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SHORT COMMUNICATION

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Generation of protective immunity against an immunogenic carcinoma requires CD40/CD40L and B7/CD28 interactions but not $CD4^+$ T cells

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Abstract Interactions between CD40 and CD40L play a central role in the regulation of both humoral and cellular immunity. Recently, interactions between these molecules have also been implicated in the generation of protective cell-mediated tumor immunity. We have generated a tumor model in which a well-understood and clearly immunostimulatory antigen, influenza hemagglutinin has been transfected into the BALB/c-derived, MHC-class-I-positive, B7-deficient murine mammary carcinoma, MT901. In this model, expression of the influenza hemagglutinin antigen does not alter tumorigenicity in naïve but serves as a tumor-rejection target in immunized mice. T-cell-depletion experiments indicate that successful tumor protection can occur following immunization in mice depleted of $CD4^+$ but not $CD8^+$ T cells, suggesting that tumor protection is largely CD8-mediated and CD4-independent. Interestingly, despite the ability of tumor protection to be generated in the absence of $CD4^+$ T cells, effective immunization was clearly dependent on CD40/CD40L as well as CD28/B7 interactions.

Key words Tumor immunity · CD40/CD40L · B7/CD28 · Lymphocyte depletion

Introduction

Experimental evidence has suggested a variety of mechanisms for the failure of tumors to generate effective immune responses, including (1) failure of the tumor to express immunostimulatory antigens, (2) active tumor-mediated suppression of the immune response, (3) defective homing of effector cells to the tumor site, and (4) inadequate antigen presentation and/or costimulation for any existing immunogenic antigen [22]. As it has become increasingly clear that most tumors express antigens to which immune responses can be elicited, including some that are also expressed on normal tissues, the role of costimulation in the generation, or prevention, of tumor-specific immune responses has become of particular interest. A number of recent studies have emphasized that providing enhanced costimulation can significantly improve tumor-specific immune responses. Transfer of B7.1 or B7.2 molecules into tumor cells has generated protective and sometimes curative immunity in a number of mouse models and blockade of B7/CD28 interactions has abrogated protection in other models [7, 17].

The interaction between CD40 and CD40L (CD154), like the interaction between the B7.1 and B7.2 molecules with their T-cell-expressed ligand CD28, has been recognized as an important element in the generation of cell-mediated as well as humoral immune responses. CD40/CD40L interactions are critical for the expansion of $CD4^+$ helper and $CD8^+$ cytotoxic T cells as well as the activation/maturation of B cells and dendritic cells [9, 13, 14]. Ligation of CD40 has also been shown to upregulate the secretion of an array of inflammatory cytokines including tumor necrosis factor, interleukin-8 (IL-8) and the Th-1-promoting cytokine IL-12 [9]. In the context of tumor immunity, transfection of tumors to express CD40L has been shown to provide potent antitumor effects reminiscent of those described for B7.1 and B7.2 transfectants, a finding that is not surprising given that the signaling through CD40 induces an increase in the expression of B7-1 and B7-2 on the surface of B cells, macrophages and dendritic cells [9]. Similarly, several experiments blocking CD40/CD40L interactions, or using CD40-deficient mice have found substantial impairment in the generation of tumor-specific immunity [12, 14].

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We have transfected a weakly immunogenic, BALB/c-derived, $B7^-$. ICAM-1^-, class I^+ tumor cell line, MT901, with the influenza hemagglutinin (HA) antigen. In this tumor model expression of HA does not significantly alter tumor growth in naïve but does serve as a rejection antigen in mice previously immunized with either irradiated HA-expressing tumor cells or influenza virus. Although $CD4^+$ T cells appear to enhance the protective immunity elicited upon immunization, substantial tumor immunity can be established in $CD4^+$ T-cell-depleted mice. However, despite the apparently limited involvement of $CD4^+$ T cells in this immunity, blockade of either CD40/CD40L or B7/CD28 interactions at the time of immunization prevented the development of tumor immunity.

Materials and methods

Animals

BALB/c mice were purchased from Harlan Laboratories (Madison, Wis.). Athymic nude and mice with severe combined immunodeficiency were obtained from The Jackson Laboratory (Bar Harbor, Me.). All mice were maintained in pathogen-free conditions and were used for experiments at 8–12 weeks of age.

Immunofluorescence and flow cytometry

Cultured tumor cells were incubated with biotinylated mAb specific for $H\text{-}2K^d$, $H\text{-}2D^d$, CD40L, B7.1, B7.2, $I\text{-}A^d$ (Pharmingen, San Diego, Calif.), or the H36-4-5.1 mAb specific to the Sb region of the hemagglutinin gene of the influenza virus A/PR/8/34 (H1N1) [23] (kindly provided by Dr. Walter Gerhard, Wistar Institute Philadelphia, Pa.) followed by steptavidin-linked fluorescein isothiocyanate (FITC) or streptavidin-linked phycoerythrin (Pharmingen) in phosphate-buffered saline (PBS) containing 5% fetal calf serum. Cells were analyzed by fluorescence-activated cell sorting using a FACScan or FACScalibur and CellQuest software (Becton Dickinson, Sunnyvale, Calif.).

Plasmid constructs

To construct the pCMV5-HA vector, the HA gene of influenza virus A/PR/8/34 (H1N1) was excised using the *Hin*dIII and *Kpn*I sites from the pT3PR8HA plasmid (kindly provided by Dr. Peter Palese, Mt. Sinai School of Medicine, New York, N.Y.) and ligated into the same sites in the polylinker of Bluescript KS+ (Stratagene, La Jolla, Calif.) to introduce flanking *Bam*HI restriction sites on either side of the HA gene insert. The HA gene was then excised a second time, using the *Bam*HI sites, and ligated into the same site immediately downstream of the cyclomegalovirus (CMV) promotor in the pCMV5 vector [1] (kindly provided by Dr. William Lee, University of Pennsylvania, Philadelphia, Pa.). The orientation of the gene was verified by restriction digest mapping. The pLXSP vector (kindly provided by Dr. William Lee) was previously created by substitution of the puromycin-resistance gene from pBABEpuro [16] for the neomycin-resistance gene in the pLXSN vector [15].

Cell lines and transfections

MT901 (MT-WT) is a weakly immunogenic, BALB/c-derived mammary carcinoma line [2], kindly provided by Dr. Alfred Chang, (University of Michigan, Ann Arbor, Mich.). CT26 is a moderately immunogenic colonic epithelial tumor derived from BALB/c mice [8] and was kindly provided by Dr. William Lee. To generate HA-expressing MT901 clones, pCMV5-HA was cotransfected with the pLXSP plasmid vector (to allow puromycin selection) using Lipofectamine (Life Technologies). A clone that maintained HA expression as well as class I, $H\text{-}2K^d$ and $H\text{-}2D^d$ expression under non-selecting conditions, as determined by FACS analysis, was expanded for use in these studies. For use as control cells, MT901 cells were transfected with pCMV5 (without the HA gene insert) and pLXSP as above and selected using 4 μg/ml puromycin. Cells from these lines, designated MT-CT, were plated at limiting dilution under puromycin selection to generate clonal populations.

Inoculations and immunizations

For primary tumor challenges, tumor cells suspended in 100 μl PBS were inoculated subcutaneously into either the right or left hindquarter of the mouse. Onset of tumor growth was scored by the presence of a palpable lump near the site of inoculation. Tumor growth was monitored at 2- to 5-day increments after inoculation by measurement in two perpendicular dimensions with a vernier caliper and the size recorded as an area. For immunization studies, mice were injected subcutanously as indicated with 10^6 irradiated (50 Gy) MT-HA cells or the control (MT-CT or MT-WT) cells in 0.1 ml PBS, as described above, or given an intranasal inoculation of 20 HAU influenza virus, A/PR/8/34 (H1N1), as allantoic fluid mixed with PBS (20 μl total volume). Mice were rechallenged by subcutaneous inoculation of non-irradiated tumor cells into the opposite flank from the immunization site as indicated, 14–42 days after immunization. Onset of tumor and tumor growth were measured as described above.

Costimulation blockade and depletion of T cell subsets in vivo

Hybridomas producing rat IgG2b mAb directed against murine CD4 (GK1.5), CD8 (2.43) antigens were obtained from the American Type Tissue Collection (Rockville, Md.). The mAb were produced as ascities and purified on protein A columns. Purified rat or Armenian hamster IgG (Jackson Immunoresearch, West Grove, Pa.) was used for the control. For CD4 and CD8 depletions, mice were given intraperitoneal (i.p.) injections of 150 μg mAb in 150 μl PBS for 3 consecutive days followed by administration of an additional 150 μg mAb every 4 days. Mice treated with this regime showed less than 2% $CD4^+$ or $CD8^+$ T cells respectively in their spleens for at least 4 days following cessation of treatment, as measured by antibody staining and flow cytometry analysis. To block the CD40/CD40L pathway, mice received i.p. injections of 200 μg the anti-CD40L mAb, MR-1 (TSD Bioservices, Germantown, N.Y.), administered in 200 μl PBS either on the day of tumor immunization or tumor challenge as indicated. This was followed by further i.p. injections of 200 μg and 100 μg 2 days and 5 days later respectively. To block the CD28/B7 pathway, mice received i.p. injections of 200 μg recombinant human CTLA4-Ig (kindly provided by Dr. Robert Peach, Bristol Myers Squibb, Princeton, N.J.) on the day of tumor inoculation and again 2 days later.

Results

HA expressed on MT901 cells does not diminish tumorigenicity but does serve as a rejection antigen in immunized mice

MT901 is a weakly immunogenic BALB/c mammary carcinoma that expresses MHC class I molecules, but does not detectably express MHC class II, B7, ICAM-1 or CD40L molecules [2]. As described in Materials and

methods, we transfected the MT901 tumor line either to express the influenza hemagglutinin as a model, strong tumor antigen (MT-HA) or with the control, empty vector (MT-CT) and compared the tumorigenicity of these cells to that of the parental line. As anticipated both MT-HA and MT-CT clones remained MHC class II^-, $B7^-$, ICAM-1^- and $CD40L^-$ and retained similar levels of class I expression to the parental (MT-WT) line (data not shown). As has been described with other tumor lines that have been transfected with strong antigens [11, 18], expression of the HA antigen by the MT901 tumor had little effect on tumorigenicity. BALB/c mice given subcutaneous rear-hindquarter inoculations of 10^6, 10^5, or 10^4 (the minimum reliable tumorigenic dose) HA-transfected (MT-HA), parental (MT-WT), or control empty-vector-transfected (MT-CT) tumor cells all exhibited indistinguishable times to tumor onset and tumor growth rates (the median time in days to tumor onset with 10^6 cells was 7.7 ± 1.9 for MT-HA, 7.1 ± 1.3 for MT-WT, and 7.0 ± 0 for MT-CT). Excised tumors remained strongly positive by FACS analysis for HA expression for at least 18 days after inoculation (data not shown), indicating that the HA antigen, while maintained on the tumor cells in vivo, had no inherent effect on tumorigenicity.

While the HA antigen did not effect tumorigenicity, it did serve as a tumor rejection antigen in immunized mice. As shown in Table 1, 100% and 83% of mice immunized with irradiated MT-HA cells or influenza virus respectively were protected against subsequent challenge with viable MT-HA cells (compared with less than 10% of mice similarly immunized and challenged with MT-WT or MT-CT cells). Protection was HA-specific, as immunization with MT-HA cells did not provide any significant cross-protection (compared to using MT-CT or MT-WT cells) against HA-negative tumor challenges nor did immunization with irradiated MT-WT or MT-CT cells provide cross-protection against challenge with MT-HA cells (compared to immunization/challenges using only MT-WT and MT-CT cells).

Table 1 Tumor incidence following immunization. BALB/c mice were either not immunized or immunized as described in Materials and methods, using either irradiated tumor cells or influenza virus as indicated. Two weeks later, mice were challenged with a subcutanous inoculation of 10^6 MT-HA or control (MT-WT and MT-CT) cells and followed for the onset of tumor growth. The percentage of mice that remained tumor-free for at least 30 days is shown. $n \geq 9$ except where indicated

Immunization	Tumor incidence (%) following challenge with:	
	Control cells	MT-HA cells
None	100	100
Control cells	90.5	92.3
MT-HA cells	88.9	0
Influenza virus	100[a]	16.7

[a] $n = 4$

Rejection of tumors is CD8-mediated and can occur in CD8-depleted mice

To evaluate the importance of $CD4^+$ and $CD8^+$ T cells in generating tumor protection following MT-HA immunization, mice were treated with CD4- or CD8- depleting mAb either before immunization of naive animals with irradiated MT-HA tumor cells or before challenge of immunized mice with non-irradiated MT-HA tumor cells. Depletions were maintained by administration of the mAb every fourth day until 16 days after tumor challenge. As shown in Fig. 1A, B, all mice depleted of $CD4^+$ T cells at the time of tumor challenge, and roughly two-thirds of mice depleted prior to immunization, were able to reject MT-HA tumor challenge. In contrast, all mice depleted of $CD8^+$ T cells, whether this was begun prior to immunization or at the time of tumor challenge, were unable to reject the tumor. These results demonstrate that (1) effective tumor inhibition required the $CD8^+$ T cells to be present during both priming and effector phases of the immune response and (2) MHC-class-II-restricted presentation of HA peptides was not essential for effective immune responses toward MT-HA tumors.

Protection in tumor-immunized mice requires CD28 /B7 and CD40/CD40L interactions

Having determined that HA could serve as a target for CD8-mediated tumor-rejecting immune responses, we then sought to examine the costimulatory requirements of this response. As shown in Fig. 2A, disruption of either B7/CD28 interactions or CD40/CD40L interactions by treatment of mice with CTLA4Ig or anti-CD40L mAb respectively during the immunization phase prevented the development of tumor immunity. In contrast, treatment of MT-HA-immunized mice with CTLA4Ig or anti-CD40L mAb at the time of tumor rechallenge did not significantly abrogate tumor immunity (Fig. 2B). These results suggest that both the B7/CD28 and CD40/CD40L interactions are required for successful immunization, but not for effector function after immunity has been established.

Discussion

Tumor-associated-antigen-specific $CD8^+$ CTL can play a central role in establishing tumor immunity and causing tumor rejection [22]. In our model, tumor rejection was dependent on $CD8^+$ T cells, as depletion of these cells prior to either immunization or rechallenge with the MT-HA tumor resulted in a complete loss of protection. Although CD4-independent CD8-mediated T cell immunity has been described in several immune responsiveness models [5, 6, 24], the initial generation of $CD8^+$ CTL effectors in most models requires the presence of $CD4^+$ T cells [3, 10]. Thus it was somewhat

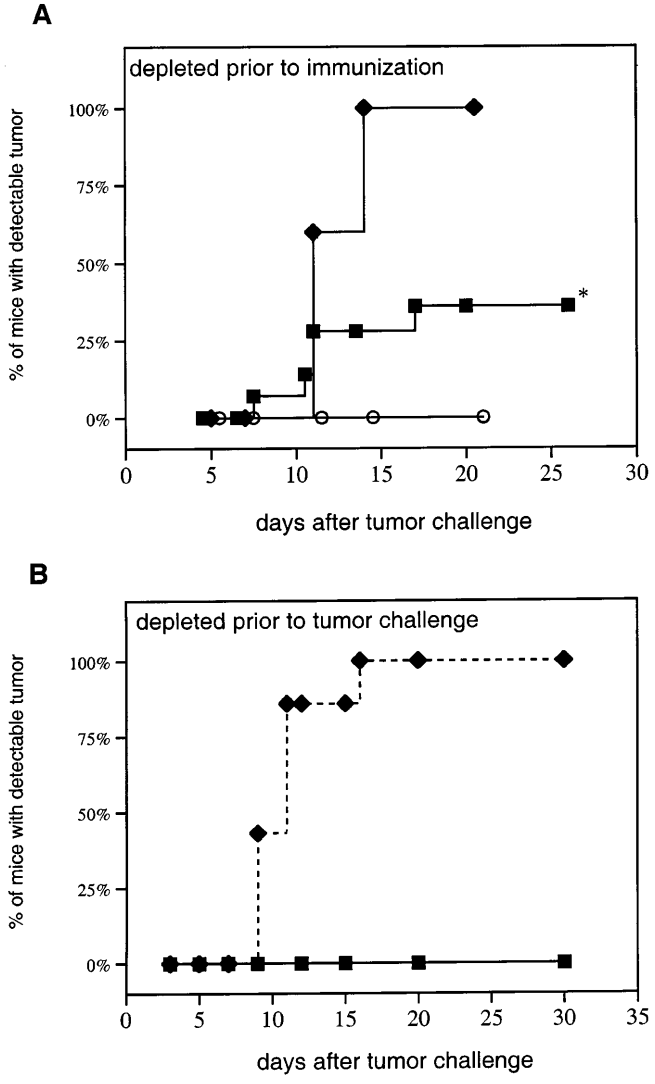

Fig. 1A, B Depletion of CD8 but not CD4 cells from MT-HA-immunized mice abrogates HA-specific tumor protection. **A** Mice were treated with mAb against CD4 or CD8 antigens, beginning 3 days prior to immunization with 10^6 irradiated (50 Gy) MT-HA cells, and maintained in a depleted state by administration of antibody every 4 days thereafter, as described in Materials and methods. Fourteen days after immunization, mice were challenged with 10^6 MT-HA cells in the opposite flank and scored for tumor onset every 2–3 days. * Eight mice from the CD4-depleted group were humanely killed between days 26 and 30, either for humanitarian reasons or for use in other experiments. Six tumor-free mice were followed for more than 40 days with no new tumor onset observed. **B** Mice were treated and tumor onset followed as above except that they were depleted of CD4 or CD8 cells, beginning 11 days after immunization with irradiated tumor cells (3 days prior to tumor challenge). ■ CD4 depleted ($n = 14$); ◆ CD8 depleted ($n = 5$); ○ control Ig treated ($n = 4$)

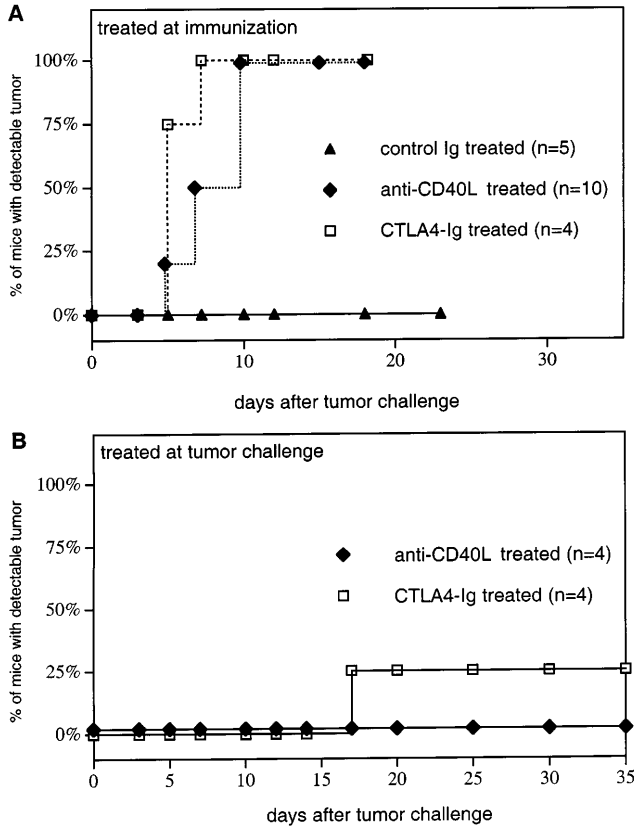

Fig. 2A, B HA-specific tumor protection requires costimulation. **A** BALB/c mice were immunized on day 0 with 10^6 irradiated (50 Gy) MT-HA cells administered subcutaneously to the left flank. On day 14, mice were challenged with 10^6 MT-HA cells and scored every 2–3 days for tumor onset. Anti-CD40L and mice treated with control Ig received three i.p. inoculations of MR-1 mAb or hamster IgG on days 0 (200 μg), 3 (200 μg) and 5 (100 μg) following tumor immunization. Mice treated with CTLA4 Ig received 200 μg recombinant murine CTLA4 Ig on days 0 and 2 following tumor immunization. **B** BALB/c mice were immunized and challenged with MT-HA cells as above. Anti-CD40L treatment was administered on days 14, 17 and 19 (0, 3 and 5 days respectively after tumor challenge) or CTLA4 Ig given on days 14 and 16 (0 and 2 days after tumor challenge). ■ CD4 depleted ($n = 7$); ◆ CD8 depleted ($n = 7$)

unexpected that nearly two-thirds of naive mice depleted of $CD4^+$ T cells prior to MT-HA immunization, as well as all of those depleted after immunization, were able to reject subsequent tumor rechallenge. It should be noted that 36% of mice depleted of $CD4^+$ T cells prior to immunization did subsequently develop tumors. Thus, $CD4^+$ T cells do appear to contribute to the establishment of immunity in this system. However, 64% of mice were able to reject tumor responses despite being depleted of $CD4^+$ T cells during both tumor immunization and challenge, strongly indicating that $CD4^+$ T cells are not obligatory for establishing the protected state. It is formally possible that our results simply reflect an incomplete depletion of $CD4^+$ T cells. However, we feel this is highly unlikely, as this method consistently depleted $CD4^+$ T cells to less than 2% of spleen cells in control CD4-depleted mice (unpublished observations). Instead, we believe that our results represent the CD4-independent generation of CD8 effector cells.

Given the ability of $CD8^+$ T cells to mediate tumor rejection in the absence of $CD4^+$ T cells in our model, we were surprised to find that anti-CD40L treatment during immunization was as effective as CTLA4 Ig treatment in preventing the generation of effective tumor

immunity. CD40/CD40L interactions have, like co-stimulatory interactions between B7 and CD28, been found to be important in the generation of tumor antigen-specific immune responses [12, 14]. However, unlike CD28, which is expressed on all T cells and can co-stimulate $CD8^+$ T cell activation, CD40L is principally expressed only on $CD4^+$ T cells and appears to act predominantly in antigen-specific activation of B cells and dendritic antigen-presenting cells [20, 21]. To our knowledge, these results mark the first demonstration of the involvement of CD40/CD40L interactions in a model where protective tumor immunity can be generated in the absence of $CD4^+$ T cells.

Although not expressed as prevalently as on $CD4^+$ T cells, inducible CD40L expression has been observed on activated $CD8^+$ T cells, human natural killer cells, mast cells, eosinophils, and B cells [9], suggesting that these cells might provide adequate CD40L stimulation in the absence of $CD4^+$ T cells. Recent experiments indicate that CD40 ligation on dendritic cells can enable these cells to directly activate naïve, antigen-specific $CD8^+$ CTL cells in the absence of $CD4^+$ T cells [4, 19, 21]. In these reports the dendritic cell "activation" was predominantly mediated by $CD4^+$ T cells, suggesting that $CD8^+$ T cell activation was temporally removed from, but not independent of, $CD4^+$ T cell help. However, it is worth noting that Ridge et al. found $CD8^+$ T cell responses to soluble antigens in some CD4-depleted mice, which disappeared when the source colonies of the mice were changed, suggesting that other environmental factors could play a significant role in activating dendritic cells in the absence of $CD4^+$ T cells [19]. In the context of immune responses to tumors, a recent report found that CD4-independent $CD8^+$ CTL responses occurred against a transfected tumor antigen when that tumor was administered by intradermal but not by intraperitoneal inoculation, suggesting that dendritic cells, as the predominant intradermal antigen-presenting cells, are capable of CD4-independent activation of $CD8^+$ CTL cells [5]. Given these findings, we feel that it is likely that dendritic cells are playing an important role in the generation of $CD8^+$ CTL cells in the absence of $CD4^+$ T cells in our model. Although unlikely, it is alternatively possible that blockade of CD40/CD40L interactions could serve to skew responses of $CD4^+$ T cells, if $CD4^+$ cells present during anti-CD40L treatments unexpectedly inhibited tumor rejection.

In summary, we have established a tumor model in which the influenza hemagglutinin molecule serves as a model tumor antigen on the weakly immunogenic MT901 tumor line. Expression of HA by MT901 does not significantly alter tumorigenicity but does serve as a tumor-rejection target for $CD8^+$ T cells in mice immunized against the HA antigen. Despite the little reliance of immunity on $CD4^+$ T cells in our model, the development of tumor immunity, but not the effector function of that immunity, was dependent on both B7/CD28 and CD40/CD40L interactions.

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