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Differential CD40/CD40L Expression Results in Counteracting Antitumor Immune Responses

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Establishment of host-protective memory T cells against tumors is the objective of an antitumor immunoprophylactic strategy such as reinforcing T cell costimulation via CD40-CD40L interaction. Previous CD40-targeted strategies assumed that T cell costimulation is an all-or-none phenomenon. It was unknown whether different levels of CD40L expression induce quantitatively and qualitatively different effector T cell responses. Using mice expressing different levels of CD40L, we demonstrated that the greater the T cell CD40 expression the less tumor growth occurred; the antitumor T cell response was host-protective. Lower levels of CD40L expression on T cells induced IL-10-mediated suppression of tumor-regressing effector CD8+ T cells and higher productions of IL-4 and IL-10. Using mice expressing different levels of CD40 or by administering different doses of anti-CD40 Ab, similar observations were recorded implying that the induction of protumor or antitumor T cell responses was a function of the extent of CD40 cross-linking. IL-10 neutralization during priming with tumor Ags resulted in a stronger tumor-regressing effector T cell response. Using IL-10−/− DC for priming of mice expressing different levels of CD40L and subsequent transfer of the T cells from the primed mice to nude mice, we demonstrated the protumor role of IL-10 in the induction of tumor-promoting T cells. Our results demonstrate that a dose-dependent cross-linking of a costimulatory molecule dictates the functional phenotype of the elicited effector T cell response. The T cell costimulation is a continuum of a function that induces not only graded T cell responses but also two counteracting responses at two extremes. The Journal of Immunology, 2007, 178: 2047–2055.

An immune response elicited by an Ag-autoantigen, foreign Ag, or a modified self-Ag such as tumor Ags is the resultant of multiparameter interactions between APCs and Ag-specific T cells (1). These two cells interact via different cell surface molecules to generate a minimum of two crucial signals in T cells. The first signal is through TCR that recognizes the Ag-MHC complex and the second or costimulatory signal is via CD28/CD152-B7 and CD40-CD40L interactions (2–4). CD40-CD40L interaction is shown to control many immune responses including antitumor immune responses (5, 6). Although the use of defined tumor Ags for the induction of protective T cells met with limited success (7–9), CD40-CD40L interaction that was proposed to induce antitumor T cells prevented tumor growth only partially (10, 11). The antitumor immune response that results in the killing of tumor cells is mediated by CTL (12). The elicitation of an efficient tumor-regressing CTL response is dependent on CD40-CD40L interaction (13, 14), particularly between T cells and dendritic cells (DC).3 The engagement of CD40 on DC by its ligand CD154, which is expressed on CD4+ T cells, is an important event during DC activation. CD40 activation results in the up-regulation of costimulatory molecules and the secretion of inflammatory cytokines that are central to the initiation of cell-mediated immune response. Among the DC-secreted cytokines, IL-12 is an important one that provides an obligatory signal for the development of Th1 cells and efficient CD8+ T cell differentiation and function, which are necessary to induce anticancer immunity. In addition, priming of CD8+ T cells in the absence of IL-12 rendered them unresponsive to the same Ag (15, 16). It has been shown that IL-12 expression in the adoptively transferred DC or IL-12 injection also induces significant antitumor CTL responses (17, 18). In addition, re-engineered CD40 receptor on DC results in significant antitumor immunity (19). It was proposed that costimulation during the presentation of an Ag to T cells induced activation, whereas the absence of costimulation induced T cell anergy (20), implying T cell costimulation as an all-or-none phenomenon. Thus, the manipulation of CD40-CD40L-mediated costimulation was a rationale for tumor immunotherapy but suffered from several inherent limitations. Firstly, it was not known whether different levels of expression of CD40L or of any costimulatory molecules would induce quantitatively and qualitatively different effector and memory T cell responses, which is the key objective to achieve in any immunoprophylaxis or immunotherapy. Secondly, most of the previous studies used CD40-expressing tumors minimizing the analysis of the effect of possibly different doses of costimulation on the antitumor T cell response. Thirdly, it is not necessary that the tumor cells express CD40. Therefore, the possibility of anti-CD40 Ab-mediated direct tumoricidal effect is limited. Finally, if all the antitumor effects of CD40 were dependent on interaction with CD40L-mediated T cell activation, no antitumor effect would be observed in mice or human subjects lacking (or expressing less) CD40L or expressing nonfunctional mutant CD40L. In addition, impaired antitumor immune responses were associated with reduced expression of CD40L on T cells or CD40 on DC (21–23). The functional changes that occurred in those DC or T cells due to low CD40 or CD40L expressions are not yet understood. All these limitations prompted us to test the effect of differential CD40-CD40L cross-linking on antitumor T cell responses. Using a model tumor induced by RM-1 cells and a C57BL/6 (B6) mouse-derived...
prostate cell line (24), we examined whether CD40-CD40L gene doses elicit different levels and functionally counteracting phenotypes of T cell responses at two extremes. Our results suggest that the activation state of the APC, which depends on the strength of its CD40-CD40L-mediated cognate interaction with CD40 T cells, plays important roles in orchestrating different outcomes of an immune response. The nature of antitumor immune response is a function of the extent of CD40 cross-linking. These observations have important implications for the immunotherapy of infectious diseases and cancer.

Materials and Methods

**RM-1 cell line and development of tumor in mice**

RM-1 cells (24) were provided by Dr. T. R. Thompson (Baylor College of Medicine, Houston, TX). These cells were maintained in Ham’s F12K complete medium supplemented with 10% FCS and antibiotics. A total of 2 × 10⁷ cells were transferred s.c. into C57BL/6 or other gene-deficient mice of C57BL/6 background (The Jackson Laboratory), as indicated. Unless otherwise mentioned, the tumor growth and T cell functions were assessed 3 wk after RM-1 cell injection in mice. All the experiments were performed according to the animal use protocols approved by the Institutional Animal Care and Use Committee following the Guidelines framed by the Committee for the Purpose of Control and Supervision of Experiments on Animals, a central authority in India that regulates animal experimentation.

**DC cultures**

DC were derived from bone marrow progenitor cells using a previously described method (25). In brief, the femoral and tibial cells were harvested in DC culture medium (RPMI 1640 medium, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME, 20 ng/ml GM-CSF, and 10 ng/ml IL-4) and seeded in 24-well plates at a density of 1 × 10⁶ cells/well. Culture medium was replaced with fresh medium every 3 days. At 5, 7, and 9 after tumor cell inoculation (BD Pharmingen).

**Preparation of RM-1 cell Ag**

The Ags were prepared by rapid freeze-thaw cycles for seven cycles, followed by sonication and clarification by microfuging. The protein content was assayed by the BCA protein assay kit (Pierce).

**Tumor Ag pulsing of DC and immunization**

Bone marrow-derived DCs were pulsed with RM-1 cell lysate Ags. The Ag-pulsed DC were harvested, washed extensively with sterile PBS, and used for in vivo priming. For prophylactic experiments, mice were immunized s.c. thrice at 7-day intervals with 3 × 10⁶ DC.

**Tumor models and Ab treatment**

A total of 2 × 10⁶ RM-1 cells were injected s.c. in the right flank of mice in 100 µl of PBS. Tumor growth was monitored and mice were sacrificed when the control mice just started to show adverse effects from tumor (difficulty in movement or any other visible distress). Mice were supervised on days 5, 7, and 9 after tumor cell inoculation. As indicated, in some experiments mice were administered with 200 µg of anti-IL-10 Ab on days 5, 7, and 9 after tumor cell inoculation (BD Pharmingen).

**T cell purification and DC-T cell coculture assay**

The splenic and lymph node T cells were isolated as previously described (26). The CD8⁺ T cells were isolated by using the CD8⁺ T cell enrichment mixture from StemCell Technologies.

The 6-day-old DC were cultured with purified T cells from naive mice in a 96-well round-bottom plate at a ratio of 1:3 in the presence or absence of anti-CD3 Ab (BD Pharmingen) for 72 h. The indicated cytokines in the culture supernatants were assayed by ELISA.

**Adaptive T cell transfer experiment**

Splenic T cells from the DC-immunized mice were isolated i.v. (10⁶ cells in 200 µl of PBS/mouse) into the recipient C57BL/6 nude mice (6- to 8-wk-old), followed by s.c. injections of 5 × 10⁶ RM-1 cells. Seven days later, established tumor-bearing mice were subjected to adoptive T cell transfer experiment.

**Cytotoxic T cell assay**

For CTL assay, splenocytes were plated at 1.5 × 10⁶ cells/well in 6-well dishes with 1.5 × 10⁶ irradiated RM-1 cells. After 5 days of coculture, viable CD8⁺ T cells were isolated and plated against thymidine incorporated target cells (RM-1) and tested for their cytolytic activity in a standard 4-h JAM test (27). In some experiments, the isolated CD8⁺ T cells from the low and high dose anti-CD40 Ab-treated animals were mixed with indicated ratio. Cells were plated against [³H]thymidine-loaded target cells (RM-1) and tested for their cytolytic activity in a standard 4-h JAM test.

**p38 MAPK activation**

For p38 MAPK expression the DC were cocultured with anti-CD3 plus anti-CD28-activated and parafomaldehyde-fixed T cells at a ratio of 1:3 for 30 min. The cells were washed twice with ice-cold TBS (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 100 mM sodium orthovanadate). The cells were then lysed in buffer (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 50 µg each of leupeptin, aprotinin, and PMSF) by incubation on ice for 30 min. Lysates were centrifuged (13,000 rpm for 30 min). The supernatants were collected, and the protein contents were determined using the BCA protein assay kit (Pierce). Obtained samples were boiled for 5 min in Laemmlli sample buffer, electrophoresed on 10% SDS-polyacrylamide, and transferred to the nitrocellulose using a Trans-Blot system (Hoefer Scientific Instruments). The nitrocellulose filter was washed and blocked with 5% BSA for 1 h at room temperature, and incubated with antiphospho-p38 MAPK Ab for 1 h at room temperature. Immunoreactive bands were visualized by the ECL system (Amersham Biosciences). Each sample was blotted with diphospho-p38 MAPK Ab to ensure equal input of protein samples onto the gel.

**Flow cytometry**

All the Abs against CD40, CD40L, TNFR1 and TNFR2 were procured from BD Pharmingen and were used for staining the cells. Cells were incubated at 4°C in 0.1% v/v BSA in PBS and surface stained with FITC-conjugated or PE-conjugated Abs for 45 min before washing three times with ice-cold PBS. Cells were then fixed in 0.1% v/v paraformaldehyde in PBS and samples were analyzed using a FACS Vantage flow cytometer (BD Biosciences) with CellQuest software.

**Cytokine ELISA**

Cytokine contents in culture supernatants were detected by a standard two-site sandwich ELISA for cytokines as described according to the BD Pharmingen manual. In brief, ELISA plates were coated with 50 µl of anti-mouse cytokine mAb in 0.1 M NaH₂PO₄ (pH 9.0) overnight at 4°C. Plates were washed three times with wash buffer (PBS, 0.05% Tween 20) and blocked with 200 µl of blocking buffer (PBS, 1% BSA, 0.05% Tween 20, and 0.05% NaNO₂) for 1 h at room temperature. Plates were washed three times with wash buffer before adding 100 µl of murine recombinant cytokine standard (BD Biosciences) or culture supernatants (PBS, 1% BSA, 0.05% Tween 20) before incubating at overnight at 4°C. Plates were washed four times with wash buffer before adding 100 µl of biotin-conjugated, anti-mouse cytokine mAb for 1 h at room temperature, then washed four times with wash buffer after addition of 100 µl of peroxidase-conjugated Streptavidin, and incubated for 45 min at room temperature. Plates were then washed six times, after which 100 µl of tetramethylbenzidine substrate (BD Pharmingen) was added to each well and allowed to develop for 10 min at room temperature before stopping the reaction with the addition of 50 ml of 1 N H₂SO₄ in double distilled H₂O.
Differential CD40L expression regulates the extent of CD40 cross-linking and CD40-induced cytokine production. A, CD40L-deficient (B6 background, CD40L<sup>−/−</sup>) mice were bred to wild-type (B6 background, CD40L<sup>+/+</sup>) mice to generate the F<sub>1</sub> (CD40L<sup>−/−</sup>) mice. The levels of CD40L expression on T cells were determined by both RT-PCR and FACS. B, The effects of the CD40L gene doses on the CD3-induced cytokine production were examined as indicated. C, p38 MAPK expression and IL-12 production by DC were assayed by coculture of CD3-specific Ab-stimulated paraformaldehyde-fixed T cells from mice expressing different levels of CD40L and B6-derived DC. The error bars represent mean ± SD. The results shown are from one of three individual experiments.

Absorbance at 450 nm was measured using an automated microplate absorbance reader (Bio-Tek Instruments).

**RT-PCR**

Total RNA was isolated from tumor cells, DC, and T cells using TRIzol (Sigma-Aldrich) according to the manufacturer’s instructions, and used for first-strand cDNA synthesis using the Thermoscript RT-PCR system (Invitrogen Life Technologies). The cDNA was then used as template for PCR amplification of mouse IL-10, IL-12, IFN-γ, IL-4, CD40, and CD40L using gene-specific primers. The primers are IL-10 (forward) 5-TCA CTC TTC ACC TGC TGC TCC AC-3, (reverse) 5-CTA TGC TGC CTG CTC TTA CTC-3; for IL-12 (forward) 5-AAA CAA GAG CGG CCC AAG AAC-3, (reverse) 5-AAA AAG CCA ACC AAG CAG AAG ACA G-3, for IFN-γ (forward) 5-CAT TGA AAG CCT AGA AAG TCT G-3, (reverse) 5-CAT TGA AAG CCT AGA AAG TCT G-3, for CD40 (forward) 5-TAG GAT CCA ATG GTG TCT TTG CCT CG-3, (reverse) 5-CTC ATG AAT CCT GTG GCA TCC A-3, (reverse) 5-CTC ATG AAT CCT GTG GCA TCC A-3. Each sample was amplified for mouse dihydrofolate reductase or β-actin to ensure equal cDNA input.

**Statistical analyses**

Each individual experiment was repeated a minimum of two times. The error bars represent the mean ± SD of replicate cultures in vitro. For in vivo experiments, error bars represent the mean ± SD of the readings from a minimum of five mice per group. In some experiments we used even eight mice per group. The significance of the difference between the mean of the control group and the mean of the experimental groups or the mean between two experimental groups was deduced by Student’s t test.

**Results**

**Differential CD40L expression regulates the cytokine production, tumor growth, and antitumor T cell response**

To assess whether different extents of CD40 ligation by a ligand such as CD40L would evoke functionally counteracting T cell responses, we cocultured B6-derived DC with T cells expressing different levels of CD40L (CD40L<sup>−/−</sup>, CD40L<sup>+/−</sup>, and CD40L<sup>+/+</sup>) (Fig. 1A) and correlated the cytokine profiles with the CD40L expression levels. We observed that with lower CD40L expression there was less production of IL-12 and IFN-γ but higher production of IL-4 and IL-10 (Fig. 1B). The T cells expressing different levels of CD40L were fixed with paraformaldehyde and were cocultured with DC from B6 mice. The coculture

**FIGURE 1.** Differential CD40L expression regulates the extent of CD40 cross-linking and CD40-induced cytokine production. A, CD40L-deficient (B6 background, CD40L<sup>−/−</sup>) mice were bred to wild-type (B6 background, CD40L<sup>+/+</sup>) mice to generate the F<sub>1</sub> (CD40L<sup>−/−</sup>) mice. The levels of CD40L expression on T cells were determined by both RT-PCR and FACS. B, The effects of the CD40L gene doses on the CD3-induced cytokine production were examined as indicated. C, p38 MAPK expression and IL-12 production by DC were assayed by coculture of CD3-specific Ab-stimulated paraformaldehyde-fixed T cells from mice expressing different levels of CD40L and B6-derived DC. The error bars represent mean ± SD. The results shown are from one of three individual experiments.

**FIGURE 2.** Differential CD40L expression regulates the tumor growth and antitumor T cell response. Mice expressing different levels of CD40L (CD40L<sup>−/−</sup>, CD40L<sup>+/−</sup>, or CD40L<sup>+/+</sup>) were s.c. injected with RM-1 cells (2 × 10<sup>5</sup>/mouse). A, The mice were then monitored for their tumor growth. The size of each tumor was assessed twice a week and was reported as the average ± SD of the tumor size in millimeters. B, In parallel experiments, the mice were sacrificed 3 wk after the tumor cell injection. The tumor from each individual mouse in each group was weighed and the average tumor weight for each group (n = 6 mice) is shown. Error bars represent mean ± SD. C, Cytokine expression in the tumor tissue; total RNA was isolated from the tumor tissue of individual animal and used for first-strand cDNA synthesis. The cDNA was then used as template for PCR amplification of mouse IL-10, IL-12, IFN-γ, and IL-4 using gene-specific primers. Results were expressed as individual data. D, Using a standard 4-h JAM test, the cytotoxic activity of the viable CD8<sup>+</sup> T cells in the splenocytes from the mice described were assessed against the [3H]thymidine-incorporated RM-1 target cells. The E/T ratio is shown. Each symbol is the mean of triplicate samples. Error bars represent mean ± SD. The results shown are from one of three individual experiments.
resulted in graded activation of p38 MAPK with higher CD40L expression with more p38 MAPK activation (Fig. 1C), suggesting that the observed alteration in T cell functions (Fig. 1B) could be due to differential triggering of CD40 signaling by T cells expressing different levels of CD40L.

Next, we examined whether the mice expressing different levels of CD40L generated functionally different effector T cell responses. The CD40L+/−, CD40L+/−, and CD40L−/− mice were injected with RM-1 cells. We observed that the mice expressing higher CD40L on their T cells had slower tumor development.

FIGURE 4. Differential CD40 expression regulates the DC activation and cytokine production. A, CD40-deficient mice (BALB/c background, CD40−/−) were bred to wild-type mice (BALB/c background, CD40+/+) to generate the F1 (CD40+/−) mice. CD40 expression profiles on DC were tested by both RT-PCR and FACS. B, The effects of gene dosages on cytokine production in CD3-specific Ab-stimulated cells were examined as indicated. C, p38 MAPK expressions and IL-12 production by DC were assayed by coculture of CD3-specific Ab-stimulated paraformaldehyde-fixed T cells from BALB/c mice. Error bars represent mean ± SD. The results shown are from one of two individual experiments.
FIGURE 5. Differential CD40 cross-linking elicits counteracting antitumor T cell responses. B6 mice were s.c. injected with RM-1 cells (2 × 10^5/mouse). The mice were sacrificed 3 wk after the tumor cell injection. A. The collected tumor sample from every individual animal of each group was weighed and the average of tumor weight in each group (n = 8 mice) is shown. Error bars represent mean ± SD of the readings from the mice in an individual group. B. Splenic T cells from five mice in the same group were cultured with RM-1 cell Ags in triplicate in 96-well plates for 48 h. Cell-free supernatants were assayed for IFN-γ production by ELISA. C. Cytokine expression in the spleen is shown. Total RNA was isolated from the splenocytes of individual animal and was used for first-strand cDNA synthesis. The cDNA was then used as template for PCR amplification of mouse IL-10, IL-12, IFN-γ, using gene-specific primers. Results are expressed as individual data. D, Using a standard 4-h JAM test, the cytotoxic activity of the viable CD8^+ T cells was assessed against [3H]thyminidine-loaded target cells (RM-1). The E:T ratio is shown. Each symbol represents the mean of triplicate samples. E, The CD8^+ T cells from the low-dose anti-CD40 (low α-CD40) and high-dose anti-CD40 Ab-treated animals were isolated. The cytotoxicity assay was set by coculturing the RM-1 target cells and the high-dose anti-CD40 CD8^+ T cells at an E:T ratio of 1:50 (0" on x-axis). In the indicated sets, low-dose anti-CD40 CD8^+ T cells were mixed with culture at the indicated number of cells, expressed as the percentage of high-dose anti-CD40 CD8^+ T cells, and the cytolytic activity was tested in a standard 4-h JAM test. Each symbol is the mean of triplicate samples. Low-dose anti-CD40 CD8^+ T cells did not show any significant cytotoxicity (data not shown). F, B6 mice were primed with tumor Ag-pulsed DC (3 × 10^5 cells/mouse) derived from the bone marrow of wild-type mice. Some of the B6 mice were treated with the indicated dose of anti-CD40 Ab during the priming with tumor Ag-pulsed wild-type DC. The mice were primed three times. Seven days after the last priming, mice were challenged with live tumor cells. Three weeks later, the mice were sacrificed and the following assays were performed. The mean tumor weight of tumor samples collected from five mice per group is shown. G, Cytotoxicity assay was performed by culturing splenocytes with irradiated tumor cells for 5 days. Later on, viable CD8^+ T cells were tested for their cytolytic activity against live tumor cells in a 4-h JAM test. The E:T ratio is shown. Each symbol represents the mean of triplicate samples. H, The CD8^+ T cells from the low (Lo-CD40) and high (Hi-CD40) doses of anti-CD40 Ab-treated animals were isolated. The cytotoxicity assay was set by coculturing the RM-1 target cells and the high-dose anti-CD40 CD8^+ T cells at an E:T ratio of 1:50 (0" on x-axis). In the indicated sets, low-dose anti-CD40 CD8^+ T cells were mixed with the above culture at the indicated number of cells, expressed as the percentage of high-dose anti-CD40 CD8^+ T cells, and the cytolytic activity was tested in a standard 4-h JAM test. Each symbol is the mean of triplicate samples. Low-dose anti-CD40 CD8^+ T cells did not show any significant cytotoxicity (data not shown). I, B6 mice were treated with the indicated dose of anti-CD40 Ab, and 24 h later the collected splenocytes were depleted of B and T cells. The resultant cells were subjected to RT-PCR analysis of cytokine expression. Results are expressed as individual data and represent one of three independent experiments. J, B6 mice were s.c. injected with RM-1 cells (2 × 10^6/mouse). The mice were i.p. injected with the indicated doses of endotoxin-free anti-CD40 mAb in 200 μl of saline on days 5, 7, and 9 after tumor cell inoculation. As indicated, some mice were coadministered 200 μg of anti-IL-10 Ab. The mice were then monitored for their tumor growth. The results shown are from one of two individual experiments.

(Fig. 2A) and less tumor weight (p < 0.001) (Fig. 2B). In accordance with the less tumor growth in CD40L^+/+ mice, the productions of IL-12 and IFN-γ (Fig. 2C), and CTL responses were higher (p < 0.001) in these mice (Fig. 2D). These observations indicate that CD40L gene dose regulates the effector antitumor T cell responses. The weaker antitumor T cell responses in CD40L^−/− and CD40L^+/− mice could be due to either lower T cell activation or differentiation of the T cells to a suppressor type that counteracted the tumor-regressing T cells.

Next, to test whether priming of the CD40L^+/+ , CD40L^−/− , and CD40L^+/− mice with tumor Ags would establish counter-effective effector T cell responses, we primed these mice by three weak injections of RM-1 Ag-pulsed, B6-derived DC. Later, the T cells were recovered from these mice and were transferred into syngenic nu/nu mice bearing tumor. It was observed that the nu/nu mice receiving CD40L^+/+ T cells showed significant inhibition in tumor growth rate (Fig. 3A) and had the smallest tumor (p < 0.001) (Fig. 3B). In accordance with the tumor growth, the nu/nu mice receiving CD40L^−/− T cells showed higher T cell proliferation (p < 0.001) (Fig. 3C), IFN-γ (p < 0.001) (Fig. 3D), and CTL responses (p < 0.001) (Fig. 3E). Interestingly, the cotransferred CD40L^+/+ and CD40L^+/− T cells inhibited the antitumor effects of CD40L^+/+ T cells in nu/nu mice (p < 0.001) (Fig. 3F). These results indicate that the effector T cells generated in CD40L^+/+ and CD40L^−/− or CD40L^+/− mice are functionally counteracting.

Differential CD40 cross-linking regulates counteracting T cell responses

To test whether differential CD40 cross-linking generates counteractive T cell responses, we cocultured CD40L^+/+ T cells with DC expressing different levels of CD40 (CD40^+/−, CD40^−/−, and CD40^+/+) (Fig. 4A) and assessed the cytokines in the culture supernatants. It was observed that the cultures with CD40^+/+ DC or CD40^+/− DC had higher IL-4 and IL-10 but less IL-12 and IFN-γ than that observed in the cultures with CD40^−/− DC (Fig. 4B). In other experiments, the T cells were stimulated overnight with anti-CD3 plus anti-CD28 to augment CD40L expression, followed by
Antibody to CD40 evokes antitumor immune responses in B6 and CD40L−/− mice. B6 and CD40L−/− mice were injected with RM-1 cells (2 × 10⁵). The mice were i.p. injected with the indicated doses of endotoxin-free anti-CD40 mAb in 200 μl of saline on days 7, 9, and 11 after tumor cell inoculation. A, The mice were monitored for their tumor growth. The size of each tumor was assessed twice a week and was reported as the average ± SD tumor size in millimeters. B, In parallel experiments, the mice were sacrificed. The collected tumor from each individual animal in each group was weighed and the average of tumor weight in each group (n = 4 mice) is shown. Error bars represent mean ± SD. C, B6 and CD40L−/− mice bone marrow-derived DC were stimulated with the indicated doses of anti-CD40 Ab for 48 h. The culture supernatant was assessed for TNF-α content by ELISA. D, The supernatants from the experiment in C were added to the RM-1 cell culture to test their antiproliferative activity. The RM-1 cell proliferation was measured by a standard [3H]thymidine ([3H]-TdR) incorporation assay. In some experiments, anti-TNF-α mAb alone or with anti-IL-10 Ab. It was observed that the mice that received the low dose of the anti-CD40 Ab had a bigger tumor than the untreated control mice (Fig. 5H). As a further control, when we added a similar number of CD8⁺ T cells from naive mice, no significant inhibition was observed (data not shown). As another control, when we added a similar number of CD8⁺ T cells from the high-dose anti-CD40 Ab recipients, we observed higher cytotoxicity (data not shown).

We also tested whether such T cell responses can be stably established as a function of the extent of CD40 ligation. We primed B6 mice by coadministering RM-1 cell lysate Ag-pulsed DC and the CD40-specific Ab (10 and 50 μg/mouse), followed by a challenge with RM-1 cells. Administration of a higher dose of the CD40-specific Ab during RM-1 Ag-pulsed DC-mediated priming resulted in a smaller tumor (p < 0.001) (Fig. 5F) and a higher CTL response (p < 0.001) (Fig. 5G), which was inhibited by the T cells from the low-dose CD40-specific Ab recipients (p < 0.001) (Fig. 5H). In addition, higher IFN-γ and IL-12 but decreased IL-10 productions were observed with increasing doses of anti-CD40 Ab (Fig. 5I). In a therapeutic model, 5 days after the injection of live RM-1 cells, the mice were treated with anti-CD40 Ab alone or with anti-IL-10 Ab. It was observed that the mice that received the low dose of the anti-CD40 Ab had a bigger tumor than the untreated control mice (Fig. 5J); the mice treated with the
higher dose of anti-CD40 Ab had a significantly smaller tumor, as compared with the control mice (p < 0.001) (Fig. 5J). In addition, the anti-IL-10 Ab coadministration not only abrogated the tumor-promoting effect of the low-dose anti-CD40 Ab treatment but also imposed a tumor-ameliorating effect onto the treatment (Fig. 5J), demonstrating the therapeutic benefit of IL-10 neutralization. The results indicate that the antitumor effect of the low-dose anti-CD40 Ab is self-limited by the CD40-induced IL-10. Our data indicate that different extents of CD40 ligation control both the amplitude and the nature of the effector T cell responses and establish functionally counteracting effector and perhaps, memory T cells.

**Anti-CD40 Ab evokes antitumor immune responses in B6 and CD40L<sup>−/−</sup> mice**

Because anti-CD40 Ab ligates CD40 on the APCs such as DC and macrophages and directly activates these cells, our results suggest that the anti-CD40 Ab may exert the antitumor effects even in CD40L<sup>−/−</sup> mice, simulating the situation observed in patients lacking CD40L or expressing nonfunctional CD40L (29). Therefore, we tested whether the anti-CD40 Ab (75 μg/mouse) would exert any antitumor effect in CD40L<sup>−/−</sup> mice. We observed that the treatment resulted in smaller tumors even in CD40L<sup>−/−</sup> mice (Fig. 6, A and B). The antitumor effect was possibly due to increased TNF-α and IL-12 production by the DC from both wild-type and CD40L<sup>−/−</sup> mice (data not shown). Indeed, CD40L<sup>−/−</sup> DC had higher TNF-α (Fig. 6C) and higher antiproliferative effect on RM-1 cells in vitro (Fig. 6D); the antiproliferative effect was prevented by TNF-α neutralization (Fig. 6E). The effect was not due to any possible direct cytoidal effect of the anti-CD40 Ab, as RM-1 cells do not express CD40 (Fig. 6E, inset). However, RM-1 cells did express TNF-α receptors (Fig. 6F) explaining the CD40-induced TNF-α-mediated antiproliferative effect on RM-1 cells.

**IL-10 regulates the CD40L-dependent T cell responses against the RM-1-induced tumor**

Because low CD40 cross-linking or less CD40L expression is associated with higher production of IL-10, a cytokine implicated in the regulation of CTL response (30), it is quite possible that higher IL-10 production during priming results in suppressed recall CTL responses. We therefore tested whether IL-10 neutralization in tumor-bearing CD40L<sup>+/+</sup> or CD40L<sup>−/−</sup> mice or during the priming of CD40L<sup>+/+</sup> and CD40L<sup>−/−</sup> mice would establish a tumor-regressing T cell response. We observed that compared with the respective untreated control mice, IL-10 neutralization reduced both tumor growth rate (p < 0.01) (Fig. 7A) and tumor weight (p < 0.01) (Fig. 7B) in CD40L<sup>+/+</sup> and CD40L<sup>−/−</sup> mice; the antitumor effect was accompanied by augmented IFN-γ production (Fig. 7C) and CTL response (Fig. 7D). These results suggest that IL-10 plays a prohibitory role in establishing antitumor recall T cell response. To test the hypothesis, these mice were primed with RM-1 lysate Ag-pulsed B6 or IL-10<sup>−/−</sup> DC. The T cells were then transferred to null mice (B6 background) and challenged with RM-1 cells 17 days later. B, The mice were monitored for their tumor growth and sacrificed when the control mice showed adverse effects from tumor, which happened three weeks after the tumor cell injection. The collected tumor sample from every individual animal of each group was weighed and the average tumor weight in each group (n = 4 mice) is shown. Error bars represent mean ± SD. C, The cytotoxic activity of the CD8<sup>+</sup> T cells was assessed by a 4-h JAM test, as described. The E:T ratio is shown. Each symbol is the mean of triplicate samples. The results shown are from one of two individual experiments.
induced antitumor immunity by impairing memory T cell generation during priming either by abrogating the clonal expansion of the Ag-specific T cells or by reducing IL-12 production. Although priming, IL-10 may anergize the T cells preventing them from responding to antigenic challenge. These anergized T cells actively suppress the other tumor-regressing T cells, a phenomenon reminiscent of infectious tolerance (39). Therefore, associating the gene dosage of a costimulatory molecule to IL-10-sensitive T cell memory serves as a basic principle of a completely preventative antitumor strategy, which may have even broader implications in infectious and autoimmune diseases.

Disclosures

The authors have no financial conflict of interest.

References


