CD28 Costimulation and Parasite Dose Combine to Influence the Susceptibility of BALB/c Mice to Infection with *Leishmania major*

Helen L. Compton and Jay P. Farrell

*J Immunol* 2002; 168:1302-1308; doi: 10.4049/jimmunol.168.3.1302

http://www.jimmunol.org/content/168/3/1302

---

**References**

This article cites 62 articles, 44 of which you can access for free at: http://www.jimmunol.org/content/168/3/1302.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CD28 Costimulation and Parasite Dose Combine to Influence the Susceptibility of BALB/c Mice to Infection with *Leishmania major*

Helen L. Compton and Jay P. Farrell

Infection with *Leishmania major* in BALB/c mice is accompanied by the development of a nonprotective Th2-type response. It has previously been shown that disease progression, and the activation of a Th2-type response, can occur in the absence of CD28 costimulation following the inoculation of high numbers of *L. major* promastigotes. In this study, we show that in the absence of CD28-B7 interactions, BALB/c mice can spontaneously resolve their infections following the inoculation of low numbers of parasites. BALB/c CD28−/+ and CD28−/− mice were inoculated with 250, 500, and 750 metacyclic parasites. The CD28−/− mice controlled their lesions, whereas the wild-type (WT) mice developed progressive nonhealing infections. Resistance to infection was associated with reduced numbers of parasites in the CD28−/− mice compared with the WT mice. Infection of the CD28−/− mice resulted in the activation of a Th1-type response as evidenced by increased levels of mRNA for IFN-γ and reduced levels of message for IL-4 and IL-10 in draining lymph nodes compared with those in WT mice. Healing of infected CD28−/− mice could also be ablated with anti-CD4 Ab treatment or treatment with anti-IFN-γ Ab. In addition, healed CD28−/− mice were resistant to a challenge infection with *L. major*. These results suggest that CD28 costimulation influences the in vivo activation of a Th2-type response in a manner that is dependent on the size of the parasite inoculum. *The Journal of Immunology, 2002, 168: 1302–1308.

M
tinite parasites (20–23). BALB/c mice given <1000 stationary
phase promastigotes develop a stable Th1 response and resist a pathologic challenge, whereas >1000 parasites result in a Th2 response and susceptibility (20). This resistance is thought to be associated with a memory state that generates an active Th1 response when challenged (21).

We are interested in whether CD28 costimulation plays a role in *Leishmania major* infections. Two signals are required for optimal T cell activation, one Ag-MHC class I- or II-dependent through the TCR and the other a secondary costimulatory signal. The interaction of CD28, which is constitutively expressed on T cells, with B7.1 (CD80) and B7.2 (CD86) on APCs provides the second signal for activation of naive T cells (reviewed in Ref. 24). CD28 costimulation is thought to help in the amplification of T cell signals, especially when Ag concentrations are low. In this aspect, CD28 is necessary for the induction of various cytokine genes, e.g. IL-4 expression (25), and the stability of mRNA transcripts, especially that of IL-2 (26), which is responsible for the clonal expansion of T cells. CD28 also provides an anti-apoptotic signal by up-regulating Bcl-xL (27, 28). In the absence of CD28, which is constitutively expressed on T cells, with B7.1 (CD80) and B7.2 (CD86) on APCs provides the second signal for activation of naive T cells (reviewed in Ref. 24). CD28 costimulation is thought to help in the amplification of T cell signals, especially when Ag concentrations are low. In this aspect, CD28 is necessary for the induction of various cytokine genes, e.g. IL-4 expression (25), and the stability of mRNA transcripts, especially that of IL-2 (26), which is responsible for the clonal expansion of T cells. CD28 also provides an anti-apoptotic signal by up-regulating Bcl-xL (27, 28). In the absence of CD28 costimulation, T cell responses are reduced with typically poor proliferation and cytokine responses (29–31) and variable levels of Ab production depending upon the antigenic stimulus. Interestingly, CD8+ T cell responses have also been shown to be less dependent on CD28 costimulation, particularly in primary viral infections (32–34) and in the activation of primed effector/memory cell responses (35).

Studies using both in vitro and in vivo models of T cell activation indicate that CD28-B7 interactions are important in the development of Th2-type immune responses (29, 36–39), though this has not proven to be a rule (30, 40, 41). Importantly, Ag dose has also been shown to help regulate Th differentiation (41–45) where a reduction in the level of antigenic stimulus for the T cell increases the requirement for CD28-B7 costimulation. These observations, combined with the contradictory findings that CD28-B7 interactions did not appear to be required for susceptibility or resistance to *L. major* infections when CD28−/− mice were used (46, 47), as opposed to the observations that BALB/c mice become resistant to *L. major* infection following treatment with CTLA4-Ig or anti-B7-2 Ab (18, 48), prompted us to re-evaluate the role of
Materials and Methods

Animals and parasites

Female BALB/cByJ and C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), BALB/cByJ CD28−/− (CD28−/−) breeding pairs were originally obtained from The Jackson Laboratory and bred and maintained in pathogen-free facilities. C57BL/6 CD28−/− female mice were a kind gift of Dr. C. Hunter (University of Pennsylvania, Philadelphia, PA). All animals were 6–8 wk old at the time of infection. The WHO MHO/MIL/80/Friedlin clone of L. major (a kind gift from D. Sacks, National Institutes of Health, Bethesda, MD) was used for all infections. Parasites were maintained in Grace’s insect cell culture medium (Life Technologies, Grand Island, NY) containing 20% FBS (HyClone Laboratories, Logan, UT), 2 mM of L-glutamine (Life Technologies), 100 μg/ml streptomycin-sulfate, and 100 U/ml penicillin-G sodium (Life Technologies).

Infections

BALB/c mice were inoculated s.c. in one hind footpad with various known numbers of metacyclic promastigotes selected from stationary phase cultures with Arachis hypogaea agglutinin (Sigma-Aldrich, St. Louis, MO) as previously described (49). In studies in C57BL/6 mice, parasites were inoculated into the ear dermis (50). Lesion development was monitored weekly with a vernier caliper and lesion size expressed as the difference in thickness between the infected and uninfected contralateral footpad, or lesion diameter in the ear-infected mice. Tissue parasites were enumerated by a limiting dilution assay in which homogenates of infected lesions were serially diluted in Grace’s insect cell culture medium and observed 5–7 days later for growth of promastigotes (51). parasite numbers are expressed as the negative log_{10} dilution at which promastigote growth is observed.

Abs for in vivo treatment

Rat anti-mouse mAb anti-IFN-γ (XMG6; Ref. 52), anti-CD4+ (GK1.5), and anti-CD8+ (H35-17.2; Ref. 53) Abs were used as indicated in Results. The control Ab was normal rat IgG (Sigma-Aldrich).

RNA purification and RNase protection assay

Total RNA was extracted from individual popliteal lymph nodes using STAT-60 (Tel-Test, Friendswood, TX) as directed by the manufacturer. RNA was resuspended in diethyl pyrocarbonate-treated water and the concentration was determined by absorbency at 260 nm. Various cytokine mRNAs were detected using a custom multiprobe RNase protection assay (BD PharMingen, San Diego, CA). The custom probe was prepared using 5′32P dITTP and hybridized to 15 μg of sample RNA. The protected probe was purified and resolved using 5% SDS PAGE (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, model GS-525, from Molecular Imager Systems (Bio-Rad, Hercules, CA). Relative quantities were determined using Multianalyst software (Bio-Rad).

Statistical analysis

Unpaired Student’s t tests were used to assess significance. Data were considered significant at p < 0.05.

Results

Absence of CD28-B7 interactions renders susceptible BALB/c mice resistant to low-dose L. major infection

Initially, we confirmed a previous observation (46) that BALB/c CD28−/− mice, when given a high dose of metacyclic promastigotes of L. major, are as susceptible to infection as their WT counterparts (data not shown). In agreement with Brown et al. (46), the absence of CD28-B7 interactions does not appear to alter the course of disease or immune response to L. major infection in BALB/c mice following infection with high parasite numbers (2 × 10^4–1 × 10^6). To determine whether CD28-B7 interactions influenced the course of disease following inoculation of low numbers of parasites, CD28−/− and BALB/c mice were inoculated in the footpad with 250, 500, or 750 metacyclic promastigotes and followed weekly. Lesions were not detected in any group of mice until wk 3–4 postinfection (Fig. 1A). In the case of CD28−/− mice, all parasite doses resulted in the production of small lesions (maximum 0.5 mm) which did not progress, whereas all BALB/c mice developed progressive lesions that increased in size in a dose-dependent manner. Mice inoculated with either 500 or 750 promastigotes were sacrificed at wk 9–11 to determine levels of infection in parasitized lesions. Limiting dilution analysis showed that parasite numbers in CD28−/− mice were significantly reduced compared with those in BALB/c control mice (Fig. 1B). Together, the development of small nonprogressive lesions and reduced numbers of lesion parasites in CD28−/− mice suggest that, unlike previous observations where mice were given a large parasite inoculum (46), the absence of CD28-B7 interactions does significantly influence the course of disease following L. major infection.

CD28 costimulation in L. major infections. We used CD28−/− mice on the susceptible BALB/c background infected with a low parasite inoculum. Our results show that in contrast to wild-type (WT)3 BALB/c mice, BALB/c CD28−/− mice are able to heal their lesions and control parasite numbers suggesting that CD28-B7 interactions play a role in susceptibility to infection.
Because both cellular proliferative and cytokine production are greatly reduced in BALB/c mice inoculated with low numbers of parasites (23), we used the RNase protection assay to examine cytokine production. At wk 7 postinfection with 750 parasites, infected BALB/c mice displayed significant levels of IL-4 and IL-10 mRNA and low levels of message for IFN-γ. In contrast, infected CD28−/− mice displayed negligible IL-4 (7-fold less) and low IL-10 (2-fold less) mRNA transcripts compared with infected BALB/c mice. Importantly, infected CD28−/− mice had 2-fold higher IFN-γ mRNA transcripts than infected BALB/c mice (Fig. 2) did. This altered pattern of cytokine production suggests that CD28 costimulation may play a role in inhibiting the development of resistance following low dose infection, but does not alter the overall pattern of infection in C57BL/6 mice. CD28−/− mice heal via an IFN-γ-dependent mechanism Because control of L. major infections in mice is associated with the production of the macrophage activating cytokine IFN-γ (1, 2), we examined whether in vivo neutralization of IFN-γ would alter the pattern of resistance in CD28−/− mice. BALB/c and CD28−/− mice were treated weekly for 4 wk with an anti-IFN-γ Ab starting at day 42 of infection. Anti-IFN-γ treatment of CD28−/− mice resulted in exaggerated lesion development compared with mice receiving control Ab. By wk 11 of infection, lesion size in anti-IFN-γ Ab-treated CD28−/− mice was equivalent to that of control WT mice (Fig. 4A). Furthermore, the anti-IFN-γ-treated CD28−/− mice had significantly more parasites within their lesions than control Ab-treated CD28−/− mice (Fig. 4B). This dramatic increase in both lesion size and parasite burden are evidence that healing occurred via an IFN-γ-dependent mechanism.

**FIGURE 2.** CD28−/− mice produce less IL-4 and IL-10, but more IFN-γ mRNA, than infected BALB/c mice. Cytokine mRNA levels in popliteal draining lymph nodes were compared between infected BALB/c and CD28−/− mice and naive BALB/c mice by RNase protection assay. Mice were infected with 750 metacyclics of L. major for 7 wk. The lanes represent two individual mice per group and are representative of other animals in the groups.

**FIGURE 3.** CD28−/− mice on the C57BL/6 background heal their lesions faster than WT mice. C57BL/6 and C57BL/6 CD28−/− mice were infected with 150 or 750 metacyclic promastigotes of L. major in the ear and the course of infection was followed for 12 wk. Lesion diameter was measured weekly. Values are the mean ± SE of five mice per group.

**Healing in CD28−/− mice is dependent on CD4+ T cells** In WT mice there is overwhelming evidence that both resistance and susceptibility to L. major infection are mediated through CD4+ T cells (1, 4, 11). However, there is also evidence that CD8+ T cells are required for immunity during reinfection (54, 55) and it has been shown in vitro that leishmanial Ag can be presented via the MHC class I pathway (56, 57). This, coupled with the fact that CD8+ T cells can be activated independently of CD28 costimulation (32–34), led us to examine whether CD4+ or CD8+ cells were required for the development of resistance in CD28−/− mice. CD28−/− and BALB/c mice infected with 750 metacyclic promastigotes were given three weekly injections of either anti-CD4 or anti-CD8 Ab starting at wk 5 postinfection. Although anti-CD4 Ab treatment had a marginal effect in WT mice, probably because it was not given early enough in the infection to dampen the developing Th2 response, it led to a dramatic increase in lesion size in CD28−/− mice (Fig. 5A). This increase in lesion size correlated with a highly significant increase in parasite numbers in the CD28−/− mice (Fig. 5B). In contrast, treatment with anti-CD8 Ab failed to significantly enhance lesion size in either WT or CD28−/− mice (Fig. 5C). Parasite burdens in anti-CD8 Ab-treated mice were also not significantly different from their untreated control group (log10 7.75 ± 1.15 vs log10 9.87 ± 1.34 for BALB/c groups and log10 1.04 ± 0.43 vs log10 1.41 ± 0.40 for CD28−/− groups). Additionally, treatment with an anti-CD8 Ab failed to alter the course of a challenge infection in CD28−/− and WT BALB/c mice (data not shown). Together, these results suggest that CD4+ T cells primarily mediate the resistance seen in CD28−/− mice.

**Healed CD28−/− mice are resistant to a challenge infection** We also examined whether the control of a primary infection in CD28−/− mice was associated with the development of resistance to reinfection. CD28−/− and WT mice were inoculated with 250 metacyclic promastigotes in one hind footpad, then challenged with either a high 20,000 or low 2,000 dose of metacyclic promastigotes delivered into the contralateral footpad 11 wk later. “Naïve” BALB/c and CD28−/− mice infected with either the high or low dose inoculum served as controls. The course of infection was followed in all mice and at wk 7 postchallenge, the animals...
were sacrificed and parasite burdens were established at the challenged site. As expected from previous observations (46), CD28−/− mice inoculated with a high dose of parasites (20,000) were as equally susceptible to infection as their BALB/c counterparts (Fig. 6). At the lower parasite dose (2,000), CD28−/− mice displayed a significant reduction in parasite load over the WT animals. For all of the challenge groups, a highly significant reduction in parasite load was found compared with the corresponding mice given a primary infection with either dose (Fig. 6), demonstrating that even WT animals, when given a low-dose primary infection, exhibited some control of a secondary infection. This is not unexpected given previous observations that BALB/c mice inoculated with low parasite numbers may exhibit a heightened Th1 response and resistance to reinfection (20). However, the degree of control in WT mice was significantly less than that in CD28−/− mice in which we observed a 10-fold log10 reduction in parasite numbers at the 20,000 challenge site and were unable to detect any parasites at the 2,000 challenge site.

Discussion
In this study, we have examined how parasite dose and CD28 costimulation interact to influence the in vivo response to infection.
with *L. major*. A number of studies have shown that optimal T cell activation requires signaling through both the TCR and a costimulatory molecule, predominantly CD28, which provides an additional stimulus for both IL-2 production as well as IL-2R expression. In part, this is because CD28-B7 interactions are required for the stabilization of mRNA transcripts, especially of IL-2 (26). CD28 costimulation also provides an anti-apoptotic signal for T cells (27, 28) to help maintain an immune response. There is considerable evidence that costimulation may influence the differentiation of naive T cells into Th1 (30, 31) or Th2 subsets (29, 37, 38), although evidence to this effect is often conflicting. In addition to costimulatory signaling, there is also evidence that the strength of the TCR signal can directly influence T cell differentiation pathways. There are several studies using naive CD4+ cells with a transgenic TCR that show that at medium-high Ag doses, T cells are induced to become Th1 cells producing IFN-γ, while very low (or extremely high) doses promote the cells to differentiate into Th2 cells and secrete IL-4 (42, 43, 45). Further, there is a threshold below which the TCR-ligand interaction fails to induce IL-2 secretion and the proliferation of T cells (44), suggesting that the Ag dose alone can dictate the extent and quality of an immune response. When Ag concentration and costimulation are combined to direct Th differentiation, naive CD4+ T cells have been shown to only be receptive to CD28-dependent IL-4 production if they receive a weak TCR signal (45). However, more importantly, costimulatory signals act to enable low doses of Ag to promote responses normally induced only by higher doses of that Ag (41).

Previous studies have independently examined the effects of costimulation and parasite dose on the immune response to *L. major*. With respect to murine cutaneous leishmaniasis, it is well known that parasite dose can influence the development of a Th1 vs a Th2 response which, in turn, determines the outcome of infection. Specifically, infection of susceptible BALB/c mice with low numbers of stationary phase promastigotes leads to the development of stable immunity while higher parasite doses lead to the development of progressive nonhealing disease (20–22). In these studies, there was some variation in the percentage of mice that generated a stable protective immune response that was resistant to a lethal challenge, possibly because stationary phase promastigotes contain a variable number of infectious parasites. To circumvent this, we inoculated only infectious metacyclic parasites. CD28-B7 interactions have been shown to be involved during the initiation of an immune response against *L. major* in either resistant or susceptible mice (58) and the use of the CTLA4-Ig Ab within the first week of infection renders susceptible BALB/c mice resistant to infection (18). However, the absence of CD28 costimulation was found to have no effect on the outcome of infection or immune response in CD28−/− mice on either a resistant or susceptible background (46, 47). Because these studies used a large parasite inoculum, we re-examined the role of CD28 costimulation in mice infected with low numbers of parasites. Our results clearly show that BALB/c CD28−/− mice inoculated with 250–750 metacyclic promastigotes control infection while WT BALB/c develop nonhealing infections characterized by continued lesion expansion. Although there is no reason to suspect that a deficiency in CD28 would alter the capacity of macrophages to support parasite growth, we have compared the ability of parasites to replicate in vitro within macrophages from WT and CD28−/− mice and observed no differences (Dr. S. Almería, unpublished observations). Importantly, we show that message levels for IL-4 are decreased and those for IFN-γ increased in CD28−/− compared with WT mice, providing evidence that the CD28−/− mice develop a more dominant Th1-type response. In addition, we show that resistance in CD28−/− mice requires the in vivo production of IFN-γ.

There are several possible reasons why CD28−/− mice might control infection with *L. major* while WT animals do not. For example, it is possible that CD8+–dependent effector mechanisms are operating in the CD28−/−–deficient mice because previous studies have shown that CD8+ T cells, under some circumstances, are less dependent on CD28 costimulation (32–35). Leishmanial Ag can be presented in association with MHC class I (56, 57) and a role has been established for CD8+ cells during *L. major* reinfection (54, 55). However, CD8+ T cell control of infection does not appear to be a primary mechanism of resistance in CD28−/− mice because treatment with neutralizing anti-CD8 Ab failed to alter the course of a primary (Fig. 5C) or secondary challenge infection (data not shown). In contrast, treatment with a neutralizing anti-CD4 Ab resulted in an exacerbated lesion size and parasite burden in CD28−/− mice (Fig. 5, A and B) indicating that as in WT animals (1, 4, 11), CD4+ T cells are critical to the development of resistance to infection.

It is also possible that CD28 interactions are required for the specific activation of Th2 CD4+ cells (29, 37, 38) and it has been shown that CD28 costimulation may be necessary for the induction of IL-4 mRNA (25). Without CD28 costimulation, a defective Th2 response may allow a Th1 response to develop. In the case of the CD28−/− mice, the loss of the CD28 pathway and the low antigenic stimulus results in a small initial immune response. In WT mice, the low infectious dose may lead to the active engagement of the CD28-B7 pathway so these mice are able to produce IL-4 leading to a nonprotective Th2 response. Such a defective Th2 response in CD28−/− mice could result in an altered disease pattern as has been observed for CD28−/− mice infected with *Schistosoma mansoni* (39). Our results are not inconsistent with this theory, but an in vivo system makes this difficult to clearly establish.

The absence of CD28 costimulation results in an overall reduction in the immune response following infection (29, 31), which could fail to trigger the early burst of IL-4 that is associated with...
susceptibility in BALB/c mice (59) thus influencing the early pro-
liferation and differentiation of T cells. In vitro studies on naive CD4+ T cells suggest that very low or very high concentrations of Ag can stimulate T cells to produce IL-4, whereas mid to high doses stimulate the production of IFN-γ (42, 43). The strength of the Ag signal also helps determine the degree of the proliferative response (44). Non-specific treatments of BALB/c mice such as in vivo administration of an Ab to CD4 (11) or IL-2 (13), sublethal gamma radiation (60), or treatment with cyclophosphamide (61), all lower the initial level of T cell activation and result in the healing of L. major infections in BALB/c mice. This theory is also not inconsistent with our results.

Whatever mechanism is in operation, we have shown that using a low parasite inoculum, CD28−/− mice are able to heal their infections in a CD4+- and IFN-γ-dependent manner. Clearly, the immune response in these mice is biased toward a Th1 phenotype. Because the general level of immune activation is small in these mice and there is evidence that the ability of CD28−/− mice to generate an effective memory response may be impaired during infection with other protozoan parasites such as Toxoplasma (62), we tested whether a protective Th1 immune response could be maintained in CD28−/− mice by reinfecting healed animals with a lethal challenge. The CD28−/− mice challenged with either the 2,000 or 20,000 metacyclics were indeed resistant to the challenge. Interestingly, the WT mice given a primary infection with 250 parasites and reinjected with either the 2,000 and 20,000 doses also showed a degree of resistance to the challenge infection by exhibiting significantly reduced parasite burdens compared with similarly infected naive mice (Fig. 6). This shows that despite the initial parasite dose generating a non-healing phenotype, some degree of cell-mediated immunity has been generated in these mice. Given that at very low parasite doses, BALB/c mice can control infection (20), it is perhaps not surprising that these mice are displaying some resistance to challenge; however, the level of resistance in WT mice was significantly less than that in CD28−/− mice showing that the absence of CD28 costimulation did not preclude the maintenance and enhancement of established resistance. In summary, we have shown that the combined effects of a low parasite inoculum and a lack of costimulation through the CD28-B7 pathway render susceptible BALB/c mice resistant to infection with L. major. This suggests that the interaction of parasite dose and costimulation can influence the differentiation of T cells and, therefore, susceptibility or resistance to disease.

Acknowledgments

We thank Drs. Phillip Scott and Christopher Hunter for their critical review of this manuscript and Laura Eustace-Berlakovich for her technical help.

References


13. Heinzel, F. P., R. M. Rerko, F. Hatam, and R. M. Locksley. 1993. IL-2 is nec-


