THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Alternatively Activated Macrophages Elicited by Helminth Infection Can Be Reprogrammed to Enable Microbial Killing

Citation for published version:
Mylonas, KJ, Nair, MG, Prieto-Lafuente, L, Paape, D & Allen, JE 2009, 'Alternatively Activated Macrophages Elicited by Helminth Infection Can Be Reprogrammed to Enable Microbial Killing' Journal of Immunology, vol. 182, no. 5, pp. 3084-3094. DOI: 10.4049/jimmunol.0803463

Digital Object Identifier (DOI):
10.4049/jimmunol.0803463

Published In:
Journal of Immunology

The Journal of Immunology

Alternatively Activated Macrophages Elicited by Helminth Infection Can Be Reprogrammed to Enable Microbial Killing

Katie J. Mylonas, Meera G. Nair, Lidia Prieto-Lafuente, Daniel Paape and Judith E. Allen

J Immunol 2009; 182:3084-3094; ;
doi: 10.4049/jimmunol.0803463
http://www.jimmunol.org/content/182/5/3084

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Alternatively Activated Macrophages Elicited by Helminth Infection Can Be Reprogrammed to Enable Microbial Killing[1]

Katie J. Mylonas,[2] Meera G. Nair,[2,3] Lidia Prieto-Lafuente, Daniel Paape, and Judith E. Allen[4]

The prime function of classically activated macrophages (activated by Th1-type signals, such as IFN-γ) is microbial destruction. Alternatively activated macrophages (activated by Th2 cytokines, such as IL-4 and IL-13) play important roles in allergy and responses to helminth infection. We utilize a murine model of filarial infection, in which adult nematodes are surgically implanted into the peritoneal cavity of mice, as an in vivo source of alternatively activated macrophages. At 3 wk postinfection, the peritoneal exudate cell population is dominated by macrophages, termed nematode-elicited macrophages (NeMϕ), that display IL-4-dependent features such as the expression of arginase 1, RELM-α (resistin-like molecule α), and Ym1. Since increasing evidence suggests that macrophages show functional adaptivity, the response of NeMϕ to proinflammatory Th1-activating signals was investigated to determine whether a switch between alternative and classical activation could occur in macrophages differentiated in an in vivo infection setting. Despite the long-term exposure to Th2 cytokines and antiinflammatory signals in vivo, we found that NeMϕ were not terminally differentiated but could develop a more classically activated phenotype in response to LPS and IFN-γ. This was reflected by a switch in the enzymatic pathway for arginine metabolism from arginase to inducible NO synthase and the reduced expression of RELM-α and Ym1. Furthermore, this enabled NeMϕ to become antimicrobial, as LPS/IFN-γ-treated NeMϕ produced NO that mediated killing of *Leishmania mexicana*. However, the adaptation to antimicrobial function did not extend to key regulatory pathways, such as IL-12 production, which remained unaltered. *The Journal of Immunology,* **2009, 182: 3084–3094.**

Signals encountered by developing macrophages (Mϕ)[5] during migration determine their functional properties at sites of inflammation or infection. Among these signals, cytokines, which can act synergistically or have opposing effects, are responsible for the development of highly divergent Mϕ phenotypes. Classical activation of Mϕ is dependent on the products of activated Th1 cells, in particular IFN-γ (1). The prime function of classically activated Mϕ (CAMϕ) is microbial destruction, which is conducted by the production of reactive oxygen and nitrogen intermediates such as NO from inducible NO synthase (iNOS/NOSII) (2). Alternatively activated Mϕ (AAMϕ), activated by the Th2 cytokines IL-4 and IL-13, play important roles in allergy and responses to parasite infection (1). AAMϕ and CAMϕ exhibit distinct L-arginine metabolism pathways. CAMϕ produce NO as a result of the up-regulation of iNOS, which is a catalyst of the L-arginine substrate (3). In AAMϕ, L-arginine is converted to L-ornithine and urea by the enzyme arginase 1 (3). The induction of either arginase or iNOS is usually coupled with suppression of the opposing enzyme (4). While the NO produced by CAMϕ has antimicrobial and cytotoxic properties, L-ornithine produced by AAMϕ can be further metabolized to proline and polyamines, which have roles in collagen production and cell proliferation, respectively. These properties have led to the hypothesis that AAMϕ are involved in wound healing (5, 6).

Although Mϕ have been usefully classified in this way, the range of actual phenotypes is likely to be much broader, with AAMϕ and CAMϕ representing two points in a wide spectrum. Unlike Th cells where activation leads to terminal differentiation into Th1 and Th2 cells, Mϕ appear to display a high degree of flexibility (7–10). Plasticity of function may be an economical strategy for the immune system since, in contrast to the high turnover of T cells, Mϕ are longer lived and may need to adapt their function to different pathogens or environments they will face during their lifespan.

Many studies have shown that Mϕ responsiveness to a given cytokine can be altered and/or suppressed by the cytokines to which it was previously exposed (11, 12). However, the flexibility of the AAMϕ phenotype is still a subject of controversy. While some reports show that IL-4 pretreatment of Mϕ renders them unresponsive to Th1 activation (4, 11–14), others have found that the previous IL-4 exposure enhanced the responsiveness to Th1-activating signals (15). As these results were obtained by in vitro activation with IL-4, the differences may be due to variation in the length and intensity of the activation stimuli. Indeed, Stout et al. demonstrated that two cytokines could have either antagonistic or synergistic effects on murine Mϕ function dependent on the order and length of each cytokine treatment (16). In another study, human Mϕ were stimulated with either pro- (e.g., TNF-α) or anti-inflammatory (e.g., IL-10) cytokines and then cultured with a

Institute of Immunology and Infection Research, The University of Edinburgh, Edinburgh, United Kingdom

Received for publication October 15, 2008. Accepted for publication January 2, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Wellcome Trust and the Medical Research Council, United Kingdom.

[2] K.J.M. and M.G.N. contributed equally to this work.

[3] Current address: Department of Pathobiology, University of Pennsylvania, Philadelphia, PA 19104.

[4] Address correspondence and reprint requests to Dr. Judith E. Allen, Institute of Immunology and Infection Research, The University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, United Kingdom. E-mail address: j.allen@ed.ac.uk

[5] Abbreviations used in this paper: Mϕ, macrophage; CAMϕ, classically activated macrophage; iNOS, inducible NO synthase; AAMϕ, alternatively activated macrophage; NeMϕ, nematode-elicited macrophage; PEC, peritoneal exudate cell; RELM-α, resistin-like molecule α; WT, wild type; BMMϕ, bone marrow-derived macrophage; PD-L, programmed death ligand; L-NMMA, N^G-monomethyl-L-arginine; nor-NOHA, N^ω-hydroxy-nor-L-arginine; CBA, cytokine bead array; ThioMϕ, thioglycollate-elicited macrophage; MFI, mean fluorescence intensity.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803463

counterstimulatory cytokine or medium alone, and it was found that Mϕ stimulated toward a specific activation state could switch their phenotype rapidly when given counterstimulatory signals or return to a quiescent state after signal arrest (9). Due to conflicting in vitro data, it is difficult to determine whether Mϕ demonstrate functional adaptivity in vivo and to ascertain the physiological relevance of this phenomenon. Investigating Mϕ plasticity in vivo could have important implications for therapeutic targeting of Mϕ in chronic diseases but also for our general understanding of how the immune system copes with multiple infections that may require differing immune responses. In support of the hypothesis that Mϕ plasticity may confer increased efficiency and flexibility for the immune response, Gratchev et al. demonstrated that in response to a second stimulation with IFN-γ, in vitro-derived human AAMϕ displayed significantly higher bactericidal activity (17). Also, pretreatment of murine macrophages with the IL-13 was found to enhance LPS-induced anti-*Toxoplasma gondii* activity (18). However, the degree of flexibility of Mϕ recruited to sites of infection is still unknown.

In this study, we have used a murine model for filarial infection as a source of Mϕ, which we have previously termed nematode-elicited Mϕ (NeMϕ) (19–21). Mice are surgically implanted i.p. with the adult stage of the nematode *Brugia malayi*. By 1 wk postinfection, the peritoneal exudate cell (PEC) population is dominated by Mϕ that display IL-4-dependent features such as the expression of arginase 1, RELM-α (resistin-like molecule α), and Ym1, as well as the ability to suppress the proliferation of neighboring cells (19, 22). This phenotype is sustained for many weeks in vivo. We have previously shown that NeMϕ share many properties with Mϕ differentiated in vitro in response to IL-4 or IL-13 (hereafter termed in vitro AAMϕ) (19). However, NeMϕ are recruited during a chronic Th2-dominated nematode infection and experience much longer term exposure to IL-4 and IL-13 than in vitro-AAMϕ. Furthermore, NeMϕ are influenced by parasite-secreted products as well as host factors other than IL-4, such as IL-10 and glucocorticoids. In light of the increasing evidence that Mϕ show functional adaptivity, we decided to study the NeMϕ response to Th1-activating signals as a model to investigate whether Mϕ differentiated in a chronic Th2 infection setting could alter their established phenotype and whether this would translate into an ability to control an intracellular microbial infection.

Despite the long-term exposure to Th2 cytokines and antiinflammatory signals in vivo, we found that NeMϕ were still responsive to LPS and IFN-γ, exhibiting dramatic changes in gene expression profile. Additionally, LPS/IFN-γ treated NeMϕ were able to effectively control infection with *Leishmania mexicana*. However, LPS/IFN-γ treated NeMϕ still maintained the IL-4-dependent capacity to suppress proliferation and failed to up-regulate IL-12. Taken together, these results demonstrate that Mϕ differentiated in vivo can make radical alterations in phenotype to accommodate a new challenge. However, functional plasticity may be limited to effector rather than to regulatory pathways.

Materials and Methods

Mice and infection

Wild-type (WT) or IL-4$^{-/-}$ mice on the C57BL/6 background were bred in house or purchased from Harlan U.K. Mice were 6–8 wk old at the start of the experiment, and all work was conducted in accordance with the Animals (Scientific Procedures) Act of 1986. Adult *B. malayi* parasites were removed from the peritoneal cavity of infected gerbils purchased from TRS Laboratories or maintained in house. Mice were surgically implanted i.p. with five to six live adult female *B. malayi*. Three weeks later, mice were euthanized and PECs harvested by washing of the peritoneal cavity with 15 ml of ice-cold DMEM. As a control for non-Th2-polarized inflammation, mice were injected i.p. with 0.8 ml of 4% thioglycollate medium, brewer modified (BD Biosciences). Three days later, the PECs were harvested, as above.

Mϕ activation

PECs were cultured in DMEM, supplemented with 10% FCS, 2 mM L-glutamine, 0.25 U/ml penicillin, and 100 mg/ml streptomycin. Thioglycollate-elicited PECs were plated in 9-cm petri dishes and left untreated or treated for 18–24 h with IL-4 (20 ng/ml; BD Pharmingen). Nonadherent cells were subsequently washed off and the remaining adherent Mϕ were left untreated or treated with LPS (100 ng/ml; *Escherichia coli* 0111:B4; Sigma-Aldrich) and IFN-γ (10 U/ml; BD Pharmingen) together or separately for 18–24 h. NeMϕ were similarly recovered and plated in medium alone or with LPS and/or IFN-γ. Following treatment, the nonadherent cells were washed off and the adherent Mϕ were recovered by a 15-min incubation at 37°C in warm 10 mM glucose and 3 mM EDTA in PBS. For cell division analysis, cells were washed in PBS and then resuspended at 1×10^7/ml in 5 μM CFSE in serum-free DMEM for 8 min at 37°C. The reaction was quenched with an equal volume of FCS and cells were washed several times in serum-free DMEM. Bone marrow-derived Mϕ (BMMϕ) were prepared by harvesting bone marrow from the femur and tibia of C57BL/6 mice. Erythrocytes were lysed using 3 ml of RBC lysis buffer (Sigma-Aldrich) for 5 min. Differentiation into Mϕ was performed according to published protocols (23). Cells were plated onto Petri dishes at 7.5×10^6 cells/plate and cultured in DMEM, supplemented with 25% FCS (Invitrogen), 25% L929 supernatant as a source of M-CSF, 2 mM L-glutamine, 0.25 U/ml penicillin, and 100 μg/ml streptomycin. The medium was replaced after 4–6 days to generate a pure population of Mϕ at day 7. BMMϕ were then plated in medium alone or with LPS and/or IFN-γ (as above).

Flow cytometry

Cells were incubated at 4°C for 15 min in blocking buffer (2% mouse serum, 10 μg/ml anti-CD16/32 in FACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA)), followed by staining for 20 min on ice with the Abs of interest at the appropriate dilution as determined by titration. Abs included tricolor-conjugated anti-F4/80 (Caltag Laboratories), PE-conjugated anti-B7.1/B7.2/MHC class II (BD Pharmingen), PE-conjugated anti-programmed death ligand (PD-L)1/PD-L2 (eBioscience), as well as appropriate isotype control Abs. Cells were washed three times in FACS buffer and fixed in 0.8% paraformaldehyde before acquisition and analysis (FACStation and FlowJo software; BD Biosciences).

Proliferation assay

Mϕ purified by adherence were cocultured (1×10^5 cells/well) in 96-well flat-bottom plates with EL-4 cells (1×10^4/well) in the presence or absence of the inhibitors N^G-monomethyl-L-arginine (L-NMMA; 100 μM; Sigma-Aldrich) or N^ω-hydroxy-nor-L-arginine (nor-NOHA; 250 μM; Sigma-Aldrich). After 48 h, 100 μl of supernatant was removed to measure NO production. For each well, 1 μCi of [^{3}H]TdR was added and plates were incubated overnight before harvesting and counting using a liquid scintillation counter (MicroBeta 1450; TriLux). Quadruplicate measurements per sample were performed. Results were plotted in cpm.

Quantification of NO production and arginase activity

NO production was assessed by nitrite accumulation in the culture media using the Griess reagent. In brief, 100 μl of culture supernatant was mixed with 100 μl of 5.8% phosphoric acid, 1% sulfanilamide, and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride. Absorbance was measured at 490 nm with background correction at 650 nm using a microplate reader. Concentration was determined according to a standard curve of sodium nitrite solution. Arginase activity was measured according to previously published protocols (3). Briefly, $1–2 \times 10^5$ cells were lysed with 100 μl of 0.1% Triton X-100. Following a 30-min incubation with shaking, 100 μl of 25 mM Tris-HCl (pH 7.2) and 20 μl of 10 mM $MnCl_2$ were added and the enzyme was activated by heating to 56°C for 10 min. L-Arginine hydrolysis was conducted by incubating 100 μl of this lysate with 100 μl of 0.5 M L-arginine (pH 9.7) at 37°C for various time points between 15 and 60 min. The reaction was then stopped with 800 μl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v), and 40 μl of 9% isonitrosopropiophenone was added, followed by heating to 99°C for 30 min before reading on the microplate reader at 540 nm. A standard curve of urea solution was used to determine concentrations. One unit of arginase enzyme activity is defined as the amount of enzyme that catalyzed the formation of 1 μmol of urea per minute at 37°C. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich.

RNA extraction and real-time RT-PCR

RNA was recovered from cells by resuspension in TRIzol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's instructions. Following DNAse1 treatment (Ambion), 1 μg of RNA was used for the synthesis of cDNA using Moloney murine leukemia virus reverse transcriptase (Stratagene). Relative quantification of the genes of interest was measured by real-time PCR, using the Roche LightCycler. PCR amplification conditions and primers have been described previously (20). Five serial 1/4 dilutions of a positive control sample of cDNA were used as a standard curve in each reaction, and the expression levels were estimated from the curve. Real-time PCR of the housekeeping gene β-actin allowed normalization of the expression of the genes of interest.

Cytokine quantification

For measurement of cytokine production, 5×10^5 PECs were plated in 24-well plates for 2–4 h followed by removal of nonadherent cells. The remaining adherent Mϕ were left untreated or were treated for 24 h with LPS/IFN-γ as described above, followed by recovery of the supernatants for cytokine quantification. TNF-α was measured using the DuoSet TNF-α ELISA kit (R&D Systems) according to the manufacturer's instructions. IL-6 and IL-12p40 were measured according to standard sandwich ELISA protocols, using Ab pairs (unconjugated and biotinylated) from BD Pharmingen, and ExtrAvidin-alkaline phosphatase conjugate in conjunction with Sigma*Fast p*-nitrophenyl phosphate tablet substrate (both from Sigma-Aldrich). IL-10 and MCP-1 were quantified using the cytokine bead array (CBA) kit (BD Pharmingen) according to the manufacturer's instructions.

Mϕ infection with L. mexicana

L. mexicana (strain MNYC/BZ/62/M379 or MNYC/BZ/62/M379 with DsRed integrated into sRNA locus) (24) promastigotes were cultured in vitro in semidefined medium (25) with 10% FCS and 1% penicillin-streptomycin (complete semidefined medium) at 26°C. Promastigotes were added to Mϕ, purified by adherence, and plated on coverslips (BDH Laboratory Supplies) in 24-well plates, at a ratio of 10:1 for 3 h or 30:1 for 3 h, in the case of the red strain. The Mϕ were then washed twice with complete DMEM to remove nonphagocytosed parasites and wells were replenished with complete medium. Where indicated, the NO inhibitor L-NMMA was added to a final concentration of 400 μM. At day 3 postinfection, cells were fixed with 2% formaldehyde for 20 min at room temperature, washed twice with PBS, and stained with 0.5–1 ml of Giemsa for 5–10 min, followed by two washes with distilled H_2O. Coverslips were removed from wells, allowed to dry, and mounted onto slides with DPX mount (BDH Laboratory Supplies) before microscopic examination. Approximately 200 cells per group were counted and the percentage infection was recorded. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. Alternatively, a leishmanicidal assay was conducted at 3 h postinfection as previously described (26). The cells were lysed using 0.01% SDS in 100 μl of DMEM (FCS free) for 30 min and pipetted up and down 5–10 times. Released amastigotes were resuspended in complete semidefined medium in a total of 600 μl per well and cultured for 72 h at 26°C. Four aliquots of 150 μl for each sample were then transferred to quadruplicate wells of a 96-well plate and pulsed with [*methyl*-^{3}H]thymidine (1 μCi/well) for a further 18 h at 26°C, and incorporation was assessed on a liquid scintillation counter (MictoBeta 1450; TriLux). Leismanicidal activity was measured as reduction in the incorporation of [^{3}H]thymidine by surviving parasites. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. For immunofluorescence assays, Mϕ infected on coverslips were fixed in 1–2 ml of 3% paraformaldehyde (in PBS) for 20 min and then washed in PBS. They were permeabilized in 1–2 ml of 0.25% Triton X-100 (in PBS) for 10 min and washed in PBS again. Blocking for 30 min was conducted with 1–2 ml of 1–3% (w/v) BSA in PBS. The primary Ab, anti-Ym1 (StemCell Technologies; 1/25 dilution in BSA/PBS) was incubated for 1 h. Coverslips were washed three times in PBS for 5 min with shaking. The secondary Ab (anti-rabbit Alexa Fluor 488 (Molecular Probes); 1/200 in BSA/PBS) was incubated for 1 h and then cells were washed in PBS. For nuclear staining, 1 μl of 10 mg/ml 4′,6-diamidino-2-phenylindole was added during the first wash and then cells washed three more times for 5 min with shaking. Coverslips were washed in dH_2O and placed on top of gel/mount on slides and allowed to dry in the dark. Cells were visualized using an Olympus BX50 microscope, and images were taken with a MicroColor RGB-MS-C camera.

Results

LPS/IFN-γ causes a switch in L-arginine metabolism from arginase to iNOS and down-regulates the expression of alternative activation markers

Preferential expression of arginase over iNOS is a consistent feature of NeMϕ (19). We decided to investigate whether the Th1-activating signals (LPS and/or IFN-γ) could alter the NeMϕ iNOS/arginase balance. Thioglycollate-elicted Mϕ (ThioMϕ) and NeMϕ were left untreated or were treated overnight with LPS and/or IFN-γ and recovered for gene expression analysis by real-time RT-PCR (Fig. 1, *A* and *B*). As expected, untreated NeMϕ showed high arginase 1 expression but low iNOS RNA levels, while untreated ThioMϕ showed none or low expression of both genes. Both IFN-γ and LPS could act alone or in concert to reduce the arginase 1 expression in NeMϕ. This decrease was paralleled by an increase in iNOS expression. In contrast, ThioMϕ required both signals for the induction of iNOS. This could reflect the less mature activation state of this Mϕ type (27), which may require signaling through a pathogen recognition receptor such as TLR4 before becoming responsive to IFN-γ. For subsequent experiments we decided to use LPS and IFN-γ together to ensure classical activation of the ThioMϕ group.

Although ThioMϕ exhibited increases in arginase 1 mRNA in response to LPS/IFN-γ, consistent with previous studies (3), this induction in gene expression did not result in increased arginase enzyme activity (Fig. 1*C*). Arginase and iNOS enzyme activities of NeMϕ were altered by LPS/IFN-γ and, although not as fully, they did reflect the gene expression data (Fig. 1, *C* and *D*). This included a down-regulation in arginase activity in LPS/IFN-γ-treated NeMϕ and an increase in NO production. In contrast to the mRNA levels, the NO produced by LPS/IFN-γ-treated NeMϕ was lower than by LPS/IFN-γ-treated ThioMϕ. These differences may reflect timing, as mRNA levels precede protein production, or other posttranslational controls. For example, the higher level of arginase in LPS/IFN-γ-treated NeMϕ may compete with iNOS for L-arginine. Nevertheless, the increase in NO production coupled by a decrease in arginase activity demonstrates that NeMϕ can alter the balance of arginine metabolism in response to proinflammatory signals.

Having identified RELM-α and Ym1 as the main IL-4-dependent genes expressed in NeMϕ (19), we investigated whether RELM-α/Ym1 expression was also altered by LPS/IFN-γ treatment. As a control for nondifferentiated Mϕ, we measured RELM-α and Ym1 expression in ThioMϕ (Fig. 1*E*). We observed induction of both genes by LPS/IFN-γ in a similar pattern to arginase 1 expression, although the expression levels were lower than in untreated NeMϕ. Upon overnight treatment with LPS/IFN-γ, RELM-α and Ym1 expression in NeMϕ was reduced by up to 90% (Fig. 1*E*). Subsequent experiments always demonstrated reduced expression of these markers following LPS/IFN-γ treatment but the extent of this reduction was variable, ranging from 30% to 90% (data not shown).

LPS/IFN-γ-induced activation switch is not due to the outgrowth of a minority cell population

To rule out the possibility that the phenotypic changes in LPS/IFN-γ-treated NeMϕ represented an outgrowth of a naive Mϕ population rather than a change in the existing NeMϕ population, we monitored the cell proliferation following treatment. Before overnight treatment with LPS/IFN-γ, cells were labeled with

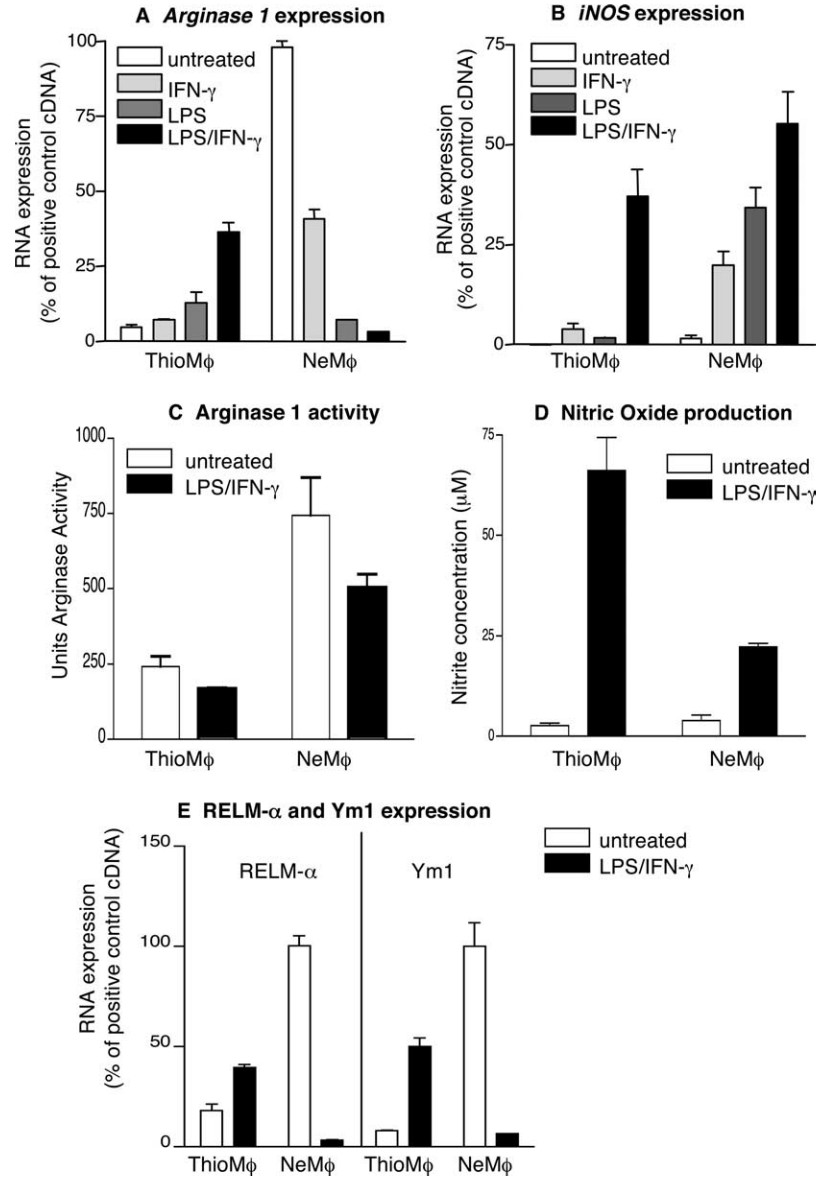

FIGURE 1. LPS/IFN-γ reduce alternative activation of NeMϕ and enhance iNOS activity. ThioMϕ or NeMϕ from C57BL/6 mice were left untreated or treated overnight with LPS and/or IFN-γ as indicated. The cells were recovered for RNA expression analysis of arginase 1 (*A*), iNOS (*B*), RELM-α, and Ym1 (*E*) by real-time RT-PCR and assessed for arginase activity (*C*). The supernatants were also recovered to assess the iNOS activity by measurement of nitrite production by the cells (*D*). Results are shown as the means of replicate samples (±SEM) and are representative of three independent experiments.

CFSE and then recovered and analyzed by flow cytometry. This allowed the comparison of proliferation between untreated and LPS/IFN-γ-treated Mϕ, as identified according to side scatter and F4/80 expression (Fig. 2*A*). Following overnight treatment with LPS/IFN-γ, ThioMϕ and NeMϕ groups did not undergo any cell divisions and remained CFSE^{high}. Thus, the switch in gene expression and iNOS/arginase activity represents a nondividing NeMϕ population and not the outgrowth of another cell population. Additionally, fluorescent staining for Ym1 and RELM-α by immunofluorescence assay (see below) or intracellular FACS (data not shown) suggested that this was a highly homogeneous starting population with all F4/80$^+$ Mϕ expressing the alternative activation markers.

The mean fluorescence intensity (MFI) for F4/80 was higher in NeMϕ (176) than in ThioMϕ (52) (Fig. 2*B*). Since F4/80 has been implicated in immunological tolerance through the generation of regulatory T cells (28), the higher surface expression on NeMϕ may point to a regulatory function. Mϕ are a prominent cell type in other filarial nematode infection models such as *Litomosoides sigmodontis*, where regulatory T cells play an important role in immunoregulation (21, 29). Interestingly, LPS/IFN-γ treatment affected F4/80 surface staining intensity, and we observed a reduction in MFI in NeMϕ (from 176 to 113) but not in ThioMϕ (from 52 to 58). Down-regulation in F4/80 expression upon Th1 activation could have an impact on the regulatory properties of NeMϕ.

Suppressive phenotype of NeMϕ is not altered by LPS/IFN-γ

Since proliferative suppression is one of the well-defined regulatory properties of NeMϕ (22, 30), we decided to investigate whether LPS/IFN-γ affected the ability of NeMϕ to suppress the proliferation of cocultured EL-4 thymoma cells. We included the NO inhibitor L-NMMA as a control since LPS/IFN-γ treatment resulted in NO production from all Mϕ groups (see Fig. 1), and we wanted to distinguish the known suppressive effects of NO (31–33) from the suppressive mechanism mediated by NeMϕ. Having previously shown that the NeMϕ suppressive phenotype is dependent on IL-4 (22), we also included NeMϕ generated in IL-4-deficient mice as a negative control for proliferative suppression. Finally, we included ThioMϕ pretreated with IL-4, allowing a comparison between in vitro-derived AAMϕ and in vivo-derived NeMϕ.

As expected, the EL-4 cell proliferation was suppressed when cocultured with NeMϕ in comparison to control ThioMϕ (Fig. 3*A*,

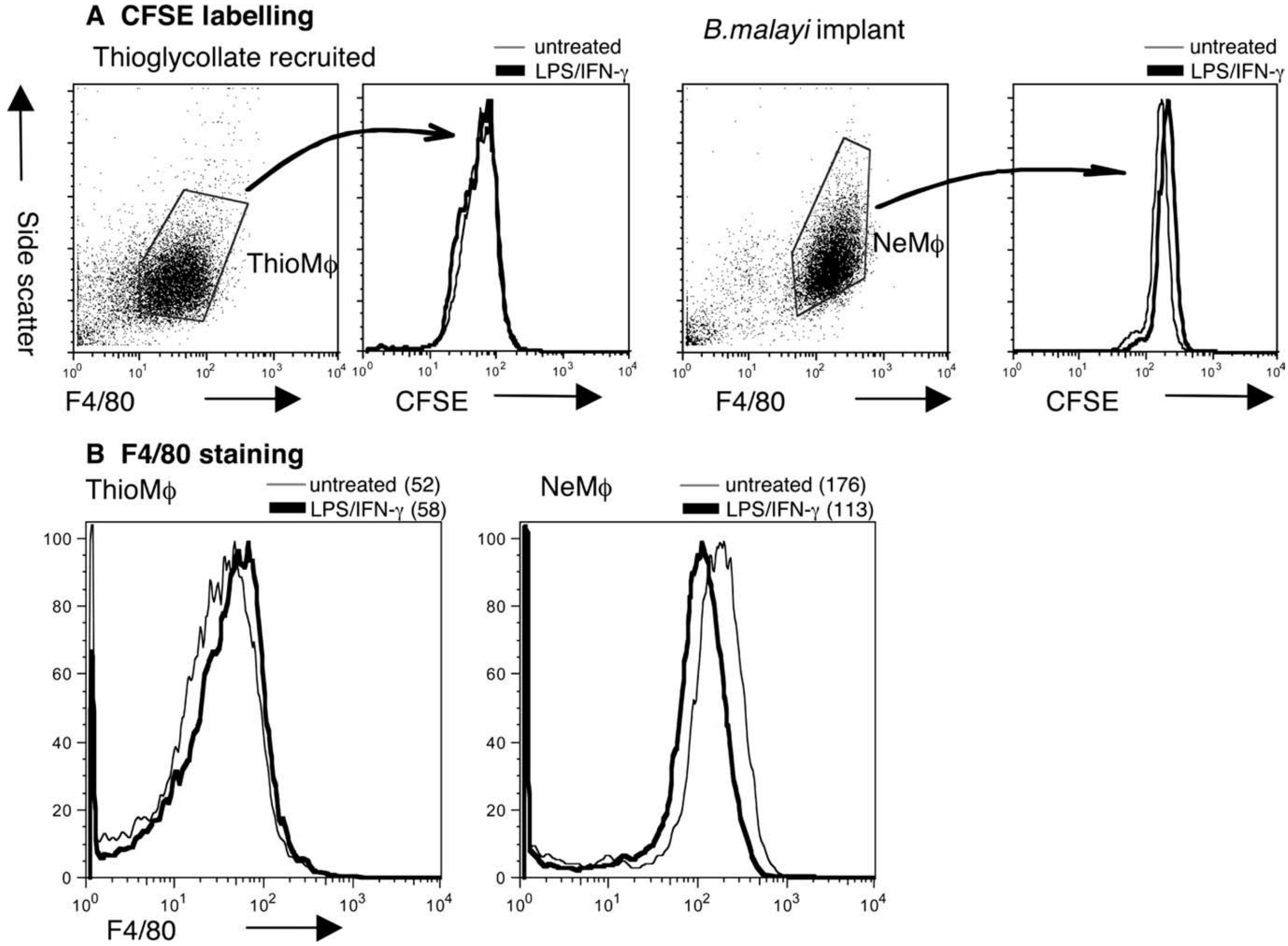

FIGURE 2. Mϕ do not divide in response to LPS/IFN-γ but down-regulate F4/80. C57BL/6 mice were implanted i.p. with *B. malayi* adult worms and the PECs were recovered 3 wk later. For comparison, C57BL/6 mice were injected i.p. with thioglycollate and the ThioMϕ were recovered at day 3. *A*, The cells were labeled with CFSE and left untreated or treated with LPS/IFN-γ overnight followed by flow cytometry analysis to identify the Mϕ population by F4/80 and side scatter and to monitor cell division according to the dilution of CFSE. *B*, The surface intensity of F4/80 in ThioMϕ and NeMϕ was also monitored for changes in response to LPS/IFN-γ treatment. Numbers in the legend represent MFI.

open bars). ThioMϕ pretreated with IL-4 (in vitro AAMϕ) were equally suppressive while IL-4$^{-/-}$ NeMϕ were unable to suppress EL-4 proliferation, confirming that IL-4 was essential for the development of this suppressive phenotype. When treated with LPS/IFN-γ (black bars), all Mϕ cell types could suppress proliferation. However, L-NMMA treatment (gray bars) reversed the LPS/IFN-γ-induced suppressive phenotype in the ThioMϕ and the IL-4$^{-/-}$ NeMϕ, demonstrating that this suppression was NO mediated. In response to LPS/IFN-γ treatment, NeMϕ and in vitro AAMϕ still suppressed EL-4 thymoma proliferation but this was not NO mediated, as it was not altered by L-NMMA. LPS/IFN-γ is thus not sufficient to reverse the suppressive function of either in vitro- or in vivo-derived AAMϕ.

While all Mϕ groups produced NO in response to LPS/IFN-γ, NeMϕ produced less NO than did ThioMϕ (Fig. 3*B*), consistent with our previous data (Fig. 1*D*). Of note, WT NeMϕ showed increased responsiveness to LPS/IFN-γ in comparison to IL-4$^{-/-}$ NeMϕ and produced higher levels of NO. This implies that activation by IL-4 may in fact enhance responsiveness to classical activation stimuli as previously reported (15, 16).

The IL-4-dependent mechanism of proliferative suppression is still unknown but one possibility is that arginase acts by depleting arginine needed for cell growth (34). To test this possibility, WT NeMϕ were treated with nor-NOHA to inhibit arginase 1 activity. This led to partial reversal of the suppressive phenotype, with nor-NOHA treatment enhancing proliferation of the EL-4 cells cocultured with NeMϕ (Fig. 3*C*). However, EL-4 cells cocultured with nor-NOHA-treated ThioMϕ also exhibited dramatically enhanced proliferation. This suggests that inhibition of even constitutive levels of arginase has significant effects on cellular proliferation. In the presence of nor-NOHA, NeMϕ permitted only one-fifth the level of EL-4 proliferation as similarly treated ThioMϕ, suggesting that arginine consumption is not the dominant mechanism of suppression in NeMϕ.

NeMϕ express cell surface activation markers that can be further up-regulated in response to LPS/IFN-γ

We studied the surface expression of MHC class II and costimulatory molecules B7.1 and B7.2 to determine the activation status of NeMϕ before and after LPS/IFN-γ treatment (Fig. 4*A*). Untreated NeMϕ expressed higher levels of MHC class II, B7.1, and B7.2 than did ThioMϕ, as shown by MFI. The high expression of these markers is consistent with the original classification of AAMϕ as "activated" (35) and the data that ThioMϕ are relatively "inactive" (27). It is also consistent with previous studies showing that NeMϕ are efficient APCs (36). Upon LPS/IFN-γ stimulation, the MFI of all markers was increased in both ThioMϕ and NeMϕ,

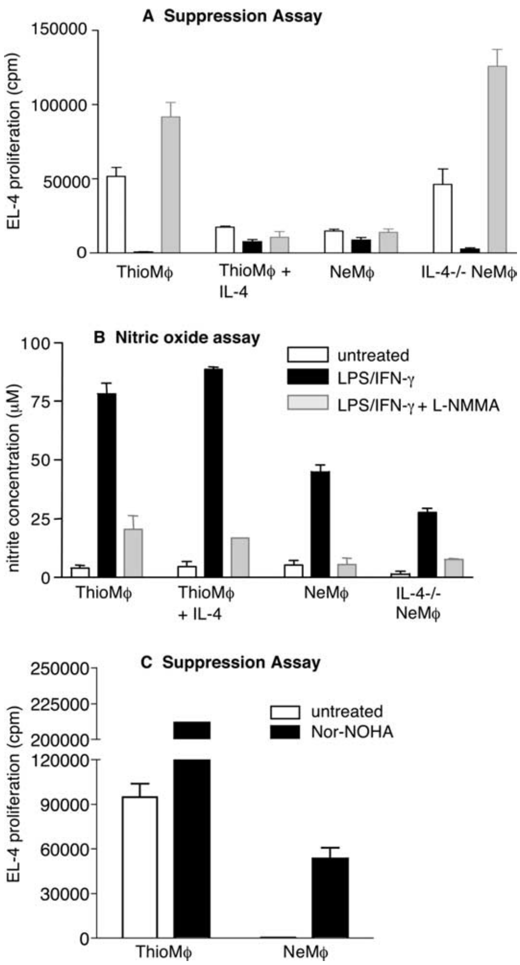

FIGURE 3. NeMϕ can produce NO in response to LPS/IFN-γ but will still retain the ability to suppress cell proliferation in a NO-independent manner. ThioMϕ, ThioMϕ + IL-4, NeMϕ, and IL-4$^{-/-}$ NeMϕ were left untreated or were treated with LPS/IFN-γ overnight followed by replacement of the medium and coculture with EL-4 thymoma cells with or without the NO inhibitor L-NMMA. After 48 h, the EL-4 cell proliferation was assessed by [^{3}H]thymidine incorporation (*A*) and supernatants were recovered for measurement of the NO levels (*B*). Data are representative of three separate experiments and are plotted as the means of triplicate wells (±SEM). In a separate experiment ThioMϕ and NeMϕ were treated with the arginase inhibitor nor-NOHA. They were cocultured with EL-4 thymoma cells and again after 48 h the EL-4 cell proliferation was assessed by [^{3}H]thymidine incorporation (*C*). EL-4 cells cultured alone proliferated at 150,000 cpm (*A*) and 250,000 cpm (*B*).

and the MFI for MHC class II and B7.2 was highest in LPS/IFN-γ treated NeMϕ.

PD-L1 and PD-L2 are members of the B7 family of costimulatory molecules and have been reported as useful markers to distinguish between classical and alternative activation of Mϕ in vitro (37). Additionally, they have been implicated in the proliferative suppression observed by Mϕ during infection with the platyhelminths *Taenia crassiceps* and *Schistosoma mansoni* (38, 39). We thus measured surface expression of both PD-L1 and PD-L2 in NeMϕ (Fig. 4*B*). PD-L1 surface expression was similar in both ThioMϕ and NeMϕ and was up-regulated in both Mϕ groups in response to LPS/IFN-γ, consistent with reports that classically activated Mϕ show increased PD-L1 expression (37). Surprisingly, despite previous studies reporting PD-L2 as a marker for in vitro AAMϕ (37), neither untreated or LPS/IFN-γ-treated NeMϕ expressed PD-L2. To confirm that the PD-L2 staining was optimal, we demonstrated that in vitro IL-4 treatment could up-regulate PD-L2 on ThioMϕ (Fig. 4*B*) as previously shown (37). Our finding that in vivo AAMϕ display different surface expression profiles to in vitro-derived AAMϕ is consistent with previous studies that reported differences in gene expression profiles and cell morphology (40), and it reiterates the importance of in vivo models for the study of these activated Mϕ subsets. Furthermore, in contrast to suppressive Mϕ found in platyhelminth infection (38, 39), these data suggest that the PD-L1/PD-L2 costimulatory molecules may not be responsible for the suppression we observe in nematode infection.

TNF-α, IL-6, and IL-10, but not IL-12p40, production is enhanced in response to LPS/IFN-γ

ThioMϕ, in vitro AAMϕ, and NeMϕ were again left untreated or were treated overnight with LPS/IFN-γ and then supernatants were taken for cytokine measurement by sandwich ELISA or CBA. Fig. 5 shows that TNF-α was absent in all groups but was induced in response to LPS/IFN-γ in both ThioMϕ and ThioMϕ plus IL-4. Although TNF-α was induced to a much lesser extent in NeMϕ than in ThioMϕ in the experiment shown, TNF is consistently up-regulated in NeMϕ by LPS/IFN-γ treatment and can sometimes exceed levels seen in similarly treated ThioMϕ (data not shown). Therefore, pretreatment with IL-4, or induction in an IL-4 environment, did not prevent TNF-α from being induced by type 1 stimuli, consistent with previous reports from Stout et al. (16). Untreated NeMϕ produced high levels of IL-6 relative to ThioMϕ, and all groups displayed an increase in IL-6 expression with LPS/IFN-γ treatment (Fig. 5).

Enhancement of TNF-α and IL-6 together provided more evidence that NeMϕ can be reprogrammed to increase their proinflammatory capacity. However, the switch to a more classical activation state was not complete. Whereas ThioMϕ and in vitro-derived AAMϕ produced IL-12p40 in response to LPS/IFN-γ, NeMϕ completely failed to do so (Fig. 5). Consistent with previous reports that IL-10 is produced by AAMϕ (41), IL-10 was produced to the greatest extent by NeMϕ and increased following LPS/IFN-γ treatment.

LPS/IFN-γ enables NeMϕ to control Leishmania *infection*

As LPS/IFN-γ treatment could induce alternatively activated NeMϕ to a produce NO and TNF, we asked whether this flexibility was reflected in the functional ability to control infection by an intracellular pathogen. To address this question, *L. mexicana* promastigotes were used to infect untreated and LPS/IFN-γ-stimulated NeMϕ and BMMϕ. Parasite survival after 3 h was determined by lysis of the Mϕ and measurement of the parasite proliferation by [^{3}H]thymidine incorporation (Fig. 6). Untreated NeMϕ had the most surviving promastigotes after 3 h. This likely correlates with enhanced parasite uptake. The NeMϕ group recorded more than 10,000 cpm compared with BMMϕ with or without LPS/IFN-γ, which had less than one-tenth the cpm (Fig. 6; 1000 cpm). NeMϕ may have encountered signals in vivo that increase expression of the receptors involved in the phagocytosis of *Leishmania* (e.g., complement receptors) (42). The thymidine count for NeMϕ treated with LPS/IFN-γ was 2500 cpm, which suggests that this group only allowed uptake of approximately one-fourth the number of promastigotes, perhaps due to the absence or down-regulation of these receptors (Fig. 6).

To assess the capacity of different Mϕ populations to control *L. mexicana* growth, we determined the percentage of Mϕ infected after 3 days by microscopy. Fig. 7*A* shows that LPS/IFN-γ treatment, and to a lesser extent IFN-γ alone, conferred resistance to

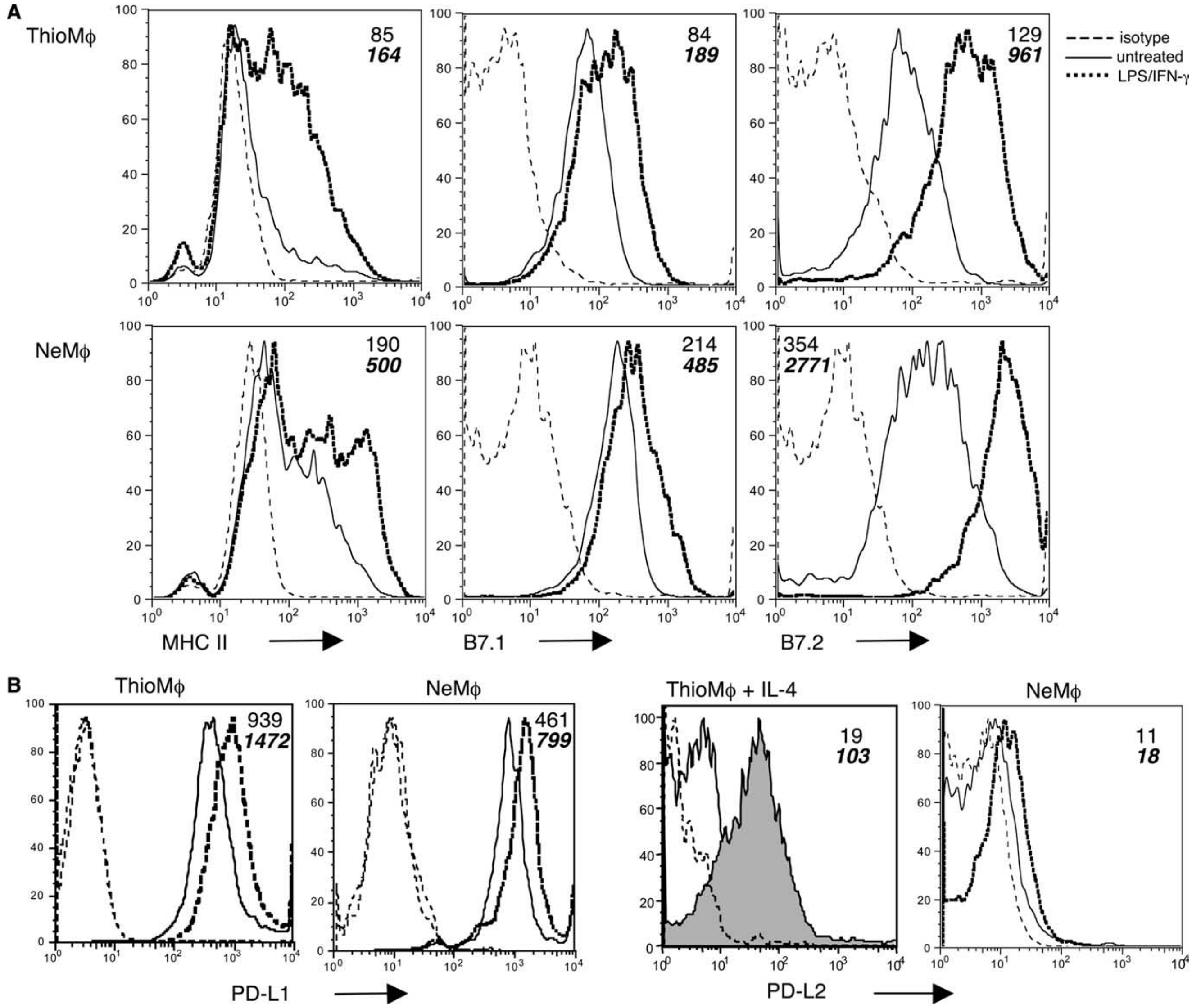

FIGURE 4. Effect of LPS/IFN-γ on the cell surface activation markers in naive Mϕ and NeMϕ. Untreated ThioMϕ, IL-4-treated ThioMϕ (tinted), or NeMϕ were left untreated or treated overnight with LPS/IFN-γ. The cells were recovered and double-stained for F4/80 and MHC class II, B7.1, B7.2 (*A*) or PD-L1 and PD-L2 (*B*). Flow cytometry graphs show histograms of F4/80-gated Mϕ, and dashed lines show the isotype control. MFI of histograms for untreated or treated Mϕ are displayed. Results are representative of three experiments.

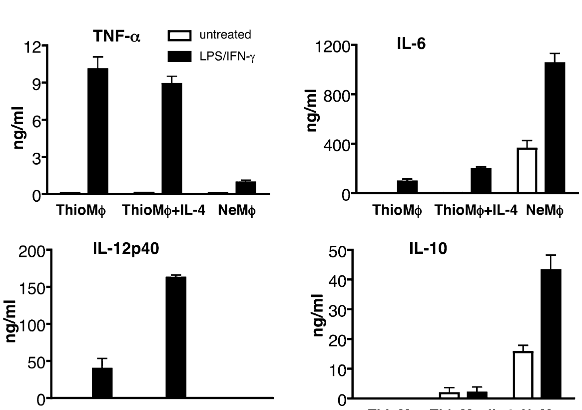

FIGURE 5. TNF-α, IL-6, and IL-10, but not IL-12p40, production is enhanced in response to LPS/IFN-γ. ThioMϕ, ThioMϕ + IL-4, and NeMϕ were untreated (open bars) or were treated overnight with LPS/IFN-γ (filled bars). Supernatants were recovered and the levels of various cytokines were measured by sandwich ELISA (TNF-α, IL-6, and IL-12p40) or CBA (IL-10). Results are shown as the mean of replicate samples (±SEM) and are representative of three experiments.

L. mexicana in both BMMϕ and NeMϕ. Indeed, NeMϕ appeared better able to clear infection when treated with IFN-γ alone than did BMMϕ.

In a separate experiment we examined the role of NO in the leishmanicidal activity observed by NeMϕ treated with LPS/IFN-γ. L-NMMA was used to inhibit iNOS activity, as verified by nitrite levels of the day 3 supernatant (Fig. 7*B*), and the Mϕ were again examined after 3 days of infection (Fig. 7*C*). In agreement with the previous experiment, LPS/IFN-γ treatment of NeMϕ conferred an increased ability to kill *Leishmania*, but in the presence of iNOS inhibitors, NeMϕ treated with LPS/IFN-γ were rendered as susceptible to infection as those not treated (Fig. 7*C*). These data demonstrate that NO is playing an important role in microbicidal activity of LPS/IFN-γ-activated NeMϕ.

To visualize these results, experiments were also conducted with promastigotes of a *L. mexicana* strain that displays red fluorescence (24). In these experiments ThioMϕ were used as controls. Fig. 7*D* shows that in the untreated groups there is an abundance of red intracellular parasites at day 3, but in all LPS/IFN-γ-treated groups we saw an almost complete absence of *L. mexicana*. Thus,

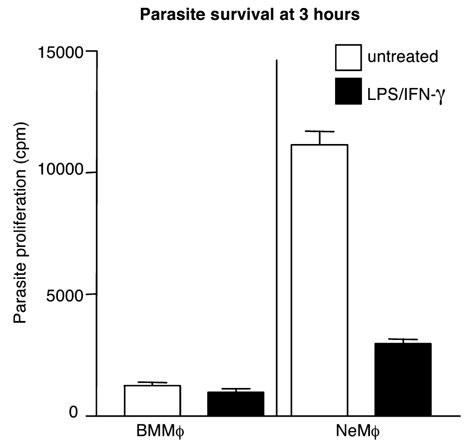

FIGURE 6. Untreated NeMϕ take up the most *L. mexicana* parasites. BMMϕ and NeMϕ were untreated (open bar) or were treated overnight with LPS/IFN-γ (filled bar) followed by infection with *L. mexicana* at 10:1 parasite/Mϕ ratio. Parasites that were not taken up were washed off 3 h postinfection, and parasite uptake was determined by lysis of the Mϕ and measurement of the parasite proliferation by [^{3}H]thymidine incorporation. Results are shown as the means of replicate samples ($\pm$SEM) and are representative of three experiments.

these type 1 stimuli again conferred full resistance to this intracellular parasite, regardless of Mϕ history. These results were confirmed by microscopic examination (Fig. 7*E*). Mϕ were also stained with anti-Ym1 to confirm their AAMϕ status before infection. To our surprise, Ym1 staining was still detectable following LPS/IFN-γ treatment (Fig. 7*D*, green). As this contrasted with our previous data in which *Ym1* mRNA decreases upon LPS/IFN-γ treatment (Fig. 1*E*), we chose to repeat these experiments to measure both protein and RNA simultaneously. The finding confirmed those in Figs. 1*E* and 7*D* by demonstrating that while mRNA levels decrease in response to LPS/IFN-γ, protein is still detectable (data not shown).

Discussion

Mϕ can be involved in both pro- and antiinflammatory responses, in tissue destructive activities as well as in restorative activities (1, 43). Increasing evidence suggests that Mϕ activated in a Th2 setting have tissue repair as a primary function. This hypothesis has been driven by the knowledge that proline, which is an important precursor of collagen and polyamines that are involved in cell proliferation, is produced by AAMϕ under the control of arginase (5) and is supported by subsequent data that AAMϕ are involved in fibrosis (6). Recent data from our laboratory strongly support this hypothesis, as the key features of alternative activation are induced solely in response to tissue injury (20, 44). It is understandable that a strong wound healing response would occur in the context of helminth infection, as tissue migratory or tissue invasive parasites often lead to physical trauma to host tissue. Importantly, the entire process of tissue repair requires the production of antiinflammatory factors (45). Consistent with this, NeMϕ produce many factors associated with tissue repair and reduced inflammation (e.g., arginase 1, IL-10 (Figs. 1*A* and 5, respectively) and TGF-β (data not shown)) (46, 47). We wanted to determine if the immune system, faced with a potentially more life-threatening situation (i.e., a bacterial pathogen), had the capacity to rapidly switch from a wound healing to a proinflammatory response. Such functional plasticity would allow AAMϕ involved in a tissue repair to respond appropriately to a microbial challenge, and this would be beneficial before the recruitment of new cells to the site of dual infection.

We show herein that a Mϕ population generated in the Th2 environment of helminth infection in vivo can respond to proinflammatory stimuli ex vivo. Following overnight incubation of NeMϕ with IFN-γ and LPS, there was reduced expression of transcripts for the alternative activation markers arginase, RELM-α, and Ym1. This reduction was accompanied by a dramatic up-regulation of iNOS mRNA. Enzyme activity changes were also seen but to a lesser degree than in control macrophages. Nonetheless, the amount of NO produced was sufficient to fully control infection

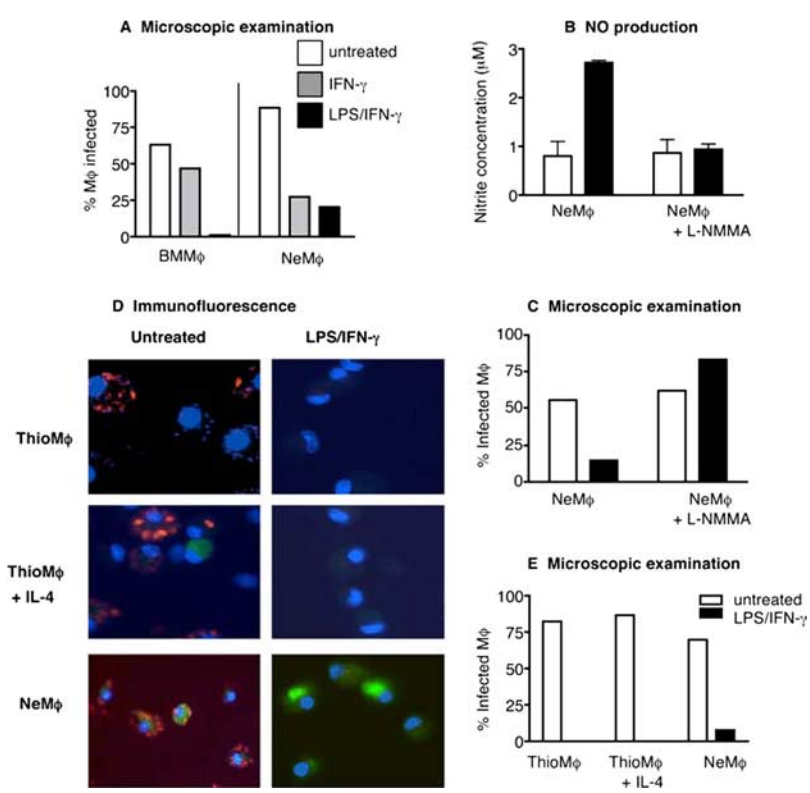

FIGURE 7. LPS/IFN-γ or IFN-γ alone confers resistance of NeMϕ to *L. mexicana* infection at day 3 postinfection. BMMϕ and NeMϕ were untreated (open bars) or treated overnight with IFN-γ (gray bars) or LPS/IFN-γ (black bars). The NO inhibitor L-NMMA was also added (*B* and *C*). Infection with *L. mexicana* was at a 10:1 parasite/Mϕ ratio. Parasite levels after 3 days were measured by microscopic examination of the number of infected Mϕ (100–200 Mϕ counted per group) (*A* and *C*). The supernatants were recovered for measurement of the NO levels (*B*). In another experiment, ThioMϕ, ThioMϕ +IL-4, and NeMϕ were treated as before followed by a 30:1 infection ratio. Immunofluoresence assays were conducted using a *L. mexicana* strain that displayed red fluorescence, along with Ym-1 staining (green) (*D*). Parasite levels after 3 days were again measured by Giemsa examination (*E*). Results are representative of three experiments.

with an intracellular pathogen, suggesting that NeMϕ can divert their resources toward antimicrobial activity if needed. We also observed an increase in surface expression of class II and costimulatory molecules (Fig. 4*A*) under the influence of LPS/IFN-γ. This suggests that these AAMϕ have enhanced APC function. However, analysis of the NeMϕ cytokine profile (Fig. 5) presented an intriguing picture with implications for APC function. Although LPS/IFN-γ treatment appeared to promote a more classically activated phenotype in the AAMϕ through elevated TNF-α, IL-6, and NO production, this switch was not complete, and they failed to produce any detectable IL-12p40 (Fig. 5). Thus, although these cells had enhanced antimicrobial killing and increased markers of classical activation, they would not induce Th1 cell development (48). Consistent with this, LPS/IFN-γ treatment caused the up-regulation of IL-10 in NeMϕ (Fig. 5), which inhibits Th1 cell responses (49). Lamina propria Mϕ from the intestine of mice, which like NeMϕ express relatively high IL-10 and TGF-β, also produce little or no IL-12 after stimulation with TLR ligands (50). Although the authors concluded that the IL-10-producing Mϕ are hyporesponsive to TLR ligands, only IL-12 was assessed, and thus it is possible that the defect, as in this study, was specific for IL-12.

Consistent with a specific defect in LPS-mediated IL-12 production but not NO production, recent work from Foster et al. has shown that TLR-induced genes with different functions may have diverse regulatory requirements (51). On an epigenetic level, gene-specific modifications of chromatin can lead to the priming of antimicrobial effectors while proinflammatory mediators, such as IL-6, are silenced. This would potentially allow the immune system to effectively deal with microbial pathogens while controlling the pathology associated with inflammation (51). The fact that we have seen the products of some genes associated with an antimicrobial response up-regulated in response to LPS/IFN-γ in NeMϕ (i.e., iNOS) but not to IL-12 suggests that these AAMϕ may have undergone similar epigenetic modifications in vivo. Indeed, a recent study by Wen et al. has shown that dendritic cells recovered from mice that have survived severe peritonitis-induced sepsis display a chronic suppression of IL-12 expression. The IL-12 deficiency of dendritic cells from this setting is due to histone modification (52). Similarly, the propensity of NeMϕ to produce large amounts of IL-10 following LPS/IFN-γ stimulation may reflect chromatin modifications to the IL-10 promoter locus, as shown for superinduction of IL-10 by immune complexes (53).

We have shown that LPS/IFN-γ treatment could confer resistance of NeMϕ to infection with *L. mexicana* promastigotes. Models of murine *Leishmania* spp. involve a dominant Th1 response leading to the classical activation of Mϕ and elimination of the parasites through the production of NO (54). NeMϕ were able to control infection almost as well as BMMϕ given the same treatment of LPS/IFN-γ (Fig. 7*A*). The ability of NeMϕ given IFN-γ alone to control parasite numbers more effectively than for BMMϕ may be explained by signals that NeMϕ encounter in vivo causing the up-regulation of the receptor for IFN-γ. Consistent with this hypothesis, NeMϕ produced more iNOS mRNA in response to IFN-γ treatment than ThioMϕ (Fig. 1*B*).

Previous work in the field has shown that alternative Mϕ activation can promote *Leishmania* growth (55, 56). This is because L-ornithine synthesized from arginase 1 activity, which is largely controlled by IL-4, can be used by the parasites to generate polyamines and to proliferate (56, 57). The role of IL-4 for promoting susceptibility in nonhealing murine *L. mexicana* infections has been demonstrated, since susceptible mice lacking IL-4 or STAT6 acquire a Th1 response and fail to develop lesions (58–60). Although these above findings may seem at odds with the results presented herein, IL-4 does not always promote susceptibility. IL-4 has been shown to provide a strong stimulus for the killing of intracellular amastigotes of *L. major*, as long as low concentrations of IFN-γ are present (61). Similarly, pretreatment with IL-13 has been shown to increase the ability of Mϕ to effectively deal with the intracellular protozoan parasite *T. gondii* after LPS activation (18), and this was found to be due to enhanced NO production (62). We found that susceptibility of NeMϕ to *L. mexicana* decreased dramatically when treated with even IFN-γ alone, as well as with LPS/IFN-γ (Fig. 7*A*). Although NeMϕ from our *B. malayi* infection model produced lower levels of NO after LPS/IFN-γ treatment than did ThioMϕ (Fig. 1*D*) or IL-4 pretreated ThioMϕ (data not shown), they were still enough to confer resistance. Even when NeMϕ were infected at a 30:1 ratio of parasites/Mϕ, they still managed to clear the parasite upon LPS/IFN-γ treatment (Fig. 7, *D* and *E*). Thus, despite extensive exposure to type 2 cytokines in vivo, the NeMϕ maintained an IFN-γ-responsive phenotype that allowed the manifestation of a fully antimicrobial phenotype. As these experiments were performed ex vivo, they do not allow us to conclude the full infection outcome in the context of a Th2-mediated response that would be maintained in vivo. As such, the data are not directly comparable to studies in which helminth infection exacerbated leishmania infection (55, 63). Nonetheless, they do allow us to dissect the potential for functional adaptation of in vivo-generated AAMϕ.

A consistent feature of NeMϕ and other helminth-induced Mϕ is that they act as potent suppressors of cellular proliferation (22, 64), and several different mechanisms for the suppression have been proposed. We recently provided evidence that TGF-β may be involved to some degree (21) and have now seen that arginase contributes to this suppression since inhibition with nor-NOHA caused a partial reversal of this phenotype. However, both arginase (Fig. 1, *A* and *C*) and TGF-β (data not shown) production were partially reversed upon treatment with LPS/IFN-γ, and yet these cells retained full suppressive abilities (Fig. 3*A*), so additional factors must be involved. PD-L1 has been implicated in the proliferative suppression observed by Mϕ during infection with the platyhelminths *T. crassiceps* and *S. mansoni* (38, 39) and would be consistent with a contact-dependent mechanism (22). However, we did not find evidence for up-regulation of PD-L1 on NeMϕ compared with the ThioMϕ controls (Fig. 4*B*). Also, NeMϕ did not up-regulate another marker of AAMϕ generated in vitro, PD-L2 (Fig. 4*B*). Data now emerging from several laboratoties would suggest that the profound suppression observed may be due to multiple antiproliferative mechanisms that in addition to PD-L interactions (38, 39) include TGF-β production (21) lipid mediator release (65) and IL-10 production (66, 67).

We have shown that despite long-term exposure to Th2 cytokines and antiinflammatory signals in vivo, Mϕ could respond to the Th1-activating signals LPS/IFN-γ and become effectively antimicrobial. These ex vivo findings may not translate directly to in vivo coinfection studies where cross-regulation will be occurring at the level of the systemic T cell response as well as local effector responses. However, they do provide evidence that Mϕ highly polarized in vivo maintain remarkable phenotypic plasticity and are very consistent with recent studies in which the abilities of tumor-promoting AAMϕ to become classically activated and promote tumor killing have been demonstrated (68, 69). Watkins et al. demonstrated that systemic treatment of tumor-bearing mice with IL-12 altered the functional response of Mϕ from a predominantly antiinflammatory to a more proinflammatory phenotype, including up-regulation of TNF-α and IL-6. Importantly, these changes were sustained over many days (70). These data support the viability of therapeutic approaches to alter Mϕ polarization in vivo (71, 72). Our data do not show that AAMϕ make a complete conversion to

CAMϕ but rather that they adopt key aspects of the CAMϕ phenotype necessary for a particular function, consistent with the partially overlapping phenotypes observed during in vitro studies (9, 16, 17). Before the therapeutic potential can be fully realized a much greater understanding of the mechanisms and limitations of Mϕ plasticity are needed.

Acknowledgments

We thank Toni Aebischer for helpful discussion, use of reagents, and critical review of the manuscript. We also thank members of the Allen and Maizels laboratories for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

References

1. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* 3: 23–35.
2. Yu, K., C. Mitchell, Y. Xing, R. S. Magliozzo, B. R. Bloom, and J. Chan. 1999. Toxicity of nitrogen oxides and related oxidants on mycobacteria: *M. tuberculosis* is resistant to peroxynitrite anion. *Tubercle Lung Dis.* 79: 191–198.
3. Munder, M., K. Eichmann, and M. Modolell. 1998. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4$^+$ T cells correlates with Th1/Th2 phenotype. *J. Immunol.* 160: 5347–5354.
4. Modolell, M., I. M. Corraliza, F. Link, G. Soler, and K. Eichmann. 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur. J. Immunol.* 25: 1101–1104.
5. Hesse, M., M. Modolell, A. C. La Flamme, M. Schito, J. M. Fuentes, A. W. Cheever, E. J. Pearce, and T. A. Wynn. 2001. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J. Immunol.* 167: 6533–6544.
6. Wynn, T. A. 2004. Fibrotic disease and the T_H1/T_H2 paradigm. *Nat. Rev. Immunol.* 4: 583–594.
7. Stout, R. D., and J. Suttles. 2004. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J. Leukocyte Biol.* 76: 509–513.
8. Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25: 677–686.
9. Porcheray, F., S. Viaud, A. C. Rimaniol, C. Leone, B. Samah, N. Dereuddre-Bosquet, D. Dormont, and G. Gras. 2005. Macrophage activation switching: an asset for the resolution of inflammation. *Clin. Exp. Immunol.* 142: 481–489.
10. Edwards, J. P., X. Zhang, K. A. Frauwirth, and D. M. Mosser. 2006. Biochemical and functional characterization of three activated macrophage populations. *J. Leukocyte Biol.* 80: 1298–1307.
11. Joyce, D. A., and J. H. Steer. 1996. IL-4, IL-10 and IFN-gamma have distinct, but interacting, effects on differentiation-induced changes in TNF-alpha and TNF receptor release by cultured human monocytes. *Cytokine* 8: 49–57.
12. Lang, R., D. Patel, J. J. Morris, R. L. Rutschman, and P. J. Murray. 2002. Shaping gene expression in activated and resting primary macrophages by IL-10. *J. Immunol.* 169: 2253–2263.
13. Erwig, L. P., D. C. Kluth, G. M. Walsh, and A. J. Rees. 1998. Initial cytokine exposure determines function of macrophages and renders them unresponsive to other cytokines. *J. Immunol.* 161: 1983–1988.
14. Herbert, D. R., C. Holscher, M. Mohrs, B. Arendse, A. Schwegmann, M. Radwanska, M. Leeto, R. Kirsch, P. Hall, H. Mossmann, et al. 2004. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity* 20: 623–635.
15. Major, J., J. E. Fletcher, and T. A. Hamilton. 2002. IL-4 pretreatment selectively enhances cytokine and chemokine production in lipopolysaccharide-stimulated mouse peritoneal macrophages. *J. Immunol.* 168: 2456–2463.
16. Stout, R. D., C. Jiang, B. Matta, I. Tietzel, S. K. Watkins, and J. Suttles. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J. Immunol.* 175: 342–349.
17. Gratchev, A., J. Kzhyshkowska, K. Kothe, I. Muller-Molinet, S. Kannookadan, J. Utikal, and S. Goerdt. 2006. Mö1 and Mö2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. *Immunobiology* 211: 473–486.
18. Authier, H., S. Cassaing, V. Bans, P. Batigne, M. H. Bessieres, and B. Pipy. 2007. IL-13 pre-treatment of murine peritoneal macrophages increases their anti-*Toxoplasma gondii* activity induced by lipopolysaccharides. *Int. J. Parasitol.* 38: 341–352.
19. Loke, P., M. G. Nair, J. Parkinson, D. Guiliano, M. Blaxter, and J. E. Allen. 2002. IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. *BMC Immunol.* 3: 7.
20. Nair, M. G., I. J. Gallagher, M. D. Taylor, P. Loke, P. S. Coulson, R. A. Wilson, R. M. Maizels, and J. E. Allen. 2005. Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infect. Immun.* 73: 385–394.
21. Taylor, M. D., A. Harris, M. G. Nair, R. M. Maizels, and J. E. Allen. 2006. F4/80$^+$ alternatively activated macrophages control CD4$^+$ T cell hyporesponsiveness at sites peripheral to filarial infection. *J. Immunol.* 176: 6918–6927.
22. Loke, P., A. S. MacDonald, A. Robb, R. M. Maizels, and J. E. Allen. 2000. Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact. *Eur. J. Immunol.* 30: 2669–2678.
23. Dransfield, I., E. Stephenson, and C. Haslett. 1996. Recognition of apoptotic cells by phagocytes. In *Techniques in Apoptosis: A User's Guide*. T. G. Cotter and S. J. Martin, eds. Portland Press, London, pp. 149–174.
24. Sorensen, M., C. Lippuner, T. Kaiser, A. Misslitz, T. Aebischer, and D. Bumann. 2003. Rapidly maturing red fluorescent protein variants with strongly enhanced brightness in bacteria. *FEBS Lett.* 552: 110–114.
25. Misslitz, A., J. C. Mottram, P. Overath, and T. Aebischer. 2000. Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in *Leishmania* amastigotes. *Mol. Biochem. Parasitol.* 107: 251–261.
26. Proudfoot, L., C. A. O'Donnell, and F. Y. Liew. 1995. Glycoinositolphospholipids of *Leishmania major* inhibit nitric oxide synthesis and reduce leishmanicidal activity in murine macrophages. *Eur. J. Immunol.* 25: 745–750.
27. Leijh, P. C., T. L. van Zwet, M. N. ter Kuile, and R. van Furth. 1984. Effect of thioglycolate on phagocytic and microbicidal activities of peritoneal macrophages. *Infect. Immun.* 46: 448–452.
28. Lin, H. H., D. E. Faunce, M. Stacey, A. Terajewicz, T. Nakamura, J. Zhang-Hoover, M. Kerley, M. L. Mucenski, S. Gordon, and J. Stein-Streilein. 2005. The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance. *J. Exp. Med.* 201: 1615–1625.
29. Taylor, M. D., L. LeGoff, A. Harris, E. Malone, J. E. Allen, and R. M. Maizels. 2005. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. *J. Immunol.* 174: 4924–4933.
30. Allen, J. E., R. A. Lawrence, and R. M. Maizels. 1996. APC from mice harbouring the filarial nematode, *Brugia malayi*, prevent cellular proliferation but not cytokine production. *Int. Immunol.* 8: 143–151.
31. Albina, J. E., J. A. Abate, and W. L. Henry, Jr. 1991. Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation: role of IFN-gamma in the induction of the nitric oxide-synthesizing pathway. *J. Immunol.* 147: 144–148.
32. Albina, J. E., and W. L. Henry, Jr. 1991. Suppression of lymphocyte proliferation through the nitric oxide synthesizing pathway. *J. Surg. Res.* 50: 403–409.
33. Mills, C. D. 1991. Molecular basis of "suppressor" macrophages: arginine metabolism via the nitric oxide synthetase pathway. *J. Immunol.* 146: 2719–2723.
34. Munder, M., H. Schneider, C. Luckner, T. Giese, C. D. Langhans, J. M. Fuentes, P. Kropf, I. Mueller, A. Kolb, M. Modolell, and A. D. Ho. 2006. Suppression of T-cell functions by human granulocyte arginase. *Blood* 108: 1627–1634.
35. Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* 176: 287–292.
36. Loke, P., A. S. MacDonald, and J. E. Allen. 2000. Antigen-presenting cells recruited by *Brugia malayi* induce Th2 differentiation of naive CD4$^+$ T cells. *Eur. J. Immunol.* 30: 1127–1135.
37. Loke, P., and J. P. Allison. 2003. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc. Natl. Acad. Sci. USA* 100: 5336–5341.
38. Terrazas, L. I., D. Montero, C. A. Terrazas, J. L. Reyes, and M. Rodriguez-Sosa. 2005. Role of the programmed death-1 pathway in the suppressive activity of alternatively activated macrophages in experimental cysticercosis. *Int. J. Parasitol.* 35: 1349–1358.
39. Smith, P., C. M. Walsh, N. E. Mangan, R. E. Fallon, J. R. Sayers, A. N. McKenzie, and P. G. Fallon. 2004. *Schistosoma mansoni* worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages. *J. Immunol.* 173: 1240–1248.
40. Nair, M. G., D. W. Cochrane, and J. E. Allen. 2003. Macrophages in chronic type 2 inflammation have a novel phenotype characterized by the abundant expression of Ym1 and Fizz1 that can be partly replicated in vitro. *Immunol. Lett.* 85: 173–180.
41. Katakura, T., M. Miyazaki, M. Kobayashi, D. N. Herndon, and F. Suzuki. 2004. CCL17 and IL-10 as effectors that enable alternatively activated macrophages to inhibit the generation of classically activated macrophages. *J. Immunol.* 172: 1407–1413.
42. Mosser, D. M., and P. J. Edelson. 1985. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania promastigotes*. *J. Immunol.* 135: 2785–2789.
43. Stout, R. D., and J. Suttles. 1997. T cell signaling of macrophage function in inflammatory disease. *Front. Biosci.* 2: d197–206.
44. Loke, P., I. Gallagher, M. G. Nair, X. Zang, F. Brombacher, M. Mohrs, J. P. Allison, and J. E. Allen. 2007. Alternative activation is an innate response to injury that requires CD4$^+$ T cells to be sustained during chronic infection. *J. Immunol.* 179: 3926–3936.
45. Eming, S. A., T. Krieg, and J. M. Davidson. 2007. Inflammation in wound repair: molecular and cellular mechanisms. *J. Invest. Dermatol.* 127: 514–525.
46. Pierce, G. F., T. A. Mustoe, J. Lingelbach, V. R. Masakowski, P. Gramates, and T. F. Deuel. 1989. Transforming growth factor beta reverses the glucocorticoid-induced wound-healing deficit in rats: possible regulation in macrophages by platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA* 86: 2229–2233.
47. Albina, J. E., C. D. Mills, W. L. Henry, Jr., and M. D. Caldwell. 1990. Temporal expression of different pathways of L-arginine metabolism in healing wounds. *J. Immunol.* 144: 3877–3880.

48. Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of TH1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 260: 547–549.
49. Hsieh, C. S., A. B. Heimberger, J. S. Gold, A. O'Garra, and K. M. Murphy. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. *Proc. Natl. Acad. Sci. USA* 89: 6065–6069.
50. Denning, T. L., Y. C. Wang, S. R. Patel, I. R. Williams, and B. Pulendran. 2007. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat. Immunol.* 8: 1086–1094.
51. Foster, S. L., D. C. Hargreaves, and R. Medzhitov. 2007. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* 447: 972–978.
52. Wen, H., Y. Dou, C. M. Hogaboam, and S. L. Kunkel. 2008. Epigenetic regulation of dendritic cell-derived interleukin-12 facilitates immunosuppression after a severe innate immune response. *Blood* 111: 1797–1804.
53. Zhang, X., J. P. Edwards, and D. M. Mosser. 2006. Dynamic and transient remodeling of the macrophage IL-10 promoter during transcription. *J. Immunol.* 177: 1282–1288.
54. Alexander, J., A. R. Satoskar, and D. G. Russell. 1999. Leishmania species: models of intracellular parasitism. *J. Cell Sci.* 112: 2993–3002.
55. Rodriguez-Sosa, M., I. Rivera-Montoya, A. Espinoza, M. Romero-Grijalva, R. Lopez-Flores, J. Gonzalez, and L. I. Terrazas. 2006. Acute cysticercosis favours rapid and more severe lesions caused by *Leishmania major* and *Leishmania mexicana* infection, a role for alternatively activated macrophages. *Cell. Immunol.* 242: 61–71.
56. Kropf, P., J. M. Fuentes, E. Fahnrich, L. Arpa, S. Herath, V. Weber, G. Soler, A. Celada, M. Modolell, and I. Muller. 2005. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. *FASEB J.* 19: 1000–1002.
57. Iniesta, V., L. C. Gomez-Nieto, I. Molano, A. Mohedano, J. Carcelen, C. Miron, C. Alonso, and I. Corraliza. 2002. Arginase I induction in macrophages, triggered by Th2-type cytokines, supports the growth of intracellular *Leishmania* parasites. *Parasite Immunol.* 24: 113–118.
58. Satoskar, A., H. Bluethmann, and J. Alexander. 1995. Disruption of the murine interleukin-4 gene inhibits disease progression during *Leishmania mexicana* infection but does not increase control of *Leishmania donovani* infection. *Infect. Immun.* 63: 4894–4899.
59. Satoskar, A., F. Brombacher, W. J. Dai, I. McInnes, F. Y. Liew, J. Alexander, and W. Walker. 1997. SCID mice reconstituted with IL-4-deficient lymphocytes, but not immunocompetent lymphocytes, are resistant to cutaneous leishmaniasis. *J. Immunol.* 159: 5005–5013.
60. Noben-Trauth, N., P. Kropf, and I. Muller. 1996. Susceptibility to *Leishmania major* infection in interleukin-4-deficient mice. *Science* 271: 987–990.
61. Bogdan, C., S. Stenger, M. Rollinghoff, and W. Solbach. 1991. Cytokine interactions in experimental cutaneous leishmaniasis: interleukin 4 synergizes with interferon-gamma to activate murine macrophages for killing of *Leishmania major* amastigotes. *Eur. J. Immunol.* 21: 327–333.
62. Authier, H., S. Cassaing, A. Coste, P. Balard, A. Gales, A. Berry, V. Bans, M. H. Bessieres, and B. Pipy. 2008. Interleukin-13 primes iNO synthase expression induced by LPS in mouse peritoneal macrophages. *Mol. Immunol.* 45: 235–243.
63. Hassan, M. F., Y. Zhang, C. R. Engwerda, P. M. Kaye, H. Sharp, and Q. D. Bickle. 2006. The *Schistosoma mansoni* hepatic egg granuloma provides a favorable microenvironment for sustained growth of *Leishmania donovani*. *Am. J. Pathol.* 169: 943–953.
64. Reyes, J. L., and L. I. Terrazas. 2007. The divergent roles of alternatively activated macrophages in helminthic infections. *Parasite Immunol.* 29: 609–619.
65. Brys, L., A. Beschin, G. Raes, G. H. Ghassabeh, W. Noel, J. Brandt, F. Brombacher, and P. De Baetselier. 2005. Reactive oxygen species and 12/15-lipoxygenase contribute to the antiproliferative capacity of alternatively activated myeloid cells elicited during helminth infection. *J. Immunol.* 174: 6095–6104.
66. Hesse, M., C. A. Piccirillo, Y. Belkaid, J. Prufer, M. Mentink-Kane, M. Leusink, A. W. Cheever, E. M. Shevach, and T. A. Wynn. 2004. The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. *J. Immunol.* 172: 3157–3166.
67. Osborne, J., and E. Devaney. 1999. Interleukin-10 and antigen-presenting cells actively suppress Th1 cells in BALB/c mice infected with the filarial parasite *Brugia pahangi*. *Infect. Immun.* 67: 1599–1605.
68. Guiducci, C., A. P. Vicari, S. Sangaletti, G. Trinchieri, and M. P. Colombo. 2005. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. *Cancer Res.* 65: 3437–3446.
69. Hagemann, T., T. Lawrence, I. McNeish, K. A. Charles, H. Kulbe, R. G. Thompson, S. C. Robinson, and F. R. Balkwill. 2008. "Re-educating" tumor-associated macrophages by targeting NF-κB. *J. Exp. Med.* 205: 1261–1268.
70. Watkins, S. K., N. K. Egilmez, J. Suttles, and R. D. Stout. 2007. IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo. *J. Immunol.* 178: 1357–1362.
71. Sica, A., P. Allavena, and A. Mantovani. 2008. Cancer related inflammation: the macrophage connection. *Cancer Lett.* 267: 204–215.
72. Sica, A., and V. Bronte. 2007. Altered macrophage differentiation and immune dysfunction in tumor development. *J. Clin. Invest.* 117: 1155–1166.