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Alternatively Activated Macrophages Elicited by Helminth Infection Can Be Reprogrammed to Enable Microbial Killing¹

Katie J. Mylonas,² Meera G. Nair,^{2,3} Lidia Prieto-Lafuente, Daniel Paape, and Judith E. Allen⁴

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 (NeM ϕ), 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 NeM ϕ 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 NeM ϕ 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 NeM ϕ to become antimicrobial, as LPS/IFN- γ -treated NeM ϕ 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 (M ϕ)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 M ϕ phenotypes. Classical activation of M ϕ is dependent on the products of activated Th1 cells, in particular IFN- γ (1). The prime function of classically activated M ϕ (CAM ϕ) 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 M ϕ (AAM ϕ), activated by the Th2 cytokines IL-4 and IL-13, play important roles in allergy and responses to parasite infection (1). AAM ϕ and CAM ϕ exhibit distinct L-arginine metabolism pathways. CAM ϕ produce

NO as a result of the up-regulation of iNOS, which is a catalyst of the L-arginine substrate (3). In AAM ϕ , 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 CAM ϕ has antimicrobial and cytotoxic properties, L-ornithine produced by AAM ϕ 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 AAM ϕ are involved in wound healing (5, 6).

Although M ϕ have been usefully classified in this way, the range of actual phenotypes is likely to be much broader, with AAM ϕ and CAM ϕ representing two points in a wide spectrum. Unlike Th cells where activation leads to terminal differentiation into Th1 and Th2 cells, M ϕ 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, M ϕ 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 M ϕ 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 AAM ϕ phenotype is still a subject of controversy. While some reports show that IL-4 pretreatment of M ϕ 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 M ϕ function dependent on the order and length of each cytokine treatment (16). In another study, human M ϕ were stimulated with either pro- (e.g., TNF- α) or anti-inflammatory (e.g., IL-10) cytokines and then cultured with a

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⁵ Abbreviations used in this paper: M ϕ , macrophage; CAM ϕ , classically activated macrophage; iNOS, inducible NO synthase; AAM ϕ , alternatively activated macrophage; NeM ϕ , nematode-elicited macrophage; PEC, peritoneal exudate cell; RELM- α , resistin-like molecule α ; WT, wild type; BMM ϕ , 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; ThioM ϕ , thioglycollate-elicited macrophage; MFI, mean fluorescence intensity.

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counterstimulatory cytokine or medium alone, and it was found that M ϕ 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 M ϕ demonstrate functional adaptivity in vivo and to ascertain the physiological relevance of this phenomenon. Investigating M ϕ plasticity in vivo could have important implications for therapeutic targeting of M ϕ 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 M ϕ 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 AAM ϕ 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 M ϕ recruited to sites of infection is still unknown.

In this study, we have used a murine model for filarial infection as a source of M ϕ , which we have previously termed nematode-elicited M ϕ (NeM ϕ) (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 M ϕ 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 NeM ϕ share many properties with M ϕ differentiated in vitro in response to IL-4 or IL-13 (hereafter termed in vitro AAM ϕ) (19). However, NeM ϕ are recruited during a chronic Th2-dominated nematode infection and experience much longer term exposure to IL-4 and IL-13 than in vitro-AAM ϕ . Furthermore, NeM ϕ 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 M ϕ show functional adaptivity, we decided to study the NeM ϕ response to Th1-activating signals as a model to investigate whether M ϕ 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 anti-inflammatory signals in vivo, we found that NeM ϕ were still responsive to LPS and IFN- γ , exhibiting dramatic changes in gene expression profile. Additionally, LPS/IFN- γ treated NeM ϕ were able to effectively control infection with *Leishmania mexicana*. However, LPS/IFN- γ treated NeM ϕ still maintained the IL-4-dependent capacity to suppress proliferation and failed to up-regulate IL-12. Taken together, these results demonstrate that M ϕ 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.

M ϕ 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 M ϕ 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. NeM ϕ 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 M ϕ 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 μ M 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 M ϕ (BMM ϕ) 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 M ϕ 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 μ g/ml streptomycin. The medium was replaced after 4–6 days to generate a pure population of M ϕ at day 7. BMM ϕ 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 μ g/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-L1)/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

M ϕ 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 μ M; Sigma-Aldrich) or *N*^ω-hydroxy-nor-L-arginine (nor-NOHA; 250 μ M; Sigma-Aldrich). After 48 h, 100 μ l of supernatant was removed to measure NO production. For each well, 1 μ Ci of [³H]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 μ l of culture supernatant was mixed with 100 μ l 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 μ l of 0.1% Triton X-100. Following a 30-min incubation with shaking, 100 μ l of 25 mM Tris-HCl (pH 7.2) and 20 μ l of 10 mM MnCl₂ were added and the enzyme was activated by heating to 56°C for 10 min. L-Arginine hydrolysis was conducted by incubating 100 μ l of this lysate with 100 μ l 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 μ l of H₂SO₄ (96%)/H₃PO₄ (85%)/H₂O (1/3/7, v/v/v), and 40 μ l 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 μ mol 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 DNaseI 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 house-keeping 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 M ϕ 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 Duo-Set 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 SigmaFast *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.

M ϕ 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 M ϕ , 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 M ϕ 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 H₂O. 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-³H]thymidine (1 µCi/well) for a further 18 h at 26°C, and incorporation was assessed on a liquid scintillation counter (MictoBeta 1450; TriLux). Leishmanicidal activity was measured as reduction in the incorporation of [³H]thymidine by surviving parasites. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. For immunofluorescence assays, M ϕ 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 dH₂O and placed on top of gel/mount on slides and allowed to dry in the dark. Cells were visual-

ized 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 NeM ϕ (19). We decided to investigate whether the Th1-activating signals (LPS and/or IFN- γ) could alter the NeM ϕ iNOS/arginase balance. Thioglycollate-elicited M ϕ (ThioM ϕ) and NeM ϕ 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 NeM ϕ showed high arginase 1 expression but low iNOS RNA levels, while untreated ThioM ϕ 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 NeM ϕ . This decrease was paralleled by an increase in iNOS expression. In contrast, ThioM ϕ required both signals for the induction of iNOS. This could reflect the less mature activation state of this M ϕ 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 ThioM ϕ group.

Although ThioM ϕ 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. 1C). Arginase and iNOS enzyme activities of NeM ϕ 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 NeM ϕ and an increase in NO production. In contrast to the mRNA levels, the NO produced by LPS/IFN- γ -treated NeM ϕ was lower than by LPS/IFN- γ -treated ThioM ϕ . 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 NeM ϕ may compete with iNOS for L-arginine. Nevertheless, the increase in NO production coupled by a decrease in arginase activity demonstrates that NeM ϕ 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 NeM ϕ (19), we investigated whether RELM- α /Ym1 expression was also altered by LPS/IFN- γ treatment. As a control for nondifferentiated M ϕ , we measured RELM- α and Ym1 expression in ThioM ϕ (Fig. 1E). 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 NeM ϕ . Upon overnight treatment with LPS/IFN- γ , RELM- α and Ym1 expression in NeM ϕ was reduced by up to 90% (Fig. 1E). 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 NeM ϕ represented an outgrowth of a naive M ϕ population rather than a change in the existing NeM ϕ population, we monitored the cell proliferation following treatment. Before overnight treatment with LPS/IFN- γ , cells were labeled with

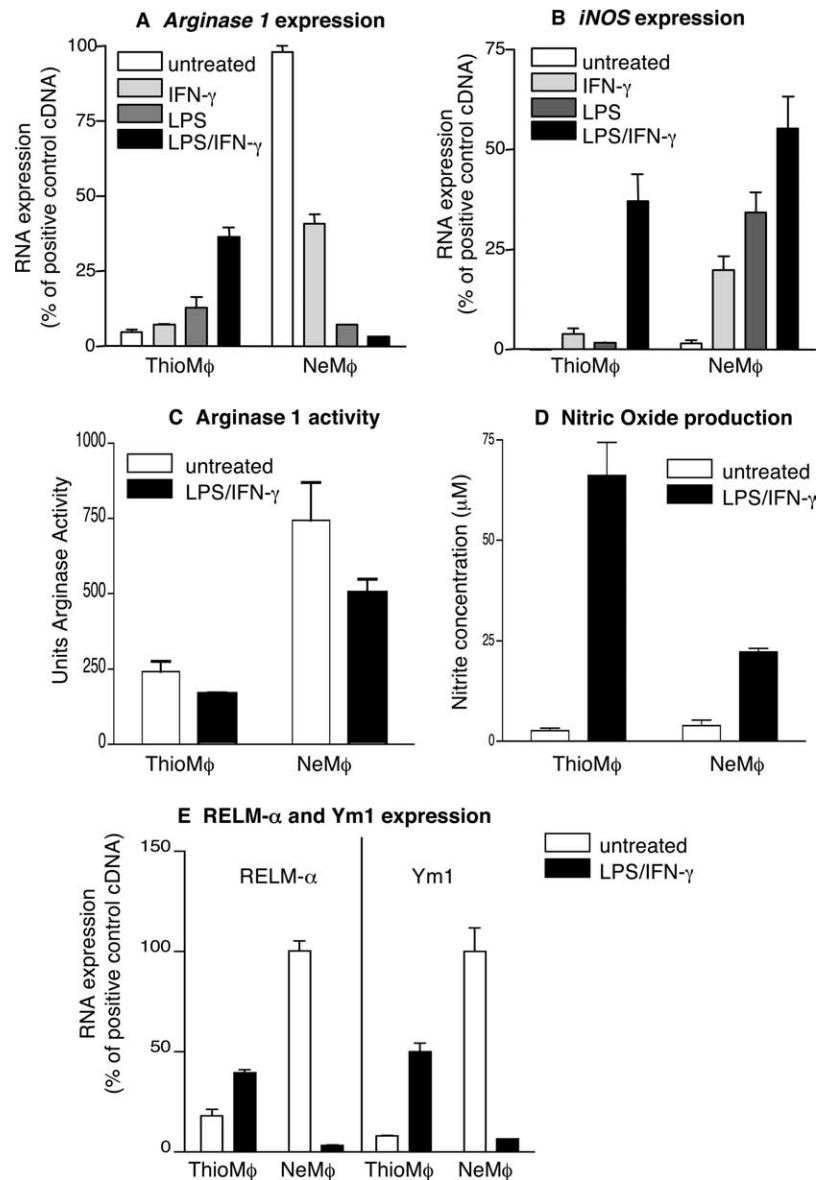


FIGURE 1. LPS/IFN- γ reduce alternative activation of NeM ϕ and enhance iNOS activity. ThioM ϕ or NeM ϕ from C57BL/6 mice were left untreated or treated overnight with LPS and/or IFN- γ as indicated. The cells were recovered for RNA expression analysis of arginase 1 (A), iNOS (B), RELM- α , and Ym1 (E) 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 (\pm 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 M ϕ , as identified according to side scatter and F4/80 expression (Fig. 2A). Following overnight treatment with LPS/IFN- γ , ThioM ϕ and NeM ϕ groups did not undergo any cell divisions and remained CFSE^{high}. Thus, the switch in gene expression and iNOS/arginase activity represents a nondividing NeM ϕ 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⁺ M ϕ expressing the alternative activation markers.

The mean fluorescence intensity (MFI) for F4/80 was higher in NeM ϕ (176) than in ThioM ϕ (52) (Fig. 2B). Since F4/80 has been implicated in immunological tolerance through the generation of regulatory T cells (28), the higher surface expression on NeM ϕ may point to a regulatory function. M ϕ 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 NeM ϕ (from 176 to 113) but not in ThioM ϕ (from 52 to 58). Down-regulation in F4/80 expression upon Th1 activation could have an impact on the regulatory properties of NeM ϕ .

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

Since proliferative suppression is one of the well-defined regulatory properties of NeM ϕ (22, 30), we decided to investigate whether LPS/IFN- γ affected the ability of NeM ϕ 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 M ϕ groups (see Fig. 1), and we wanted to distinguish the known suppressive effects of NO (31–33) from the suppressive mechanism mediated by NeM ϕ . Having previously shown that the NeM ϕ suppressive phenotype is dependent on IL-4 (22), we also included NeM ϕ generated in IL-4-deficient mice as a negative control for proliferative suppression. Finally, we included ThioM ϕ pre-treated with IL-4, allowing a comparison between in vitro-derived AAM ϕ and in vivo-derived NeM ϕ .

As expected, the EL-4 cell proliferation was suppressed when cocultured with NeM ϕ in comparison to control ThioM ϕ (Fig. 3A,

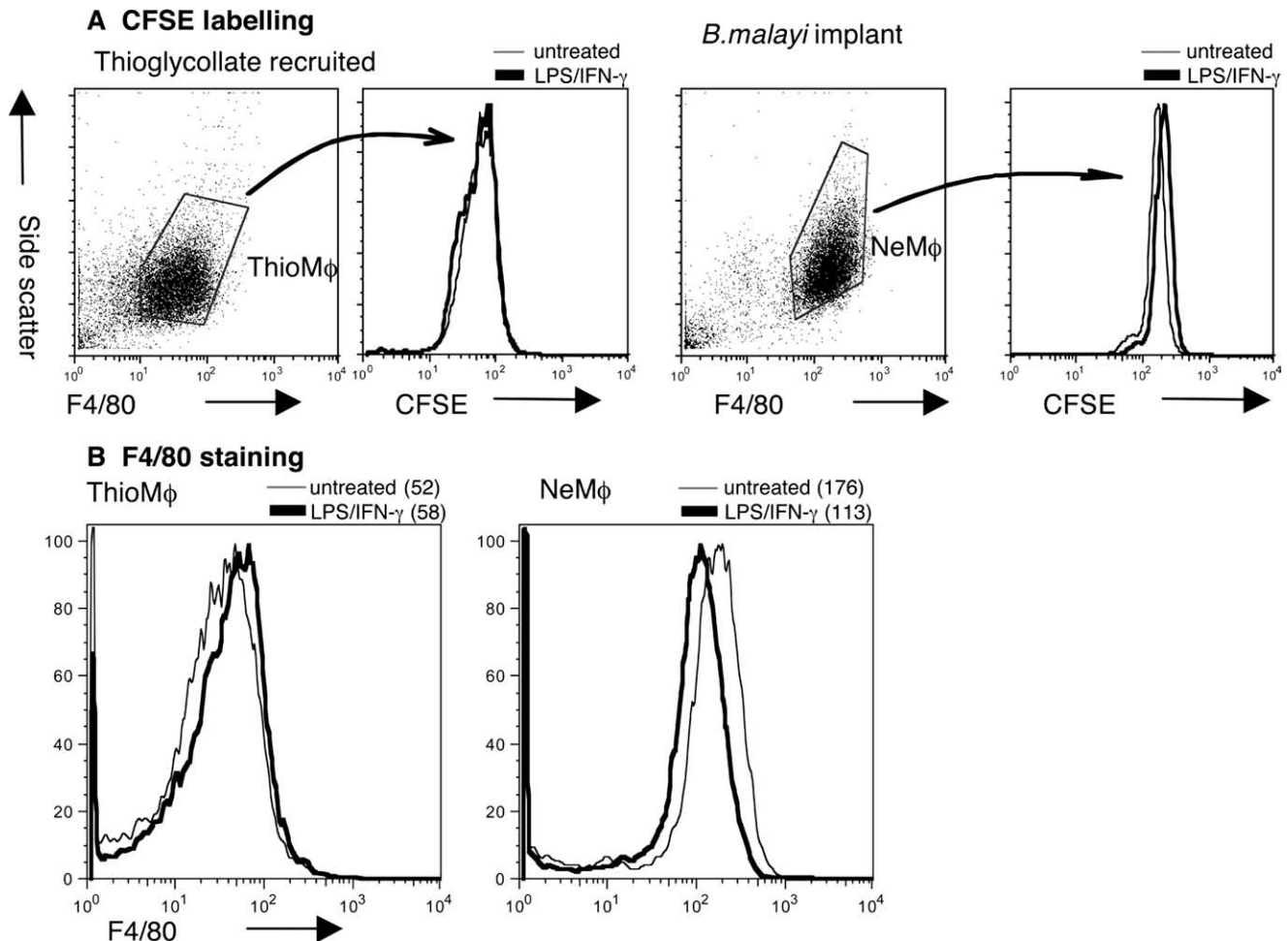


FIGURE 2. M ϕ do not divide in response to LPS/IFN- γ but down-regulate F4/80. C57BL/6 mice were implanted i.p. with *B. malayi* adult worms and the PECs were recovered 3 wk later. For comparison, C57BL/6 mice were injected i.p. with thioglycollate and the ThioM ϕ were recovered at day 3. **A**, The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

open bars). ThioM ϕ pretreated with IL-4 (in vitro AAM ϕ) were equally suppressive while IL-4^{-/-} NeM ϕ 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 M ϕ cell types could suppress proliferation. However, L-NMMA treatment (gray bars) reversed the LPS/IFN- γ -induced suppressive phenotype in the ThioM ϕ and the IL-4^{-/-} NeM ϕ , demonstrating that this suppression was NO mediated. In response to LPS/IFN- γ treatment, NeM ϕ and in vitro AAM ϕ 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 AAM ϕ .

While all M ϕ groups produced NO in response to LPS/IFN- γ , NeM ϕ produced less NO than did ThioM ϕ (Fig. 3B), consistent with our previous data (Fig. 1D). Of note, WT NeM ϕ showed increased responsiveness to LPS/IFN- γ in comparison to IL-4^{-/-} NeM ϕ 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 NeM ϕ 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 NeM ϕ (Fig. 3C). However, EL-4 cells cocultured with nor-NOHA-treated ThioM ϕ 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, NeM ϕ permitted only one-fifth the level of EL-4 proliferation as similarly treated ThioM ϕ , suggesting that arginine consumption is not the dominant mechanism of suppression in NeM ϕ .

NeM ϕ 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 NeM ϕ before and after LPS/IFN- γ treatment (Fig. 4A). Untreated NeM ϕ expressed higher levels of MHC class II, B7.1, and B7.2 than did ThioM ϕ , as shown by MFI. The high expression of these markers is consistent with the original classification of AAM ϕ as “activated” (35) and the data that ThioM ϕ are relatively “inactive” (27). It is also consistent with previous studies showing that NeM ϕ are efficient APCs (36). Upon LPS/IFN- γ stimulation, the MFI of all markers was increased in both ThioM ϕ and NeM ϕ ,

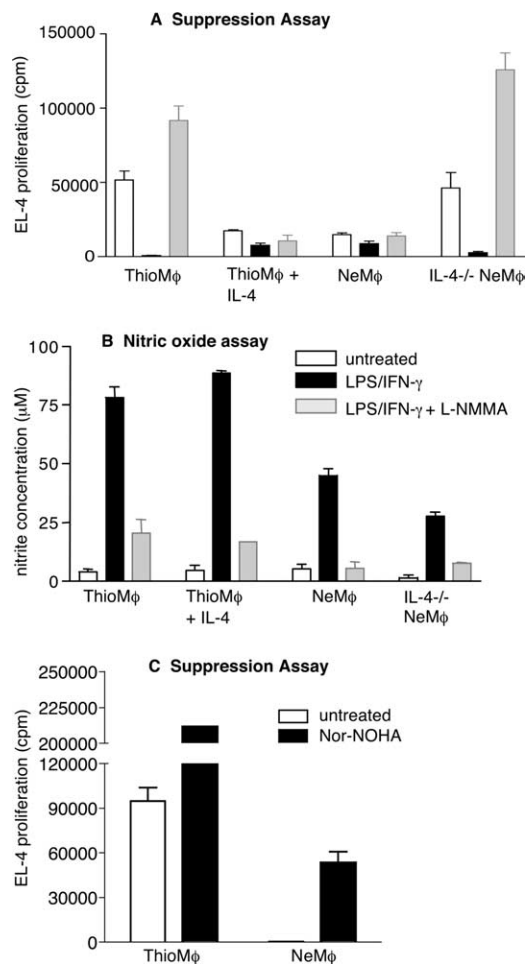


FIGURE 3. NeMφ can produce NO in response to LPS/IFN-γ but will still retain the ability to suppress cell proliferation in a NO-independent manner. ThioMφ, ThioMφ + IL-4, NeMφ, and IL-4^{-/-} NeMφ were left untreated or were treated with LPS/IFN-γ overnight followed by replacement of the medium and coculture with EL-4 thymoma cells with or without the NO inhibitor L-NMMA. After 48 h, the EL-4 cell proliferation was assessed by [³H]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 [³H]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. 4B). 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. 4B) 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 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 [³H]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. 7A shows that LPS/IFN-γ treatment, and to a lesser extent IFN-γ alone, conferred resistance to

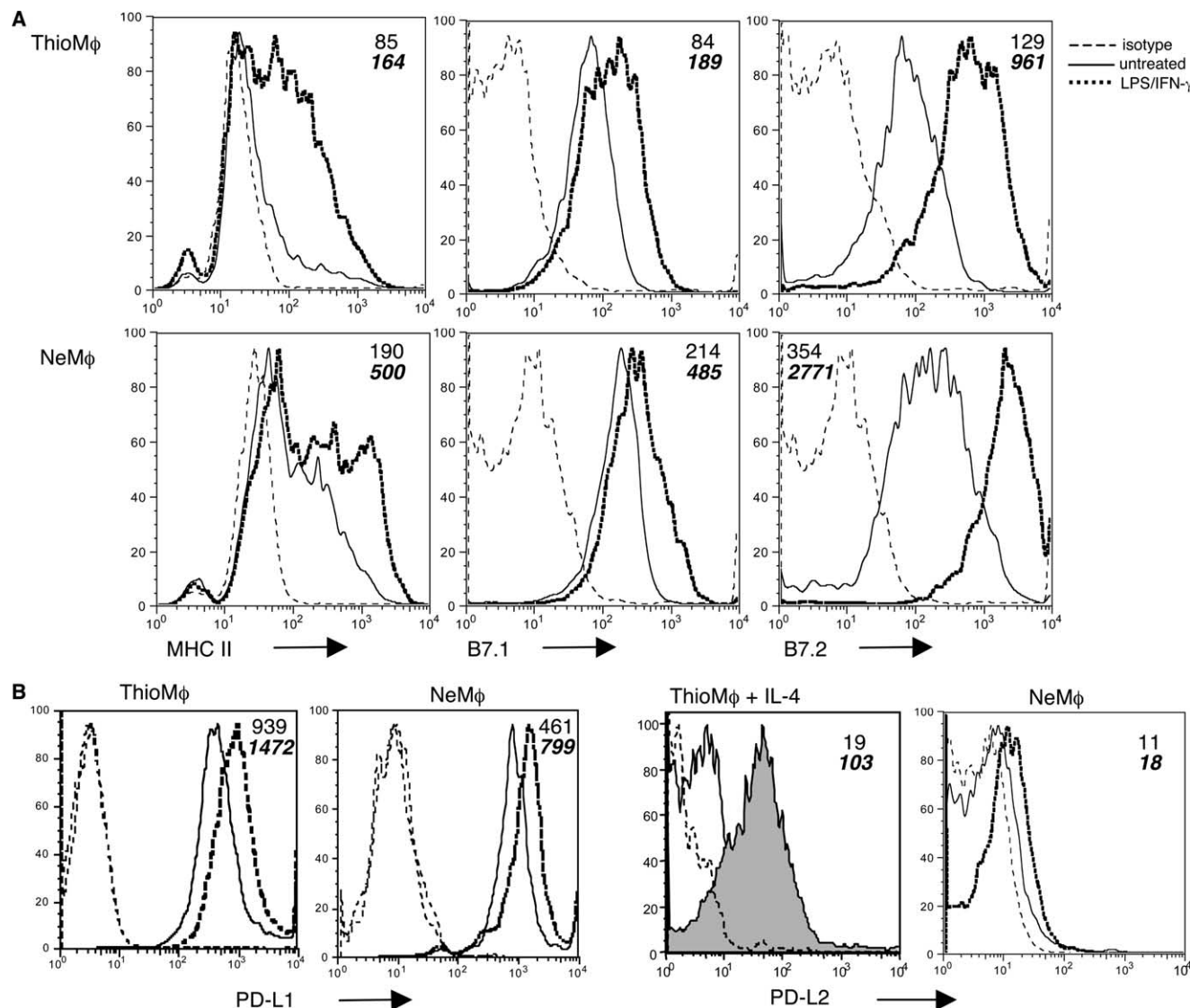


FIGURE 4. Effect of LPS/IFN- γ on the cell surface activation markers in naive M ϕ and NeM ϕ . Untreated ThioM ϕ , IL-4-treated ThioM ϕ (tinted), or NeM ϕ were left untreated or treated overnight with LPS/IFN- γ . The cells were recovered and double-stained for F4/80 and MHC class II, B7.1, B7.2 (A)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

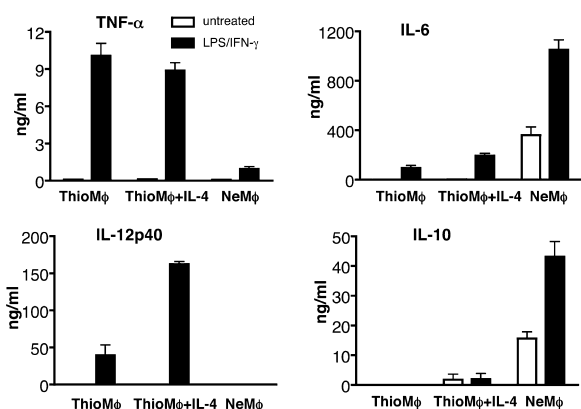


FIGURE 5. TNF- α , IL-6, and IL-10, but not IL-12p40, production is enhanced in response to LPS/IFN- γ . ThioM ϕ , ThioM ϕ + IL-4, and NeM ϕ were untreated (open bars) or were treated overnight with LPS/IFN- γ (filled bars). Supernatants were recovered and the levels of various cytokines were measured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

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

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

To visualize these results, experiments were also conducted with promastigotes of a *L. mexicana* strain that displays red fluorescence (24). In these experiments ThioM ϕ were used as controls. Fig. 7D 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,

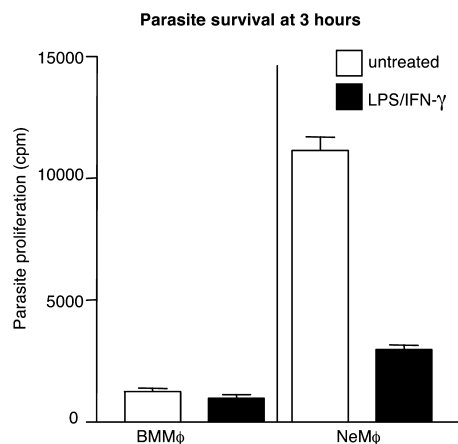


FIGURE 6. Untreated NeMφ take up the most *L. mexicana* parasites. BMMφ and NeMφ were untreated (open bar) or were treated overnight with LPS/IFN-γ (filled bar) followed by infection with *L. mexicana* at 10:1 parasite/Mφ ratio. Parasites that were not taken up were washed off 3 h postinfection, and parasite uptake was determined by lysis of the Mφ and measurement of the parasite proliferation by [³H]thymidine incorporation. Results are shown as the means of replicate samples (±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. 7E). 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. 7D, green). As this contrasted with our previous data in which *Ym1* mRNA decreases upon LPS/IFN-γ treatment (Fig. 1E), we chose to repeat these experiments to measure both protein and RNA simultaneously. The finding confirmed those in Figs. 1E and 7D 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 anti-inflammatory factors (45). Consistent with this, NeMφ produce many factors associated with tissue repair and reduced inflammation (e.g., arginase 1, IL-10 (Figs. 1A 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

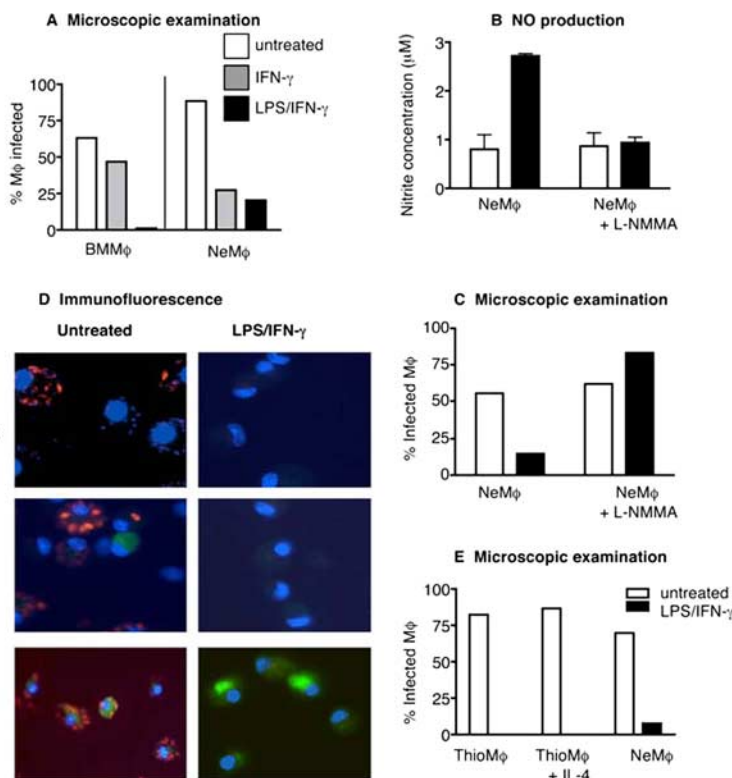


FIGURE 7. LPS/IFN-γ or IFN-γ alone confers resistance of NeMφ to *L. mexicana* infection at day 3 postinfection. BMMφ and NeMφ were untreated (open bars) or treated overnight with IFN-γ (gray bars) or LPS/IFN-γ (black bars). The NO inhibitor L-NMMA was also added (B and C). Infection with *L. mexicana* 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. Immunofluorescence assays were conducted using a *L. mexicana* strain that displayed red fluorescence, along with Ym1 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 NeM ϕ can divert their resources toward antimicrobial activity if needed. We also observed an increase in surface expression of class II and costimulatory molecules (Fig. 4A) under the influence of LPS/IFN- γ . This suggests that these AAM ϕ have enhanced APC function. However, analysis of the NeM ϕ 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 AAM ϕ 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 NeM ϕ (Fig. 5), which inhibits Th1 cell responses (49). Lamina propria M ϕ from the intestine of mice, which like NeM ϕ 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 M ϕ 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 NeM ϕ (i.e., iNOS) but not to IL-12 suggests that these AAM ϕ 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 NeM ϕ 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 NeM ϕ to infection with *L. mexicana* promastigotes. Models of murine *Leishmania* spp. involve a dominant Th1 response leading to the classical activation of M ϕ and elimination of the parasites through the production of NO (54). NeM ϕ were able to control infection almost as well as BMM ϕ given the same treatment of LPS/IFN- γ (Fig. 7A). The ability of NeM ϕ given IFN- γ alone to control parasite numbers more effectively than for BMM ϕ may be explained by signals that NeM ϕ encounter in vivo causing the up-regulation of the receptor for IFN- γ . Consistent with this hypothesis, NeM ϕ produced more iNOS mRNA in response to IFN- γ treatment than ThioM ϕ (Fig. 1B).

Previous work in the field has shown that alternative M ϕ 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 M ϕ 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 NeM ϕ to *L. mexicana* decreased dramatically when treated with even IFN- γ alone, as well as with LPS/IFN- γ (Fig. 7A). Although NeM ϕ from our *B. malayi* infection model produced lower levels of NO after LPS/IFN- γ treatment than did ThioM ϕ (Fig. 1D) or IL-4 pretreated ThioM ϕ (data not shown), they were still enough to confer resistance. Even when NeM ϕ were infected at a 30:1 ratio of parasites/M ϕ , 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 NeM ϕ 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 AAM ϕ .

A consistent feature of NeM ϕ and other helminth-induced M ϕ 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. 3A), so additional factors must be involved. PD-L1 has been implicated in the proliferative suppression observed by M ϕ 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 NeM ϕ compared with the ThioM ϕ controls (Fig. 4B). Also, NeM ϕ did not up-regulate another marker of AAM ϕ generated in vitro, PD-L2 (Fig. 4B). Data now emerging from several laboratories 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, M ϕ 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 M ϕ highly polarized in vivo maintain remarkable phenotypic plasticity and are very consistent with recent studies in which the abilities of tumor-promoting AAM ϕ 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 M ϕ 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 M ϕ polarization in vivo (71, 72). Our data do not show that AAM ϕ make a complete conversion to

CAM ϕ but rather that they adopt key aspects of the CAM ϕ 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 M ϕ plasticity are needed.

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Disclosures

The authors have no financial conflicts of interest.

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