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An Immunomodulatory Function for Neutrophils During the Induction of a CD4$^+$ Th2 Response in BALB/c Mice Infected with *Leishmania major*[1]

Fabienne Tacchini-Cottier,[2]* C. Zweifel,* Y. Belkaid,† C. Mukankundiye,* M. Vasei,* P. Launois,[3]* G. Milon,† and J. A. Louis*

The possible immunomodulatory role of polymorphonuclear leukocytes (PMN) in CD4$^+$ T lymphocyte differentiation in mice was examined by studying the effect of transient depletion of PMN during the early phase after *Leishmania major* delivery. A single injection of the PMN-depleting NIMP-R14 mAb 6 h before infection with *L. major* prevented the early burst of IL-4 mRNA transcription otherwise occurring in the draining lymph node of susceptible BALB/c mice. Since this early burst of IL-4 mRNA transcripts had previously been shown to instruct Th2 differentiation in mice from this strain, we examined the effect of PMN depletion on Th subset differentiation at later time points after infection. The transient depletion of PMN in BALB/c mice was sufficient to inhibit Th2 cell development otherwise occurring after *L. major* infection. Decreased Th2 responses were paralleled with partial resolution of the footpad lesions induced by *L. major*. Furthermore, draining lymph node-derived CD4$^+$ T cells from PMN-depleted mice remained responsive to IL-12 after *L. major* infection, unlike those of infected BALB/c mice receiving control Ab. PMN depletion had no effect when the NIMP-R14 mAb was injected 24 h postinfection. The protective effect of PMN depletion was shown to be IL-12 dependent, as concomitant neutralization of IL-12 reversed the protective effect of PMN depletion. These results suggest a role for an early wave of PMN in the development of the Th2 response characteristic of mice susceptible to infection with *L. major*. *The Journal of Immunology*, 2000, 165: 2628–2636.

Resistance and susceptibility to infection with the protozoan parasite *Leishmania major* have been correlated with the sustained activation of parasite-reactive CD4$^+$ Th1 or Th2 cells, respectively (reviewed in Ref. 1). Cytokines such as IL-12 and IL-4 have been shown to play a predominant role in directing the functional differentiation of Ag-reactive CD4$^+$ T cell precursors, including *L. major*-reactive cells (2–8). We have previously reported that in susceptible BALB/c mice, an early and transient production of IL-4 by LACK-reactive CD4$^+$ T cells during the first 48 h of infection drives the subsequent development of a Th2 response (reviewed in Ref. 9). This early IL-4 production was also shown to induce a state of unresponsiveness to IL-12 in parasite-reactive CD4$^+$ T lymphocytes present in the draining lymph node of susceptible mice (10). This IL-12 unresponsiveness of CD4$^+$ T cells was further shown to correlate with the nondetection of the IL-12R β2-chain transcripts in CD4$^+$ T cells 5 days or more after infection with *L. major* (11).

Altogether, these observations converged to indicate that events occurring during the first days following infection with *L. major* are crucial in instructing subsequent Th differentiation. Thus, it is likely that the early transient waves of leukocyte trafficking between the s.c. site of parasite delivery and the draining lymph node could, through the secretion of various factors, influence early events occurring during this period.

Polymorphonuclear leukocytes (PMN)[4] are professional phagocytes present only within the blood during steady state conditions. Once the steady state of a peripheral tissue is disrupted, PMN are the first cells recruited within the tissues. Early PMN infiltrate at the site of infection with *L. major* has been reported, with qualitative and quantitative differences between susceptible BALB/c and resistant C57BL/6 mice. In BALB/c mice, characteristics of an acute inflammatory process, such as persistent elevated numbers of neutrophils, are sustained, whereas this is not the case in mice of resistant strains such as C57BL/6 (12).

Recently, several studies have demonstrated that PMN, once exposed to inflammatory signals, transcribe many genes coding for cytokines and actively synthesize several cytokines, including IL-12 and TGF-β1 (13–15). These two cytokines have been reported to influence Ag-specific CD4$^+$ Th cell differentiation. The role of IL-12 in promoting Th1 differentiation has been well established by experiments performed both in vitro and in the murine model of *L. major* infection (2–4, 7, 8, 10). The role of TGF-β in Th differentiation, however, is less clear; depending on the levels present, it may promote either CD4$^+$ Th1 or Th2 differentiation (16–19).

An immunomodulatory role for PMN has been recently reported in experimental infections with fungi and *Toxoplasma gondii* where the IL-12 produced by neutrophils was shown to be the initiator of Th1 cell maturation (20, 21). In these models of infection, depletion of PMN correlated with the development of a Th2

*World Health Organization Immunology Research and Training Center, Institute of Biochemistry, University of Lausanne, Epalinges, Switzerland; and †Pasteur Institute, Paris, France

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[2] Address correspondence and reprint requests to Dr. Fabienne Tacchini-Cottier, World Health Organization Immunology Research and Training Center, Institute of Biochemistry, University of Lausanne, 155 chemin des Boveresses, CH-1066 Epalinges, Switzerland. E-mail address: Fabienne.Tacchini-Cottier@unil.ch

[3] Current address: Pasteur Institute, Cayenne, French Guyane.

[4] Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; MPO, myeloperoxidase; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

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response (20) and enhanced mortality (21). These results indicate that, in response to micro-organisms, neutrophils can produce cytokines with immunoregulatory properties that could influence the outcome of the subsequent T cell-dependent immune response. Furthermore, secretion of minute quantities of cytokines by PMN could be relevant due to the elevated number of PMN present within sites of micro-organism inoculation. We therefore investigated whether mAb-mediated depletion of PMN could influence the pathway of Th cell development in mice infected with *L. major*. The results presented in this report show that depletion of PMN in susceptible (BALB/c) mice infected with *L. major* hampered the development of a polarized Th2 response. In contrast such treatment did not significantly affect the development of a Th1 response in resistant (C57BL/6) mice. These results suggest that PMN could play an early role in the induction of the Th2 response that develops in BALB/c mice following infection with *L. major*.

Material and Methods

Mice

BALB/c, C57BL/6, and C3H/HeJ mice were obtained from Harlan (Horst, The Netherlands) and housed in pathogen-free facilities at the Epalinges Center.

Parasites, infection, and treatment of mice

L. major (LV 39 MRHO/Sv/59/P strain) were maintained in vivo and grown in vitro as previously described (22). Mice were injected in the hind footpads with 3×10^6 stationary promastigotes in a final volume of 50 μl. Affinity protein A column-purified NIMP-R14, a rat IgG2b mAb that selectively binds to mouse neutrophils (23), was given i.p. at a dose of 1 mg, 6 h before infection with *L. major*. Rat anti human CEA (gift from Dr. J.-P. Mach, Institute of Biochemistry, Epalinges, Switzerland) was used as control mAb. In some experiments involving only BALB/c mice an IgG2b mAb against the Vα3.2 chain of the TCR (a TCR not present in BALB/c mice) was also used as control Ab (gift from Dr. R. MacDonald, Ludwig Institute for Cancer Research, Epalinges, Switzerland). The RB6-8C5 mAb (IgG2b) staining mouse neutrophils and eosinophils (24) was also used in some experiments (0.5 mg, administrated i.p. 6 h prior to infection with *L. major*).

Treatments with anti IL-12 mAb were performed using a combination of 250 μg of C17.8 and 250 μg of C17.15 mAbs (gift from Dr. G. Trinchieri, The Wistar Institute, Philadelphia, PA) administered -16, -4, and 0 h and 2 days following *L. major* infection. Treatment with the anti-IFN-γ mAb XMG 1.2 (25) was performed using a single injection of 1 mg of mAb 2 h before infection with *L. major*. Following injection with *L. major*, lesion progression was monitored using a metric caliper to quantitate footpad thickness.

Detection of neutrophils within blood and footpads

Neutrophils within the sites of *L. major* inoculation were detected by microscopic analysis (magnification, $\times$40/high power field) of hematoxylin-eosin-stained sections prepared from paraffin-embedded tissue samples. In selected cases visualization was confirmed by indirect immunostaining with NIMP-R14 mAb followed by streptavidin-PE (PharMingen, San Diego, CA; Becton Dickinson, Mountain View, CA).

PMN depletion following injection of the NIMP-R14 mAb was assessed before and on days 2, 3, and 4 after mAb injection (i.p.) by the analysis of peripheral blood smears stained with DIFFquick (Dade, Düdingen, Switzerland), and/or by FACS analysis, using the biotin-labeled RB6-8C5 mAb staining mouse neutrophils and eosinophils (24) as primary mAb followed by streptavidin-PE or streptavidin-FITC (Becton Dickinson) for PMN detection. Four or 5 h after a single injection of NIMP-R14 mAb the number of circulating neutrophils was dramatically reduced. This treatment led to a transient depletion of PMN, which returned to normal levels in the blood 3–5 days after the injection. It is noteworthy that 21 h following treatment with this mAb, no significant differences were observed in other leukocyte populations within the draining lymph node of mice. The percentage of cells in lymph nodes of mice treated with NIMP-R14 mAb and with the control mAb were as follows: 49 vs 43% of $CD4^+$ T cells, 18 vs 23% of $CD8^+$ T cells, and 33 vs 31% of B cells, respectively.

The numbers of neutrophils within the lesions were estimated using the myeloperoxidase (MPO) assay (26). MPO is a neutrophil enzyme absent in resident tissue macrophages. MPO was measured during the first week following infection with *L. major* at a time when no increase in footpad thickness was detectable in either resistant or susceptible mice. Briefly, footpads were weighed and homogenized in 1 ml of 100 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide as detergent and 5 mM EDTA. The homogenate was sonicated and centrifuged at $13{,}000 \times g$ for 15 min. The supernatant (0.1 ml) was diluted 1/15 in the above buffer, and 0.03 ml of 10 mg/ml of a solution of *O*-dianisidine hydrochloride and 0.001 ml of 0.3% H_2O_2 was added. The increase in A_{460} was measured over 2 min. MPO activity is expressed as the change in absorbance units. During the time after infection presently studied, the weights of the footpads of susceptible and resistant mice were equivalent.

Depletion following NIMP-R14 mAb treatment was also assessed in the mouse footpad using this MPO assay (26). Histological analysis of 5-μm sections of footpads stained with hematoxylin/eosin was performed to confirm the results obtained by MPO analysis. The specificity of this treatment in depleting PMN was further assessed by analyzing peripheral blood, the site of *L. major* injection, and the draining lymph node in mAb treated mice 4 h after infection with *L. major*. Treatment with the NIMP-R14 mAb resulted in the disappearance of PMN in the three compartments, without modifying the percentage of eosinophils present (Y. Belkaid and G. Milon, unpublished observations).

Detection of cytokines in lymphocyte culture supernatants

Mice infected with *L. major* were sacrificed at given times after infection, and 5×10^6 lymph node cells from the draining popliteal lymph node were restimulated in vitro in the presence of UV-irradiated *L. major* promastigotes in a final volume of 1 ml. Cells were cultured in the presence of DMEM supplemented with 5% heat-inactivated FCS, L-glutamine (216 μg/ml), 5×10^{-5} 2-ME, and 10 mM HEPES in 7% CO_2. Supernatants were collected after 72 h of culture. IFN-γ was measured in supernatant by ELISA as previously described (27). Mouse rIFN-γ, used as a standard, was the supernatant from L1210 cells transfected with the murine IFN-γ gene (gift from Dr. Y. Wanabe, Kyoto University, Kyoto, Japan). The limit of detection is 10 IU/ml. IL-4 was detected by a bioassay using the CTL.44.A cell line (gift from Dr. P. Erb, University of Basel, Basel, Switzerland). Recombinant murine IL-4 expressed in X63Ag-653 (gift from F. Melchers, Basel Institute of Immunology, Basel, Switzerland) was used as a standard. The limit of detection of this assay is 20 pg/ml. $CD4^+$ T cells were purified from the draining popliteal lymph node by magnetic cell sorting (Miltenyi Biotech, Bergish-Gladbach, Germany) according to the manufacturer's procedure. Cells (5×10^5) were stimulated with UV-irradiated promastigotes (1×10^6) in the presence of 5×10^6 irradiated (3000 rad) spleen cells from normal mice.

RT-PCR

Total RNA was purified from the popliteal lymph node of infected or uninfected mice, and cDNA synthesis was performed as previously described (28). The semiquantitative PCR developed by Reiner et al. with the use of primers for hypoxanthine guanidine phosphoribosyl transferase, IL-4, and IFN-γ in the presence of a polycompetitor was used as previously described (28). Results are expressed as the fold increase in mRNA transcripts in the lymph nodes of mice infected with *L. major* compared with that in noninfected mice.

The presence of IL-12R β2 mRNA was monitored using qualitative PCR assay as previously described (11). Briefly, HPRT levels for each sample were assessed by the semiquantitative PCR method of Reiner et al. (28). All samples were normalized with respect to their HPRT content and further subjected to IL-12Rβ2 PCR.

Quantitation of parasites

The number of parasites per lesion was evaluated by limiting dilution analysis (29). The estimation of the frequency was calculated by the Taswell method using the program Estimfree (30).

Statistical analysis

Statistical analysis was conducted using the *t* test for unpaired data.

Results

Recruitment of neutrophils in the lesions of mice infected with L. major *and effectiveness of treatment with the mAb NIMP-R14 in preventing PMN accumulation in the lesions*

Before evaluating a potential role for neutrophils during infection with *L. major*, we first ascertained whether PMN were recruited within the site of parasite delivery (footpad). Using the MPO assay

as a correlate to PMN number (26), PMN were detected in both resistant and susceptible mice as early as 1 h following injection of 3×10^6 stationary phase promastigotes. A significant ($p < 0.05$) decrease in MPO activity was observed 72 h after infection in C57BL/6 mice, whereas high MPO activity persisted for up to 6 days after infection in susceptible BALB/c mice, reflecting the sustained presence of neutrophils within the site of parasite inoculation in these mice (Fig. 1). Administration 6 h prior to infection with *L. major* of 1 mg of the PMN-depleting mAb NIMP-R14 prevented PMN accumulation within tissues following *L. major* infection, as reflected by a decrease in MPO activity. The effect of the mAb on the presence of PMN infiltration 16 h postinfection is shown in Fig. 1, *right*. These results were confirmed by histological analysis of hematoxylin-eosin-stained sections of *L. major*-infected footpads (data not shown). A significant difference was thus detected in the neutrophil content of the infiltrate between mice of susceptible (BALB/c) and resistant (C57BL/6) phenotypes.

A single injection of the PMN-depleting NIMPR-14 mAb before infection with L. major *modifies the course of infection in BALB/c mice*

Groups of BALB/c and C57BL/6 mice were injected i.p. with the PMN-depleting mAb NIMP-R14 6 h before infection with 3×10^6 *L. major* stationary phase promastigotes. BALB/c and C57BL/6 mice injected with a control mAb and infected similarly were used as control groups. The results in Fig. 2 show that, in contrast to BALB/c mice, BALB/c mice depleted of neutrophils develop lesions, but only small and nonprogressing ones at least during the period under study, i.e., 60 days after parasite injection (Fig. 2).

To assess whether the BALB/c mice depleted of neutrophils, showing reduced lesion development, also contained parasite growth within their lesions, the number of parasites in their footpads was estimated by limiting dilution analysis in two independent experiments. Thirty-five days after infection the lesions of BALB/c mice depleted in PMN by a single injection of NIMP-R14 6 h before infection contained 1000 × fewer parasites than control infected BALB/c mice (Table I). Sixty-five days postinfection, while untreated BALB/c mice had to be sacrificed on day 38 due to the appearance of necrotic tissues, footpads from NIMP-R14-treated mice showed only 800 parasites/footpad. The decreased

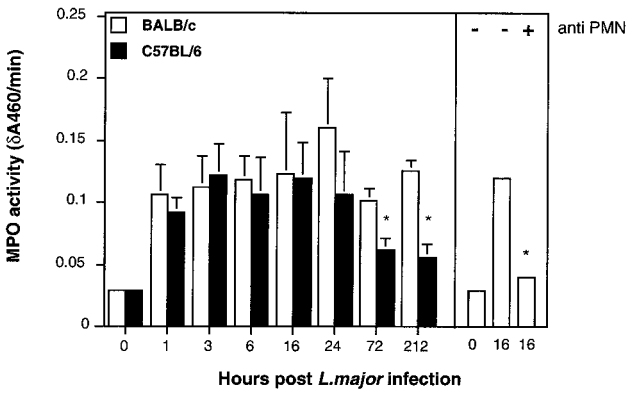

FIGURE 1. Number of PMN as determined by their MPO activity in the footpads of BALB/c and C57BL/6 mice infected with *L. major*. Footpads were collected at different times postinjection with 3×10^6 *L. major* promastigotes, and their MPO activity was measured as described in *Materials and Methods*. The mean enzymatic activity ± SD at a particular time point following *L. major* infection from three different experiments including four to six footpads per time point is represented. *, $p < 0.05$. *Right panel*, Mean MPO activity from footpads isolated from PMN-depleted or control BALB/c mice 16 h after infection with 3×10^6 *L. major* promastigotes.

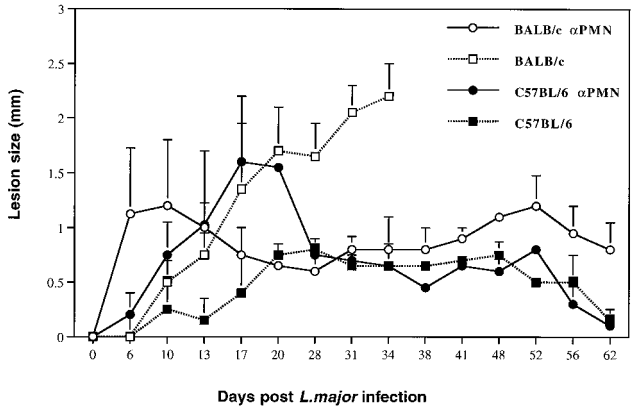

FIGURE 2. Effect of the injection of the NIMP-R14 PMN-depleting mAb on cutaneous lesion progression in BALB/c mice infected with *L. major*. A single injection i.p of 1 mg of NIMP-R14 mAb or control mAb in BALB/c or C57BL/6 mice was given 6 h before infection of the mice with 3×10^6 *L. major* (six mice per group). Footpad thickness increase was monitored by weekly measurements. The size of the lesion was determined by subtracting the values obtained from the uninfected footpad from those from the infected one. Bars represent the SD of the mean size of footpad thickness increase.

number of parasites present in footpads of mice treated with the NIMP-R14 mAb probably reflects enhanced macrophage activation at the site of *L. major* inoculation.

C57BL/6 mice treated, or not, with the NIMP-R14 mAb resolved completely their lesions by 62 days postinfection (Fig. 2). As complete resolution of the lesions was also observed in treated mice, the effect of multiple injections (6 h before and 3 and 6 h after *L. major* infection) of the NIMP-R14 mAb was studied. This treatment was also ineffective in changing the final outcome of infection, and these mice ultimately resolved their lesions. Depletion of PMN also had no effect on the final resolution of lesions by mice from another resistant strain, i.e., C3H/HeJ (data not shown). C57BL/6 mice depleted in neutrophils showed a 5-fold increase in parasite number within their lesions 35 days postinfection, but controlled their lesions equally well at 65 days (Table I). Thus, mAb depletion of PMN resulted in statistically significant impairment of the development of lesions and significantly lower parasite load within the infected site in BALB/c mice. In contrast, such treatment did not alter the final outcome of infection in either C57BL/6 or C3H resistant mice.

Th2 cell maturation is impaired in BALB/c mice depleted in neutrophils before injection with L. major

To evaluate whether the inhibition of lesion progression and parasite growth in BALB/c mice depleted of neutrophils was correlated with a shift in the pathway of Th subset differentiation, IFN-γ

Table I. *Parasite load in footpads of* L. major*-infected mice depleted or not from PMN 6 h before infection*[a]

Mice	Parasites/Footpad	
	35 days postinfection	65 days postinfection
BALB/c	5×10^5	[b]
BALB/c anti-PMN	460	800
C57BL/6	7	50
C57BL/6 anti-PMN	35	48

[a] Values pooled from footpads of three mice represent the mean number of viable parasites per footpad as determined by limiting dilution assay.

[b], Mice were sacrificed at day 35 when the footpad thickness increase reached >2.5 mm.

and IL-4 mRNA transcripts as well as protein production were analyzed 10 days after infection, a time when differentiation of the Th subset is established, in the draining lymph node cells of mice depleted, or not, of PMN before infection with *L. major*.

As expected, an increase (24 times) in IL-4 mRNA expression was observed in control BALB/c mice infected with *L. major*. Mice treated with the anti-PMN mAb showed only a 5-fold increase in IL-4 mRNA compared with uninfected NIMP-R14-treated mice, a value considered not significant with the semiquantitative PCR assay used (Fig. 3*A*). Levels of IFN-γ transcripts were very low and were comparable in both PMN-depleted and control BALB/c mice, but significantly increased (25 times) in C57BL/6 mice. Injection with the NIMP-R14 mAb resulted in a further increase (10 times) of IFN-γ transcripts within the lymph node of *L. major*-infected C57BL/6 mice. Comparable results were obtained with $CD4^+$ T cells isolated from the draining lymph node; a 15-fold increase in IL-4 mRNA was observed in $CD4^+$ cells from BALB/c mice, while a 4-fold increase was observed in NIMPR14-depleted mice 10 days after infection with *L. major*.

The lower levels of IL-4 mRNA in lymph node cells from BALB/c mice depleted in PMN correlated to the lower level of bioactive IL-4 released by these cells following specific restimulation in vitro. The IL-4 production by lymph node cells isolated from BALB/c mice depleted of PMN was significantly lower (~3 times) than that of control infected BALB/c mice (Fig. 3*B*). Levels of IFN-γ, however, were similar (Fig. 3). Draining lymph node cells from C57BL/6 mice depleted of PMN retained a typical Th1 cytokine profile, with no IL-4 production and an increase (1.6 times) in IFN-γ proteins (Fig. 3*B*). Treatment with the RB6−8C5 mAb, an mAb depleting both neutrophils and eosinophils, given 6 h before infection had a similar effect on the decreased IL-4 production by $CD4^+$ T cells (260 vs 6120 pg/ml), while levels of IFN-γ were more elevated (2548 vs 638 IU/ml) in RB6−8C5 vs control treated BALB/c mice, respectively.

A similar analysis was performed 62 days after infection in mice that had been injected with the NIMPR-14 6 h prior to infection. Indeed, in this experiment control infected BALB/c mice had to be sacrificed 32 days postinfection due to the development of necrosing lesions. *L. major*-stimulated draining lymph node cells from BALB/c mice depleted in PMN produced only 800 pg/ml of IL-4, while LNC from control untreated mice produced 2000 pg/ml of bioactive IL-4 34 days following infection (data not shown). The level of IFN-γ was comparable in NIMPP R14-treated or untreated BALB/c and C57BL/6 mice. Similar differences were noted in a separate experiment in which IL-4 production was measured in both groups of mice 35 days after infection with *L. major* (data not shown). These results show that a single injection of the PMN-depleting NIMP-R14 mAb 6 h prior infection is sufficient to prevent their subsequent differentiation along the Th2 pathway normally observed in BALB/c mice infected with *L. major*.

Effect of PMN depletion on the early cytokine transcript profile within the draining lymph node of L. major*-infected mice*

To evaluate whether the presence of PMN could modulate the early events preceding Th differentiation, BALB/c and C57BL/6 mice were depleted of PMN by a single injection of the mAb NIMP-R14 followed 6 h later by the s.c. injection of 3×10^6 *L. major* promastigotes. The levels of IL-4 mRNA in the draining lymph node were analyzed 16 h following infection and compared with those in untreated, similarly infected mice. Treatment with the PMN-depleting mAb prevented the early IL-4 burst of mRNA transcription normally observed in infected BALB/c mice (Fig. 4). PMN depletion did not induce IL-4 mRNA transcription in C57BL/6 mice (Fig. 4). The early IL-4 mRNA transcription levels seen in BALB/c mice within 1 day of infection with *L. major* have been reported to decrease to values observed in uninfected mice 48 h postinfection. Treatment with the anti-PMN mAb did not modify the kinetics of the early IL-4 mRNA expression or allow its expression in C57BL/6 mice (Fig. 4). Levels of IFN-γ mRNA were similar and low in both treated and untreated groups of BALB/c and C57BL/6 mice at these time points (data not shown). These results show that a single injection of the NIMP-R14 PMN-depleting mAb 6 h prior infection is sufficient to inhibit the early IL-4 mRNA burst and the subsequent IL-4 production normally observed in BALB/c mice infected with *L. major*.

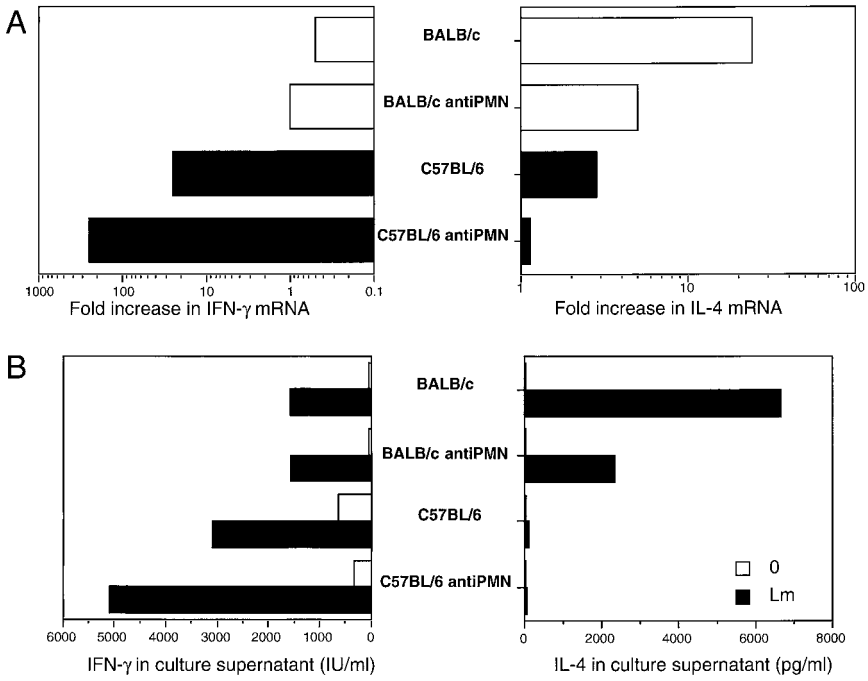

FIGURE 3. *A*, Detection 10 days postinfection with *L. major* of IFN-γ and IL-4 cytokines in popliteal lymph node cells of BALB/c and C57BL/6 mice treated with the NIMP-R14 mAb. Cells were isolated from popliteal lymph nodes of BALB/c mice infected with *L. major* receiving 1 mg of the NIMP-R14 mAb or control Ab 6 h prior to infection. As a control, cells were isolated from noninfected mice similarly treated. mRNA was extracted, and IFN-γ and IL-4 mRNA levels were determined by semiquantitative RT-PCR. Results are expressed as the fold increase over similarly treated noninfected mice. Data are representative of three independent experiments. *B*, Popliteal lymph node cells were isolated 10 days after *L. major* infection, and 5×10^5 cells from mice of each group (six mice per group) were stimulated in vitro with 10^6 UV irradiated *L. major* for 72 h. IFN-γ and IL-4 production was evaluated in the supernatant as described in *Materials and Methods*. These data are representative of three different experiments.

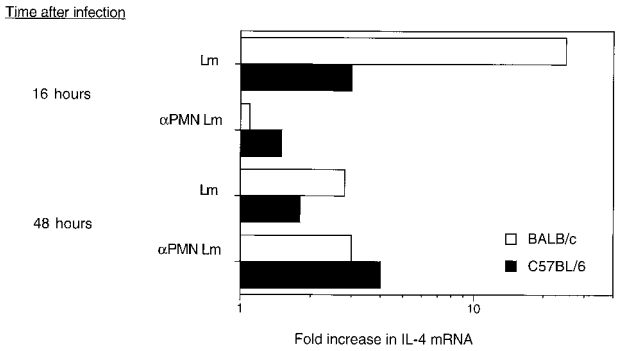

FIGURE 4. Effect of anti-PMN treatment on early IL-4 mRNA burst in BALB/c and C57BL/6 mice. Cells were isolated from popliteal lymph nodes 16 h following the injection of 3×10^6 *L. major* promastigotes. mRNA was extracted, and the levels of IL-4 mRNA were determined by semiquantitative RT-PCR. Results are expressed as the fold increase in IL-4 mRNA compared with that in noninfected similarly treated mice. Results are representative of two independent experiments.

Detection of neutrophils within the draining lymph nodes of mice early after infection with L. major

The presence of neutrophils within the draining lymph nodes during the first 72 h following infection with *L. major* was assessed by histological examination of hematoxylin-eosin-stained sections. In BALB/c mice, the presence of PMN within the draining lymph node was transiently detected, occurring mostly during the first 24 h following infection, with the highest number present 16 h or less postinfection. In C57BL/6-resistant mice, PMN were also detectable in the subcapsular space at similar time points postinfection, but in significantly lower number (Fig. 5). These results reveal that PMN are present transiently in the subcapsular space of draining lymph node within hours following *L. major* infection and thereby could affect processes involved in the early IL-4 mRNA transcription observed 16 h following *L. major* infection of BALB/c mice.

Maintenance of IL-12 responsiveness in lymph node cells from BALB/c mice depleted in PMN before infection with L. major

It was previously reported that as soon as 3 days after infection with *L. major*, $CD4^+$ T cells from the draining lymph node of BALB/c mice, unlike those from resistant mice, lose their capacity to respond to IL-12 in terms of IFN-γ production (31). This unresponsiveness was shown to proceed from the down-regulation of the IL-12 β2-receptor expression on their $CD4^+$ T cells (12, 13). To assess the IL-12 responsiveness of $CD4^+$ T lymphocytes in PMN-depleted mice, lymph node cells of PMN-depleted or undepleted BALB/c mice were isolated 5 days after *L. major* infection and stimulated with *L. major* in vitro in the presence or the absence of rIL-12, and IFN-γ production was measured. We confirmed that $CD4^+$ T cells isolated from the draining lymph nodes of BALB/c mice 5 days after infection with *L. major* were unresponsive to IL-12 in terms of IFN-γ production (Fig. 6). In contrast, similar to those from C57BL/6 mice, lymph node cells from BALB/c mice depleted in PMN exhibited enhanced IFN-γ production in response to IL-12 (Fig. 6*A*). Consistently, both C57BL/6 and anti-PMN-treated BALB/c mice had similar high levels of IL-12Rβ2 mRNA in their draining lymph nodes 5 days postinfection with *L. major* (Fig. 6*B*). Together with results showing an absence of early IL-4 mRNA burst in PMN-depleted BALB/c mice, these data indicate that early events occurring in these mice following infection with *L. major* correspond to those observed in resistant mice.

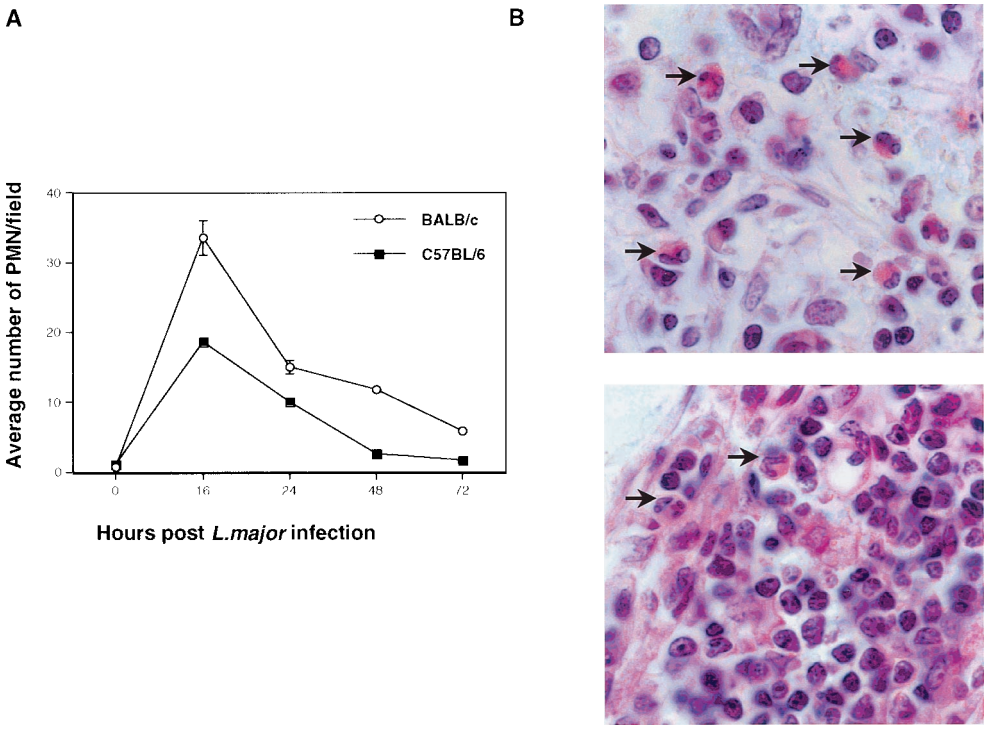

FIGURE 5. Determination of the average number of PMN in the cortex area of lymph nodes from mice 0, 16, 24, 48, and 72 h following infection with *L. major*. *A*, Popliteal lymph nodes from four to six mice were isolated, embedded in paraffin, cut (5 μm), and stained with hematoxylin-eosin. The number of PMN was evaluated by microscopic analysis. The results represent the mean ± SD number of PMN scored per 10 high power fields (HPF). *B*, Detection of PMN (arrow) in the cortex area of the lymph node of mice 16 h after *L. major* infection. Paraffin sections of lymph nodes were stained with hematoxylin-eosin. A representative area is shown. Arrows point to neutrophils.

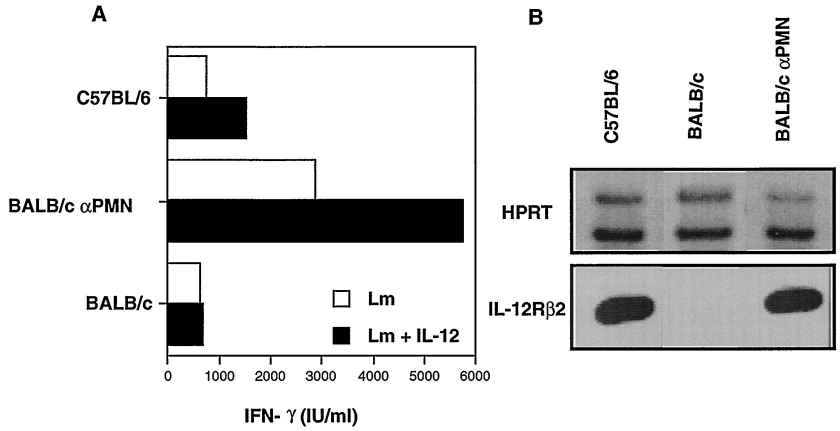

FIGURE 6. IL-12 responsiveness of lymph node cells of PMN-depleted BALB/c mice maintain IL-12 responsiveness 5 days following infection with 3×10^6 *L. major* promastigotes. *A*, Five days after *L. major* infection, cells from popliteal lymph node were isolated, pooled (three or four mice per group), and stimulated with 10^6 UV-irradiated parasites in the presence or the absence of IL-12 (10 ng/ml). After 72 h of culture in vitro, IFN-γ production in culture supernatants was measured by ELISA as described in *Materials and Methods.* Levels of IFN-γ detected in cultures performed in the absence of UV-irradiated *L. major* were subtracted. This is a representative experiment of three performed. *B*, Five days after infection with *L. major*, mRNA was extracted from popliteal lymph nodes of infected BALB/c mice that had been depleted of PMN, control BALB/c, and C57BL/6 mice (three mice per group). Expression of IL-12R β2-chain mRNA was monitored by RT-PCR as described in *Materials and Methods*. All samples were normalized with respect to HPRT content. Data are the results from one of two experiments.

Neutralization of IL-12 abolishes the effect of PMN depletion

To monitor whether the shift toward a Th1 pattern following PMN depletion in BALB/c mice was mediated by IL-12, the effect of the simultaneous depletion of PMN and neutralization of IL-12 was studied. Mice were sacrificed 30 days postinfection, and their draining lymph nodes were isolated for cytokine mRNA analysis. As observed previously, a single dose of the NIMP-R14 mAb was sufficient to significantly inhibit the development of lesions. Treatment with mAb against IL-12 abolished the protective effect resulting from PMN depletion (Fig. 7*A*). Thirty days after infection with *L. major*, analysis of draining lymph node cytokine mRNA revealed high levels of IL-4 mRNA in BALB/c mice treated, or not, with mAb against IL-12 (~40 times more IL-4 than those from the uninfected, similarly treated mice). Draining lymph nodes of BALB/c mice depleted of PMN showed very low levels of IL-4 mRNA, not significantly different from those in mAb-treated uninfected mice. However, when IL-12 was neutralized by injection of mAb in PMN-depleted mice, the IL-4 mRNA transcripts were detected at levels comparable to those in untreated BALB/c mice not depleted in PMN (Fig. 7*B*). Under these conditions, no IL-4 transcript was detected in the draining lymph node of C57BL/6 resistant mice as previously described for mice developing Th1 responses. Accordingly, treatment with the αIL-12 mAb significantly the levels of bioactive IL-4 detected in draining lymph node of cells from BALB/c mice depleted of PMN (1250 vs 230 pg/ml) as shown in Fig. 7*C*.

It is noteworthy that treatment with an anti-IFN-γ mAb had a similar effect, since it also suppressed the decrease in IL-4 production normally observed 10 days after *L. major* infection in the draining lymph nodes of mice treated with a single injection of NIMP-R14 mAb. A nonsignificant 1.8-fold increase in IL-4 mRNA was seen in the draining lymph nodes of anti-PMN mice, whereas a 40-fold increase in IL-4 mRNA was detected in lymph nodes of mice simultaneously treated with both αIFN and αPMN. Furthermore, measurement of IL-4 in supernatants of specifically stimulated LN cells substantiated these results by showing 10 times more IL-4 in supernatants of LN cells of mice treated with both αPMN and αIFN-γ (6154 vs 62 pg/ml).

Discussion

PMNs have been associated with efficient innate defense mechanisms in several experimental models of bacterial and fungal infection, such as *Listeria* and *Candida*, and in infections with protozoan parasites, such as *T. gondii*, where neutrophilia was associated with resistance to infection. In these models of infection, mAb-mediated depletion of PMN resulted in increased susceptibility to these pathogens (20, 21, 32–36). In contrast, PMNs have been associated with immunopathologic manifestations during infections with *Plasmodium berghei*, where they were shown to contribute to the microvascular lesions in a murine model of cerebral malaria. Treatment with mAb that deplete PMN prevented mortality in these mice (37). These examples illustrate one of the paradoxes of neutrophil function; although they are actively involved in primary host defense, they may also be involved in the pathology characterizing various acute inflammatory conditions.

In an attempt to assess the role of neutrophils in mice infected with *L. major*, we have investigated their recruitment within the site of parasite inoculation and their potential role in shaping the *L. major*-specific T cell response. Profound quantitative differences in the number of PMN recruited were observed between susceptible (BALB/c) and resistant (C57BL/6) mice. Although neutrophils appeared at the site of infection within 1 h in mice from both strains, their number dropped significantly 3 days following infection in resistant mice. In BALB/c mice, their number remained elevated, as estimated by myeloperoxidase activity and histology. These results are in agreement with previous studies using electron microscopic analysis where it was reported that in mice infected with 2×10^7 *L. major* parasites, the proportion of PMN in the cellular infiltrate in BALB/c mice dropped from 90 to 50% 3 days following infection, persisting for at least 8 days following infection. The percentage of neutrophils in the cellular infiltrate of C57BL/6 mice infected with *L. major* dropped from 60 to <10% 72 h after infection (12).

The molecular mechanisms underlying the presently observed qualitative and quantitative differences in the number of PMN recruited in the lesions between mice of both strains are not yet deciphered. Differential regulation of adhesion molecules such as

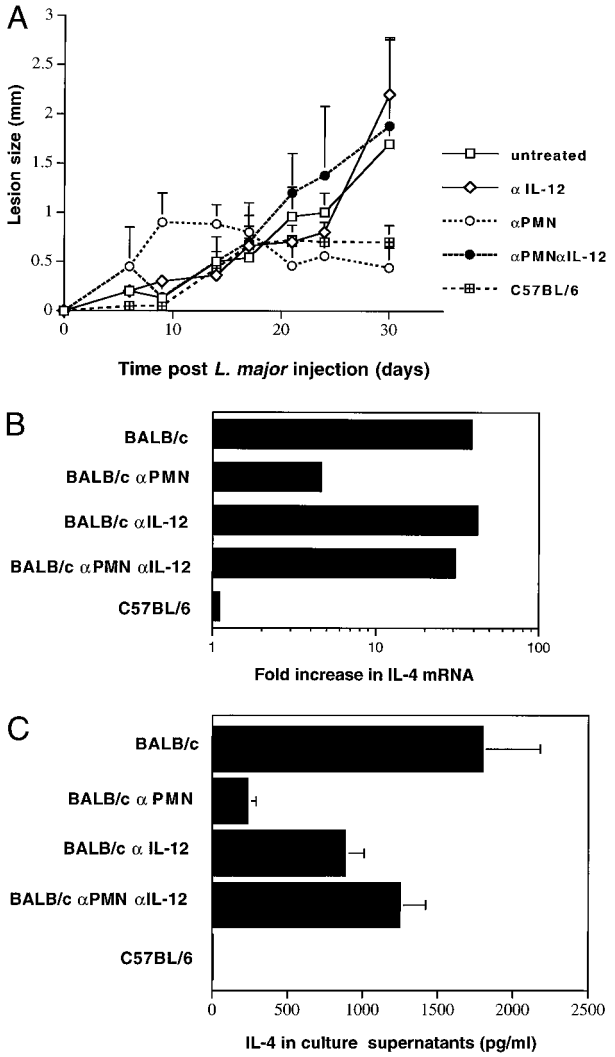

FIGURE 7. Injection of IL-12-neutralizing mAb prevents the protective effect resulting from PMN depletion. *A*, Development of cutaneous lesions in BALB/c mice infected with 3×10^6 *L. major* promastigotes, untreated, treated with anti-IL-12 (αIL-12), treated with anti-PMN (αPMN), or treated with both (αPMNαIL-12) was evaluated by subtracting the size of the noninfected footpad from that of the infected one. For comparison, cutaneous lesion development was measured in resistant *L. major*-infected C57BL/6 mice. Bars represent the SD of the mean size lesion in each group of seven mice. This is a representative experiment of two performed. *B*, IL-4 mRNA was isolated from the draining lymph node of mice 30 days after infection with *L. major* and subjected to semiquantitative PCR analysis as described in *Materials and Methods.* Results are expressed as the fold increase in IL-4 mRNA compared with those obtained from noninfected, similarly treated mice. Results are representative of two independent experiments. *C*, Production of bioactive IL-4 was evaluated in culture supernatants of cells isolated from popliteal lymph nodes of each group of mice, 30 days after *L. major* infection. Cells (5×10^6) were cultured with 10^6 UV-irradiated *L. major* parasites for 72 h, and IL-4 levels were determined by biological assay as described in *Materials and Methods.* Bars represent the SD of the mean of triplicate determinations.

VCAM-1/VLA-4 and β_2 integrins/ICAM-1 in susceptible and resistant mice could result in distinct patterns of PMN migration through the endothelial cell of postcapillary venules. Differences in apoptotic rate could also exist between resistant and susceptible strains of mice; mature neutrophils have a short lifespan and constitutively undergo apoptosis. Their lifespan can be increased to days upon recruitment into tissues, presumably due to the actions of particular cytokines. The apoptosis of neutrophil in vitro has indeed been reported to be delayed in the presence of various cytokines, including IL-4 (38, 39). Accordingly, differences in the local cytokine environment between susceptible and resistant mice as a result of infection with *L. major* could also contribute to the sustained presence of neutrophils within lesions of BALB/c mice and their earlier clearance in C57BL/6. Finally, differences in the maturation of precursor cells in the bone marrow could be involved.

Notable differences have been observed between the ability of PMN from susceptible or resistant mice to destroy intracellular *L. major*. PMN from BALB/c mice were reported to contain *L. major* in higher proportion that those from C57BL/6 mice (12). Furthermore, during the first 2 days following infection, parasites within the PMN of BALB/c mice were reported to be intact. In contrast, very low numbers of intact *L. major* were noted in PMN of C57BL/6 (12) (Y. Belkaid and G. Milon, unpublished observations).

A recent study showed increased numbers of *L. major* in the footpads of C57BL/6 mice treated with a mAb that depleted both PMN and eosinophils (40). In contrast, no decrease in parasite number was seen in similarly treated BALB/c mice that developed lesions similar to those in control untreated mice. These results differ from those presented here and may be accounted for by the different strains of parasite used, the important amounts of mAb injected repeatedly in their study (a total of 6 mg given 3 and 0 days before and 24 h after *L. major* infection), and the different spectra of cells recognized by both mAb. In the study byLima et al. the number of PMN in the peripheral blood from untreated BALB/c mice was quite elevated ($13.2 \pm 1.2 \times 10^6$ vs $1.2 \pm 0.6 \times 10^6$ in the BALB/c mice used in our study). The observed decrease in IL-4 we also observed 10 days after infection with *L. major* in mice treated with a single dose of the RB6−8C5 mAb 6 h before infection suggests that the differences observed may not be due to the different spectra of cells recognized by both mAb. Furthermore, it has been reported that the number of eosinophils is very low in the lesions of BALB/c mice during the first 3 days after infection (5). Thus, the regimen of mAb might at least in part explain the differences in the results obtained.

We also analyzed the number of neutrophils present within the draining popliteal lymph nodes of infected mice during the first 3 days following inoculation of *L. major*. Histological examination of paraffin-embedded sections revealed in BALB/c mice a transient increase in the number of neutrophils during the first 24 h after *L. major* infection. PMN were also detectable in C57BL/6 mice with similar kinetics but in lower numbers. Most neutrophils were located in the subcapsular zone of popliteal lymph nodes of BALB/c mice. The presence of PMN within the lymph node of *L. major*-infected mice very early following infection indirectly suggests that these cells may contribute to the early events that can influence the pathway of Th cell differentiation. The observation mentioned above that PMN within the lesions of BALB/c mice contain poorly degraded or intact *Leishmania* raises the possibility that these parasites or parasite fragments could be transported by neutrophils into the lymph nodes, where they could play a role in Ag presentation. Previous studies have shown that human neutrophils can be induced to express MHC class II molecules in vitro (41, 42), and it has been reported recently that human neutrophils can be driven to acquire dendritic cell characteristics in vitro (43). Although in humans PMN have been reported in one study to present superantigens, they did not present classical Ags (42). Thus, the possibility exists that PMN within the lymph nodes of mice infected with *L. major* could play a role in early Ag presentation either by delivering immunogenic molecules to dendritic cells otherwise known to take up apoptotic cells or, less likely, by

presenting the Ags themselves, an issue that deserves further experimental testing.

A role for cytokines secreted by PMN in Th cell differentiation has been reported in several models of infection. The IL-12 secreted by neutrophils has been reported to be associated with the development of a protective Th1 response during infections with *Candida albicans* and *Toxoplasma gondii*, whereas secretion of IL-10 by neutrophils was associated with the maturation of deleterious Th2 cells (20, 32, 36, 44). We showed here that depletion of neutrophils before infection with *L. major* significantly modified the development of lesions and the type of Th response developing in BALB/c mice. The mAb NIMP-R14, which appears to specifically target PMN, has also been reported to prevent both the local and systemic toxicity of LPS due at least in part to the activity of PMN (45).

Prior studies from this laboratory have shown that lymph node cells from BALB/c mice, in contrast to lymph node cells from mice of resistant strains, exhibited a burst of IL-4 mRNA transcripts in $CD4^+$ T cells from the draining lymph nodes within 1 day after infection with *L. major* (46). This early burst of IL-4 expression occurred in a restricted population of $V\beta4V\alpha8$ $CD4^+$ T cells after cognate recognition of a single epitope of the *Leishmania* homologue of mammalian RACK1 (47) designated LACK. The IL-4 produced rapidly by these cells has further been shown to set in motion the molecular events, including prevention of the IL-12R β2-chain expression in $CD4^+$ T cells, ultimately resulting in Th2 cell maturation and susceptibility to infection in BALB/c mice (11, 47). The results reported here, showing that depletion of PMN in BALB/c mice hampers the expression of the early IL-4 burst normally occurring in $V\beta4V\alpha8$ $CD4^+$ T cells within 1 day of infection with *L. major*, strongly suggest that PMN contribute to the early events instructing Th2 development in these mice. The maintenance of the IL-12R β2-chain expression and the responsiveness to IL-12 in $CD4^+$ cells from PMN-depleted BALB/c mice further strengthen the crucial role of the IL-4 normally produced in these mice during the initial phase of infection in subsequent Th2 cell maturation.

The precise mechanism(s) accounting for the immunomodulatory role of PMN observed in BALB/c mice infected with *L. major* are not known. It has been suggested that the function of the IL-12 p40 produced in BALB/c mice early after infection was inhibited by other cytokines produced simultaneously (48). Therefore, it is possible that PMN participate in the production of these cytokines. This hypothesis is supported by previous observations that IL-12 is capable of down-regulating the early IL-4 production seen in BALB/c mice within 1 day of infection with *L. major* (31). The absence of the early burst in BALB/c mice depleted of PMN could indeed allow expression of the functional activity of IL-12 in absence of inhibitory cytokines.

Two cytokines, IL-10 and TGF-β, that are secreted by murine neutrophils could potentially play such an inhibitory role. Both of these cytokines have been reported to counteract IL-12-mediated effects in Th differentiation. During infections with *L. amazonensis*, the production of endogenous TGF-β correlates with the development of a Th2 response and its associated susceptibility to infection. Less virulent disease associated with the development of a Th1 pattern occurs in animals treated with an mAb against TGF-β, while more virulent disease occurs in animals given rTGF-β (49). Furthermore, TGF-β has been shown to inhibit the IL-12-induced Th1 development of naive $CD4^+$ T cells in vitro (16). Interestingly low concentrations of TGF-β during primary activation of $CD4^+$T cells inhibit their IL-12 responsiveness (19). Depleting PMN in BALB/c mice could therefore reduce local TGF-β concentrations and consequently allow the development and sustained responsiveness to IL-12 in $CD4^+$ T cells. The role of neutrophils and their secreted TGF-β in *L. major* infections is currently being investigated. IL-10 has also been shown to inhibit IL-12 production during primary Ag response, preventing the development of Th1 responses (50).

PMN have been shown to be involved in the polarization of Th2 immune responses in another experimental model involving artificially induced neutrophilia using G-CSF. Furthermore, in a model of acute graft-vs-host disease the longer survival of recipient of allogeneic cells from G-CSF-treated donors was attributed to the development of a Th2 response, preventing acute graft-vs-host disease (51).

We also show here that in resistant C57BL/6 mice, neutrophils present during the first 3 days of infection do not participate to the development of a Th1 response. These results differ from those obtained in mice infected with *C. albicans*, in which it was shown that neutrophils, through IL-12 secretion, participate in Th1 cell differentiation (32). Interestingly, bone marrow-derived PMN and *L. major*-elicited peritoneal PMN obtained from C57BL/6 mice did not show either an increase in IL-12 p40 mRNA or significant IL-12 production following incubation with *L. major* in vitro (C. Zweifel, J. A. Louis, and F. Tacchini-Cottier, unpublished observations).

In conclusion, the present study shows that neutrophils are recruited early during the first days following infection with *L. major* in lesions of both resistant and susceptible mice, with a sustained presence only within lesions of susceptible mice. The results presently reported strongly suggest that neutrophils contribute to the development of lesions in susceptible BALB/c mice. Thus, it appears that neutrophils, in addition to contributing to early containment of microorganisms, could also account at least in part for the influence that the innate immune system exerts on the development of adaptive effector T cell responses.

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