CD40 Ligand (CD154) Does Not Contribute to Lymphocyte-Mediated Inhibition of Virulent Mycobacterium tuberculosis within Human Monocytes

Rhonda Larkin, Christopher D. Benjamin, Yen-Ming Hsu, Qing Li, Lynn Zukowski, and Richard F. Silver

Divisions of Pulmonary and Critical Care Medicine* and Infectious Diseases, Case Western Reserve University School of Medicine, and University Hospitals of Cleveland, Cleveland, Ohio, and Biogen, Inc., Cambridge, Massachusetts

Received 16 May 2001/Returned for modification 26 June 2001/Accepted 15 April 2002

Human monocytes displayed increased expression of CD40 following infection with virulent Mycobacterium tuberculosis. Nevertheless, soluble CD40 ligand (CD40L; also designated CD154) had no effect on the intracellular growth of the organism. Restriction of the intracellular growth of M. tuberculosis by peripheral blood lymphocytes and antigen-specific CD4⁺ T-cell lines likewise was not reduced by blocking anti-CD40L monoclonal antibody 5c8.

The susceptibility of individuals with defective function of receptors for gamma interferon (IFN-γ) and interleukin-12 (IL-12) to disseminated infections with mycobacterial species suggests that Th1 responses are central to protective human immunity against Mycobacterium tuberculosis (reviewed in reference 7). Nevertheless, the ability of IFN-γ to directly activate infected human mononuclear phagocytes to kill intracellular M. tuberculosis appears to be much more limited than that described for murine models of tuberculosis (5, 8, 9, 18). We previously described an in vitro model of infection of human monocytes (MN) with virulent M. tuberculosis strain H37Rv in which the ability of peripheral blood lymphocytes (PBL) to inhibit intracellular growth of the organism could be demonstrated and in which PBL-mediated inhibition of M. tuberculosis was much more effective than that mediated by transferred supernatants of these cocultures (22). These findings suggested a role for direct cell-to-cell contact in lymphocyte-mediated activation of MN to contain intracellular M. tuberculosis.

The interaction of CD40 ligand (CD40L; also designated CD154) and CD40 represents one pairing of cell surface ligands which could play a role in the contact-mediated inhibition of intracellular M. tuberculosis. CD40L was first recognized as a CD4⁺ T-cell surface ligand that plays an essential role in isotype switching from immunoglobulin M (IgM) to other immunoglobulins (13). However, individuals with hyper-IgM syndrome resulting from functional deficiency of CD40L also exhibit an increased incidence of infections characteristically observed in T-cell deficiency states (16). This observation has been explained by the subsequent discovery that the interaction of CD40L with CD40 contributes to the development of cell-mediated immune responses via several mechanisms. These include stimulation of the production of IL-12 and, thus, promotion of Th1 responses (20) and upregulation of costimulatory molecules to provide for maximal T-cell proliferation and cytokine production (1, 14). Of particular interest for the present study is the fact that the interaction of CD40L with CD40 on mononuclear phagocytes also results in direct activation of MN to produce numerous proinflammatory cytokines, such as IL-1 and tumor necrosis factor alpha (1, 12). We therefore assessed the possible role of the CD40L-CD40 interaction in directly activating human MN to inhibit intracellular growth of M. tuberculosis.

The present study made use of our previously reported in vitro model of lymphocyte-mediated limitation of intracellular bacterial growth. All cells were obtained from healthy subjects aged 22 to 50 according to protocols approved by the Institutional Review Board of Case Western Reserve University and University Hospitals of Cleveland. For studies involving lymphocyte populations, subjects with a history of positive skin test responses to purified protein derivative of M. tuberculosis were specifically recruited. Peripheral blood was obtained by venipuncture, and peripheral blood mononuclear cells (PBMC) were isolated by density sedimentation. MN were separated from PBL by plastic adherence as previously described (22). Infections were performed with the virulent M. tuberculosis strain H37Rv (no. 25618; American Tissue Type Collection, Rockville, Md.). In preparation for the infection of MN, mycobacteria were processed by using a series of mechanical disruptions and centrifugations to minimize bacterial clumping and provide for accurate quantification of the inoculum as previously described (22).

Infection with M. tuberculosis increases MN surface expression of CD40. Because any effect of CD40L on M. tuberculosis-infected MN would require the presence of CD40 on MN, we first examined the effects of infection with M. tuberculosis on MN surface expression of this molecule. Following infection with a 5:1 bacteria-to-cell ratio of M. tuberculosis, MN were incubated with murine anti-human CD40 (no. 33071A; Pharmingen) followed by fluorescein isothiocyanate-conjugated antimurine IgG (no. 38475; Cappel, Durham, N.C.). In three separate experiments, expression of CD40 was observed on 29.6%
rsCD40L does not activate human MN to kill intracellular *M. tuberculosis* H37Rv. Recombinant soluble human CD40L (rsCD40L; kindly provided by Biogen, Inc., Cambridge, Mass.) was then used to assess the ability of CD40L to limit the intracellular growth of H37Rv. The purity of the reagent was assessed by limulus lysate assay, which indicated that, with dilution of the ligand to the working concentrations of 2, 10, and 25 μg/ml used in this study, final lipopolysaccharide concentrations within our cultures were 0.003, 0.015, and 0.038 endotoxin units (EU)/ml, respectively. To confirm the bioactivity of rsCD40L, we assessed the ability of the ligand to induce IL-12 production from MN-derived dendritic cells (DC) (4). MN were incubated with 1,000 U of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, no. 300-03; Pepro-Tech, Rocky Hill, N.J.) and 1,000 U of recombinant human IL-4 (no. 200-04; Pepro-Tech) for 6 days as previously described (19). Following counting and allocation into 48-well plates, DC were then incubated with rsCD40L for 48 h. Supernatants were then collected, and IL-12 was measured with a commercially available enzyme-linked immunosorbent assay kit (no. EH21L12T; Endogen, Cambridge, Mass.). As shown in Fig. 2, concentrations of 2, 10, and 25 μg of rsCD40L/ml each induced significant production of IL-12 from DC (P = 0.041, 0.010, and 0.019, respectively, by paired t test).

These same concentrations of rsCD40L were then added to cultures of *M. tuberculosis*-infected MN. MN infections were established in microtiter well format as previously described in detail (22). In brief, 10⁵ MN were plated into each of the triplicate wells and infected during a 1-h incubation with H37Rv at a 1:1 bacteria-to-cell ratio. Autologous PBL were added to some wells at a ratio of 10 lymphocytes per MN as a positive control for growth limitation. At time points of interest, supernatants were removed and cell pellets were lysed, pooled, and plated onto Middlebrook 7H10 agar with oleic acid-albumin-dextrose-catalase enrichment (Difco). Viable intracellular organisms were determined by colony counting following 2 to 3 weeks of incubation and expressed as CFU per milliliter of cell lysate. The number of CFU of supernatants was determined separately and was at least 10-fold lower than that of pellets of the same culture in each case, indicating that relatively few bacteria were released into the supernatant following initial infection and rinsing of nonphagocytosed organisms. In cocultures in which PBL were added to *M. tuberculosis*-infected MN, the separate assessment of CFU within culture supernatants confirmed that reductions in intracellular CFU observed in this system predominantly reflected the killing rather than the release of intracellular organisms, as previously described (21).

As shown in Fig. 3, PBL mediated a significant reduction in the intracellular growth of H37Rv compared to that within MN alone at each of the time points studied (P < 0.0001, P = 0.002, and P = 0.003 at 1, 4, and 7 days, respectively). In contrast, the addition of rsCD40L in concentrations of 2, 10, and 25 μg/ml did not significantly reduce the viability of intracellular *M. tuberculosis*.

Blocking the CD40L-CD40 interaction does not reduce the ability of PBL to limit intracellular growth of *M. tuberculosis* H37Rv. One potential explanation of our inability to demonstrate an effect of rsCD40L on limiting the intracellular growth
of *M. tuberculosis* was the need for additional cell surface signals to work in combination with this ligand to contain the organism. To investigate this possibility, we studied the ability of blocking anti-CD40L monoclonal antibody (MAb) 5c8 (kindly provided by Biogen, Inc.) to inhibit lymphocyte-mediated limitation of the growth of *H37Rv* within MN.

To assess the efficacy of blocking of the CD40L-CD40 interaction in our culture system, we first measured IFN-γ production in cocultures of PBL and *M. tuberculosis*-infected MN in the presence of 5c8. Consistent with the costimulatory role of CD40L and CD40 in lymphocyte activation, 5c8 partially inhibited IFN-γ production in a dose-dependent fashion, as illustrated in Fig. 4 (15). The reduction in IFN-γ production was statistically significant for 1-, 10-, and 100-μg/ml concentrations of 5c8 (*P* = 0.001, 0.021, and 0.007, respectively)

The same concentrations of 5c8 were then added to cocultured *M. tuberculosis*-infected MN and PBL. As illustrated in Fig. 5, PBL mediated a significant reduction in intracellular *H37Rv* at days 1, 4, and 7 of coculture (*P* < 0.0001, *P* < 0.0001, and *P* = 0.007, respectively). The addition of 5c8 in concentrations of 1, 10, and 100 μg/ml had no significant effect on the ability of PBL to limit the intracellular growth of *M. tuberculosis* at any of the time points studied, as shown.

**Blocking anti-CD40L antibody does not interfere with inhibition of intracellular *M. tuberculosis* by short-term CD4+ T-cell lines.** Because of the heterogeneity of PBL, the ability of these cells to inhibit the intracellular growth of *M. tuberculosis* could result from the combined actions of several effector mechanisms. We therefore investigated the role of the CD40L-CD40 interaction in the containment of intracellular *M. tuberculosis* by a more well-defined lymphocyte population, that of short-term *M. tuberculosis*-specific CD4+ T-cell lines. This population was selected for study because CD4+ T cells are thought to be an essential component of acquired immunity to *M. tuberculosis* and because CD40L-expressing CD4+ T cells mediate the most well-characterized function of CD40L, that of costimulation of B cells to initiate immunoglobulin isotype switching.

*M. tuberculosis*-specific CD4+ T-cell lines were developed by incubation of PBMC of purified protein derivative-positive donors with avirulent *M. tuberculosis* strain H37Ra (no. 25177; American Tissue Type Collection). Aliquots of 2 × 10^6 PBMC were incubated with H37Ra in a 24-well plate format. Following 4 days of culture, 10 U of IL-2 (no. 3001050; Advanced Biotech, Columbia, Md.)/ml was added to each well. On day 7, cells were harvested and CD4+ T cells were isolated by positive selection with magnetized anti-CD4 antibodies (Dynabeads M-450 with DETACHaBEAD; Dynal, Lake Success, N.Y.) as previously described (23). Short-term CD4+ T-cell lines were then added to *H37Rv*-infected MN at a 5:1 ratio. As illustrated in Fig. 6, *M. tuberculosis*-specific short-term CD4+ T-cell lines mediated significant killing of *M. tuberculosis* compared to the growth within MN alone at each time point (*P* = 0.019, 0.010, and 0.020 at 1, 4, and 7 days, respectively). As with PBL, however, the ability of antigen-specific CD4+ T cells to limit
FIG. 5. Blocking anti-CD40L MAb 5c8 has no effect on the ability of PBL to limit the intracellular growth of M. tuberculosis H37Rv. The figure illustrates CFU of H37Rv within MN immediately following infection and at 1, 4, and 7 days. Infected MN were cocultured with PBL alone and with PBL plus 5c8 in concentrations of 1, 10, and 100 μg/ml. Anti-CD40L did not significantly alter the ability of PBL to limit the intracellular growth of M. tuberculosis at any of the time points. Results illustrate means and standard deviations of results for cells from 10 donors.

FIG. 6. Anti-CD40L MAb does not inhibit killing of intracellular M. tuberculosis by antigen-specific CD4+ T cells. CD4+ T cells were isolated from PBMC that had been stimulated for 7 days by incubation with avirulent M. tuberculosis H37Ra. As illustrated, the addition of these cells in a 5:1 ratio relative to infected MN resulted in a significant reduction of intracellular M. tuberculosis at 1, 4, and 7 days of coculture. The addition of 5c8 in concentrations of 10 and 100 μg/ml had no effect on CD4+ T-cell-mediated killing of H37Rv.

We thank David Canaday of Case Western Reserve University for assistance with protocols for preparation of short-term CD4+ T-cell lines. We also thank W. Henry Boom of Case Western Reserve University for many helpful discussions.

This work was supported by National Institutes of Health grants HL 59858 and AI 35027 and by American Lung Association research grant RG-1489-N. R. F. Silver was also supported by a Parker B. Francis Fellowship in Pulmonary Research sponsored by the Francis Families Foundation. The infections described in this manuscript were performed within the Elizabeth A. Rich Biosafety Level 3 Facility, which is a core facility of the Case Western Reserve University Center for AIDS Research (NIH AI-36219).

REFERENCES


Editor: S. H. E. Kaufmann