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Proinflammatory Clearance of Apoptotic Neutrophils Induces an IL-12^{low}IL-10^{high} Regulatory Phenotype in Macrophages

Alessandra A. Filardy,* Dayana R. Pires,* Marise P. Nunes,† Christina M. Takiya,‡ Celio G. Freire-de-Lima,* Flavia L. Ribeiro-Gomes,* and George A. DosReis*,§

Clearance of apoptotic exudate neutrophils (efferocytosis) induces either pro- or anti-inflammatory responses in mouse macrophages depending on host genetic background. In this study, we investigated whether neutrophil efferocytosis induces a stable macrophage phenotype that could be recalled by late restimulation with LPS. Bone marrow-derived macrophages previously stimulated by pro- but not anti-inflammatory neutrophil efferocytosis expressed a regulatory/M2b phenotype characterized by low IL-12 and high IL-10 production following restimulation, increased expression of LIGHT/TNF superfamily 14, Th2-biased T cell responses, and permissive replication of *Leishmania major*. Induction of regulatory/M2b macrophages required neutrophil elastase activity and was partially dependent on TLR4 signaling. These results suggested that macrophage differentiation to a regulatory phenotype plays a role in resolution of inflammation but could contribute to increased humoral Ab responses and parasite persistence in the infected host. ***The Journal of Immunology*****, 2010, 185: 2044–2050.**

Neutrophils provide the first line of defense against infection but release mediators that can lead to tissue injury (1). Therefore, resolution of inflammation requires removal of neutrophils. Neutrophils undergo constitutive apoptosis and are phagocytosed by tissue macrophages in the course of immune responses (2). Phagocytic clearance of apoptotic cells—or efferocytosis (3)—can either suppress or trigger proinflammatory responses, depending on additional innate immune stimuli (4–7). In addition, efferocytosis regulates macrophage arginine metabolism (8) and the intracellular growth of protozoan pathogens such as *Leishmania major*, *Trypanosoma cruzi*, and *Leishmania amazonensis* (7–9).

Macrophage responses to environmental stimuli are plastic, leading to discrete functional states (10–12). Polarized macrophage phenotypes play a role in chronification of parasitic and inflammatory diseases (13–16). Classically activated or M1 macrophages are induced by IFN-γ in concert with microbial stimuli. M1 macrophages have an IL-12^{high}, IL-10^{low} phenotype, produce high amounts of inflammatory cytokines, and participate in Th1 responses (10, 12). Alternatively activated or M2a macrophages are induced by Th2 cytokines IL-4 and IL-13 (11). These macrophages have an IL-12^{low}, IL-10^{high} phenotype, express high levels of mannose and scavenger receptors, and produce ornithine and polyamines via arginase (11). M2a macrophages participate in Th2 responses and express immunoregulatory functions (10–12). A third population, termed regulatory or M2b macrophages (10, 12), is induced by ligation of Fc, complement, or scavenger receptors (12, 17). Regulatory macrophages more closely resemble M1 than M2a macrophages (18). Regulatory macrophages produce TNF-α and NO and have low arginase activity (18). Distinct from M1 macrophages, regulatory macrophages express an IL-12^{low}, IL-10^{high} phenotype and support the differentiation of Th2 cells (18). In addition, regulatory macrophages express increased amounts of LIGHT/TNF superfamily (TNFSF)14 (18). Generation of regulatory macrophages correlates with suppression of proinflammatory responses (19), successful establishment of *Leishmania* infection (20), and remission of lupus nephritis (21).

Efferocytosis of exudate neutrophils can be either pro- or anti-inflammatory, depending on host genetic background, and lead to opposing outcomes of *Leishmania* replication in mouse macrophages (7). Whether changes in macrophage metabolism and immune activation are transient or sustained is unknown. In this study, we sought to investigate functional and biochemical evidences of a stable macrophage phenotype induced by neutrophil efferocytosis. To this end, we developed a two-step system of macrophage culture with apoptotic neutrophils (APO PMNs), followed by a subsequent challenge with bacterial LPS. Our results indicated that pro- but not anti-inflammatory efferocytosis induces a stable regulatory/M2b phenotype in macrophages characterized by a low IL-12, high IL-10 cytokine profile, Th2-biased T cell responses, increased LIGHT expression, and permissive replication of *L. major*. These results suggested that neutrophil efferocytosis plays a role in the resolution of inflammation but could contribute to increased humoral Ab responses and parasite persistence through production of regulatory macrophages.

Materials and Methods

Mice and parasite

C57BL/6 (B6) and BALB/c mice were from Oswaldo Cruz Institute (Rio de Janeiro, Brazil). All animal work was approved by an Institutional Review Board. *L. major* strain LV39 (MRHO/Sv/59/P) was isolated from BALB/c mice and maintained in vitro for up to 4 wk (22).

*Instituto de Biofísica Carlos Chagas Filho and ‡Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro; †Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro; and §Instituto Nacional de Saúde e Ambiente na Região Amazônica, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazília, Brazil

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Address correspondence and reprint requests to Dr. George A. DosReis, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco G, Ilha do Fundão, Rio de Janeiro, RJ, 21941-900, Brazil. E-mail address: gdosreis@biof.ufrj.br

Abbreviations used in this paper: APO PMN, apoptotic neutrophil; B6, C57BL/6; BMDM, bone marrow-derived macrophage; MeOSuc-AAPV-cmk, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone; NE, neutrophil elastase; SPHK1, sphingosine kinase 1; TNFSF, TNF superfamily.

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Abs and chemicals

The following reagents were used: IFN-γ, IL-2, IL-4, IL-10, Annexin V, propidium iodide, and anti-mouse LIGHT/TNFSF14 mAb 261639 (R&D Systems, Minneapolis, MN); purified human neutrophil elastase (NE), NE inhibitor methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MeOSuc-AAPV-cmk), and collagenase inhibitor Z-Pro-D-Leu-D-Ala-NHOH (both inhibitors were used at 20 μg/ml; Calbiochem, San Diego, CA); DMSO and LPS from *Salmonella enterica* serotype *typhimurium* (Sigma-Aldrich, St. Louis, MO); anti-CD16/CD32, allophycocyanin-labeled anti–Gr-1 mAb RB6-8C5, rat IgG1 isotype control, and hamster anti-mouse CD3ε mAb 145.2C11 (BD Biosciences); neutralizing anti-TLR4 mAb MTS510 and control rat IgG2a (eBioscience, San Diego, CA; and anti-sphingosine kinase 1 (SPHK1) and control rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA). All Abs were used at 10 μg/ml. RPMI 1640 medium and DMEM were supplemented with glutamine, 2-ME, gentamycin, sodium pyruvate, MEM nonessential amino acids, HEPES buffer, and 10% FCS or 1% Nutridoma.

Apoptotic cells

Neutrophils were obtained 7 h after i.p. injection of 1 ml 3% thioglycollate broth (Sigma-Aldrich) or 4 h after i.p. injection of 60 ng MIP-2 (R&D Systems), as described previously (23). Unless otherwise stated, neutrophils elicited by thioglycollate were used. Exudate cells were incubated in DMEM-FCS at 37°C for 1 h in 250-ml flasks. Nonadherent cells contained 90% neutrophils. APO PMNs were obtained by overnight incubation (aging) in the absence of serum (1). Aged Gr-1$^+$ cells contained >90% Annexin V$^+$, propidium iodide-negative cells. Apoptotic cells were washed in cold medium before use.

Macrophage differentiation assays

Bone marrow cells were cultured (5×10^4 cells/well) in 24-well vessels with 1 ml supplemented RPMI 1640 medium-FCS containing 20% v/v L929 cell-conditioned medium as a source of M-CSF (24). After 7 d, cultures contained 2×10^5 bone marrow-derived macrophages (BMDMs). BMDMs were treated overnight with 0.5 ng/ml IFN-γ and recultured with medium, 10 ng/ml LPS, 5 ng/ml IL-4, APO PMNs (1×10^6/well), or latex beads (10^6/well; Sigma-Aldrich). FCS was replaced by 1% v/v Nutridoma-SP (Roche, Basel, Switzerland). Cultures were kept at 37°C and 7% CO_2. After 3 d, monolayers were washed and recultured with medium or 1 μg/ml LPS. After 48 h, supernatants were collected and assayed for cytokine and nitrite content. Expression of macrophage differentiation markers LIGHT and SPHK1 was evaluated by cellular ELISA (25) by a modified technique suitable for macrophages, as described previously (26). Briefly, adherent BMDMs (10^4) previously cultured in 96-well plates (0.2 ml) were primed overnight with IFN-γ and cultured for 3 d with medium, IL-4, or neutrophils (2×10^5) as above. Residual nonadherent cells were removed, and monolayers were fixed with 1% paraformaldehyde for 30 min and treated with PBS-10% FCS plus 2% mouse serum for 60 min for blockade of FcRs. Monolayers were washed with PBS-0.05% Tween 20 and incubated overnight with 5 μg/ml anti-mouse LIGHT/TNFSF14 mAb 261639 (R&D Systems) or anti-SPHK1 Abs (Santa Cruz Biotechnology). Plates were washed, and the reaction was developed with 5 μg/ml biotinylated anti-rat IgG (Vector Laboratories, Burlingame, CA), streptavidin-alkaline phosphatase conjugate, and *p*-nitrophenyl phosphate as substrate. Results are mean and SE of absorbance readings of triplicate cultures.

Infection with L. major *in vitro and assessment of parasite load*

BMDMs (2×10^5) were primed with IFN-γ, washed, and mixed with 2×10^6 *L. major* promastigotes in RPMI 1640 medium-10% FCS. After 4 h, extracellular parasites were removed, and BMDMs were cultured with medium, LPS (10 ng/ml), IL-4, or APO PMNs for 3 d. Alternatively, primed BMDMs were cultured for 3 d with the same stimuli as above, washed, and then infected with *L. major* for 4 h, followed by reculture for an additional 3 d. All monolayers were transferred to Schneider medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 20% FCS and 2% human urine (27). Monolayers were cultured in Schneider medium at 26°C for an additional 3 d. The relative intracellular load of *L. major* amastigotes was assessed by counting the number of motile extracellular promastigotes released (7, 27).

Treatment with APO PMNs in vivo

Mice (B6) were injected with 2×10^6 *L. major* promastigotes, either with medium (left footpad) or with an additional injection of 2×10^6 apoptotic B6 neutrophils (right footpad). Parasite loads and cellularity of left and right draining lymph nodes from individual mice were separately determined after 15 d by promastigote production in Schneider medium (7, 27). In addition, B6 mice were first injected with medium (left footpad) or 2×10^6 apoptotic B6 neutrophils (right footpad). After 3 d, animals were injected in both footpads with 2×10^6 promastigotes. Parasite loads and cellularity of left and right draining lymph nodes from individual mice were determined after 15 d by promastigote production in Schneider medium. Results were presented as number of promastigotes produced for 10^5 lymph node cells.

T cell stimulation assay

BMDMs (10^5/well) were cultured with highly purified (magnetic cell sorting) allogeneic splenic CD4$^+$ T cells (5×10^5/well) for 7 d. After 4 d, IL-2 (1 ng/ml) was added. Primed T cells were recovered and recultured (2×10^5) with plate-bound anti-CD3 (10 μg/ml). After 48 h, supernatants were collected and assayed for IFN-γ, IL-4, and IL-10 contents.

Release of cytokines and nitrites

Supernatants were assayed for IL-12p40, IL-12p70, IL-10, TNF-α, IFN-γ, and IL-4 content by sandwich ELISA, according to the manufacturer instructions (R&D Systems). Results are mean and SE of triplicate cultures. Nitrite production was assessed by the Griess reaction (28).

Statistical analysis

All experiments shown are representative of two or more independent experiments with similar results. In vitro data were analyzed by Student *t* test for independent samples, using SigmaPlot for Windows. In vivo data were analyzed by paired *t* test. Differences with $p < 0.05$ or lower were considered significant.

Results

Pro- but not anti-inflammatory neutrophil efferocytosis induced an IL-12^{low}IL-10^{high} phenotype in macrophages

Efferocytosis of exudate neutrophils induces a proinflammatory response in B6 macrophages (7). To investigate whether proinflammatory efferocytosis imprints a stable phenotype in macrophages, B6 BMDMs were primed with IFN-γ, cultured for 3 d with different stimuli, and challenged or not with LPS. Cytokine and NO profiles were determined. Primed BMDMs cultured with low doses of LPS developed endotoxin tolerance, as indicated by reduced secretion of TNF-α, plus failure to secrete IL-12p70 and NO following challenge with LPS (Fig. 1). Previous studies indicate that IL-4 synergizes with IFN-γ for induction of leishmanicidal activity and IL-12 production by macrophages (29, 30). Our results confirmed that BMDMs primed with IFN-γ and cultured with

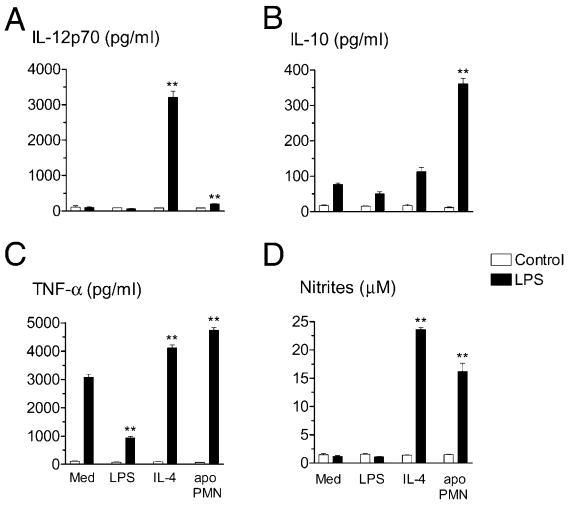

FIGURE 1. Neutrophil efferocytosis induces an IL-12^{low}IL-10^{high}NO^{high} secretory profile in B6 BMDMs. B6 BMDMs were primed with IFN-γ, and cultured with medium, LPS (10 ng/ml), IL-4, or APO PMNs, as indicated in *bottom panels*. After 3 d, BMDMs were restimulated with medium (Med;□) or LPS, 1 μg/ml (■). Supernatants were collected after 48 h and assayed for IL-12p70 (*A*), IL-10 (*B*), TNF-α (*C*), and nitrites (*D*). Results are mean and SE of triplicate cultures. Results are representative of five experiments with similar results. $**p < 0.01$, compared with treatment with medium.

IL-4 secreted high amounts of IL-12p70, TNF-α, and NO but low amounts of IL-10 following challenge with LPS (Fig. 1). However, when BMDMs were primed with IFN-γ and cultured with apoptotic B6 neutrophils, they secreted little, if any, IL-12p70 but high amounts of TNF-α, IL-10 and NO following challenge with LPS (Fig. 1). This phenotype characterizes regulatory/M2b macrophages (12, 18). Induction of regulatory phenotype was not restricted to thioglycollate-induced neutrophils, because efferocytosis of neutrophils recruited by MIP-2 (60 ng per animal i.p.) also induced regulatory/M2b macrophages (data not shown). Furthermore, culture with apoptotic B6 neutrophils, but not with latex beads, induced a low IL-12, high IL-10, high NO regulatory profile on primed B6 BMDMs (Fig. 2). This result indicates that phagocytosis alone was not sufficient to induce the regulatory/M2b phenotype. Besides BMDMs, peritoneal B6 macrophages also expressed a regulatory/M2b phenotype following culture with APO PMNs (data not shown).

Efferocytosis of exudate neutrophils induces an anti-inflammatory response in BALB/c macrophages (7). To investigate the effect of anti-inflammatory neutrophil efferocytosis on macrophage differentiation, B6 and BALB/c BMDMs were primed with IFN-γ, cultured for 3 d with medium or APO PMNs, and challenged with LPS. However, BALB/c BMDMs cultured with apoptotic BALB/c neutrophils did not express a polarized phenotype. Compared with B6 BMDMs (Fig. 3, *left panels*), BALB/c BMDMs secreted IL-10 and NO in a manner similar to control BMDMs (Fig. 3, *right panels*). These results indicated that anti-inflammatory efferocytosis does not induce a regulatory phenotype in macrophages.

Role of NE and TLR4

Efferocytosis of exudate neutrophils by B6 macrophages induces a proinflammatory response, which requires NE and TLR4, and

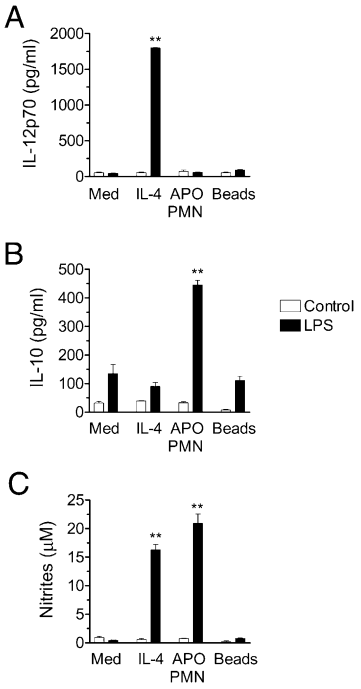

FIGURE 2. Phagocytosis of latex beads fails to induce a regulatory phenotype. B6 BMDMs were primed with IFN-γ and cultured with medium (Med), IL-4, APO PMNs, or latex beads, as indicated. After 3 d, BMDMs were restimulated with medium (control; □) or LPS (■). Supernatants were collected after 48 h and assayed for IL-12p70 (*A*), IL-10 (*B*), or nitrites (*C*). Results are representative of two experiments with similar results. $^{**}p < 0.01$, compared with treatment with medium.

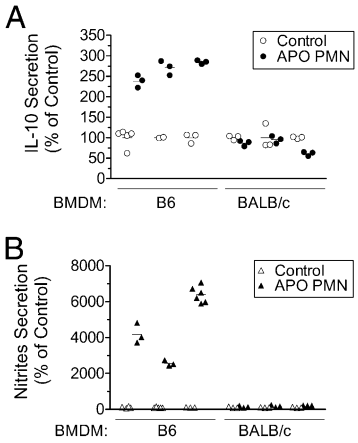

FIGURE 3. Neutrophil efferocytosis does not induce a regulatory phenotype in BALB/c BMDMs. B6 and BALB/c BMDMs were primed with IFN-γ and cultured for 3 d with either medium (control; open symbols) or syngeneic APO PMNs (filled symbols). BMDMs were restimulated with either medium or LPS for 48 h, and supernatants were assayed for secretion of either IL-10 (*A*) or nitrites (*B*). Graphs indicate LPS responses from three independent experiments for both B6 and BALB/c cells. Data were normalized by showing LPS results as percentage of control, and the mean response to medium alone was taken as 100%. $p < 0.01$ (*A*); $p < 0.05$ (*B*).

results in killing of *L. major* (7, 31). To investigate the role of NE in macrophage differentiation, primed B6 BMDMs were cultured with medium or APO PMNs in the presence of the NE inhibitor MeO-Suc-AAPV-cmk, or control collagenase inhibitor Z-Pro-D-Leu-D-Ala-NHOH for 3 d, and challenged with LPS. Treatment with NE inhibitor, but not with control inhibitor, markedly reduced the ability of primed BMDMs to secrete IL-10 and NO (Fig. 4), indicating that regulatory/M2b differentiation was blocked. Next, BMDMs were cultured with APO PMNs in the presence of neutralizing anti-TLR4 mAb or an isotype control and challenged with LPS. Previous treatment with anti-TLR4 completely blocked the ability of primed BMDMs to upregulate IL-10 secretion, compared with isotype control (Fig. 5*A*). However, the ability to secrete NO was not blocked by anti-TLR4 (Fig. 5*B*). These results suggested that NE plays an important role and that TLR4 signaling was involved, at least in part, in induction of the M2b phenotype.

BMDM induced by B6 APO PMNs expressed increased amounts of LIGHT

Regulatory/M2b macrophages express increased amounts of LIGHT and SPHK1 (12, 18). We investigated expression of LIGHT and SPHK1 induced by neutrophil efferocytosis. Primed B6 BMDMs cultured with medium, IL-4, or apoptotic allogeneic BALB/c neutrophils expressed low amounts of LIGHT (Fig. 6*A*). However, BMDMs cultured with apoptotic B6 neutrophils expressed increased amounts of LIGHT, as measured by cellular ELISA (Fig. 6*A*). Culture with apoptotic B6 neutrophils also increased SPHK1 expression by BMDMs (Fig. 6*B*). Increased expression of LIGHT was confirmed by immunofluorescence (data not shown). These results further indicated that proinflammatory efferocytosis induces a regulatory/M2b phenotype.

BMDMs induced by B6 APO PMNs promoted a Th2-biased T cell response

We investigated whether induced regulatory macrophages favored Th2 responses. Primed BMDMs were cultured with either medium or APO PMNs and recultured with allogeneic $CD4^+$ T cells plus IL-2. After 7 d, $CD4^+$ T cells were removed and restimulated with anti-CD3. Secretion of IFN-γ, IL-4, and IL-10 were evaluated

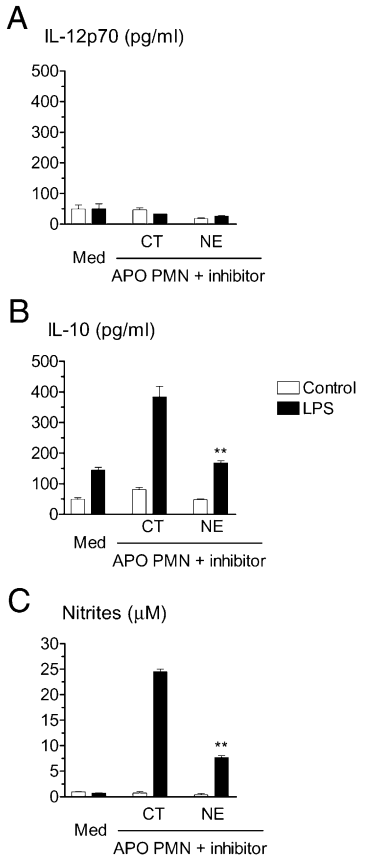

FIGURE 4. NE inhibitory peptide blocks induction of regulatory phenotype. B6 BMDMs were primed with IFN-γ, and cultured with medium (Med) or APO PMNs in the presence of control peptide or NE inhibitory peptide MeOSuc-AAPV-cmk. All cultures received the same amount of solvent (DMSO). After 3 d, BMDMs were restimulated with medium (□) or LPS (■). Supernatants were collected after 48 h, and assayed for IL-12p70 (*A*), IL-10 (*B*), and nitrites (*C*). Results are representative of two experiments with similar results. $^{**}p<0.01$, compared with APO PMNs in the presence of control peptide.

(Fig. 7). Compared with control BMDMs, BMDMs treated with APO PMNs induced a 3.2-fold increase in IL-4 (Fig. 7*B*) and a 3.5-fold increase in IL-10 secretion (Fig. 7*C*), compared with a 1.4-fold increase in IFN-γ secretion (Fig. 7*A*). These results characterized a Th2-biased cytokine response in $CD4^+$ T cells.

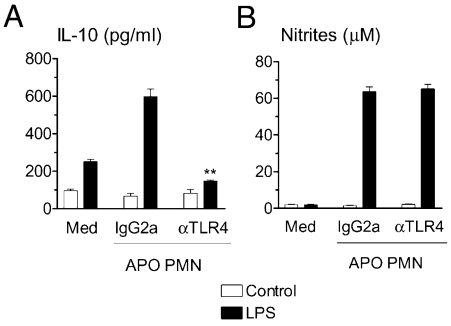

FIGURE 5. Anti-TLR4 partially blocks induction of regulatory phenotype. B6 BMDMs were primed with IFN-γ and cultured with medium (Med) or APO PMNs in the presence of anti-TLR4 mAb or rat IgG2a. After 3 d, BMDMs were restimulated with medium (□) or LPS (■). Supernatants were collected after 48 h and assayed for IL-10 (*A*), and nitrites (*B*). Results are representative of two experiments with similar results. $^{**}p < 0.01$, compared with APO PMNs in the presence of rat IgG2a.

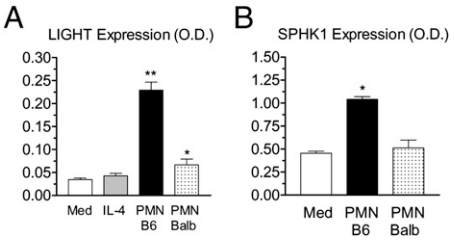

FIGURE 6. BMDMs differentiated by APO PMNs express increased amounts of LIGHT and SPHK1. B6 BMDMs were primed with IFN-γ and cultured with medium (Med; open bars), IL-4 (gray bars), B6 APO PMNs (PMN B6; closed bars), or BALB/c APO PMNs (PMN BALB; dotted bars). After 3 d, BMDMs were washed and assayed for expression of LIGHT/TNFSF14 (*A*) or SPHK1 (*B*) by cellular ELISA. $^{*}p < 0.05$; $^{**}p < 0.01$, compared with medium. Results are representative of seven (LIGHT) and three independent experiments (SPHK1), respectively, with similar results.

BMDMs induced by B6 APO PMNs were permissive to Leishmania *replication*

Neutrophil efferocytosis induces a proinflammatory response in B6 macrophages, which results in killing of *L. major* (7). These results were confirmed with BMDMs. B6 BMDMs were primed, infected with *L. major*, and cultured with medium, LPS, IL-4, or apoptotic B6 neutrophils. Intracellular parasite burden was evaluated after 3 d. Culture with LPS, IL-4 or APO PMNs induced marked killing of *L. major* (Fig. 8*A*). We then investigated the effect of differentiation to the regulatory phenotype. Primed BMDMs were cultured with medium, LPS, IL-4, or APO PMNs for 3 d, infected with *L. major*, and recultured for an additional 3 d to evaluate parasite burden. Previous culture with LPS resulted in a marked increase of

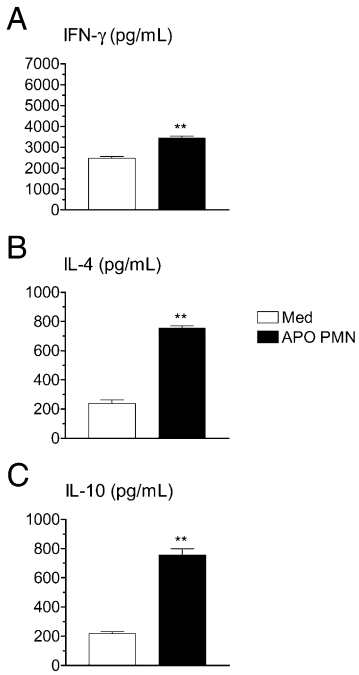

FIGURE 7. BMDMs differentiated by APO PMNs induce a Th2-biased T cell response. B6 BMDMs were primed with IFN-γ and cultured with medium (Med; □) or APO PMNs (■). After 3 d, BMDMs were cocultured with BALB/c splenic $CD4^+$ T cells and IL-2. After 7 d, primed T cells were restimulated with immobilized anti-CD3. Supernatants were collected after 48 h and assayed for IFN-γ (*A*), IL-4 (*B*), and IL-10 (*C*). Results are representative of two experiments with similar results. Note the greater increase in secretion of type 2 cytokines IL-4 and IL-10. $^{**}p < 0.01$, compared with BMDMs induced with medium.

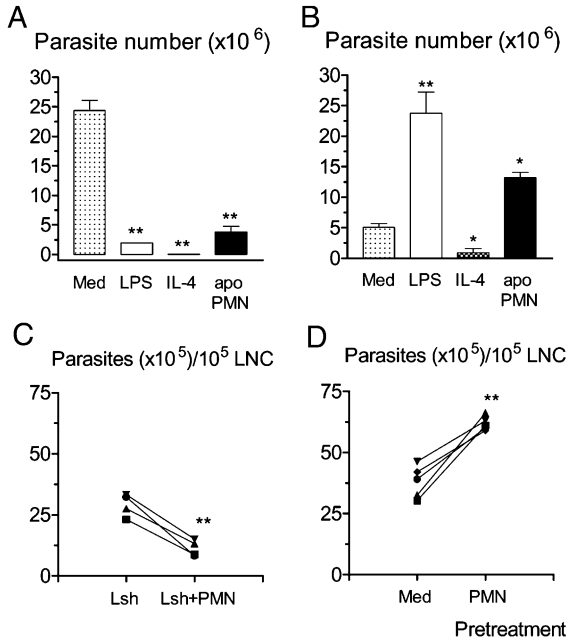

FIGURE 8. Regulatory macrophages are permissive to replication of *L. major*. *A*, B6 BMDMs were primed with IFN-γ, infected with *L. major*, and cultured with medium (Med), LPS (10 ng/ml), IL-4, or APO PMNs. After 3 d, intracellular load of parasites was measured. *B*, B6 BMDMs were primed with IFN-γ and cultured with medium (Med), LPS (10 ng/ml), IL-4, or APO PMNs. After 3 d, BMDMs were infected with *L. major* promastigotes. Intracellular load of parasites was measured after an additional 3 d. Results are representative of three experiments with similar results. $*p < 0.05$; $**p < 0.01$, compared with treatment with medium. *C*, Injection of APO PMNs together with *L. major* (Lsh) in the footpad reduced parasite loads in draining lymph nodes of B6 mice ($**p < 0.01$). Each symbol represents individual mice. *D*, Injection of APO PMNs 3 d before *L. major* in the footpad increased parasite loads ($**p < 0.01$) in draining lymph nodes of B6 mice, compared with injection of medium alone (Med). Each symbol represents individual mice.

parasite replication, perhaps as a result of endotoxin tolerance (Fig. 8*B*). Previous culture with IL-4 induced a stable microbicidal state in BMDMs, as shown by marked killing of *L. major* after 3 d (Fig. 8*B*). However, previous culture with APO PMNs induced a permissive state in BMDMs, as shown by increased replication of *L. major* (Fig. 8*B*). We also measured cytokine and NO production elicited by *Leishmania* infection in the distinct macrophage subsets. However, infection did not elicit any change in IL-12p70, TNF-α, IL-10, or NO levels, compared with controls (data not shown). We then investigated how treatment with APO PMNs regulates *L. major* infection in vivo. In agreement with previous studies (7), injection of APO PMNs together with *L. major* in the footpad reduced subsequent parasite load in draining lymph nodes from B6 mice (Fig. 8*C*). In contrast, injection of APO PMNs 3 d before injecting *L. major* resulted in increased parasite loads (Fig. 8*D*). These results agreed with in vitro studies and suggested that, different from the acute effects of efferocytosis, regulatory macrophages differentiated by efferocytosis become permissive to infection by *L. major*.

Discussion

Neutrophils and macrophages play concerted and complementary roles in innate immune responses to infection (32). During *Leishmania* infection, inflammatory neutrophils and monocytes are recruited to sites of parasite replication (33). Neutrophils play an important regulatory role in the initial phase of *Leishmania* infection (34, 35). However, little is known regarding the cell interactions underlying such regulatory function. In this study, our data have indicated that proinflammatory efferocytosis of neutrophils induces a stable regulatory/M2b phenotype in macrophages characterized by low IL-12, high IL-10 secretion, high TNF-α, high NO secretion, increased Th2 responses, increased expression of LIGHT/TNFSF 14, and permissive replication of *Leishmania*.

Previous studies suggest that efferocytosis induces an alternative activation phenotype in macrophages (36, 37). Efferocytosis increases the amounts of arginase (38), ornithine decarboxylase (8), and TGF-β (7–9, 39) expressed by phagocytosing macrophages. In this study, we have investigated whether efferocytosis imprints a macrophage phenotype that can be recalled by LPS after 3 d. Our data have indicated that proinflammatory efferocytosis of neutrophils induces a regulatory IL-12^{low},IL-10^{high} phenotype in macrophages, which can be recalled by LPS after a delay of 3 d. Proinflammatory efferocytosis also increased expression of LIGHT and favored Th2 responses, which was in agreement with induction of a regulatory/M2b phenotype (12, 18). In contrast, our data suggested that anti-inflammatory efferocytosis does not imprint any particular phenotype recalled by LPS restimulation. At present, the reason for this difference is not clear. It is possible that the regulatory phenotype requires a previous proinflammatory stimulus to be induced. In agreement, efferocytosis of B6, but not BALB/c neutrophils, induces TNF-α secretion (7) and engages TLR4 in syngeneic macrophages (31). However, we cannot discard the possibility that anti-inflammatory efferocytosis blocks induction of the regulatory phenotype through secretion of TGF-β or other suppressive mediators.

Granule proteins released by activated neutrophils regulate macrophage function (40). Specifically, NE activates TLR4 (41) and induces leishmanicidal activity through TLR4 (31). Apoptotic B6, but not BALB/c, neutrophils induce leishmanicidal activity in B6 macrophages (7). This proinflammatory effect correlates with increased NE release, requires NE activity, and depends on TLR4 as a downstream effector (31). In this paper, our results have indicated that induction of the regulatory/M2b phenotype also required NE and TLR4 activities. Increases of both IL-10 and NO secretion required NE activity. In contrast, IL-10 secretion, but not NO production, required TLR4 function. These results suggested that NE induces macrophage differentiation through both TLR4-dependent and TLR4-independent pathways. However, purified NE alone failed to induce the regulatory phenotype (data not shown). This observation is not surprising, because induction of the regulatory/M2b phenotype requires two distinct signals (12). The involvement of TLR4 was in agreement with a role for TLR signaling in induction of the regulatory phenotype (12). The second signal was not identified in the current study but was likely provided by the apoptotic cell. Macrophages interact with apoptotic cells through multiple receptors that mediate intracellular signaling (42). Noteworthy, interactions with apoptotic cells both reduce IL-12 and increase IL-10 secretion by monocytes (43), a response similar to the ones elicited by engagement of Fc or iC3b receptors in monocyte/macrophages (19, 44). These data suggest that apoptotic cells could potentially initiate intracellular signaling required for regulatory/M2b differentiation. We also compared the microbicidal effect of efferocytosis either before or after induction of the regulatory/M2b phenotype. Treatment with low doses of LPS induced intracellular killing of *Leishmania* but rendered macrophages highly permissive to parasite replication upon a late challenge. These results confirmed the deleterious role of endotoxin tolerance on intracellular growth of *Leishmania* (45). Similar to previous studies (7, 29), both neutrophil efferocytosis

and IL-4 induced leishmanicidal activities in primed BMDMs. In addition, our data showed that primed BMDMs already differentiated by IL-4 retained leishmanicidal activity. However, primed BMDMs already differentiated by efferocytosis lost their leishmanicidal activity. These results indicated that regulatory/M2b macrophages are permissive to intracellular growth of *L. major*. Accordingly, treatment of B6 mice in vivo with APO PMNs 3 d before infection resulted in increased parasitic loads. These latter results were compatible with the in vitro assays, because mice were infected when regulatory/M2b macrophages were expected to be already induced by efferocytosis. Our results agreed with deleterious roles for regulatory macrophages (20) and for IL-10 (20, 46) in *L. major* infection. In addition, paracrine IL-10 produced by myeloid cells is important to maintain Foxp3 expression and suppressive function of regulatory T cells (47). This finding suggests another potential role for IL-10 in supporting parasite persistence during *Leishmania* infection.

The anti-inflammatory effects of efferocytosis provide a physiological pathway to resolve inflammation. However, as shown in several models, efferocytosis can also be proinflammatory (4–7). Our results indicated that anti-inflammatory efferocytosis does not affect subsequent macrophage phenotype. In contrast, proinflammatory efferocytosis induces differentiation to the regulatory/M2b phenotype, perhaps as a safe mechanism to stop inflammation. In this context, the fate of intracellular *Leishmania* in host macrophages would depend on the timing between infection and phagocytosis of senescent neutrophils. Although regulatory macrophages help to resolve inflammation, they might also function as permissive niches for parasite persistence.

Disclosures

The authors have no financial conflicts of interest.

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