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Immortalized Murine Macrophage Cell Line as a Model for Macrophage Polarization into Classically Activated M(IFNγ+LPS) or Alternatively Activated M(IL-4) Macrophages

Andra Banete, Paulina Achita, Katherine Harding, Rylend Mulder and Sameh Basta*

Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Canada

***Corresponding author:** Samesh Basta, Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Ontario, Canada, Tel: 613-533-6648; E-mail: bastas@queensu.ca

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Abstract

Objective: Macrophages (Mϕ) represent a link between the innate and adaptive arms of the immune system. Generally, Mϕ are classified into two major subsets after stimulation; either ascribed classically (M1), or more specifically M(IFNγ+LPS) based on the activating condition, or alternatively (M2), or more specifically M(IL-4) activated cells. The purpose of the study was to evaluate an immortalized murine Mϕ cell line (BMA) as an *in vitro* model for Mϕ polarization into M(IFNγ+LPS) and M(IL-4) phenotypes to facilitate the progress in this exciting research field.

Methods: The BMA cell line was stimulated with either IFNγ and LPS or IL-4 to induce cellular polarization. The cells were characterized using multi-parameter analyses employing phenotypic and functional assays, and compared to bone-marrow derived macrophages (BMDM).

Results: The BMA cell line was found to differentiate into either M(IFNγ+LPS) Mϕ, characterized by production of inflammatory cytokines and up-regulation of inducible nitric oxide synthase (iNOS) or M(IL-4) cells with high Arginase-1 activity. Furthermore, polarized BMA cells were found to have a differential expression of cell surface markers.

Conclusion: These findings demonstrate that the BMA cell line can be polarized into M(IFNγ+LPS)/M(IL-4) phenotypes, and can therefore be used as a model for *in vitro* Mϕ polarization reducing the need for primary Mϕ isolation when investigating biological phenomena related to their polarization.

Keywords: Lipopolysaccharide; Pro-inflammatory; Arginase; Nitric oxide synthase; MHC; Interleukin-4; Interferon-gamma

Introduction

Monocytes and Mϕ play a critical role in innate immunity, and the regulation of the adaptive immune response. Mϕ are heterogeneous populations with a variety of functional phenotypes depending on the presence of different stimuli in their local environment [1]. Mϕ can present both endogenous and exogenous antigens to cytotoxic T lymphocytes [2,3]. The phenotype of a Mϕ following differentiation may dictate its ability to present antigens to T lymphocytes [4]. Generally, Mϕ phenotypes were ascribed classically (M1-pro-inflammatory) or alternatively (M2-anti-inflammatory) activated status [5]. However, this system has expanded to include the different subsets of M2 Mϕ (M2a, M2b, M2c, and M2d) [1]. Recently, a more specific nomenclature system to define Mϕ subpopulations based on the activation condition has been proposed [6], which we will employ to describe activated Mϕ in this current report.

Stimulation with lipopolysaccharide (LPS) in the presence of interferon-gamma (IFNγ) induces a pro-inflammatory phenotype [5]. M(IFNγ+LPS) Mϕ are associated with the production of pro-inflammatory cytokines and reactive nitrogen and oxygen, and have a high microbicidal activity [7-9]. An important marker for the pro-inflammatory phenotype is the up-regulation of inducible nitric oxide synthase (iNOS), resulting in the production of nitric oxide (NO) from L-arginine [10]. In contrast, Mϕ stimulated by interleukin-4 (IL-4) and IL-13 function as anti-inflammatory cells, and promote tissue repair [11]. These M(IL-4) Mϕ are characterized by the secretion of the anti-inflammatory cytokine IL-10. Moreover, IL-4 also regulates the genes for Arginase-1 (Arg1), and Mannose Receptor-1 (CD206) [12,13]. Arg1 is a direct competitor of iNOS, utilizing the substrate L-arginine to produce polyamines associated with cell growth and proliferation [5]. In mouse models, the M(IL-4) phenotype is associated with the induction of resistin-like-α (also known as FIZZ1), and chitinase 3-like 3 (also known as Ym1) [14].

The aim of this study was to determine if immortalized murine Mϕ cell line (BMA) can be polarized into M(IFNγ+LPS) and M(IL-4) phenotypes by evaluating cytokine and surface marker expression, arginase activity, and nitric oxide (NO) production. Immortalized murine Mϕ cell line (BMA) was found to be able to differentiate into either M(IFNγ+LPS) cells, characterized by production of the inflammatory cytokines IL-1β, IL-6, IL-12, and tumour necrosis factor (TNF)-α, and up-regulation of inducible nitric oxide synthase (iNOS), or M(IL-4) Mϕ with their canonical high Arg1 activity.

Materials and Methods

Macrophage preparations

The BM A3.1A7 (BMA) murine Mϕ cell line is an adherent Mϕ cell line derived from the bone marrow of adult female C57BL/6 mice immortalized by overexpressing *raf* and *myc* oncogenes (provided by Dr. Ken L. Rock, University of Massachusetts Medical School, Worcester, MA) [2]. BMA cells were cultured in RPMI media supplemented with 5% fetal calf serum (FCS), and incubated at 37°C, 6% CO_2.

Bone marrow-derived macrophages (BMDM) were extracted as described previously [15]. Bone marrow from the femurs and tibia of 6-8 week old C57BL/6 (H-2b) mice (Charles River, St. Constant, QC, Canada) was flushed with PBS. Cells were incubated with red cell lysis buffer (1.66% ammonium chloride) for 5 minutes at room temperature. Afterwards, cells were cultured in a 6-well tissue culture plate in RPMI containing 10% FCS (Fisher Scientific), 20% supernatant from MCSF-secreting L929 fibroblasts, and 50μg/mL gentamycin. Non-adherent cells were removed after 3 days and fresh media was added. Cells were used after 7 days in culture.

To induce an M(IFNγ+LPS) phenotype, cells were stimulated with RPMI (5% FCS) containing IFNγ (25 ng/ml; Bioscience) for 6 hours, after which LPS (10 or 100 ng/ml; E. coli O55:B5, Sigma-Aldrich), was added for a total of 24 or 48 hours. To induce an M(IL-4) phenotype, the Mϕ were cultured in RPMI (5% FCS) supplemented with IL-4 (20 ng/ml; Bioscience) for 24 or 48 h.

Flow cytometry analysis

Polarized BMA and BMDM cells (2 $\times 10^5$) were harvested and washed with cold 1 x PBS, then transferred to a round-bottom 96-well plate. Fluorochrome-labeled monoclonal antibodies were used for cell surface marker staining. Cells were either left unstained or stained with FITC anti-CD206, clone MR5D3 (AbD Serotech), FITC anti-CD86, clone RMMP-2 (Cedarlane), PE anti-MHC I, clone 28-8-6 (Biolegend), or FITC anti-MHCII, clone: M5/114.15.2 (ebioscience). Following incubation at 4°C for 20 minutes, cells were resuspended in FACS buffer (0.5% sodium azide in PBS), then sorted by flow cytometry (Epics XL-MCL). The data was then analyzed using the Expo 32 Software package (Beckman Coulter).

RT-PCR

Polarized cells (5 × 10^6) were harvested and total RNA was extracted using TRI reagent (Sigma-Aldrich, Oakville, ON). Reverse transcription (RT-step) was performed by mixing random primers, dNTP mix, 5x RT buffer, RNase inhibitor, and M-MULV enzyme (GeneDireX), and diluted RNA samples. The cDNA obtained from the RT-step was used for the PCR step by adding it to the following gene-specific primers (IDT, Coraville, Iowa) and i-Taq 5x Master Mix (New England Biolabs): iNOS 5′-CCTTGTTCAGCTACGCCTTC-3′, 5′-AAGGCCAAACACAGCATACC-3′; Arg1 5′- CAGAAGAATGGAA GAGTCAG-3′, 5′- CAGATATGCAGGGAGTCACC-3′[16]; IL-1β 5'-CAACCAACAAGTGATATTCTCCATG -3', 5'- GATCCACACTCTC CAGCTGCA-3'; IL-6 5'-CAGAATTGCCATCGTACAACTCTTTTC TCA-3', 5'-AAGTGCATCATCGTTGTTCATACA-3'; IL-12p40 5'-GAGGTGGACTGGACTCCCGA -3', 5'-CAAGTTCTTGGGCGGGT TGGGCGGGTCTG3'; TNF-α 5'-CATCTTCTCAAAATT CGAGTGA CAA - 3', 5'-TGGGAGTAGACAAGGTACAACCC - 3'; FIZZ1 5'-T CCCAGTGAATACTGATGAGA -3', 5'- CCACTCTGGATCTCCCAA GA-3'[14]; Ym1 5′-GGGCATACCTTTATCCTGAG-3′,5′- CCACTGA AGTCATCCATGTC-3′[14], and as a control 18S rRNA was used; 5′-AA ACGGCTACCACATCCAAG-3′-,5′ CCTCCAATGGATCCTCGTTA-3′.

Arginase assay

To evaluate polarization to a M(IL-4) phenotype, arginase activity was determined as described elsewhere [12,15]. BMA cells (1 × 10^6) were stimulated as described above. Cells were resuspended in lysis buffer containing protease inhibitors (leupeptin (8 μg/ml) and PMSF (100 μM). Samples were incubated at 4°C for 30 min, and protein concentration in the supernatant was determined. The hydrolysis of arginine to ornithine and urea was conducted by incubating the lysates with 0.5M L-arginine at 37°C for 2 hours. Urea concentration was measured at 550nm using a Varioskan spectrophotometric microplate reader.

Nitric oxide assay

To evaluate polarization to an M(IFNγ+LPS) state, nitric oxide production was indirectly assessed by detection of nitrites in cell culture supernatants with Griess reagent (Sigma-Aldrich; Oakville, ON) as previously described [17]. A standard curve was obtained using 0-100 μM sodium nitrite (Fisher Scientific, Whitby, ON) in PBS. BMA cells 1 × 10^6 were polarized as previously-described) in phenol-red free media (Gibco, Life Technologies). Following treatment, 100 μl of cell culture supernatant was transferred to a 96-well flat-bottom plate, and 50 μl of sulfanilamide solution (1% w/v sulfanilamide in 5% w/v phosphoric acid) was added to each well. The plate was incubated in the dark at room temperature for 10 minutes, after which 50 μl of photometric NED solution (0.1% w/v N-1-napthylethylenediamine dihydrochloride in water) was then added to each well and again incubated in the dark at room temperature for 10 minutes. Absorbance values were measured at 540 nm using a Varioskan spectrophotometric microplate reader.

Microscopy

Morphological analyses using light microscopy were performed on BMA cells that were cultured overnight in media supplemented with IFN-γ plus LPS or IL-4 as described above. Cells were seeded into 24-well plate onto 12 mm circular glass cover slips (Fisher Scientific, Ontario, Canada) at a density of 1 × 10^5/well. The samples were observed under a light microscope (Leica DM IRE2, Germany) using 20X and 40X magnifications. Images were acquired using Leica DFC340 cooled monochrome digital camera.

Statistical analysis

Statistical significances were determined using unpaired, two-tailed Student's t test. Values of $p<0.005$ were considered statistically significant. All values are reported as mean ± SD of three replicates.

Results

iNOS and *Arg1* are differentially expressed in BMA cells following treatment with LPS, IFNγ+LPS or IL-4

The effect of stimulation with LPS alone, IFNγ+LPS or IL-4 on the expression of the *iNOS* and *Arg1* genes was determined, compared to non-treated (NT) cells. BMA cells were treated for 24h with LPS, IFNγ +LPS, IL-4, or grown in media only. Total RNA was isolated from BMA cells and reverse-transcribed into cDNA prior to PCR amplification. Expression of the 18S ribosomal RNA was used as a loading control, and expression levels for each gene were normalized using densitometry analysis and expressed as fold-change (Figure 1). Treatment with LPS induced a 6-fold increase in transcription of iNOS compared to control levels (Figure 1A, top) and treatment with both IFNγ+LPS resulted in a 7-fold increase (Figure 1B, top). No significant expression could be detected following IL-4 treatment (Figures 1A and 1B). Expression of *Arg1* was more than 2-fold higher in IL-4 treated cells than control cells (Figures 1A and 1B), but expression was repressed with LPS treatment (Figure 1A, middle; 0.3-fold expression compared to control). No repression of *Arg1* was observed when cells were pre-treated with IFNγ prior to LPS treatment. These results indicate that at the genetic level, three distinct Mϕ populations can be obtained by stimulating with LPS (with or without IFNγ) or IL-4, corresponding to the expected M(LPS), M(IFNγ+LPS) and M(IL-4) phenotypes.

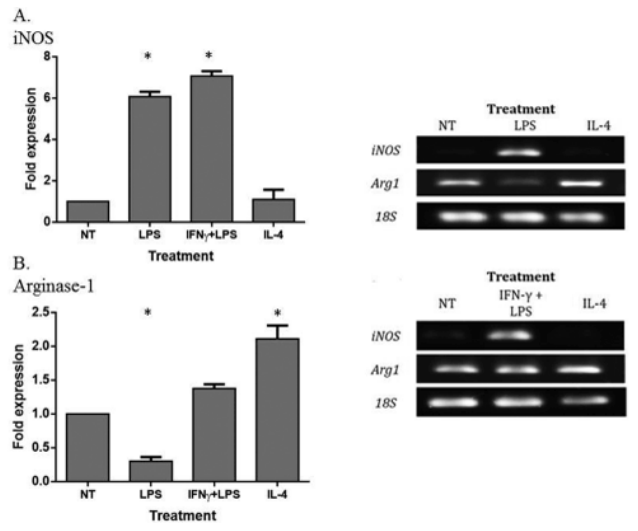

Figure 1: Expression of the iNOS and Arg1 genes in BMA cells as indicators of their polarization states following treatment with LPS, IFNγ+LPS or IL-4. Transcriptional expression of genetic markers in BMA macrophages was determined following treatment and analyzed by densitometry. A. Expression of iNOS was found to increase following LPS and IFNγ+LPS treatments, respectively. Treatment with IL-4 reduced iNOS expression. B. Arg1 expression was found to decrease following LPS treatment, while treatment with IFNγ+LPS and IL-4 produced increased it, with IL-4-treated cells having higher expression than IFNγ+LPS-treated BMAs. This is one representative experiment from 5 independent trials.

Morphological comparison of polarized BMA cells

M(IFNγ+LPS) and M(IL-4) Mϕ were obtained by stimulating BMA cells *in vitro* and observed under light microscope at 20x or 40x magnification (Figure 2). The majority of M (IFNγ+LPS) Mϕ appeared more round when compared to M (NT) or M(IL-4) cells. Moreover, M (IFNγ+LPS) Mϕ had a flattened, "fried-egg" morphology and an abundance of vesicles. On the contrary, M (IL-4) Mϕ were more elongated than the other two populations, and stretched, spindle-like cytoplasmic projections at the poles of the cell were observed. This indicates that polarized BMA cells can be easily distinguished under the microscope.

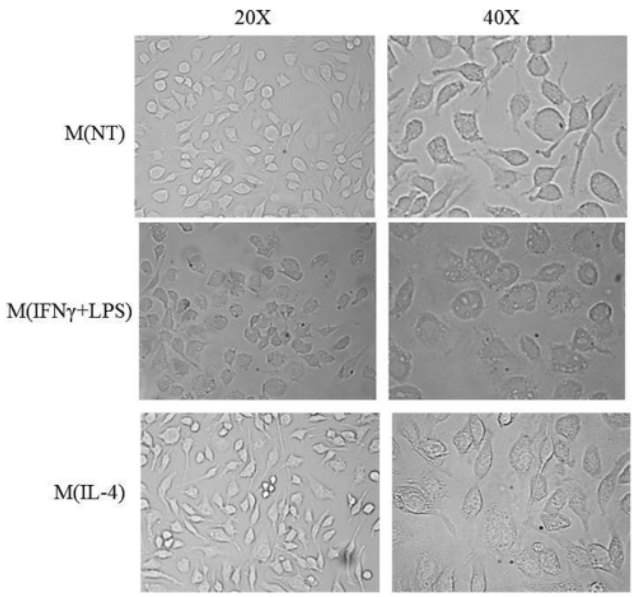

Figure 2: Morphology of polarized BMA cells. Stimulation of BMA cells for 24 h with IFNγ+LPS or IL-4 induced morphological changes that were visible under a light microscope at 20X and 40X magnification. M(IFNγ+LPS) macrophages exhibit an irregular phenotype represented by a rounded shape and abundance of vesicles. M(IL-4) macrophages are similar in appearance to untreated BMA cells [M(NT)]. The cells were examined via microscopy (Leica DM IRE2, bright field). One representative experiment is shown in this figure.

Polarization of BMA cells results in differential expression of cell surface markers classically associated with M(IFNγ +LPS)/M(IL-4) phenotypes

Mϕ polarization is associated with changes in extra-cellular marker expression, contributing to their specialized function. The M(IFNγ +LPS) phenotype is characterized by high surface expression of the co-stimulatory molecules CD80 and CD86, and an up-regulation of MHC class I and II, and can therefore efficiently present antigens to T cells. On the other hand, the tissue-healing M(IL-4) phenotype is characterized by an up-regulation of mannose receptor (CD206), which is involved in phagocytosis. We observed a significant increase in surface marker expression of both CD86 and MHC I in cells treated with IFNγ and LPS (100 ng) (data not shown for 10ng LPS) compared to unstimulated cells (Figures 3A and 3B, respectively), while BMA

Mϕ treated with IL-4 showed similar expression levels compared to control. Interestingly, both M(IFNγ+LPS) and M(IL-4) cells showed a similar increase in MHC II expression compared to control (Figure 3C). Moreover, we observed a significant increase in surface expression of CD206 after treatment with IL-4 (48 h) compared to control, while expression is suppressed in cells treated with IFNγ+LPS (Figure 3D). Therefore, these results provide evidence of subsets of polarized Mϕ, indicating polarization of BMA cells towards M(IFNγ +LPS) and M(IL-4) phenotypes. Surface marker expression on polarized BMA cells closely parallels that on BMDM (Figure 3E, 3F, 3G and 3H), demonstrating that the BMA cell line is a suitable substitute for primary cells.

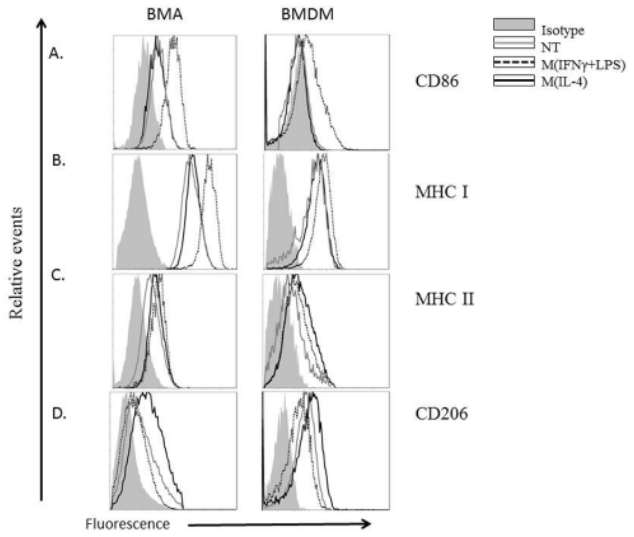

Figure 3: Cell surface marker expression profiles for polarized macrophages. Flow cytometry analyses of the profile of BMA and BMDM treated with either IFNγ+LPS or IL-4. Changes in expression are assessed by comparison against non-treated (NT) cells. Histograms show surface staining for CD86, MHC I, MHC II, and CD206 and their differential expression in activated Mϕ. Figures are representative of one out of three independent experiments.

BMA macrophages show increased Arg1 activity following IL-4 treatment

Mϕ polarization into M(IFNγ+LPS) and M(IL-4) phenotypes can be assessed by investigating the pathways involved in the metabolism of the substrate L-arginine. M(IFNγ+LPS) Mϕ use L-arginine to synthesize nitric oxide (NO) in the process catalyzed by the enzyme inducible nitric oxide synthase (iNOS). In contrast, M(IL-4) Mϕ strongly suppress NO production and utilize L-arginine via arginase 1 (Arg1), producing polyamines associated with cell growth and proliferation, and the metabolic by product urea. Firstly, we assessed production of NO as an indicator of the M(IFNγ+LPS), pro-inflammatory phenotype. Following treatment, the supernatants were collected to determine the concentration of soluble nitrites produced by Griess reaction. BMA cells treated with both IFNγ and LPS show a significant increase in the production of NO compared to control, indicating high levels of iNOS activity (Figure 4A). LPS alone induced low levels of NO production and NO was undetectable following treatment with IFNγ. IL-4-treated cells had very low levels of NO production, comparable to non-treated cells.

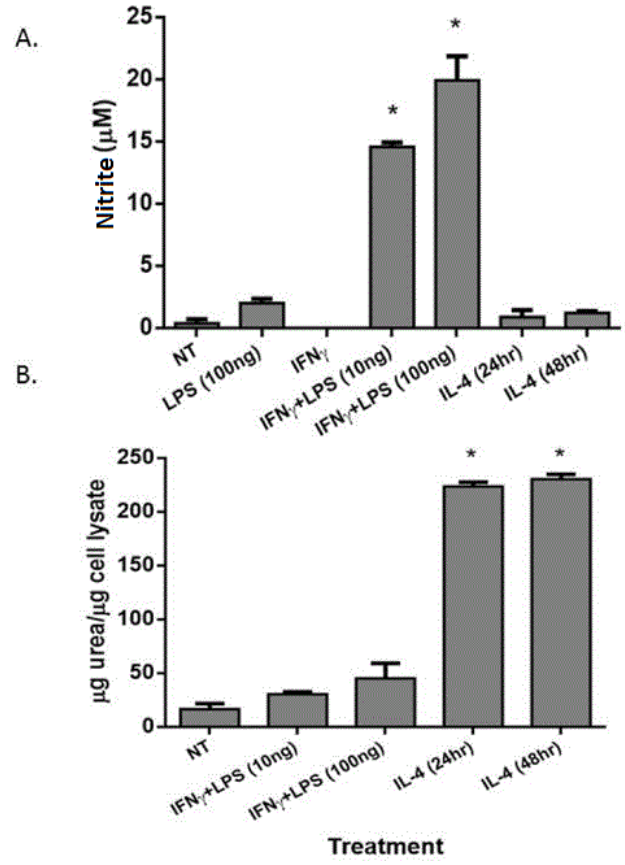

Figure 4: Urea and nitrite production in polarized macrophages. A. Nitrite production in BMA cells. Following treatment, the supernatant was collected to determine the amount of NO produced. Treatment with IFNγ+LPS induced a significant production of NO in BMA cells. Data shown represents the average of 3 replicates; error bars represent the standard deviation. B. Production of urea from BMA cell lysates was measured to detect arginase activity. Cells were treated with IFNγ+LPS, IL-4, or grown in media only (NT, non-treated). Treatment with IL-4 was found to significantly increase urea production compared to both untreated and IFNγ+LPS treated cells. Values are represented as μg urea adjusted to μg cell lysate. Data shown are mean ± SD of three representative experiments ($p<0.005$).

Furthermore, we determined urea concentrations as an indirect measure of Arg1 activity. Cells treated with IL-4 for 24 and 48 h show a significant increase in urea levels in cell lysates (Figure 4B). No significant urea production was detected in BMA cells treated with IFNγ+LPS compared to unstimulated control cells. Together, these results provide strong evidence that BMA cells are readily polarized into the pro-inflammatory M(IFNγ+LPS) phenotype and the anti-inflammatory M(IL-4) phenotype following treatment.

Expression of pro- and anti-inflammatory cytokine genes in polarized BMA cells

BMA cells were treated with IFNγ and LPS (10 and 100 ng) or IL-4 (24 and 48 h) to induce an activated phenotype. Immortalized murine Mϕ cell line (BMA) was shown to be able to differentiate into either M(IFNγ+LPS) Mϕ, characterized by production of the inflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α, as well as up-regulation of iNOS, or M(IL-4) Mϕ with high Arginase-1 activity (Figure 5). The M(IL-4) murine Mϕ marker Ym1 was up-regulated in cells stimulated with IL-4 for 48 h, but not 24 h.

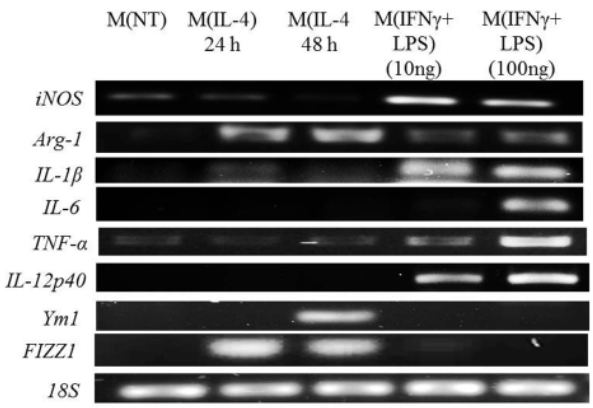

Figure 5: Transcriptional gene expression of cytokines and specific markers in M(IFNγ+LPS) and M(IL-4) macrophages. BMA cells were treated for 24 or 48 h with IFNγ+LPS or IL-4 to induce an activated phenotype. Total RNA was extracted from cell lysates, and RT-PCR for specific markers was carried out. The cytokines (IL-1β, IL-6, TNF-α and IL-12p40) expression, as well as inducible NO synthase (iNOS) was up-regulated in BMAs stimulated with IFNγ +LPS. iNOS gene expression is a marker for classically activated macrophages. Arg-1, FIZZ1 and Ym1 are markers for alternatively activated murine macrophages, and their expression was up-regulated in IL-4 stimulated BMAs. Ribosomal 18S was used as a loading control.

Discussion

Paralleling T helper cell polarization into Th1 and Th2 cells, activated macrophages were categorized as either M1(associated with the Th1 cytokine IFNγ) and M2 (associated with the Th2 cytokine IL-4) cells [18]. However, the M2 designation has been further subdivided into three different subtypes (M2a, b, and c) based on their gene expression profiles [1]. The M2a subtype is induced by IL-4 or IL-13, and they play a role in Th2 immune responses against parasitic infections such as helminths [19]. M2b cells are induced by exposure to IL-1R ligands in the presence of TLR agonists such as LPS or immune complexes (Ic), and have immune regulatory functions [1]. The M2c phenotype is induced by IL-10, TGF-β, and glucocorticoid (GC) hormones, and has a role in tissue healing and remodeling [20]. Although this classification system is still widely used, Murray et al. have proposed to describe activated Mϕ based on their culture conditions to ensure consistent terminology [6]. Moreover, recent studies have demonstrated *in vitro* that macrophages are capable of repolarizing from one phenotype to another in response to their cytokine environment [15,21]. Furthermore, studies show that besides cytokines, factors such as microRNAs (miRNAs) [22-24] and enhancer RNAs [25,26] play a significant role in macrophage polarization.

The aim of our study was to evaluate for the first time a cell line of bone marrow-derived Mϕ from C57/BL6 mice (H2b haplotype) as an *in vitro* model for Mϕ polarization, as the only other macrophage cell lines currently available are derived from BALB/c mice (H2d haplotype) [27,28]. Stimulation with IFNγ and LPS induced the production of pro-inflammatory cytokines, as well as iNOS, indicating an M(IFNγ+LPS) phenotype. Transcription of the iNOS gene was up-regulated with LPS alone and with both IFNγ and LPS, but NO production required both IFNγ and LPS, as LPS alone induced low levels of NO production, and NO was undetectable following treatment with IFNγ. This may be because the murine iNOS promoter contains binding sites for transcription factors associated with both the IFNγ and LPS signaling pathways, and both signals may be required for translation of this gene [29]. The activation of these distinct transcription pathways through the addition of IFNγ and LPS has a synergistic effect on the expression of iNOS [30], which is seen in the BMA cell line when stimulated with both the IFNγ and LPS as compared to either one alone.

We also examined the morphology of polarized BMA cells and found it to be quite distinct after treatment. M(IFNγ+LPS) cells had an irregular shape with many visible intracellular vacuoles, while M(IL-4) cells had an elongated morphology. These finding parallel the observations made recently by Reichard et al. in the murine J774A.1 cell line [27], and in human M(IFNγ+LPS) and M(IL-4) macrophages [31,32].

Furthermore, the induction of the M(IL-4) murine Mϕ marker Ym1 (a chitinase-like, secretory lectin), as well as expression of CD206 were observed only after 48 hour of stimulation with IL-4. This indicates that a longer polarization time is needed to fully commit BMA cells towards an M(IL-4) phenotype. However, treatment with IL-4 increased Arg1 and FIZZ1 (found in the inflammatory zone; a resistin-like secreted anti-inflammatory molecule) [14] expression levels after both 24 and 48 h of stimulation. Urea production, corresponding to arginase activity, was also higher in IL-4 treated cells. Arginase is thought to deplete the substrate pool of arginine in alternatively activated Mϕ, reducing its conversion to NO [33,34]. This would have a suppressive effect on inflammation by limiting the potential for the production of inflammatory mediators. However, we found that cells treated with IFNγ and LPS showed higher expression of Arg1 and urea than non-treated cells. Although expression of Arg1 is one of the most well-known markers for M(IL-4) Mϕ, LPS has also been shown to induce its expression, and M(IFNγ+LPS) cells are able to express Arg1 and iNOS simultaneously [35]. Such findings indicate that the balance between iNOS and arginase *in vivo* may be more dynamic than what can be replicated *in vitro*. Clearly, solely analyzing the balance between arginase and iNOS, or IL-12 versus IL-10 production is not sufficient to fully identify the many subpopulations of macrophages [6].

As a result, the need for reassessing the categorization and nomenclature of macrophage polarization to accommodate novel findings has been discussed [6,36,37]. One major obstacle in the revision of macrophage terminology is the lack of defined macrophage subsets in disease. *In vivo*, macrophages can develop mixed phenotypes under certain conditions [38,39]. The incredible plasticity of macrophages is also evident in their responses to different

pathogens. Recent studies using human immunodeficiency virus 1 (HIV-1) and human cytomegalovirus (HCMV) have found that infection can induce M(IFNγ+LPS)-like properties in M(IL-4) macrophages, and that polarization status alters macrophage susceptibility to viral infection [16]. However, little is known about the roles of M(Ic), M(IL-10), M(GC+TGFβ), and M(GC) macrophage subsets in infection. As the BMA cell line was easily polarized using either IFNγ and LPS or IL-4, behaving both phenotypically and functionally according to the revised classification proposed by Murray et. Al [6] for M(IFNγ+LPS) and M(IL-4) macrophages, it would be a useful model to study polarization to M(Ic), M(IL-10), M(GC+TGFβ), and M(GC) subtypes and biological phenomena associated with their activation status.

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