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Human Neutrophil-Expressed CD28 Interacts with Macrophage B7 to Induce Phosphatidylinositol 3-Kinase-Dependent IFN-γ Secretion and Restriction of *Leishmania* Growth[1]

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We previously showed that CD28 is expressed on human peripheral blood neutrophils and plays an important role in CXCR-1 expression and IL-8-induced neutrophil migration. In this work we demonstrate that *Leishmania major* infection of macrophages results in parasite dose-dependent IL-8 secretion in vitro and in IL-8-directed neutrophil migration, as blocked by both anti-IL-8 and anti-IL-8R Abs, toward the *L. major*-infected macrophages. In the neutrophil-macrophage cocultures, both CTLA4-Ig, a fusion protein that blocks CD28-CD80/CD86 interaction, and a neutralizing anti-IFN-γ Ab inhibit the anti-leishmanial function of neutrophils, suggesting that the neutrophil-macrophage interaction via CD28-CD80/CD86 plays an important role in the IFN-γ-dependent restriction of the parasite growth. Cross-linking of neutrophil-expressed CD28 by monoclonal anti-CD28 Ab or B7.1-Ig or B7.2-Ig results in phosphatidylinositol 3-kinase association with CD28 and in wortmannin-sensitive but cyclosporin A-resistant induction and secretion of IFN-γ. Whereas the neutrophils secrete IFN-γ with CD28 signal alone, the T cells do not secrete the cytokine in detectable amounts with the same signal. Thus, neutrophil-expressed CD28 modulates not only the granulocyte migration but also induction and secretion of IFN-γ at the site of infection where it migrates from the circulation. ***The Journal of Immunology*, 2002, 169: 920–928.**

Introduction of foreign materials, including infectious agents, into tissue sites elicits inflammatory reactions that are initially characterized by infiltration of cells, chiefly neutrophils (1). At the site of inflammation neutrophils perform many important functions, such as control of bacterial and parasitic infections, by releasing inflammatory mediators and different cytokines (2, 3). In experimental *Leishmania donovani* infection, for example, 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). Although these findings suggest that neutrophil migration to the site of *L. donovani* infection may play an important role in controlling the anti-leishmanial immune response, the stimuli required for cytokine secretion by neutrophils and their mechanism of anti-leishmanial action remain to be established.

It was reported earlier that neutrophils clear *Leishmania* infection by virtue of their phagocytic function (6). Because *Leishmania* is a protozoan parasite that resides and replicates within the macrophages, the parasite clearance by phagocytosis alone as described in previous studies limits the role of neutrophils in *Leishmania* infection. It was proposed recently that neutrophils may interact with macrophages through cell surface molecules like LFA-1 during infection and inflammatory reactions (7). The macrophage surface molecules, CD80 and CD86, the receptors for CD28/CD152 (CTLA-4) on T cells, were shown to play important roles in T cell response against both *Leishmania major* and *L. donovani* infections (8, 9). Because we have already demonstrated CD28 expression on human peripheral blood neutrophils and CD28-regulated neutrophil migration in response to IL-8 (10), we investigated whether neutrophil-expressed CD28 interacts with CD80/CD86 on macrophages, resulting in alteration of cellular functions.

In this work we demonstrate that *L. major* infection of macrophages results in increased IL-8 secretion and IL-8-directed migration of neutrophil toward the *L. major*-infected macrophages. The neutrophils interact with the macrophages through the CD28-CD80/CD86 pathway, resulting in phosphatidylinositol 3-kinase (PI3-kinase)[3]-dependent induction and secretion of IFN-γ, which in turn controls *L. major* growth in the macrophages. In addition, IFN-γ induction in neutrophils does not require a primary signal as in T cells or NK cells. Therefore, taken together, our results suggest that the neutrophil-expressed CD28 serves an anti-leishmanial function by interacting with the *Leishmania*-infected macrophages.

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, Oslo, Norway) as described previously (10). PBMCs were plated at a final concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS. Nonadherent lymphocytes were washed after 12 h. Monocytes were allowed to differentiate to macrophages for 72 h with washing every 24 h to remove the

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[3] Abbreviation used in this paper: PI3-kinase, phosphatidylinositol 3-kinase.

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nonadherent cells. The cells recovered after the end of culture were >99% macrophages as judged by morphology, histochemistry, and FACS analysis (data not shown). Characterization of the neutrophils by morphology and histochemistry showed that >99.5% of the isolated cells were polymorphonuclear and that no $CD3^+$ T cells were detectable (10). Cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) with 10% FCS.

$CD4^+$ T cells were isolated from the PBMC fraction by affinity chromatography using the IsoCell human T cell isolation kit (Pierce, Rockford, IL), and the purity (>99.5%) was confirmed by FACS analysis of the anti-CD3-FITC-stained cells in FACSVantage (BD Biosciences, Mountain View, CA).

Quantitation of IL-8 in L. major*-infected macrophage culture supernatant*

Macrophages were infected with different doses of *L. major* promastigote (strain MHOM/SU/73/5ASKH). Extracellular parasites were washed after 4 h. The culture supernatant collected at different intervals after infection was assayed for IL-8 content by ELISA using a human IL-8 ELISA kit (Amersham Life Sciences, Little Chalfont, U.K.) following the manufacturer's instructions.

Leishmanicidal assay

Human monocyte-derived macrophages were infected with *L. major* at a 1:5 ratio for 4 h and were cocultured with neutrophils at a 1:4 ratio in the presence of neutralizing anti-IFN-γ Ab, CTLA-4-Ig (R&D Systems, Minneapolis, MN), control human Ig, or other treatments, as indicated, for 36 h. The intracellular parasite load was determined after staining the cells with Giemsa stain. Five hundred cells per culture were counted. The percentage of infection and number of parasites per 100 infected macrophages were calculated.

Neutrophil transmigration assay

Neutrophil transmigration assay was performed using 24-well transwell inserts of 3-μm pore size (Falcon; BD Labware, Franklin Lakes, NJ), as described earlier (10). In brief, uninfected and *L. major*-infected macrophages were cultured in 24-well plates for 12 h. In some experiments, neutralizing anti-IL-8 Ab or control isotype (BD PharMingen, San Diego, CA) (10 μg/ml) were added to the lower well 30 min before placing the transwell insert. A total of 10^5 neutrophils, pretreated with anti-IL-8R A and anti-IL-8R B or control isotype (BD PharMingen) at 4°C for 30 min, were added to each insert and were allowed to migrate for 60 min. The filter was stained with Giemsa and cells in the lower surface were counted by randomly selecting 10 fields at ×40 magnification (10).

Quantitation of IFN-γ in the culture supernatants by ELISA

CD28 on neutrophils was cross-linked using anti-CD28 Ab or control isotype (BD PharMingen) as described earlier (10), and the cells were cultured in the presence of indicated concentrations of wortmannin (ICN Biomedicals, Costa Mesa, CA) or cyclosporin A (Sigma-Aldrich, St. Louis, MO). The culture supernatant was assayed for IFN-γ content by ELISA using the OptEIA kit (BD PharMingen) following the manufacturer's recommendations. $CD4^+$ T cells were cultured either in the presence of only anti-CD28 (5 μg/ml) or along with immobilized anti-CD3 Ab (1 μg/ml). Supernatant was collected at different time points and ELISA was performed as described above. In parallel experiments neutrophils were also stimulated either with CD28 alone or with anti-CD3 and anti-CD28 mAbs. To compare the effect of IL-12 on IFN-γ induction by neutrophils and T cells, cells were stimulated with indicated concentrations of IL-12 and anti-CD28 mAbs. Culture supernatant was collected at different time points and ELISA was performed using the OptEIA kit (BD PharMingen) following the manufacturer's recommendations.

Flow cytometry

Human monocyte-derived macrophages were infected with *L. major* at a 1:5 ratio. Six hours after infection, the cells were incubated with anti-human CD80-PE, anti-human CD86-FITC, and isotype-matched Abs (BD PharMingen) for 30 min in staining buffer (PBS containing 2% FCS and 0.1% sodium azide) at 4°C. This was followed by two washes in the staining buffer. The samples were analyzed using a FACSVantage flow cytometer.

For intracellular staining, the wells in a 96-well tissue culture plate were coated with anti-CD28 mAb (10). Neutrophils were plated to cross-link with the plate-bound anti-CD28 Ab (10). Neutrophils were recovered from the wells, fixed and permeabilized using Cytofix/Cytoperm Plus kit (BD PharMingen), and stained for IFN-γ with FITC-labeled anti-IFN-γ Ab (BD PharMingen). An isotype-matched FITC-conjugated Ab was used as isotype control. The samples were analyzed using a FACSVantage flow cytometer.

RT-PCR

Using TRIzol (Life Technologies), total RNA was isolated from neutrophils after 4 h of incubation with medium alone or stimulation with anti-CD28 mAb or isotype-matched Ab in the presence or absence of wortmannin (10 ng/ml). For cDNA synthesis, 1 μg of total RNA from each sample was incubated with random primer, 0.1 M DTT, 500 μM dNTPs, 40 U RNase inhibitor, and 1 μl (400 U) of Moloney murine leukemia virus reverse transcriptase (Life Technologies). Samples were then incubated at 37°C for 1 h followed by a 5-min incubation at 95°C. Amplification of synthesized cDNA from each sample was conducted with DNAzyme DNA polymerase (Finnzymer, Espoo, Finland) in 50 μl under following conditions: 95°C for 2 min, 94°C 1 min, 55°C for 1 min, and 72°C for 1 min for a total of 35 cycles. Specific primers were designed to amplify the human IFN-γ coding region (sense, 5′-GCAGGTCATTCAGATGTAGC-3′; antisense, 5′-GGCCCCTGAGATAAAGCC-3′). Each sample was also amplified for either GAPDH or human β-actin to ensure equal input of cDNA.

CD28 immunoprecipitation and immunoblotting of PI3-kinase

Purified neutrophils were stimulated with immobilized anti-CD28 Ab (10 μg/ml) for 10 min at 37°C. The reaction was stopped by adding Triton X-100 lysis buffer (pH 7.5) containing 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin for 1 h at 4°C. The lysates were centrifuged at 10,000 × *g* at 4°C for 30 min. Supernatants were precleared with 5 μg of mouse IgG and 30 μl of protein G-Sepharose (Life Technologies) and were incubated with 5 μg monoclonal anti-CD28 Ab overnight at 4°C. Immune complexes were recovered by incubation with 30 μl protein G-Sepharose for 1 h at 4°C. The beads were washed five times with the lysis buffer and the immune complexes were resuspended in reducing Laemmli sample buffer (Sigma-Aldrich). The samples were heated to 95°C for 3 min and run on 10% SDS-PAGE. The separated proteins were transferred onto a nitrocellulose membrane. The membrane was incubated with blocking buffer containing 2% BSA and 0.1% Tween 20 in TBS (100 mM Tris-HCl (pH 7.5), 0.9% NaCl) for 1 h at room temperature, followed by incubation with rabbit anti-PI3-kinase polyclonal Ab at a 1/500 dilution in the same buffer containing 1% BSA and 0.1% Tween for 1 h. After washing, the blot was incubated for 1 h with HRP-conjugated anti-rabbit Ab at 1/2000 dilutions. Immunoblot signal was detected by using the ECL kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.) following the manufacturer's instructions.

Confocal microscopy

Confocal microscopy of neutrophils for checking IFN-γ production was performed following the methods of Yeaman et al. (11). Highly purified neutrophils were cross-linked with anti-CD28 or isotype control Abs in the presence or absence of wortmannin (10 ng/ml) for 5 h. The culture was continued for an additional 4 h with brefeldin A. The cells were then stained with 1 μg of FITC-labeled anti-human IFN-γ Ab. After counterstaining the nuclei with propidium iodide, cells were examined with a Zeiss LSM 510 confocal microscope equipped with argon and helium lasers (Zeiss, Jena, Germany). Laser power, photomultiplier tube gains, and confocal thresholds were set using long-pass and band-pass filters. The physical parameters defined as above were kept constant throughout the acquisition and analysis of the samples.

Statistical significance

The raw scores were analyzed using SigmaStat software program (Binary Semantics, Pune, India). The data are presented as mean ± SD of triplicates in one representative of at least three individual experiments. The fiducial limit for considering any difference of mean as significant is fixed at 0.01. The correlation coefficient for the association between parasite dose and IL-8 secretion was determined by Pearson's *r* test.

Results

L. major *infection of macrophages induced parasite dose-dependent IL-8 secretion, resulting in IL-8-directed neutrophil infiltration*

Because *Leishmania* infection is known to induce neutrophil infiltration to the site of infection (4), we investigated whether *L.*

major-infected macrophages secreted IL-8, a major neutrophil-activating chemokine. Whereas infection of macrophages with *L. major* promastigotes at ratios of 1:5, 1:10, and 1:20 resulted in the peak IL-8 secretion ~6 h after infection, infection at a 1:1 ratio yielded the peak IL-8 concentration 24 h after infection (Fig. 1*A*). With 1:1 and 1:5 macrophage:*Leishmania* infection ratios, the level of IL-8 in the macrophage culture supernatant was undetectable 3 h after infection. At higher macrophage:parasite ratios, the chemokine was detectable after 3 h but was still significantly less than the value observed 6 h after *Leishmania* infection (data not shown). Because the peak IL-8 concentrations 6 h after infection were directly proportional to the load of infection (correlation coefficient = +0.954), the IL-8 secretion by *L. major*-infected macrophages was parasite dose dependent. Thus, our data are consistent with a previous observation describing the appearance of IL-8 mRNA by RT-PCR 1 h after *L. major* infection (12).

In a transwell plate, where macrophages were cultured in the lower chamber with or without *L. major* infection, it was found that more neutrophils migrated to the wells containing *L. major*-infected macrophages than the uninfected macrophages (Fig. 1*B*). The neutrophil migration was significantly inhibited by both anti-IL-8 (Fig. 1*B*; $p < 0.01$) and anti-IL-8R (Fig. 1*C*; $p < 0.001$) Abs, suggesting that IL-8 secreted by the *L. major*-infected macrophages chemoattract neutrophils toward the site of infection. However, the incomplete inhibition of neutrophil migration in both anti-IL-8 and anti-IL-8R blockade could be due to chemoattraction effected by other chemokines released by the infected macrophages and IL-8R recycling to the surface, as the migration assay was performed at 37°C. Nevertheless, 65% inhibition of neutrophil migration by IL-8 neutralization or by blocking IL-8Rs identified IL-8 as the major neutrophil chemoattractant released by the *L. major*-infected macrophages.

Neutrophils interact with macrophages via CD28-CD80/CD86

It is known that T cell-expressed CD28 interacts with CD80 or CD86, also called B7.1 and B7.2, respectively, to generate the CD28 signal in T cells (13). Likewise, neutrophil-expressed CD28 may also interact with CD80/CD86 on macrophages. To test that hypothesis, we cocultured neutrophils with *L. major*-infected macrophages in the presence or absence of CTLA4-Ig, a fusion protein that blocks the CD28-CD80/CD86 interaction (14). The culture containing CTLA4-Ig had higher number of parasites (Fig. 2, *A* and *B*) and less IFN-γ (Fig. 2*C*), suggesting that the blockade of CD28-CD80/CD86 interaction resulted in the inhibition of the neutrophils' anti-leishmanial function. The inhibition of the leishmanicidal effect of neutrophils by anti-IFN-γ Ab suggested that the CD28 signaling in neutrophils resulted in the secretion of IFN-γ, which mediated the anti-leishmanial function of neutrophils. In addition, the supernatant from CD28-cross-linked neutrophil culture restricted the growth of *L. major* in macrophages, and neutralization of IFN-γ in the supernatant reduced the anti-leishmanial effect of the supernatant significantly (data not shown).

We previously showed that the chronic *Leishmania* infection of murine macrophages resulted in down-regulation of the ligands for CD28 (9). Therefore, we tested CD80 and CD86 expression on *L. major*-infected macrophages. It was observed that 6 h after *L. major* infection CD86 expression was marginally increased, whereas CD80 expression remained unaltered (Fig. 2*D*). Thus, the profile of CD80 and CD86 expression early after *L. major* infection validates our observation that the neutrophils and macrophages interact through CD28-CD80/CD86 to express the IFN-γ-dependent anti-leishmanial function.

It has been argued that neutrophils, being active phagocytes, control *Leishmania* infection by phagocytosis (6). In this study, phagocytosis of *Leishmania* was not a significant factor, because neutrophils were added to the culture after extracellular parasites were washed off following the infection of macrophages with *Leishmania*. We also investigated whether CD28 cross-linking, which imparts anti-leishmanial function to neutrophils by inducing cytokine secretion, increased phagocytosis. Using *Plasmodium*-infected human RBC and *Leishmania* as the models, we did not observe any difference between phagocytosis by CD28 and isotype cross-linked neutrophils (data not shown). This suggests that CD28-induced *Leishmania* killing by neutrophils is not via parasite phagocytosis. Instead, the fact that the parasite growth is inhibited by anti-IFN-γ Ab demonstrates the IFN-γ-dependent control of *Leishmania* growth within macrophages.

Neutrophil-expressed CD28 interacts comparably with CD80 and CD86 to induce IFN-γ

Because *Leishmania*-infected macrophages showed an increase in CD86 but not CD80 expression (Fig. 2*D*), and because the regulation of T cell function was reported to be differentially modulated

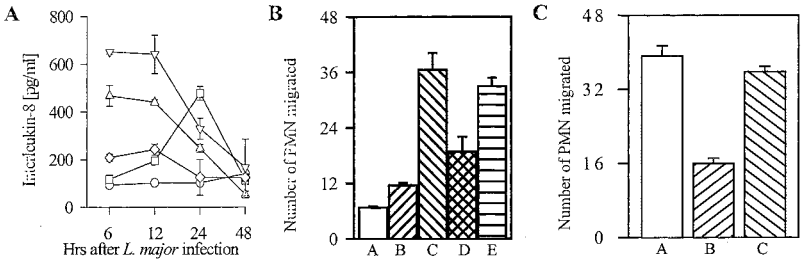

FIGURE 1. *L. major*-infected macrophages produce IL-8 to induce IL-8-dependent neutrophil migration. *A*, IL-8 was produced by human monocyte-derived macrophages. PBMC were isolated and cultured in a 24-well plate. Nonadherent cells were washed out and the adherent macrophages were infected with *L. major* promastigotes at the indicated macrophage:parasite ratios (uninfected, ○; 1:1, □; 1:5, ◇; 1:10, △; and 1:20, ▽) for 2 h. The extracellular parasites were washed out and the culture supernatants were collected at different periods of time for IL-8 ELISA. *B*, Effect of IL-8 neutralization on the migration of neutrophils toward macrophages. In a transwell setup, the macrophages were infected with *L. major* promastigotes and cultured for 6 h with or without anti-IL-8 or isotype-matched control Ab. The neutrophils were put on the upper chamber of the transwell and the migration assay was performed. *Lane A*, Medium; *lane B*, uninfected macrophages; *lane C*, infected macrophages; *lane D*, infected macrophages plus anti-IL-8 Ab; *lane E*, infected macrophages plus isotype Ab. *C*, Effect of IL-8R blockade on neutrophil migration. In a transwell culture, the macrophages were infected with *L. major* promastigotes and cultured for 6 h. The neutrophils were treated with anti-IL-8RA and anti-IL-8RB Abs and seeded on the transwell insert. The migration assay was performed as described in *Materials and Methods*. *Lane A*, Untreated neutrophils; *lane B*, anti-IL-8RA- and anti-IL-8RB-treated neutrophils; *lane C*, control isotype Ab-treated neutrophils. The results shown are from one representative experiment that was conducted three times.

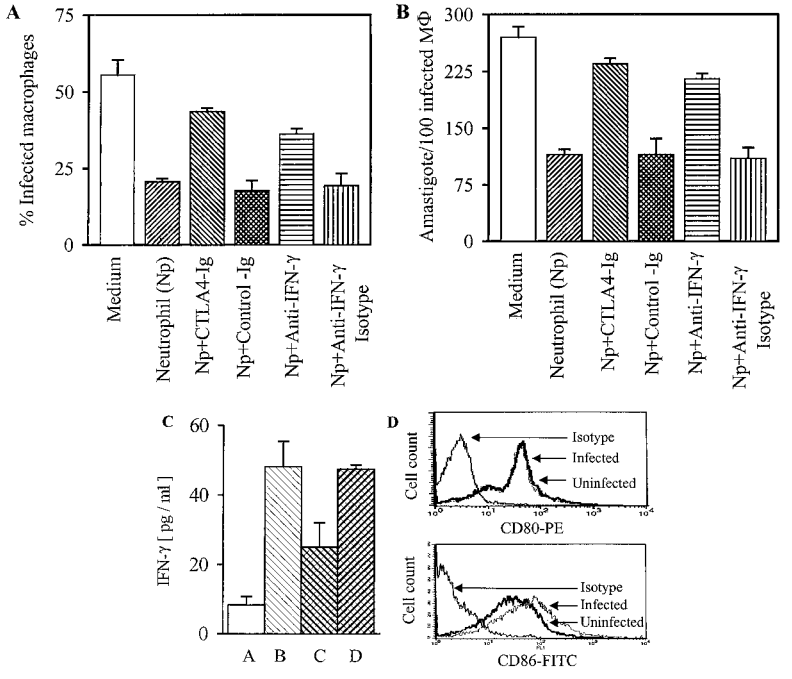

FIGURE 2. Neutrophil-expressed CD28 interacts with macrophage-expressed B7. *A* and *B*, Neutrophils were added to the macrophage cultures in the presence of CTLA4-Ig or control human Ig as shown. The parasite load was expressed in terms of the percentage of infected macrophages (*A*) and amastigotes per 100 infected macrophages (*B*). The leishmanicidal activity was performed as described in *Materials and Methods*. *C*, The presence of IFN-γ in the supernatant of macrophage-neutrophil coculture. The supernatant was collected from *L. major*-infected macrophages (*lane A*), *L. major*-infected macrophage and neutrophil cocultures (*lane B*), or *L. major*-infected macrophage and neutrophil cocultures in presence of either CTLA4-Ig (*lane C*) or human Ig (*lane D*). *D*, CD80 and CD86 expression on the *Leishmania*-infected and uninfected macrophages. *L. major*-infected human macrophages were stained with FITC-labeled anti-CD80, PE-labeled anti-CD86, and isotype-matched Abs after 6 h of infection and analyzed by a flow cytometer as described in *Materials and Methods*. The results shown are from one representative experiment that was conducted three times.

by CD80 and CD86 (15, 16), we examined whether neutrophil-expressed CD28 preferred CD86 to CD80 for inducing IFN-γ production. CD28 on neutrophils was cross-linked with immobilized B7.1-Ig-Fc and B7.2-Ig-Fc chimeras, the fusion proteins created by fusion of the extracellular domains of either B7.1 or B7.2 to the Fc portion of human IgG1, and assayed for IFN-γ in the supernatant after 12 h. Stimulation of neutrophils with human B7.1-Ig-Fc chimera or B7.2-Ig-Fc chimera did not show a significant difference in either IFN-γ secretion (Fig. 3*A*) or IFN-γ mRNA induction (Fig. 3*B*). Similarly, although both the anti-CD80 and anti-CD86 Abs alone prevented the IFN-γ-mediated restriction of parasite load in the neutrophil-macrophage coculture, they did not show a marked difference in their ability to prevent anti-leishmanial activity (data not shown). Our result thus indicates that neutrophils do not show any preference for either of the two B7 molecules.

CD28 cross-linking of neutrophils induces wortmannin-sensitive but cyclosporin A-resistant IFN-γ secretion

Because the above experiments suggested that, upon CD28 cross-linking, neutrophils secrete IFN-γ, we investigated the signaling involved in CD28-induced IFN-γ secretion. We found that cross-linking with anti-CD28 Ab but not with the control isotype resulted in IFN-γ secretion, which peaked within 6 h of stimulation (Fig. 4*A*).

It is known that T cell-expressed CD28 results in cytokine production that is sensitive to wortmannin, a PI3-kinase inhibitor (13, 17), but resistant to cyclosporin A (13). To test whether PI3-kinase was involved in CD28-induced IFN-γ secretion from neutrophils, the cells were incubated with different doses of wortmannin, followed by CD28 cross-linking. It was recorded that the CD28-induced IFN-γ secretion was inhibited by wortmannin in a dose-dependent manner (Fig. 4*A*, inset), suggesting a PI3-kinase-dependent IFN-γ secretion. The observation was confirmed by confocal microscopy, which showed that the CD28 cross-linked neutrophils expressed more IFN-γ after 9 h than the isotype cross-linked neutrophils and that wortmannin treatment of the CD28 cross-linked neutrophils inhibited IFN-γ production (Fig. 4*B*). We investigated whether CD28 was physically associated with PI3-kinase after cross-linking, as described in case of T cells (17). Immunoprecipitation of CD28 from the CD28 cross-linked neutrophils followed by Western blot with PI3-kinase Ab showed the presence of the reported PI3-kinase protein (18–20) only in CD28 cross-linked but not in the isotype cross-linked or unstimulated neutrophils (Fig. 4*C*). The result

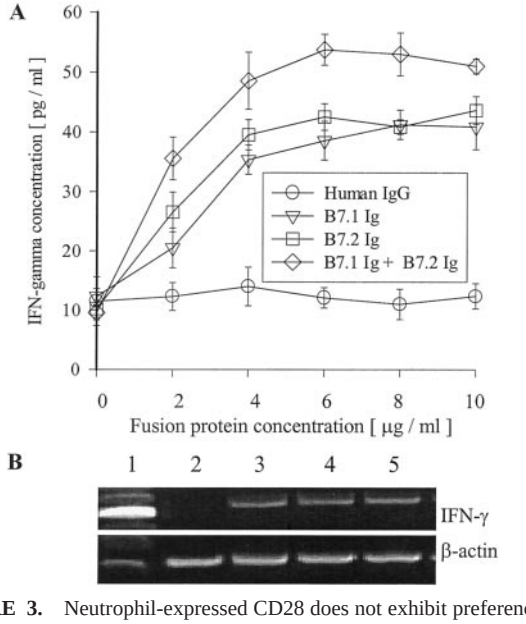

FIGURE 3. Neutrophil-expressed CD28 does not exhibit preference for either CD80 (B7.1) or CD86 (B7.2) to induce IFN-γ production. *A*, Freshly isolated neutrophils were cultured for 12 h in the presence of indicated concentrations of B7.1 Ig, B7.2 Ig, or both; human IgG was used as control. The supernatants were harvested and assayed for IFN-γ using an IFN-γ ELISA kit. The results shown are from one representative experiment that was conducted three times. *B*, Detection of IFN-γ transcripts by RT-PCR. Total RNA was isolated from neutrophils. RT-PCR was performed using gene-specific primers as described in *Materials and Methods*. *Lane 1*, A 100-bp ladder; *lane 2*, RNA processed from neutrophils stimulated with human IgG; *lane 3*, RNA processed from neutrophils stimulated with B7.1 Ig (5 μg/ml); *lane 4*, RNA processed from neutrophils stimulated with B7.2 Ig (5 μg/ml); *lane 5*, RNA processed from neutrophils stimulated with both B7.1 Ig and B7.2 Ig. The results shown are from one representative experiment that was conducted three times.

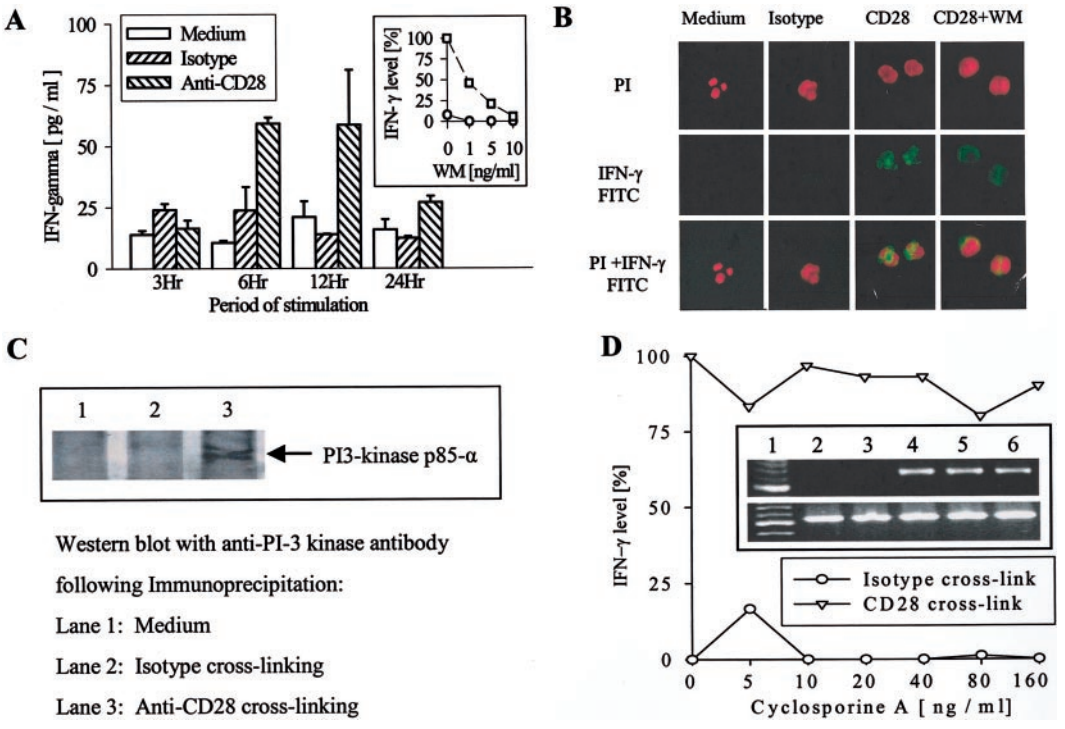

FIGURE 4. Cross-linking of CD28 on neutrophils results in wortmannin-sensitive but cyclosporin A-resistant IFN-γ production. *A*, CD28 cross-linking induced IFN-γ production by neutrophils. Neutrophils were cross-linked with anti-CD28 or isotype-matched control Ab and were cultured for different periods of time as described in *Materials and Methods*. At the end of culture, the supernatants were harvested and assayed for IFN-γ using an IFN-γ ELISA kit. Inset, Induction of IFN-γ in neutrophils by CD28 signaling through PI3-kinase. In parallel to the above experiments, neutrophils were preincubated with the indicated concentrations of wortmannin, a PI3-kinase inhibitor. The neutrophils were cultured for 12 h after cross-linking with either anti-CD28 (□) or isotype-matched Ab (○). At the end of culture, supernatants were harvested and measured for IFN-γ content. The wortmannin-untreated set of anti-CD28 Ab-treated neutrophils was considered as the 100% value for IFN-γ expression. The percentage of IFN-γ expression was obtained by comparing the values in the wortmannin-treated and untreated sets. The background IFN-γ value, i.e., IFN-γ in the supernatant of untreated neutrophils, was deducted from those of anti-CD28 or isotype Ab cross-linked sets. *B*, Confocal microscopic study showing the CD28-induced wortmannin-sensitive IFN-γ induction in neutrophils. Peripheral blood neutrophils were cultured in medium alone (Medium) or cross-linked with the isotype-matched Ab (Isotype), with anti-CD28 Ab (CD28), or with anti-CD28 Ab in the presence of wortmannin (CD28 + WM). Cells were stained for intracellular IFN-γ (IFN-γ FITC) and counterstained with propidium iodide (PI). IFN-γ was found within the neutrophils (stained green with FITC-labeled anti-IFN-γ Ab). Propidium iodide staining (red) revealed the nuclear morphology. *C*, Association of PI3-kinase with CD28 in the CD28-cross-linked neutrophils. Using a monoclonal anti-CD28 Ab, CD28 was immunoprecipitated from the untreated (*lane 1*), isotype-matched Ab-treated (*lane 2*), or anti-CD28 Ab-treated (*lane 3*) neutrophils. The immunoprecipitated products were further developed with anti-PI3-kinase Ab in the Western blot. *D*, CD28-induced IFN-γ induction and secretion is cyclosporin A (CsA) resistant. Neutrophils stimulated with anti-CD28 Ab were cultured in the presence of the indicated concentrations of cyclosporin A. The culture supernatant was assayed for IFN-γ by ELISA. Inset, Induction of IFN-γ transcription was assessed by RT-PCR as described in *Materials and Methods*. *Lane 1*, A 100-bp DNA ladder; *lane 2*, medium; *lane 3*, isotype; *lane 3*, anti-CD28; *lane 4*, anti-CD28 plus cyclosporin A (50 ng/ml); *lane 5*, anti-CD28 plus cyclosporin A (100 ng/ml). The results shown are from one representative experiment that was conducted three times.

suggested that neutrophil-expressed CD28 associated with PI3-kinase after CD28 cross-linking.

Although IFN-γ secretion was wortmannin sensitive, addition of different concentrations of cyclosporin A to the culture did not inhibit either transcription (Fig. 4*D*, inset) or secretion (Fig. 4*D*) of IFN-γ, indicating the cyclosporin A insensitivity of CD28-induced IFN-γ production in neutrophils as reported in case of T cells (13). The observation thus demonstrated that upon CD28 cross-linking PI3-kinase was associated with CD28 to transmit the signal inducing IFN-γ secretion.

Neutrophils do not require a primary signal for CD28-induced IFN-γ secretion

Because CD28 enhances the TCR-induced cytokine mRNA in T cells (21), we checked whether CD28-induced IFN-γ secretion was enhanced by a concomitant stimulation with IL-12, an IFN-γ-inducing cytokine. IL-12 addition to the anti-CD28-stimulated neutrophil culture did not augment CD28-induced IFN-γ secretion from neutrophils (Fig. 5*A*). Addition of anti-CD3 to neutrophils had no effect on anti-CD28-induced IFN-γ secretion (Fig. 5*B*). In T cells, CD28-derived signal alone failed to induce IFN-γ (Fig. 5*C*), although it enhanced anti-CD3-induced IFN-γ production (Fig. 5*D*). A similar kind of synergism between the CD28 signal and LPS or IL-8, the known neutrophil activators, in IFN-γ induction was not observed in neutrophils (data not shown). Besides differential signaling requirements for IFN-γ production, the results described in this work showed two other differences in the cytokine secretion by T cells and neutrophils. Whereas the neutrophil IFN-γ secretion peaked 6–9 h after stimulation, the T cell cytokine secretion peaked ~48 h after stimulation. Moreover, IFN-γ produced by T cells was 20 times more than that produced by neutrophils.

CD28 signaling through PI3-kinase increases IFN-γ gene transcription

Because IFN-γ secretion peaked rapidly after CD28 signaling, it was possible that CD28 signaling through PI3-kinase induced IFN-γ secretion from its preformed pool. Therefore, we stained intracellular IFN-γ at various time points after CD28 cross-linking.

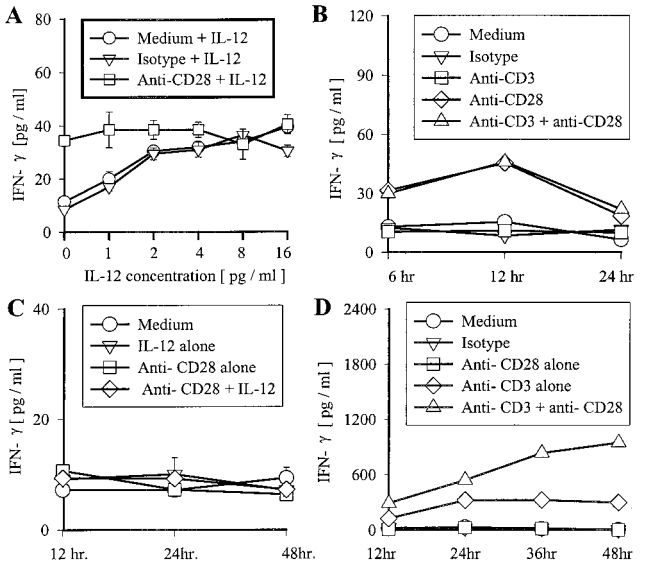

FIGURE 5. Neutrophil-expressed CD28 does not require the first signal. *A*, Neutrophils stimulated with anti-CD28 Ab or control Ab were cultured in the presence of indicated concentrations of IL-12. IFN-γ released was quantitated as described in *Materials and Methods*. *B*, Neutrophils stimulated with anti-CD28 Ab alone, anti-CD3 alone, or anti-CD3 plus anti-CD28 Ab were cultured for 12 h and IFN-γ released was quantitated. *C*, $CD4^+$ T cells isolated form the human PBMCs were stimulated with IL-12 alone, with anti-CD28 alone, or with both IL-12 and anti-CD28. Culture supernatant was harvested after 12 h and IFN-γ content was assessed. *D*, T cells were stimulated with CD3 alone, CD28 alone, or both CD3 and CD28, and the culture supernatant was checked for IFN-γ at indicated time points. The results shown are from one representative experiment that was conducted three times.

We observed that the intracellular IFN-γ staining was not detectable above the background level until 3 h after CD28 signaling, suggesting that the cytokine was induced and secreted but not released from the preformed pool (Fig. 6*A*).

To investigate whether the PI3-kinase signaling was inducing IFN-γ gene transcription, the neutrophils were stimulated with anti-CD28 Ab in the presence of different doses of actinomycin D, an RNA transcription inhibitor (22). We observed an actinomycin D dose-dependent inhibition of IFN-γ production (Fig. 6*B*). In other experiments, the optimum dose of actinomycin D (50 ng/ml) was added to the anti-CD28-stimulated neutrophil cultures at different time points after initiating the culture as indicated in Fig. 6*C*. Addition of the transcription inhibitor 3 h after initiation of culture had little effect on the cytokine production (Fig. 6*C*). These experiments suggested that transcription was necessary for IFN-γ production after CD28 signaling.

To determine whether the PI3-kinase signaling was inducing *IFN-γ* gene, RNA was prepared from the neutrophils cross-linked with CD28 in the presence or absence of wortmannin. Subsequent reverse transcription of the RNA, followed by PCR, showed that the message for IFN-γ was extremely low in the unstimulated neutrophils and that the message was increased significantly after CD28 cross-linking but not isotype cross-linking (Fig. 6*D*). Addition of wortmannin to the CD28 cross-linked neutrophils reduced IFN-γ RNA levels to that found in unstimulated neutrophils (Fig. 6*D*). These observations together indicate that the *IFN-γ* gene is induced by CD28 signaling through PI3-kinase and that the transcription is an obligatory prerequisite for the cytokine production.

Discussion

Leishmania is a dimorphic protozoan parasite. Flagellated promastigotes are introduced into the host during the blood meal of the sandfly vector. Parasites then invade the cells of the monocyte-macrophage lineage of the mammalian host and are transformed into aflagellate amastigotes. It has been proposed that the host's reaction in response to the infection initiated at this point is mediated by a monoclonal T cell repertoire of $V\beta4^+V\alpha8^+$ T cells (23), which produces IL-4, to set the bias toward Th2 cells in the susceptible host (24). In contrast, an earlier suggestion was that the initial T cell activation results in the production of a wide array of cytokines (25) that control parasite growth by regulating the macrophage activation or inactivation (26) or by setting a Th subset bias (24). 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. Among the non-T cells, neutrophils are known to infiltrate the site of infection as early as 21 h after s.c. introduction of the parasite in mice (our unpublished observation) and are present in the lesions of cutaneous leishmaniasis patients (27). A possible role of neutrophils at the site of lesion is phagocytosis of *Leishmania* (6). Therefore, we tested the hypothesis that neutrophils may play a crucial role in the initiation of anti-leishmanial immune response.

In this paper, we describe how the inflammatory reaction against *Leishmania* infection is initiated with the release of chemokines such as IL-8 by *Leishmania*-infected macrophages, followed by IL-8-dependent recruitment of neutrophils to the site of infection. CD28 on neutrophils interacts with CD80/CD86 on macrophages to generate CD28 signal-inducing IFN-γ secretion. In addition to restriction of *Leishmania* growth, IFN-γ may alter the cytokine microenvironment at the site of infection, affecting the IL-4- and IFN-γ-secreting T cell differentiation as a consequence (4, 28). Although neutrophils have already been reported to secrete IFN-γ (11), our study provides the first mechanistic description of how a signal generated by CD28 on cell surface regulates the cytokine gene transcription and secretion in neutrophils. While observations reported in this work indicate the significance of CD28 signaling in neutrophil-mediated induction of anti-leishmanial immune response, earlier observations on murine *L. major* infection in CD28-deficient mice suggested a limited role for CD28 in Th subset differentiation during the infection (29, 30). The apparent discrepancy between these observations can be explained by a possible absence of CD28 on murine neutrophils, as casein-elicited early peritoneal exudate cells do not stain for CD28 (data not shown). Thus, although the current hypothesis for initiation of the disease leishmaniasis by setting a Th subset bias relies heavily on T cell-secreted cytokines (31), our observations define for the first time a significant role of neutrophils in the afferent limb of the anti-leishmanial immune response, especially that which precedes the T cell response (32).

In the afferent arm of an immune response, interaction between the cells of the adaptive immune system such as T cells and B cells is a well-documented fact, but such interactions between two different types of cells of the innate immune system has not been shown previously. The results described in this work show that the neutrophils and macrophages interact through CD28-B7, which are known to be the key regulatory molecules for cytokine production by T cells. We have identified three major differences between T cell and neutrophil IFN-γ secretion. First, while T cells require the first signal through the Ag-specific TCR to induce the cytokine gene transcription, the message of which is stabilized by CD28 signaling (33), neutrophils do not require any primary signal to

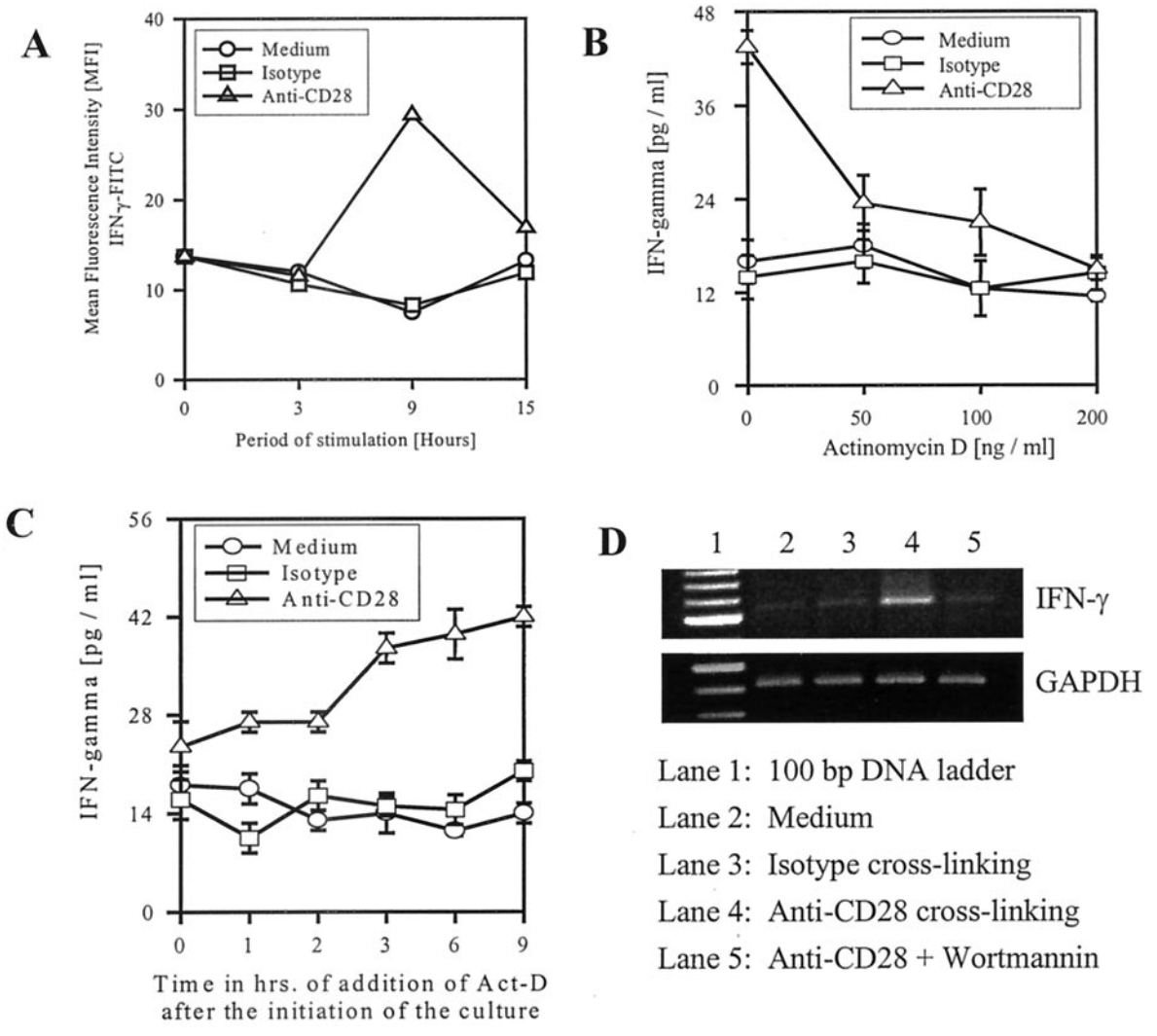

FIGURE 6. CD28 signaling through PI3-kinase increases IFN-γ gene transcription. *A*, Intracellular staining of IFN-γ in neutrophils. Neutrophils without treatment or cross-linked with anti-CD28 mAb or isotype-matched control Ab were cultured for different time points, stained intracellularly for IFN-γ, and analyzed by FACSVantage. *B*, Actinomycin D inhibits CD28-induced IFN-γ production. Neutrophils were stimulated by cross-linking anti-CD28 or isotype-matched Abs in the presence of indicated concentrations of actinomycin D. The cell culture supernatants were collected 12 h after the initiation of culture and assayed for IFN-γ by ELISA. *C*, Actinomycin D has to be added to the cultures at the initiation to inhibit CD28-induced IFN-γ production. Actinomycin D was added to the anti-CD28 or isotype cross-linked neutrophil cultures at different periods of time after the initiation of the cultures. Culture supernatants were collected 12 h after the initiation of the cultures and assayed for IFN-γ content. *D*, RT-PCR for IFN-γ message in neutrophils. RNA was isolated from neutrophils 4 h after incubation with medium alone (*lane 2*), or isotype cross-linking (*lane 3*) or CD28 cross-linking in the absence (*lane 4*) or in the presence of 10 ng/ml wortmannin (*lane 5*). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and PCR was performed as described in *Materials and Methods*. Equal loading of RNA was assured by running a parallel reaction for GAPDH. The product size was determined by comparison with the 100-bp DNA ladder (*lane 1*). The results shown are from one representative experiment that was conducted three times.

accomplish the same function. Absence of such a requirement for the Ag-specific signal in neutrophil also justifies its Ag nonspecific function during an inflammatory response. However, it is possible (but remains to be tested) that neutrophils express a pattern recognition receptor that signals, as TCRs do in T cells, to induce more IFN-γ. Second, the kinetics of IFN-γ production in neutrophils is different from that of T cells. While in T cells IFN-γ production peaks ~48 h after anti-CD3 plus anti-CD28 stimulation, in neutrophils IFN-γ production peaks between 6 and 12 h after stimulation. This rapid production of the cytokine correlates with the observation that the neutrophils are the first cells to migrate to the site of infection to encounter foreign materials (1), favoring the age-old paradigm that the cells of the innate immune system unleash the first assault on invaders. Third, neutrophils produce significantly less IFN-γ than T cells ($p < 0.001$). A recent report shows that cytokine levels as low as 50 pg/ml induce Th cell differentiation (34). This reported level of the cytokine is equal to the quantity of IFN-γ that we measured. The observation implies that, despite low IFN-γ concentration, the cytokine does not lose its functional significance, which is amply demonstrated in our anti-leishmanial assays. Besides the differences in the biological functions of these two cell types, they also vary in cell surface costimulatory molecule expression. While T cells express both CD28 and CTLA-4, which signal in a reasonably counteracting manner in regulating IL-4 and IFN-γ secretion from T cells (35), the neutrophils express only CD28 and not CTLA-4 (data not shown).

When considered from the evolutionary perspective, the marked quantitative and qualitative differences in the cytokine produced by T cells and neutrophils suggest some interesting possibilities. The

Ag recognition mechanisms, perhaps the first form of Ag recognition using simple patterns on the Ag, came to existence in phagocytes much earlier than in T cells (36, 37). Therefore, as T cells evolved, the complex MHC-restricted Ag recognition system, which is much finer and detailed, covering wider range of Ags, came into operation (38). Because Igs, MHC molecules, TCR, CD28, and CTLA-4 belong to the Ig superfamily (39), it is possible that CD28 came earlier than the TCR and might have added a finer operational flexibility to the neutrophils. However, phylogenetic studies on these molecules are required to lend further support to this notion.

Because neutrophils are the first cells to encounter invading pathogen, their primary function is to restrict further invasion and mobilize a finer course of immune response. It is possible that a higher amount of IFN-γ production by neutrophils may lead to destruction of host tissue. In contrast, a much higher quantity of IFN-γ from T cells provides a wider window for finer modulation of the cytokine production under different conditions. Host tissue destruction by a high quantity of IFN-γ may be significantly inhibited by the counteracting T cell cytokines, such as IL-4 and IL-10 (40, 41), which are not secreted by neutrophils (data not shown). However, neutrophils do secrete T cell chemotactic factors, which may draw the T cells to the site of infection, and can modulate Th subset differentiation (Refs. 5 and 42 and K. Venuprasad, S. Chattopadhyay, and B. Saha, manuscript in preparation). Thus, the phylogeny of functions of these two cells is mirrored during the progress of an Ag-specific immune response in a two-tiered system. In the first tier, at the beginning of the response, the neutrophils not only encounter the Ag directly, as demonstrated using *Leishmania* infection here, but also lead to the second tier, where T cells dictate the final outcome of the infection (42).

In conclusion, neutrophils play a crucial role in the initiation of anti-leishmanial immune response by secreting IFN-γ and restricting parasite growth. The results described in this paper reveal some novel facts about the differential regulation of T cell and neutrophil IFN-γ production that affects the course of *Leishmania* infection and perhaps other intracellular infections as well; they can serve as the basis for further exploration of the role of neutrophils in Th subset differentiation.

Acknowledgments

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References

1. Woodman, R. C., B. Johnston, M. J. Hickey, D. Teoh, P. Reinhardt, P. Y. Boon, and P. Kubes. 1998. The functional paradox of CD43 in leukocyte recruitment: a study using CD43-deficient mice. *J. Exp. Med. 188:2181.*
2. Matsukawa, A., and M. Yoshinaga. 1999. Neutrophils as a source of cytokines in inflammation. *Histol. Histopathol. 14:511.*
3. Bliss, S. K., A. J. Marshall, Y. Zhang, and E. Y. Denkers. 1999. Human polymorphonuclear leukocytes produce IL-12, TNF-α, and chemokines macrophage inflammatory protien-1α and -1β in response to *Toxoplasma gondii* antigens. *J. Immunol. 162:7369.*
4. Cotterell, S. E. J., C. R. Engwerda, and P. M. Kaye. 1999. *Leishmania donovani* infection initiates T cell-independent chemokine responses, which are subsequently amplified in a T cell-dependent manner. *Eur. J. Immunol. 29:203.*
5. Smelt, S. C., S. E. Cotterell, C. R. Engwerda, and P. M. Kaye. 2000. B cell-deficient mice are highly resistant to *Leishmania donovani* infection but develop neutrophil-mediated tissue pathology. *J. Immunol. 164:3681.*
6. Chang, K. P. 1981. Leishmanicidal mechanisms of human polymorphonuclear phagocytes. *Am. J. Trop. Med. Hyg. 30:322.*
7. Magnarin, M., P. Spessotto, M. R. Soranzo, A. Pontillo, and G. Zabucchi. 2000. Human neutrophils specifically interact with human monocyte-derived macrophage monolayers. *Inflammation 24:89.*
8. Corry, D., S. L. Reiner, P. S. Linsley, and R. M. Locksley. 1994. Differential blockade of CD28–B7 on the development of Th1 and Th2 effector cells in experimental leishmaniasis. *J. Immunol. 153:4142.*
9. Saha, B., G. Das, H. Vohra, N. K. Ganguly, and G. C. Mishra. 1995. Macrophage-T cell interaction in experimental visceral leishmaniasis: failure to express costimulatory molecules on *Leishmania*-infected macrophages and its implication in the suppression of cell-mediated immunity. *Eur. J. Immunol. 25:2492.*
10. Venuprasad, K., P. B. Parab, D. V. R. Prasad, S. Sharma, P. P. Banerjee, M. Deshpande, D. K. Mitra, S. Pal, R. Bhadra, D. Mitra, and B. Saha. 2001. Immunobiology of CD28 expression on human neutrophils. I. CD28 regulates neutrophil migration by modulating CXCR-1 expression. *Eur. J. Immunol. 31: 1536.*
11. Yeaman, G. R., J. E. Collins, J. K. Currie, P. M. Guyre, C. R. Wira, and M. W. Fanger. 1998. IFN-γ is produced by polymorphonuclear neutrophils in human uterine endometrium and by cultured peripheral blood polymorphonuclear neutrophils. *J. Immunol. 160:5145.*
12. Badolato, R., D. L. Sacks, D. Savoia, and T. Musso. 1996. *Leishmania major* infection of human monocytes induces expression of IL-8 and MCAF. *Exp. Parasitol. 82:21.*
13. June, C. H., J. A. Bluestone, L. M. Nadler, and C. B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol. Today 15:321.*
14. Linsley, P. S., W. Brady, M. Urnes, L. S. Grosmaire, N. K. Damle, and J. A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med. 174:561.*
15. Freeman, G. J., V. A. Boussiotis, A. Anumanthan, G. M. Bernstein, X. Y. Ke, P. D. Rennert, G. S. Gray, J. G. Gribben, and L. M. Nadler. 1995. B7.1 and B7.2 do not deliver identical costimulatory signals, as B7.2 but not B7.1 preferentially costimulates the initial production of IL-4. *Immunity 2:523.*
16. Kuchroo, V. K., M. P. Das, J. A. Brown, A. M. Ranger, S. S. Zamvil, R. A. Sobel, H. L. Weiner, N. Nabavi, and L. H. Glimcher. 1995. B7.1 and B7.2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell 80:707.*
17. Ward, S. G., A. Wilson, L. Turner, J. Westwick, and D. M. Sansom. 1995. Inhibition of CD28-mediated T cell costimulation by the phosphatidylinositol 3-kinase inhibitor wortmannin. *Eur. J. Immunol. 25:526.*
18. Ghiotto-Ragueneau, M., M. Battifora, A. Truneh, M. D. Waterfield, and D. Olive. 1996. Comparison of CD28–B7.1 and B7.2 functional interaction in resting human T cells: PI 3-kinase association to CD28 and cytokine production. *Eur. J. Immunol. 26:34.*
19. Pages, F., M. Ragueneau, R. Rottapel, A. Truneth, J. Nunes, J. Imbert, and D. Olive. 1994. Binding of phosphatidyl-inositol 3-OH kinase to CD28 is required for T cell signaling. *Nature 369:327.*
20. Truitt, K. E., C. M. Hicks, and J. B. Imboden. 1994. Stimulation of CD28 triggers an association between CD28 and phosphatidyl inositol 3-kinase in Jurkat T cells. *J. Exp. Med. 179:1071.*
21. Fraser, J. D., D. Straus, and A. Weiss. 1993. Signal transduction events leading to T cell gene expression. *Immunol. Today 14:357.*
22. Feng, B., D. C. Hilt, and S. R. Max. 1990. Transcriptional regulation of glutamine synthetase gene expression by dexamethasone in muscle cells. *J. Biol. Chem. 265:18702.*
23. Reiner, S. L., D. J. Fowell, N. H. Moskowitz, K. Sweir, D. R. Brown, C. R. Brown, C. W. Turck, P. A. Scott, N. Killeen, and R. M. Locksley. 1998. Control of *Leishmania major* by a monoclonal $\alpha\beta$ T cell repertoire. *J. Immunol 160:884.*
24. Launois, P., I. Maillard, S. Pingel, K. G. Swihart, I. Xenarios, H. Acha-Orbea, H. Diggelmann, R. M. Locksley, H. R. MacDonald, and J. A. Louis. 1997. IL-4 rapidly produced by Vβ4Vα8 CD4$^+$ T cells instructs Th2 development and susceptibility to *Leishmania major* in BALB/c mice. *Immunity 6:541.*
25. Reiner, S. L., S. Zheng, Z.-E. Wang, L. Stowring, and R. M. Locksley. 1994. *Leishmania* promastigotes evade interleukin-12 (IL-12) by macrophages and stimulate a broad range of cytokine from CD4$^+$ T cells during initiation of infection. *J. Exp. Med. 179:447.*
26. Assreuy, J., F. Q. Cunha, M. Epperlein, A. Noronha-Dutra, C. A. O'Donnell, F. Y. Liew, and S. Moncada. 1994. Production of nitric oxide and superoxide by activated macrophages and killing of *Leishmania major*. *Eur. J. Immunol. 24: 672.*
27. Peltier, E., P. Wolkenstein, M. Deniau, E. S. Zafrani, and J. Wechsler. 1996. Caseous necrosis in cutaneous leishmaniasis. *J. Clin. Pathol. 49:517.*
28. Krummel, M. F., and J. P. Allison. 1996. CTLA-4 engagement inhibits IL-12 accumulation and cell cycle progression upon activation of resting T cells. *J. Exp. Med. 183:2533.*
29. Brown, D. R., J. M. Green, N. H. Moskowitz, M. Davis, C. B. Thompson, and S. L. Reiner. 1996. Limited role of CD28-mediated signals in T helper subset differentiation. *J. Exp. Med. 184:803.*
30. Saha, B., S. Chattopadhyay, R. Germond, D. M. Harlan, and P. J. Perrin. 1998. CTLA4 (CD152) modulates the Th subset response and alters the course of experimental *Leishmania major* infection. *Eur. J. Immunol. 28:4213.*
31. Himmelrich, H., P. Launois, I. Maillard, T. Biedermann, F. Tacchini-Cottier, R. M. Locksley, M. Rocken, and J. A. Louis. 2000. In BALB/c mice, IL-4 production during the initial phase of infection with *Leishmania major* is necessary

and sufficient to instruct Th2 cell development resulting in progressive disease. *J. Immunol. 164:4819.*
32. Lloyd, A. R., and J. J. Oppenheim. 1992. Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. *Immunol. Today 13:169.*
33. Lindstein, T., C. H. June, J. A. Ledbetter, G. Stella, and C. B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science 244:339.*
34. Noben-Trauth, N., J. Hu-Li, and W. E. Paul. 2000. Conventional, naive $CD4^+$ T cells provide an initial source of IL-4 during Th2 differentiation. *J. Immunol. 165:3620.*
35. Khattri, R., J. A. Auger, M. D. Griffin, A. H. Sharpe, and J. A. Bluestone. 1999. Lymphoproliferative disorder in CTLA-4 knockout mice is characterized by CD28-regulated activation of Th2 response. *J. Immunol. 162:5784.*
36. Imler, J. L., and J. A. Hoffmann. 2001. Toll receptors in innate immunity. *Trends Cell Biol. 11:304.*
37. Mushegian, A., and R. Medzhitov. 2001. Evolutionary perspective on innate immune recognition. *J. Cell. Biol. 155:705.*
38. Little, A. M., and P. Parham. 1999. Polymorphism and evolution of HLA class I and II genes and molecules. *Rev. Immunogenet. 1:105.*
39. Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily: domains for cell surface recognition. *Annu. Rev. Immunol. 6:381.*
40. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol. 136:2348.*
41. Jankovic, D., Z. Liu, and W. C. Gause. 2001. Th1 and Th2 cell commitment during infectious disease: asymmetry in divergent pathways. *Trends Immunol. 22:450.*
42. Tacchini-Cottier, F., C. Zweifel, Y. Belkaid, C. Mukankundiye, M. Vasei, P. Launois, G. Milon, and J. A. Louis. 2000. An immunomodulatory function for neutrophils during the induction of a $CD4^+$ Th2 response in BALB/c mice infected with *Leishmania major*. *J. Immunol. 165:2628.*