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Simultaneous Targeting of CD3 on T Cells and CD40 on B or Dendritic Cells Augments the Antitumor Reactivity of Tumor-Primed Lymph Node Cells

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To date, molecular targets chosen for Ab activation to generate antitumor effector cells have been confined on T cells, such as TCR/CD3, CD28, CD137 (4-1BB), CD134 (OX40), and inducible costimulator. In this report we investigated the immune function of murine tumor-draining lymph node (TDLN) cells after simultaneous Ab targeting of CD3 on T cells and CD40 on APCs. Anti-CD3 plus anti-CD40-activated TDLN cells secreted significantly higher amounts of IFN-γ, but less IL-10, compared with anti-CD3-activated cells. In adoptive immunotherapy, ligation of CD3 and CD40 resulted in the generation of more potent effector cells in mediating tumor regression. Freshly harvested TDLN cells were composed of ~60% CD3+ T cells, 30–35% CD19+ B cells, 5% CD11c+ dendritic cells (DC), and few CD14+ or NK cells (each <3%). CD40 was distributed predominantly on B cells and DCs. Cell depletion indicated that simultaneous targeting was toward CD3 on T cells and CD40 on APCs, respectively. Elimination of APCs completely abrogated the augmented antitumor responses induced by anti-CD40. Either B cell or DC removal partially, but significantly, reduced the therapeutic efficacy conferred by CD40 engagement. Furthermore, the immunomodulation function of anti-CD40 was associated with its capability to increase IL-12 secretion while inhibiting IL-4 production. Our study establishes a role for CD40 expressed on B cells or DCs in the costimulation of TDLN cells. Eliciting antitumor activity via simultaneous targeting of CD3 on T cells and CD40 on APCs is relevant for the design of effective T cell-based cancer immunotherapy. The Journal of Immunology, 2005, 175: 1424–1432.

Generation of tumor-reactive T cells is critical for the success of adoptive immunotherapy of cancer. Toward this goal, our efforts have been concentrated on the use of tumor-draining lymph nodes (TDLNs)4 that harbor pre-effector cells potentially capable of recognizing and destroying tumors (1, 2). Recent attempts in adoptive immunotherapy have aimed at seeking novel protocols to transform these pre-effector cells into tumor-reactive effector T cells in hope that the latter can be used successfully in clinical cancer therapy.

We as well as the other laboratories have previously shown that TDLN cells activated with anti-CD3 mAb and expanded with IL-2 result in competent effector T cells capable of eradicating tumor upon adoptive transfer (1–3). This method preferentially activates CD8+ cells. The effector cells manifest their antitumor effects by the release of cytokines, e.g., IFN-γ and GM-CSF, in response to tumor Ags. The optimal activation of T lymphocytes requires engagement of both the TCR complex (i.e., first signal) as well as a costimulatory molecule, such as CD28, which acts as the second signal (4). We reported that in vitro activation of human vaccine-primed lymph node cells with an anti-CD3/anti-CD28 mAb combination significantly enhanced cell proliferation and cytokine secretion compared with anti-CD3 alone (5). In that model, anti-CD3 and anti-CD28 mAbs were used to supply the first and second signals for T cell activation, respectively. In another report the antitumor reactivity of anti-CD3/anti-CD28 activated TDLN cells was evaluated in vivo in a syngeneic animal tumor model (6). Distinct differences of the immune function of these doubly activated cells were found compared with activation with anti-CD3 alone. The combined use of anti-CD3 and anti-CD28 mAbs for in vitro activation of TDLN cells promoted the generation of tumor-reactive CD4+ cells as well as CD8+ cells. As a result, effector cells can be generated with significantly improved antitumor efficacy using costimulatory pathways.

Another important costimulatory pathway that has been documented to be required in the induction of tumor immunity is the CD40:CD40L (CD154) interactions (7, 8). In vivo administration of anti-CD40 mAb has been reported to induce proliferation of memory CD8 T cells (9) and enhance Ag-presenting Langerhans cell priming of IFN-γ-producing CD4+ and CD8+ T cells (10). Studies have suggested that binding of CD4+ cells to CD40 expressed on dendritic cells (DC) will activate the CD4+ cells to provide help in the sensitization of CD8+ cells (11–13). This three-cell collaboration does not need to occur simultaneously for CD8+ cells to mature into CTL, nor do CD4+ cells need to activate DC; the function of CD4+ cells can be bypassed by ligation of CD40 with an anti-CD40 mAb, FGK45 (13).

We speculated that simultaneous engagement of CD40 and CD3 ligation in TDLN cells may be vital for the induction of effective antitumor responses and may act coordinately to enhance the adaptive immunity against cancer. In this study we evaluated the costimulatory effects of anti-CD40 mAb in concert with anti-CD3...
mAb on modulating the antitumor activity of pre-effector TDLN cells in animal models. We found that engagement of both TCR and CD40 using anti-CD3 and anti-CD40 Abs led to the generation of more potent effector T cells that secreted significantly higher levels of IFN-γ and mediated tumor regression more efficiently than cells activated with anti-CD3 alone. This study provides evidence of an interactive effect between Ab activation of two collaborative cell populations that involves targeting CD3 on T cells and CD40 on B cells and/or DCs, respectively.

Materials and Methods

Mice

Female C57Bl/6 (B6) mice and IFN-γ−/− knockout mice on the B6 background from The Jackson Laboratory were maintained in a pathogen-free environment and used at 8 wk of age or older. Principles of laboratory animal care (National Institutes of Health) were followed, and all animal protocols were approved by the University of Michigan Laboratory of Animal Medicine.

Tumor cells

Murine tumor MCA 205 is a methylcholanthrene-induced, weakly immunogenic fibrosarcoma that is syngeneic to C57Bl/6 mice (14). It was maintained in vivo by s.c. transplantation in B6 mice and was used within the eighth generation. Tumor cells were isolated from solid tumors and resuspended in PBS for administration into mice or in complete medium for in vitro assays as previously described (6).

TDLN

To induce TDLN, 1 × 10⁵ MCA 205 cells in 0.1 ml of PBS were injected s.c. into the flanks of B6 mice or IFN-γ−/− knockout mice. The draining inguinal lymph nodes were collected 9 days later; processed using mechanical dissociation, then filtered through nylon mesh and washed in HBSS. Multiple inguinal TDLN were pooled from groups of mice for lymphoid cell suspension preparation.

Cell depletion

To eliminate APCs, TDLN T cells were enriched by passing the single-cell suspension prepared above through 1-μm sterilized nylon wool (Cellular Products) column, using a 45-min absorption period at 37°C, followed by elution of the nonadherent T cells with warm RPMI 1640 medium without additives. The resultant APC− TDLN cells were 95% CD3⁺, as indicated by FACS analysis (data not shown). To remove B cells and/or DCs, TDLN cells were treated with superparamagnetic microbeads conjugated with anti-mouse CD19 and/or anti-mouse CD11c mAbs, followed by cell separation using the MACS separator (Miltenyi Biotec). The efficiency of cell depletion was confirmed by FACS staining (>90% CD19⁺ or CD11c⁺ cells were depleted; data not shown).

Ab activation of TDLN cells

TDLN cells were activated with anti-CD3 mAb alone or anti-CD3 plus anti-CD40, respectively. In certain experiments, anti-CD3 plus anti-CD40L was used as a control. Abs were immobilized on 24-well tissue culture plates (Costar). Anti-CD3 mAb (2C11) ascites (1/100 dilution) and anti-CD40 (FGK45) mAb ascites (1/100 dilution) were used. Purified anti-CD40L mAb (MR1; obtained from Dr. K. Bishop, University of Michigan, Ann Arbor, MI) was used at 10 μg/ml. The anti-CD3 and anti-CD40 anti-bodies were produced by using 14.5C11 and FGK45 hybridoma cells (American Type Culture Collection). The 14.5C11 hybridoma cells produce hamster IgG mAb against the CD3 ε-chain of the murine TCR/CD3 complex. The ascites was partially purified by 50% ammonium sulfate precipitation, and the IgG content was determined by ELISA. The 1/100 dilution is equivalent to ∼1.0 μg/ml anti-CD3. This was the concentration we used in our laboratory in previous studies. The use of anti-CD40 ascites at a 1/100 dilution was determined by previous titrating tests to be optimal for cell expansion in combination with anti-CD3. We also determined the IgG content in FGK45 ascites using ELISA and found that the 1/100 dilution is equivalent to an IgG concentration of 0.2–0.5 μg/ml. Immobilization was achieved by placing 1 ml of Ab solution in PBS in the wells of the plates and incubating overnight at 4°C or at room temperature for 5 h. The coated plates were washed with PBS and then used for TDLN cell activation at a concentration of 4 × 10⁶ cells/2 ml/well. X-Vivo-15 medium (BioWhittaker) was used for T cell activation in the absence of IL-2. These cells were activated at 37°C with 5% CO₂ for 2 days. Activated TDLN cells were collected, washed, counted, and then expanded in complete medium containing human rIL-2 (60 IU/ml; Chiron Therapeutics) for 3 days. Ab-activated and IL-2-expanded TDLN cells were used for cytokine secretion evaluation, phenotype analysis, and adoptive immunotherapy.

Cytokine release

After activation and expansion, 1 × 10⁶ TDLN cells were cocultured with 2.5 × 10⁶ irradiated (6000 cGy) MCA 205 tumor stimulator cells in 24-well tissue culture plates for 24 h at 37°C with 5% CO₂. The supernatant was then collected and analyzed for the secretion of IFN-γ, IL-10, IL-12, and IL-4 by ELISA (BD Pharmingen).

Adoptive immunotherapy

Pulmonary metastases were induced by tail vein injection of 2 × 10⁵ MCA 205 cells in B6 mice. Three days after tumor inoculation, the tumor-bearing mice were treated with Ab-activated TDLN cells by tail vein injection. Commencing on the day of cell transfer, i.p. injections of IL-2 (4200–60000 IU) were administered in 0.5 ml of PBS and continued twice daily for eight doses. Approximately 14 days after T cell transfer, all mice were randomized and killed, and lungs were harvested for enumeration of pulmonary metastatic nodules as previously described (6). Five mice were used in each experimental group.

Phenotypic analysis

Phenotypes of TDLN cell populations were analyzed by immunofluorescence assay and flow cytometry using a FACScan flow microfluorometer (BD Biosciences). Fluorescence profiles were generated by analyzing 10,000 cells and displayed as logarithmically increased fluorescence intensity vs cell numbers. TDLN cells were analyzed for CD3, CD4, CD8, CD40, CD40L, CD11c, CD19, and CD14, and NK1.1 markers. All FITC-conjugated Abs were purchased from BD Pharmingen. Two-color staining was performed to analyze CD40 distribution on different cell subsets. In brief, 1 million viable TDLN cells in 100 μl of PBS were blocked for 15 min with 0.5 μg/ml anti-mouse CD3/CD16 (Fe-roKIII; BD Pharmingen) to reduce background IR-mediated nonspecific binding. The cells were then stained for 20 min with 0.5 μg of the following FITC-conjugated Abs: anti-mouse CD3, anti-mouse CD19, anti-mouse CD11c, and PE-conjugated anti-mouse CD40 (all from BD Pharmingen). The stained cells were washed and fixed with 1% paraformaldehyde in PBS. FACS analysis was performed using the above-mentioned FACScan flow cytometer and CellQuest software (BD Biosciences).

RT-PCR

RNA was isolated from 5 × 10⁶ Ab-activated TDLN cells using a RNA minikit (Qiagen). RNA was eluted with RNase-free water to a final volume of 50 μl, and an aliquot was used to determine the purity and concentration. An A₂₆₀/A₃₂₀ ≥ 1.8 was measured for all samples. Two micrograms of total RNA was reverse transcribed using oligo(dT) primer and SuperScript reverse transcriptase (Invitrogen Life Technologies) following the manufacturer’s recommended protocol. The final 20-μl product was diluted to 100 μl in Tris-EDTA buffer before proceeding. PCR was performed in 25-μl volumes containing 6 μl of cDNA and 19 μl of Master Mix constructed to deliver final concentrations of 200 μM of each dNTP, 250 nM of each primer, 4 mM MgCl₂, SYBR Green 1 (20,000), and 0.45 U of Taq in 20 mM Tris, pH 8.4. An initial GAPDH PCR was performed to equilibrate cDNA input for further tests. PCR primers used were as follows: control GAPDH forward, 5'-TGTCTTCCACCTCTTGAT-3'; reverse, 5'-TGCTTACACCCCTCTTGAT-3'; IL-12(p35): forward, 5'-TGTTTTACCCCAGCTTGTG-3'; reverse, 5'-TGTTTTACCCCAGCTTGTG-3'; IL-12(p40): forward, 5'-TACAATTCCTCGTCAGCACA-3'; reverse, 5'-TCCACCTTTCCTTTCCTC-3'. All primer pairs were designed to span at least one intron. PCR was performed on a Cepheid SmartCycler using an initial 4-min denaturation at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C (GAPDH was limited to 25 cycles). A final melt analysis was performed to ensure that product accumulation was target specific. PCR products were additionally analyzed by visualization on 1.2% agarose gel and ethidium bromide staining.

Band densitometry was performed using a Chemilumager 4400 (Alpha Innotech) and AlphaEase software. Band densities for each target were calculated using the nonparametric Wilcoxon rank-sum test. Two-sided P <
Results
Simultaneous targeting of CD3 and CD40 during in vitro TDLN cell activation boosts the antitumor activity of the effector T cells

In vivo administration of agonistic anti-CD40 mAb has been shown to enhance antitumor activity (15, 16). To evaluate the effects of CD40 ligation during the activation of TDLN cells in vitro, we examined the effector T cells generated by TDLN cell activation using anti-CD3 plus anti-CD40 mAbs. MCA 205 TDLN cells were activated with anti-CD3 alone or anti-CD3 plus anti-CD40 mAb, followed by IL-2 expansion as described in Materials and Methods. The activated and expanded MCA 205 TDLN cells were then cocultured with irradiated MCA 205 tumor cells for cytokine release measurement. As shown in Fig. 1, TDLN cells activated by the anti-CD3/anti-CD40 combination resulted in IFN-γ secretion to a significantly higher level than anti-CD3 alone (p < 0.05), whereas the production of IL-10 was reduced. Therefore, targeting CD40 by an agonistic anti-CD40 Ab during TDLN cell activation by anti-CD3 in vitro lead to the generation of effector T cells with augmented type 1 cytokine (i.e., IFN-γ) production, but decreased type 2 cytokine (i.e., IL-10) secretion. In these experiments we also used anti-CD40 alone as a control. TDLN cells activated with anti-CD40 alone, followed by culture in IL-2, did not grow (data not shown). These experiments thus indicated that it was required to stimulate T cells with anti-CD3 to achieve the augmenting effect of anti-CD40; ligation of CD40 alone was not sufficient to stimulate TDLN cells.

The therapeutic efficacy of anti-CD3/anti-CD40-activated TDLN cells was examined in the adoptive immunotherapy of 3-day established pulmonary MCA 205 metastases. As evident in Fig. 2A, transfer of Ab-activated TDLN cells mediated tumor regression in a dose-dependent manner. At a high dose (2.5 × 10⁶), both groups of T cells mediated efficient tumor regression. However, there was a statistically significant difference in tumor regression between anti-CD3-activated TDLN cells and anti-CD3/anti-CD40-activated TDLN cells (p < 0.05) when the animals received only 1 × 10⁶ activated cells. Notably, the mean number of pulmonary metastases was significantly reduced after the transfer of anti-CD3/anti-CD40-activated TDLN cells compared with

FIGURE 2. In vivo antitumor reactivity of anti-CD3/anti-CD40-activated TDLN cells. A, Anti-CD3/anti-CD40-activated TDLN cells mediated tumor regressions more effectively than TDLN cells activated with anti-CD3 alone. TDLN cells were activated with anti-CD3 alone or anti-CD3 plus anti-CD40, followed by IL-2 expansion. B6 mice bearing 3-day established MCA 205 pulmonary metastases were treated by the adoptive transfer of Ab activated MCA 205 TDLN cells at various doses followed by IL-2 administration. Therapeutic efficacy was compared with control (no treatment) or IL-2 only groups as indicated. IL-2 (60,000 IU) was administrated i.p. twice daily for eight doses after cell transfer. The results of one representative experiment of four performed are shown. ∗, p < 0.05 compared with and equal number (1 × 10⁶) of cells transferred. B, IFN-γ was one of the mediators involved in the augmented therapeutic efficacy induced by CD40 engagement. TDLN cells were induced from MCA 205-bearing IFN-γ−/− KO mice and were activated with anti-CD3 alone or anti-CD3 plus anti-CD40, followed by IL-2 expansion. Wt B6 mice bearing 3-day established MCA 205 pulmonary metastases were treated by the adoptive transfer of activated IFN-γ−/− TDLN cells as described in A. IL-2 (42,000 IU) was administrated i.p. twice daily for eight doses after cell transfer. Therapeutic efficacy was compared with activated wt TDLN cells as indicated. The data were obtained from two independent experiments. ∗, p < 0.05 compared with an equal number (1 × 10⁶) of anti-CD3-activated wt TDLN cells.

FIGURE 1. CD40 ligation using agonistic anti-CD40 mAb in combination with anti-CD3 significantly enhanced IFN-γ secretion of TDLN cells. TDLN were induced by inoculation of B6 mice with MCA 205 tumors. TDLN cells were then prepared and activated with anti-CD3 alone (□) or anti-CD3 plus anti-CD40 (●), followed by expansion in IL-2. Activated TDLN cells (1 × 10⁶) were then cocultured with 2.5 × 10⁶ irradiated MCA 205 tumor cells. The supernatants were collected and analyzed for cytokine production. Data represent the mean of six independent experiments ± SEM. ∗, p < 0.05 compared with anti-CD3 alone.
Approximately 40% of fresh TDLN cells express CD40, but CD40 expression on CD19

Although at a high dose (3

animals (Fig. 2

cacy was markedly reduced compared with activated wild-type

responses of tumor-primed lymphoid cells both in vitro and in

and CD40 during TDLN cell activation can boost the antitumor

B cells (33%; Table I).

Although anti-CD3 alone activation resulted in

15% CD4

CD40-positive cells decreased significantly after Ab activation. As shown in Fig. 4, although anti-CD40 sig-
nificant decrease in the percentage of CD40-expressing cells.

TDLN cells. In an adoptive transfer model (Fig. 5 ), at a

CD3

T cells within the freshly harvested TDLN cells were

composed of half CD4

and half CD8

T cells. Anti-CD3 alone or anti-CD3 plus anti-CD40 activation both result-
ed in >95% CD3

T cells. However, Ab activation changed the percentage of CD4

and CD8

T cells within the resultant

CD3

CD4

and CD8

cells within the TDLN cell population are potential targets for the anti-CD40 mAb used.

CD3

T cells within the freshly harvested TDLN cells were

an equal number (1 × 10

6) of anti-CD3-activated cells. The results therefore indicated that anti-CD3/anti-CD40-activated TDLN cells were more potent, on a per cell basis, than TDLN cells activated

with anti-CD3 alone in mediating tumor eradication.

The above results suggested that IFN-γ plays an important role in tumor regression mediated by anti-CD3/anti-CD40-activated TDLN cells. To investigate this, we used IFN-γ

knockout (KO) mice for donor cells in subsequent experiments. TDLN cells were induced in IFN-γ

KO mice and activated with anti-CD3 or anti-CD3 plus anti-CD40. These TDLN cells had no detectable IFN-γ secretion in vitro (data not shown). Their therapeutic efficacy was markedly reduced compared with activated wild-type (wt) TDLN cells when adoptively transferred into tumor-bearing animals (Fig. 2B).

Although at a high dose (3 × 10

6) these TDLN cells mediated tumor regression to a moderate extent, they showed no antitumor effect at a lower dose (1 × 10

6). Importantly, they demonstrated no increased activity after anti-CD3 plus anti-CD40 treatment compared with anti-CD3 alone, in contrast to wt TDLN cells. These data thus support the role of IFN-γ in the enhancement of antitumor reactivity induced by CD40 ligation.

Together, these data indicate that simultaneous targeting of CD3 and CD40 during TDLN cell activation can boost the antitumor responses of tumor-primed lymphoid cells both in vitro and in vivo, and IFN-γ is one of the major mediators involved.

CD40 expression on CD19

B cells, CD11c

DCs, as well as T

cells in TDLN

The expression of CD3, CD4, CD8, CD40, and CD40L on TDLN cells was determined by FACS both before and after Ab activation. The freshly harvested, nonactivated TDLN cells were composed mostly of CD3

T cells (57%) and CD19

B cells (33%; Table I). Approximately 40% of fresh TDLN cells express CD40, but CD40L expression was low (<5%). Through double staining, we tested the distribution of CD40 molecules on TDLN cells before Ab activation (Fig. 3). CD40 was expressed on nearly 20% of CD3

T cells, whereas almost all CD19

B cells (95.1%) and CD11c

DCs (94.5%) were CD40 positive. Although CD40 was also found on portions of CD14

and NK cells (data not shown), considering the low percentages of these cells within the TDLN (each <3%; Table I), the CD14

CD40

or CD40

NK cells within the TDLN were actually <1% of the TDLN cells (data not shown) or negligible. Therefore, CD19

B cells and CD11c

DCs as well as a small portion of T cells within the TDLN cell population are potential targets for the anti-CD40 mAb used.

Anti-CD40 mAb augments the antitumor activity of anti-CD3-activated TDLN cells by targeting CD40 on APCs

Based on the above observations, CD19

B cells, CD11c

DCs, as well as a small portion of T cells within the TDLN cells could respond by binding with anti-CD40 mAb. We then performed corollary studies in nylon wool-treated TDLN cells to eliminate APCs. TDLN cells were put into a nylon wool column to remove APCs. FACS analysis indicated that the resultant APC

TDLN cells were >95% CD3

cells with low CD40 expression (<10% CD3

cells; data not shown). Using these cells in parallel with unfractionated TDLN cells, we measured their cytokine secretion after Ab activation. As shown in Fig. 4, although anti-CD40 significantly up-regulated IFN-γ secretion of anti-CD3-activated unfractionated TDLN cells compared with anti-CD3 alone (p < 0.05), it exhibited no effect on anti-CD3-activated APC

TDLN cells. In contrast, anti-CD40 down-regulated IL-10 secretion of anti-CD3-activated unfractionated TDLN cells, but, again, not on APC

TDLN cells. In an adoptive transfer model (Fig. 5), at a very low dosage (0.5 × 10

6) of T cells transferred, anti-CD40


cells; data not shown).

| CD3 | 57.0 ± 3.0 | 98.0 ± 1.0 | 97.0 ± 1.0 |
| CD4 | 29.0 ± 3.0 | 14.0 ± 1.0 | 24.0 ± 2.0 |
| CD8 | 28.0 ± 3.0 | 81.0 ± 1.0 | 60.0 ± 4.0 |
| CD40 | 43.0 ± 7.0 | 0.3 ± 0.3 | 0.3 ± 0.3 |
| CD40L | 4.8 ± 0.6 | 2.7 ± 1.0 | 0.3 ± 0.3 |
| CD11c | 5.0 ± 0.5 | nd | nd |
| CD19 | 33.0 ± 3.0 | nd | nd |
| CD14 | 2.5 ± 0.3 | nd | nd |
| NK | 1.5 ± 0.3 | nd | nd |

* Data are expressed as mean ± SE percentage of positive cells.

* Data are representative of 3 independent experiments.

* Data are representative of 14 independent experiments.

* Data are representative of 8 independent experiments; nd, not done.

* p < 0.05 compared with CD4 cells generated by 2C11 activation.

**TABLE 1. Phenotype analysis of TDLN cells before and after Ab activation**
significantly enhanced the therapeutic efficacy of anti-CD3-activated unfractionated TDLN cells, but it exhibited no immunomodulatory function on TDLN cells in the absence of APCs. The adoptive immunotherapy data correlate well with the in vitro finding that anti-CD40 mAb could significantly increase type 1 (e.g., IFN-γ) cytokine production of anti-CD3-activated TDLN cells only with the presence of APCs. Collectively, these data indicate that anti-CD40 mAb delivered the costimulatory signal by targeting CD40 on APCs.

We proceeded by respective B cell or DC depletion from TDLN cells using MACS beads coupled with anti-CD19 or anti-CD11c. In some groups both B cells and DCs were depleted. Unfractionated or B cell− and/or DC− MCA 205 TDLN cells were activated either with anti-CD3 alone or with anti-CD3 plus anti-CD40, followed by IL-2 expansion, then analyzed for IFN-γ secretion (Fig. 6A) and therapeutic efficacy (Fig. 6B). Anti-CD40 significantly increased IFN-γ secretion of unfractionated MCA 205 TDLN cells after anti-CD3 activation in a tumor Ag-specific fashion, but not in the absence of B cells and/or DCs (Fig. 6A). Depletion of either B cells or DCs partially, but significantly, abrogated the therapeutic efficacy mediated by anti-CD3/anti-CD40 activation (Fig. 6B). When both B cells and DCs were removed, the antitumor activity was completely eliminated. These data thus establish a role for both CD40-expressing B cells and DCs in TDLN cell costimulation through CD40 ligation in concert with CD3 engagement. B cells and DCs seemed to contribute additively in this event.

**Simultaneous CD3 and CD40 ligation correlates with up-regulated IL-12 production, but down-regulated IL-4 secretion**

It has been well documented that IL-12 is the driving force for type 1 phenotype differentiation, and IL-4 is one of the key factors leading to type 2 responses (17, 18). Our studies demonstrated that CD3 and CD40 costimulation could generate more potent effector cells with a type 1 cytokine profile. One of the assumptions for the shifting of the TDLN cells toward a type 1 pattern would be the regulation of IL-12 and IL-4 upon ligation of CD40 in addition to CD3. We therefore examined the effects of simultaneous targeting of CD3 and CD40 on IL-12 and IL-4 production by Ab-activated TDLN cells. TDLN cells activated with anti-CD3 alone or anti-CD3 plus anti-CD40 were used for the extraction of mRNA and were analyzed for IL-12 expression using RT-PCR (Fig. 7A). Both p35 and p40 subunits of IL-12 were expressed at higher levels in anti-CD3/anti-CD40-activated TDLN cells (lane 3) than that in anti-CD3 alone-activated cells (lane 1). Anti-CD3/anti-CD40L-activated cells (lane 2) were used as a control. To quantitatively demonstrate the difference of p35, p40 expression, we generated a bar graph using the data obtained by densitometric analysis of the bands of the agarose gel electrophoresis of PCR products (Fig. 7A). Compared with anti-CD3 activation alone, anti-CD3/anti-CD40 increased p35 and p40 expression by ~50% and 4-fold, respectively. To confirm this finding, supernatants collected at the end of IL-2 expansion after Ab activation of TDLN cells were tested for IL-12 and IL-4 production using ELISAs. IL-12 secretion by anti-CD3/anti-CD40-activated TDLN cells was significantly higher than that by anti-CD3-activated cells (Fig. 7B). By contrast, IL-4 secretion by anti-CD3/anti-CD40-activated TDLN cells was markedly lower compared with that after anti-CD3 activation alone.
Discussion

In this report we examined the role of simultaneous ligation of CD3 and CD40 on TDLN cells in generating antitumor effector T cells. Targeting of CD3 and CD40 concurrently with anti-CD3 and anti-CD40 mAbs led to an increased production of IFN-γ that correlated with significantly augmented therapeutic efficacy in adoptive transfer compared with anti-CD3-activated TDLN cells. We believe that this activation procedure may have potential use in clinical adoptive immunotherapy of cancer. The use of anti-CD3 in generating effector cells for human cancer adoptive T cell therapy has been reported by several groups with some success (2, 3). The addition of a second Ab to simultaneously involve APCs may provide a rational strategy to enhance the antitumor reactivity of the effector T cells.

We previously reported that in vitro activation of human vaccine-primed lymph node cells with an anti-CD3/anti-CD28 mAb combination significantly enhanced cell proliferation and cytokine secretion compared with anti-CD3 alone (5). On a per cell basis, doubly activated TDLN cells were more efficient in mediating tumor regression compared with TDLN cells activated only by anti-CD3 (6). In addition, costimulatory signaling via CD40 has been shown to play a central role in the regulation of cell-mediated antitumor (19–21) as well as anti-infectious disease (22) responses. Together, these studies suggest the involvement of a second signaling during TCR/CD3 activation of T cells to generate potent effectors.

Our data revealed that CD40 is predominantly expressed on CD19+ B cells as well as on CD11c+ DCs and T cells in TDLN. Ligation of CD40 is essential for primary B cell activation and expansion (23). Hwu et al. (24) reported that B lymphocytes activated through CD40 ligation could efficiently process and present tumor Ags to specific CD4+ T cell clones. This suggested that CD40-activated B cells have the functional and molecular competence to present epitopes, thereby making them a relevant source of APCs to generate T cells. In another study, B cells activated by LPS or by anti-CD40 mAb exhibited distinctly different outcomes. Although LPS-activated B cells led to anergy in CD8+ T cells, anti-CD40 Ab-activated B cells resulted in proliferation, cytokine secretion, and cytotoxic ability of CD8+ T cells (25). Furthermore, CD40 engagement was found to lead to NF-κB activation and cell cycle progression in B cells (26). Moreover, ligation of CD40 has demonstrated suppressive or apoptotic effects on CD40-expressing hematological malignancies, such as mouse (27) and human (23) B cell lymphomas. In contrast to its inhibitory effect on B cell-derived tumors, anti-CD40 stimulated the growth of primary B cells, and the stimulation was greatly enhanced in the presence of CD40L-expressing cells (23). Our study provides evidence that targeting both CD3 and CD40 can potentiate the antitumor effect.

FIGURE 6. Anti-CD40 mAb augmented antitumor responses of anti-CD3-activated TDLN cells via ligation to CD40 on both B cells and DCs. B cells and/or DCs were depleted from MCA 205 TDLN cells using anti-CD19- and/or anti-CD11c-coupled magnetic beads before Ab activation. Unfractionated or B+ and/or DC− TDLN cells were activated with anti-CD3 alone or anti-CD3 plus anti-CD40, respectively, followed by expansion in IL-2. A. Activated cells were cocultured with MCA 205 tumor cells to determine IFN-γ production. MCA 207 was used as a specificity control. Data are representative of two separate experiments. * p < 0.05 compared with any other group. B, Activated cells prepared as described above were adoptively transferred into B6 mice bearing 3-day established MCA 205 metastases at 1 × 106 cells/mouse. Therapeutic efficacy mediated by unfractionated cells was compared with an equal number of anti-CD3/anti-CD40-activated B+ and/or DC− MCA 205 TDLN cells. IL-2 was administrated as previously described. This study was repeated in a second experiment. * p < 0.05 compared with any other group.
of activated TDLN cells. Simultaneous targeting of CD3 and CD40 occurred on T cells and APCs (including B cells and DCs), respectively, as suggested by CD3 and CD40 distribution analysis and supported by the findings obtained from APC depletion and B cell and/or DC removal experiments.

CD40 signaling is reported to be crucial for optimal Ab production, isotype switching, and humoral memory response, although the molecular mechanisms responsible for these reactions are incompletely understood (28–30). Our study exploring the roles of CD40 in augmenting the antitumor responses of TDLN cells suggests that CD40 engagement on B cells in TDLN enhanced the antitumor activity by modulating both cellular responses and humoral responses, the latter of which warrants additional investigation, particularly in the presence of synergy between CD40 and BCR signaling using anti-Ig Abs. This study was performed using TDLN cells, which leaves open the possibility that we may observe similar or even superior augmenting effects of anti-CD40 when using splenocytes due to the fact that, in general, larger numbers of APCs (B cells and DCs) are present in the spleen than in the lymph nodes. Current studies are focused on addressing these possibilities.

Murphy et al. (15) investigated the synergistic antitumor responses after administration of agonistic Abs to CD40 and IL-2 and reported coordination of DC and CD8<sup>+</sup> cell responses. They found that the combination of anti-CD40 and IL-2 resulted in significant increases in DC and CD8<sup>+</sup> T cell numbers and induced complete regression of metastatic renal tumor in the majority of treated mice. Additional studies presented evidence that CD40L

![Figure 7](image-url)
induced DC maturation (31) and activation (32) and augmented DC production of inflammatory cytokines, e.g., of IL-12 and IL-8 (33). Our study demonstrated the involvement and contribution of DC cells during the anti-CD3/anti-CD40 activation of TDLN cells in vitro.

The mechanisms involved in mediating tumor rejection by the adaptively transferred cells are dependent upon the elaboration of cytokines (18, 34). We have previously reported that the in vitro cytokine profile of activated lymphocytes secreted in response to tumor Ag correlated with the in vivo antitumor activity of the cells in adoptive immunotherapy (34). Specifically, effector cells that elaborated IFN-γ (type 1) or GM-CSF in response to tumor mediated tumor regression in vivo. By contrast, elaboration of IL-10 (type 2) is associated with a suppressive antitumor effect. Fox et al. (18) also found elaboration of IL-4 by lymphoid cells in response to tumor to suppress their antitumor reactivity in vivo. Cosstimulation of TDLN cells by anti-CD40 was found in this study to significantly enhance the elaboration of IFN-γ. The superior therapeutic efficacy against established tumors mediated by anti-CD3/anti-CD40-activated TDLN cells correlated with their higher levels of IFN-γ secretion and decreased IL-10 production. Efforts at promoting the expansion of T cells expressing a type 1 cytokine profile would be relevant in developing more effective adoptive immunotherapy strategies. Toward that aim, we have reported that costimulation of TDLN cells through newly induced 4-IBB and CD3/CD28 signaling can significantly increase antitumor reactivity by shifting T cell responses toward a type 1 cytokine pattern (35).

In this study the immunomodulation of anti-CD40 on antitumor activity was found to be associated with its ability to significantly boost the IL-12 secretion of anti-CD3-activated TDLN cells while inhibiting IL-4 production. Ligation of CD40 on macrophages has been shown to be a potent stimulus for the production of IL-12 (36). DCs (37) and B cells (38, 39) can also produce IL-12 in response to activation. Using ELISA, we found that elicited IFN-γ production by anti-CD3/anti-CD40 activation was associated with higher levels of IL-12 and lower levels of IL-4. Concurrently, RT-PCR revealed up-regulated expression of IL-12 subunits p35 and p40 at the molecular level after anti-CD3/anti-CD40 activation. Modulation of IL-12 and IL-4 production by simultaneous targeting of CD3 and CD40 on T cells and B cells and/or DCs in TDLN may serve as oblige signals for the generation of type 1 cytokine responses that lead to augmented antitumor efficacy. The mechanism underlying this observation has yet to be elucidated. The augmenting effects are most likely through cytokine secretion, as demonstrated by IL-12 and IL-4 modulation. Our findings, however, do not exclude the possibility that cell contact is required during the stimulation phase.

We demonstrated that simultaneous ligation of CD3 and CD40 on TDLN cells enabled them to secrete IFN-γ and mediate tumor regression to an extent not accomplished through CD3 ligation alone. Simultaneous targeting of CD3 and CD40 takes place by Ab binding to CD3 on T cells and CD40 on B cells and/or DCs, respectively. Our data are relevant for the design of more effective T cell-based immunotherapies.

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Disclosures
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