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CD40 Ligand-Dependent T Cell Activation: Requirement of B7-CD28 Signaling Through CD40

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The role of CD40 ligand (CD40L) in the primary activation of T cells is not clear. The cellular and humoral immune responses to adenoviral vectors in a murine model of liver-directed gene transfer were studied to define the mechanisms responsible for CD40L-dependent T cell priming. CD40L-deficient mice did not develop effective cytotoxic T cells to transduced hepatocytes, and T cell–dependent B cell responses were absent. Full reconstitution of cellular and humoral immunity was achieved in CD40L-deficient mice by administration of an activating antibody to CD40 that increased expression of B7.2 on spleen cells. Wild-type mice could be made nonresponsive to vector by administration of antibodies to B7. Thus, CD40L-dependent activation of T cells occurs through signaling of CD40 in the antigen-presenting cell to enhance requisite costimulatory pathways that include B7.

The role of CD40L in humoral immunity is illustrated by the multiple defects in B cell activation that characterize its genetic deficiency in mice and humans, including a failure to form germinal centers, activate memory B cells, and class switch (*1*). Experiments in knockout mice have implicated CD40L in the antigen-specific priming of T cells, although the precise mechanism by which this occurs is unclear (*2*). Enhanced susceptibility of CD40L-deficient ($CD40L^{-/-}$) mice to leishmania infection is consistent with an important role of this molecule in cellular immunity (*3*). The relation between CD40L and other T cell costimulatory pathways such as B7-CD28 is unclear; in models of allograft rejection, these signaling pathways appear uncoupled (*4*).

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Adenoviral vectors are being tested as a possible approach to gene therapy, but the cytotoxic T lymphocyte (CTL) and B cell responses to the viral proteins and transgene products make this strategy less tenable (*5*). Activation of $CD4^+$ T cells to input viral capsid proteins, which requires stimulation through the CD40L-CD40 and B7-CD28 pathways, is necessary for both the $CD8^+$ T cell (that is, CTL) and B cell (that is, neutralizing antibody) effects (*6*– *8*). If one could minimize the immune response, the adenovirus becomes a viable option. We have used this well-studied immune response to further elucidate the mechanisms of CD40L-dependent activation of T cells (*9*).

Infusion of adenovirus missing the early (E1) genes and containing the *lacZ* gene into C57BL/6 mice led to transgene expression in 93% of hepatocytes in liver harvested 3 days later that diminished to undetectable levels by day 24 (Table 1). Analysis of $CD4^+$ lymphocytes in vitro demonstrated activation of T helper (T_H) cells to viral antigens of both the T_H1 [that is, secretion of interferon-γ (IFN-γ) and interleukin-2 (IL-2)] and T_H2 (that is, secretion of IL-4 and IL-10) subsets (Fig. 1A) (*10*). Chromium release assays showed the presence of CTLs to viral-infected targets in splenocytes harvested 10 days after gene transfer (Fig. 1B) (*10*). Infusion of vector stimulated the development of germinal centers [20.4 ± 1.5 per section (*11*)] and the formation of antiviral antibodies of immunoglobulin M (IgM), IgG1, and IgG2 isotypes (Fig. 2) that are neutralizing (Table 1).

Similar studies performed in mice genetically deficient in CD40L demonstrated the requirement of CD40L-CD40 interactions in the full spectrum of cellular and humoral immune responses to adenoviral vectors in mouse liver; transgene expression was stable for 24 days in $CD40L^{-/-}$ mice (82% of hepatocytes still express *lacZ*, Table 1), and activation of CTLs to viral-infected cells was markedly blunted (*8*) (Fig. 1B). $CD4^+$ T cells harvested 10 days after gene transfer failed to respond to viral antigens; however, the basal secretion of cytokines was increased as compared with that observed in C57BL/6 mice (Fig. 1A). $CD40L^{-/-}$ mice failed to develop germinal centers [that is, no germinal centers were detected in four sections from two mice (*11*)] or neutralizing antibodies (Table 1). Antiviral IgM was formed, but class switch to IgG1 or IgG2a was virtually absent (Fig. 2).

Several models have been suggested to explain the dependent role of CD40L on T cell priming. A critical question is whether CD40L directly transduces an activating signal to the T cell at the time of engagement with its receptor CD40, or whether the role of CD40L is indirect, effecting T cell activation through CD40-mediated signaling in the antigen-presenting cell (APC) that leads to enhanced costimulation of downstream pathways. Previous in vivo studies in the $CD40L^{-/-}$ mouse documented a primary defect in T cell priming to soluble antigens without clarification of the mechanisms (*2*), although in vitro studies have shown that wild-type but not CD40L-deficient T cells activate costimulatory activity in B cells (*12*). Activation of CD40L with soluble CD40 in the $CD40L^{-/-}$ mice partially reconstituted formation of germinal centers, although isotype switching was not observed (*2*); thus,

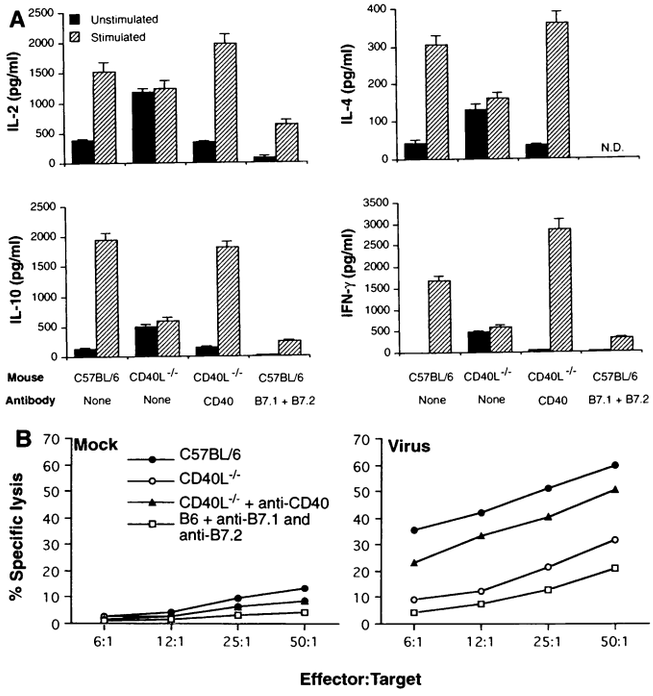

Fig. 1. T helper functions. (**A**) Splenocytes (6×10^6) harvested from mice 10 days after infection were cultured with (stimulated) or without (unstimulated) antigen (that is, *lacZ* virus) in 24-well plates for 48 hours. C57BL/6 mice were left untreated or treated with a mixture of antibodies to B7.1 and B7.2. CD40L-deficient ($CD40L^{-/-}$) mice were left untreated or treated with antibody to CD40 (anti-CD40). Culture supernatants were assayed for the production of IL-2, IL-4, IL-10, and IFN-γ (picograms per milliliter). (**B**) Splenocytes from the following mice—normal C57BL/6 (filled circles), $CD40L^{-/-}$ (open circles), $CD40L^{-/-}$ treated with anti-CD40 (filled triangles), and C57BL/6 (B6) mice treated with anti-B7.1 and anti-B7.2 (open squares) 10 days after administration of virus—were restimulated in vitro for 5 days and tested for specific lysis on mock-infected (left) or virus-infected (right) C57SV cells in a 6-hour ^{51}Cr release assay.

Table 1. Morphometric analysis of liver sections for transgene expression and neutralizing antibody titer. Data were quantified with Leica Quantimet 500+ by analyzing a total of 15 lobes from three mice for the *lacZ*-expressing hepatocytes at days 3 and 24 and are represented as the mean ± SD. Neutralizing antibody titer (NAT) was determined by assessing the ability of sera to block transduction of 293 cells with the E1-deleted *lacZ* virus. The data are represented as the reciprocal dilution of serum samples harvested at day 24 with the end point defined as the dilution that inhibits transduction by 50%.

Mice and treatment	*lacZ*-expressing hepatocytes (%)		NAT
	Day 3	Day 24	
C57BL/6 (B6)	93.2±1.6	0	640
$CD40L^{-/-}$	90.5±3.8	82.4±5.2	<20
$CD40L^{-/-}$ + anti-CD40	92.9±4.5	0	320
B6+anti-B7.1 and anti-B7.2	91.4±3.5	82.3±2.8	40

the costimulation of T cells through CD40L might activate T cells that help B cells to make mature antibody responses and to generate memory populations.

We used a model of adenoviral vectors in murine liver to further clarify the role of CD40L in T cell activation. The contribution of direct T cell activation by CD40L signaling was separated from its indirect activation through CD40 signaling in the APCs by administering repeated injections of an activating monoclonal antibody to CD40 (*13*) to vector-treated $CD40L^{-/-}$ animals. This resulted in the reconstitution of T cell–dependent B cell functions, including the formation of germinal centers [12.5 ± 1.2 per section (*11*)] and the isotype switch of antiviral antibodies from IgM to IgG1 and IgG2a (Fig. 2) that were neutralizing (Table 1). Direct analysis of T cell function confirmed that CD40 signaling reconstitutes antigen-specific T cell activation. The defects in $CD4^+$ T cell proliferation (Fig. 1A) and CTL function (Fig. 1B) observed in $CD40L^{-/-}$ mice were normalized in these animals after injection with the CD40-activating monoclonal antibody; furthermore, transgene expression was no longer stabilized, confirming the reconstitution of $CD4^+$ and $CD8^+$ T cell function (Table 1).

Our studies suggest that the requirement of CD40L for activation of T cells occurs through CD40-mediated signaling in the APC, presumably by modulating important downstream costimulatory pathways. One potential downstream event is the up-regulation of B7.2 on the APC, a step necessary for the priming of T cells through interactions of B7.2 with CD28 (*14*). Mononuclear cells harvested from spleen 10 days after vector administration were analyzed for expression of B7.2 by flow cytometry (Fig. 3). The number of B7.2-expressing cells increased from 0.4% (Fig. 3A) to 42% (Fig. 3B) after gene transfer in C57BL/6 mice.

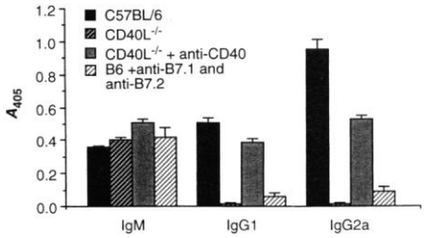

Fig. 2. The formation of antiviral antibody. Serum samples (diluted 1:200) from normal (C57BL/6) mice, $CD40L^{-/-}$ mice either untreated or treated with anti-CD40, and C57BL/6 mice treated with anti-B7.1 and anti-B7.2 were harvested 24 days after infection and analyzed for the presence of adenovirus-specific IgM, IgG1, and IgG2a by isotype-specific ELISA (*15*). Absorbance was measured at a wavelength of 405 nm (A_{405}) on a Bio-Rad model 450-microplate reader.

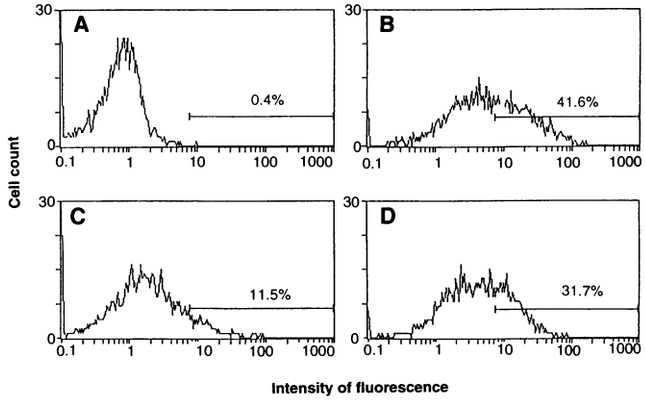

Fig. 3. Regulation of B7.2 expression. Single-cell suspensions of spleen were prepared from immunized (day 10) or naïve mice. Before staining, all cell preparations were spun through Ficoll-Hypaque. Cells were stained by incubation for 1 hour at 4°C with FITC-labeled anti-B7.2 (Pharmingen, San Diego, California) followed by washes. Cells were subsequently fixed in 1% formaldehyde in PBS and analyzed on a Coulter EPICS XL-MCL flow cytometer. A minimum of 10,000 cells were collected for each sample. The bar indicates the percent of B7.2-positive cells. Splenocytes were prepared from naïve mice (**A**) or immunized mice on day 10, including $CD40L^{+/+}$ (**B**), $CD40L^{-/-}$ (**C**), and $CD40L^{-/-}$ mice treated with anti-CD40 (**D**). Cells were stained with FITC-labeled anti-B7.2 and analyzed for B7.2 expression by fluorescence-activated cell sorting.

This activation was blunted to 12% of cells in CD40L$^{-/-}$ mice (Fig. 3C); activation of CD40 with antibody in the CD40L$^{-/-}$ mice increased B7.2 expression to 32% (Fig. 3D).

We studied the functional importance of B7-mediated costimulation of T cells to vector in wild-type C57BL/6 mice by injecting blocking antibodies to B7.1 and B7.2 at the time of vector administration. T cells from these animals failed to secrete T_H1- and T_H2-specific cytokines in response to antigen in vitro (Fig. 1A), and CTL activity to viral-infected targets was markedly diminished (Fig. 1B). This response was associated with the stabilization of transgene expression, with 82% of hepatocytes expressing *lacZ* at day 24 (Table 1). T cell–dependent B cell responses were also blunted, with diminished formation of germinal centers [20.4 ± 1.5 per section without anti-B7 and 2.5 ± 0.7 per section with anti-B7 (*11*)], less antiviral neutralizing antibody (Table 1), and a notable absence of class switching from IgM to IgG1 and IgG2a (Fig. 2). Another inhibitor of this pathway, CTLA4-Ig, has a similar effect on the cellular responses to vector in liver (*7*).

The well-characterized T and B cell responses to adenoviral vectors were useful in defining the biology of CD40L in T cell activation (*5*). Full immune competence was achieved in the absence of CD40L by activating CD40, thereby ruling out a direct effect of CD40L on the T cell. We show that CD40 signals an up-regulation of B7.2 on the APC that is necessary for T cell activation, presumably through its interaction with CD28. The interdependence of the CD40 and CD28 pathways in this system differs from the situation in models of allograft rejection where redundancies appear to exist (*4*). Our studies suggest that pharmacologic subversion of the CD40 pathway may be effective in abrogating problematic host responses to vectors, a concept that has been recently validated in murine models of liver and lung gene transfer (*8*).

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9. CD40L-deficient (CD40L$^{-/-}$) mice and their control litter mates (CD40L$^{+/+}$) were obtained from Jackson Laboratory (Bar Harbor, ME). Mice (female, 7 to 8 week old) were immunized with the recombinant adenovirus H5.010CMV*lacZ* [2 × 10^9 plaque-forming units (pfu)] through the tail vein and killed 3, 10, and 24 days later. This replication-defective vector is deleted of the early genes *E1a* and *E1b* and expresses *lacZ* from a cytomegalovirus (CMV) promoter. Liver tissues were harvested for morphological analysis, and spleens and blood samples were saved for immunological assays. Some CD40L$^{-/-}$ mice were also treated with a monoclonal antibody to CD40 (anti-CD40) (100 μg/day, Pharmingen, San Diego, CA) for 7 days after immunization, and C57BL/6 mice were treated with monoclonal antibodies to B7.1 and B7.2 (100 μg/day; Pharmingen, San Diego, CA) every 3 days after immunization for the duration of the experiment.
10. Splenocytes harvested from day 10 mice were cultured either for 2 days for analysis of T cell proliferation to antigen or for 5 days in preparation for CTL assays. The cells (6 × 10^6 cells per well) were cultured in 1.6 ml of Dulbecco's minimum essential medium supplemented with 5% fetal bovine serum and 50 μM 2-mercaptoethanol in the presence of *lacZ* virus at a multiplicity of infection of one in 24-well Costar plates. After the 48-hour incubation with antigen, supernatants were analyzed for IL-2, IL-4, IL-10, and IFN-γ by cytokine-specific enzyme-linked immunosorbent assays (ELISAs). A standard 6-hour ^{51}Cr release assay was performed subsequently with different ratios of effector to target cells (C57SV, H-2^b). These target cells were mock-infected or infected with *lacZ* virus for 18 hours and labeled with ^{51}Cr. After incubation for 6 hours, 100-μl samples of supernatant were removed for counting in a gamma counter. The percentage of specific ^{51}Cr release was calculated as follows: [(cpm of sample − cpm of spontaneous release)/(cpm of maximal release − cpm of spontaneous release)] × 100. Spontaneous release was determined by culturing target cells in medium, and maximal release was established by culturing target cells in a 1% solution of SDS. The percentage of specific lysis is expressed as a function of different effector-to-target ratios (6:1, 12:1, 25:1, and 50:1).
11. Spleens from C57BL/6, CD40L$^{-/-}$, and CD40L$^{-/-}$ mice treated with anti-CD40 and C57BL/6 mice treated with anti-B7.1 and anti-B7.2 were harvested 10 days after infusion of H5.010CMV*lacZ* virus, and the frozen sections were analyzed for germinal center formation. Frozen sections were fixed with methanol for 10 min at −20°C, air-dried, and rehydrated with phosphate-buffered saline (PBS). After blocking with 10% goat serum for 20 min, sections were incubated with peanut agglutinin conjugated to fluorescein isothiocyanate (FITC) (Sigma; 20 μg/ml) for 1 hour at room temperature. Sections were washed and mounted with Citiflour (Canterbury, UK). Four spleen sections from two mice (*2*) were stained for the presence of germinal centers (GCs) and analyzed. Data are represented as the number of GCs per section (mean ± SD).
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15. Antiviral antibodies were evaluated with an ELISA assay in which microtiter plates were coated with 200 ng of viral antigen in 100 μl of PBS per well overnight at 4°C, washed three times in PBS, and blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C. Serum samples diluted 1:200 were added to antigen-coated plates and incubated for 4 hours at 37°C. Plates were washed three times with 0.05% Tween-20 in PBS and incubated with biotin-labeled goat antibody to mouse IgG1, IgG2a, or IgM (Pharmingen, San Diego, CA) at 1:1000 dilution overnight at 4°C. Plates were washed as above and anti-biotin phosphatase (Sigma) was added to each well at 1:30,000 dilution for 1 hour at 37°C. Wells were washed again as above and *p*-nitrophenyl phosphate substrate was added.

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Requirement for CD40 Ligand in Costimulation Induction, T Cell Activation, and Experimental Allergic Encephalomyelitis

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The mechanism of CD40 ligand (CD40L)–mediated in vivo activation of CD4$^+$ T cells was examined by investigation of the development of experimental allergic encephalomyelitis (EAE) in CD40L-deficient mice that carried a transgenic T cell receptor specific for myelin basic protein. These mice failed to develop EAE after priming with antigen, and CD4$^+$ T cells remained quiescent and produced no interferon-γ (IFN-γ). T cells were primed to make IFN-γ and induce EAE by providing these mice with B7.1$^+$ antigen-presenting cells (APCs). Thus, CD40L is required to induce costimulatory activity on APCs for in vivo activation of CD4$^+$ T cells to produce IFN-γ and to evoke autoimmunity.

CD40 ligand is preferentially expressed on the surface of activated CD4$^+$ T cells and is critical for effective humoral immunity (*1*). The receptor for CD40L, CD40, is expressed on various APCs, such as B cells, dendritic cells, and macrophages, and in vitro experimental evidence has shown that CD40-CD40L interaction induces up-regulation of major histocompatibility complex class II, B7, and other molecules that po-