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Differential Requirements of T Cell Subsets for CD40 Costimulation in Immunity to Blastomyces dermatitidis

Marcel Wüthrich,* Phil L. Fisette,* Hanna I. Filutowicz,* and Bruce S. Klein2*†‡§

Cell-mediated immunity and production of type 1 cytokines are the main defenses against pathogenic fungi. Ligation of CD40 by CD40L on T cells is critical for the induction of these immune responses in vivo. We explored the role of CD40/CD40L interactions in vaccine immunity to Blastomyces dermatitidis by immunizing CD40−/− and CD40L−/− mice and analyzing their resistance to reinfection in a murine pulmonary model. In the absence of CD40 or CD40L, CD4+ cells failed to get primed or produce type 1 cytokine and impaired the generation of CD8+ T1 cells. The CD8+ T cell defect was not due to regulatory T cells or impaired APC maturation or Ag presentation to T cells. If CD4+ cells were first eliminated, vaccination of CD40−/− and CD40L−/− mice restored priming of CD8+ cells, type 1 cytokine production, and resistance. Hence, CD4+ and CD8+ cells differ sharply in their requirement for CD40/CD40L interaction during the generation of antifungal immunity. Despite the plasticity of T cell subsets in vaccine immunity, in absence of CD40/CD40L interaction, CD4+ cells may impede the priming of CD8+ cells at the cost of host survival against a lethal infectious disease. The Journal of Immunology, 2006, 176: 5538–5547.

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Abbreviations used in this paper: CMI, cell-mediated immunity; LCMV, lymphocytic choriomeningitis virus; DC, dendritic cell; T Reg, regulatory T cell; CW/M, cell-wall/membrane; MFI, mean fluorescent intensity; BM, bone marrow.

CD40/CD40L interaction is required to mount a protective CMI response to infection with intracellular parasites such as Leishmania spp. and Trypanosoma cruzi (11, 12, 21, 22). CD40−/− and CD40L−/− mice fail to control the growth of these parasites and succumb to infection. Conversely, infection with Mycobacterium tuberculosis, Listeria monocytogenes, Toxoplasma gondii, lymphocytic choriomeningitis virus (LCMV), and Histoplasma capsulatum is sufficient to activate APC, thus bypassing the need for costimulation (23–30). In the absence of CD40L, these pathogens can directly induce IL-12 and/or elicit protective CD8+ T cells and the mice are not more vulnerable than wild-type littermates. In contrast to the resistance of CD40L−/− mice, CD40−/− mice are susceptible to aerosol infection with M. tuberculosis because infected APC fail to produce IL-12 and prime IFN-γ-producing T cells (26). This disparity in the outcome of infection between CD40−/− and CD40L−/− mice is likely due to M. tuberculosis surface heat shock protein 70, which serves as an alternative ligand for CD40L in engaging CD40 and conditioning of APC.

In studying requisite and dispensable elements of immunity to B. dermatitidis, we have observed remarkable plasticity in the cellular and molecular elements of vaccine resistance (4, 5). Herein, we explored the role of CD40/CD40L interaction. We postulated that CD40L interaction with CD40 on APC would be dispensable for the induction of vaccine immunity for the following reasons: 1) B. dermatitidis infection of bone marrow (BM)-derived dendritic cells (DC) efficiently up-regulates Ag-presenting and costimulatory molecules and induces IL-12 and TNF-α production (our unpublished data presented in this article); 2) T1 CD8+ T cells can mediate vaccine resistance when generated in the absence of CD4+ T cells and T cell help (5); and 3) primary and secondary infection with the related dimorphic fungus H. capsulatum does not require CD40 or CD40L (29, 30).

We report here that, unexpectedly, CD40 and CD40L were required for the induction of T1 CD4+ T cells. CD8+ T cells surprisingly failed to compensate for CD4+ cells in the absence of CD40 or CD40L costimulation, if CD4+ cells were present. In contrast, when CD4+ T cells were eliminated, the induction of T1 CD8+ T cells and acquisition of resistance was independent of CD40 or CD40L costimulation. Hence, CD4+ and CD8+ T cells are differentially dependent on CD40/CD40L interaction during...
the induction of vaccine immunity to this pathogenic fungus. To our knowledge, we describe a novel event whereby CD4+ cells impede the priming of CD8+ cells at the cost of host resistance against a lethal infectious disease.

Materials and Methods

Fungi

Strains used were American Type Culture Collection (ATCC) 26199 (31), a wild-type virulent strain, and the isogenic, attenuated-mutant-lacking BAD1, designated strain no. 55 (32). Isolates of B. dermatitidis were maintained as yeast on Middlebrook 7H10 agar with oleic acid–albumin complex (Sigma-Aldrich) at 39°C. In some assays, the attenuated strain was cultured with a red fluorescent linker PKH26 for in vivo cell tracking, using the PKH26GL kit from Sigma-Aldrich (33–35).

Inbred strains of mice including thymectomized and nonthymectomized C57BL/6, CD40L-deficient B6.129S2-Tnfsf5tm1nos stock no. 2770 (11, 36, 37) and CD40-deficient B6.129P2-Tnfsf5tm1ks stock no. 2928 mice (38) were obtained from The Jackson Laboratory. Male mice were 7–8 wk of age at the time of these experiments. Mice were housed and cared for according to guidelines of the University of Wisconsin Animal Care Committee, who approved all aspects of this work.

In vivo cell depletion

CD4+ and CD8+ T cells were depleted by mAb treatment as previously described (4, 5). Mice received 100 μg of anti-CD4 (mAb GK1.5 rat IgG2b), anti-CD8 (mAb 2.43 rat IgG2b), or rat IgG as a control i.v. 1 day before vaccination or infection and weekly afterward. Cell depletion was analyzed by FACS and showed >95% depletion of desired subsets in the peripheral blood and lung (data not shown). For regulatory T cell (T Reg) depletion in recipients was stopped 2 wk before adoptive transfer of CD4+ cells during vaccination to foster activation of CD8+ cells to prevent the elimination of transplanted cells.

Real-time RT-PCR

Lung cells of individual mice (six to eight per group) were harvested 48–60 h postinfection with 2 × 106 26199 yeast and total RNA was isolated using the RNeasy Mini kit (Qiagen) as described previously (5). A 1 μl of RNA in a final volume of 20 μl was reverse-transcribed using random hexamers and the TaqMan RT-PCR kit (Applied Biosystems). The PKH26-labeled with a red fluorescent linker PKH26 for in vivo cell tracking, using the PKH26GL kit from Sigma-Aldrich (33–35).

Transcript quantity was calculated using the comparative cycle threshold (Ct) method (39) and reported as fold difference relative to a calibrator cDNA (i.e., sample from unvaccinated mice). Data are an average of two independent experiments.

Intracellular cytokine staining

Lung cells from individual mice were harvested at day 4 postinfection with 2 × 106 26199 yeast. The isolated lung cells (0.5 × 106 cells/ml) were stimulated for 4 h with anti-CD3 (clone 145-2C11; 0.1 μg/ml) and anti-CD28 (clone 37.51; 1 μg/ml) in the presence of 2 μM monensin (Sigma-Aldrich) to halt gress of cytokines from the cells. After cells were washed and stained for surface CD4 and CD8 using anti-CD4 FITC, anti-CD8 CyChrome, anti-CD44-allophycocyanin, and anti-CD25 mAbs (clones H129.19, 53-6.7, IM7, and 7D4; BD Pharmingen), they were fixed in 2% paraformaldehyde at 4°C overnight and permeabilized the next day with 0.1% saponin in PBS containing 0.1% BSA and 0.1% sodium azide. Permeabilized cells were stained with PE-conjugated mAbs and isotype controls (BD Pharmingen) for IFN-γ (clone XMG1.2), TNF-α (clone MP6-XT22), GM-CSF (clone MP1-22E9), IL-4 (11B11), and IL-10 (JES5-16E3) in 20% mouse serum for 30 min at 4°C, washed, and analyzed by FACS. Lymphocytes were gated on CD4 or CD8 and CD4+ or CD8+, and cytokine expression in each gate was analyzed. The number of cytokine-producing CD4+ and CD8+ T cells per lung was calculated by multiplying the percentage of cytokine-producing cells by the number of CD4+ and CD8+ cells in the lung.

Cytokine protein measurements

Cell-culture supernatants were generated in 24-well plates in 1 ml containing 5 × 106 splenocytes and lymph node cells and 5 μg/ml Con A or 12.5 μg/ml Blastomyces yeast cell-wall/membrane (CW/M) Ag (3). CW/M Ag contained <0.1 endotoxin unit/ml. Supernatants were collected after 96 h of coculture. IFN-γ, GM-CSF, IL-12 p40, IL-4, IL-10, and TGF-β (R&D Systems) were measured by ELISA according to manufacturer’s specifications (detection limits were 0.05, 0.02, 0.05, 0.05, and 0.05 ng/ml, respectively).

Vaccination and experimental infection with B. dermatitidis

Mice were vaccinated as described (3) twice, 2 wk apart, each time receiving a s.c. injection of 106 attenuated vaccine strain no. 55 yeast at each of two sites, dorsally and at the base of the tail, unless otherwise stated. Mice were infected intratracheally with 2 × 105 isogenic wild-type strain 26199 yeast as described (3). Infected mice were monitored for survival or analyzed 2 wk after infection for extent of lung infection, determined by plating of homogenized lung and enumeration of CFU on brain heart infusion (Difco) agar. To distinguish wild-type strain 26199 yeast from vaccine strain no. 55 (containing a selectable marker) in tissue homogenates, samples were plated on brain heart infusion with and without 100 μg/ml hygromycin B (32).

Generation and adoptive transfer of immune CD4+ T cells into CD4-depleted recipients

To generate immune CD4+ cells for transfer, mice were vaccinated with attenuated yeast as above. Skin draining lymph nodes (brachial and inguinal) and splenocytes were harvested; CD4+ T cells were purified using anti-CD4-coated immunomagnetic beads (BD Pharmingen) and shown to be >95% pure by flow cytometry. Purified CD4+ cells were adoptively transferred into vaccinated wild-type mice that had been depleted of CD4+ cells during vaccination to foster activation of CD8+ effector cells. CD4+ depletion in recipients was stopped 2 wk before adoptive transfer of CD4+ cells to prevent the elimination of transferred cells.

Enrichment of Ag-loaded APCs

Male C57BL/6 mice were injected s.c. with PKH26-labeled attenuated yeast (2 × 107 yeast/mouse). Skin draining lymph nodes (brachial and inguinal) and spleen were harvested 48–72 h postinfection from naive or vaccinated mice and treated with 1 mg/ml collagenase D and 50 μg/ml DNase I (Roche) in PBS containing 10 mM HEPEs (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 0.5% BSA for 60 min at 37°C. After inhibition of collagenase activity with 50 mM EDTA in PBS, the lymph node cells were washed through 40-μm cell strainers into 50 mM EDTA, washed again through 40-μm cell strainers into 40 mM EDTA. Single-cell suspensions were prepared and incubated with MACS CD11b (Miltenyi Biotec) microbeads according to the manufacturer’s instructions. CD11b+ cells were positively selected.
with high speed MACS (AutoMACS; Miltenyi Biotec). The purity of CD11b+ cells preparations was determined using allophycocyanin-Cy7 conjugated anti-CD11b mAb (clone M1/70) and was 95–99%. Surface staining with allophycocyanin-conjugated anti-CD11c mAb (clone HL3) revealed that 90% of CD11b+ cells were also positive for CD11c indicating that enriched cells contained chiefly DCs. The number of APC that had taken up PKH26-labeled yeast was calculated by multiplying the percent of PKH26 labeled-positive cells within the CD11b+ gate by the total number of CD11b+ cells enriched from the draining lymph nodes.

**Maturation of in vivo-loaded APC**

The activation status of Ag-loaded APC was analyzed by quantifying the expression of surface markers CD80 (B7-1), CD86 (B7-2), CD40, and MHC class II with biotinylated Abs (clone 16-10A1, clone GL1, clone 3/23, clone AF6-120.1, respectively) and streptavidin PerCP-Cy5.5. The relative increase in activation markers on Ag-loaded vs nonloaded CD11b-enriched APC was calculated by dividing the mean fluorescent intensities (MFI) of PKH26+ cells by that of PKH26− cells harvested from vaccinated mice or naive mice.

In functional tests of in vivo-loaded APC, we cocultured CD11b-enriched APC with T cells in vitro and measured the amount of IFN-γ produced in the cell-culture supernatant and frequency of IFN-γ-producing CD4+ T cells. CD4+ responder cells were purified using anti-CD4-coated immunomagnetic beads and seeded at 3 × 10^5 cells/250 μl in a 96-well plate. CD11b-enriched cells as APC were added at 5 × 10^4 cells/well. After 24 h of coculture, T cells were harvested and stained for intracellular cytokine. IFN-γ was measured in supernatant harvested after 4 days of coculture.

**Ag-induced maturation of BM-derived DC and triggering of T cells**

BM-derived DC were established by growth for 10–13 days in the presence of 5 μg/ml GM-CSF (a gift from G. S. Deepe, University of Cincinnati College of Medicine, Cincinnati, OH) as described (40).

To induce maturation, DC were washed twice and cultured with 5 μg/ml CW/M Ag or 3 × 10^5 attenuated yeast/0.5 ml in a 48-well plate or 10^5 yeast/1 ml in a 24-well plate (DC:yeast ratio = 1:2). Cell-culture supernatant was harvested after 24 h and assayed for IL-12 and TNF-α by ELISA (R&D Systems). DC were analyzed for surface expression of B7-1 (CD80), B7-2 (CD86), CD40, and MHC class II (I-Ab) as above.

For functional assays, BM-derived DC were loaded with CW/M Ag or yeast as above; 24 h later, responder T cells were added at 3 × 10^5 cells/ml in a 24-well plate or at 10^6 cells/0.5 ml in a 48-well plate. After 24 h of coculture, T cells were harvested, stained for intracellular cytokine, and analyzed by flow cytometry to calculate the percentage of cytokine-positive T cells. After 72 h of coculture, supernatants were collected and IFN-γ measured by ELISA.

**Statistical analysis**

Kaplan-Meier survival curves were generated (41). Survival times of infected mice alive by the end of the study were regarded as censored. Time data were analyzed by the log-rank statistic (Mantel-Haenszel test) (42) and exact p values were computed using the statistical package Stat Xact-3 by CYTEL Software. The number of cytokine-producing CD4+ and CD8+ T cells, relative changes in cytokine transcripts, and differences in number of CFU were analyzed using the Wilcoxon rank test for nonparametric data (41). A p value of <0.05 is considered statistically significant.

**Results**

**CD40−/− and CD40L−/− mice fail to acquire vaccine immunity in the presence of CD4+ cells**

Immunity to primary infection with the intracellular parasites *Leishmania spp.*, *T. cruzi* and aerosol-administered *M. tuberculosis* requires the presence of CD40 on APC (11, 12, 21, 22). To explore whether CD40 is obligate for vaccine immunity against *B. dermatitidis* infection, we immunized CD40−/− and wild-type mice and analyzed their ability to resist infection in a lethal pulmonary model. Vaccinated CD40−/− mice failed to resist infection, whereas vaccinated wild-type controls were highly resistant and reduced lung CFU by >6 logs (Fig. 1A). Because CD40L−/− mice resist aerosol *M. tuberculosis* infection, unlike CD40−/− mice (26), and because CD40L−/− mice also resist primary and secondary infection with *H. capsulatum* (29, 30), we tested whether CD40L is dispensable in the induction of vaccine resistance to *B. dermatitidis*. Similar to CD40−/− mice, vaccinated CD40L−/− mice failed to acquire resistance and had a burden of lung infection comparable to unvaccinated controls (Fig. 1B). Hence, when CD4+ cells are present and are the “drivers” of vaccine resistance (4, 5), neither CD40−/− nor CD40L−/− mice are able to acquire vaccine immunity.

**CD40 costimulation is dispensable for vaccine induction of protective CD8+ cells**

Because CD4+ cells and CD4 help are dispensable for vaccine resistance and activation of protective CD8+ cells (5), we postulated that CD8+ cells might not require CD40 costimulation. To test this hypothesis, we vaccinated CD4−/− and CD40L−/− mice depleted of CD4+ cells (this drives resistance by CD8+ cells (5)) and analyzed their resistance phenotype upon a lethal infection with wild-type yeast. Vaccinated, CD4-depleted CD40−/− and CD40L−/− mice each reduced the burden of lung infection by nearly 5 logs, compared with vaccinated knockout mice that were not depleted and nonvaccinated controls (Fig. 1). Vaccinated, CD4-depleted CD40L−/− mice that were depleted of CD8+ cells during the effector phase (after lung infection) had 600-fold more lung CFU than did CD4-depleted controls, indicating that CD8+ cells acquire and confer protection independently of CD40 costimulation. CD4+ cells and CD8+ cells are therefore differentially dependent on CD40 costimulation. Why do CD8+ cells fail to mediate vaccine resistance in the absence of CD40 costimulation when CD4+ cells are present?

**CD4 T cells and T<sub>Reg</sub> do not suppress CD8+ T cells during the effector phase**

To explore whether CD4+ cells and T<sub>Reg</sub> interfere with CD8+ T cell-mediated resistance during the effector/expression phase (after
To understand the role of CD4+ cells in CD40L−/− mice and the mechanisms by which they contribute to resistance, we undertook two approaches. First, we vaccinated CD40L−/− and wild-type mice and adoptively transferred purified CD4+ cells from these animals into vaccinated, CD4-depleted wild-type mice in whom resistance is mediated by CD8+ cells (5). Adoptive transfer of CD4+ cells from vaccinated CD40L−/− mice into CD4-depleted wild-type mice did not reduce resistance mediated by CD8+ cells, as compared with the effect of naive CD4+ cells; in contrast, depletion of CD8+ cells increased lung CFU significantly (Fig. 2A).

In a second approach, we vaccinated CD40L−/− mice (and wild-type controls) and depleted CD4− cells during the effector/expression phase (Fig. 2B). This maneuver had no significant effect on resistance in CD40L−/− mice, whereas it sharply curtailed resistance in wild-type mice. Hence, CD4− cells from CD40L−/− mice consisting of both TReg and T effector cells did not blunt CD8+−mediated resistance.

**Depletion of TReg during vaccination fails to restore resistance in CD40L−/− mice**

Because regulatory CD4+ CD25+ T cells (TReg) can suppress activation of APC (43) and CD8+ T cells (44, 45), we explored the possibility that TReg in vaccinated CD40L−/− mice might interfere with the priming of naive CD8+ cells and acquisition of resistance. We pursued two approaches. First, we prevented the induction of TReg by a combination of anti-CD25 and anti-CTLA-4 mAb treatment during the period of vaccination. Because CD25 is not only a marker of TReg, but also an activation marker on CD4+ and CD8+ T cells, we treated mice here (and in the second approach below) only at days −7 and −4 before vaccination so that CD25 depletion would not eliminate Ag-experienced, protective T cells elicited by the vaccine (44). CD25 depletion reduced lung CFU in vaccinated CD40L−/− mice by 1 log as compared with vaccinated controls treated with rat IgG (Fig. 2C). After receipt of both anti-CD25 and anti-CTLA-4 mAb during vaccination, mice had >3 logs less lung CFU than rat IgG-treated control mice, indicating that TReg cells might have altered the acquisition of vaccine-induced resistance (Fig. 2B). Because in vivo blockade of CTLA-4 by neutralizing mAb can directly enhance proliferation and activation of CD8+ T cells (46), increased resistance in anti-CTLA-4-treated mice could have been due to either elimination of TReg or direct stimulation of T cells. To distinguish between these possibilities, we sought to eliminate TReg without the activating effects of anti-CTLA-4.

In a second approach, we thymectomized CD40L−/− and wild-type mice to prevent repopulation of T cells and depleted CD25+ cells from them before vaccination. Depletion of CD25+ cells sharply reduced the percentage of total and primed (CD4+ and CD8+) TReg throughout the vaccination period in thymectomized CD40L−/− and wild-type mice, as compared with rat IgG-treated controls (Fig. 3A, left panel). Depletion of TReg in thymectomized, vaccinated wild-type mice significantly increased the percentages of primed CD4+ and CD8+ T cells (Fig. 3A, center and right panel) and the production in vitro of Ag-specific IFN-γ and GM-CSF by T cells harvested from infected mice (Fig. 3B). However, depletion of TReg in thymectomized CD40L−/− mice did not increase the pool of primed CD4+ and CD8+ cells or the amounts of infection), we undertook two approaches. First, we vaccinated CD40L−/− and wild-type mice and adoptively transferred purified CD4+ cells from these animals into vaccinated, CD4-depleted wild-type mice in whom resistance is mediated by CD8+ cells (5). Adoptive transfer of CD4+ cells from vaccinated CD40L−/− mice into CD4-depleted wild-type mice did not reduce resistance mediated by CD8+ cells, as compared with the effect of naive CD4+ cells; in contrast, depletion of CD8+ cells increased lung CFU significantly (Fig. 2A).

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Ag-specific type 1 cytokines produced in vitro (Fig. 3, A and B). On challenge of thymectomized mice, vaccinated CD40L−/− mice depleted of TReg had lung CFU values similar to nondepleted controls, whereas vaccinated wild-type mice cleared the infection (Fig. 3). Thus, elimination of TReg in vaccinated CD40L−/− mice did not restore T cell priming during the afferent phase or resistance.

BM-derived DC from CD40L−/− mice have no intrinsic defect in Ag-induced maturation
CD40L expressed on activated T cells triggers CD40 on macrophages and DC, leading to up-regulation of costimulatory molecules and expression of IL-12 in these APC (13–15). We explored whether Ag-induced maturation of APC was impaired in mice lacking CD40L. We first tested whether APC from CD40L−/− mice have an intrinsic defect in activation after they encounter fungal Ag. After 24-h exposure of DC with yeast or CW/M Ag, expression of surface B7-1, B7-2, CD40, and MHC class II, and production of IL-12 and TNF-α were similarly increased in CD40L−/− vs wild-type mice, compared with DC cultured in medium alone (Table I). To assess function of Ag-loaded DC, they were cocultured in vitro with CD4+ cells isolated from vaccinated or naive wild-type mice. Loaded DC from CD40L−/− mice and wild-type mice stimulated similar numbers of Ag-specific CD4+ cells and elicited similar levels of IFN-γ (Table I). Thus, BM-derived DC from CD40L−/− mice show no intrinsic defect in becoming conditioned in vitro by Ag and stimulating Ag-specific CD4+ cells.

FIGURE 3. Elimination of TReg in thymectomized mice excludes their role in the failure of CD8 T cell-mediated resistance in CD40L−/− mice. A, Depletion of TReg in thymectomized mice. Thymectomized CD40L−/− and wild-type mice were depleted with anti-CD25 mAb (PC61) at days −7 and −4 before vaccination. At the start of (day 2) and after (day 34) vaccination, the percentage of CD4+CD25+ (Total TReg) cells (left panel), and the percentages of CD4+CD44+ (primed CD4; middle panel), and CD8+CD44+ (primed CD8; right panel) cells were determined in PBMC by flow cytometry. *, p < 0.05 vs nondepleted controls. B, CD4+ cells were purified from the spleen and skin draining lymph nodes (brachial and inguinal) of thymectomized mice in C 16 days postinfection, and stimulated with CW/M Ag in vitro. Data are the mean ± SEM of two pools of four to five mice per group. *, p < 0.05 vs vaccinated wild-type mice that got rag IgG (C), or vaccinated CD40L−/− mice that got anti-CD25. C, Burden of infection. On day 35 postvaccine, mice were infected as above and analyzed for lung CFU 16 days later. Data are geometric mean ± SEM; n = 8–10 mice/group.
We next explored the function of APC removed from the vaccine mature and functional
CD40L associated with resistance (3–5), we explored whether T cells in
Depletion of CD4 cells in CD40L or presentation by the APC.

Ag-loaded APC explanted from vaccinated CD40L−/− mice are mature and functional
We next explored the function of APC removed from the vaccine site of immunized CD40L−/− and wild-type mice. We vaccinated mice with PKH-26 labeled yeast and compared the number of Ag-loaded APC harvested from skin draining lymph nodes (brachial and inguinal) at days 2 and 4 postvaccination, which coincides with the peak influx of skin draining dermal and Langerhans DC, respectively (47). Comparable numbers of Ag-loaded APC were detected in vaccinated CD40L−/− and wild-type mice at day 2 postvaccination and these numbers fell off by day 4 (Fig. 4A, upper panel). In vivo-loaded APC from CD40L−/− and wild-type mice showed similar increases in surface expression of B7-1, B7-2, CD40, and MHC class II at days 2 and 4 postvaccination, as compared with naive APC (Fig. 4A, lower panel) or unloaded APC (data not shown). In vivo-loaded APC from CD40L−/− and wild-type mice triggered similar percentages (6–8%) of Ag-specific CD4+ cells to produce IFN-γ (Fig. 4B), and elicited comparable levels of IFN-γ from Ag-specific CD4+ cells (Fig. 4C). Thus, in CD40L−/− mice, APC at the site of vaccination phagocytose the yeast, display maturation markers, and trigger Ag-specific CD4+ cells. Thus, impaired resistance is not due to a defect in maturation or presentation by the APC.

Depletion of CD4 cells in CD40L−/− mice restores priming and T1 polarization of CD8 cells
CD40/CD40L interactions are pivotal for priming and expansion of CD4+ cells. We investigated priming of CD8+ cells in CD40L−/− mice in the presence and absence of CD4+ cells. CD44 staining of CD4+ and CD8+ cells was sharply reduced in vaccinated CD40L−/− mice compared with vaccinated, wild-type controls (Fig. 5A), indicating that CD8+ T cell priming and expansion was markedly impaired when CD4+ cells were present. Conversely, priming of CD8+ cells was restored fully by the elimination of CD4+ cells in CD40L−/− mice.

We also investigated polarization of T cells in CD40L−/− mice in the presence and absence of CD4+ cells. Because T1 cells are associated with resistance (3–5), we explored whether T cells in nondepleted CD40L−/− mice switched to a T2 or TReg phenotype after vaccination. We used several approaches including analysis of lung T cells ex vivo for cytokine transcript and intracellular protein expression, and splenocyte and lymph node cells in vitro for cytokine response to Ag stimulation.

We measured total lung cell cytokine transcript 60 h postinfection because IFN-γ mRNA increases by 48–72 h after infection in association with vaccine resistance (5). Here, transcripts for IFN-γ, IL-13, and IL-4 were elevated in vaccinated wild-type (CD4-depleted and nondepleted mice) vs unvaccinated mice, whereas none of the cytokines and other products assayed (IFN-γ, TNF-α, GM-CSF, IL-4, IL-5, IL-10, IL-13, TGF-β1, FoxP3, Lag3, and Grail) was significantly increased in vaccinated CD40L−/− mice (non-CD4-depleted) vs unvaccinated controls (Fig. 5B). This initial screen pointed to a failure of T1 differentiation, rather than T2 polarization, and confirmed the absence of TReg transcripts in CD40L−/− mice vaccinated in the presence of CD4+ cells. In contrast, CD4 depletion in CD40L−/− mice was associated with increased amounts of IFN-γ and IL-13 transcript, similar to those in vaccinated wild-type mice. Hence, eliminating CD4+ cells from vaccinated CD40L−/− mice leads to marked T1 differentiation, based on lung cell transcripts.

We analyzed intracellular cytokine in lung T cells. The numbers of CD4+ and CD8+ T cells that produced type 1 cytokines IFN-γ, TNF-α, and GM-CSF was sharply diminished in vaccinated CD40L−/− mice vs wild-type controls (Fig. 5C). T cells from vaccinated CD40L−/− mice and wild-type controls did not produce type 2 cytokines IL-4 and IL-10. When CD4+ cells were eliminated from CD40L−/− mice during vaccination, the numbers of CD8+ cells producing T1 cytokines increased markedly, comparable to those in wild-type controls (Fig. 5, C and D). Hence, CD4+ cells strictly depended on CD40L to acquire a T1 phenotype, but T1 CD8+ cells could be induced without CD40 costimulation when CD4+ cells were absent.

Analysis of splenocyte and lymph node cell responses to fungal Ag in vitro yielded similar results. IFN-γ, GM-CSF, and IL-10 levels were reduced in vaccinated CD40L−/− mice vs wild-type controls, without T2 polarization and production of IL-4 or TGF-β1 (data not shown).

Discussion
In this study, we explored the role of CD40/CD40L interactions for the generation of vaccine immunity to B. dermatitidis mediated by CD4+ and CD8+ T cells. We found that CD40/CD40L interaction is dispensable for the generation CD8+ T cell resistance when CD4+ cells are eliminated during vaccination. In contrast, CD40/CD40L interactions are requisite for CD4+ T cell resistance; without them, CD4+ cells could not be primed and expressed neither a T1, T2, nor a TReg phenotype. Hence, CD4+ and CD8+ T cells differ markedly in their requirements for CD40 costimulation during vaccine-induced immunity to B. dermatitidis.

Our findings are distinctly different from those reported for immunity to H. capsulatum. CD40-CD40L interactions are dispensable in primary and secondary immunity in an i.v. model of H. capsulatum infection (30), and in a pulmonary model described by Wheat et al. (29). Studies by G. S. Deepe (unpublished observations) confirmed those results, showing no loss of immunity in either CD40L−/− or CD40−/− mice in primary and secondary infection in a pulmonary model. In both models, fungal clearance is mediated chiefly by CD4+ cells; however, elimination of CD4+ or CD8+ cells led to accelerated mortality in CD40L−/− mice during a primary infection (30), suggesting that CD4+ and CD8+ cells are primed to protect against H. capsulatum infection in the absence of

Table I. Ag-induced maturation and function of DC from CD40L−/− and wild-type mice

<table>
<thead>
<tr>
<th>Strain of Mouse</th>
<th>CD40L−/−</th>
<th>Wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maturation (fold increase MFI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7.1</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>B7.2</td>
<td>2.6 ± 0.4</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>CD40</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Class II</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Cytokine production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12 (ng/ml)</td>
<td>6.6 ± 3.8</td>
<td>7.8 ± 4.3</td>
</tr>
<tr>
<td>TNF-α (ng/ml)</td>
<td>67.7 ± 10</td>
<td>51.9 ± 9.7</td>
</tr>
<tr>
<td>T cell stimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ T cells (%)</td>
<td>1.7 ± 0.6</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>IFN-γ (ng/ml)</td>
<td>22.1 ± 3</td>
<td>18.3 ± 5.3</td>
</tr>
</tbody>
</table>

*BM-derived DC from CD40L−/− or wild-type mice were cocultured with attenuated yeast. After 24 h of coculture, supernatant was harvested and analyzed for IL-12 p40 and TNF-α. Marker expression levels were reduced in vaccinated CD40L−/− mice (non-CD4-depleted) vs unvaccinated controls (Fig. 5B). This initial screen pointed to a failure of T1 differentiation, rather than T2 polarization, and confirmed the absence of TReg transcripts in CD40L−/− mice vaccinated in the presence of CD4+ cells. In contrast, CD4 depletion in CD40L−/− mice was associated with increased amounts of IFN-γ and IL-13 transcript, similar to those in vaccinated wild-type mice. Hence, eliminating CD4+ cells from vaccinated CD40L−/− mice leads to marked T1 differentiation, based on lung cell transcripts.

We analyzed intracellular cytokine in lung T cells. The numbers of CD4+ and CD8+ T cells that produced type 1 cytokines IFN-γ, TNF-α, and GM-CSF was sharply diminished in vaccinated CD40L−/− mice vs wild-type controls (Fig. 5C). T cells from vaccinated CD40L−/− mice and wild-type controls did not produce type 2 cytokines IL-4 and IL-10. When CD4+ cells were eliminated from CD40L−/− mice during vaccination, the numbers of CD8+ cells producing T1 cytokines increased markedly, comparable to those in wild-type controls (Fig. 5, C and D). Hence, CD4+ cells strictly depended on CD40L to acquire a T1 phenotype, but T1 CD8+ cells could be induced without CD40 costimulation when CD4+ cells were absent.

Analysis of splenocyte and lymph node cell responses to fungal Ag in vitro yielded similar results. IFN-γ, GM-CSF, and IL-10 levels were reduced in vaccinated CD40L−/− mice vs wild-type controls, without T2 polarization and production of IL-4 or TGF-β1 (data not shown).
CD40L. T cell-mediated resistance to *H. capsulatum* and *B. dermatitidis* thus differ in the requirement for CD40 costimulation. It is unclear what accounts for this difference, but the intracellular lifestyle of *H. capsulatum* is likely to be important in efficient priming of APC and could diminish the requirements for particular costimulatory molecules.

The ability of CD40L−/− mice to generate functional T1 CD8+ T cells but not CD4+ T cells is a striking finding in our study. What are potential explanations for the differential dependence on CD40/CD40L for the two T cell subsets? Whitmire et al. (48) recently reported that antiviral CD4+ and CD8+ T cells also differ in their requirements for CD40-CD40L costimulation. CD40L−/− mice made potent CD8+ T cell responses to dominant and subdominant epitopes that were independent of CD4+ Th cells following infection with LCMV. In contrast, virus-specific CD4+ T cells in the mice were compromised both for T1 and T2 responses. The authors offered possible explanations for the observed dichotomy. First, MHC class I-restricted LCMV peptides could provide a stronger TCR signal for CD8+ cells than MHC class II-restricted LCMV-peptides do for CD4+ cells, and thereby obviate the need for costimulation (49, 50). A weaker CD4 coreceptor or TCR signal may require more costimulation such as CD40L signaling to activate CD4+ T cells. The authors provided circumstantial evidence for that idea by showing that CD4+ cells contained higher levels of CD40L than CD8+ cells (48).

A second possible explanation is that during LCMV infection, CD4+ cells do not encounter sufficient Ag to activate them, whereas CD8+ cells do encounter sufficient amounts. Hence, CD4+ cells may not reach an activation threshold without additional costimulatory signals. This idea is supported by other studies on cross-presentation of cell-associated OVA in vivo (51). An equivalent amount of cell-associated OVA was 100-fold more efficient at activating epitope-specific CD8+ (OT-I) cells than CD4+ (OT-II) cells.

There may be an anatomical explanation. Whitmire et al. (48) reported that the follicular architecture of the spleen of CD40L−/− mice is abnormal, with little or no migration of B7.1+ cells migrating into the follicles (where CD4+ cells are located) after LCMV infection. Hence, CD4+ cells may miss out on CD28/B7 interactions (52), whereas CD8+ cells that are localized outside the follicles, in the marginal zone and red pulp, may not be affected by the altered lymphoid architecture. A final reason could be that CD8+ T cells use alternate or additional costimulatory molecules (e.g., 4-1BB-4-1BBL) to acquire antiviral activity (53, 54).

A finding that distinguishes our study from the Whitmire study is the behavior of antifungal CD8 cells in CD40L−/− mice when CD4+ cells are present. In our study, antifungal CD8+ T cells failed to get activated or mediate resistance in CD40L−/− and CD40L−/− mice when CD4+ cells were present. Conversely, elimination of CD8+ cells from these mice restored priming and resistance mediated by CD8+ cells. Selective depletion of Treg during the vaccine induction phase or adoptive transfer of CD4+ T cells from vaccinated CD40L−/− mice into CD4-depleted wild-type mice during the effector phase did not restore resistance. Both in vitro- and in vivo-loaded APC from CD40L−/− mice failed to produce IFN-γ and yielded values similar to those for naive wild-type mice (data not shown). Data in A and B represent the mean ± SEM of two independent experiments.

**FIGURE 4.** Ag uptake and maturation by APC in vivo remain intact in vaccinated CD40L−/− mice. Two and 4 days after i.c. delivery of PKH-26-labeled yeast, CD11b+ cells were isolated from skin draining lymph nodes (brachial and inguinal) from CD40L−/− and wild-type mice and analyzed for Ag-uptake and maturation phenotype. A. The number (upper panel) and maturation phenotype (lower panel) of red fluorescent PKH-26+ APC are depicted. To determine the relative increase in MFI for each maturation marker, MFI of Ag-uptake loaded APC was divided by MFI of naive (or non-Ag-loaded) APC. Data represent the mean ± SEM of 3 (CD40L−/− mice) to 12 (wild-type) experiments, with *n* = 5 mice/group. B and C. In vivo-loaded APC from CD40L−/− mice trigger primed CD4+ cells. CD11b+ enriched cells from skin draining lymph nodes were cocultured in vitro with CD4+ cells from vaccinated and naive wild-type mice.
mice demonstrated “normal” Ag-induced maturation and presentation to primed T cells. Thus, the failure of CD8 cells to mediate vaccine resistance in CD40L−/− mice with CD4+ cells present could not be explained by T_{Reg} activity or defects in DC maturation and Ag presentation. Nevertheless, the presence of CD4+ cells impedes priming of CD8+ cells, which can otherwise be accomplished in the absence of costimulation.

We used two strategies to analyze whether T_{Reg} suppressed CD8+ -mediated resistance in the presence of CD4+ cells. Depletion of CD25+ cells in thymectomized CD40L−/− mice failed to restore resistance, whereas combined treatment with anti-CD25 and anti-CTLA-4 mAb partially did so. Combined treatment has been used in some studies to ensure the complete elimination of T_{Reg}, without repopulation, in mice that are not thymectomized. In our study, addition of anti-CTLA-4 significantly enhanced resistance. We attribute this result to the ability of anti-CTLA-4 mAb to directly activate CD8+ cells in the absence of CD4 help, as described by McCoy et al. (46) who reported that anti-CTLA-4 regulates DC activation of CD8+ cells without CD4 help. The effect of anti-CTLA-4 in our system suggests that antifungal CD8+ cells simply failed to get activated when CD4+ cells were present and were not instead under control of regulatory cells or products, or alternatively, that a positive signal via anti-CLTA-4 was sufficient to overcome it. Because CD25 depletion did not restore resistance in thymectomized CD40L−/− mice, we considered the possibility that CD25 CD4+ regulatory T cells interfere with the acquisition of resistance and priming of CD8+ T cells. Fontenot et al. (55) recently found that Foxp3 is the T_{Reg} lineage-specification factor and not CD25. They provided compelling evidence for the existence of two T_{Reg} populations, both with potent suppressive activity: CD4+CD25lowFoxp3+ and CD4+CD25highFoxp3+ T cells. The latter population comprised >50% of the tissue-infiltrating, Foxp3-expressing T cells in the lungs of uninfected and M. tuberculosis-infected mice. In addition to Foxp3, both subsets displayed increased expression of IL-10 and CTLA-4. Because there is no good marker available to deplete CD4+CD25highFoxp3+...


**References**


