CD40 Ligand Promotes Priming of Fully Potent Antitumor CD4⁺ T Cells in Draining Lymph Nodes in the Presence of Apoptotic Tumor Cells


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CD40 Ligand Promotes Priming of Fully Potent Antitumor CD4⁺ T Cells in Draining Lymph Nodes in the Presence of Apoptotic Tumor Cells

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The presence or absence of CD4⁺ T cell help can determine the direction of adaptive immune responses toward either cross-priming or cross-tolerance. It has been demonstrated that interactions of CD40-CD40 ligand can replace CD4⁺ T cell help and enable dendritic cells to prime cytotoxic T cells. Here, we demonstrate that antitumor reactivity induced in regional lymph nodes (LN)s by s.c. injection of CD40 ligand (CD40L)-transduced tumor (MCA205 CD40L) showed far superior therapeutic efficacy against established brain tumors of a weakly immunogenic fibrosarcoma, MCA205, when adaptively transferred. Coinjection of apoptotic, but not necrotic parental tumor cells with CD40L-expressing tumor cells caused a strong synergistic induction of antitumor reactivity in tumor-draining LNs. Freshly isolated T cells from LNs immunized with apoptotic parental tumor cells and MCA205 CD40L were capable of mediating regression of the parental tumor in vivo. In contrast, T cells derived from LNs immunized without MCA205 CD40L required ex vivo anti-CD3/IL-2 activation to elicit therapeutic activity. On anti-CD3/IL-2 activation, cells from LNs immunized with MCA205 CD40L exhibited superior per cell antitumor reactivity. An in vitro depletion study revealed that either CD4⁺ or CD8⁺ T cells could mediate therapeutic efficacy but that the antitumor efficacy mediated by CD4⁺ T cells was far superior. Cytosolic flow cytometric analyses indicated that priming of CD4⁺ cells in LNs draining CD40L-expressing tumors was polarized to the Th1 type. This is the first report that fully potent antitumor CD4⁺ T cell priming was promoted by s.c. injection of CD40L-transduced tumor in the presence of apoptotic tumor cells. The Journal of Immunology, 2001, 167: 5678–5688.

Several mechanisms have been reported to explain how tumor cells can escape from the immune surveillance mechanisms that detect and delete non-self (1–6). One of the possible mechanisms is that tumors differ antigenically only slightly from self-Ags, and therefore peripheral tolerance mechanisms are activated, although tumor cells have altered Ags recognizable by T cells. Recently, it was reported that peripheral tolerance against self somatic tissues is maintained by active cross-presentation of MHC class I-restricted Ags by dendritic cells (DCs), which results in activation induced cell death of effector T cells (7). In contrast, CD4⁺ T cell help can totally change the function of DCs, which cross-prime effector T cells, resulting in the loss of tolerance (8, 9). Thus, it is likely that regulation of the state of DCs by CD4⁺ cells acts as a switch to determine the outcome of immune responses. We and others previously demonstrated that CD4⁺ T cell function is depressed in tumor-bearing hosts and that the magnitude of suppression is correlated with the tumor burden (10, 11). Indeed, genetically modified tumor cells that secrete IFN-γ and express B7.1 and thus restore the suppressed function of CD4⁺ cells successfully enhanced the generation of antitumor effector T cells (12).

It has been well documented that the major help signals of CD4⁺ cells are provided by interactions between CD40 on DCs and CD40 ligand (CD40L) on CD4⁺ T cells (13, 14). CD40L is a 33-kDa type II membrane protein and a member of the TNF gene family and is transiently expressed on CD4⁺ T cells after TCR engagement (15). Disruption of the CD40-CD40L interaction renders mice unable to mount effective immunological responses against allo gene-transplanted tissues (16) and infectious agents (17, 18). In contrast, continuous expression of CD40L on CD4⁺ T cells leads to autoimmune disease (19).

APC can acquire soluble antigenic proteins by endocytosis. However, it has been demonstrated DCs engulf dying cells and cell fragments that contain antigenic protein via an active process mediated by receptors on cell membranes such as CD36 and αβ2 (20). Moreover, antigenic proteins engulfed as dead cells are 1,000–10,000 times more efficient in generating MHC-peptide complexes than the same proteins administered as a soluble Ag pulse (21). Thus, it has been believed that the major sources of Ags are dead cells themselves. Several reports showed that DCs acquire Ags from apoptotic but not from necrotic cells to cross-prime T cells in regional lymph nodes (LNs) (22, 23). In contrast, other reports demonstrated that Ags derived from apoptotic cells induce tolerance rather than positive immune responses and that necrotic cells act as danger signals to promote cross-priming (24–27). Thus, it is still uncertain whether apoptotic or necrotic tumor cells

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‡ Abbreviations used in this paper: DC, dendritic cell; LN, lymph node; CM, complete medium; m-, murine; CD40L, CD40 ligand; HSV-4k, HSV thymidine kinase; BM-DC, bone marrow-derived DC.
are superior for cross-priming to induce antitumor immune responses. To determine whether apoptotic or necrotic tumor cells are the better source of tumor-associated Ag in the presence of CD40L-transduced cells as CD4+ T cell help, we tested γ-ray-irradiated tumor cells, thymidine kinase-transduced tumor cells treated with ganciclovir and tumor cells treated by hypotonic shock. In this report, we show that the antitumor reactivity induced in regional LNs was enhanced both quantitatively and qualitatively by tumors genetically modified to express CD40L (MCA205 CD40L), and that the enhanced antitumor efficacy was mediated by CD4+ T cells. Furthermore, we found that only apoptotic tumor cells had a synergistic effect with CD40L-expressing tumor to induce antitumor efficacy in draining LNs.

Materials and Methods

Mice

Female C57BL/6j (B6) mice were purchased from CLEA Laboratory (Tokyo, Japan). They were maintained in a specific pathogen-free environment and were used for experiments at the age of 8–10 wk.

Tumors

MCA205 and MCA102 are antigenically distinct fibrosarcomas of B6 origin induced by i.m. injection of 3-methylcholanthrene (28). Single-cell suspensions were prepared from solid tumors by enzymatic digestion as described previously (29). An MCA205 tumor cell line was established and maintained in vitro.

Expression vectors

The eukaryotic cDNA expression vector BCMGSNeo, conferring neomycin resistance (NeoR), was kindly supplied by Dr. H. Karasuyama (Basel Institute for Immunology, Basel, Switzerland) (30). The cDNA encoding murine CD40L (mCD40L) was kindly supplied by Dr. A. Shimpil (Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany) (31). The cDNAs encoding mCD40L and the HSV thymidine kinase (HSV-tk) were introduced into the XhoI and NorI site of BCMGSNeo.

Gene transfection

BCMGSNeo, mCD40L cDNA-containing BCMGSNeo plasmids were transfected into MCA205 tumor cells using the lipofectin reagent (Life Technologies, Gaithersburg, MD). MCA205 tumor cells were plated in a 100-mm tissue culture dish in RPMI 1640 supplemented with 10% heat-inactivated FCS and cultured until the cells were 40–60% confluent. The lipofectin–DNA complexes were overlaid onto the cells for a 12-h incubation period at 37°C in a CO2 incubator. After the DNA-containing medium was replaced with RPMI 1640 containing 10% FCS, cells were incubated for an additional 48 h. The transfectants MCA205 CD40L and MCA205 HSV-tk were selected by supplementation of the medium for 14 days with 400–1000 μg/ml of the neomycin analogue G418 (Life Technologies).

These genetically modified tumor cells were maintained as monolayer cultures in complete medium (CM). CM consists of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (all from Life Technologies) and 5 × 10−5 M 2-ME (Sigma, St. Louis, MO).

mAbs and flow cytometry

Hybridomas producing mAbs against the murine CD3ε chain (145-2C11), CD4 (GK1.5, L3T4), CD8 (2.43, Lyt-2), and CD62L (MEL14) were obtained from the American Type Culture Collection (Manassas, VA). Anti-CD3 mAb was harvested as a supernatant of an in vitro culture of hybridoma cells and then partially purified by 50% ammonium sulfate precipitation, and the IgG content was determined by ELISA. Anti-CD4 mAb, anti-CD8 mAb, and anti-CD62L mAb were produced as ascites fluid from sublethally irradiated (500 Gy) DBA/2 mice. FITC-conjugated anti-CD4 (L3T4), PE-conjugated anti-CD8 (Lyt-2), PE-conjugated anti-H-2Kk (AF6-88.5), PE-conjugated anti-I-Ak (AF6-120.1), PE-conjugated anti-B7.1 (16-10A), PE-conjugated anti-B7.2 (GL1), FITC-conjugated anti-Thy-1.2 (30-H12), PE-conjugated anti-CD11b (M1/70), FITC-conjugated anti-CD11c (HL3), PE-conjugated anti-CD62L (MEL-14), and PE-conjugated anti-CD40L (MR1) were purchased from BD PharMingen (San Diego, CA). Cell surface phenotypes were analyzed by direct immunofluorescence staining of 0.5–1 × 106 cells with conjugated mAbs. In each sample, 10,000 cells were analyzed using a FACScan flow microfluorometer (BD Biosciences, Sunnyvale, CA). PE-conjugated subclass-matched Abs used as isotype controls (M5/14.15.2, G155-178, 2C11) were also purchased from BD PharMingen.

Cytokine ELISA

Two million cells were stimulated with immobilized anti-CD3 mAb for 24 h in a 24-well plate containing 2 ml CM. Supernant was harvested and assayed for IL-4 or IFN-γ content by a quantitative “sandwich” ELISA using a mIL-4 ELISA kit (Endogen, Boston, MA) or a mIFN-γ ELISA kit (Genzyme, Cambridge, MA).

Induction of apoptosis or necrosis

To induce apoptosis, MCA205 tumor cells were irradiated at 50 Gy using a cesium irradiator (Rx205), and MCA205 HSV-tk cells were incubated in 10 μM ganciclovir for 24 h at 37°C (TK205). Apoptotic death was confirmed using an apoptosis detection kit (Sigma). Cells were stained with annexin V–FITC and propidium iodide. Early apoptosis was defined by annexin V+/propidium iodide− staining as determined by FACScan (BD Biosciences) analysis. Necrosis was induced by incubating cells in HzO for 30 min at 37°C (Hypo205), or treating cells with a repeated freeze and thaw technique using liquid nitrogen (F&T205) after which all the cells incorporated trypan blue.

Immunization models

B6 mice were immunized intradermally in the left flank with 1 × 107 Rx205, Hypo205, or F&T205 in the presence or absence of 1 × 106 MCA205 CD40L. Twenty-one days after immunization, these mice were inoculated s.c. on the midline of the abdomen with 3 × 105 MCA205 tumor cells or 1 × 106 MCA102 tumor cells. Diameters of skin tumors were measured twice weekly with a caliper, and size was recorded as the average of two perpendicular diameters. The number of mice with tumor growth was counted.

Tumor-draining LN cells

B6 mice were inoculated s.c. with 1 × 106 dying MCA205 tumor cells with or without 1 × 106 MCA205 CD40L on bilateral flanks. Inguinal LNs draining dying tumor cells admixed with MCA205 CD40L were harvested 7 days after s.c. tumor inoculation, and LNs draining only dying tumor cells were harvested 12 days after s.c. tumor inoculation. Single-cell suspensions were prepared mechanically as described previously (29).

Anti-CD3/IL-2-2 activation

The LN cells were activated in vitro by incubating 3–4 × 106 cells in a 24-well plate precoated with anti-CD3 mAb containing 2 ml CM. After 2 days of incubation at 37°C in a 5% CO2/95% air atmosphere, activated cells were harvested, washed, and further cultured at a concentration of 2 × 107/ml in CM containing 40 U/ml IL-2 for 3 days.

Fractionation of T cells

T cells in the LN cell suspension were concentrated by passing through nylon wool columns (Wako Pure Chemical Industries, Osaka, Japan). After a 45-min incubation at 37°C, the first 15 ml passsthrough fraction contained 90–95% T cells. Purified T cells were further fractionated into two subpopulations based on the expression of CD62L. Cells were first incubated for 30 min at 4°C with MEL14 hybridoma ascites fluid at a 1/1000 dilution. The cells were then washed free of unbound Ab. In all, 6–8 × 107 cells in 12 ml CM were plated in a T-75 flask which was precoated with goat anti-rat Ig Ab (Jackson ImmunoresearchLaboratories, West Grove, PA). After 1 h incubation at 4°C, nonadherent cells were collected by gentle rocking. These cells were then treated with sheep anti-rat Ig Ab-coated DynaBeads M-450 (Dynal, Oslo, Norway) at a 4:1 bead-cell ratio, according to the manufacturer’s instructions, to yield highly purified (>90%) cells that down-regulated CD62L expression (CD62Llow). In some experiments, CD62Llow cells were further separated into CD4+ and CD8+ cells by using magnetic beads as described previously (32).

Adoptive immunotherapy

B6 mice were inoculated intracranially in the right hemisphere with 0.8–1 × 106 MCA205 tumor cells in 10 μl HBSS to establish brain metastases (33). Three days after tumor inoculation, mice were sublethally irradiated (500 Gy) and then infused i.v. with effector T cells. Mice were followed for evidence of intracerebral tumor growth, and survival time was recorded. The significance of differences of survival time between groups

The Journal of Immunology 5679

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Bone marrow-derived DCs and cytokine ELISA

DCs were obtained from bone marrow precursors by 6-day culture in CM containing 10 ng/ml mGM-CSF (gift from KIRIN, Tokyo, Japan) and 10 ng/ml mIL-4 (Serotec, Oxford, U.K.). After culturing, flow cytometric analyses revealed that the bone marrow-derived cells contained ~80% CD11c<sup>+</sup>CD11b<sup>+</sup> cells and 20% CD11c<sup>-</sup>CD11b<sup>-</sup> cells. Two million bone marrow-derived DCs (BM-DCs) were stimulated with 2 × 10<sup>6</sup> MCA205 tumor cells, MCA205 CD40L tumor cells or 10 ng/ml LPS (Sigma) for 24 h in 2 ml CM in 24-well plates at 37°C. Supernatants were harvested, and the concentration of IL-12 was measured using a mIL-12 ELISA kit (BioSource International, Camarillo, CA) according to the manufacturer’s instructions.

Cytoplasmic cytokine FACS

After 5 h stimulation with 500 ng/ml ionomycin and 50 ng/ml PMA in the presence of brefeldin A, cells were stained with perCP-conjugated anti-CD4 (GK1.5) mAb and then treated with 1% paraformaldehyde. Cytoplasmic cytokines were double-stained with FITC-labeled anti-IL-4 (11B11), and PE-conjugated anti-IFN-γ (XMG1.2) mAb. Cells were analyzed by flow cytometry with a FACScan immediately.

Results

MHC class I and II Ag, costimulatory molecule expression, and IL-12 production of BM-DC cocultured with MCA205 CD40L tumor cells

To establish tumor cells that constitutively express CD40L, MCA205 tumor cells were transfected with cDNA encoding murine CD40L using the lipofection method. Flow cytometric analysis of MCA205 CD40L revealed strong expression of CD40L (Fig. 1).

To verify the bioactivity of CD40L expressed on MCA205 CD40L, the phenotype and cytokine production of BM-DCs was analyzed after coculture with the tumor cells. Immature BM-DCs expressed low levels of B7.1, B7.2, and MHC class II Ag before stimulation. After the 48-h coculture with MCA205 CD40L, a subpopulation with increased expression of B7.1, B7.2, and MHC class II Ag was observed, whereas no up-regulation of MHC class I or II Ag or of costimulatory molecules on BM-DCs was observed with parental MCA205 tumor cells (Fig. 2). Immature BM-DCs produced a small amount of IL-12 without stimulation. Coculturing with MCA205 CD40L tumor cells resulted in a marked increase of IL-12 production (Fig. 3).

Kinetics analysis of LNs draining MCA205 CD40L tumor cells

It is well documented that s.c. injected Ags are ferried to draining LNs by DCs to prime T lymphocytes (34, 35). Although many molecules can affect DC migration (36–41), the interactions of CD40 and CD40L play a central role in regulating the migration of Ag-bearing DCs (42). To test the hypothesis that priming of T lymphocytes in LNs draining CD40L-expressing tumors is accelerated by enhancing the migration capacity of DCs, we examined inguinal LNs every 3 days after s.c. infections of tumor cells on bilateral flanks. MCA205 CD40L tumors injected s.c. grew once to form nodules but disappeared by the 14th day after injection (data not shown).

The increase of LN cells was accelerated with CD40L stimulation (Fig. 4). The number of LN cells reached its peak 7 days after s.c. tumor inoculation, with ~26 × 10<sup>6</sup> cells per LN, when irradiated MCA205 tumor cells were injected with MCA205 CD40L, whereas the number of cell in LNs draining only irradiated parental MCA205 tumor cells reached its peak, with ~17 × 10<sup>6</sup> cells per LN, 12 days after s.c. tumor injection.

Previously, we reported down-regulation of the LN homing molecule CD62L, which serves as a surrogate marker for tumor

![FIGURE 1. CD40L expression on gene-transduced MCA205 tumor cells. Tumor cells were stained with PE-conjugated anti-CD40L (MR1) mAb. CD40L expression was analyzed by flow cytometry. A total of 10<sup>6</sup> cells were analyzed for each sample. Each frame consists of 10,000 cells.](http://www.jimmunol.org/)

![FIGURE 2. MHC class I, MHC class II, B7.1, and B7.2 expression of BM-DCs. Two million BM-DCs were cultured with 2 × 10<sup>6</sup> MCA205 CD40L or MCA205 tumor cells for 24 h in 2 ml CM in 24-well plates. Cells were double-stained with FITC-labeled anti-CD11c (HL.3), and PE-conjugated anti-I-<A><sup>+</sup></A> (AF6-120.1), PE-conjugated anti-H-<K><sup>+</sup></K> (AF6-88.5), PE-conjugated anti-B7.1 (16-10A), PE-conjugated anti-B7.2 (GL1), or PE-conjugated isotype control Abs. CD11c<sup>-</sup> cells were gated for analyses. A total of 10<sup>5</sup> cells were analyzed for each sample. Thin lines indicate fluorescence intensity of BM-DCs stained with PE-conjugated subclass matched isotype control Abs; thick lines indicate fluorescence intensity of cells stained with indicated PE-conjugated Ab. Each frame consists of 10,000 cells.](http://www.jimmunol.org/)
Thus, to elucidate whether T cell priming with Ags is facilitated in LNs draining CD40L-transduced tumor cells, we examined the percentage of CD62Llow T lymphocytes among the total Thy-1.2^+ cells in draining LNs. The percentage of CD62Llow T lymphocytes draining irradiated tumor cells admixed with CD40L-expressing tumor cells increased up to 26.5% by the 7th day after tumor inoculation, whereas the percentage of CD62Llow T lymphocytes reached 18% on the 12th day when draining only irradiated tumor cells.

### Potent antitumor immunity is induced by s.c. injection of apoptotic tumor cells admixed with MCA205 CD40L tumor cells

To examine whether protective immunity was induced by s.c. injection of MCA205 CD40L, mice immunized by s.c. tumor cell injection were challenged with 3 × 10^6 parental MCA205 tumor cells. Contrary to our expectations, MCA205 CD40L alone could not induce protective immunity against the parental tumor (Fig. 5). It has been reported that the level of Ags in peripheral tissues must be relatively high for them to be cross-presented by DCs. It is likely that macrophages activated by inflammatory cytokines scavenged tumor Ags (44, 45). Thus, we speculated that the dose of tumor Ags had been insufficient for cross-priming and was ignored. To test this hypothesis, we immunized mice with MCA205 CD40L admixed with 1 × 10^7 apoptotic or necrotic MCA205 tumor cells. Tumor cell apoptosis was induced by γ-ray irradiation at 50 Gy, and necrotic cell death was induced by either incubating MCA205 tumor cells in H_2O for 30 min at 37°C or treating with repeated freeze-thawing. Apoptosis was confirmed by external phosphatidylserine exposure (Fig. 6). All of mice immunized with MCA205 CD40L and apoptotic MCA205 tumor cells rejected parental tumor cells challenge, whereas no protective immunity was observed in mice immunized with MCA205 CD40L and necrotic tumor cells (Fig. 5a, Table I). To determine whether the induced protective immunity is tumor specific, mice immunized with irradiated MCA205 tumor cells admixed with MCA205 CD40L tumor cells were challenged with 1 × 10^6 MCA102 tumor cells. No difference was observed in skin tumor growth between mice without immunization and mice immunized with Rx205/MCA205 CD40L (Fig. 5b).

### CD62Llow T cells freshly isolated from LNs draining apoptotic tumor cells admixed with MCA205 CD40L have potent antitumor reactivity

We previously demonstrated that T cells primed in LNs draining a weakly immunogenic tumor alone could not elicit therapeutic activity without ex vivo activation (29, 33, 43, 46). To elucidate whether Ag-primed LN T cells achieved qualitative difference by...
immunization with CD40L-expressing tumor cells, the therapeutic efficacy of freshly isolated CD62Llow T cells from tumor-draining LNs was examined (Fig. 7). We harvested LNs draining Rx205, TK205, Rx205/MCA205 CD40L, TK205/MCA205 CD40L, or Hypo205/MCA205 CD40L. Because our data from the kinetics study showed that T cell priming in regional LNs immunized with MCA205 CD40L was accelerated, we examined the therapeutic efficacy of LN cells on the days when the total number of cells and

FIGURE 5. Challenge of mice with MCA205 tumor cells after immunization with $1 \times 10^6$ apoptotic or necrotic tumor cells in the presence or absence of $1 \times 10^8$ MCA205 CD40L tumor cells or mock transfectant tumor cells. a. Mice were inoculated s.c. with $3 \times 10^6$ parental MCA205 tumor cells 21 days after immunization. b. Mice immunized with Rx205 and MCA205 CD40L were challenged with $1 \times 10^6$ MCA102 tumor cell inoculation. Age-matched naive mice were used as a control.

FIGURE 6. Apoptosis was induced by γ-ray irradiation of MCA205 tumor cells (e) or in vitro GCV treatment of MCA205 HSV-tk cells (d). Necrotic cell death was achieved by incubating MCA205 tumor cells in H$_2$O$_2$ (b) or treating with repeated freeze-thawing (c). Untreated MCA205 tumor cells were stained as a control (a). Cells were stained with FITC-conjugated annexin V and propidium iodide (Sigma). Early apoptotic cells are defined as the annexin V$^+$, propidium iodide$^-$ population.
There was no significant difference between the group that received cells derived from LNs draining Rx205 and the no-treatment group. In experiment 2, established intracranial MCA205 metastases were infused i.v. with $10^6$ purified CD62Llow T cells freshly isolated from LNs draining Rx205 and MCA205 CD40L. The purity of both Thy-1.2+ and CD62Llow cells as assessed by flow cytometric analyses was ~95% (data not shown). Purified cells were adoptively transferred to 3-day established brain tumor-bearing mice. In experiment 1, the survival of the group that received CD62Llow T cells from LNs draining Rx205 and MCA205 CD40L was significantly ($p < 0.01$) superior to that of all other groups. There was no significant difference between the no-treatment group and the group that received cells derived from LNs draining Rx205. In experiment 2, T cells from LNs immunized with TK205 and MCA205 CD40L exhibited significantly ($p < 0.01$) superior therapeutic efficacy. There was no significant difference between the no-treatment group and the group that received cells from LNs draining Hypo205 with MCA205 CD40L, thus indicating that T cells with fully potent effector functions are induced by immunization with apoptotic tumor cells and CD40L-expressing tumor cells. CD62Lhigh LN T cells that were positively isolated by panning showed no antitumor reactivity (data not shown).

Activated LN cells immunized with apoptotic tumor cells and MCA205 CD40L can mediate antitumor reactivity when adoptively transferred into 3-day established brain tumor-bearing mice

We have reported that LNs draining a growing tumor are an excellent source of tumor-sensitized T cells. Tumor-draining LN cells readily acquire therapeutic efficacy after ex vivo activation, even though freshly isolated LN cells exhibit no antitumor reactivity (29, 33, 43, 46). To examine the antitumor reactivity induced in tumor draining LNs on ex vivo activation, cells from LNs draining s.c. MCA205 CD40L, Rx205, Rx205/MCA205 CD40L, Hypo205/MCA205 CD40L, or live MCA205 tumors were adoptively transferred after ex vivo activation by the anti-CD3/IL-2 method. There was no significant difference of cell proliferation among these cells during culture. In experiment 1, $2 \times 10^7$ activated cells were infused i.v. into mice bearing 3-day established brain tumors, whereas in experiment 2, $1 \times 10^7$ activated cells were infused. Although activated T cells derived from LNs draining Rx205-mediated antitumor reactivity, the therapeutic efficacy of LN cells immunized with Rx205 and MCA205 CD40L was significantly ($p < 0.03$ in experiment 1; $p < 0.05$ in experiment 2) superior (Fig. 8). In experiment 1, all of the mice infused with activated LN cells immunized with Rx205 and MCA205 CD40L survived, whereas two of five mice that received activated cells from LNs draining live MCA205 tumor cells died of brain tumors. There was no significant difference between the no-treatment group and the group that received activated LN cells immunized with Hypo205 and MCA205 CD40L.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** Antitumor effects of freshly isolated CD62Llow T cells from LNs draining apoptotic tumor cells with MCA205 CD40L. Mice bearing 3-day established intracranial MCA205 metastases were infused i.v. with $2 \times 10^6$ purified CD62Llow T cells freshly isolated from LNs draining Rx205, TK205, or Hypo205 with or without MCA205 CD40L or mock transfectant tumor cells after sublethal whole body irradiation. In experiment 1, the survival of the group that received CD62Llow T cells from LNs draining Rx205 and MCA205 CD40L was significantly ($p < 0.01$) different from that of all other groups. There was no significant difference between the group that received cells derived from LNs draining Rx205 and the no-treatment group. In experiment 2, the survival of the group that received T cells from LNs draining TK205 and MCA205 CD40L was significantly ($p < 0.01$) different from that of all other groups. The survival of the group that received cells from LNs draining TK205 was significantly ($p < 0.05$) different from that of the no-treatment group. There was no significant difference between the group that received cells from LNs draining Hypo205 with MCA205 CD40L and the no-treatment group.

The Journal of Immunology

5683

Table I. Challenge of mice with parental MCA205 tumor cells after immunization with apoptotic or necrotic MCA205 tumor cells in the presence or absence of MCA205 CD40L tumor cells

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<tbody>
<tr>
<td>No treatment</td>
<td>5/5</td>
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CD4⁺CD62Llow T cells derived from LN draining apoptotic tumor cells with MCA205 CD40L can mediate potent antitumor reactivity

To identify the T cell subset population(s) with potent antitumor efficacy induced in LNs draining apoptotic tumor cells and MCA205 CD40L, we depleted either the CD4⁺ or CD8⁺ T cell subset population in vitro with magnetic beads coated with mAb after T cell enrichment with nylon wool columns. In experiment 1, 2 × 10⁶ LN cells immunized with Rx205 and MCA205 CD40L tumor cells were isolated and activated i.v. to 3-day brain metastasis-bearing mice. Although CD8⁺CD62Llow T cells elicited significant (p < 0.05) prolongation of survival, the antitumor efficacy of CD4⁺CD62Llow T cells was even greater (Fig. 9). In experiment 2, 1 × 10⁶ CD4⁺CD62Llow T cells derived from LN immunized by Rx205 with or without MCA205 CD40L were adoptively infused into 3-day brain metastasis-bearing mice. Although CD4⁺ T cells from LN immunized with Rx205 alone showed no therapeutic efficacy, CD4⁺ T cells from LNs immunized with Rx205 and MCA205 CD40L-mediated significant (p < 0.02) antitumor reactivity.

Th1 CD4⁺ cell priming is promoted in LNs draining apoptotic tumor cells admixed with CD40L-transduced tumor

To determine the cytokine production profile of CD4⁺CD62Llow T cells derived from LNs draining MCA205 CD40L, we performed flow cytometric analysis of cytotoxic cytokines. CD4⁺CD62Llow T cells derived from LNs draining Rx205/MCA205 CD40L contained 6.5% IFN-γ⁺ cells, whereas cells from LNs draining Rx205 contained only 1.4% IFN-γ⁺ cells (Fig. 10). No IL-4⁺ cells were observed (data not shown). CD4⁺CD62Llow T cells derived from LNs draining Rx205/MCA205 CD40L produced 4 times more IFN-γ than cells from LNs draining Rx205 when stimulated with immobilized anti-CD3 mAb (Fig. 11), whereas both types of cells produced almost the same amount of IL-4 on stimulation.

Discussion

Classically, antitumor T cells are thought to be primed by tumor cells themselves by recognizing peptides expressed on cell surfaces as complexes with class I MHC Ag. However, in many kinds of systems, including immune responses against virus-infected cells, transplanted allogeneic tissues and tumor cells, it has been demonstrated that class I-restricted Ags as well as MHC class II-restricted Ags are acquired and presented after processing by professional APCs such as DCs (34). It is now believed that cross-presentation in secondary lymphoid tissues plays a crucial role in regulating adaptive immune responses.

Immature DCs capture Ags and then migrate to regional LNs to present Ags to T cells. However, they express minimal amounts of surface activation molecules (47). Ag presentation to naive T cells, in the absence of costimulatory molecules, leads to anergy of T cells (48). Moreover, cross-presentation by DCs without CD4⁺ T cell help results in peripheral deletion of Ag-specific T cells in regional LNs (7). In contrast, in the presence of CD4⁺ T cell help, DCs totally change their functions to cross-prime T cells, resulting in clonal expansion of effector T cells (9). This CD4⁺ T cell help can be replaced with CD40-CD40L interactions (13, 49).

Our data have shown that the antitumor efficacy induced in LNs draining apoptotic tumor cells was greatly enhanced by coinjection of CD40L-transduced tumor cells both quantitatively and qualitatively. Freshly isolated T cells from LNs draining a weakly immunogenic fibrosarcoma, the MCA205 tumor, did not demonstrate any detectable antitumor reactivity against visceral organ metastasis models without ex vivo activation by agents such as anti-CD3/IL-2 or staphylococcal enterotoxin A/IL-2 (29, 33, 43, 46). Surprisingly, T cells derived from LNs immunized with MCA205 CD40L and apoptotic MCA205 tumor cells did not require ex vivo activation to elicit therapeutic efficacy (Fig. 7). Moreover, on activation, LN cells immunized with MCA205 CD40L and apoptotic
tumor cells showed per cell therapeutic efficacy superior to that of LN cells immunized with apoptotic tumor cells alone (Fig. 8).

Either CD4$^+$ or CD8$^+$ T cells freshly isolated from LNs immunized with Rx205 and MCA205 CD40L tumor cells could mediate tumor regression. However, the antitumor reactivity of CD4$^+$ cells was far superior to that of CD8$^+$ cells when adoptively transferred (Fig. 9). We previously reported that CD4$^+$CD62L low T cells derived from LNs draining a growing MCA205 tumor can mediate marked antitumor reactivity on ex vivo activation (32). Two million of those cells were sufficient to cure 3-day established brain tumor. In this study, 2 × 10^6 CD4$^+$CD62L low T cells freshly isolated from LNs immunized with Rx205/MCA205 CD40L cured four of five mice bearing 3-day established brain metastases; therefore, the therapeutic efficacy was almost equivalent to that of activated CD4$^+$CD62L low T cells from LNs draining a growing MCA205 tumor. Thus, it seems that cross-priming by APCs that received help signals through CD40L-CD40 interaction resulted in induction of antitumor CD4$^+$ cells with full effector functions.

Because MCA205 tumor cells do not express MHC class II molecules, it is unlikely that transferred CD4$^+$ T cells can directly recognize tumor cells. Our data showed that neither 2 × 10^6 CD4$^+$ nor CD8$^+$CD62L low T cells derived from LNs were sufficient to cure all the mice. However, mice infused with 2 × 10^6 CD62L low T cells consisting of 1 × 10^6 CD8$^+$ and 1 × 10^6 CD4$^+$ cells successfully cured all of the mice (Fig. 9). In vivo depletion of CD8$^+$ cells with anti-CD8 mAb partially abrogated the antitumor efficacy of transferred CD4$^+$ cells (data not shown). It is well known that DCs that have received help signals from CD4$^+$ cells...
have the capacity to induce and maintain the CTL function of Ag-primed CD8\(^+\) cells. It is likely that synergistic antitumor effects of CD4\(^+\) and CD8\(^+\) cells are mediated by activation of APCs. Recently, it was shown using bone marrow chimeric mice that antitumor CD4\(^+\) cells could mediate regression of established tumors in vivo without direct tumor recognition (50). It was suggested that transferred CD4\(^+\) T cells that recognized Ags cross-presented on DCs secreted proinflammatory cytokines and that activated macrophages and NK cells directly mediated tumor regression. Thus, multiple killing mechanisms involving CD8\(^+\) CTL work in the presence of APCs when fully potent antitumor CD4\(^+\) cells are adoptively transferred, and that is why CD4\(^+\) T cells much more efficiently mediate antitumor reactivity.

As we have shown, DCs stimulated with CD40L express high levels of costimulatory molecules and MHC class II Ag (Fig. 2). On the other hand, CD40L is transiently expressed on CD4\(^+\) T cells after TCR engagement with complexes consisting of antigenic peptides and MHC class II Ag on DCs. Thus, there is a positive feedback mechanism between DCs and CD4\(^+\) T cells, in which CD40-CD40L interactions play an essential role. Indeed, our data have shown that use of CD40L-expressing tumor as the help of CD4\(^+\) cells resulted in induction of antitumor CD4\(^+\) T cells in draining LNs, which could mediate antitumor efficacy by activating APCs. It is likely that this activation loop involving APCs and CD4\(^+\) T cells orchestrates immune responses by regulating the activation state of APCs carrying specific Ags.

A recent study indicated that signaling through CD40 plays an important role not only in maturation but also in inducing the migration ability of DCs to regional LNs (42). Our findings that the total cell number in LNs draining irradiated MCA205 tumor cells admixed with MCA205 CD40L increased much earlier than that in LNs draining irradiated tumor cells alone supports this conclusion (Fig. 4). Moreover, the percentage of CD62L\(^{low}\) T cells also increased earlier in LNs draining CD40L-transduced tumor cells. CD62L is expressed on naive T cells at a high level to maintain the homing capacity to secondary lymphoid tissues. Naive T cells that encounter specific Ags presented on APCs immediately down-regulate their CD62L expression; hence, CD62L\(^{low}\) is a surrogate marker of primed T cells (51). We reported that all the antitumor reactivity in tumor-draining LNs exclusively resided in the small subpopulation with down-regulated CD62L expression (43). Thus, we can conclude that priming of effector T cells with tumor-associated Ags is accelerated in LNs immunized with CD40L-transduced tumors.

Our data have shown that apoptotic but not necrotic tumor cells synergistically induce antitumor reactivity when coinjected with CD40L-transduced tumor cells (Fig. 5). Although freshly isolated LN cells immunized with MCA205 CD40L and apoptotic tumor cells showed potent therapeutic efficacy, LN cells immunized with MCA205 CD40L and necrotic tumor cells failed to mediate antitumor reactivity with or without ex vivo activation (Figs. 7 and 8). The synergistic effects were elicited by apoptotic tumor cells in our system because in the presence of CD40L as CD4 help, DCs require an Ag source but no longer require stimulatory signals. It is possible that early apoptotic cells that proceeding toward programmed death have preserved Ags. In contrast, necrosis induced by tissue distortion or nutritional starvation results in denatured proteins, which are good as danger signals but not suitable as an Ag source. We used two methods, freeze and thaw technique or hypotonic shock, to induce necrosis. In the presence of CD40L-expressing tumor, necrotic tumor cells induced by these particular technique failed to provide tumor Ag to APCs. Thus, it is likely that DCs that are undergoing maturation to become immunostimulatory APCs via CD40-CD40L interaction capture Ags mostly from apoptotic tumor cells but not from necrotic cells.

Both irradiated MCA205 tumor cells and MCA205 HSV-tk treated with ganciclovir in vitro synergistic effects with CD40L-expressing tumors on induction of tumor-sensitized T cells in tumor-draining LNs; however, MCA205 HSV-tk was not as effective as RxA205. Moreover, when s.c. inoculated MCA205 HSV-tk was treated with GCV in vivo, no antitumor reactivity was effective as Rx205. Moreover, when s.c. inoculated MCA205 HSV-tk was treated with GCV in vivo, no antitumor reactivity was effective as Rx205. Moreover, when s.c. inoculated MCA205 HSV-tk was treated with GCV in vivo, no antitumor reactivity was effective as Rx205.

Although apoptotic tumors admixed with MCA205 CD40L successfully induced antitumor reactivity, inoculation of MCA205
CD40L tumor cells was not sufficient for induction of specific antitumor reactivity against the parental tumor. There seem to be three possible reasons why s.c. injection of MCA205 CD40L alone could not evoke adaptive immune responses against parental MCA205 tumor cells: 1) the cloned MCA205 CD40L tumor has already lost all the tumor-associated Ags that are shared with parental tumor cells. However, this explanation is unlikely because T cells derived from LN-draining parental MCA205 tumor cells secreted IFN-γ when cocultured with MCA205 CD40L (data not shown); 2) the MCA205 CD40L tumor is killed by activated macrophages and NK cells before it grows to provide sufficient tumor-associated Ags, because DCs and macrophages stimulated with CD40L secrete cytokines, which activate macrophages and NK cells. The activated macrophages then scavenge tumor Ags, so that DCs cannot acquire sufficient amounts of tumor Ags to prime T cells in regional LNs. This explanation is compatible with the previous report that cross-presentation requires relatively high doses of Ags (44, 52); 3) immediately after tumor inoculation, MCA205 CD40L activates maturation of DCs; however, matured DCs do not have the ability to capture Ags. Thus, sufficient tumor Ags are secreted at the time when MCA205 CD40L tumor cells are inoculated. In contrast, irradiated tumor cells alone failed to evoke adaptive immune responses against specific Ags by localization. Indeed, immunization by s.c. injection of MCA205 CD40L-deficient tumor cells show deficient in antiviral immunity and have an impaired memory CD8+ T cell response. J. Exp. Med. 183:2129.

This is the first report that antitumor CD4+ T cells that have potent therapeutic efficacy when adoptively transferred are induced by immunization with CD40L-transduced tumor and apoptotic tumor cells. Because autoimmune responses are evoked by continuous expression of CD40L on CD4+ T cells that is independent of TCR engagement, continuous stimulation of DCs via CD40 with anti-CD40 mAb or soluble form CD40L has the risk of unexpected activation of immune responses. In contrast, membrane-bound type CD40L is applicable to enhance immune responses against specific Ags by localization. Indeed, immunization by s.c. injection of CD40L-transduced tumor cells did not make any difference in cell number or composition of counterlateral inguinal LNs (data not shown). Thus, membrane-bound type CD40L is promising adjuvant for clinical immunotherapy for cancer.

References

Migration of Langerhans cells and dermal dendritic cells in skin organ cultures: augmentation by TNF-α and IL-1β. J. Leukocyte Biol. 66:462.


