ELSEVIER

CD28 Signaling in Neutrophil Induces T-Cell Chemotactic Factor(s) Modulating T-Cell Response

K. Venuprasad, Subhasis Chattopadhyay, and Bhaskar Saha

ABSTRACT: We previously reported that human peripheral blood neutrophils express CD28 and interact with macrophage B7 to generate CD28 signaling through PI-3 kinase. Here, we demonstrate that crosslinking of CD28 on neutrophils results in the release of IFN-γ, which restricts amastigote growth and modulates $CD4^+$ T cells cytokine secretion. CD28 crosslinking also induces a T-cell chemotactic factor (TCF) that induces chemotactic migration of $CD4^+$ T cells. Based on our previous and the current set of data, we propose an operational model explaining how neutrophils are involved in *Leishmania* infection and how the reported effect of neutrophils on the control of infection is mediated by alteration of T-cell function. *Human Immunology 64, 38–43 (2003).* © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Science Inc.

KEYWORDS: neutrophils; CD28; *Leishmania*; Th subset differentiation

ABBREVIATIONS

PBMC peripheral blood mononuclear cells

TCF T-cell chemotactic factor

INTRODUCTION

Leishmania is a protozoan parasite that resides and replicates within the cells of monocyte-macrophage lineage of a mammalian host. The susceptibility or resistance to the infection is proposed to be mediated by selective expansion of the T-helper (Th) subsets [1–3]. The Th1 subset secretes IFN-γ, which activates macrophages to eliminate the intracellular amastigotes, and thereby ameliorates the infection. In contrast, the Th2 subset secretes IL-4 and IL-10, which deactivate macrophages and promote parasite growth to exacerbate the infection. On the other hand, recent studies report that in experimental *Leishmania donovani* infection, the anti-leishmanial immune response begins with the release of chemokines [4] and the elimination of neutrophils increases the parasite load in hepatic tissue [5]. Another report suggests that in experimental *L. major* infection neutrophils may regulate Th subset cytokine profile [6]. In addition, neutrophils are revealed to be the very first cells to migrate into the site of inflammation [7]. Therefore, we investigated whether or not neutrophils regulate the Th response. Here, we report that CD28 signaling in neutrophil results in the production of IFN-γ that restricts amastigote growth, and T-cell chemotactic factor(s) that chemoattracts T cells. The neutrophil secreted IFN-γ induces a skewing towards Th1 subset. Based on our previous observations [8] and the data presented in this report, we propose a model demonstrating how neutrophils play an important role in the afferent limb of the anti-leishmanial immune response to set a Th subset bias.

MATERIALS AND METHODS

Isolation of Neutrophils, Macrophages, and T Cells

Following approval from the Institutional Ethics Committee, venous blood from healthy volunteers was collected in heparin, and cells were isolated from the blood using polymorphoprep (Nycomed Pharma AS, Oslo, Norway) as described previously [8]. Peripheral blood mononuclear cells (PBMCs) were plated at a final concentration of 2×10^6 cells/ml in RPMI-1640 supplemented with 10% FCS. Nonadherent lymphocytes were

From the National Centre for Cell Science, Ganeshkhind, Pune, India.

Address reprint requests to: Dr. Bhaskar Saha, National Centre for Cell Science, Ganeshkhind, Pune 411007, India; Tel: +91 (20) 5690922; Fax: +91 (20) 5692259; E-mail: sierra112233@hotmail.com, sahab@nccs.res.in.

Received May 31, 2002; revised August 19, 2002; accepted August 22, 2002.

0198-8859/03/$–see front matter
PII S0198-8859(02)00689-4

washed after 12 hours. Monocytes were allowed to differentiate to macrophages for 72 hours with washing every 24 hours to remove the nonadherent cells. The cells recovered after the end of culture were >99% macrophages as judged by morphology, histochemistry, and fluorescence activated cell sorter (FACS) analysis (data not shown). Characterization of the neutrophils by morphology and histochemistry revealed that >99.5% of the isolated cells were neutrophils and that no $CD3^+$ T cells were detectable [8]. Cells were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) with 10% fetal calf serum.

$CD4^+$ T cells were isolated from the PBMC fraction by affinity chromatography [8] using the IsoCell human T-cell isolation kit (Pierce, Rockford, IL, USA) and the purity (>99.5%) was confirmed by FACS analysis of the anti-CD3-FITC stained cells in FACSvantage (Becton Dickinson, Gaithersburg, MD, USA).

Generation of Neutrophil Culture Supernatant

The 96-well tissue culture plates were coated with anti-CD28 (10 μg/ml) or isotype control for anti-CD28 antibody (10 μg/ml). The plates were washed with RPMI-1640 medium and the isolated neutrophils were seeded at a density of 3×10^5 per well. The cells were cultured for 12 hours at 37°C in a CO_2 incubator. The supernatants were harvested at the end of culture.

Leishmanicidal Assay

Human monocyte-derived macrophages were infected with *L. major* strains (HOMOM/SU/73/5ASKH) at a 1:5 ratio for 4 hours and were cultured with neutrophil supernatant generated by isotype or CD28 crosslinking as described above, for 72 hours at 34°C. In some experiments, neutralizing anti-IFN-γ antibody (PharMingen, San Diego, CA, USA), as indicated, was added to the culture. The intracellular parasite load was determined by examination under a light microscope (E600, Nikon, Tokyo, Japan) as described earlier [8].

T-Cell Chemotaxis Assay

T-cell chemotaxis was assayed using a Boyden chamber [8] (Neuro Probe, Inc., Franklin Lakes, NJ, USA). Briefly, freshly isolated T cells were suspended in RPMI-1640 with 10% FCS. Cell culture supernatants from normal neutrophils, CD28 crosslinked neutrophils, and isotype crosslinked neutrophils were added to the lower wells of Boyden chamber. T cells (1.5×10^5/well) were put in the upper chamber. The upper and lower wells of the chamber were separated by 3 μm polycarbonate filters (Neuro Probe, Inc.). Migration was proceeded in a CO_2 incubator at 37°C for 90 minutes. At the end of incubation period, cells migrated to the lower wells were counted.

T-Cell Culture and Quantitation of IFN-γ in the Culture Supernatants by ELISA

$CD4^+$ T cells were purified from human volunteers [9] and were incubated for 12 hours with the neutrophil supernatants generated by isotype or CD28 crosslinking. Following the preincubation, the T cells were washed and cultured with immobilized anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) antibodies in a CO_2 incubator at 37°C. The culture supernatants were assayed for IFN-γ content by enzyme-linked immunoabsorbent assay (ELISA) using OptEIA Kit (PharMingen) following manufacturer's recommendations.

Statistical Significance

The raw scores were analyzed by using Sigma Stat software program (Sigma, St. Louis, MO, USA). The data are presented as mean ± standard deviation of triplicates in one representative of at least three individual experiments.

RESULTS

CD28 Crosslinked Neutrophils Secreted IFN-γ that Induced Leishmanicidal Activity

We found that the culture supernatant of CD28-crosslinked neutrophils inhibited intracellular *L. major* growth in the infected macrophages as compared to the respective controls ($p < 0.001$). The restricted parasite growth was reversed by neutralizing IFN-γ in the supernatant with a monoclonal anti-IFN-γ antibody. In contrast, the control supernatants, medium and the isotype control for anti-IFN-γ antibody, had no effects on amastigote growth (Figure 1) suggesting that the IFN-γ secreted by CD28-crosslinked neutrophils induced leishmanicidal activity.

Anti-CD28 Activated Neutrophil Supernatant Induced Chemotactic Migration of $CD4^+$ T Cells

It is known that neutrophils are the very first cells to migrate to the site of inflammation [7]. Subsequent to the initial neutrophil reaction, T cells migrate to the site of infection. Therefore, we tested whether or not the CD28-activated neutrophil supernatant induced T-cell chemotaxis. We found that the supernatant generated by crosslinking CD28 on neutrophils significantly increased $CD4^+$ T-cell transmigration ($p < 0.01$; Figure 2A). In contrast, in the control sets, fewer $CD4^+$ T cells migrated (Figure 2A). In order to identify the chemokine responsible for the T-cell chemotaxis, we performed assays for the known T-cell chemoattractants, MIP-1α and MCP-1, in the CD28 activated neutrophil supernatant. We did not observe any detectable levels of the chemokines (data not shown).

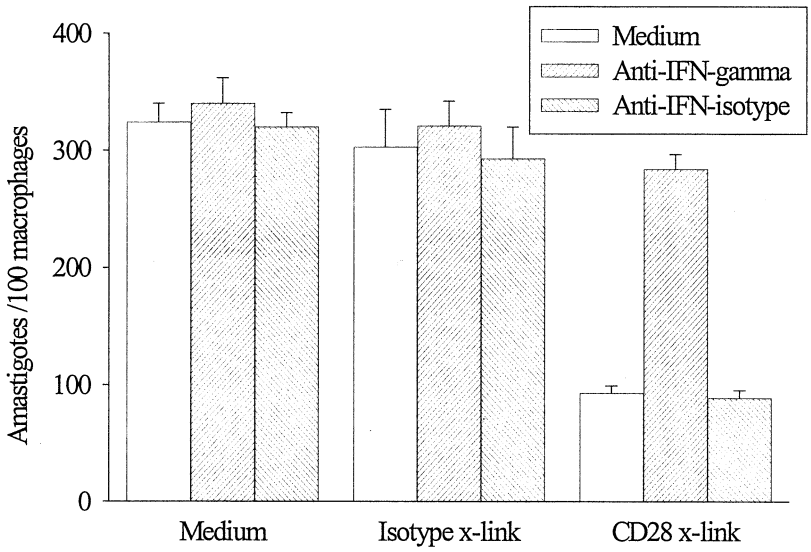

FIGURE 1 CD28 crosslinked neutrophils induced leishmanicidal activity. Human monocyte-derived macrophages were infected with *L. major* at a 1:5 ratio for 4 hours and were cocultured with neutrophil supernatant generated by isotype or CD28 crosslinking for 12 hours at 37°C. In some experiments, neutralizing anti-IFN-γ antibody (Pharmingen) or control isotype or other treatments, as indicated, was added to the culture. The intracellular parasite load was determined by light microscopy. The experiment was carried out three times and the data from one representative experiments are illustrated here.

CD28 Crosslinked Neutrophils Induced IFN-γ Production From $CD4^+$ T Cells

The $CD4^+$ T cells preincubated with the culture supernatants of CD28-activated neutrophils secreted more IFN-γ but less IL-4 upon subsequent activation with anti-CD3+anti-CD28 antibodies (Figure 2B). In contrast, in controls, where T cells were preincubated with the supernatants of unstimulated or isotype crosslinked neutrophils, such an enhancement of IFN-γ secretion was not observed (Figure 2B). However, the culture supernatants of CD28-activated neutrophils failed to enhance IFN-γ secretion by T cells when neutralizing anti-IFN-γ antibody was added to the T-cell culture during preincubation with the neutrophil supernatant (Figure 2B). This observation suggests that the CD28-activated neutrophils secrete IFN-γ and that neutrophil secreted IFN-γ enhanced the $CD4^+$ T cell's IFN-γ secretion.

DISCUSSION

Leishmania is a dimorphic protozoan parasite. The flagellated promastigotes are introduced into the host during the blood meal of the sandfly vector. The promastigotes invade the cells of the monocyte-macrophage lineage of its mammalian host and is transformed into aflagellate amastigotes. It is proposed that in a susceptible murine host, the infection initiated at this point is mediated by a monoclonal T-cell repertoire of $V\beta4^+V\alpha8^+$ T cells, which produce IL-4 to set the bias towards Th2 cells in the susceptible host [10–12]. In contrast, earlier proposition was that the initial T-cell activation results in the production of a wide array of cytokines [13], which control parasite growth by regulating the macrophage activation or deactivation [14] or by setting a Th subset bias [15]. Therefore, the time of involvement of T cells in the initiation of the anti-leishmanial immune response is not well defined, raising a possibility of involvement of non-T cells in the initiation of anti-leishmanial immune response [4]. Neutrophils, among the non-T cells but not lymphocytes, are reported to infiltrate the site of infection as early as 12-hours after subcutaneous introduction of the parasite in mice (Saha *et al.*, unpublished observation), and were demonstrated to be present in the lesion of cutaneous leishmaniasis patients [16]. Therefore, we tested whether or not neutrophils might play a crucial role in the initiation of anti-leishmanial immune response.

We report that the culture supernatant of CD28-crosslinked neutrophils inhibited intracellular *L. major* growth in the infected macrophages in an IFN-γ dependent manner. The CD28-activated neutrophils also secreted a factor(s), which chemoattracted $CD4^+$ T cells. At this time, however, the identity of the chemotactic factor remains unknown because MIP-1α or MCP-1, the chemokines described to induce T-cell chemotaxis [17, 18], was undetectable in the CD28-activated neutrophil culture supernatant, and addition of anti-MIP-1α and

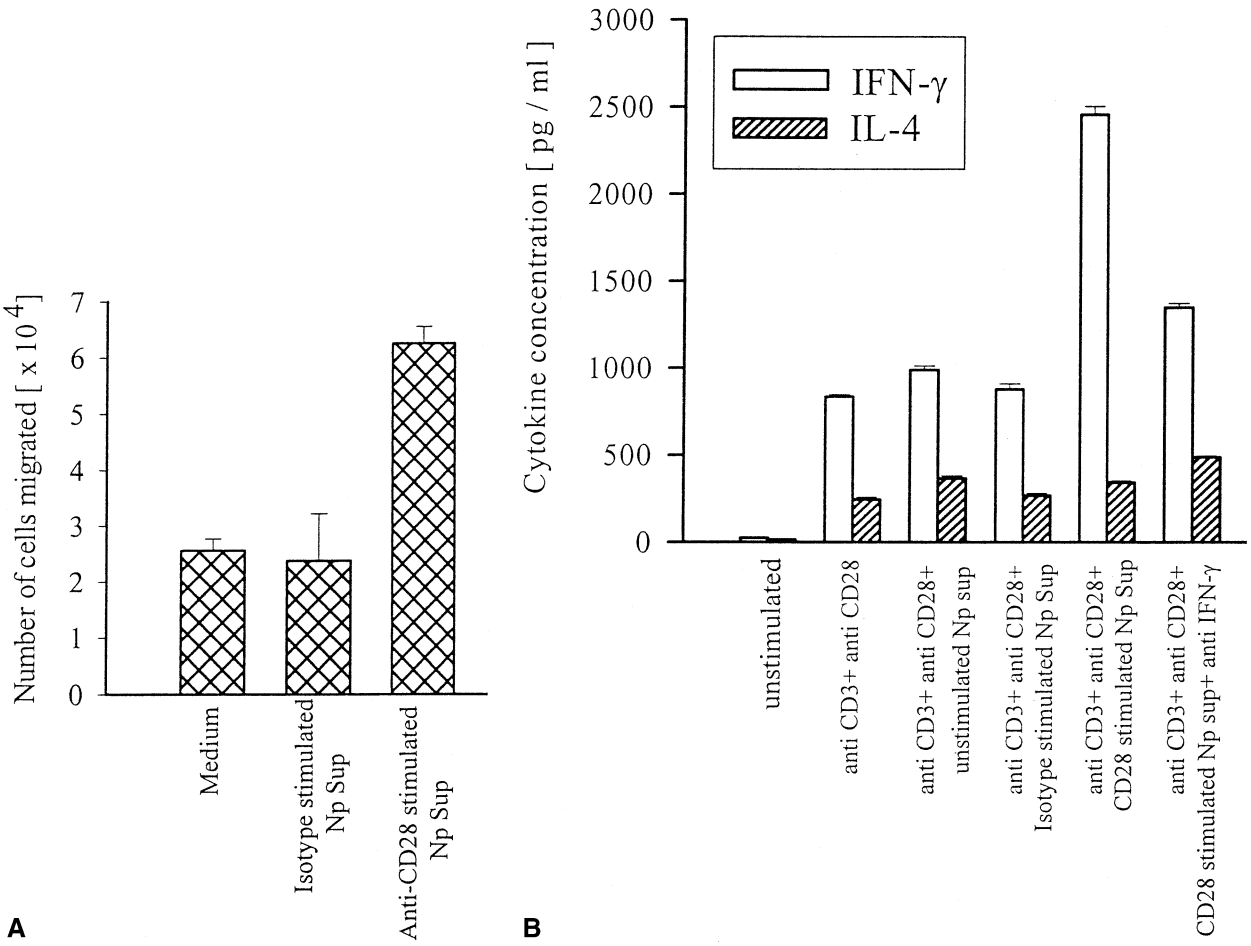

FIGURE 2 CD28-activated neutrophil supernatant induces T-cell chemotaxis and modulates $CD4^+$ T-cell cytokine profile. (A) Neutrophil-secreted factor induces T-cell migration. The culture supernatants were generated by culturing neutrophils (Np) in medium or crosslinked using anti-CD28 antibody or isotype control. The supernatants were tested for their ability to chemoattract T cells in Boyden chamber as described in materials and methods. (B) Neutrophil derived IFN-γ modulates $CD4^+$ T-cell cytokine profile. $CD4^+$ T cells from human peripheral blood mononuclear cells were purified and incubated for 12 hours with the neutrophil supernatants as indicated. T cells were then stimulated with anti-CD3 and anti-CD28 for 48 hours and the culture supernatants were assayed for IFN-γ and IL-4. In some experiments, CD28-activated neutrophil culture supernatants were neutralized with anti-IFN-γ antibody before treatment of $CD4^+$ T cells. The results described represent one of three individual experiments.

anti-MCP-1 antibodies to the CD28-activated neutrophil culture supernatant had no effect on the T-cell chemotaxis against the supernatant (data not shown). Because IFN-γ was present in the CD28-activated neutrophil supernatant, we tested whether or not IFN-γ induced the $CD4^+$ T-cell chemotaxis. We did not observe any significant chemotaxis of $CD4^+$ T cells in response to IFN-γ (data not shown). Because the cytokine microenvironment is proposed to effect the Th subset polarization (reviewed in Jankovic *et al.* [19]), we investigated whether or not the CD28-activated neutrophil supernatant had any effect on $CD4^+$ T-cell cytokine secretion profile. In this study we report that the $CD4^+$ T cells preincubated with the CD28-activated neutrophil culture supernatant secreted more IFN-γ but less IL-4 upon subsequent activation. IFN-γ neutralization in the supernatant inhibited the augmented T-cell IFN-γ secretion suggesting that IFN-γ secreted by neutrophils plays an important role in setting a Th subset bias. Similar observation was reported where natural killer cell secreted IFN-γ sets a Th1 bias [20]. Because IL-12 is known to induce IFN-γ, we tested for IL-12 content in the neutrophil supernatant. We could not find any detectable level of IL-12 in the CD28-activated neutrophil supernatant (data not shown). Based on our previous and present observations, we propose the model illustrated in Figure 3, which defines the role of neutrophils in the afferent limb of the anti-leishmanial immune response.

We have previously reported that the inflammatory reaction against *Leishmania* infection is initiated with the

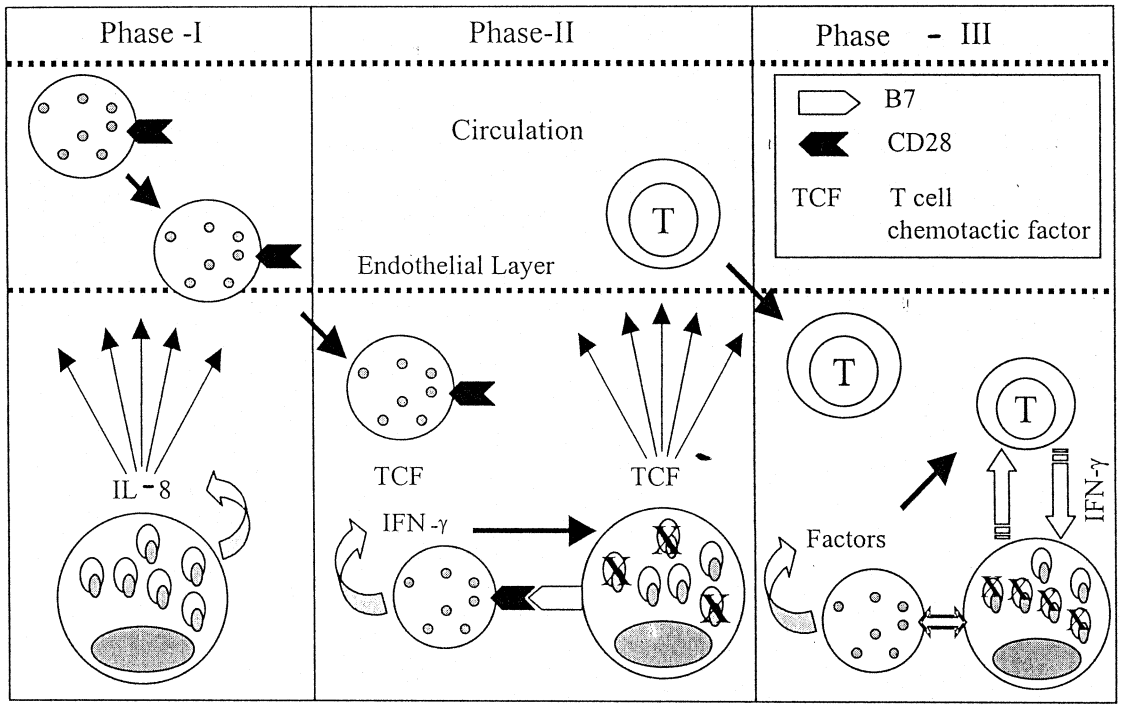

FIGURE 3 The model proposes the role of neutrophils in the afferent limb of an immune response. In phase I, neutrophils are chemoattracted to the site of infection by IL-8 secreted from *Leishmania*-infected macrophages. In phase II, the migrated neutrophils interact with macrophages to generate CD28-dependent IFN-γ, killing amastigotes and T-cell chemotactic factor (TCF), chemoattracting T cells. In phase III, migrated T cells control the infection.

release of chemokines, such as IL-8, by *Leishmania*-infected macrophages, followed by IL-8-dependent neutrophil recruitment to the site of infection [8, 21]. CD28 on neutrophils engages B7 on macrophages to generate CD28 signal, which induces IFN-γ secretion [8, 22]. Besides restricting the *Leishmania* growth, neutrophil secreted IFN-γ alters the cytokine microenvironment at the site of infection, affecting the IL-4 and IFN-γ secreting T-cell differentiation as a consequence. Thus, although the current hypothesis for initiation of the disease Leishmaniasis by setting a Th subset bias relies heavily on T-cell secreted cytokines, our observations define a significant role of neutrophils in the afferent limb of the anti-leishmanial immune response [23], especially that which precedes T-cell response.

ACKNOWLEDGMENTS

The authors extend their sincere thanks to Dr. G.C. Mishra (Director, National Centre for Cell Science) for encouragement and constructive criticism, and to Mr. Atul Suple for technical assistance. This work was supported by the Department of Science and Technology, Department of Biotechnology, and Life Science Research Board, Government of India.

REFERENCES

1. Launois P, Tacchini Cottier F, Parra Lopez C, Louis JA: Cytokines in parasitic diseases: the example of cutaneous leishmaniasis. Int Rev Immunol 17:157, 1998.
2. Reiner SL, Locksley RM: The regulation of immunity to Leishmania major. Annu Rev Immunol 13:151, 1995.
3. Locksley RM, Wakil AE, Corry DB, Pingel S, Bix M, Fowell DJ: The development of effector T cell subsets in murine *L. major* infection. Ciba Found Symp 195:110, 1995.
4. Cotterell SEJ, Engwerda CR, Kaye PM: *Leishmania donovani* infection initiates T cell independent chemokine responses, which are subsequently amplified in a Tcell-dependent manner. Eur J Immunol 29:203, 1999.
5. Smelt SC, Cotterell SE, Engwerda CR, Kaye PM: B cell-deficient mice are highly resistant to Leishmania donovani infection, but develop neutrophil-mediated tissue pathology. J Immunol 164:3681, 2000.
6. Tacchini-Cottier F, Zweifel C, Belkaid Y, Mukankundiye C, Vasei M, Launois P, Milon G, Louis JA: An immunomodulatory function for neutrophils during the induction of a $CD4^+$ Th2 response in BALB/c mice infected with *L. major*. J Immunol 165:2628, 2000.
7. Woodman RC, Johnston B, Hickey MJ, Teoh D, Reinhardt P, Boon PY, Kubes P: The functional paradox of

CD43 in leukocyte recruitment: a study using CD43-deficient mice. J Exp Med 188:2181, 1998.

8. Venuprasad K, Banerjee PP, Chattopadhyay S, Sharma S, Pal S, Parab PB, Mitra D, Saha B: Human neutrophil expressed CD28 interacts with macrophage B7 to induce PI3-kinase dependent IFN-γ secretion and restriction of *Leishmania* growth. J Immunol 169:920, 2002.
9. Deshpande M, Venuprasad K, Parab PB, Saha B, Mitra D: A novel CD28 mRNA variant and simultaneous presence of various CD28 mRNA isoforms in human T lymphocytes. Hum Immunol 63:20, 2002.
10. Reiner SL, Fowell DJ, Moskowitz NH, Swier K, Brown DR, Brown CR, Turck CW, Scott PA, Killeen N, Locksley RM: Control of *Leishmania major* by a monoclonal alpha beta T cell repertoire. J Immunol 160:884, 1998.
11. Launois P, Maillard I, Pingel S, Swihart KG, Xenarios I, Acha Orbea H, Diggelmann H, Locksley RM, MacDonald HR, Louis JA: IL-4 rapidly produced by V beta 4 V alpha 8 $CD4^+$ T cells instructs Th2 development and susceptibility to *Leishmania major* in BALB/c mice. Immunity 6:541, 1997.
12. Launois P, Swihart KG, Milon G, Louis JA: Early production of IL-4 in susceptible mice infected with *Leishmania major* rapidly induces IL-12 unresponsiveness. J Immunol 158:3317, 1997.
13. Reiner SL, Zheng S, Wang ZE, Stowring L, Locksley RM: *Leishmania* promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from $CD4^+$ T cells during initiation of infection. J Exp Med 179:447, 1994.
14. Assreuy J, Cunha FQ, Epperlein M, Noronha-Dutra A, O'Donnell CA, Liew FY, Moncada S: Production of nitric oxide and superoxide by activated macrophages and killing of *Leishmania major*. Eur J Immunol 24:672, 1994.
15. Reiner SL, Locksley RM: Cytokines in the differentiation of Th1/Th2 $CD4^+$ subsets in leishmaniasis. J Cell Biochem 53:323, 1993.
16. Peltier E, Wolkenstein P, Deniau M, Zafrani ES, Wechsler J: Caseous necrosis in cutaneous leishmaniasis. J Clin Pathol 49:517, 1996.
17. Loetscher P, Seitz M, Clark Lewis I, Baggiolini M, Moser B: Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human $CD4^+$ and $CD8^+$ T lymphocytes. FASEB J 8:1055, 1994.
18. Karpus WJ, Lukacs NW, Kennedy KJ, Smith WS, Hurst SD, Barrett TA: Differential CC chemokine-induced enhancement of T helper cell cytokine production. J Immunol 158:4129, 1997.
19. Jankovic D, Liu Z, Gause WC: Th1 and Th2 cell committment during infectious disease: asymmetry in divergent pathways. Trends Immunol 28:450, 2001.
20. Scharton Kersten T, Scott P: The role of the innate immune response in Th1 cell development following Leishmania major infection. J Leukoc Biol 57:515, 1995.
21. Badolato R, Sacks DL, Savoia D, Musso T: *Leishmania major* infection of human monocytes induces expression of IL-8 and MCAF. Exp Parasitol 82:21, 1996.
22. Venuprasad K, Parab PB, Prasad DVR, Sharma S, Banerjee PP, Deshpande M, Mitra DK, Pal S, Bhadra R, Mitra D, Saha B: Immunobiology of CD28 expression on human neutrophils. I. CD28 regulates neutrophil migration by modulating CXCR-1 expression. Eur J Immunol 31:1536, 2001.
23. Lloyd AR, Oppenheim JJ: Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. Immunol Today 13:169, 1992.