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The Development of a Th1-Type Response and Resistance to Leishmania major Infection in the Absence of CD40-CD40L Costimulation

Udaikumar M. Padigel,* Peter J. Perrin,‡ and Jay P. Farrell§

CD40-CD40L interactions have been shown to be essential for the production of IL-12 and IFN-γ and control of L. major infection. In contrast, C57BL/6 mice deficient in CD28 develop a dominant Th1-type response and heal infection. In this study, we investigate the effects of a deficiency in both CD40L and CD28 molecules on the immune response and the course of L. major infection. We compared infection in mice genetically lacking CD40L (CD40L−/−), CD28 (CD28−/−), or both (CD40L−/−CD28−/−), and in C57BL/6 mice, all on a resistant background. Although CD40L−/− mice failed to control infection, CD28−/− and CD40L−/−CD28−/− mice, as well as C57BL/6 mice, spontaneously resolved their infections. Healing mice had reduced numbers of lesion parasites compared with nonhealing CD40L−/− mice. At wk 9 of infection, we detected similar levels of IL-4, IFN-γ, IL-12p40, and IL-12Rβ2 mRNA in draining lymph nodes of healing C57BL/6, CD28−/−, and CD40L−/−CD28−/− mice, whereas CD40L−/− mice had increased mRNA levels for IL-4 but reduced levels for IFN-γ, IL-12p40, and IL-12Rβ2. In a separate experiment, blocking of the CD40-CD40L pathway using Ab to CD40L led to an exacerbation of infection in C57BL/6 mice, but had little or no effect on infection in CD28−/− mice. Together, these results demonstrate that in the absence of CD28 costimulation, CD40-CD40L interaction is not required for the development of a protective Th1-type response. The expression of IL-12p40, IL-12Rβ2, and IFN-γ in CD40L−/−CD28−/− mice further suggests the presence of an additional stimulus capable of regulating IL-12 and its receptors in absence of CD40-CD40L interactions.

Infections with Leishmania major in inbred mice have been used extensively to study the in vivo regulation of Th1-and Th2-type responses. Numerous studies have shown that mice that develop self-limiting L. major infections do so because they generate an immunological response in which the Th1 subset of CD4+ cells predominates (reviewed in Ref. 1). The association between Th1-type response and protection is due primarily to the ability of Th1 cells to produce the macrophage-activating cytokine IFN-γ (2). In contrast, nonhealing infections, such as those that occur in BALB/c mice, are characterized by the development of dominant Th2-like response in which parasite-specific CD4+ cells produce high levels of IL-4 and other Th2-associated cytokines but little IFN-γ. In the past few years, a number of studies have examined how signaling through costimulatory molecules affects cytokine production and the subsequent development of resistance to cutaneous leishmaniasis. These studies have shown that CD40-CD40L interactions appear to be essential for induction of a protective cell-mediated immunity to both L. major and the related protozoan parasite, Leishmania amazonensis. CD40−/− mice on a resistant C57BL/6 × 129J background are markedly impaired in their production of IL-12 and IFN-γ and fail to control their infections (3–5). In contrast, studies with other intracellular pathogens like Histoplasma (6), Mycobacterium (7), and Toxoplasma (8) have shown that the CD40-CD40L interactions are not essential for initiating a protective Th1-type response, although in the case of Toxoplasma, CD40-CD40L costimulation may be required for limiting chronic brain encephalitis. The role of B7-CD28 costimulation in cutaneous leishmaniasis has also been extensively examined. Treatment of BALB/c mice with CTLA4Ig, which blocks interactions between B7.1/B7.2 on APCs and both CD28 and CTLA-4 expressed on T cells, will promote healing if given before infection, but will block healing if administered continuously through infection (9). However, CD28−/− mice bred onto either the BALB/c or C57BL/6 background do not exhibit altered patterns of infection or cytokine production following infection with L. major, suggesting that CD28-B7 interactions are not absolutely required for the development of Th2- vs Th1-type responses (10).

In the present study, we further examined the role of costimulation during infection using mice that lack either CD40L or CD28 molecules and mice lacking both CD40L and CD28 molecules. Our results show that CD40L−/−CD28−/− mice, in contrast to CD40L−/− mice, control infection. In addition, these double knockout (DKO) mice fail to exhibit the defects in IFN-γ production characteristic of infection in CD40L−/− mice, suggesting that CD40-CD40L interaction is not the only pathway for inducing IFN-γ production during this infection. Furthermore, in a separate set of experiments, we show that reduction in the magnitude of the response by lowering the number of parasites used to initiate infection leads to the activation of a dominant Th1 response in

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4 Abbreviations used in this paper: DKO, double knockout; RPA, RNase protection assay; SLA, soluble leishmanial Ag.
CD40L−/− mice. These results suggest that parasite dose as well as costimulatory pathways can influence the pattern of response to *L. major* infection.

**Materials and Methods**

**Parasites and animals**

Female BALB/c, C57BL/6, and CD40L−/− mice (B6:129S-Tgfs2m1Hnes) were purchased from The Jackson Laboratory (Bar Harbor, ME). Dr. C. Hunter at the University of Pennsylvania (Philadelphia, PA) provided CD28 KO mice breeding pairs that were used for all experiments. The original CD40L KO mice (mixed 129J × C57BL/6 background) were obtained from The Jackson Laboratory. The original CD28 KO mice were on the C57BL/6 background. The CD40L−/− CD28−/− mice used in these studies were the F4 generation. The genotype of the knockouts was verified by PCR screening for the wild-type and KO locus, and confirmed by FACS analysis of splenic T cells for the absence of CD28 and CD40L. All mice were 6–10 wk old at the time of infection. *L. major* (WHO MHOM/IL/80/Friedlin) was maintained in an insect cell culture medium (Life Technologies, Grand Island, NY) containing 20% FBS, 2 mM L-glutamine, 100 µg of streptomycin, and 100 µU of penicillin G sodium per milliliter.

**Infections**

Mice were inoculated into one hind footpad with either 1 × 10^5 stationary-phase *L. major* promastigotes. In experiments where we used low doses of parasites at 50 infections, mice were inoculated with 1 × 10^5 to 2 × 10^4 metacyclic promastigotes selected from stationary phase cultures by negative selection using peanut agglutinin (Sigma-Aldrich, St. Louis, MO) as described previously (11). Lesion size was measured with Vernier calipers and expressed as the difference in thickness between the infected and the uninfected contralateral footpads. Parasites were enumerated by a limiting dilution assay as described previously (12). In brief, the homogenates of infected lesions were serially diluted in Grace's insect cell culture medium plus 20% FBS and observed 5–7 days later for growth of promastigotes. Parasite numbers are expressed as the negative log_{10} dilution at which promastigotes growth was observed.

**Anti-CD40L mAb treatment protocol**

CD28−/− mice and C57BL/6 mice were treated i.p. with anti-CD40L mAb (MR1; TSD Biosciences, Newark, DE) on day 0 (200 µg/mouse) and then on days 5, 10, and 15 (100 µg/mouse) of infection. Control mice were treated with normal rat Ig.

**RNase protection assay**

Total RNA was isolated from popliteal draining lymph nodes using RNA STAT-60 (Tel-Test, Friendswood, TX) as directed by the manufacturer. mRNA was quantified by RNase protection assay (RPA) using a Riboquant mRNA kit (BD PharMingen, San Diego, CA) as directed. A custom probe from BD PharMingen was prepared using [32P]UTP and hybridized to 15 µg of each sample RNA. The protected probe was purified and resolved on 5% denaturing polyacrylamide gels using Ultra Pure Sequagel reagents (National Diagnostics, Atlanta, GA). Dried gels were exposed to a phosphor imaging screen and protected fragments were visualized using a phosphor imager GS-525 Molecular Imager System (Bio-Rad, Richmond, CA).

**ELISPOT assay**

The number of IL-12p40 secreting cells in lymph node and spleen cell suspensions was determined using an ELISPOT assay as previously described (13). The mAbs C17.8 and biotinylated C15.6 were generously provided by Dr. C. Hunter (University of Pennsylvania). IL-12 secreting cells were determined in cell cultures following overnight stimulation with 50 µg/ml soluble leishmanial Ag (SLA).

**Cell culture and ELISA**

Single-cell suspensions of spleens were cultured at 5 × 10^6 cells/ml in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 5 × 10^–5 M 2-ME in the presence of 50 µg/ml SLA (prepared as described previously; Ref. 14). Supernatants were collected at 72 h and assayed for IFN-γ by ELISA as previously described (15). rIFN-γ (generously provided by Dr. P. Scott, University of Pennsylvania) was used as standard.

**Statistical analysis**

Statistically significant differences between groups were determined using the unpaired Student t test. Significance was assumed if *p* < 0.05.

**Results**

**CD40L−/− CD28−/− mice are resistant to *L. major* infection**

To determine whether combined defects in both CD40-CD40L and CD28-B7 signaling pathways would alter the response of mice to infection with *L. major*, we initially compared the course of infection in CD40L−/− CD28−/− mice with that in CD28−/−, CD40L−/−, and C57BL/6 mice following inoculation of 1 × 10^6 stationary-phase promastigotes into a hind footpad. As can be seen in Fig. 1, CD40L−/− mice, in confirmation of previous reports, failed to control infection, as evidenced by continued expansion of lesion size through wk 9 of infection. As expected, C57BL/6 wild-type as well as CD28−/− mice developed small lesions, which ultimately resolved. However, unexpectedly, CD40L−/− CD28−/− mice resolved their infection in a manner similar to that in CD28−/− and C57BL/6 wild-type mice. When lesion parasite numbers were determined at wk 9 of infection, CD40L−/− CD28−/− mice were found to harbor ~5 log fewer lesion parasites than CD40L−/− mice, while C57BL/6 and CD28−/− mice cleared their infections and had few detectable lesion parasites (Fig. 2).

**CD40L−/− CD28−/− mice develop immune responses similar to those in CD28−/− and C57BL/6 wild-type mice**

To assess the immune response of the various groups of mice during infection, we analyzed mRNA levels for cytokines and cytokine receptors by RPA. The mRNA expression levels of IL-2, IL-4, IFN-γ and IL-12 were not analyzed, as these cytokines were measured quantitatively by ELISA. As can be seen in Fig. 3, CD40L−/− CD28−/− mice expressed mRNA levels for IL-12p40, IL-12Rβ1, and IL-12Rβ2, as well as IFN-γ and IL-4 in draining lymph nodes were compared in CD40L−/− CD28−/−, C57BL/6, CD28−/−, and CD40L−/− mice at wk 9 of infection. As can be seen in Fig. 3, CD40L−/− CD28−/− mice expressed mRNA levels for IL-12p40, IL-12Rβ2, and IFN-γ similar to those expressed by CD28−/− and C57BL/6 mice. Message for IL-4 in CD40L−/− CD28−/− mice, as well as in C57BL/6 wild-type and CD28−/− mice, was not detected. In contrast, lymph nodes for CD40L−/− had reduced mRNA levels for IFN-γ, IL-12p40, and IL-12Rβ2 receptor, whereas the message levels for IL-4 were enhanced compared with those in the other groups of mice. When compared with uninfected C57BL/6, message levels for

![FIGURE 1](http://www.jimmunol.org/)

CD40L−/− CD28−/− mice are resistant to *L. major* infection. CD40L−/−, CD28−/−, CD40L−/− CD28−/−, and C57BL/6 wild-type mice were infected with *L. major* promastigotes (1 × 10^6) and followed for 9 wk. Lesion size was expressed as the difference in thickness between the infected and the uninfected contralateral footpads. Values are the mean ± SD of four to five mice per group and are representative of results of three separate experiments.
IFN-γ, IL-12p40, and IL-12Rβ2 in draining lymph nodes were increased 4- to 5-fold during infection with *L. major* (data not shown).

We extended this analysis to determine whether lymph node and spleen cells from CD40L−/−/CD28−/− mice produce IL-12p40 protein. We determined the frequency of IL-12p40-secreting cells in lymph node and spleen of these mice using an ELISPOT assay. CD40L−/−/CD28−/− mice along with C57BL/6 mice were infected with 1 × 10⁶ *L. major* promastigotes and sacrificed at wk 2 of infection. Lymph node and spleen cells from naive CD40L−/−/CD28−/− and C57BL/6 mice were used as control. IL-12p40-secreting cells from lymph node were ∼5- to 6-fold higher in infected CD40L−/−/CD28−/− and C57BL/6 mice compared with their respective naive controls (Fig. 4). Similar results were obtained with spleen cells of these mice (data not shown).

**Effect of anti-CD40L mAb treatment in C57BL/6 and CD28−/− mice**

We next investigated the effect of blockade of the CD40-CD40L interactions on the outcome of *L. major* infection in CD28−/− mice. We injected CD28−/− mice as well as C57BL/6 mice i.p. with anti-CD40L mAb on day 0 (200 μg/mouse), and then on days 5, 10, and 15 (100 μg/mouse) after infection, and monitored lesion size of these mice for 9 wk. CD28−/− and C57BL/6 mice were used as controls to compare the course of infection in anti-CD40L mAb-treated groups. As can be seen in Fig. 5, treatment with anti-CD40L mAb exacerbated infection in C57BL/6 mice, but not in CD28−/− mice. At wk 9 of infection, anti-CD40L mAb-treated CD28−/− mice had a reduced number of lesion parasites compared with anti-CD40L mAb-treated C57BL/6 mice (Fig. 6).

**Course of infection and immune response in mice infected with low numbers of parasites**

Because the absence of CD28 costimulation could influence the magnitude of the response to infection, we examined whether a similar effect would occur in mice inoculated with a lower number of promastigotes. CD40L−/− mice were inoculated with 2 × 10⁴ metacyclic promastigotes and lesion size was monitored for 8 wk. C57BL/6 and BALB/c mice infected with same dose were used as controls. As can be seen in Fig. 7, BALB/c mice, as expected, failed to control infection and developed progressive disease. However, CD40L−/− mice developed small lesions that ultimately resolved in a manner similar to those in C57BL/6 mice. Similar results were obtained when mice were infected with 5 × 10⁵ or 1 × 10⁵ parasites (data not shown). Mice were sacrificed at wk 15 of infection to determine lesion parasite numbers and cytokine production. Lesions from BALB/c mice, as expected, contained high numbers of parasites, whereas lesions from CD40L−/− and C57BL/6 mice were negative for parasites, suggesting that they had completely cleared their infections (data not shown). Spleen

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**FIGURE 3.** mRNA levels for cytokines and cytokine receptors in draining lymph nodes were determined by RPA at wk 9 of infection. The lanes represent two individual mice from each mutant group and one control C57BL/6 mouse.

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**FIGURE 4.** Lymph node cells from naive and infected DKO and C57BL/6 mice were assayed for IL-12p40 production by direct ELISPOT assay. The data are expressed as mean frequency per 10⁶ lymph node cells ± SE of three or more mice per group.

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**FIGURE 5.** The effect of anti-CD40L mAb (MR1) treatment on the course of *L. major* infection in CD28−/− and C57BL/6 mice. CD28−/− mice and C57BL/6 mice were treated i.p. with anti-CD40L mAb on day 0, and on days 5, 10, and 15 after infection. Values are the mean lesion size ± SD of five mice per group.
cells from these CD40L−/− and C57BL/6 mice produced significant levels of IFN-γ, whereas little IFN-γ was produced by cells from BALB/c mice (Fig. 8).

Discussion

The development of resistance to L. major in mice is dependent upon the generation of a cellular immune response in which a population of Th1-type cells produces the macrophage-activating cytokine IFN-γ. The development of a dominant Th1-type response, in turn, is dependent on the presence of IL-12 during the initial stage of T cell activation. Thus, deletion of the IL-12 gene or treatment of susceptible strains of mice with anti-IL-12 or resistance strains with anti-IL-12 Ab can reverse the expected pattern of infection with L. major (16–19). Conversely, mice treated with anti-IL-4 Ab or mice deficient in the gene for IL-4 or IL-4R are resistant to infection, consistent with a role for IL-4 in promoting a Th2-type response (20–22). In BALB/c mice, IL-4 production during early stages of infection is believed to down-regulate IL-12Rβ2 chain expression, resulting in progressive unresponsiveness to IL-12 (23–25). In addition to specific treatments which alter levels of IL-4 or IL-12, or nonspecific treatments such as sublethal irradiation or in vivo administration of CTLA4Ig or Abs to CD4 or IL-2, can also promote healing and the activation of a dominant Th1-type response in susceptible BALB/c mice (9, 26–28). Such nonspecific treatments may reduce the initial level of T cell activation, which, in susceptible mice such as the BALB/c, would result in reduced activation of IL-4-producing Th2-type cells.

Because costimulatory molecules play an important role in T cell activation, it is not surprising that they are equally important in regulating the in vivo response to L. major. Cells from BALB/c mice treated with an agonistic Ab to CD40 produce increased amounts of IL-12 and control infection (29). Conversely, mice genetically deficient in either CD40 or CD40L, or mice treated with neutralizing Abs to CD40L, have been shown to be highly susceptible to infection with either L. major and L. amazonensis (3–5, 30). Susceptibility has been linked to decreased levels of IL-12 during infection, suggesting that CD40-CD40L interaction, a known stimulus for IL-12 production (31), is a major stimulus for in vivo IL-12 production. Kamanaka and colleagues (3) have observed significantly less expression of IL-12p40 mRNA in cells from CD40−/− mice compared with those cells from wild-type mice following infection with L. major. Another study with L. amazonensis reported a 10-fold reduction in IFN-γ mRNA in cells from CD40L−/− mice compared with IFN-γ mRNA levels from cells from wild-type mice (5). In the present study, we confirm that cells from infected, nonhealing CD40L−/− mice express elevated mRNA levels of IL-4, but little for IL-12 or IFN-γ. In addition, message levels for IL-12Rβ2 in these mice were low compared with those in wild-type mice, which would be consistent with the development of a Th2-type response. In contrast, we show that mice lacking both the genes for CD40L and CD28 can control infection and, importantly, express mRNA levels of IL-12, IL-12Rβ2, and IFN-γ comparable to those in healing C57BL/6 mice. Furthermore, using an ELISPOP assay, we demonstrate that these mice have numbers of IL-12p40-producing cells during early stages of infection comparable to those by C57BL/6 mice. In contrast to infection in CD40L−/− mice, we observed no evidence of increased IL-4 production in CD40L−/−CD28−/− mice, showing that these mice did not mount a demonstrable Th2-type response.

Further support for the concept that a deficiency in both CD40L and CD28 costimulation promotes resistance comes from our observation that treatment of C57BL/6 wild-type mice, but not CD28−/− mice, with Ab to CD40L led to increased susceptibility to infection as assessed by increased lesion size. Lesions in Ab-treated wild-type mice ultimately started to heal, which is not unexpected, because anti-CD40L Ab was administered during only the first 2 wk of infection. Heinzel and colleagues (30) have shown similar effects of anti-CD40L mAb on the course of L. major infection in C57BL/6 mice. When parasite numbers were assessed at wk 9, Ab-treated wild-type mice still harbored significantly more lesion parasites than Ab-treated CD28−/− mice.
Our findings on Th1-type cytokine production in CD40L−/− mice differ from previous observations which suggest that CD40-CD40L interaction may be required for protective cell-mediated immunity to *L. major*. They also suggest that an alternative mechanism for inducing IL-12 production may exist in these mice. In contrast to other intracellular pathogens such as *Histoplasma*, *Mycobacterium*, and *Toxoplasma* (6–8), *Leishmania* is not thought to directly activate IL-12 production by infected macrophages (32). However, recent studies have shown that dendritic cells infected in vitro with *L. major* or *donovani* do produce IL-12 (33, 34). In addition, it has been suggested that a protein from *L. major*, termed Leif, can directly stimulate IL-12 production by human monocytes (35). Thus, it is probable that stimuli other than CD40-CD40L interactions can promote the production of this critical cytokine. Why IL-12 production is up-regulated in DKO mice, but not in CD40L−/− mice, is unclear. However, we have noted that low mRNA levels for both IFN-γ and IL-12 can be detected during early stages (wk 4) of infection in CD40L−/− mice (data not shown), suggesting that these mice may produce a weak Th1-type response which is ultimately down-regulated by an emerging Th2-type response.

In contrast to CD40L−/− mice infected with a high parasite dose (1 × 10^7) which developed nonhealing lesions and harbored high numbers of parasites, CD40L−/− mice infected with a low dose of parasites (2 × 10^6) or fewer promastigotes) developed small lesions and ultimately resolved infection consistent with their development of a dominant Th1 response. These results suggest that parasite doses and the level of T cell activation during infection are contributing factors influencing whether costimulatory interactions come into play and alter the pattern of response to infection. Our observations with low-dose experiments differ from previous findings in which intradermal inoculation of 100 *L. major* metacyclics promastigotes into the ear resulted in the development of nonhealing infections in CD40L−/− mice (36). A possible reason for these differing results may be either the different sites of infection or, more likely, the different genetic background of mice used in these two studies. CD40L−/− mice used in our study were on a mixed background (129 × C57BL/6) and back-crossed on C57BL/6 mice for fewer than five generations, whereas Belkaid and her colleagues (36) used CD40L−/− mice on the C57BL/6 background, which are known to be more susceptible to *L. major* infection (4).

The mechanism by which a deficiency in CD28−/− costimulation influences cytokine production in DKO mice is unclear. As noted above, CD28−/− mice on a resistant background produce normal levels of Ag-specific IFN-γ protein in comparison to wild-type C57BL/6 mice (10). Several studies suggest that CD8+ cells can be activated and differentiated into effector cells in the absence of signals delivered through CD28 (37, 38). In this regard, we tested whether treatment of DKO mice with anti-CD8 Ab through the first 5 wk of infection would alter the course of disease, but we noted no differences in lesion size between treated and untreated mice through wk 10 of infection, and anti-CD8-treated mice healed in a timely manner (data not shown). Recent studies have reported that maximal responsiveness to IL-12 is dependent on stimulation through CD28, which results in up-regulation of the IL-12R (39, 40). Lindsten and colleagues (41) have reported that CD28 not only up-regulates IL-12R expression but also results in increased stability of IFN-γ mRNA, which enhances production of IFN-γ protein. However, CD28 costimulation has also been implicated in the activation of Th2 cells (42), and an essential role of CD28 in Th2 differentiation has been described previously, although the operative mechanism is not known (43). Up-regulation in IL-4 mRNA level in cells from CD40L−/− mice, but not in those from DKO mice, is consistent with a role for CD28-mediated costimulation in promoting the dominant Th2 responses during *Leishmania* infection in CD40L−/− mice. Thus, it is possible that increasing production of IL-4, in association with reduced production of IL-12, contributes to the inability of these mice to control infection. Because stimulation through CD40 can enhance the expression of CD80 and CD86 expression (44, 45), it is also possible that reduced levels of CD80/CD86 molecules on APCs may contribute to the reduced level of CD28 costimulation required to drive expansion of IL-4-producing T cells in mice inoculated with a low dose of parasites. In the absence of high levels of IL-4 production, it is possible that sufficient levels of IL-12 and IFN-γ are produced in both DKO and CD40L−/− mice to compensate for the deficiency in IL-12 production that would normally be induced following CD40-CD40L interaction.

In summary, our results reconfirm the central role of IL-12 in resistance to *Leishmania* infection but suggest that the requirements for IL-12 production may differ depending on the costimulatory pathways available to individual mice. Whether CD40L−/−CD28−/− mice ultimately control infection because they use alternate costimulatory pathways such as TRANCE-TRANCE-R (46) to induce a dominant Th1-type response or because the absence of costimulation hinders the development of a dominant Th2-type response remains to be determined. However, our results suggest that the reduced level of responsiveness in CD28-deficient mice might be a contributing factor governing whether CD40-CD40L interactions are required for the development of resistance. This hypothesis is supported by results from our low-dose experiments that show that a reduction in the magnitude of the response leads to the activation of a dominant Th1 response in the absence of CD40-CD40L interactions.

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