CD28, TNF Receptor, and IL-12 Are Critical for CD4-Independent Cross-Priming of Therapeutic Antitumor CD8+ T Cells

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CD28, TNF Receptor, and IL-12 Are Critical for CD4-Independent Cross-Priming of Therapeutic Antitumor CD8+ T Cells

Hong-Ming Hu,2*† Hauke Winter,2,3* Jun Ma,*‡§ Michael Croft,‖ Walter J. Urba,*§ and Bernard A. Fox4*†‡§

Previously, we have shown that priming of therapeutic CD8+ T cells in tumor vaccine-draining lymph node nodes of mice vaccinated with GM-CSF secreting B16BL6 melanoma cells occurs independent of CD4 T cell help. In this study, we examined the contribution of the major costimulatory molecules, CD40 ligand (CD40L), CD80, and CD86, in the priming of CD8+ T cells. Priming of therapeutic CD8+ T cells by a GM-CSF-transduced tumor vaccine did not require CD40 and CD40L interactions, as therapeutic T cells could be generated from mice injected with anti-CD40L Ab and from CD40L knockout mice. However, costimulation via either CD80 or CD86 was required, as therapeutic T cells could be generated from mice injected with either anti-CD80 or anti-CD86 Ab alone, but administration of both Abs completely inhibited the priming of therapeutic T cells. Blocking experiments also identified that priming of therapeutic T cells in MHC class II-deficient mice required TNFR and IL-12 signaling, but signaling through CD40, lymphotoxin-βR, or receptor activator of NF-κB was not essential. Thus, cross-priming of therapeutic CD8+ T cells by a tumor vaccine transduced with GM-CSF requires TNFR, IL-12, and CD28 signaling. The Journal of Immunology, 2002, 169: 4897--4904.

Generation of tumor-specific CD8+ T cells via CD4 T cell-dependent cross-priming of tumor Ags by professional APCs, such as dendritic cells or macrophages, is well established (1–4). After capturing Ags released from tumor cells, dendritic cells must undergo a maturation process before they are able to prime tumor-specific naive T cells (1–4). Dendritic cell maturation occurs following engagement of CD40 on dendritic cells with CD40 ligand (CD40L)5 on specific CD4 T cells (5–10). Major consequences of this interaction are the up-regulation of costimulatory molecules CD80 and CD86 on dendritic cells and the release of inflammatory cytokines, IL-12 and TNF-α.

GM-CSF gene modification of tumor cells has proven to be one of the most effective vaccine adjuvants for priming antitumor T cells in naive mice (11). Optimal priming of antitumor CD8+ T cells by a GM-CSF gene-modified whole cell tumor vaccine requires cross-presentation of tumor Ags by host bone marrow-derived APC, e.g., dendritic cells (12–14). However, we have recently shown that the cross-priming of tumor-specific CD8+ T cells can occur without CD4+ T cell help. Vaccination of animals depleted of CD4+ T cells, as a result of mAb treatment or by targeted mutation of class II, led to the priming of tumor-specific CD8+ T cells, as evidenced by cytotoxicity and IFN-γ release assays (15). Additionally, the tumor-specific CD8+ T cells primed in CD4-deficient animals were highly therapeutic, reducing pulmonary metastases and curing animals with established systemic tumor. These results suggest that molecular interactions other than CD40 and CD40L are involved in the maturation of effective APC when a GM-CSF-secreting tumor is used as a vaccine.

One way a tumor may escape immune destruction is by inducing anergy or tolerance in tumor-specific CD4+ T cells; effectively blocking the priming of therapeutic T cells (16–18). Thus, effective vaccine strategies will need to provide CD4 help or develop CD4-independent approaches to prime therapeutic CD8+ T cells. Our efforts have been focused on the latter, delineating the mechanisms by which CD8+ T cells can be primed without CD4 T cell help. To better understand the mechanisms for CD4-independent priming, we examined the role of the important costimulatory molecules, CD40L, CD154, CD80, and CD86, and the inflammatory cytokines IL-12 and TNF during the priming phase of our adoptive immunotherapy model.

Materials and Methods

Mice

Female C57BL/6J mice and CD40L knockout (KO) mice (B6; 129S-Tnfsf51tm1ums) were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC class II KO mice (C57BL/6Tg-Abbml1 N5) were purchased

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from Taconic (Germantown, NY). Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996), and all animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee.

Tumor cell lines and mAbs

D5 is a poorly immunogenic subclone of the spontaneously arising B16BL6 melanoma (19). D5-G6 is a stable murine GM-CSF-transduced D5 clone, which secretes GM-CSF at 400 ng/ml/10^6 cells/24 h (15). Both were provided by S. Shu (Cleveland Clinic Foundation, Cleveland, OH). Tumor cells and T cells were cultured in complete medium (CM), which consisted of RPMI 1640 (BioWhittaker, Walkersville, MD) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin sulfate. This was further supplemented with 50 μM 2-ME (Aldrich, Milwaukee, WI) and 10% FBS (Life Technologies, Grand Island, NY). Tumor cells were harvested two to three times per week by brief trypsinization and maintained in T-150 or T-225 culture flasks.

Purified rat Ig (500 μg; Sigma-Aldrich I-4131, St. Louis, MO) was used as the control Ab. Hamster anti-mouse CD40L (MR1), anti-mouse CD80 (16-10A1), and rat anti-mouse CD86 (GL1) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Ascites fluid was prepared in primine-primed DBA mice. Hamster anti-mouse CD3 (2c11) and CD28 (PV1) mAbs were purified from culture supernatants by ammonium sulfate precipitation and ion exchange chromatography. Recombinant soluble TNFR (TNFR-Fc, Enbrel, etanercept) were obtained from Immunex (Seattle, WA). Soluble recombinant receptor activator of NF-κB (RANK) human Ig Fc fusion protein was a gift from A. Weinberg. (Earle A. Chiles Research Institute, Portland OR) and soluble rLT-R human Ig Fc fusion protein was a gift from C. Ware (La Jolla Institute for Allergy and Immunology, San Diego, CA).

In vivo priming and in vitro activation

In vivo priming of therapeutic T cells was achieved by s.c. injection of D5-G6 tumor cells. Four aliquots of 1 x 10^6 D5-G6 tumor cells were injected into both the fore and hind flanks of recipient wild-type (wt), MHC II KO, or CD40L KO mice. When blocking Ab or recombinant fusion proteins were used, they were injected according to the dose and schedule indicated in each table. Eight days after vaccination, enlarged vaccine-draining superficial inguinal and axillary lymph nodes (LN) were collected, and single cell suspensions were prepared by grinding the LN with a pair of sterile glass slides. The resulting cell suspension was washed in HBSS, counted, and activated to generate effector T cells. In some experiments, fresh tumor vaccine-draining LN (TVDLN) were phenotyped, as specified below. Activation was accomplished by cultured TVDLN in CM at 2 x 10^6 cells/ml in 24-well plates with 5 μg/ml 2c11 Ab (anti-CD3). In some experiments, 10 μg/ml anti-CD28 mAb was added together with the anti-CD3. After 2 days of activation, the T cells were harvested by centrifugation, washed once, and subsequently expanded at 0.1 x 10^6 cells/ml in CM containing 60 IU/ml IL-2 (kind gift of M. Giedlin, Chiron, Emeryville, CA) in B16 BL6 melanoma culture flasks (Nexell Therapeutics, Irvine, CA) for 3 additional days. Cultured cells were then harvested by centrifugation, washed twice in HBSS, counted, and used in adoptive transfer, cytotoxicity, and cytokine release assays.

Adoptive immunotherapy

Pulmonary metastases were generated by tail vein injection of 0.2 x 10^6 tumor cells.

T cells were adoptively transferred i.v. into B6 mice with 3-day experimentally established D5 pulmonary metastases (five mice per group, unless indicated otherwise).

Starting on the day of T cell infusion, mice received 90,000 IU IL-2 i.p. once per day for 2 days. Animals were sacrificed 11–13 days following tumor inoculation by CO2 narcosis. Lungs were resected and fixed in Fekete’s solution. The number of pulmonary metastases was counted in a blinded fashion. Metastases that were too numerous to count accurately were assigned an arbitrary value of 250. In some experiments, effector T cells were transferred into mice injected with 50 μl CD80 and CD86 mAbs ascites diluted in 500 ml HBSS 1 day before and 2 days after T cell transfer.

Flow cytometric analysis

Flow cytometry was performed on a B-D FACSscan, and data were analyzed with CellQuest software. FITC- or PE-conjugated anti-CD4, CD8, CD11c, CD69, CD80, and CD86 mAbs were purchased from BD PharMingen (San Diego, CA). FITC-conjugated anti-mouse OX-40 mAb was prepared in our lab with purified OX86 Ab (gift of A. Weinberg). Purified anti-CD16/CD32 mAb, which was prepared from culture supernatant of the 2G42 hybridoma (ATCC), was used to block nonspecific binding to FcR.

Results

Priming of therapeutic T cells is independent of CD40/CD40L interaction

Previously, we documented that priming of therapeutic CD8⁺ T cells occurred without CD4⁺ T cell help, if mice were vaccinated with the GM-CSF gene-modified tumor cell line, D5-G6 (15). It is thought that the key contribution of CD4⁺ T cells is the licensing of APCs that occurs following an interaction between CD40 and CD40L (10, 18). This interaction between the CD4⁺ T cells and APC results in up-regulation of costimulatory molecules (CD80 and CD86) and production of inflammatory molecules, IL-12 and TNF-α. Therefore, we sought to determine whether the CD4-independent priming of therapeutic T cells in our model was also CD40L independent. To test this hypothesis, priming with D5-G6 was performed in mice injected with the anti-CD40L blocking Ab, MR-1, using a dose and administration schedule that has been shown to effectively block CD40L function in vivo (20). The results in Table I clearly show that blocking the interaction between CD40 and CD40L did not inhibit the priming of therapeutic T cells. Complete regression of pulmonary metastases was observed in mice receiving effector T cells generated from either control mice or mice treated with Abs to CD40L (Table I).

Because there was no control to prove the CD40/CD40L interaction was blocked in Ab-treated mice, we sought to ascertain further the contribution of CD40L in the priming of therapeutic T cells by vaccinating CD40L-deficient mice. A critical role for CD40L in both humoral responses and cell-mediated immunity has been documented using these same CD40L knockout mice (5, 8, 9, 21–25). TVDLN T cells from both wt and KO mice were activated in vitro by either anti-CD3 alone or anti-CD3 plus anti-CD28, and subsequently expanded in medium containing low dose IL-2. Anti-CD28 Ab was included in the experiment to examine whether in vitro costimulation would effect the generation of therapeutic T cells from wt or CD40L KO mice. Results from Table II showed that effector T cells generated from both wt and CD40L KO mice were capable of eradicating 3-day established pulmonary metastases (Table II). Although effector T cells generated by stimulation

<table>
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<tr>
<th>Host*</th>
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<th>T cells*</th>
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<th>Expt. 2</th>
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<td>Anti-CD154</td>
<td>70 x 10^6</td>
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</tr>
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</table>

*Mice were vaccinated with a s.c. dose of 1 x 10^6 D5G6 tumor cells.

T cells were harvested by centrifugation, washed twice in HBSS, counted, and used in adoptive transfer, cytotoxicity, and cytokine release assays.

**Two days before and three and six days after vaccination, 50 μl of anti-CD40L (MR1-1) Ab (1/10 dilution in HBSS) or control hamster IgG mAb (500 μg) was administered i.p.

†Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2 x 10^6 cells/ml with anti-CD3 (2c11) for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5 x 10^6 cells/ml in CM supplemented with 10 IU/ml IL-2. A total of 35 x 10^6 and 70 x 10^6 cells were adoptively transferred into C57BL/6 mice bearing 3-day D5 pulmonary metastases. A total of 90,000 IU IL-2 was administered i.p. daily for 3 days after transfer of effector T cells.

Values of p < 0.01 compared with IL-2-only treatment group.
with either anti-CD3 or anti-CD3/CD28 appeared to be equally effective on a per cell basis, stimulation with both anti-CD3 and anti-CD3/CD28 appeared to double the effector T cell yield.

Costimulation by either CD80 or CD86 is critical for priming therapeutic T cells

One major consequence of the interaction between CD40 and CD40L expressed on dendritic cells and CD4 T cells is the up-regulation of CD80 and CD86 expression on the dendritic cell surface. Engagement of CD28 and CD80, or CD86, has been shown to be the critical costimulatory signal for in vivo T cell activation in many different Ag systems. However, there are exceptions. For example, generation of the primary CTL response to lymphocytic choriomeningitis virus (LCMV) has been shown to be CD40L independent (21–23, 26, 27). To investigate the contribution of CD80 and CD86 to T cell priming in our model, we vaccinated mice in which CD80 and CD86 were blocked alone or in combination with Abs to mouse CD80 (10-A1) and/or CD86 (GL1). The ability to generate therapeutic T cells was not affected by blocking either CD80 or CD86 alone. Complete tumor regression was observed in mice receiving effector T cells generated from either control mice or mice blocked with anti-CD80 or anti-CD86 in three of three experiments (Table III). However, when mice were vaccinated in the presence of both anti-CD80 and anti-CD86 Abs, therapeutic T cells were not generated by in vitro activation and expansion of these TVDLNs (Table IV). This strongly suggested that costimulation via CD28, by CD80 or CD86, is critical for the priming of therapeutic T cells, even in a situation that does not require interaction between CD40L and CD40.

Blocking costimulatory molecules reduces expression of T cell activation markers

If treatment with Abs to both CD80 and CD86 prevented T cell priming in TVDLN, we postulated that the expression of the early T cell activation marker, CD69, would be affected. Therefore, the expression of CD69 was analyzed by flow cytometry. The average percentage of CD69+ T cells in naive LNs in three independent experiments was 6.9%. In TVDLNs from D5-G6-vaccinated mice, the average percentage of CD69+ T cells increased to 12.3% (Fig. 1). This represented a significant increase compared with naive mice (p < 0.05). Blocking both CD80 and CD86 resulted in levels of CD69 expression on CD8+ T cells that were equal to that expressed by naive lymphocytes. Blocking CD80 or CD86 alone had little effect on CD69 expression in vaccinated mice. Furthermore, treatment with anti-CD40L Ab did not affect the expression of CD69 on T cells. These results are consistent with the notion that CD28, but not CD40 signaling, is critical for T cell priming in our model.

IL-12 and TNF are required for priming therapeutic T cells in MHC class II-deficient mice

Previously, we showed that D5-G6 was able to prime therapeutic CD8 effector T cells in MHC II KO mice (15). Ag-loaded MHC II-deficient dendritic cells have been shown to be ineffective vaccines unless pretreated with CD40L (28). Because CD40L is found on mast cells (24), eosinophils (29), and platelets (30), we wanted to rule out the possibility that these cell types could provide CD40 costimulation in MHC class II-deficient mice. Therefore, we performed vaccination and adoptive immunotherapy in MHC class II KO mice that were treated with anti-CD40L blocking Ab. Data presented in Table V clearly demonstrate that CD40L is not required for the generation of therapeutic T cells in the MHC II KO mice. We also examined the requirement for IL-12, because its production by dendritic cells has been shown to be essential for the generation of antitumor immunity (4, 31). Treatment of mice

<table>
<thead>
<tr>
<th>Vaccination</th>
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<tr>
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<td>CD40L KO</td>
<td>CD3/CD28</td>
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</table>

Values of p < 0.01 compared with IL-2-only treatment group.

Table III. Anti-CD80 or anti-CD86 does not inhibit T cell priming

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</thead>
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<td>Host</td>
<td>Ab</td>
<td>T cells</td>
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<tr>
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<td>35</td>
</tr>
<tr>
<td>wt</td>
<td>Anti-CD86</td>
<td>70</td>
</tr>
</tbody>
</table>

Values of p < 0.01 compared with IL-2-only treatment group.
CD4-independent priming of therapeutic T cells, soluble TNFR I, CD40L were from one experiment. Expression on CD8 T cells was analyzed by vaccination and 3 days later. TVDLN were harvested on day 8, and CD69 anti-CD80, anti-CD86 Ab, or a combination of both Abs on the day of cinemation with D5-G6 tumor cells and treated with nothing, anti-CD40L, RANK-IgFc is able to neutralize LIGHT signaling (42, 43). RANK-IgFc is able to neutralize RANK ligand signaling (32). Although the number of TVDLN and the presence of dendritic cells in the spleen (38–34)–I and RANK have been implicated in dendritic cell activation (32, 33). Specifically, the following candidates were examined: TNFR I, lymphotixin (LT)-βR, and RANK. Both TNFR I and RANK have been implicated in dendritic cell activation (32, 34–37), while LT-βR signaling is critical for LN organogenesis and the presence of dendritic cells in the spleen (38–41).

To determine whether any of these molecules are involved in CD4-independent priming of therapeutic T cells, soluble TNFR I, LT-βR, or RANK human IgFc fusion proteins were used to prevent signaling by TNF-α, LT-αβ, LT-αβ, LIGHT, and RANK ligand (TNF-related activation-induced cytokine). The soluble TNFR I human IgFc protein will block both TNF-α and LT-α signaling, while soluble LT-βR-IgFc blocks both LT-αβ and LIGHT signaling (42, 43). RANK-IgFc is able to neutralize RANK ligand signaling (32). Although the number of TVDLN cells recovered from MHC II KO mice treated with these blocking agents was not different from the control IgG-treated group (data not shown), a reduction in the percentage of mature dendritic cells (CD11c and CD80 or CD86 positive) was observed in TVDLN from mice treated with soluble TNFR I-IgFc (7.8%) compared with control human IgG (11.04%), LT-βR-IgFc (11.08%), or RANK-IgFc (11.05%)-treated mice. As a point of reference, mature dendritic cells make up ~1% of the cells from a naive LN.

Unlike treatment with both anti-CD80 and anti-CD86 Abs, the expression of early T cell activation markers (CD69, CD25, or OX40) was not different among control and treated groups (data not shown). Effector T cells were generated from each group as described, and their therapeutic activity was determined by adoptive transfer into wt mice bearing 3-day established pulmonary metastases. The T cells generated from vaccinated mice that received soluble TNFR I-IgFc fusion protein failed to mediate tumor regression, while T cells generated from vaccinated mice receiving LT-βR or RANK Fc fusion proteins were highly therapeutic (Table VI). These data suggest that the TNFR, rather than CD40 signaling, is critical for cross-priming of therapeutic T cells in MHC class II-deficient mice vaccinated with GM-CSF-producing tumor cells. However, it is difficult to appreciate how this happens given the mAb against the IL-12 p70 heterodimer following vaccination with D5-G6 completely prevented the priming of therapeutic cells (Table V).

Because something other than CD40L must have been inducing costimulatory molecules and triggering IL-12 production by dendritic cells in MHC II KO mice, we sought to determine whether another member of the TNFR superfamily could replace the function of CD40 in our model. We focused on the TNFR superfamily because recent reports have suggested that its members can enhance dendritic cell function as well as T cell expansion and growth (32, 33). Specifically, the following candidates were examined: TNFR I, lymphotixin (LT)-βR, and RANK. Both TNFR I and RANK have been implicated in dendritic cell activation (32, 34–37), while LT-βR signaling is critical for LN organogenesis and the presence of dendritic cells in the spleen (38–41).

Table IV. Anti-CD80 and anti-CD86 inhibit T cell priming

<table>
<thead>
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<th>Vaccination</th>
<th>Immunotherapy</th>
<th>Mean No. of Pulmonary Metastases (SE)</th>
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<td>T cells&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>wt</td>
<td>Anti-CD80 and 86</td>
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<td>Anti-CD80 and 86</td>
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<td>wt</td>
<td>Anti-CD80 and 86</td>
<td>70 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice were vaccinated with a s.c. dose of 1 × 10<sup>8</sup> DSG6 tumor cells.

<sup>b</sup> Two days before and three and five days after vaccination, 100 μl of a combination of anti-CD80 and anti-CD86 mAb (mouse B7.1 clone 16-10A1 ascites and mouse B7.2 clone GL1 1/10 dilution in HBSS) or 500 μg of control rat IgG plus 500 μg of hamster IgG was administered i.p.

<sup>c</sup> Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2 × 10<sup>6</sup> cells/ml with anti-CD3 (2C11) for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5 × 10<sup>5</sup> cells/ml in CM supplemented with 10 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day D5 pulmonary metastases. A total of 90,000 IU rIL-2 was administered i.p. daily for 3 days after transfer of effector T cells.

<sup>d</sup> Values of p < 0.01 compared with IL-2-only treatment group.

Table V. IL-12, but not CD40L, is required for T cell priming in MHC II KO mice

<table>
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<tr>
<th>Vaccination</th>
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<td>MHC II KO</td>
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<td>IL-12 p70</td>
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<sup>a</sup> C57BL/6 MHC II KO mice were vaccinated with a s.c. dose of 1 × 10<sup>8</sup> DSG6 tumor cells.

<sup>b</sup> On the day of vaccination, and 3 days later, mice were treated with 50 μl of anti-CD40L ascites, anti-IL-12 p70 ascites, 500 μg of control rat Ig, or 500 μg of hamster Ig.

<sup>c</sup> Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2 × 10<sup>6</sup> cells/ml with anti-CD3 (2C11) for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5 × 10<sup>5</sup> cells/ml in CM supplemented with 10 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day D5 pulmonary metastases. A total of 90,000 IU rIL-2 was administered i.p. daily for 3 days after transfer of effector T cells.

<sup>d</sup> Values of p < 0.01 compared with IL-2-only treatment group.
only a slight decrease in the number of dendritic cells and the similarities in the T cell activation phenotype of TVDLN from animals treated with TNFR I-IgFc fusion protein.

We hypothesized that treatment with TNFR I-IgFc fusion protein might have interfered with priming of therapeutic CD8+ T cells by blocking production of IL-12 in the TVDLN. To address this, we examined day 8 TVDLN for the presence of mRNA for IL-12 p35 and p40. Although naive LN cells express a very low level of message for p40 and message for p35, day 8 TVDLN exhibited substantial mRNA for both p35 and p40. Surprisingly, we saw no difference in the level of IL-12 mRNA in TVDLN from control mice or animals treated with TNFR I-IgFc fusion protein (data not shown). Thus, the ability of TNFR I blockade to eliminate the priming of T cells with therapeutic activity cannot be explained by an effect on IL-12.

Costimulation by CD80 and CD86 is not required for T cell effector function

Recently, we have reported that the adoptive transfer of in vitro activated TVDLN from D5-G6-vaccinated mice can mediate regression of pulmonary metastases directly, without participation of host lymphocytes (15). However, we could not differentiate between the direct cytotoxicity of transferred T cells and the indirect activation of host phagocytes, such as macrophages, as the final effector mechanism responsible for tumor cell killing. Our data indicate that adoptive transfer of CD8+ effector T cells can induce tumor regression in tumor-bearing MHC class I- and class II-deficient mice, thereby implying that direct killing mechanisms are most likely operational in our model. Although we demonstrated that costimulation by either CD80 or CD86 is critical for the priming phase of therapeutic T cells in this model, the role for costimulation during the effector phase of T cell-mediated tumor regression is not known. Where it has been studied, the requirement for costimulation during the effector phase has been reported to vary among systems (44–51). To address the contribution of CD80 and CD86 in the effector phase of our adoptive immunotherapy model, therapeutic T cells were transferred into tumor-bearing mice that received either control Ig, or anti-mouse CD80 and anti-mouse CD86 mAbs, before and following T cell transfer. In each case, the T cells were highly therapeutic, suggesting that CD80 and CD86 play a minimal role, if any, in the effector phase of our model (Table VII).

Discussion

Our discovery that priming of therapeutic T cells occurs normally in both CD40L KO mice and mice receiving anti-CD40L blocking mAb demonstrates that priming of therapeutic CD8+ T cells by D5-G6 tumor cells does not require interaction between CD40 and CD40L. This is consistent with our previous report that CD4+ T cells, which express the CD40L, are not necessary in this model (15). Priming of therapeutic T cells was also normal in mice in which costimulation by either CD80 or CD86 was blocked by mAb treatment. However, complete inhibition of priming was observed if CD28 signaling was prevented by administration of mAbs to both CD80 and CD86. This suggests that costimulation via the CD28 pathway is critical for priming of therapeutic T cells, even when a CD40 signal is not required. Furthermore, blocking experiments showed that IL-12 and TNFR signaling are critical for priming therapeutic T cells in CD4-deficient MHC II KO mice, suggesting that TNFR signaling can replace CD40 to activate APCs in our model. In contrast to the priming phase, CD80 and CD86 costimulation is not important during the effector phase of tumor regression, because complete tumor regression occurred in mice receiving T cells in the presence of mAbs to both CD80 and CD86.

The lack of a strong antitumor T cell immune response has been attributed to the lack of expression of CD80 and CD86 on tumor cells. A large number of studies have shown that increased antitumor T cell responses to tumor Ags can be achieved by expressing CD80 or CD86 on tumor cells (52–54). The efficacy of this approach varies between different tumor models, but the consensus is that protective immune responses can be augmented by exogenous expression of costimulatory molecules only in immunogenic tumor models (55). These findings support a role for tumor cells in direct presentation to T cells. However, most studies show that host professional APC rather than tumor cells are the major presenters of tumor Ags. Therefore, several laboratories have focused on inhibiting CTLA-4 (56) or enhancing CD80 and CD86 expression on dendritic cells to improve antitumor immunity (4, 16, 18).

Recently, Lu et al. (57) have demonstrated that CD4-dependent cross-priming of CTL can occur in a CD40/CD40L-independent fashion. Zhan et al. (58) recently reported that CD4-independent priming of allogenic tumor-specific CTL is both CD40L and CD28 costimulation dependent. Our data demonstrate that CD4-independent priming of therapeutic CD8+ T cells by a GM-CSF-producing tumor vaccine is CD40L independent, but CD28 dependent. Therefore,
cross-priming of CD8+ effector T cells can be either: 1) dependent on both CD4 Th cells and CD40L; 2) CD4 Th cell dependent, but CD40L independent; 3) CD4 Th independent and CD40L dependent; or 4) independent of both CD4 Th cells and CD40L. In most cases, costimulation via either CD80 or CD86 is critical for cross-priming of CD8+ effector T cells, regardless of whether CD4 Th cells or CD40L are required. However, the Th-independent primary CTL response to LCMV has been found to be both CD40L and CD28 independent (21–23, 26). This observation may be explained by the possibility that a prolonged signal 1 can overcome the requirement for signal 2 (27).

Upon infection with LCMV, the maturation of dendritic cells was found to be CD40 independent while CD8 T cell dependent (59). Consistent with the hypothesis that the strength of signal 1 determines the requirement for costimulation and CD4 Th cells, Franco et al. (60) recently reported that high affinity, but not low affinity MHC class I peptide was able to prime CTLs without CD4 Th cell help. Taken together, there is no generalized requirement for CD4+ T cells and costimulation in CTL cross-priming; the requirement is influenced by multiple factors, including the strength of signal 1 and the cytokine environment.

In this study, we also reported that TNFR and IL-12, but not RANK and LT-βR, are involved in the CD4-independent priming of therapeutic CD8 T cells in mice vaccinated with a GM-CSF-transduced tumor vaccine. A similar requirement for TNFR and IL-12 has also been shown for priming therapeutic antitumor T cells by dendritic cells pulsed with tumor peptides (31). The critical role for TNFR signaling in cross-priming may be explained by either a direct effect on T cells (61, 62), or indirect effect on dendritic cells, including enhanced induction of IL-12 production (63–65). This latter explanation seems less likely given our observation that a similar number of T cells with an activated phenotype, and thus presumably responding to tumor, were recovered from TVDLN of both control and TNFR I-IgFc-treated animals. This argument is further buttressed by the detection of similar levels of IL-12 mRNA in TVDLN of both control and TNFR I-IgFc-treated animals.

A more critical question should be how GM-CSF transfection of tumor cells leads to the activation of dendritic cells for the production of IL-12 and TNF. Unfortunately, our studies have not been able to identify which component is responsible for activating the dendritic cells in this model in which high levels of GM-CSF are secreted by the vaccine. In this regard, the following postulation may be feasible. The most likely suspects responsible for activating the dendritic cells in this model are heat shock proteins (hsp). Cross-priming of CTLs by tumor vaccines has been shown to occur through dendritic cell uptake of either apoptotic cells (2, 66) or hsp released from necrotic tumor (67–71). Interestingly, the hsp gp96 can mediate dendritic cell maturation in a CD4-independent fashion (69, 72), and induce inflammatory cytokines such as IL-12 and TNF-α (70, 71). Further studies will be required to directly investigate whether hsp actually function as an interplayer between GM-CSF transfection and dendritic cell activation. The similarities between the requirements for costimulatory molecules and inflammatory cytokines in our model and that for cross-priming by hsp are so striking that it raises the intriguing possibility that priming by a whole cell vaccine may occur through the hsp presentation pathways. In fact, overexpression of hsp70 on B16 melanoma cells not only induced MHC class I expression on the tumor cell surface, it also rendered the B16 melanoma immunogenic (73–75). Combining this with the capacity of hsp gp96 to induce IL-12 and TNF-α (70, 71) provides a possible pathway for...
CD4-independent cross-priming of tumor-reactive CD8 T cells by dendritic cells (76).

Finally, our studies in animals deficient of MHC class II-restricted T cells or lacking CD40L are likely to be important given the observations that tumor-bearing mice and some humans have significant defects in their CD4 T cell populations. Therefore, dissecting the critical elements for CD4-independent priming of therapeutic CD8+ T cells may provide a basic paradigm for a therapeutic vaccine strategy for patients with cancer. These findings summarized in Fig. 2, outline the critical signaling pathways that are operational in this mouse model. It is interesting to note that all three appear to exert their effect directly on the effector T cell, and interfering with any one eliminates the generation of therapeutic CD8+ T cells. Clearly, the costimulatory and maturational effects of these molecules play an essential role in antitumor immunity. These findings provide a strong rationale for determining the level of expression of these molecules in TVLN of patients on clinical vaccine trials. Characterization of these parameters, in addition to monitoring for tumor-specific T cell responses, may provide correlates for objective clinical responses as well as critical insights into possible reasons that vaccines fail. If these characterizations identify defects in CD80/CD86 expression, IL-12 or TNF production, in addition to deficiencies in CD4 T cells, strategies to overcome the defect(s) or supply the appropriate signals will need to be pursued. However, even if CD4-independent cross-priming of therapeutic CD8+ T cells occurs, the effect may not prove curative if CD4 help cannot be induced to maintain the tumor-specific CD8 memory response (15).

Overall, our results suggest that in patients in which CD4 responses may be inadequate or deficient, the priming of therapeutic tumor-reactive CD8 T cells can be achieved by augmenting host APC function via local production of GM-CSF. Alternatively, increasing the concentration of peptides chaperoned by hsp may also prime therapeutic CD8+ T cells in the absence of an optimal CD4 response. We are currently engaged in clinical trials of both strategies.

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References


