INFECTION AND IMMUNITY, Feb. 2001, p. 665–672 Vol. 69, No. 2
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.2.665–672.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

Influence of Costimulatory Molecules on Immune Response to *Leishmania major* by Human Cells In Vitro

CLAUDIA I. BRODSKYN,† GREGORY K. DEKREY,

virulence. Stationary metacyclic promastigotes were isolated by negative selection using peanut agglutinin as described previously (38).

Reagents. The following reagents were used in cell cultures: human CTLA-4–Fc chimera (R&D Systems, Minneapolis, Minn.) at 10 μg/ml; neutralizing anti-human CD80 and CD86 monoclonal antibodies (MAbs) (mouse immuno-

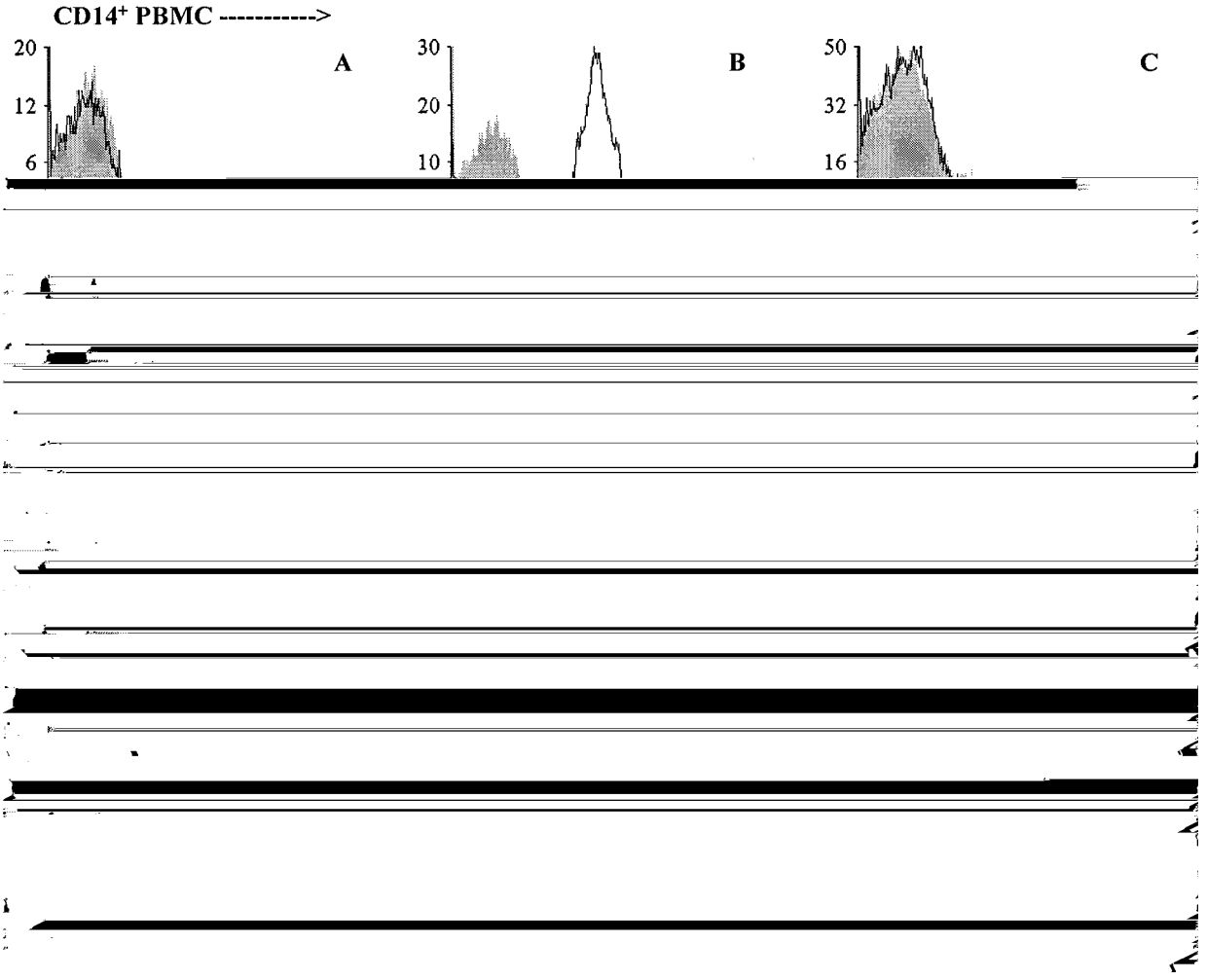

FIG. 1. Expression of CD80, CD86, and CD40 on monocytes and macrophages. Three different populations of monocytes/macrophages were assessed for expression of CD80 (A, D, and G), CD86 (B, E, and H), and CD40 (C, F, and I) by flow cytometry before or after culture. (A to C) Expression on freshly isolated $CD14^+$ PBMC. (D to F) Expression on MHC class II^+ adherent cells (macrophages [see Materials and Methods]) for cells exposed (gray line) or not exposed (black line) to *L. major* during the last 24 h of 7-day cultures. Similar results were obtained for macrophages cultured for a total of 14 days. (G to I) Expression on MHC class II^+ adherent cells exposed (gray line) or not exposed (black line) to *L. major* and cultured with PBL for an additional 7 days (14-day total culture). In all panels, shaded histograms represent nonspecific fluorescence of cells stained with an isotype control antibody. The data presented are representative of five individual donors.

taining isotype-matched control antibody. This difference in cytokine levels was also observed following blast cell restimulation even though no anti-CD154 was present in these cultures (Fig. 2C and D). Interestingly, no change in IL-10 levels was detected in primary (Fig. 3A) or secondary (data not shown) cultures as a result of anti-CD154 exposure, whereas anti-CD154 exposure reduced the amount of IL-12 to undetectable levels in both primary (Fig. 3B) and secondary (data not shown) cultures.

CD80 and/or CD86 blockade. The influence of CD80 and CD86 expression on *L. major*-specific cytokine production was examined by blocking B7-CD28 and B7–CTLA-4 interactions with CTLA-4Ig. As shown in Table 1, the presence of CTLA-4Ig during primary stimulation of PBL resulted in the reduction of IFN-γ and IL-5 to below detectable levels. Cytokine levels in cultures containing control Ig were comparable to those seen in previous control cultures (see above) and cultures containing no control reagent (Table 1). The reduction of cytokine levels resulting from CTLA-4Ig exposure in primary cultures persisted in cultures of restimulated blast cells (Table 1). To investigate the contribution of individual B7 subtypes to cytokine production, we blocked CD80/CD86-CD28 and CD80/CD86–CTLA-4 interactions with anti-CD80 and anti-CD86 antibodies. As shown in Fig. 4, the presence of anti-CD80 during primary stimulation of PBL resulted in a slight but nonsignificant reduction of IFN-γ (Fig. 4A) and IL-5 (Fig. 4B) levels compared to cultures containing isotype-matched control antibody. The presence of anti-CD86 significantly reduced both IFN-γ and IL-5 levels, an effect that was greater than that observed for anti-CD80 exposure. The presence of both anti-CD80 and anti-CD86 during primary stimulation led to a significant and near-complete elimination of detectable cytokine levels (Fig. 4A and B, respectively), results similar to those obtained when CTLA-4Ig was used. Similar reductions

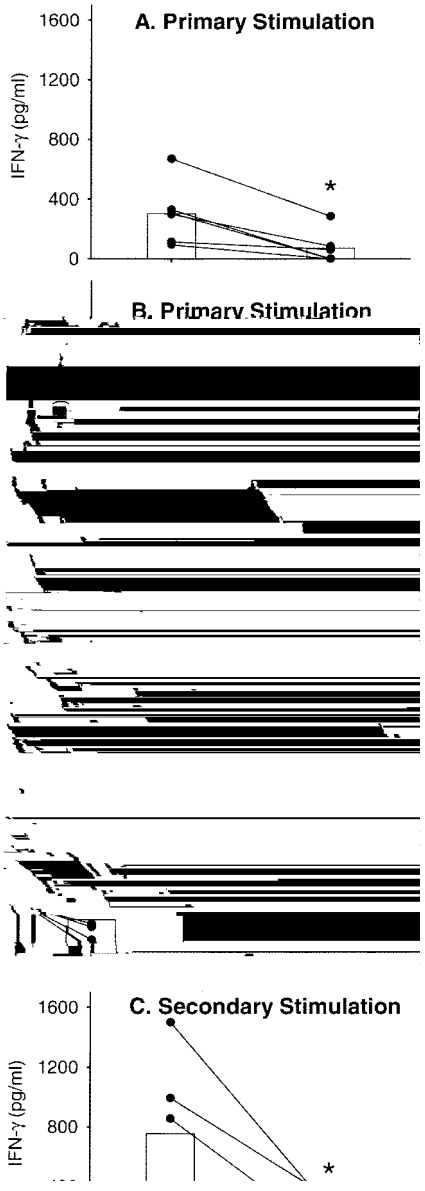

FIG. 2. Blockade of CD40-CD154 interaction reduces IFN-γ and IL-5 production after primary and secondary stimulation. PBL were stimulated with autologous *L. major*-infected adherent cells (macrophages) in the presence or absence of anti-CD154 or an isotype control antibody (mouse IgG1). After 7 days, supernatants were harvested and concentrations of IFN-γ (A) and IL-5 (B) were determined by ELISA. The blast cells from these cultures were isolated and restimulated using autologous *L. major*-infected adherent cells. After 48 h, supernatants were harvested and concentrations of IFN-γ (C) and IL-5 (D) were determined. The data for individual donors (●) and means (bars) are presented with background cytokine levels subtracted. Background equaled the concentration of cytokine when PBL were stimulated with uninfected adherent cells and ranged <10 to 270 pg/ml for IFN-γ and <2.5 to 5.5 pg/ml for IL-5. The concentrations of IFN-γ and IL-5 observed in cultures treated with isotype control antibody were equivalent to those in cultures with no added antibody (data not shown). p, statistically different ($P < 0.05$).

of IFN-γ and IL-5 levels resulting from anti-CD80 and anti-CD86 exposure persisted in cultures of restimulated blast cells and were similarly statistically significant (Fig. 4C and D). No consistent effect of anti-CD80 or anti-CD86 on IL-10 levels

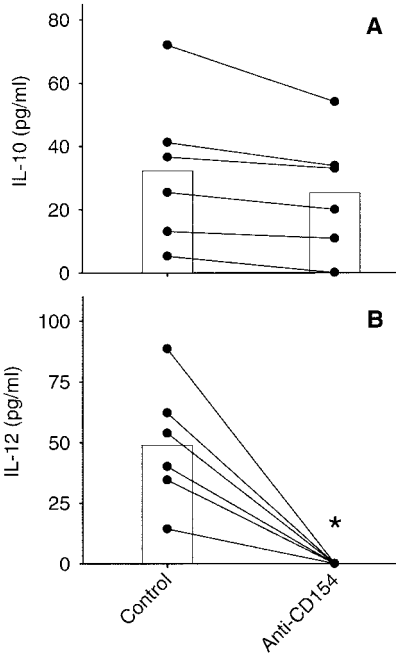

FIG. 3. Effects of CD40-CD154 blockade on IL-10 and IL-12 levels after primary stimulation. PBL were stimulated with autologous *L. major*-infected adherent cells (macrophages) in the presence or absence of anti-CD154 or an isotype control antibody (mouse IgG1). After 7 days, supernatants were harvested and concentrations of IL-10 (A) and IL-12 (B) were determined by ELISA. The data for individual donors (●) and means (bars) are presented with background cytokine levels subtracted. Background equaled the concentration of cytokine when PBL were stimulated with uninfected adherent cells and ranged 10 to 30 pg/ml for both IL-10 and IL-12. The concentrations of IL-10 and IL-12 observed in cultures treated with isotype control antibody were equivalent to those in cultures with no added antibody (data not shown). p, statistically different ($P < 0.05$).

was detected in primary (Fig. 5A) or secondary (data not shown) cultures. However, a slight but increasingly significant decrease in IL-12 levels resulting from exposure to anti-CD80, anti-CD86, or both antibodies, similar to that seen for IFN-γ and IL-5, was observed in both primary (Fig. 5B) and secondary (data not shown) cultures.

TABLE 1. CTLA-4Ig blocks the production of cytokines in response to *L. major*[a]

Treatment	1st stimulation		2nd stimulation	
	IFN-γ	IL-5	IFN-γ	IL-5
L. major	77 ± 10	14 ± 2	134 ± 11	18 ± 3
L. major + CTLA-4Ig	<10	<10	<10	<10
L. major + control Ig	90 ± 17	14 ± 1	142 ± 6	17 ± 2

[a] PBL were stimulated with autologous *L. major*-infected adherent cells (macrophages) in the presence of CTLA-4Ig or control Ig. After 7 days (primary stimulation), supernatants and blast cells were harvested. Blast cells were restimulated using autologous *L. major*-infected adherent cells without additional CTLA-4Ig. Supernatants were harvested after 48 h (secondary stimulation). Cytokine concentrations in culture supernatants were determined by ELISA. Values are presented as mean ± standard error for three donors, with background cytokine levels subtracted. Cytokine levels for PBL cultured with uninfected adherent cells (background levels) ranged <10 to 35 pg/ml for IFN-γ and <2.0 to 5.5 pg/ml for IL-5.

FIG. 4. B7 blockade reduces IFN-γ and IL-5 production after primary and secondary stimulation. PBL were stimulated with autologous *L. major*-infected adherent cells (macrophages) in the presence or absence of anti-CD80, anti-CD86, both antibodies, or an isotype con-

DISCUSSION

The importance of various costimulatory molecules (e.g., CD40, CD80, and CD86) to the outcome of disease following *Leishmania* infection of mice has been examined. CD40 has been associated with resistance (6, 22, 26, 45), whereas the role of CD80 and CD86 is unclear (3, 4, 9, 12). Infection of mouse macrophages can alter costimulatory molecule expression, at least in vitro (27, 36, 41, 49). However, the impact on host resistance, if any, of this potential subversion of immune function is unclear. Although evidence suggests that dendritic cells play the major role in priming antileishmanial responses (31, 49), macrophages too are host cells for *Leishmania* and play the major role in killing these parasites (33, 34, 48). This parasitocidal activity is likely dependent, in part, on the macrophage's interaction with T cells (35). Little is known of the role that costimulation plays in directing anti-*Leishmania* responses in humans. Therefore, we undertook the study presented here to examine (i) the influence of *L. major* on costimulatory molecule expression on human monocyte-derived macrophages and (ii) the interactions of these macrophages with *L. major*-activated T cells.

Because costimulatory molecule expression has been shown to be modulated on nonhuman APCs following *Leishmania* infection in vitro (27, 36, 41, 49), we first determined if the

expression of these molecules on cultured human macrophages was modulated following infection with *L. major*. CD86 expression was slightly decreased by *L. major* infection, but this effect was not observed with all donors, and we found no change in the expression of CD40 or CD80 (Fig. 1). Thus, no marked alteration of costimulatory molecule expression was observed on *L. major*-infected human macrophages. These results are similar to those for C57BL/6 mouse macrophages: no change in CD80 expression was observed following infection with *L. donovani* (41), and no change in CD40, CD80 or CD86 expression was observed following infection with *L. major* (49). However, *Leishmania*-induced changes in expression of costimulatory molecules has been observed on macrophages from BALB/c mice and dogs: decreased CD80 expression was observed following infection with *L. donovani* (27, 41) and *L. infantum* (36), respectively. From a comparison of the results shown here for humans, and shown by others in animal models, it seems reasonable to predict that the sensitivity of macrophages to modulation of costimulatory molecule expression following *Leishmania* infection may be dependent on the host's genetic background.

Because T cells may interact directly with infected macrophages through costimulatory molecules to promote parasite killing (35), we also examined the influence of T cells on the expression of costimulatory molecules by *L. major*-infected and uninfected macrophages. Infection with *L. major* had no effect on CD86 expression by macrophages cocultured with PBL (Fig. 1). In contrast, coculture with PBL led to an increase in CD40 and CD80 expression on *L. major*-infected macrophages (Fig. 1). These findings are significant because they demonstrate the feedback interactions between APCs and effector cells and suggest that the effects of *L. major* on macrophages, at least in part, require such interactions. These interactions may include indirect mechanisms, such as IFN-γ, which is known to induce expression of CD80 and to up-regulate expression of CD86 on monocytes (11, 14), or direct mechanisms such as stimulation of CD40 by CD154, which has been shown to up-regulate both CD40 and CD86 (28). A combination of direct and indirect mechanisms seems likely in this system.

This is the first report that live *L. major* modulates costimulatory molecule expression on human macrophages, but it is not the first such report regarding intracellular parasites. For example, Subauste et al. observed that infection of human monocytes with tachyzoites of *Trypanosoma gondii* caused a rapid induction of CD80 expression and up-regulation of CD86 (46). On the other hand, infection of mouse macrophages with *L. donovani* or *Mycobacterium tuberculosis* failed to up-regulate or actually decreased the expression of CD80 and CD86 (27, 40). These effects on costimulatory molecule expression may represent strategies used by pathogens to induce anergy or otherwise cause advantageous immune suppression. Interestingly, the results presented here contrast with those of Probst et al. (37), who cultured human macrophages (without PBL) in the presence of a purified *Leishmania* protein, LeIF, and showed an increase in both CD40 and CD80 expression. This difference in results may be due to confounding interactions between the multitude of potentially immunomodulatory factors released by live parasites versus a single purified protein, or a difference in the concentrations of LeIF present in the macrophage cultures.

We had predicted that primary human responses to *L. major* would be dependent on CD40 costimulation and influenced by CD80 and CD86 costimulation. These predictions were shown to be mostly true. Blocking CD40 or CD86 significantly reduced the production of IFN-γ, IL-12, and IL-5 following stimulation with *L. major*, whereas the only significant effect of blocking CD80 was a reduction in IL-12 production. Thus, of these three costimulatory molecules, CD40 and CD86 played the greatest role in initiating human in vitro anti-*L. major* responses. The relatively small influence of CD80 in this system is an interesting finding in light of the marked up-regulation of CD80 on macrophages cultured with PBL and *L. major*. The mechanism(s) behind this lack of effect is unclear, but a low sensitivity to CD80 costimulation may be a characteristic of PBMC, as suggested by others (2, 23).

Our prediction that CD40 would be essential to human in vitro responses to *L. major* was based on well-established pathways: (i) CD40 ligation leads to IL-12 production which leads to IFN-γ production and (ii) CD40 induces up-regulation of B7 molecules on APCs (for reviews, see references 15 and 18). Thus, our finding that blocking CD40 ligation reduced IL-12 and IFN-γ production is not unique to anti-*Leishmania* responses, but it is important to know that in human cells, as in mouse cells, *L. major* does not alter the predictability of this costimulatory pathway. The greater effect of CD40 blockade over B7 blockade could be explained, in part, by a double effect on cytokines and B7 expression when CD40 ligation was inhibited.

An unexpected observation was the reduction of IL-5 production in macrophage+*L. major*+PBL cultures following blockade of CD40 and CD86. IL-5 was used as an indicator of Th2 cell activity. The importance of CD40 to the development of Th1 responses has been well established, but the role of this costimulatory pathway in the development of Th2 responses is less well studied. The results of the studies presented here suggest that CD40, as well as CD86, can costimulate for both Th1 and Th2 responses. Similar findings in another model (44, 47) suggest that, at least for CD40, this may be a common phenomenon.

As shown here, IL-10 production was not significantly altered by blocking CD40 or the B7 molecules, unlike what was observed for IL-5, IL-12, and IFN-γ. The IL-10 levels measured in these studies were lower than those observed by others following mitogen or MAb stimulation (5, 10, 43) and were more in line with spontaneous production levels (5, 25). This can be explained by the use of metacyclic *L. major* in the study presented here. Metacyclic *L. major* has been shown to be a poor stimulator of IL-10 production from human PBMC, unlike the log-phase promastigote form of the parasite (42). Because blocking costimulation did not alter IL-10 levels, these results suggest that spontaneous or metacyclic *L. major*-stimulated IL-10 production is independent of the costimulatory molecules examined. Others have shown that IL-10 production can be modulated by IL-12 and IFN-γ (1, 8, 10, 15). Therefore, it was interesting that IL-10 levels were not changed in the face of markedly lower IL-12 and IFN-γ levels. These data suggest that spontaneous or metacyclic *L. major*-stimulated IL-10 production is also independent of modulation by IL-12 and IFN-γ. This possibility is supported for IL-12 by the results of Sartori et al. (42), who showed that IL-10 production by metacyclic *L.*

genesis modulates the ability of *Leishmania* promastigotes to induce IL-12 production in human mononuclear cells. J. Immunol. **159:**2849–2857.
43. **Sch ar , M., O. Majdic, W. Knapp, and W. Holter.** 1995. High-level IL-10 production by monoclonal antibody-stimulated human T cells. Immunology **86:**364–371.
44. **Shi, F. D., B. He, H. Li, D. Matuse icius, H. Link, and H. G. Ljunggren.** 1998. Differential requirements for CD28 and CD40 ligand in the induction of experimental autoimmune myasthenia gravis. Eur. J. Immunol. **28:**3587–3593.
45. **Soong, L., J. C. Xu, I. S. Gre al, P. Kima, J. Sun, B. J. Longle Jr., N. H. Ruddle, D. McMahon-Pratt, and R. A. Fla ell.** 1996. Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to *Leishmania amazonensis* infection. Immunity **4:**263–273.
46. **Subauste, C. S., R. De Wall Malef t, and F. Fuh.** 1998. Role of CD80 (B7.1) and CD86 (B7.2) in the immune response to an intracellular pathogen. J. Immunol. **160:**1831–1840.
47. **Ta lor, P. A., A. Panoskaltsis-Mortari, R. J. Noelle, and B. R. Bla ar.** 2000. Analysis of the requirements for the induction of CD4+ T cell alloantigen hyporesponsiveness by ex vivo anti-CD40 ligand antibody. J. Immunol. **164:** 612–622.
48. **Titus, R. G., A. Kelso, and J. A. Louis.** 1984. Intracellular destruction of *Leishmania tropica* by macrophages activated with macrophage activating factor/interferon. Clin. Exp. Immunol. **55:**157–165.
49. **on Stebut, E., Y. Belkaid, T. Jakob, D. L. Sacks, and M. C. Ude .** 1998. Uptake of *Leishmania major* amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-*Leishmania* immunity. J. Exp. Med. **188:**1547–1552.

Editor: W. A. Petri, Jr.