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TRANCE-RANK Costimulation is Required for IL-12 Production and the Initiation of a Th1-Type Response to Leishmania major Infection in CD40L-Deficient Mice

Udaikumar M. Padigel,* Nacksung Kim,† Yongwon Choi,† and Jay P. Farrell2*

Blockade of TNF-related activation-induced cytokine (TRANCE)-receptor activator of NF-κB (RANK) interaction reverses healing in CD40L−/− mice infected with Leishmania major. Although previous studies demonstrated a requirement for CD40-CD40L interaction in production of IL-12 and the development of resistance to Leishmania infection, we recently showed that CD40L−/− mice control infection when inoculated with low numbers of parasites and that cells from these mice produce IL-12. Here, we show that in vivo treatment with a TRANCE receptor fusion protein results in a decrease in numbers of IL-12 producing cells as well as a shift from a dominant Th1 to Th2 type response in infected mice. These results demonstrate that CD40L−/− mice use the TRANCE-RANK costimulatory pathway to promote IL-12 production and the activation of a protective Th1 type response. The Journal of Immunology, 2003, 171: 5437–5441.

Resistance to Leishmania major in mice requires the activation of a dominant Th1 type response in which CD4+ effector T cells produce the macrophage activating cytokine, IFN-γ (1, 2). In contrast, non-healing infections are characterized by the development of a 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 (2, 3). The development of a dominant Th1 response is dependent upon the proinflammatory cytokine IL-12 as evidenced by the inability of animals lacking the IL-12 gene (4) or mice treated with Ab to IL-12 (5, 6) to resolve infection, and by the demonstration that treatment with recombinant IL-12 promotes healing in highly susceptible BALB/c mice (7, 8). In addition to its role in promoting the development of a dominant Th1 type response, IL-12 is also required for the maintenance of an established Th1 response. Thus, in the absence of endogenous IL-12 production, Th1-type responses decline and Th2-type responses emerge in IL-12−/− mice induced to heal by IL-12 treatment given early during L. major infection (9, 10). A recent study suggests that maintenance of IL-12 production throughout infection may be required to prevent the loss of Th1 cells rather than prevent the expansion of a population or parasite-specific Th2 cells (11).

Given the critical role of IL-12 in directing and regulating the immune response, identifying the stimuli required for its induction is central to understanding the immune response to a L. major infection. Unlike pathogens such as Listeria which induce macrophages to produce IL-12 (12), direct infection of macrophages by Leishmania fails to elicit IL-12 production; rather, the ability of macrophages to produce IL-12 in response to other proinflammatory stimuli appears to be impaired (13, 14). However, dendritic cells (DCs)3 infected in vitro with either L. major or Leishmania donovani are induced to produce measurable levels of IL-12 p40 (15, 16, 17) and both human and mouse DCs have been shown to rapidly release preformed stores of IL-12p70 following contact with L. donovani (18). Still, direct infection is not always a sufficient stimulus to induce production of this cytokine as evidenced by the observation that infection of human myeloid DCs with L. major failed to directly induce IL-12 production, although infection did up-regulate levels of MHC class II and costimulatory molecules. However, following interaction with CD40 ligand, infected DCs did produce increased levels IL-12p70 over that produced by uninfected DCs (19) although the capacity to stimulate IL-12 production varied depending on the species or strain of Leishmania used to stimulate cells (20). The concept that CD40-CD40L interactions provide a necessary stimulus for IL-12 production during cutaneous leishmaniasis is strongly supported by in vivo studies showing that CD40 (21) and CD40L (22, 23) deficient mice as well as mice treated with neutralizing anti-CD40L Ab (24) fail to develop a protective cell mediated immune response following infection with L. major and the related protozoan parasite, Leishmania amazonensis. Infected CD40L−/− or CD40−/− mice on a resistant C57BL/6 × 129J background exhibit a marked reduction in both IL-12 and IFN-γ production (21, 22); however, IFN-γ production and the ability to control infection is fully restored following treatment with exogenous IL-12 (22). It is logical to conclude from these studies that the interaction of host APCs with CD40L expressing T cells is critical to the production of IL-12 and activation of a protective Th1 effector cells. However, we have recently shown that CD40-CD40L costimulatory pathway of IL-12 production is not always required for the development of a protective Th1 response since CD40L−/− mice, when inoculated with reduced numbers of L. major promastigotes, can resolve a primary infection (25, 26). Thus, an alternate pathway for IL-12 production must be used in these healing CD40L−/− mice. One alternative

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3 Abbreviations used in this paper: DC, dendritic cell; TRANCE, TNF-related activator-induced cytokine; RANK, receptor activator of NF-κB; TR-Fc, TRANCE receptor fusion protein; SLA, soluble leishmanial Ag.

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mechanism of IL-12 production involves the interaction of the TNF-family molecule TRANCE (TNF-related activation-induced cytokine also known as RANK-L) and its receptor RANK (TRANCE-R) which are important regulators of bone remodeling and essential in the development and activation of osteoclasts (27). TRANCE, expressed on activated T cells, can induce IL-12 production via its interaction with RANK expressed on DCs and also enhance DC survival (28). In this study, we show that the TRANCE-RANK costimulatory pathway is required for the development of a Th1 response and healing of L. major infections in CD40L−/− mice.

Materials and Methods

Parasites and animals

Female B6;129 and CD40L−/− mice (B6;129S-Tnfsf5tm1Tes) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were 6–10 wk old at the time of infection. L. major (WHO MHOM/IL/80/L. major) was maintained in Grace’s insect cell culture medium (Life Technologies, Grand Island, NY) containing 20% FBS, 2 mM l-glutamine, 100 μg of streptomycin, and 100 μg of penicillin G-sodium per milliliter.

Infections and treatments

Mice were inoculated into one hind footpad with 2 × 10⁴ metacyclic promastigotes selected from stationary phase cultures by negative selection using peanut agglutinin (Sigma-Aldrich, St. Louis, MO) as described previously (29). Lesion size was measured with a Vernier caliper 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 (30). In brief, the homogenates of infected lesions were serially diluted in Grace’s insect cell culture medium plus 2-ME in the presence of 50 μg/ml. Limiting dilution assay as described previously (30). In brief, the homogenates of infected lesions were serially diluted in Grace’s insect cell culture medium plus 2-ME in the presence of 50 μl/ml. Lesion size was measured with a Vernier caliper and expressed as the difference in thickness between the infected and the uninfected contralateral footpads. Additional control mice receiving normal human IgG1 (IgG1) had the same course of infection as untreated mice (not shown). Values are the mean ± SD of four to five mice per group and are representative of results of two separate experiments.

Cell culture and ELISA

Single cell suspensions of lymph nodes were cultured at 5 × 10⁶ cells/ml in DMEM containing 10% FBS, 2 mM glutamine, 100 U penicillin G-sodium per milliliter, 100 μg streptomycin sulfate/ml, and 5 × 10⁻³ M 2-ME in the presence of 50 μg/ml/milliliter soluble leishmanial Ag (SLA) prepared as previously described (32). Supernatants were collected at 72 h and assayed for IFN-γ by ELISA as previously described (32). Recombinant IFN-γ (PeproTech, Rocky Hill, NJ) was used as standard.

ELISPOT assay

The number of IL-12p40 and IL-4 secreting cells in lymph node suspensions was determined using an ELISPOT assay as previously described (33, 34). The mAbs C17.8 and biotinylated C15.6, generously provided by Dr. C. Hunter at the University of Pennsylvania (Philadelphia, PA) were used for IL-12 ELISPOT assay. IL-4 producing cells were detected using mAbs 1B11 and biotinylated BD6-24G2. IL-12 and IL-4 secreting cells were determined in cell cultures following overnight stimulation with SLA (50 μg/ml).

Statistical analysis

Statistically significant differences between groups were determined using the unpaired Student’s t test. Significance was assumed if p < 0.05.

Results

Treatment with TR-Fc reverses healing in CD40L−/− mice

Previous studies have shown that the inability of CD40 deficient and CD40L deficient mice to control infection with L. major is their inability to produce sufficient amounts of IL-12 required to activate a dominant Th1 response (21, 22). However, these studies used a high parasite dose and we have shown that the ability to heal may be directly related to the parasite dose used to initiate infection. Thus, we observed that mice inoculated with 1 × 10⁶ promastigotes developed progressive disease, whereas mice inoculated with 2 × 10⁶ promastigotes resolved their infections (25). We also showed that healing CD40L deficient mice have numbers of IL-12 producing cells similar to those in control mice suggesting that an alternate pathway of IL-12 production may operate in the absence of CD40-CD40L costimulation (26). To examine whether TRANCE-RANK interaction is involved in activation of a protective response, we inoculated CD40L deficient mice with 2 × 10⁶ promastigotes to produce a healing infection. Mice were then treated with TR-Fc every 4 days through day 12 of infection and lesion size was measured weekly to assess the effects on the course of disease. As can be seen in Fig. 1, treatment with TR-Fc reversed the ability of CD40L deficient mice to control infection. Analysis of lesion parasite numbers at week 8 revealed that mice treated with TR-Fc exhibited significantly elevated levels of infection compared with control mice treated with normal Ig (Fig. 2).

In vivo blockade of TRANCE-RANK alters the phenotype of the immune response in infected mice

To determine whether the inability of TR-Fc treated CD40L−/− mice correlated with an altered immune response, we analyzed IFN-γ production by Ag stimulated lymph node cells from mice at week 8 of infection. In addition, the number of IL-4 producing cells was determined following Ag stimulation using the ELISPOT technique. As can be seen in Fig. 3, cells from TR-Fc treated mice produced ~50% less IFN-γ than control mice. In contrast, we detected ~3-fold more IL-4 producing cells in the TR-Fc group.
SLA and cell supernatants were harvested at 72 h and assayed for IFN-γ/H9253 as mean frequency per 10^6 lymph node cells at wk 8 of infection by direct ELISPOT assay. The data are expressed as mean frequency per 10^6 lymph node cells ± SD of three to four mice per group and are representative of results of two separate experiments.

(Fig. 4). Together, these results are evidence that TR-Fc treatment resulted in a shift from a dominant Th1 type response to one characteristic of Th2 dominance.

**Blockade of TRANCE-RANK results in decreased numbers of IL-12 producing cells**

Our previous studies showed that numbers of IL-12 producing cells in healing CD40L-/- mice were similar to those infected wild-type control mice. To determine whether TRANCE-RANK interaction may contribute to IL-12 production in the absence of CD40-CD40L costimulation, we enumerated IL-12p40 producing cells in draining lymph nodes of infected mice. As can be seen in Fig. 5, TR-Fc treated mice had ~3-fold fewer IL-12 producing cells than control mice at week 8 of infection. Since the TR-Fc treated mice exhibited a dominant Th2 type response by week 8 of infection and IL-4 is known to suppress IL-12 production, we also assessed the number of IL-12 producing cells in treated and control mice at day 12 of infection to assess whether TR-Fc treatment altered early up-regulation of IL-12 production. As can be seen in Fig. 6, numbers of IL-12 producing cells in TR-Fc treated mice did not differ from those observed in uninfected mice as opposed to a 3-fold increase in IL-12 producing cells in infected mice treated with normal Ig. This observation is consistent with the hypothesis that TRANCE-RANK costimulation is an important in vivo stimulus for IL-12 production and is required for the development of a protective Th1 response.

**Discussion**

Considerable evidence suggests that IL-12 is required for both the development and maintenance of a protective Th1 response in mice infected with *L. major* (4, 5, 6, 9, 10). Given the critical role of IL-12 in the activation and maintenance of a population of IFN-γ producing CD4+ effector cells, identifying the in vivo stimuli for IL-12 production is central to understanding the events that regulate resistance to infection. Multiple signaling pathways are known to stimulate the production of IL-12 by DCs and/or macrophages. For example, Toll-like receptors on APCs can recognize pathogen-associated pattern recognition molecules such as bacterial LPS, peptoglycans, and lipoproteins as well as GPI anchors of Trypanosoma cruzi (35). Although direct infection of macrophages by *Leishmania* does not appear to elicit IL-12 production (14), several studies have shown that DCs infected in vitro with *Leishmania* do produce IL-12 (15, 17) and at least one in vivo study has shown that *L. donovani* stimulates splenic DCs to produce IL-12 (16). In addition, a *Leishmania* protein termed LeIF can directly stimulate IL-12 production by human monocytes (36), although the receptor recognizing this parasite product has not been identified. Whether any of these *Leishmania*-host cell interactions involved in IL-12 production require stimulation of Toll-like receptors has yet to be established.

Although it is possible that direct stimulation of APCs by parasites or their products may contribute to in vivo IL-12 production in *Leishmania*-infected mice, the primary stimulus for IL-12 production during infection appears to be the interaction of CD40L on activated T cells with CD40 expressed on DCs and macrophages. This is evidenced by several observations showing that disruption of the CD40:CD40L costimulatory pathway reduces IL-12 production and reverses the normal healing pattern of an *L. major* infection in a resistant strain of mouse (21, 22). In addition, recent...
studies have shown that infection-induced IL-12 production by 
*Leishmania*-infected DCs requires additional signaling through the CD40-CD40L pathway (20, 19). The basis of this current study is our previous observation that CD40L-/- mice can control *L. major* infection and develop a dominant Th1 response following inoculation of a reduced number of parasites (25). Thus, an alternate pathway other than CD40-CD40L costimulation must be used in these mice to promote IL-12 production. Our results suggest that this alternative stimulus for IL-12 production involves TRANCE-RANK costimulation. TRANCE is expressed by activated CD4+ and CD8+ T cells and is induced by Ag receptor engagement (28). Indeed, we can detect enhanced TRANCE expression by LN cells from *L. major* infected mice following Ag stimulation (not shown). Engagement of RANK on DCs by TRANCE enhances DC survival by regulating the expression of the anti-apoptotic molecule, Bcl-xL (37). In addition, TRANCE promotes the production of various cytokines such as IL-12, IL-15, IL-1, and IL-6 (28). Thus, TRANCE has some functional similarities to CD40L since both are expressed on activated T cells and enhance the activation, survival, and production of cytokines by DCs. However, the presence of CD40 signaling limits the physiological importance of TRANCE-RANK interactions and signaling through CD40-CD40L on DCs appears to recapitulate any effect induced by TRANCE in vitro (38). In this regard, we currently have no evidence that inhibition of this pathway alters the pattern of *L. major* infection in intact mice. However, our observation that treatment of infected CD40L deficient mice with TR-Fc results in a significant reduction in numbers of IL-12 producing cells is consistent with the idea that TRANCE expresses on activated T cells in infected mice interacts with RANK on DCs to induce the production of IL-12. It is also possible that TRANCE activates cytokine production by infected macrophages, although in contrast to CD40L/CD40, TRANCE/RANK signaling does not appear to alter the expression of cell surface MHC class II, CD80, or CD86 molecules in macrophages. TRANCE stimulation also fails to promote NO production by IFN-γ-primed macrophages (39) so it is less likely that TRANCE expressing T cells directly induces microbical activity in *Leishmania*-infected macrophages. However, TRANCE effects on macrophages cannot be totally ruled out since it has been recently shown that preincubation of human monocytes with either soluble TRANCE or CD40L before stimulation with IFN-γ plus LPS suppresses IL-12 production suggesting that both TRANCE and CD40L may share some properties relevant to macrophage signaling (40). Inhibition of TRANCE in vivo using soluble TR-Fc has been previously shown to dramatically reduce the T cell proliferative response in CD40L deficient mice infected with LCMV and almost totally abrogate the production of IFN-γ, suggesting that TRANCE-RANK interaction may provide an independent costimulatory pathway involved in T cell activation (38). Our results expand on observations in CD40 deficient mice to show that in vivo blockade of TRANCE-RANK interaction cannot only result in reduced production of IFN-γ production but also promote a shift from a dominant Th1 to a Th2 type response and reverse the ability of CD40L−/− mice to control infection. Given the requirement for IL-12 in the development of a protective Th1 type response, our observations are consistent with the hypothesis that TRANCE-RANK interaction may be a required stimulus for IL-12 production in the absence of CD40-CD40L interaction in mice infected with *L. major*.

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