CD40 Ligand Is Not Essential for Induction of Type 1 Cytokine Responses or Protective Immunity after Primary or Secondary Infection With *Histoplasma capsulatum*

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Summary

The induction of type 1 immune responses (interleukin [IL]-12, interferon [IFN]-γ) has been shown to be important in mediating protection against many intracellular infections including *Histoplasma capsulatum*. Costimulatory molecules such as CD40 ligand (CD40L) have been shown to be a central regulator of type 1 responses in vivo. To study the role of CD40L in mediating protection against infection with *H. capsulatum*, CD40L-deficient (CD40L$^{-/-}$) and CD40L$^{+/+}$ mice were infected with *H. capsulatum* and assessed for various parameters. After a lethal challenge of *H. capsulatum*, CD40L$^{-/-}$ mice were not substantially different from CD40L$^{+/+}$ mice in terms of mortality, fungal burden, or production of IFN-γ, IL-12, nitric oxide, or tumor necrosis factor α. Moreover, CD40L$^{-/-}$ mice treated with anti–IFN-γ or anti–IL-12 at the time of infection had accelerated mortality, providing further evidence that IL-12 and IFN-γ are produced in vivo in the absence of CD40L. In addition, CD40L$^{-/-}$ mice infected with a sublethal dose of *H. capsulatum* survived infection, whereas all mice infected with the same dose and treated with anti–IFN-γ had accelerated mortality, demonstrating that IFN-γ but not CD40L was essential for primary immunity to *H. capsulatum* infection. Interestingly, depletion of either CD4$^+$ or CD8$^+$ T cells resulted in accelerated mortality in CD40L$^{-/-}$ mice, suggesting a critical role for these cells in response to infection. Finally, CD40L$^{-/-}$ mice initially infected with a sublethal dose of *H. capsulatum* were protected from secondary infection with a lethal dose of *H. capsulatum*, demonstrating that CD40L is not required for the maintenance of memory immunity.

The role of CD40L–CD40 costimulation in mediating T cell responses in vivo was first shown in studies using CD40L-deficient (CD40L$^{-/-}$) mice. In these studies, T cell activation and production of cytokines such as IFN-γ were markedly impaired in response to protein antigens in vivo (1). This ability of CD40L–CD40 stimulation to regulate Th1 (IFN-γ) or type 1 (IFN-γ, IL-12) cytokine responses in vivo was subsequently shown to occur through at least two mechanisms (2), the first through the induction of IL-12 from APCs such as macrophages and dendritic cells (2–6) and the second by the ability of CD40L–CD40 stimulation to enhance expression of costimulatory cell surface molecules (e.g., B7-1, B7-2) on APCs, leading to increased T cell stimulation and production of IFN-γ (7–13). Based on these data, since CD40L–CD40 costimulation appears to be a key regulator of CD4$^+$ T cell–mediated type 1 responses in vivo, the role and mechanism by which CD40L regulates immunity to infectious pathogens is of great interest.

The first studies to examine the role of CD40L in regulating immune responses to infection were done using murine models of parasitic and viral infections. In studies of viral infection, CD40L$^{-/-}$ mice exposed to lymphocytic choriomeningitis virus had normal primary CTL responses with clearance of infection (14–16); however, memory CTL responses were impaired, suggesting that CD40L was important in maintaining a memory cellular (CTL) response (14). With regard to the role of CD40L in an infectious model requiring type 1 cytokine production, a series of studies were done using the well-established murine model of *Leishmania* infection (17–19). In these experiments, CD40$^{-/-}$ or CD40L$^{-/-}$ mice on a resistant background were markedly impaired in production of IL-12 (17) and IFN-γ (17–19), correlating with enhanced susceptibility to infection (17, 19) or exacerbation of infection (18).

The aforementioned studies provided strong evidence that CD40L–CD40 interactions were important in mediating CD4$^+$ T cell–dependent production of IL-12 to either protein antigens or *Leishmania* infection in vivo; however, DeKruyff et al. then showed a CD40L-independent path-

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[1] *Abbreviations used in this paper:* CD40L, CD40 ligand; NO, nitric oxide.

way for IL-12 production from mononuclear cells stimulated in vitro with either LPS or heat-killed *Listeria monocytogenes* (20). These latter data suggested that certain intracellular pathogens can directly induce IL-12 in vitro and raised the question as to whether these or other pathogens would elicit functional type 1 immune responses in vivo in the absence of CD40L.

In this report, we addressed the role of CD40L in regulating type 1 cytokine responses in vivo using a murine model of disseminated histoplasmosis. *Histoplasma capsulatum* is a dimorphic fungus found in the soil in distinct geographic regions around the world. Primary infection occurring through inhalation of conidial or mycelial fragments often results in a self-limited upper respiratory infection in immunocompetent hosts. By contrast, in immunocompromised hosts, disseminated infection can occur in multiple organs either through primary infection as described above or by recurrence of a previous infection (21–23). Protective immunity is achieved by the interaction of T cells and macrophages through the generation of a type 1 immune response characterized by production of IL-12 leading to IFN-γ induction (24, 25). Additional factors such as TNF-α and nitric oxide have also been shown to be important in mediating protection against primary infection (26). The studies presented here examined the role of CD40L in the generation of an immune response after both primary and secondary infection with *H. capsulatum* using CD40L$^{-/-}$ mice. The results show that CD40L$^{-/-}$ mice are not substantially different from CD40L$^{+/+}$ mice in terms of mortality, fungal burden, or the ability to develop a functional type 1 cytokine response in vivo compared with control CD40L$^{+/+}$ mice after infection with *H. capsulatum*. Furthermore, although both CD40L$^{-/-}$ and CD40L$^{+/+}$ mice infected with a sublethal dose of *H. capsulatum* survived infection and developed sterilizing immunity, all mice infected with the same dose and treated with anti–IFN-γ at the time of infection had accelerated mortality. These data provide clear evidence that IFN-γ but not CD40L is essential for protective immunity to primary *H. capsulatum* infection. Finally, of interest was the observation that CD40L$^{-/-}$ mice depleted of either CD4$^+$ or CD8$^+$ T cells had accelerated mortality and increased fungal burden after primary infection. Overall, these studies demonstrate that CD40L$^{-/-}$ mice develop relatively intact type 1 cytokine responses to *H. capsulatum* and suggest a differential requirement for CD40L in the generation of this type of immune responses after infection to *H. capsulatum* versus *Leishmania major*.

Materials and Methods

Mice and Infection. CD40L$^{-/-}$ mice generated on C57BL/6J × 129/J background (27) were obtained from The Jackson Laboratory (Bar Harbor, ME). As a control, C57BL/6 × 129(F2), also purchased from The Jackson Laboratory, were used and designated as CD40L$^{+/+}$ mice. In some experiments, CD40L$^{-/-}$ mice obtained from Immunex (Seattle, WA) were bred on a C57BL/6 background (greater than six generations). Virus-free female C57BL/6 mice purchased from Division of Cancer Treatment, National Cancer Institute (Frederick, MD) were used as controls for these experiments. In all experiments, mice were between 5 and 10 wk of age. Mice were inoculated intravenously in 0.5 ml sterile PBS with varying doses of *H. capsulatum* yeast cells. In one experiment, CD40L$^{-/-}$ mice were challenged with 10^5 metacyclic promastigotes *L. major* (WHOM/IR/-/173) in their hind footpads as previously described (28). Weekly footpad swelling measurements were recorded using a caliper.

Media. HBSS (Biofluids, Inc., Rockville, MD) was used as a wash medium. Complete medium: RPMI 1640 (Biofluids, Inc.) supplemented with 10% fetal bovine serum (Biofluids, Inc.), penicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (2 mM), sodium pyruvate (1 mM), and 2-ME (0.05 mM) was used for culturing spleen cells.

Preparation and Quantitation of H. capsulatum. The yeast phase of *H. capsulatum* (strain GS-57) was used in all experiments, and quantitation of *H. capsulatum* was performed as previously described (26). In brief, spleens from mice infected with *H. capsulatum* and/or treated with various cytokine antagonists were killed at various times after infection. In most experiments, three individual spleens from each group were quantitated for CFUs. In some experiments, one-third of each spleen from two or three individual animals in each group were combined and homogenized in a sterile mortar using PBS to prepare a 1:10 wt/vol suspension. 10-fold dilutions in PBS were plated in duplicate at 0.05 ml/plate on BHI-SAGC medium and incubated for 7 d at 30°C. Colonies were enumerated and the counts were recorded as CFUs.

In Vivo Treatment of Mice. Most antibodies were purified from ascites by ammonium sulfate precipitation. Rat anti–mouse IFN-γ mAb (XMG1.2; reference 29), anti-CD4 (GK1.5; reference 30), and anti-CD8 (2.43; reference 31) were used to neutralize IFN-γ and to deplete CD4$^+$ and CD8$^+$ T cells, respectively. Purified mAbs against murine IL-12 (C17.8; reference 32) were obtained from Dr. Giorgio Trinchieri (Wistar Institute, Philadelphia, PA) and have been shown to be effective in neutralizing IL-12 in vivo. Mice were injected with anti–IFN-γ (1 mg) intraperitoneally at the time of primary infection. Anti-CD4 and anti-CD8 antibodies were injected 3 d before, at the time of, and 5–7 d after infection. This treatment resulted in a >95% depletion of CD4$^+$ or CD8$^+$ T cells from spleen at 1 wk after infection, as assessed by FACS®.

Cytokine mRNA Measurement. Cytokine mRNA levels were determined by semiquantitative reverse transcription PCR techniques as previously described (26). In brief, total RNA was isolated from spleen cells by resuspending in RNAzol B (Tel-Test, Friendswood, TX) and recovering the aqueous phage after addition of chloroform. RNA was precipitated with alcohol and resuspended in RNase-free H$_2$O. Total RNA (1 μg) was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The reaction mixture was then diluted 1:8, and 10 μl was used for specific semiquantitative amplification of cytokine mRNA with Taq DNA polymerase (Promega, Madison, WI) and specific cytokine sense and antisense primers. The number of amplification cycles was as follows: 24 (hypoxanthine phosphoribosyltransferase), 25 (IFN-γ), 29 (IL-12 p40), and 33 (NO and TNF-α). Southern transfers of PCR products were subsequently probed with internal cytokine-specific oligonucleotides and visualized using the ECL chemiluminescent detection system (Amersham Corp., Arlington Heights, IL).

Statistics. Statistical evaluation of differences between means of experimental groups was done by analyses of variance and multiple Student's *t* tests. The log-rank was used for statistical

analysis of mortality. A value of $P < 0.05$ was considered to be significant.

Results

CD40L^{−/−} Mice Are More Susceptible to Infection with L. major but Not H. capsulatum Compared with CD40L^{+/+} Mice.   Recent work by several groups has shown that CD40^{−/−} or CD40L^{−/−} mice were susceptible to *Leishmania* infection due to diminished production of type 1 cytokines (17–19). In other studies, mice infected with *H. capsulatum* (6×10^5) and treated with anti–IL-12 or anti–IFN-γ have accelerated mortality, demonstrating a critical role for these cytokines in primary infection to *H. capsulatum* (24, 25). Thus, the role of CD40L in mediating protective immunity and the generation of type 1 cytokine production in response to *H. capsulatum* infection was evaluated. As shown in Fig. 1 *A*, the rate at which CD40L^{−/−} mice succumbed to infection (14.67 ± 3.82 d) was not different than that for CD40L^{+/+} mice (14.75 ± 3.84 d). By contrast, in the same experiment, CD40L^{−/−} mice infected with *L. major* were found to be susceptible to infection compared with control CD40L^{+/+} mice (Fig. 1 *B*). These data suggest that CD40–CD40L stimulation, although es-

sential for induction of type 1 responses to *L. major* (17–19), may not be required for eliciting type 1 cytokine responses to *H. capsulatum*.

CD40L^{−/−} Mice on a C57BL/6 × 129 Background Are Resistant to Sublethal Infection with H. capsulatum.   In addition to the experiments shown above using CD40L^{−/−} mice bred several generations (greater than six) onto a C57BL/6 background, a series of experiments using CD40L^{−/−} mice on a C57BL/6 × 129 background were initiated. It should be noted that in these experiments, the control CD40L^{+/+} mice are C57BL/6 × 129(F2), approximating the background of the CD40L^{−/−} mice. As shown in Fig. 2 in data combined from two independent experiments, infection of CD40L^{−/−} mice with *H. capsulatum* (6×10^5) showed a modest but not statistically significant increase in the rate of mortality (35.6 ± 20.4 d) compared with the control CD40L^{+/+} mice (41.5 ± 21.3 d). In addition, all mice infected with a sublethal dose (6×10^4 or 6×10^3) survived infection. Thus, these data support a CD40L-independent role in protective immunity.

Quantitative Burden of H. capsulatum in CD40L^{−/−} and CD40L^{+/+} Mice on Different Backgrounds.   To correlate whether the fatal outcome shown above was due to an increase in the infectious burden of *H. capsulatum*, quantitative cultures were set up from spleen cells at various times after infection. In the same experiment as shown in Fig. 2, spleen cells of mice were harvested 7 d after infection and CFUs for *H. capsulatum* were determined (Table 1). CD40L^{−/−} mice infected with 6×10^5 yeast cells had a twofold increase in infectious burden at 7 d after infection compared with control mice. Furthermore, CD40L^{−/−} mice infected with lower doses (6×10^4, 6×10^3) had almost identical CFUs for *H. capsulatum* compared with control mice. These data further support the contention that CD40L is dispensable for this infection.

CD40L^{−/−} Mice Have Similar mRNA Expression of Inflammatory Cytokines as Do CD40L^{+/+} Mice.   In the studies ex-
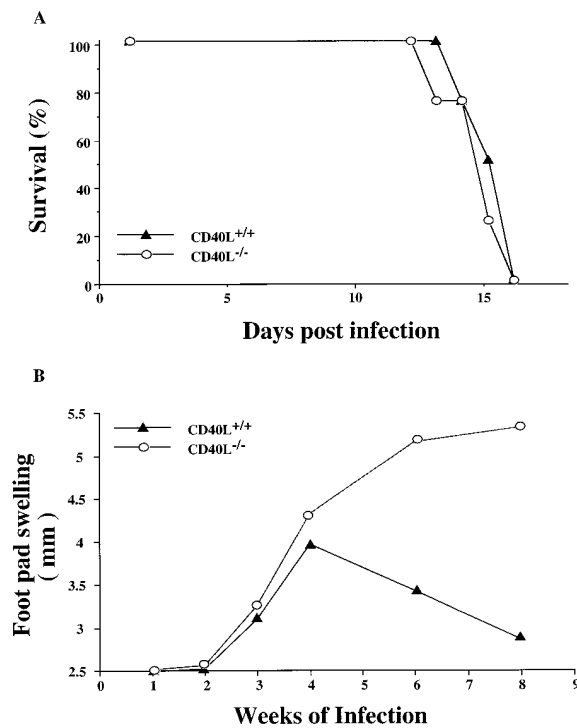

Figure 1.   CD40L^{−/−} mice on a C57BL/6 background are more susceptible to infection with *L. major* but not *H. capsulatum* compared with CD40L^{+/+} mice. (*A*) CD40L^{−/−} or CD40L^{+/+} mice (6–10 mice/group) on a C57BL/6 background were injected intravenously with *H. capsulatum* yeast cells (6×10^5) and followed for mortality. (*B*) In a parallel experiment, CD40L^{−/−} and CD40L^{+/+} mice were challenged in the hind footpad with 10^5 live *L. major* metacyclic promastigotes. Weekly footpad measurements represent the average footpad scores $\pm$ SEM. Similar results were noted in a second experiment.
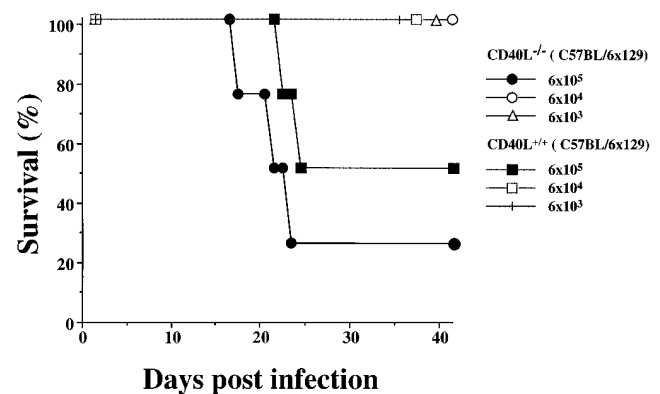

Figure 2.   CD40L^{−/−} mice are resistant to sublethal infection with *H. capsulatum*. CD40L^{−/−} or CD40L^{+/+} mice (4–6 mice/group) on a C57BL/6 × 129 background were injected intravenously with varying doses of *H. capsulatum* yeast cells (6×10^5, 6×10^4, 6×10^3) and followed for mortality. Results are combined from two individual experiments.

Table 1. *Quantitative Burden of H. capsulatum in CD40L$^{-/-}$ and CD40L$^{+/+}$ Mice After Primary Infection*[*]

	H. capsulatum, day 7
	CFUs
CD40L$^{-/-}$ (C57BL/6 × 129)	
HC 6 × 10^5	3.3 × 10^6 ± 0.18 × 10^6
HC 6 × 10^4	6.5 × 10^5 ± 0.21 × 10^5
HC 6 × 10^3	3.5 × 10^5 ± 1.21 × 10^{5‡}
CD40L$^{+/+}$ (C57BL/6 × 129)	
HC 6 × 10^5	1.4 × 10^6 ± 0.12 × 10^6
HC 6 × 10^4	6.7 × 10^5 ± 0.12 × 10^5
HC 6 × 10^3	2.7 × 10^5 ± 0.35 × 10^{5‡}

[*]Mice were infected with *H. capsulatum* with various doses (6 × 10^5, 6 × 10^4, or 6 × 10^3), and quantitative burden of *H. capsulatum* from spleen cells of individual mice ($n = 3$) were determined 7 d after infection as described in Materials and Methods. 1 wk later, colonies were counted as CFUs.
[‡]Results are statistically different ($P < 0.001$) from those of mice infected with *H. capsulatum* (6 × 10^5).

amining cytokine production in CD40$^{-/-}$ or CD40L$^{-/-}$ mice in response to leishmanial infection, it was clear that there was a marked deficiency in production of IFN-γ (17–19), IL-12 (17, 19), and nitric oxide (NO) (18). To determine whether any qualitative or quantitative changes in cytokine production occurred in CD40L$^{-/-}$ mice compared with control CD40L$^{+/+}$ mice after *H. capsulatum* infection, mRNA expression for several cytokines previously found to be important in regulating primary immunity to *H. capsulatum* was assessed by semiquantitative PCR at various time points after infection. As shown in Fig. 3, mRNA for IFN-γ, IL-12 p40, and TNF-α were expressed from spleen cells of CD40L$^{-/-}$ and CD40L$^{+/+}$ mice 7 and 20 d after infection with any of the doses tested. In addi-

tion, mRNA for NO was expressed from both types of mice 7 d after infection. As a control, there was minimal mRNA expression for any of the cytokines from spleen cells of uninfected mice prepared at day 7 after infection. Thus, these data confirm that type 1 cytokines are induced in CD40L$^{-/-}$ mice after infection with *H. capsulatum*. Finally, it should be noted that similar amounts (4–7 ng/ml) of IL-12 protein (p40 + p70) were detected from serum 3 and 7 d after infection from both CD40L$^{-/-}$ and CD40L$^{+/+}$ mice (data not shown).

In Vivo Depletion of IFN-γ or IL-12 Results in Accelerated Mortality in CD40L$^{-/-}$ Mice in Response to Infection with H. capsulatum. The data shown above suggest that CD40L$^{-/-}$ and CD40L$^{+/+}$ mice induce comparable type 1 immune responses that might provide some immunity against a lethal challenge with *H. capsulatum*. Functional evidence showing that CD40L$^{-/-}$ mice are capable of producing IL-12 and IFN-γ in vivo was demonstrated by treating mice with neutralizing antibodies against IL-12 or IFN-γ at the time of infection and assessing their outcome. As shown in Fig. 4, CD40L$^{-/-}$ mice treated with either anti–IL-12 (8.6 ± 1.3 d) or anti–IFN-γ (8.8 ± 1.1 d) had accelerated mortality compared with mice infected with only CD40L$^{-/-}$ (31.0 ± 15.1 d; $P < 0.05$). Similar results were seen from CD40L$^{+/+}$ mice after infection. Neutralization of IL-12 or IFN-γ also resulted in a 3–10-fold increase in infectious burden of *H. capsulatum* compared with infected-only mice ($P < 0.001$), providing further evidence that CD40L$^{-/-}$ mice are capable of making IL-12 and IFN-γ in vivo.

CD40L Is Not Required for Protective Immunity against H. capsulatum. Although CD40L$^{-/-}$ mice had similar outcomes to control mice infected with a lethal dose of *H. capsulatum*, most of them still succumbed to infection. By contrast, as demonstrated in Fig. 2, CD40L$^{-/-}$ mice infected with 6 × 10^4 yeast cells were able to control infection, suggesting that CD40L is not essential for protective immunity at this infective dose. Since we had shown in a previous study that IFN-γ$^{-/-}$ mice infected with 6 × 10^4 or 6 × 10^3 yeast cells succumbed to infection (26), the relative dis-
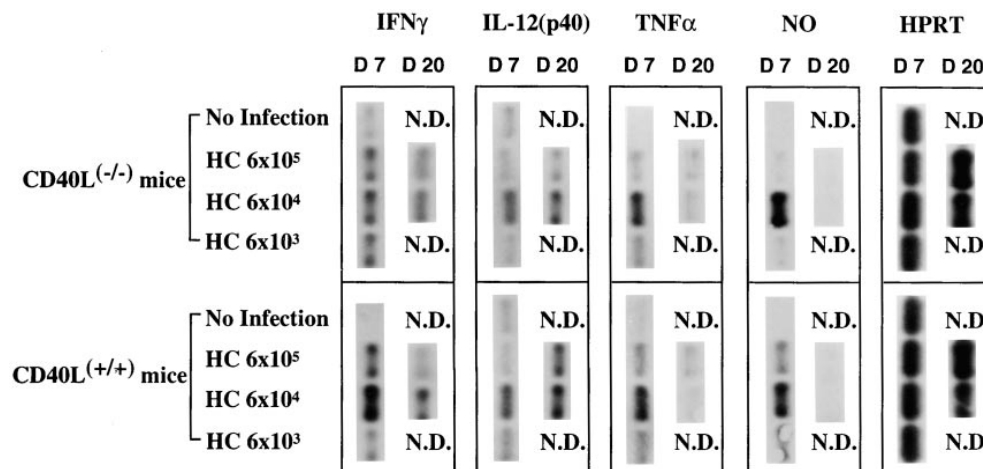

Figure 3. CD40L$^{-/-}$ mice have similar mRNA expression for type 1 cytokines as CD40L$^{+/+}$ mice. mRNA was isolated from spleen cells combined from three individual spleens of mice at 7 and 20 d after primary infection with *H. capsulatum*. As a control, mRNA was prepared from uninfected mice at 7 d after infection. Cytokine mRNA was subsequently determined for IFN-γ, IL-12 p40, TNF-α, and NO by semiquantitative reverse transcription PCR as described in Materials and Methods. N.D., not done.

Days post infection
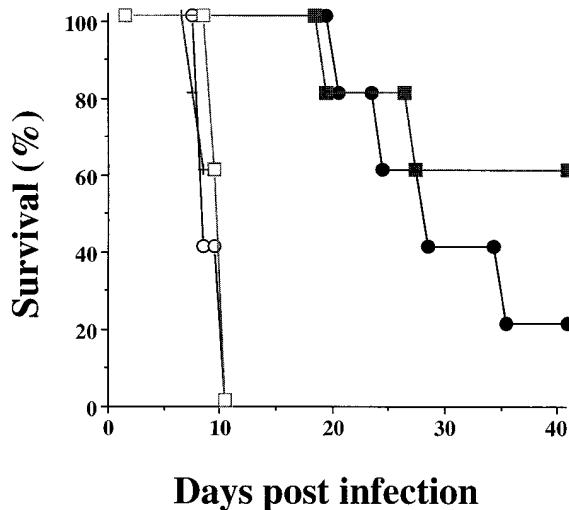
Quantitation	*H. capsulatum* (cfu) Day 7
CD40L$^{-/-}$	
—●— HC	$2.6 \times 10^6 + 0.16 \times 10^6$
—○— HC+aIFNγ	$2.3 \times 10^7 + 0.21 \times 10^7$ *
—+— HC+aIL-12	$1.9 \times 10^7 + 0.13 \times 10^7$ *
CD40L$^{+/+}$	
—■— HC	$4.8 \times 10^6 + 0.60 \times 10^6$
—□— HC+aIFNγ	$1.7 \times 10^7 + 0.13 \times 10^7$*

Figure 4. Neutralization of endogenous IFN-γ or IL-12 results in accelerated mortality in CD40L$^{-/-}$ and CD40L$^{+/+}$ mice after infection with *H. capsulatum*. CD40L$^{-/-}$ and CD40$^{+/+}$ (C57BL/6 × 129) mice (4–6 mice/group) were injected intravenously with *H. capsulatum* yeast cells (6 × 10^5) and followed for mortality. Additional groups of mice were treated with anti–IFN-γ (1 mg) or anti–IL-12 (1 mg) at the time of reinfection. Results are combined from two independent experiments. To assess quantitative burden of *H. capsulatum*, mice were killed 7 d after infection, and spleen cells from individual mice (*n* = 3) were plated at various concentrations on agar plates as described in Materials and Methods. 1 wk later, colonies were counted as CFUs. Asterisk indicates that results are statistically different (*P* <0.001) from those of mice infected with *H. capsulatum* alone.

pensability of CD40L compared with IFN-γ in developing effective immunity against primary infection with a sublethal dose of *H. capsulatum* (6 × 10^4) was assessed. As shown in Fig. 5, all CD40L$^{-/-}$ and CD40L$^{+/+}$ mice survived infection with 6 × 10^4 yeast cells, whereas all mice treated with anti–IFN-γ had a fatal outcome with accelerated mortality. These experiments show conclusively that effective primary immunity to *H. capsulatum* occurs in an IFN-γ–dependent, CD40L-independent manner.

In the Absence of CD40L, CD4$^+$ or CD8$^+$ T Cells Are Required for Effective Immunity to Infection with H. capsulatum. Both CD4$^+$ and CD8$^+$ T cells have been shown to be important in primary immunity against *H. capsulatum* (33, 34). To assess the role of CD4$^+$ and CD8$^+$ T cells in CD40L$^{-/-}$ mice infected with *H. capsulatum*, animals were treated with antibodies against CD4$^+$ and CD8$^+$ T cells before, at the time of, and after infection to ensure depletion (>95%
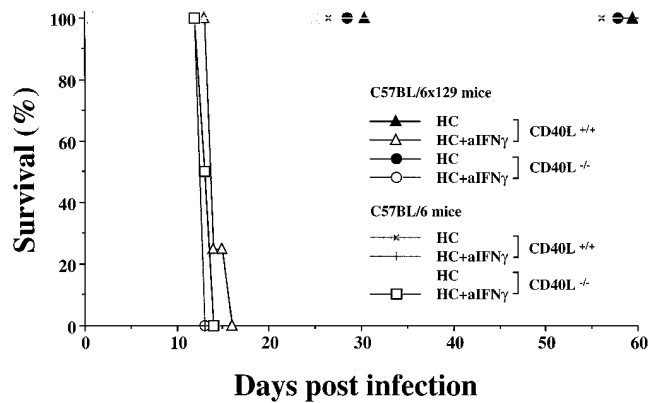

Figure 5. IFN-γ but not CD40L is required for protective immunity after sublethal infection to *H. capsulatum*. CD40L$^{-/-}$ and CD40L$^{+/+}$ mice on a C57BL/6 × 129 or C57BL/6 background were infected intravenously with a sublethal dose of *H. capsulatum* yeast cells (6 × 10^4) and treated with anti–IFN-γ (1 mg) at the time of infection. Mice were followed for mortality.

by FACS®). As shown in Fig. 6 in data combined from two independent experiments, depletion of either CD4$^+$ (12.8 ± 0.9 d) or CD8$^+$ T cells (12.3 ± 0.05 d) caused accelerated mortality compared with infected controls (34.8 ± 19.6 d). This increase in mortality was associated with a three- to fourfold increase in CFUs for *H. capsulatum* from spleen cells of mice depleted of either CD4$^+$ or CD8$^+$ T cells (*P* <0.001). Thus, in the absence of CD40L, CD4$^+$ or CD8$^+$ T cells play a critical role in protective immunity.

CD40L Is Not Required for Protective Immunity after Secondary Infection to H. capsulatum. One final aspect of these studies was to examine the requirement for CD40L in a memory or secondary immune response to *H. capsulatum*. To assess the role of CD40L$^{-/-}$ in the maintenance of immunity after secondary infection, CD40L$^{-/-}$ mice were
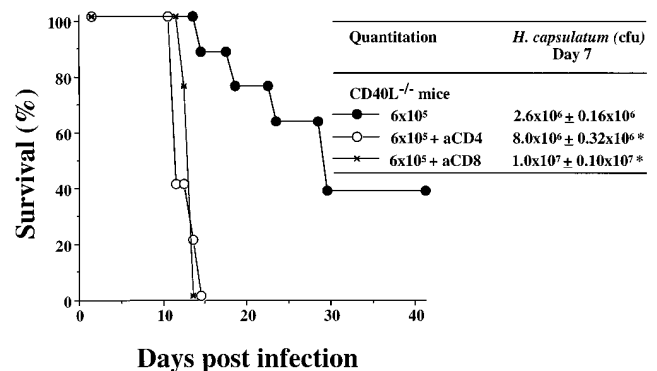

Days post infection

Quantitation	*H. capsulatum* (cfu) Day 7
CD40L$^{-/-}$ mice	
—●— 6×10^5	$2.6 \times 10^6 \pm 0.16 \times 10^6$
—○— 6×10^5 + aCD4	$8.0 \times 10^6 \pm 0.32 \times 10^6$ *
—×— 6×10^5 + aCD8	$1.0 \times 10^7 \pm 0.10 \times 10^7$ *

Figure 6. CD4 or CD8$^+$ T cells are required for effective immunity to *H. capsulatum* infection in CD40L$^{-/-}$ mice. In results combined from two independent experiments, CD40L$^{-/-}$ and CD40L$^{+/+}$ mice (4–6 mice/group) intravenously infected with *H. capsulatum* yeast cells (6 × 10^5) were treated with anti-CD4 (1 mg) or anti-CD8 (1 mg) 3 d before, at the time of, and 5–7 days after infection and were followed for mortality. Quantitative burden of *H. capsulatum* was assessed as described in Fig. 4. Similar results were noted in a second experiment.
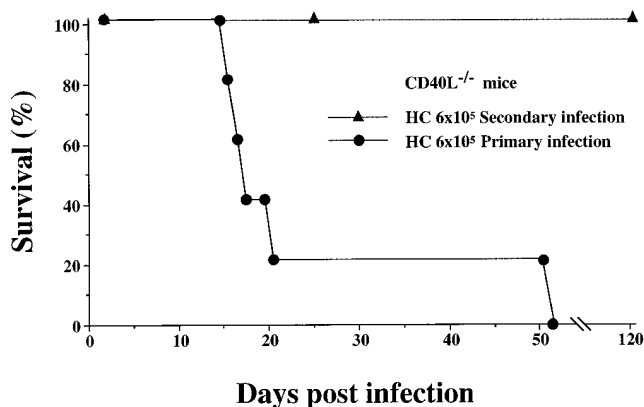
Figure 7. CD40L is not required for effective immunity after secondary infection with a lethal challenge of *H. capsulatum*. In results combined from two independent experiments, CD40L$^{-/-}$ mice initially were infected intravenously with 6×10^4 yeast cells and then reinfected (secondary infection) 3 wk later with *H. capsulatum* yeast cells (6×10^5). As a control, CD40L$^{-/-}$ mice were infected primarily with 6×10^5 yeast cells at the same time mice were undergoing secondary infection.

initially infected with a sublethal dose of *H. capsulatum* (6×10^4) and then reinfected 3–4 wk later with a lethal dose (6×10^5). As shown in Fig. 7 in data combined from two independent experiments, all CD40L$^{-/-}$ mice reinfected with a lethal dose (6×10^5) survived and remained healthy up to 120 d after infection. As a control, a majority of the CD40L$^{-/-}$ mice undergoing primary infection (6×10^5) at the same time succumbed within 20–30 d, consistent with the data shown in the previous figures.

To determine whether effective immunity to a secondary challenge was due to control of *H. capsulatum* in vivo, the infectious burden of *H. capsulatum* was assessed from spleen cells at various time points after reinfection. Mice originally infected with 6×10^4 yeast cells had low but detectable amounts of *H. capsulatum* at the time of reinfection (day 0) in one of the experiments (Table 2, Exp. 1) and no detectable amounts in the other experiment (Table 2, Exp. 2). When assessed 7 d after reinfection, previously infected

CD40L$^{-/-}$ mice had a 3-log reduction ($P < 0.001$) in *H. capsulatum* compared with CD40L$^{-/-}$ mice undergoing primary infection (Table 2, Exp. 2) at the same time. Moreover, in neither experiment was there any *H. capsulatum* detected from spleen cells at 60 or 90 d after reinfection, demonstrating that CD40L$^{-/-}$ is not essential for the development of sterilizing immunity after reinfection.

Discussion

CD40L Is Not Essential for Effective Primary Immunity to H. capsulatum. CD40L–CD40 interactions have a central role in the induction of humoral and cellular immune responses (35, 36). With regard to its effects on the cellular immune response, CD40L–CD40 induction of inflammatory cytokines and costimulatory molecules from macrophages and dendritic cells has been shown to enhance the cellular immune response with production of type 1 cytokines (35). Since IL-12–dependent production of IFN-γ was shown to be essential for protective immunity after primary infection to the intracellular fungi *H. capsulatum* (24), it was of interest to determine the requirement for CD40L in the generation of type 1 cytokine responses after primary and secondary infection with *H. capsulatum*. The initial experiments using CD40L$^{-/-}$ mice bred onto a C57BL/6 background (>6 generations) showed that they did not have accelerated mortality or increased fungal burden (data not shown) compared with CD40L$^{+/+}$ mice (Fig. 1). In several additional experiments using CD40L$^{-/-}$ mice on a C57BL/6 × 129 background, there did appear to be a modest increase in the rate of mortality (Figs. 2 and 4) and the fungal burden (Table 1) compared with the control CD40L$^{+/+}$ mice in response to a lethal dose (6×10^5 yeast) of *H. capsulatum*. However, it should be noted that CD40L$^{-/-}$ or CD40L$^{+/+}$ mice infected with a sublethal amount of *H. capsulatum* (6×10^4) had identical outcomes. Furthermore, the data showing that mRNA for IL-12, IFN-γ, TNF-α, and NO are similar between the CD40L$^{-/-}$ and CD40L$^{+/+}$ mice, combined with the fact that in vivo neutralization with anti–IFN-γ or anti–IL-12 caused accelerated mortality and

Table 2. *CD40L$^{-/-}$ Mice Develop Sterilizing Immunity after a Secondary Challenge with H. capsulatum***

	H. capsulatum (CFU)		
Experiment 1	Day 0	Day 7	Day 90
Secondary infection: HC 6×10^5	$4.6 \times 10^2 \pm 0.03 \times 10^2$	ND	<10
Experiment 2	Day 0	Day 7	Day 60
Secondary infection: HC 6×10^5	<10	$8 \times 10^2 \pm 0.84 \times 10^2$	<10
Primary infection: HC 6×10^5	ND	$5.3 \times 10^6 \pm 0.25 \times 10^{6\ddagger}$	Died

*In two independent experiments, CD40L$^{-/-}$ mice were initially infected with 6×10^4 yeast cells and then reinfected (secondary infection) 3–4 wk later with *H. capsulatum* (6×10^5). At the time of secondary infection (day 0), spleens of CD40L$^{-/-}$ mice were assessed for *H. capsulatum* CFUs. Quantitative burden of *H. capsulatum* was also assessed from spleens of mice at various times after secondary infection. In addition, quantitation was done from CD40L$^{-/-}$ undergoing primary infection at the same time mice were reinfected.
‡Results are statistically different ($P < 0.001$) from those of mice undergoing primary infection with *H. capsulatum*.

increased fungal burden, provides strong evidence that CD40L is not required for the induction of type 1 responses after infection with *H. capsulatum.* Finally, the demonstration that all CD40L$^{-/-}$ mice survived infection with a sublethal dose of *H. capsulatum* (Fig. 5) but all mice treated with an anti–IFN-γ antibody had accelerated mortality and increased fungal burden provided conclusive evidence that there is a CD40L-independent pathway for IFN-γ production.

Differential Role for CD40L in the Induction of Type 1 Cytokine Production in Response to H. capsulatum and Other Intracellular Infections versus L. major. The ability of CD40L$^{-/-}$ mice to generate functional Th1 responses in vivo is in contrast to the studies previously alluded to in which CD40$^{-/-}$ or CD40L$^{-/-}$ mice had a striking deficiency in production of IL-12 or IFN-γ after infection with *L. major* or *Leishmania amazonensis* (17–19). Several potential mechanisms could account for this failure of these deficient mice to develop functional type 1 responses in response to leishmanial infection. One is that *L. major* is a relatively poor direct inducer of IL-12 production or other inflammatory mediators compared with other infectious pathogens. This is supported by previous studies showing that *Leishmania* promastigotes are able to evade or inhibit IL-12 production (37, 38). In data not shown, we found that peritoneal macrophages from CD40L$^{-/-}$ mice produced comparable amounts of IL-12, TNF-α, and NO in vitro as CD40L$^{+/+}$ mice in response to *Staphylococcus aureus* Cowan strain, *Toxoplasma gondii*, and *H. capsulatum*; however, there was no induction of these cytokines in response to *L. major* promastigotes (data not shown). These data are consistent with a recent report by DeKruyff et al. showing that heat-killed *Listeria monocytogenes* induces IL-12 production in a CD40L-independent manner in vitro (20). In addition, the demonstration that SCID mice infected with *H. capsulatum* (39), *L. monocytogenes* (40), or *T. gondii* (32) all had accelerated mortality when treated at the time of infection with anti–IL-12 provides additional evidence that these pathogens can induce IL-12 directly or at least in the absence of T cells. Taking these data together, we would conclude that, in addition to *H. capsulatum, L. monocytogenes* and *T. gondii* would also induce IL-12 in vivo independently of CD40L. Finally, it should be noted that although *L. major* may be a relatively poor direct inducer of IL-12, mice on a resistant C57BL/6 background develop effective immunity after infection with *L. major* in an IL-12–dependent manner, suggesting additional factors to explain the enhanced susceptibility of CD40L$^{-/-}$ mice to *L. major* (see below).

A second mechanism to explain why CD40L$^{-/-}$ mice fail to induce IL-12 in response to *L. major* infection is that this experimental model is highly reliant on MHC class II–dependent CD4 responses for primary immunity (41–43). Furthermore, based on in vivo (1) and in vitro (6, 20) evidence showing that CD4$^+$ T cell–mediated production of type 1 cytokines is CD40L dependent in response to protein antigens, it is possible that antigens that require CD4 and/or are relatively poor direct inducers of IL-12 (i.e., *L. major*) would require CD40L for a functional IL-12 re-

sponse. This relative CD4 dependence in the *Leishmania* model would be contrasted to experimental models of listeriosis, toxoplasmosis, and histoplasmosis in which both CD4$^+$ and CD8$^+$ T cells have a role in primary immunity (see below). Moreover, *L. monocytogenes, T. gondii,* and *H. capsulatum* are all potent inducers of IFN-γ from NK cells through induction of IL-12. Thus, in these latter models, CD8$^+$ T cells or NK cells may provide a sufficient amount of IFN-γ or other proinflammatory mediators that could help control infection directly and/or potentiate further production of IL-12 by a positive feedback mechanism (44, 45).

In the Absence of CD40L, CD4$^+$ or CD8$^+$ Cells Are Required for Effective Primary Immunity. As noted above, there is evidence that CD8$^+$ T cells also play an important role in primary immunity against a variety of intracellular pathogens including *T. gondii* (46), *Mycobacterium bovis* (47), *Mycobacterium tuberculosis* (48), and *H. capsulatum* (34). It has been shown that the ability of CD8$^+$ T cells to influence immunity is through at least two independent mechanisms. For secondary *Listeria* infection, CD8$^+$ T cells have been shown to protect mice through a cytolytic mechanism independent of IFN-γ (49, 50). By contrast, in murine models of *L. monocytogenes* (50), *M. tuberculosis* (51, 52), *T. gondii* (53), or *H. capsulatum* infection (Zhou, P., manuscript in preparation), primary immunity is maintained in the absence of perforin- or granzyme-mediated cytolysis. Since IFN-γ is required for effective primary immunity to all of these infections, these data suggest that IFN-γ produced from CD8$^+$ T cells is responsible for the effector function at this phase of the response.

In the experiments reported here, CD40L$^{-/-}$ mice depleted of either CD4$^+$ or CD8$^+$ T cells had similar outcomes in terms of accelerated mortality and increased fungal burden after primary infection with *H. capsulatum.* Explanations for these data follow. (*a*) There is a quantitative threshold for cytokine (i.e., IFN-γ) production necessary for effective primary immunity in the absence of CD40L requiring both CD4$^+$ and CD8$^+$ T cells. In preliminary data, CD40L$^{-/-}$ mice depleted of CD4$^+$ T cells at the time of infection had markedly diminished production of IFN-γ (data not shown). (*b*) Depletion of either CD4$^+$ or CD8$^+$ T cells changes the qualitative cytokine response. This is consistent with a striking enhancement of the Th1 counterregulatory cytokine IL-10 noted from mice depleted of CD4$^+$ or CD8$^+$ T cells (data not shown). (*c*) There are recent reports showing that CD4$^+$ T cells are required for CD8$^+$ T cell function (54, 55). This dependence on CD4$^+$ T cells could occur through modification of the antigen presenting cell (i.e., CD40L-inducing activation and/or cytokine production) or to provide a cytokine-rich environment (i.e., IL-2) for the CD8$^+$ T cells. These explanations may relate to the studies reported here in which the absence of both CD4$^+$ T cells and CD40L may not provide help sufficient for CD8$^+$ T cell activation. Experiments are now in progress to determine the immune mechanism by which CD4 and CD8 depletion worsens the course of infection in these mice.

CD40L Is Not Required for Secondary Immunity to H. capsulatum. In a previous study, we showed that effective primary immunity to systemic infection requires a coordinated immune response requiring many factors including IL-12, IFN-γ, TNF-α, or NO. By contrast, none of these factors alone, including IFN-γ, were required for the maintenance of immunity after secondary challenge to *H. capsulatum* (26); however, treatment of mice with both anti–IFN-γ and anti–TNF-α at the time of reinfection did result in a fatal outcome. The only studies of the role of CD40L in memory immunity were done in experimental murine models of viral infection. In these studies, CD40L$^{-/-}$ mice had relatively normal primary CTL responses but had markedly diminished memory responses (14). In this report, CD40L$^{-/-}$ mice initially infected with a sublethal dose of *H. capsulatum* and then infected 3 wk later with a lethal dose remain alive more than 90 d after infection with no detectable *H. capsulatum* from spleen cells. These results demonstrate that CD40L is not required for effective secondary immunity.

CD40L-independent Induction of Type 1 Cytokine Responses Has Clinical Application for Infectious and Autoimmune Disease. To summarize, *L. major* may in fact be one of the few intracellular infections requiring CD40–CD40L stimulation for the induction of functional type 1 cytokine responses. Infection with other pathogens such as *L. monocytogenes*, *T. gondii*, and *M. tuberculosis*, which directly induce IL-12 and/or have a role for CD8$^+$ T cells in immunity, would not show increased susceptibility in the absence of CD40L. This is supported by the observations in studying patients with hyper-IgM syndrome (56). In a large review of such patients, a minority of patients were reported to

have increased susceptibility to opportunistic infections associated with T cell deficiencies such as *Pneumocystis carinii* (<10%) and *Cryptosporidium* (<2%), whereas there were no cases described for other infections mediated by type 1 cellular immune responses such as *T. gondii*, *M. tuberculosis*, or *H. capsulatum*. These latter findings would be consistent with the ability of these infectious pathogens to induce type 1 cytokine responses by the mechanisms listed above. In addition, since exogenous B7 costimulation has been shown to restore IFN-γ production in CD40L$^{-/-}$ mice (12, 13), it is possible that these various pathogens may differ in their capacity to activate accessory cells in vivo, providing a mechanism to activate T cells independently of CD40L–CD40.

Finally, with regard to autoimmune disease, since CD40L–CD40 stimulation has been shown to be critical for both IL-12 production and T cell activation in response to protein antigens in vivo, inhibition of CD40L–CD40 may be a potent treatment for certain organ-specific autoimmune diseases. This is supported by murine models of experimental autoimmune encephalitis and inflammatory colitis in which treatment with anti–IL-12 (57, 58) or anti-CD40L (59, 60) was shown to ameliorate disease. One cause of concern from chronic treatment with either of these inhibitors would be increased susceptibility to intracellular infection. The ability to sustain and generate an effective immune response to certain intracellular pathogens independently of CD40L may lessen this potential increase in susceptibility to these particular infections by anti–CD40L–CD40 treatment. By contrast, treatments directed at inhibiting IL-12 while ameliorating autoimmune disease could still increase vulnerability to the aforementioned intracellular infections.

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