested and cultured with either MuLV gag peptide (1  $\mu$ g/mL; B, C) or irradiated D5 cells. Control spleen cells from naive BKO mice were also cultured with MuLV gag peptide or irradiated D5 cells. After 4 days, cells were restimulated with either MuLV gag peptide or nonirradiated D5 cells (D). C, cells were gated on the NK-1.1<sup>+</sup> (B and D) or CD8 $\alpha$ <sup>+</sup> (C) populations. Numbers, percentage of IFN- $\gamma$ <sup>+</sup> cells. Results are representative of two experiments.

stimulation is operative in the early antitumor responses. Although CD40-stimulated B cells can either up-regulate or down-regulate antitumor responses, inhibitory effects of B cells are likely to predominate under physiologic conditions if stimulated by CD40L-expressing tumor cells.

MCA304 sarcoma cells did not induce IFN- $\gamma$  production from BKO spleen cells or IL-10 production by WT B cells. IFN- $\gamma$  production in NK and CD8 T cells was achieved by a CD40 agonist antibody bound to MCA304 cells. Furthermore, MCA304 sarcoma cells grew equally well in BKO and WT mice. These results suggest that not only IL-10 but also IFN- $\gamma$  production in this model requires CD40-CD40L interactions. Transgenic CD40L expression in murine tumor cells enhances antitumor immunity (43, 44). This is relevant to clinical data showing that CD40L expression is significantly associated with improved prognosis in bladder cancer patients (35). Our data show that CD40-CD40L is associated not only with down-regulatory effects of B cells on NK cells and T cells but also with the potential for stimulating antitumor immunity, such as IFN- $\gamma$  production. Therefore, benefi-

cial effects of B-cell depletion in this model may be limited to cytokine-sensitive tumors.

In conclusion, B cells can function as regulatory cells in some specific tumor settings. B-cell depletion is achievable in humans with antibodies, and under such conditions, strong tumor-specific T-cell responses have been shown in patients with cancer, such as B-cell lymphoma (45), renal cell carcinoma, and melanoma (30). Our results suggest that temporary B-cell depletion has a potential use in cancer immunotherapy, and we show for the first time a CD40L-based mechanism responsible for this phenomenon.

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