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# Characterization of *In vitro* Generated Human Polarized Macrophages

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## Abstract

**Objective:** Contact with invading pathogens and/or tissue injury leads to the polarization of macrophages into either a M1 or a M2 state which is further divided into M2a, M2b and M2c subsets. The human macrophage subsets have been poorly characterized. The present study was undertaken to characterize macrophage polarization using a non-exhaustive panel of surface markers with respect to M1, M2a, M2b and M2c macrophages and production of pro- and anti-inflammatory cytokines in response to various toll-like receptors (TLR), ligands.

**Methods:** We generated various macrophage subsets by treating monocyte-derived macrophages (MDMs) with IFNγ (M1), IL-4 (M2a), LPS and IL-1β (M2b) or IL-10 (M2c) followed by stimulation with toll-like receptor (TLR)-2, TLR-3 and TLR-4 agonists and analysis of surface marker and cytokines expression was carried out by flow cytometry and ELISA, respectively.

**Results:** M2a subset was characterized by $CD14^{low}$, $CD163^{low}$ and $TLR4^{low}$ phenotype and produced high levels of IL-10. M2b subset was characterized by $CD14^{high}$, $CD80^{high}$ and $CD200R^{low}$ phenotype and produced IL-6 prior to stimulation. M2c subset displayed a $CD86^{low}$, $CD163^{high}$ phenotype and produced high levels of IL-10. M1 subset was characterized by $CD80^{high}$, $CD86^{high}$, $CD163^{low}$ and $TLR4^{high}$ phenotype and produced high levels of proinflammatory IFN-g, IL-12, TNFα and IL-23 following stimulation.

**Conclusion:** This study characterizes all four polarization states in human macrophages. Each polarization state demonstrated a unique cell surface marker profile and cytokine profile. These phenotypic markers can be used to characterize macrophage populations in tissue inflammatory disease conditions *in vivo* to further understand disease pathogenesis.

**Keywords:** Macrophage polarization; Cytokines; Surface markers

## Introduction

Monocytes released from the bone marrow circulate in the bloodstream for 3 days before infiltrating tissues to maintain tissue resident macrophage populations [1] such as osteoclasts in the bone or microglia in the brain [2]. Macrophages operate at the interphase of the innate and adaptive immunity and through their plasticity can display polarized states. These polarization states are acquired due to a change in type, timing or amount of micro-environmental signals [3].

Macrophages have been classified as being either M1 or M2 polarized in a linear representation where the two polarization states are at opposite ends of the scale. M1 polarization is used to describe classically activated macrophages [4]. These macrophages play an important role in clearing intracellular pathogens [5]. Alternatively activated macrophages or M2 polarized macrophages denote a more heterogeneous polarization state that includes several subtypes: M2a, M2b and M2c [4]. This classification of M1 and M2 has persisted despite evidence demonstrating biochemical and physiological differences between M2a, M2b and M2c polarized subtypes [6]. M2a macrophages or wound healing macrophages arise in response to IL-4 or IL-13 which are produced during tissue injury [7]. M2b and M2c are regulatory macrophages that are generated in the later stages of the adaptive immune response, serve to limit inflammation and dampen the immune response [8].

The majority of studies on macrophage polarization have been conducted in the murine model. Murine macrophages are well characterized with distinct markers that delineate polarization states in mice such as arginase-1 and Ym1 in M2 polarized states [9]. However human polarized macrophages are poorly characterized. There are no such phenotypic markers that are only present in one polarization state of human macrophages. Furthermore, studies exploring polarization are limited to M1 and M2a macrophages. In addition, markers alone are not sufficient in characterizing macrophage polarization states [10].

Macrophages are a major source of cytokines produced in the body and play a central role in the immune response, hematopoiesis and inflammation by virtue of their cytokine production [11]. Macrophages express pattern recognition receptors (PRRs) that can recognize conserved repeated motifs on a variety of pathogens called pathogen-associated molecular patterns (PAMPs). Activation of PRRs, such as Toll-like receptors (TLRs) by virtue of their agonists leads to the production of pro-inflammatory cytokines as well as destruction of the pathogen via phagocytosis or release of cytotoxic agents [12]. There are at least 13 TLRs that have been discovered and each has their own specificity to microbial ligands [13]. Therefore, the present study was undertaken to characterize macrophage polarization using a restricted panel of surface markers with respect to M1, M2a, M2b and M2c macrophages and production of pro- and anti-inflammatory cytokines in response to various TLR ligands. TLR 2, 3 and 4 ligands

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reflect majority of bacterial ligands and thus were used to characterize the cytokine profile of the human polarized macrophages.

## Materials and Methods

### Generation of polarized macrophages

Blood was obtained from healthy donors as per the protocol approved by the Ethics Review Committee of The Ottawa Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated by collecting the buffy coat generated after Ficoll Paque (GE Healthcare, Buckinghamshire, UK) density centrifugation. PBMCs were re-suspended ($4 \times 10^6$/ml) in Iscove's Modified DMEM 1X media (Sigma-Aldrich) St. Louis, MO and seeded into 12 well polystyrene plates (Thermoscientific, Rochester, New York) to isolate monocytes by adherence method. Monocytes were allowed to adhere at 37°C, 5% $CO_2$ for 3 h. Non-adherent cells were washed off using Iscove's Modified DMEM 1X media. The adherent monocytes were cultured for 6 days in Iscove's Modified DMEM 1X media supplemented with 10% FBS (GE Healthcare), 10 units/ml of penicillin/gentamicin (Sigma-Aldrich) and 10 ng/ml recombinant MCSF (R&D Systems) to generate monocyte-derived-macrophages (MDMs). The media was changed every second day. MDMs were polarized using appropriate stimuli for 2 days: IFNγ (20 ng/ml) (Thermoscientific, Rochester, New York) for M1 macrophages, IL-4 (20 ng/ml) (R&D Systems, Minneapolis, Minnesota) for M2a macrophages, LPS (1 μg/ml) (Sigma-Aldrich) and IL-1β (10 ng/ml) (R&D Systems) for M2b macrophages and IL-10 (10 ng/ml) (R&D Systems) for M2c macrophages.

### Cell surface marker analysis by flow cytometry

Polarized macrophages were washed with PBS and stained using phycoerythrin-conjugated antibodies for CD80, CD86, CD14, TLR4, CD163, CD200R and HLADR (BD Biosciences, Franklin Lakes, New Jersey) for 1 h at 4°C in the dark as described earlier. The cells were washed with 1X PBS (Sigma-Aldrich) to remove excess antibodies. Fluorescence was measured *via* flow cytometry using a FACSCanto flow cytometer and FACSDiva software (BD Biosciences, Franklin Lakes, NJ). Histograms were plotted using WinMDI version 2.8 software (J. Trotter, Scripps Institute, San Diego, CA).

### Cytokine analysis by flow cytometry

Cytokine secretion was measured using Human Th1/Th2/Th9/Th17/Th22 13plex FlowCytomix Multiplex from affymetrix eBioscience (San Diego, CA) according to manufacturer's instructions. The bead-based immunoassay is designed to detect multiple analytes in the same sample. In this assay, the beads are coated with antibodies to the respective analyte and can be distinguished by their size and spectral address. 25 μl of the sample supernatant was incubated with the bead mixture and then incubated with a mixture of biotin-labelled antibodies against the analytes to be measured. Streptavidin-Phycoerythrin, which binds to the biotin and emits fluorescence, was then added to the mixture. Fluorescence was measured *via* flow cytometry using a FACSCanto flow cytometer and FACSDiva software (BD Biosciences, Franklin Lakes, NJ).

### Cytokine analysis by ELISA

**IL-23:** Human IL-23 Duoset (R&D Systems), a plate based ELISA, was used to measure IL-23 in macrophage supernatants. Briefly, the Costar high binding 96 well ELISA plates (Corning Incorporated, Corning, NY) were coated overnight at 4°C with the capture antibody (60 μg/ml) in PBS. The following day, plates were washed with 0.05% Tween 20 (Biorad laboratories, Saint Laurent, QC, Canada) and blocked with 1% Bovine serum albumin (BSA) for 2 h at room temperature. The following day the detection antibody (400 ng/ml) in 1% BSA was added for 2 h followed by the addition of Streptavidin-HRP in 1% BSA for 30 min and substrate (BioFX Labs, Owing Mills, MD) for another 30 min. The reaction was stopped by Stop Solution (BioFX Labs). The plates were read at 490 nm using iMark Microplate reader (Biorad) and data was processed using Micro Plate Manager 6 software.

**IFNγ:** Human IFNγ Duoset (R&D Systems, Minneapolis, MN) ELISA was used to measure IFNγ levels in macrophage supernatants. The plates were coated overnight at 4°C with 100 μl/well of capture antibody (4 μg/ml) in PBS. The plates were washed with 0.05% Tween 20 and blocked with 1% BSA for 2 h at room temperature. The detection antibody (200 ng/ml) in 1% BSA and 2% normal goat serum was added to the plates at room temperature for 2 h. Subsequently, Streptavidin-HRP in 1% BSA was added and incubated at room temperature for 30 min followed by the addition of substrate (BioFX Labs) for another 30 min. The reaction was stopped by the Stop Solution (BioFX Labs). The plates were read at 490 nm using iMark Microplate reader (Biorad) and the data was processed using Micro Plate Manager 6 software.

## Results

### Distinct cell surface marker profile of polarized macrophages

Macrophages subsets were characterized by a differential expression of the cell surface molecules that are generally present on macrophages, including CD80, CD86, HLA-DR, CD14, TLR4, CD163 and CD200R. This restricted panel was selected based on literature reports, and the involvement of these molecules in macrophage activation [14-16]. The expression of the cell surface molecules was determined by flow cytometry analyses setting the expression levels in unpolarized MDMs as the base line. M1 macrophages were found to have significantly higher CD80 and CD86 expression compared to MDMs. M2a macrophages exhibited a significantly higher expression of CD86 and CD200R and significantly lower expression of CD14 and TLR4 as compared to MDMs. M2b macrophages had significantly higher CD80 and CD14 expression and lower HLA-DR expression as compared to MDMs. M2c macrophages were characterized by a significant decrease in CD86 and HLA-DR expression and a significant increase in CD163 expression compared to MDMs. The expression levels of other cell surface markers were similar to that of unpolarized MDMs (Figure 1). In summary, M1 macrophages are $CD80^{high}$ and $CD86^{high}$, M2a macrophages are $CD86^{high}$, $CD200R^{high}$, $TLR4^{low}$ and $CD14^{low}$, M2b macrophages are $HLA\text{-}DR^{low}$, $CD80^{high}$ and $CD14^{high}$, and M2c macrophages are $CD86^{low}$, $HLA\text{-}DR^{low}$, $TLR4^{high}$, and $CD163^{high}$.

### Basal cytokine profile of polarized macrophages subsets

To analyze the cytokine profile, the polarizing stimulus was completely removed using aspiration after 48 h, fresh media was added and the cells were incubated for additional 24 h. The cell culture supernatants were examined for the production of key cytokines involved in the immune responses including: IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p70, IL-13, IL-17A, IL-22, IL-23 and TNFα using the FlowCytomix kit. The results showed that IL-2, IL-4, IL-9, IL-13 or IL-17A cytokines were not produced by any of the polarized states (Figure 2). Unstimulated M1 macrophages did not produce significant levels of any of the cytokines. In contrast, the M2b macrophages produced significant levels of TNFα and IL-6 compared to the other macrophage populations analyzed. The M2a and M2c macrophages exhibited a similar cytokine profile manifested

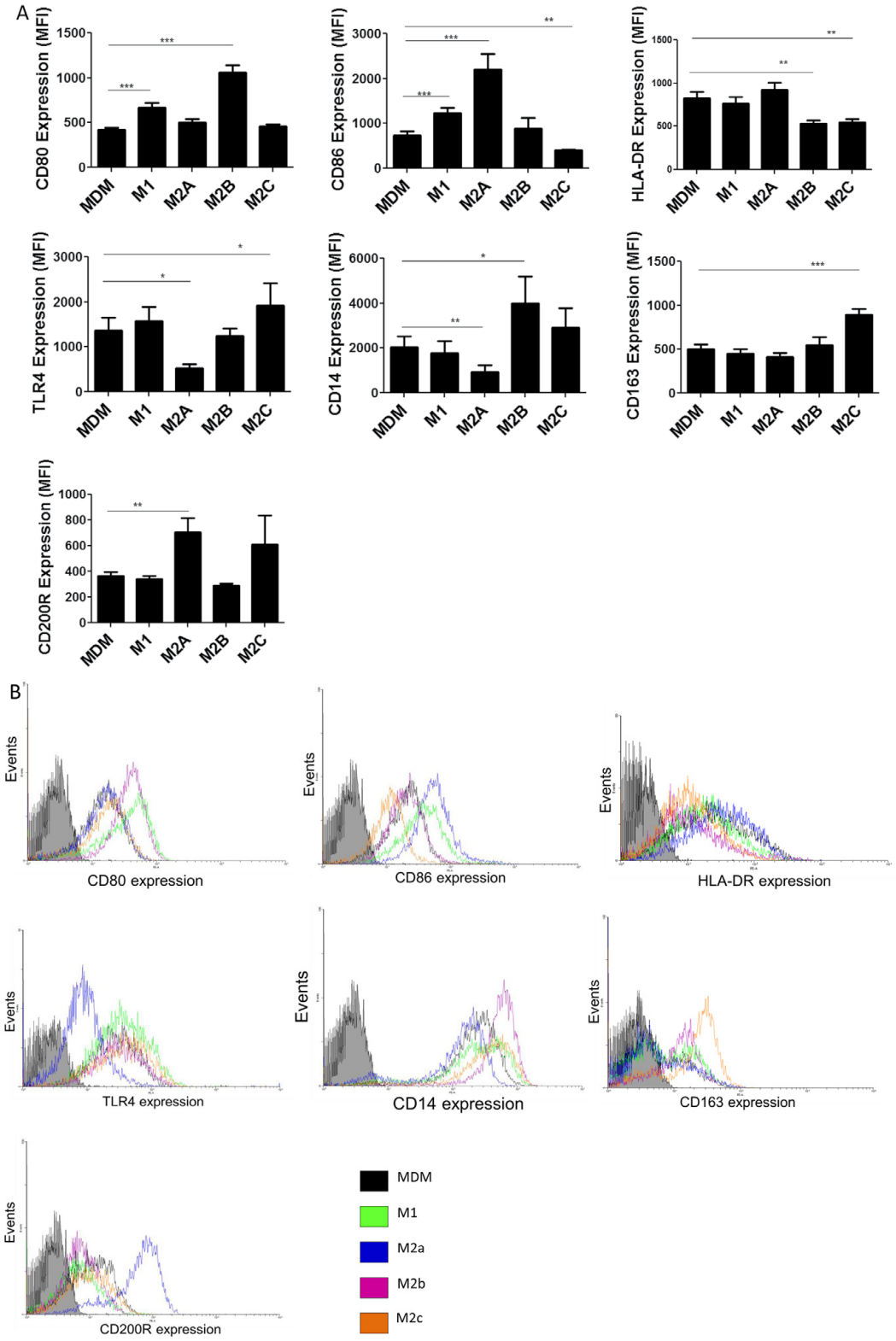


**Figure 1: Cell surface marker characterization of polarized macrophages.** MDMs were polarized using indicated stimuli for 2 days: IFNγ (20 ng/ml) for M1 macrophages, IL-4 (20 ng/ml) for M2a macrophages, LPS (1 μg/ml) and IL-1β (10 ng/ml) for M2b macrophages and IL-10 (10 ng/ml) for M2c macrophages. Polarized macrophages were stained with antibodies against the mentioned cell surface molecules and fluorescence was measured by flow cytometry. MFI values **(A)** were obtained using FACSDiva software and histograms **(B)** from one representative experiment are shown. Bar graphs represent mean ± SEM, $^{*}p \leq 0.05$; $^{**}p \leq 0.005$; $^{***}p \leq 0.0005$ with n=10 (CD80, CD86, HLA-DR, CD14, CD163 and CD200R) and n=4 (TLR4).

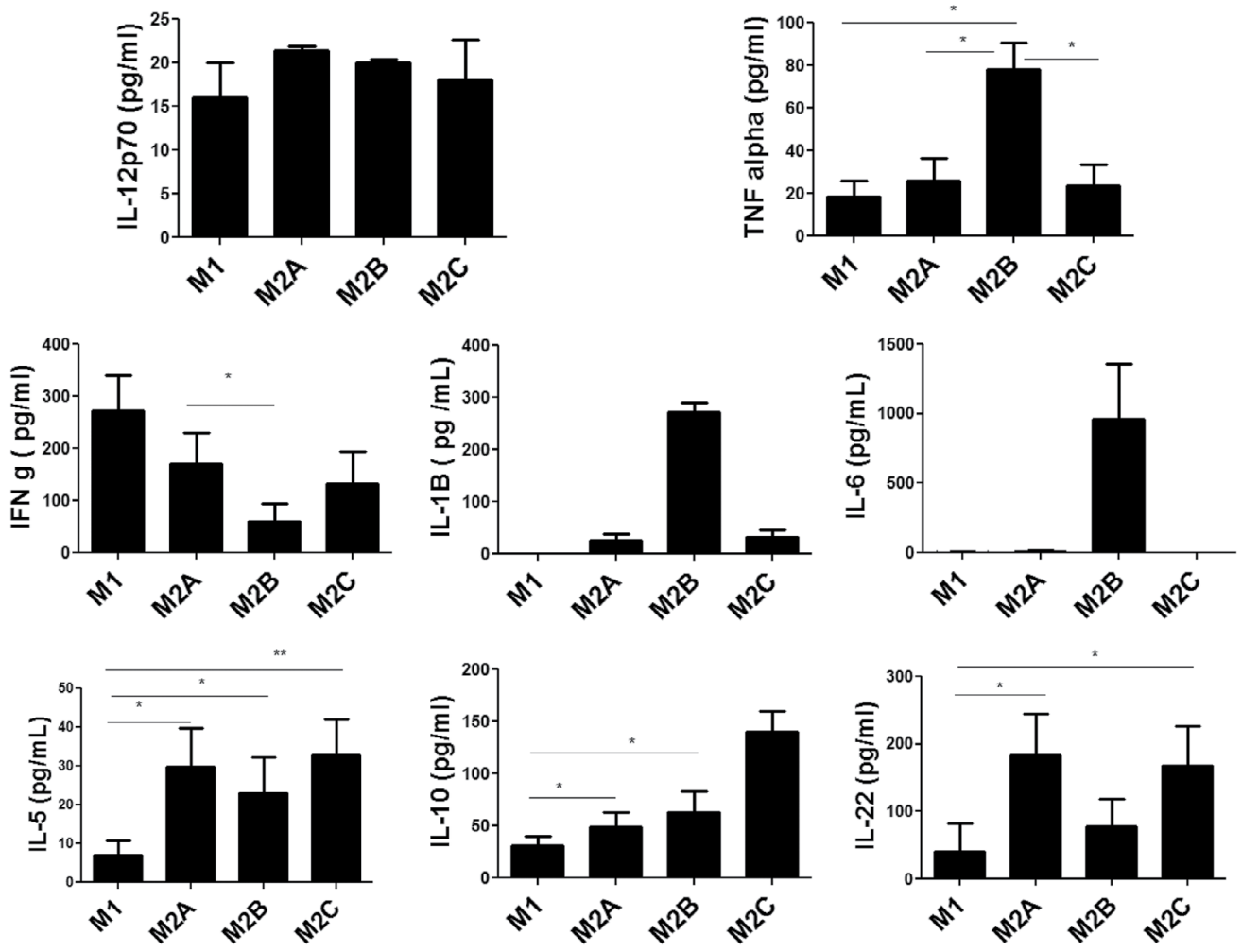


**Figure 2: Basal cytokine production in polarized macrophages.** MDMs were polarized using indicated stimuli for 2 days: IFNγ (20 ng/ml) for M1 macrophages, IL-4 (20 ng/ml) for M2a macrophages, LPS (1 μg/ml) and IL-1β (10 ng/ml) for M2b macrophages and IL-10 (10 ng/ml) for M2c macrophages. Polarizing stimuli were removed after the 2 days, fresh media was added and the cells were incubated for another 24 h. Cytokine levels in the culture supernatants were measured using flow cytometry as described in Materials and Methods. Bar graphs represent mean ± SEM, *p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005 with at least n=5.

by enhanced production of IL-22 and IL-5 and low levels of IL-1β and IL-6. All the M2 macrophages subsets including M2a, M2b and M2c produced enhanced levels of IL-10 as compared to M1 macrophages. Significance was not calculated for the M2c macrophages since IL-10 was used to generate these macrophages. Thus, M2c macrophages were excluded when calculating significance for IL-10. Similar levels of IL-12p70 were produced by all polarized macrophages.

## Cytokine profile of polarized macrophages after TLR 2, 3 and 4 stimulation

Macrophage subsets may produce distinct cytokine profiles in response to inflammatory stimuli such as following activation of receptors, such as TLRs, known to be recognized by bacterial and viral pathogens. Polarized macrophages were stimulated for 24 h with ligands against two cell surface bound TLRs, LPS (TLR4), and LTA (TLR2), and one against intracellular membrane bound TLR, Poly I:C (TLR3). The M1 macrophages secreted predominately and significantly higher levels of pro-inflammatory cytokines IL-23, IL-12p70, TNFα and IFNγ upon stimulation with LPS (Figure 3), LTA (Figure 4), and Poly I:C (Figure 5) as compared to unstimulated cells. In contrast, none of the M2 macrophage subsets (M2a, M2b and M2c) produced significant levels of IL-23, IL-12p70, TNFα or IFNγ upon stimulation. Although stimulation of M1 macrophages with different TLR ligands exhibited similar profiles of cytokine production, there were also some differences. For example, LPS stimulation of M1 macrophages also resulted in the induction of IL-1β, IL-6, IL-5, IL-10 and IL-22 (Figure 3). LTA stimulation of M1 macrophages resulted in the induction of IL-1β, IL-6, IL-5 and IL-10 (Figure 4) and Poly I:C stimulation led to the induction of IL-6, IL-5 and IL-10 (Figure 5).

M2b macrophages were poor inducers of the cytokines tested, regardless of the TLR stimulation used, with the exception of IL-10 and IL-5. IL-10 and IL-5 were significantly induced in M2b macrophages following LPS stimulation (Figure 3). Stimulation of M2a and M2c macrophages with LPS induced IL-1β, IL-6, IL-5 and IL-10 (Figure 3), while stimulation with LTA led to the induction of IL-1β, IL-6 and IL-10 (Figure 4), and stimulation with Poly I:C resulted in IL-10 production and a decrease in IL-22 secretion (Figure 5). Moreover, there were several differences between the M2a and M2c macrophages

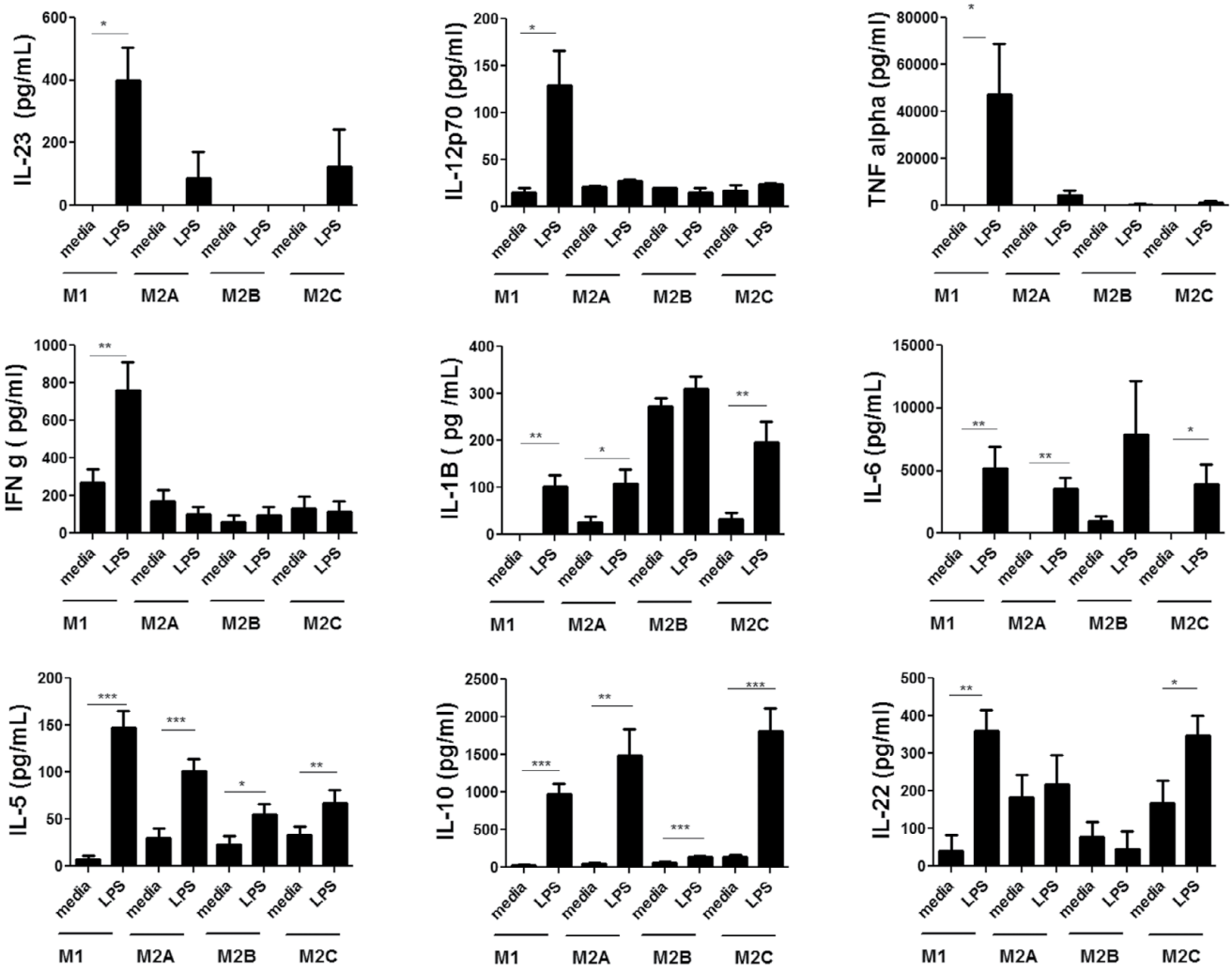


**Figure 3: Cytokine production in polarized macrophages following LPS stimulation.** MDMs were polarized using appropriate stimuli for 2 days: IFNγ (20 ng/ml) for M1 macrophages, IL-4 (20 ng/ml) for M2a macrophages, LPS (1 μg/ml) and IL-1β (10 ng/ml) for M2b macrophages and IL-10 (10 ng/ml) for M2c macrophages. Polarizing stimuli were removed after the 2 days and polarized macrophages were treated with LPS (1 μg/ml) for 24 h. Cytokine levels in the supernatants were measured using flow cytometry as described in Materials and Methods. Bar graphs represent mean ± SEM, *p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005 with at least n=5.

with respect to their response to TLR agonists. In particular, M2c but not M2a macrophages induced IL-22 upon LPS stimulation (Figure 3). However, M2a but not M2c macrophages induced IL-22 and IL-5 upon LTA stimulation (Figure 4). In addition, M2c macrophages induced high levels of IL-6 and IL-10 when stimulated with Poly I:C (Figure 5).

## Discussion

In innate or adaptive immunity, differentiated hematopoietic cells must regulate their functional response in such a way as to limit damage while clearing infection or inflammation in the surrounding tissue. One mechanism by which this is carried out is fine tuning of the immune response influenced by the milieu to achieve a polarized state of macrophage cells that can effectively contribute to the re-establishment of homeostasis. In this study, we characterize the polarization states of macrophages using *in vitro* generated human MDMs with respect to expression of surface markers and their cytokine profile.

Our results show that M1 macrophages generated following IFN-γ stimulation express higher levels of CD80, CD86 and TLR-4 and exhibit a proinflammatory cytokine profile as compared to MDMs and suggest that these cells are capable of responding efficiently to microbial/endotoxin challenge through enhanced expression of co-stimulatory molecules and proinflammatory cytokines [17].

M2a macrophages exhibited a distinctly higher CD200R expression as compared to MDMs. However, murine macrophages do not express CD200R following IL-4 stimulation [18]. Binding of CD200 to CD200R present on endothelial cells delivers a down-regulatory signal and exerts an inhibitory effect on macrophage activation. Expression of viral homologues of CD200, such as herpesvirus 8 K14 protein [19], cytomegalovirus e127 [20] protein and myxoma virus M141R [21] in the Herpesviridae and Poxviridae families [22], results in asymptomatic infection and inhibition of macrophage function. This suggests that enhanced expression of CD200R by M2a macrophages may contribute to viral pathology. M2a macrophages also expressed significantly higher levels of CD86 compared to the other subsets. CD86 has been shown to promote Th2 cell differentiation [18] and CD86 co-stimulation leads to IL-4 production by Th2 cells [23]. Thus, IL-4 promotes M2a

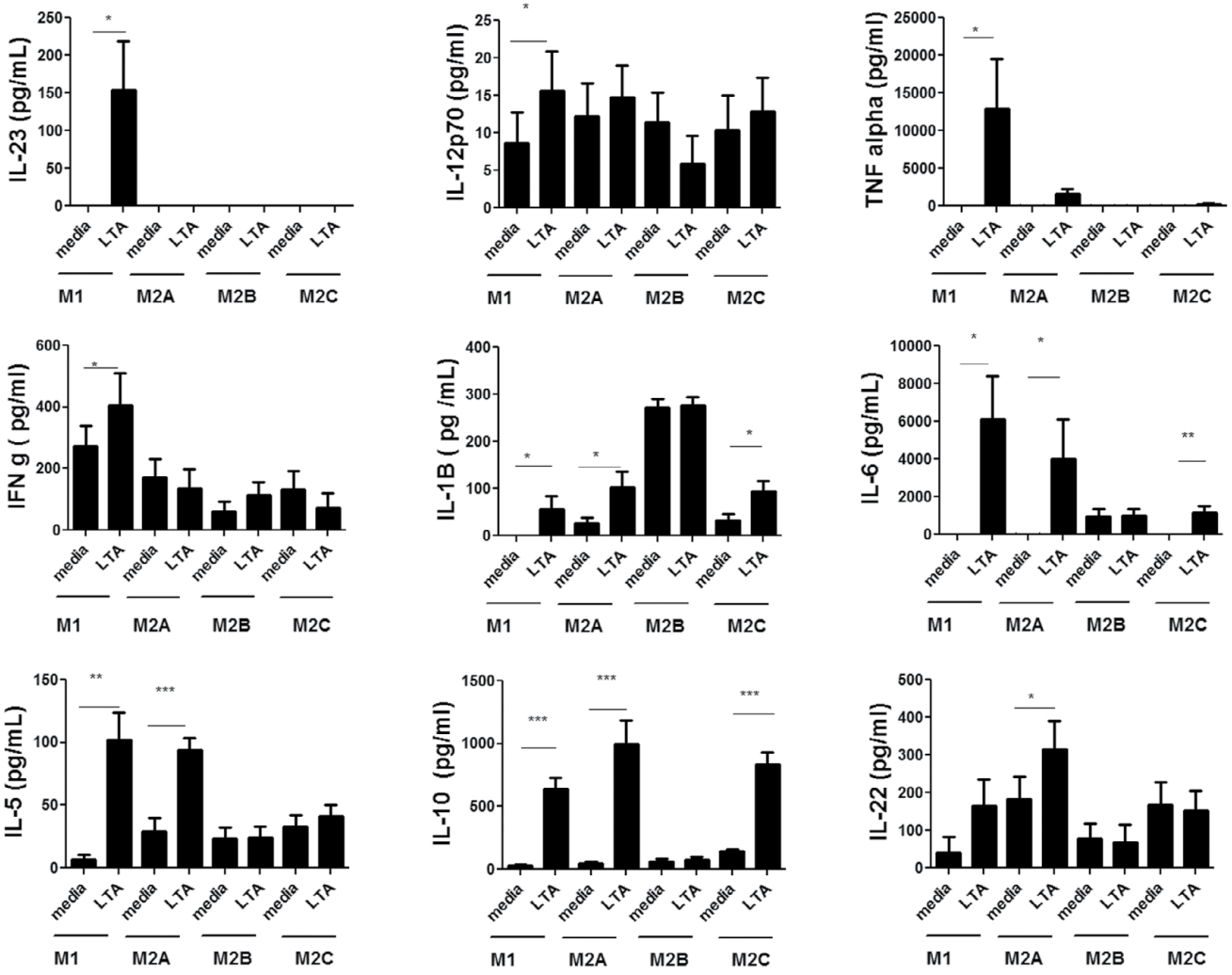


**Figure 4. Cytokine production in polarized macrophages following LTA stimulation.** MDMs were polarized as mentioned previously. Polarizing stimuli were removed after the 2 days and polarized macrophages were treated with LTA (5 µg/ml) for 24 h. Cytokine levels in the supernatants were measured using flow cytometry as described in Materials and Methods. Bar graphs represent mean ± SEM, *p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005 with at least n=5.

polarization which feeds back and may drive the Th2 response by virtue of CD86 engagement. Human infection with *Ascaris lumbricoides* was shown to drive a Th2 response [24]. Indeed, it has been demonstrated that M2a macrophages are essential for survival during helminth infection [25]. It has also been reported that M2a macrophages express low levels of CD86 [15]. The contrasting CD86 expression levels in these macrophages may be due to different stimulation protocols employed in these studies.

M2b and M2c macrophages expressed low levels of HLA-DR as compared to MDMs. Lower expression levels of HLA-DR have been linked with immune responses mediated by stress, such as trauma, burns, surgery and sepsis [26]. Low HLA-DR expression is in agreement with the role of M2b and M2c macrophages to dampen the immune response. M2c macrophages also expressed high levels of CD163 as compared to the other polarization states. High CD163 expression in macrophages has been linked to inflammatory disease, including liver cirrhosis, type 2 diabetes, macrophage activation syndrome, sepsis, rheumatoid arthritis and Hodgkin Lymphoma [27]. CD163 plays a role in preventing tissue inflammation by clearing hemoglobin/ haptoglobin complexes and inducing antioxidative enzyme heme oxygenase-1 [28]. The soluble form of CD163 plays a role in preventing inflammation by preventing T cell activation [28,29]. Thus, high expression of CD163 by M2c macrophages highlights their role in preventing tissue inflammation.

We characterized cytokine profiles exhibited by polarized macrophages by examining production of various immunoregulatory cytokines before and after TLR-2, -3 and -4 agonist stimulation. Most studies thus far have characterized cytokines in polarized macrophages by mRNA analysis [14]. Recent developments in mRNA/protein analysis techniques have demonstrated the correlation between mRNA and protein expression is rather poor [30,31]. Fournier et al., demonstrated that for 26 out of 56 proteins, an increase in protein expression did not correspond to an increase in mRNA expression, and some of these proteins actually demonstrated a decrease in mRNA expression [30]. Our results show lack of IL-2, IL-4, IL-9, IL-13 or IL-17A production by all polarized states. None of the polarized macrophages produced IL-23 and IL-12p70 at the basal level prior to stimulation. At the basal level, M2b macrophages predominately produced higher levels of

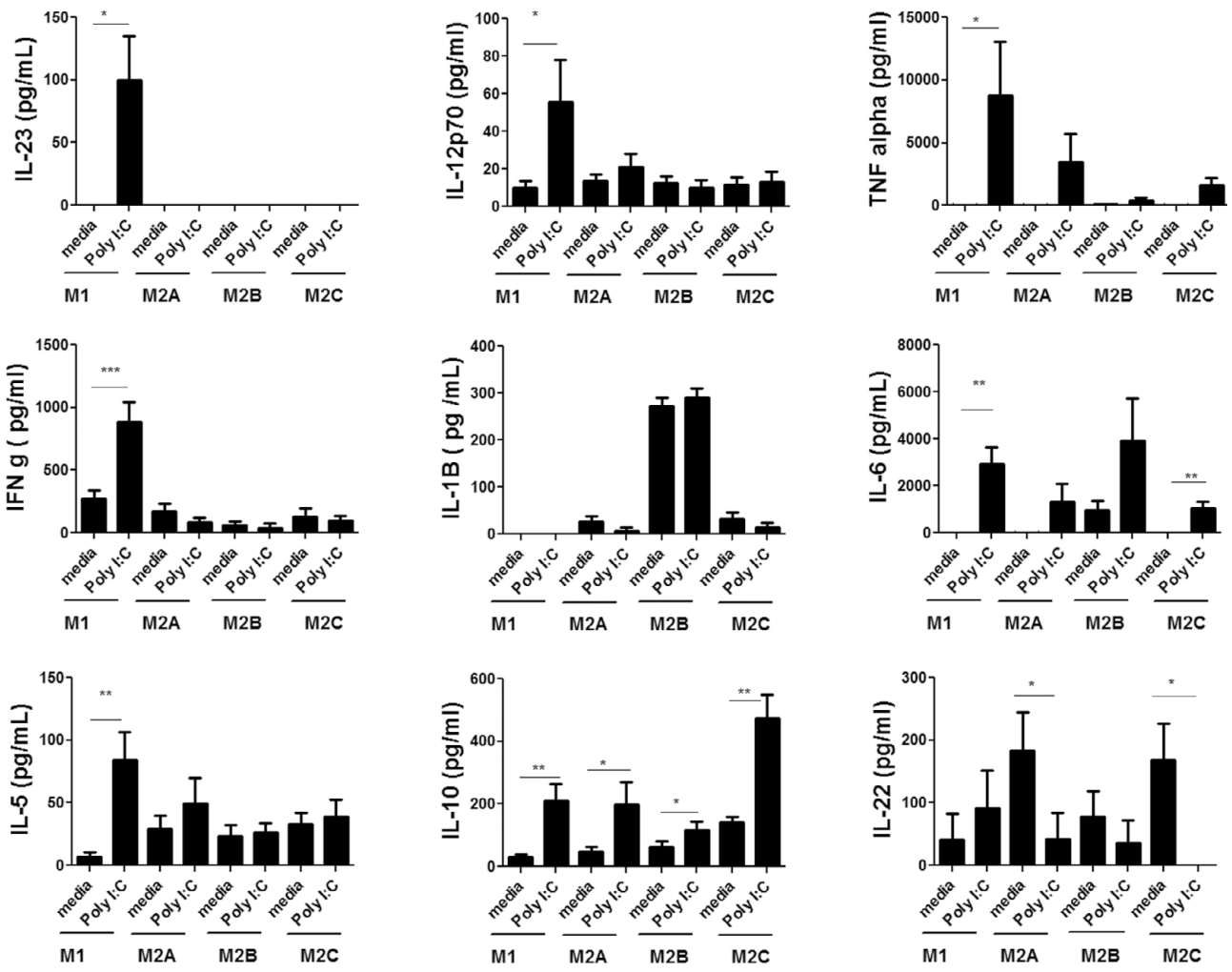


**Figure 5: Cytokine production in polarized macrophages following Poly I:C stimulation.** MDMs were polarized as described previously. Polarizing stimuli were removed after the 2 days and polarized macrophages were treated with Poly I:C (50 μg/ml) for 24 h. Cytokine levels in the supernatants were measured using flow cytometry as described in Materials and Methods. Bar graphs represent mean ± SEM, *p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005 with at least n=5.

IL-6 and TNFα. This is in agreement with the murine model where macrophages, classified as type II and polarized with TLR agonists were reported to secrete TNFα and IL-6 [32]. TNFα and IL-6 are considered as biomarkers for inflammation and sepsis [33]. This is consistent with M2b macrophages being implicated in sepsis and inflammatory response [34]. Moreover, M2b macrophages become tolerant to further inflammatory stimuli [34]. Consistent with literature reports, the M2 polarized macrophages were found to produce more IL-10 than M1 macrophages [35]. M2a and M2c macrophages had a similar cytokine profile suggesting shared transcriptional responses by the polarization stimuli.

TLR-2, -3 and -4 ligands reflect a broad range of microbial ligands and thus were used to characterize the cytokine profile of the polarized macrophages. Activation of the IRF5 transcription factor has been linked to induction of IL-12p40, a subunit of IL-12 and IL-23 as well as to M1 polarization [36]. Consistent with this, M1 macrophages predominately produced IL-23, IL-12p70 and TNFα when stimulated with LPS, LTA or Poly I:C compared to M2 macrophages. This is in contrast to a previous report demonstrating lack of IL-23 and IL-12 production in M1 macrophages [37]. This could be a result of the difference in macrophage generation as macrophages were generated for 6 days in the presence of M-CSF and followed by co-stimulation with LPS and IFNγ for 24 h [37].

Our results show that M2b macrophages did not induce significant levels of pro-inflammatory cytokines, including IL-23, IL-12p70, TNFα, in response to either TLR 2, 3 or 4 stimulation. These results may be explained by observations showing M2b macrophages being tolerant to inflammatory stimuli such as TLR agonists [34]. In contrast, M2b macrophages produced high levels of IL-10, an anti-inflammatory cytokine important in limiting inflammation-mediated tissue injury [38]. M2a and M2c macrophages exhibited similar cytokine profiles prior to and after TLR stimulation although they were polarized using IL-4 and IL-10, respectively. This phenomenon is analogous to what has been observed with M2a macrophages polarized by either IL-4 or IL-13. Though IL-13 and IL-4 are different cytokines they both polarize macrophages to the M2a state and share a similar transcriptional response by engagement of signal transduction pathways culminating in STAT6 phosphorylation [39]. Similarly, IL-10 and IL-4 could share

a transcriptional profile allowing similar cytokine profile of M2a and M2c macrophages.

In summary, this study characterizes all four polarization states in human macrophages with respect to their surface marker and cytokine profile. Phenotypic markers can be used to characterize macrophage populations in tissue inflammatory disease conditions *in vivo* to further understand disease pathogenesis.

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