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Temporal Phenotypic Features Distinguish Polarized Macrophages *In Vitro*

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Abstract

Macrophages are important in vascular inflammation and environmental factors influence macrophage plasticity. Macrophage transitions into pro-inflammatory (M1) or anti-inflammatory (M2) states have been defined predominately by measuring cytokines in culture media (CM). However, temporal relationships between cellular and secreted cytokines have not been established. We measured phenotypic markers and cytokines in cellular and CM of murine bone marrow-derived macrophages at multiple time points following stimulation with IFN- γ +LPS (M1), IL-4 (M2a), or IL-10 (M2c). Cytokines/proteins in M1-polarized macrophages exhibited two distinct temporal patterns; an early (0.5–3 hr), transient increase in cellular cytokines (GM-CSF, KC-GRO, MIP-2, IP-10 and MIP-1 β) and a delayed (3–6 hrs) response that was more sustained [IL-3, regulated on activation normal T cell expressed and secreted (RANTES), and tissue inhibitor of metalloproteinases 1 (TIMP-1)]. M2a-related cytokine/cell markers (IGF-1, *Fizz1*, and *Ym1*) were progressively (3–24 hrs) increased post-stimulation. Additionally, novel patterns were observed. First, and unexpectedly, cellular pro-inflammatory chemokines, MCP-1 and MCP-3 but not MCP-5, were comparably increased in M1 and M2a macrophages. Second, *Vegfr1* mRNA was decreased in M1 and increased in M2a macrophages. Finally, VEGF-A was increased in the CM of M1 cultures and strikingly reduced in M2a coinciding with increased *Vegfr1* expression, suggesting decreased VEGF-A in M2a CM was secondary to increased soluble VEGFR1. In conclusion, macrophage cytokine production and marker expression were temporally

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regulated and relative levels compared across polarizing conditions were highly dependent upon the timing and location (cellular vs. CM) of the sample collection. For most cytokines, cellular production preceded increases in the CM suggesting that cellular regulatory pathways should be studied within 6 hours of stimulation. The divergent polarization-dependent expression of *Vegfr1* may be essential to controlling VEGF potentially regulating angiogenesis and inflammatory cell infiltration in the vascular niche. The current study expands the repertoire of cytokines produced by polarized macrophages and provides insights into the dynamic regulation of macrophage polarization and resulting cytokines, proteins, and gene expression that influence vascular inflammation.

Keywords

chemokine; cytokine; macrophage; polarization; temporal

Introduction

Vascular inflammation is defined by the infiltration of the vessel wall by inflammatory cells (lymphocytes, neutrophils, and monocytes/macrophages) and their complex interactions with vessel endothelial cells, fibroblasts, vascular smooth muscle cells, and the extracellular matrix. Vascular inflammation is a central characteristic in several pathologies such as atherosclerosis, vasculitis, and aneurysmal disease. Pro-inflammatory cytokines are mediators of disease progression and monocytes/macrophages are primary sources of many of these cytokines (1, 2). Macrophages and the pro-inflammatory cytokines they produce have been implicated in atherosclerosis (3), Kawasaki disease (4), giant cell arteritis (5), abdominal aortic aneurysms (6), and aortic dissections (7). Interestingly, macrophages are capable of interrupting this chronic pro-inflammatory condition through a functional change characterized by diminished pro-inflammatory and increased anti-inflammatory cytokine production. This therapeutic strategy has been identified in the treatment of Kawasaki disease (4) and atherosclerosis (3). The potential of macrophages to achieve such diversity in functional phenotype and the utility for treating multiple diseases is gaining attention in multiple fields but remains relatively unexplored in many vascular inflammatory conditions.

A better understanding of macrophage diversity could provide therapeutic targets to alter the course of many diseases. However, attempts to define macrophage populations *in vivo* have failed to fully characterize this complexity. Given that environmental cues affect macrophage responses, a more simplistic, agonist-dependent *in vitro* model of macrophage activation was devised. This model has provided insight into distinct macrophage phenotypes known as macrophage polarization states (8).

Macrophage polarization has been defined by *in vitro* culture systems to ascertain the effect of various stimuli on macrophage activation and the functional outcome. The first polarization state, defined as ‘classical’ activation or M1, alludes to the Th1 cytokine interferon- γ (IFN- γ) that induced pro-inflammatory, pathogen killing, and reactive oxygen species producing macrophage phenotype.(9, 10) However, the full activation of M1 macrophages was achieved when IFN- γ was accompanied or followed by tumor necrosis

factor- α (TNF- α) or toll-like receptor (TLR) ligation, such as lipopolysaccharide (LPS) binding to TLR4, resulting in TNF- α production (11, 12).

Siamon Gordon's group soon discovered that a very different phenotype was observed when macrophages were exposed to interleukin (IL) 4 or 13. These 'alternatively' activated or M2 macrophages, alluding to the Th2 cytokines IL-4 & IL-13, cleared mannosylated ligands by increased expression of a macrophage mannose receptor, increased MHC II expression, and reduced pro-inflammatory cytokines.(13, 14) The M2 classification has been further subdivided to account for other agonist-dependent phenotypes that also counter the pro-inflammatory nature of M1 macrophages. These subtypes are M2a alternative/wound healing macrophage (IL-4 or IL-13 mediated activation), M2b Type 2 immunoregulatory macrophage (LPS + immune complex activation)(15), and M2c regulatory/deactivated macrophage (IL-10 or glucocorticoid activation) (16, 17).

Polarization phenotypes represent a continuum on which M1 and M2 form the extremes. M1 macrophages are characterized by high IL-12, IL-6, IL-1, IL-23, TNF- α , nitric oxide production, and increased intracellular pathogen killing (11, 12, 17–19). Other cytokines produced by pro-inflammatory monocytes/macrophages include regulated on activation normal T cell expressed and secreted (RANTES) (20, 21), macrophage inflammatory protein-1 β (MIP-1 β) (22), macrophage inflammatory protein 2 (MIP-2) (23), tissue inhibitor of metalloproteinase 1 (TIMP-1) (24), interferon- γ -induced protein 10 (IP-10) (17, 25), keratinocyte-derived cytokine (KC-GRO) (23), monocyte chemotactic protein (MCP) MCP-1 (26–29), MCP-3 (27, 29), and MCP-5 (30). Cytokines are generally classified as pro-inflammatory due to their production by immune cells in response to inflammatory stimuli but also by their capability to promote inflammation. The best example is TNF- α ; which is both produced by macrophages in response to LPS and promotes fever (31), vasodilation, leukocyte vascular adhesion, and immune cell recruitment (32). Other inflammatory monocyte/macrophage-related cytokines also have pro-inflammatory functions including IL-12 inducing Th1 cell maturation/activation (33), IL-1 (34) and IL-6 (31) causing fevers, IL-6 stimulating the acute phase response (35), and immune cell recruitment by multiple cytokines: MCP-1 (26–29), MCP-3 (27, 29), MCP-5 (30), IP-10 (36), KC-GRO (17, 37), MIP-1 β (17, 38, 39), MIP-2 (23), and RANTES (20, 21).

M2a macrophages are defined by increased scavenger receptor (SR-A), arginase, decoy IL-1 receptor, growth factors [e.g. transforming growth factor- β (TGF- β) and insulin-like growth factor 1 (IGF-1)] (11, 12, 17–19) as well as expression of mRNAs [mannose receptor (*Cd206*) (11), chitinase-like 3 (*Chil3* aka *Ym1*) (40, 41), and resistin-like α (*Retnla* aka *Fizz1*) (40, 41)]. While M2b macrophages produced pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 they also produced large amounts of IL-10 and lower levels of IL-12 (15, 42). Given this mixed phenotype, M2b macrophages may represent an intermediate between M1 and M2a polarization states. M2c macrophages are primarily characterized by the dramatic reduction of pro-inflammatory cytokine/chemokine expression and production of IL-10 and TGF- β (12, 17, 21). The apparent simplicity of the *in vitro* model and the identification of intermediate phenotypes reflect the complex nature of macrophage plasticity.

The complexity of macrophage polarization has contributed to controversies within the literature. For example, IL-10, a potent anti-inflammatory cytokine, has been identified by several groups as a M2 marker of macrophage polarization (19, 42, 45–48). However, IL-10 was also produced in copious levels from M1 polarized macrophages (49–52). Although these studies provided conflicting results, assumptions utilized in the *in vitro* system, especially without comparing polarization conditions within the same experiment, may have contributed to the differing conclusions and may further complicate work to define mechanisms involved in macrophage polarization.

Multiple efforts have been focused on dissecting regulatory mechanisms that underlie the complex phenotypes defining macrophage polarization. Essential pathways,(53) transcription factors,(54, 55) epigenetic modifications,(56) and post-transcriptional regulatory mechanisms, such as microRNA (57, 58) have been identified but their role in macrophage polarization in the *in vitro* system is poorly understood.

One challenge to understanding regulatory pathways is the limitation of measuring cytokines in macrophage culture media (CM). Widespread studies have defined polarization-related cytokines by measuring levels in the CM, often days after macrophage stimulation. While cytokines may accumulate and remain in CM, the events leading to cellular cytokine production may occur earlier than previously studied. The stability of cytokines in CM has been demonstrated by measuring both cytokine mRNA in the macrophages and cytokine proteins in CM. These studies demonstrated that cellular mRNA was decreased when cytokine protein in CM was maximal (26, 50, 59). For example, in both Wong *et al.* (59) and Huang *et al.* (26), TNF- α cellular mRNA and CM protein were measured at the same time points, demonstrating three important issues; 1) TNF- α protein was stable in the CM, 2) TNF- α protein in the CM did not reflect temporal changes in the expression of cellular TNF- α mRNA, 3) sustained elevations of TNF- α in the CM were associated with decreased cellular mRNA possibly due to feedback inhibitory mechanisms (60). Similar observations were also present with IL-6 (26) and IL-12p40 (50). Thus, activation of pathways necessary to create the cytokines in the CM likely occurred earlier than the rise in cytokines in the CM, suggesting that signal transduction pathways and release kinetics of cytokines should be studied early in the time course of the experiments after identification of the critical time points for each cytokine.

Another pitfall is the reliance on mRNA expression/abundance changes to define dynamic regulation of polarization without protein measurement (26, 50, 61, 62). Over the last decade, the central dogma of molecular biology of DNA to RNA to protein has been amended to account for other regulatory mechanisms such as translational control which occurs independent of transcription regulation (63, 64). Thus, elevated mRNA may not result in increased protein. A better indication of the dynamic regulation of polarization may be cellular cytokines, comprehensive characterization of cellular cytokines following macrophage polarization comprises a significant gap in current literature.

Lastly, the dependence of *in vitro* observations upon timing has not been thoroughly appreciated. Many studies have characterized cytokines after polarization with or without additional perturbations at one time point following stimulation (50, 65, 66). To illustrate,

Wu *et al.* showed decreased vascular endothelial growth factor (VEGF) in the CM while *Vegf* mRNA was increased in the cells of IFN- γ stimulated macrophages, all measured at one time point. Whether this represents a disconnect between mRNA and protein or if a later time point would have revealed increased CM VEGF was not determined (65). Studies that accounted for timing in *in vitro* experiments have identified observations that would have been lost if conducted in a single timeframe (26, 59, 61, 62, 67, 68). For example, if time course studies were not conducted it is uncertain that Lucas *et al.* would have identified the diminished level of TNF- α in the CM of LPS-stimulated macrophages co-cultured with apoptotic cells (67) or if Islam *et al.* would have identified age-related differences in IL-10 and IFN- γ expression/production in LPS-stimulated macrophage cultures (68). Time is a critical variable that likely contributes to confusion in the literature and must be accounted for within this *in vitro* model.

Given the issues with timing of macrophage cytokine production versus stability of the cytokine in CM, the controversies regarding cytokine production in different polarization states, and the use of cytokine mRNA as a surrogate for protein expression as markers of macrophage polarization states, the current study addresses these important gaps in the literature by directly comparing multiple cytokines in both cellular and CM, using control and three stimulating conditions, and multiple time points. We hypothesize that the characterization of macrophage polarization markers temporally, across multiple stimulating conditions, and in the cellular vs. CM will reveal distinct time- and location-dependent patterns that can be used to further study the regulatory mechanisms and transcription pathways in macrophage plasticity.

Methods

Bone marrow-derived macrophage cultures

Bone marrow was harvested bilaterally from the femurs and tibias of 3–4 month old C57BL/6J male mice (Jackson Labs, Bar Harbor, ME). All procedures complied with the National Institutes of Health Animal Care and Use Guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and the South Texas Veterans Health Care Systems, San Antonio, TX, Protocol #1107-002 (12025x).

Cell cultures were maintained at 37°C in 5% CO₂. L929-conditioned media was created by plating 4×10^5 L929 cells (ATCC, Manassas, VA) in 50 mL of media [DMEM/F12 + 10% FBS + 1% penicillin/streptomycin (Life Technologies, Grand Island, NY)] in a 175 mm culture flask (Corning, Corning, NY) for 10 days. L929-conditioned media were filtered (0.22 μ m) and stored at -30°C. Bone marrow-derived macrophages (BMDM) were obtained from bone marrow cells after culture at 5×10^6 cells/100 mm non-tissue culture treated petri dish (BD Bioscience, San Jose, CA) in 10 mL bone marrow macrophage media #1 (BMM#1; 30% L929-conditioned media + DMEM/F12 + 10% FBS + 1% penicillin/streptomycin); after day 3, an additional 5 mL of BMM#1 was added to each plate and incubated for another 3 days. BMDM were subcultured into 60 mm non-tissue culture treated petri dishes (BD Bioscience) at 5×10^6 cells/plate in 3 mL of bone marrow macrophage media #2 (BMM#2; 10% L929-conditioned media + DMEM/F12 + 10% FBS +

1% penicillin/streptomycin) and allowed to adhere and proliferate for 48 hours with a media change every 24 hours. After 48 hours, a single 60mm plate of subcultured BMDMs was collected, counted and cellular lysates prepared at 10^7 cells/mL in a lysate buffer previously described (69); lysates were aliquoted and stored at -80°C as a 0 hour time point cellular lysate. Concurrent with the 0 hour lysate preparation, media was replaced in replicate cultures with media (BMM#2) supplemented with IFN- γ (20 ng/mL from Miltenyi Biotech, San Diego, CA; prepared in PBS + 1% FBS) + LPS (1000 ng/mL from Invivogen San Diego, CA; prepared in PBS + 1% FBS) for M1 polarized macrophages, IL-4 (40 ng/mL from Miltenyi Biotech; prepared in PBS + 1% FBS) for M2a polarized cells, or IL-10 (20 ng/mL from Miltenyi Biotech; prepared in PBS + 1% FBS) for M2c polarized macrophages, or media only (BMM#2 only) as a control. Media prepared for this experiment (BMM#2 only) was aliquoted as labeled as “Media” on the figures to determine cytokines present in the BMM#2 media before exposure to macrophages. Cultures were incubated for 0.5, 1, 3, 6, 12, or 24 hours before collection. At each time point, the cell culture media was removed from each condition, centrifuged at $1000\times g$ 4°C for 10 min., the supernatant was aliquoted, and stored at -80°C as cell culture media (CM). In parallel, cells were lysed in the plate for RNA isolation using Qiazol according to the manufacturers specifications (Qiagen, Valencia, CA), or cells were collected, counted and cellular lysates prepared at 10^7 cells/mL in a lysate buffer previously described (69); lysates were aliquoted and stored at -80°C as cellular lysate samples (Cellular).

Cellular and CM cytokine measurement

Cellular protein lysates (Cellular) and paired cell-free culture media (CM) collected from the macrophage cultures were submitted for cytokine profiling by Myriad RBM (Austin, TX) using a microsphere-based immuno-multiplexing technology to quantify analyte concentrations. In addition, cytokines present in the L929 conditioned media and FBS used in the BMM#2 were also measured to determine cytokines in the media prior to exposure to the macrophage cultures. Twenty four different cytokines/chemokines/proteins were quantified including: fibroblast growth factor 9 (FGF-9), granulocyte macrophage colony-stimulating factor (GM-CSF), keratinocyte-derived cytokine (KC or GRO α , aka KC-GRO), interferon- γ (IFN- γ), interleukin (IL) IL-2, IL-3, IL-4, IL-7, IL-10, IL-11, IL-12 (p70), IL-17A, interferon- γ -induced protein 10 (IP-10), lymphotactin, monocyte chemotactic protein (MCP) MCP-1, MCP-3, MCP-5, macrophage inflammatory protein (MIP) MIP-1 β , MIP-2, oncostatin (OSM), stem-cell factor (SCF), regulated on activation normal T cell expressed and secreted (RANTES), tissue inhibitor of metalloproteinase 1 (TIMP-1), and vascular endothelial growth factor A (VEGF-A). Data were reported as concentration in pg/mL.

Insulin-like growth factor 1 (IGF-1) enzyme-linked immunosorbent assay

A colorimetric enzyme-linked immunosorbent assay, Quantikine[®] (R&D Systems, Minneapolis, MN), was used to quantify IGF-1 protein concentrations in cellular protein lysates according to manufacturer's recommendations. Data were reported as concentration in pg/mL.

Quantitative Real Time RT-PCR

RNA was extracted from Qiazol-lysed cells with a miRNeasy Mini Kit (Qiagen) without DNase treatment. Purity of RNA was confirmed in a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) by an A260/A280 ratio of ~2. RNA quality was confirmed by gel electrophoresis in an Agilent Bioanalyzer 2100 (Santa Clara, CA) as indicated by RNA Integrity Numbers (RINs) of > 9. RNA was converted to cDNA by use of a High Capacity cDNA Reverse Transcriptase Kit (Life Technologies). TaqMan® (Life Technologies) primer/probe gene expression assays used were designed across multiple exons for *Gapdh* (ID# Mm99999915_g1), *Chil3* (*Ym1*, ID# Mm00657889_mH), *Retnla* (*Fizz1*, ID# Mm00445109_m1), and *Flt1* (*Vegfr1*, ID# Mm00438980_m1) were used to quantify mRNAs, normalized to *Gapdh*, and analyzed with the $\Delta\Delta$ Ct method described by the manufacturer. Data were reported as $\Delta\Delta$ Ct $\pm$ SD. Reporting adhered to and exceeded the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (70).

Data analysis

All data were analyzed with two-way ANOVA with Dunnet's multiple comparison test. Results below the level of detection were assigned a value equivalent to the square root of the lowest detectable limit (71). Normality of cellular cytokine protein data was determined with a Kolmogorov-Smirnov test; non-normally distributed data including the CM data was log transformed before statistical analysis. Dunnet's test adjustment for multiple comparisons was performed to compare result from each stimulating condition (M1, M2a, M2c) to the media only control at the corresponding time point with a two-sided significance threshold of $p < 0.01$; data presented as mean $\pm$ standard deviation (SD). Statistical calculations were performed with GraphPad Prism 6 v.6.03 (La Jolla, CA).

Results

Twenty-four cytokines were measured in cellular protein lysates (Cellular) or in the cell culture media (CM) of parallel cultures at various time points for up to 24 hours following stimulation with agonists commonly used for macrophage polarization, *i.e.*, IFN- γ + LPS (M1), IL-4 (M2a), and IL-10 (M2c). Some macrophages were lysed for protein or RNA just before replicate cultures were stimulated (0 time point) to determine cytokines in the macrophages prior to stimulation. In addition, the BMM#2 used in the experiment was also measured to determine cytokines present prior to contact with macrophages and labeled as "Media" on the figures. Comparisons of cytokines were made between each polarization condition (M1, M2a, and M2c) and the media only control at each corresponding time point. Cytokine assessment included cytokines added to the media to achieve macrophage polarization (Fig. 1) to explore the interplay between polarizing cytokines and the relationship between cellular and CM levels.

Cellular IFN- γ (Fig. 1A) progressively increased ($p < 0.001$) with marked and sustained increases from 1–24 hours in M1 polarized macrophages. Small amounts of IFN- γ were present in the other 3 conditions (control, M2a and M2c) with modest increases ($p < 0.007$) in cellular M2a and M2c compared to controls. A small, but notable elevation ($p < 0.008$) of

IFN- γ was also present in the CM of the M2a condition at all time points compared to control. In contrast, there was an interesting pattern of IFN- γ in the CM of M1 macrophages; IFN- γ was minimal at 3 hours and continued to increase ($p < 0.001$) through 12–24 hours (Fig. 1A) despite adding IFN- γ to the media. In separate studies, exogenous, recombinant IFN- γ added to the media, without exposure to cells, was not detected in the microsphere-based immuno-multiplexing technology used to quantify analytes (20,000 pg/mL expected; 11 ± 3 pg/mL, $n=3$). These findings suggest that the recombinant IFN- γ added to the media was not detected by the assay and the measured IFN- γ reflected endogenous IFN- γ production. Thus, despite IFN- γ added to the media, M1 polarizing conditions (IFN- γ + LPS) resulted in sustained production of IFN- γ .

In contrast to IFN- γ , cellular IL-4 (Fig. 1B) in M2a polarized cells was immediately elevated and sustained ($p < 0.001$) at 0.5 – 6 hours and precipitously declined at 12 and 24 hours despite continuously elevated ($p < 0.001$) IL-4 in the CM, suggesting the IL-4 elevations in the CM may have resulted in feedback inhibition of cellular IL-4. IL-4 was detectable, but lower than the control, M1 and M2c conditions in both the cell lysates and CM compared to M2a. Interestingly, IL-4 was elevated ($p = 0.01$) in the CM of the M1 condition at 12 & 24 hours.

While IFN- γ and IL-4 predominated in M1 and M2a macrophages, respectively, IL-10 exhibited interesting patterns in M1 and M2c macrophages. Cellular IL-10 (Fig. 1C) was maximal ($p < 0.001$) at 0.5 - 1 hour and decreased thereafter in M2c macrophages while IL-10 in CM was maximal ($p < 0.001$) at the earliest time point (3 hours) and progressively decreased through 12 – 24 hours although remaining elevated ($p < 0.001$) compared to control CM. Interestingly, cellular IL-10 in M1 macrophages was increased ($p = 0.003$) at all time points; peaking at 1 hour and decreased thereafter while IL-10 in the M1 CM exhibited a sustained elevation ($p < 0.001$). Decreased IL-10 was evident in the control and M2a conditions in both the cell lysates and CM with elevations ($p = 0.001$) discernable in the M2a condition at 3 & 24 hours. While cellular IL-10 was higher in M1 compared to M2c polarized macrophages, the CM had the opposite pattern with the most dramatic level present in M2c macrophage CM, which had received exogenous IL-10. Although these cytokines (IFN- γ , IL-4, and IL-10) were utilized to polarize macrophages into their respective conditions (M1, M2a, and M2c, respectively), comparison of levels across all conditions and time points revealed unexpected patterns.

Cellular levels of cytokines indicate at least two distinct temporal patterns in M1 polarized macrophages: early-transient and late-sustained

Among the 24 cytokine and other protein markers that were assessed, several were selectively increased in M1 polarized macrophages and released into the CM. Subsets of these cytokines had distinct temporal patterns based upon cellular levels. An early-transient pattern (Fig 2) of cellular cytokine production was observed for GM-CSF, KC-GRO, MIP-2, IP-10 and MIP-1 β . These 5 cytokines demonstrated early cellular increases ($p < 0.001$) between 0.5 – 3 hours after stimulation with IFN- γ + LPS that diminished, sometimes rapidly (i.e. GM-CSF), thereafter. CM levels for these 5 cytokines were 1] elevated and sustained (GM-CSF, $p < 0.001$), 2] progressively increased ($p < 0.001$) over the 24 hours

(KC-GRO, MIP-2, and IP-10) or 3] maximal at 12 hours with a precipitous decrease at 24 hours (MIP-1 β , $p < 0.001$). Of note, these 5 cytokines were either not detectable or present at very low concentrations in the cellular lysates and CM of the control, M2a and M2c conditions. Compared to control, cellular and CM levels of MIP-2 (in M2a & M2c) and IP-10 (in M2a) were decreased ($p = 0.009$). In contrast, MIP-1 β was elevated ($p = 0.002$) in the cellular and CM of M2a and M2c compared to control. The pattern of cellular changes (early 0.5 – 3 hour elevation and rapid decline) and the relative M1-specificity of GM-CSF, KC-GRO, MIP-2, IP-10, and MIP-1 β characterized the ‘early-transient’ group of M1 cytokines.

A second cellular pattern defined as late-sustained described another set of M1 associated proteins. The cellular patterns of IL-3, RANTES, and TIMP-1 were characterized by maximum and sustained levels between 3 – 12 hours ($p < 0.001$) after IFN- γ + LPS stimulation (Fig. 3). Cytokine/TIMP-1 in the CM were increased ($p = 0.01$) at 3 hours compared to control and were maximal and sustained at 12–24 hours ($p < 0.001$). Small, but detectable increases of these cytokines/TIMP-1 were present in the control, M2a, and M2c conditions in the cell lysates and CM. Peak cellular levels of these cytokines/TIMP-1 were delayed relative to the early-transient group and were sustained for ≥ 6 hours, therefore these markers were defined as late-sustained markers of M1 macrophage polarization.

While the early-transient and late-sustained markers were clearly elevated in M1 versus the other conditions, three other cellular cytokines did not differentiate M1 macrophages from other macrophage polarization states; these included FGF-9, IL-2, and IL-17A. Nevertheless, these cytokines in the CM clearly distinguished M1 polarized cells with >2 fold increased levels compared to M2a, M2c or control cells; data not shown.

Lastly, five cytokines were present in controls, M2a and M2c in relatively equivalent levels and were moderately elevated ($< 2x$ difference) in M1 macrophages in the cell lysates and CMs. These included IL-7, IL-11, OSM, SCF, and lymphotactin (data not shown). Finally, IL-12(p70), a well described M1 cytokine,(26) was also elevated similarly in the M2a condition. Further exploration revealed cross-reactivity in the antibody between IL-12(p70) and the recombinant IL-4 used to achieve M2a polarization (data not shown).

Cellular levels of M2a-related markers have later and more gradual temporal patterns than M1-related markers

M2a-related markers, mRNAs *Fizz1* & *Ym1* and IGF-1 cellular protein, were also measured in these studies (Fig. 4). M2a polarization promoted an increase in cellular IGF-1, *Fizz1* mRNA, and *Ym1* mRNA as early as 0.5 hour (for *Fizz1*, $p = 0.01$) but most readily by 3 – 6 hours ($p < 0.001$) and progressively increased through 24 hours. Interestingly, *Ym1* mRNA expression was decreased ($p < 0.001$) by 24 hours in M1 polarized macrophages indicating a novel, divergent pattern compared to the M2a condition. Collectively, M2a markers exhibited a more delayed and gradual pattern than M1 markers.

MCP-1 and MCP-3, but not MCP-5, have similar temporal patterns in M1 and M2a polarized macrophages

MCPs cellular and CM levels demonstrated unexpected temporal patterns across polarization conditions (Fig. 5). Cellular MCP-1 were increased ($p = 0.008$) 1 – 12 hours in M1 macrophages compared to media only controls at the relevant time point, but surprisingly, MCP-1 were also comparably increased ($p < 0.001$ vs. control; no significant difference vs. M1 condition at relevant time points) by 1 – 3 hours in M2a polarized macrophages (Fig. 5A). While MCP-1 in the CM was increased ($p < 0.001$) in both M1 and M2a conditions 3 – 24 hours compared to control, MCP-1 was increased ($p = 0.007$) in M1 versus M2a CM at 12–24 hours. Similar patterns were present with MCP-3 (Fig. 5B) with elevations ($p < 0.001$) in the cellular and CM of M1 and M2a compared to controls. Surprisingly, cellular MCP-3 was increased ($p < 0.001$) in M2a at 0.5 to 3 hours compared to M1 macrophages while MCP-3 in the CM was mostly comparable between M1 and M2a conditions.

In contrast to the mostly comparable MCP-1 and MCP-3 elevations in M1 and M2a macrophages, while cellular and CM MCP-5 (Fig. 5C) were increased ($p = 0.01$) in all three conditions compared to controls, the greatest elevations ($p = 0.01$) occurred in the M1 compared to M2a and M2c macrophages. Interestingly, MCP-3 and MCP-5 in the CM of M2c macrophages were increased ($p = 0.006$) compared to control. Thus MCPs were produced by all three polarizing conditions.

VEGF-A was differentially expressed in M1 and M2a macrophages

Vascular endothelial growth factor (VEGF-A) has been previously characterized as a pro-inflammatory cytokine (65). As expected, cellular and CM VEGF-A were increased ($p < 0.001$) in M1 macrophages at 24 hours (Fig. 6A). Consistent with previous reports,(72) the L-929 conditioned media used in our experiments contained VEGF-A; permitting the detection of the dramatic reduction ($p < 0.001$) in VEGF-A in the CM in the M2a cultures at 12 – 24 hours. This 20-fold reduction of VEGF-A in the M2a CM was accompanied by an increased expression ($p = 0.01$) of the VEGF receptor 1 (*Vegfr1* aka *Flt1*) by 3 hours and sustained throughout the experiment (Fig. 6B). Conversely, the expression of *Vegfr1* in the M1 macrophages decreased ($p = 0.005$) by 24 hours (Fig. 6B) coinciding with the increased VEGF-A in the CM (Fig. 6A). Given that the cellular VEGF-A in M2a macrophages was similar to control (Fig. 6A) with minimal changes over the time course of the experiment, the drastic decrease of VEGF-A in the CM might be secondary to increased expression and secretion of a soluble VEGF receptor 1 induced by IL-4 stimulation.

Discussion and Conclusions

The wide tissue distribution and the ever-expanding functional capabilities of macrophages demonstrate their complexity and importance to biological processes. Unfortunately, macrophages have also been causally implicated in tissue injury and contributing to many diverse diseases that further depict their complex nature such as metabolic disorders (73, 74), cancer (75, 76), autoimmune diseases (77) and are especially important in vascular inflammatory diseases including atherosclerosis (3) and abdominal aortic aneurysms (6).

Therefore, a better understanding of macrophage diversity may lead to new therapies based upon controlling macrophage functional capabilities.

Advances in understanding the complexity of macrophage activation and the resulting diversity of phenotypes were made possible by the use of *in vitro* models. In this study, cytokines were measured in both CM and the cellular compartment at multiple time points and across multiple polarizing conditions. This approach provided better resolution of the *in vitro* system and identified three critical aspects that should be considered: 1) the stability of cytokines in the CM is advantageous in defining markers of macrophage polarization but does not reflect dynamic changes in cellular cytokine expression/production, 2) understanding the time course of each is a critical parameter in macrophage polarization and should be considered when designing experiments to study pathway regulation, 3) markers of macrophage polarization, such as cytokines, should be defined across multiple polarization conditions within the same experimental context to allow accurate comparisons between polarization states.

The ease of measuring markers such as cytokines in the CM of stimulated macrophages has become a widely used method of characterizing macrophage polarization (20, 21, 50, 78). Measurement in the CM remains an effective means of determining whether a polarized macrophage population produces a particular cytokine. However, the limitations of this approach have not been fully appreciated. Cytokines were measured in the CM and cell lysates across multiple time points and conditions to define the temporal interplay in cytokines between the cellular compartment and CM. It was anticipated that cellular cytokines would reflect the consistently elevated CM levels. However, cellular levels for many of the cytokines diminished precipitously by 6 hours of stimulation revealing a disconnection between the chronically elevated CM levels and dynamic cellular cytokines.

A prime example of the uncoupled dynamic patterns between the CM and cellular cytokines was provided by GM-CSF. GM-CSF (Fig. 2A) in the CM was elevated throughout the 3–24 hour time course of the experiment while a transient peak response at 1 hour occurred in the cellular compartment. The transient pattern of cellular GM-CSF was not paralleled in the CM likely due to the stability and retention of GM-CSF in the media and autocrine feedback mechanisms. Regarding studies of molecular mechanisms employed to regulate GM-CSF production; the cellular levels would provide more information and determine the best time points to study the regulation of GM-CSF production. Interestingly, GM-CSF has been shown to promote a greater M1 phenotype relative to M-CSF (50, 79–81). Some studies rely strictly on GM-CSF to induce an M1 polarization state (81, 82) and yet the current study has demonstrated that GM-CSF was produced in a L929-based culture system in response to IFN- γ + LPS stimulation. Conversely, exogenous GM-CSF-stimulation of human monocyte derived macrophages resulted in a 10-fold increase in the expression of *CSF1* (M-CSF mRNA) (83). Therefore, macrophages constantly revise their microenvironment through mediators such as cytokines and these mediators through autocrine/paracrine mechanisms can further direct macrophage responses (84). Due to the stability of GM-CSF and its propensity to direct macrophage responses, measurement of GM-CSF in the CM was not reflective of the dynamic changes occurring within macrophages.

Another compelling observation that supported measuring cellular cytokines in conjunction with CM cytokines was the discrepancy between cellular and CM MCP-1 and MCP-3 identified in this study (Fig. 5 A & B). While MCP-1 and MCP-3 in CM in the M1 condition were greater than or equivalent to the M2a condition, the cellular levels tell a slightly different story. Cellular MCP-1 in the M2a and M1 conditions were equivalent whereas cellular MCP-3 was increased in M2a compared to M1 conditions. The distinction between dynamic cellular MCP and levels in the CM has never been reported and suggests that extracellular MCP-1 and MCP-3 may be regulated at the level of secretion. The MCP observations also suggest that measuring CM cytokine in tandem with cellular levels would be important in studying secretory regulatory mechanisms.

Timing is a parameter of macrophage polarization that has been poorly studied and is a major impediment to comparing studies and defining regulatory mechanisms. Though a handful of studies have temporally defined a few macrophage polarization markers (78, 79, 85), none have expanded this approach to widely survey multiple markers. The present study focused on time points that precede or were concurrent with the earliest changes in macrophage polarization markers. Since CM changes could be detectable by 3 hours, time points were chosen to study cellular cytokines that may have preceded the 3 hour time point (0.5, 1, 3, 6, 12 hours) and to remain consistent with many other studies (21, 50, 78, 86) the 24 hour time point was also used. Interestingly, even the 0.5 hour time point was not early enough to detect the initial change in the M1 cellular cytokines KC-GRO (Fig. 2B) and MIP-2 (Fig. 2C) as levels were already maximal at even this very early time point. Changes in protein were not expected this quickly after stimulation. Most cellular protein markers were appreciably elevated by 3 hours following stimulation. Notable exceptions include VEGF-A and IGF-1, supporting the need to fully explore the dynamics in protein production for each individual cytokine/marker prior to studying regulatory pathways.

Although cytokines in the CM tend to remain elevated, a few of the markers diminished drastically over time, as exemplified by MIP-1 β (Fig. 2E). MIP-1 β in the CM of M1 and M2a conditions at 24 hours was much less distinctive, 23050 ± 9455 pg/mL versus 6975 ± 1340 pg/mL, than at 12 hours, 204250 ± 37942 pg/mL versus 4245 ± 1115 pg/mL, respectively due to the nearly 10 fold drop in the CM of the M1 condition. VEGF-A was another cytokine with a critical time point. VEGF-A changes in the M1 cellular and CM could only be detected by 24 hours (Fig. 6A) consistent with a previous study (52). While convention and convenience have dominated the use of a single time point, typically 24 hours after stimulation for cytokine/marker levels in macrophage polarization experiments, the current study provides evidence of complex, dynamic patterns that are specific to each cytokine/marker and support measuring cytokines in the CM using multiple time points.

Temporally limited studies may miss key biological observations across various stimulatory conditions. This was best demonstrated in the divergent behavior of *Ym1* identified in the current study (Fig. 4C). At 24 hours, *Ym1* mRNA was greatly down-regulated in the M1 and up-regulated in M2a conditions. While previous studies reported increased expression of *Ym1* in M2a macrophages (40, 62) and demonstrated the capability of IFN- γ to antagonize IL-4-mediated *Ym1* expression (41), the down-regulation in the M1 condition has not been previously reported. In contrast to the very early increases in cytokines in M1 conditions at

0.5 hours (KC-GRO, MIP-2, IP-10, MIP-1 β), M2a cellular markers exhibited initial increases at later time points and progressively increase over time (Fig 4). The delayed expression/production of the other M2a markers in this study (*Fizz1* mRNA and IGF-1 protein) verify and extend the delayed pattern of these markers in previous studies (40, 62, 87). Another mRNA with divergent expression between M1 and M2a was *Vegfr1* (Fig. 6B) at 24 hours. The *Vegfr1* expression in M2a polarized macrophages suggested a role in managing VEGF-A as its peak expression precedes a dramatic drop in VEGF-A in the CM (Fig. 6A) with no discernable change in cellular VEGF-A. This suggests that the M2a polarization condition up-regulates and secretes the soluble VEGF receptor 1 to regulate extracellular VEGF-A levels; a mechanism known to exist for other cell types (88). Concurrent with our study, another group demonstrated that IL-4 was capable of increasing the production of VEGFR-1 from RAW264.7 macrophages consistent with the expression of *Vegfr1* in the current study (89). Our study has expanded this definition to demonstrate the divergent pattern of *Vegfr1* across macrophage polarization; decreased expression of *Vegfr1* and the consistent increase in VEGF-A in the M1 polarization condition. The possible *in vivo* relevance was suggested by the up-regulation of *Vegfr1* induced by IL-4. Therein, Gundra *et al.* elicited peritoneal macrophage activation with IL-4 and through *ex vivo* analysis of gene expression reported *Flt1* (aka *Vegfr1*) was highly up-regulated (90). However, increased soluble VEGF receptor 1 has been reported in monocytes/macrophages stimulated with GM-CSF (89, 91). As discussed earlier, GM-CSF was elevated in the media of M1 macrophages but in the present study VEGF-A actually increased in the CM and *Vegfr1* was down-regulated in a temporally-dependent manner in M1 macrophages.

Observations regarding VEGF-A in this study are potentially at odds with previous work characterizing the M2 macrophages as pro-angiogenic (93, 94). These previous studies characterized M2 macrophages as pro-angiogenic based upon factors that they produced and by co-culture with endothelial cells. *In vitro* functional studies suggest that M1 and M2c macrophages can promote vessel-associated stem cell migration to a greater extent than M2a macrophages, partially due to VEGF (52) further complicating the assignment of pro- or anti-angiogenic potential to macrophage polarization. In addition to VEGF-A, other M1-specific cytokines identified in the current study have also been reported to have angiogenic properties such as KC-GRO (Fig. 2A; aka CXCL1 or GRO α), MIP-2 (Fig. 2C aka CXCL2 or GRO β), and IL-3 (Fig. 3A) (94–96). Conversely, some of the M1-specific proteins identified in the current study were reported to have anti-angiogenic properties such as IP-10 (Fig 2D) and TIMP-1 (Fig 3C) (97–99). However, in addition to promoting angiogenesis, VEGF-A has other functions such as monocyte chemotaxis (100, 101) and increasing vascular permeability (102). Whether these *in vitro* findings occur *in vivo* producing a biologically relevant effect is unknown. Nevertheless, the VEGF-A and *Vegfr1* observations were dependent upon timing; if focused on any time point prior to 24 hours these observations may have not been appreciated. Collectively, these results exhibit the time-dependence of cytokine/marker levels and that timing may be an overlooked variable in the characterization of macrophage polarization.

Our approach identified that assessment of cytokine expression/production in macrophage polarization should be compared across multiple polarizing conditions to determine

specificity and relative expression/production. By adhering to this principle, the current study has identified novel polarization-specific markers and unexpected patterns.

M1 polarized macrophages exhibited two distinct temporal patterns of polarization-specific cellular cytokines: early-transient and late-sustained. Early-transient cellular cytokines included (Fig. 2) KC-GRO, GM-CSF, MIP-2, IP-10, and MIP-1 β . The cytokines that comprised the early-transient group have recognized roles in macrophage activation and immune cell chemotaxis. GM-CSF (50, 79–81) promotes an M1 phenotype and may play an important pro-inflammatory role in our system. Cytokines KC-GRO and MIP-2 promote neutrophil chemotaxis.(17, 37), IP-10 induces T cells chemotaxis,(17, 25), and MIP-1 β (17, 38, 39) stimulates monocyte and T cell chemotaxis. Therefore, M1 macrophages may promote early but temporally limited recruitment and activation of immune cells by the production of these early-transient cytokines.

Unexpectedly, cellular IL-10 in the M1 condition also fit an early-transient pattern. The capability of LPS or LPS + IFN- γ stimulated macrophages to produce IL-10 has been previously reported (50, 52, 103–105) and contributes to the controversy of IL-10 as a marker of M2 macrophage polarization. The evidence for IL-10 as a M2 marker centers around the larger production of IL-10 from M-CSF cultured macrophages (M2-like) following stimulation with LPS compared to GM-CSF cultured macrophages (M1-like) (46). However, in the absence of exogenously added M-CSF or GM-CSF, other macrophage *in vitro* systems, RAW264.7 cell line (103) and thioglycollate-elicited peritoneal macrophages (104), also produced IL-10 in response to LPS stimulation. As IL-10 may diminish cytokine expression, this cytokine may play a role in buffering pro-inflammatory responses in the context of M1 macrophage polarization.

Late-sustained M1-specific cellular cytokines/proteins included IL-3, TIMP-1, and RANTES. Cytokines/proteins in this group were involved in T cell and monocyte recruitment & activation and regulation of angiogenesis. IL-3, especially in synergism with GM-CSF, promoted hematopoiesis with a shift towards myeloid populations (106); macrophage activation and IL-1 production (107); monocyte phagocytosis (108); and endothelial cell migration, proliferation, and tube formation *in vitro* (96). Counter to previous literature that IL-3 may promote an M1 phenotype (107), a more recent study suggested a shift toward an M2 state (109) in which *Ym1* was used to confirm an M2 phenotype. However, in our study, *Ym1* mRNA was actually significantly decreased (Fig. 4C) in the M1 population despite elevated IL-3. Considering that IL-3 was endogenously produced by M1 macrophages, evident from the current study, and exogenous IL-3 modulates macrophage polarization, further study into the role of IL-3 in macrophage polarization is warranted. To our knowledge, the increased production of IL-3 by M1 macrophages has not been previously reported. TIMP-1 production has been reported in TNF- α , GM-CSF, and IL-1 β -stimulated monocytes (24). Consistent with the inhibitory effect that the TIMPs have on matrix metalloproteinases, TIMP-1 diminished proMMP-9 mediated angiogenesis in M1 polarized macrophages (99). M1 macrophages produce RANTES (20, 21), and RANTES recruits monocytes & T cells (17, 110), and activates T cells (110). While the early-transient markers are involved in chemotaxis of myeloid cells (KC-GRO, MIP-2, GM-CSF, MIP-1 β) & T cells (IP-10 & MIP-1 β) and activating

monocyte/macrophages (GM-CSF), the late-sustained group may further recruit/activate monocyte/macrophages (IL-3) & T cells (RANTES) and regulate angiogenesis (IL-3 & TIMP-1).

Another unexpected observation was provided by the comparative survey of cytokines produced by each macrophage polarization condition, the production of MCP-1 & 3 by M2a polarized macrophages. The elevated levels of these chemokines in the M2a condition were surprising because MCP-3 and especially MCP-1 have been characterized as pro-inflammatory mediators primarily due to their production from macrophages exposed to inflammatory stimuli (i.e. LPS & IFN- γ) (26–29) and their potent ability to recruit immune cells to inflammatory sites (17, 111). However, evidence of MCP-1 & 3 expression/production strictly from inflammatory macrophages lacks some consistency in the literature. Contrary reports include a lack of difference across multiple macrophage polarization conditions (50, 52) and a strong up-regulation in *ex vivo* peritoneal macrophages following IL-4 stimulation (90). Another aspect that questions the ‘pro-inflammatory’ nature of MCP-1 involves its propensity to promote M2 polarization marker expression and the diminished expression of M1 markers in its absence (29, 112). Further evaluation of MCP production and influence in macrophage polarization is needed and should be assessed in each polarization condition intra-experimentally.

Unlike the M1 polarization condition, the M2a polarization markers (IGF-1 cellular protein and *Ym1* & *Fizz1* mRNAs) exhibited similar temporal patterns. All three markers (Fig. 4) gradually increased over the time course of the experiment with maximal expression at 24 hours. Previous studies have suggested the delayed nature of YM1 and FIZZ1 expression/production through limited time course studies (40, 62) but the current study has extended and increased the resolution of their temporal expression. This delayed, gradual pattern was consistent with the onset of M2 phenotypes both *in vitro* and *in vivo* suggesting this timing was controlled on a cellular/molecular level. Of these markers, IGF-1 was the most characterized for its expression in M2a macrophages (113), its capability in promoting cell proliferation/growth (114), and its promotion of angiogenesis (93, 115). *Ym1* and *Fizz1* are established markers of M2a macrophage polarization (41, 116). Some biological roles of *Fizz1* have been explored; angiogenesis (117, 118), dendritic cell chemotaxis (119), feedback inhibit Th2 responses (120–122), and even promotion of IL-6 and TNF- α production from LPS-stimulated bone marrow-derived macrophages (123). The biological function of *Ym1* is not as clear but may be important to promoting Th2 cytokine expression (124) which might explain the reduced expression in the M1 macrophages (Fig 4C).

Most of the cytokines measured across each polarization condition tended to either mildly increase in the cellular and CM or remain unchanged relative to the media only controls in the M2a condition. However, IP-10 (Fig. 2D) was the only cytokine in this study that was consistently decreased in the cellular compartment and CM of the M2a condition suggesting a possible role of IL-4 in regulating IP-10.

Similar to the M2a condition, M2c polarized macrophages tended to exhibit a small increase or comparable levels relative to media only controls in the cellular compartment and CM for many cytokines. Notable differences between M2a and M2c included IP-10 (Fig. 2D),

MCP-3 (Fig. 5B), IGF-1, *Fizz1*, and *Ym1* (Fig. 4). Consistent with previous literature characterizing IL-10 stimulation of macrophages (M2c) as capable of diminishing cytokine expression (43), specific markers in this study were not identified. One possible exception was IL-10 production evident by elevated cellular levels (Fig. 1C) which supports previous reports suggesting IL-10 can promote its own expression (45).

In conclusion, the current study presents aspects of macrophage polarization that have been overlooked in the literature: the value of marker measurement in the cellular compartment, the contribution of time, importance of comparison across multiple polarization conditions and the potential for missing key observations due to any of these factors. Additionally, temporal measurements in the cellular compartment revealed several previously undefined patterns. At least two temporal cytokine/chemokine patterns were discovered in the M1 condition; early-transient and late-sustained. Pro-inflammatory chemokines MCP-3 had comparable CM and increased cellular levels in M2a versus M1 macrophages. Divergent expression of *Ym1* and *Vegfr1* mRNAs were found between the M1 and M2a conditions. Lastly, VEGF-A in the culture media was increased in M1 and drastically decreased in M2a, corresponding to increased *Vegfr1* mRNA. All of these observations were dependent upon comparing across time points and multiple polarization conditions in both the cellular compartment and the CM. The results of this study argue this approach was essential to provide clarity needed to fully characterize the complexity of macrophage polarization, especially as the field advances in defining regulatory mechanisms. Taken altogether, these results demonstrate cytokine production by macrophages was temporally regulated and that controversies regarding specific cytokine markers in different activation states may have resulted from issues related to timing; for most cytokines, cellular production precedes increases in the media and should be studied at time points within the first 6 hours of stimulation. Interestingly, many cytokines that affect T and B cell functions were predominately produced by M1 macrophages. The current study expands the defined repertoire of cytokines produced by macrophage polarization states and provides insights into the complex regulation of macrophages that can influence vascular inflammation. A better understanding of macrophage plasticity and interactions with adaptive immune cells could lead to improved therapeutics for atherosclerosis, vasculitis and other diseases resulting from vascular inflammation.

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Figure 1. Interplay between cellular and culture media (CM) temporal changes in response to exogenous cytokines added to create differing polarizing conditions in murine macrophages Control (media only), M1 (IFN- γ + LPS), M2a (IL-4) or M2c (IL-10) stimulating conditions were used to achieve macrophage polarization. Cytokines in cellular protein lysates (Cellular, left column), including a 0 hour time point prior to stimulation, and in the culture media (right column), including the conditioned media (BMM#2, Media) used prior to placing on the cells, were measured at various time points after macrophage polarization. Protein levels of (A) IFN- γ , (B) IL-4, or (C) IL-10 in pg/mL presented as the mean $\pm$ SD of results derived from four independent cell cultures; # $p < 0.01$ compared to controls at the corresponding time point.

Figure 3. Late-sustained cytokine/TIMP-1 pattern of M1-polarized murine macrophages
Control (media only), M1 (IFN- γ + LPS), M2a (IL-4) or M2c (IL-10) stimulating conditions were used to achieve macrophage polarization. Cytokines and other proteins in the cellular protein lysates (Cellular, left column), including a 0 hour time point prior to stimulation, and in the culture media (right column), including the conditioned media (BMM#2, Media) used prior to placing on the cells, were measured at various time points after macrophage polarization. Protein levels of (A) IL-3, (B) RANTES, and (C) TIMP-1 in pg/mL presented as the mean $\pm$ SD of results derived from four independent cell cultures; # $p < 0.01$ compared to controls at the corresponding time point.

Figure 4. Delayed, gradual pattern of markers in M2a-polarized murine macrophages Control (media only), M1 (IFN- γ + LPS), M2a (IL-4) or M2c (IL-10) stimulating conditions were used to achieve macrophage polarization. A protein (IGF-1) and two mRNAs (*Fizz1* and *Ym1*) markers in the cellular protein or RNA lysates (Cellular), including a 0 hour time point prior to stimulation, were measured at various time points after macrophage polarization. Protein levels were reported for (A) IGF-1 in pg/mL. RNA levels of (B) *Fizz1* and (C) *Ym1* expression with a range of raw Ct values as 12.6 – 35 and 21.0 – 34.5, respectively, normalized to Gapdh with a range of raw Ct values of 15.1 – 17.6, and set relative to the 0 hour time point were presented as – Ct. Data presented as the mean $\pm$ SD of results derived from four independent cell cultures; # $p < 0.01$ compared to controls at the corresponding time point.

Figure 5. MCP cytokine patterns in cellular and culture media (CM) of polarized murine macrophages

Control (media only), M1 (IFN- γ + LPS), M2a (IL-4) or M2c (IL-10) stimulating conditions were used to achieve macrophage polarization. Cytokines in the cellular protein lysates (Cellular, left column), including a 0 hour time point prior to stimulation, and in the culture media (right column), including the conditioned media (BMM#2, Media) used prior to placing on the cells, were measured at various time points after macrophage polarization. Protein levels of (A) MCP-1, (B) MCP-3, and (C) MCP-5 in pg/mL presented as the mean $\pm$ SD of results derived from four independent cell cultures; # $p < 0.01$ compared to controls at the corresponding time point, * $p < 0.01$ compared to M1 at the corresponding time point.

Figure 6. VEGF-A and *Vegfr1* mRNA expression in polarized murine macrophages

Control (media only), M1 (IFN- γ + LPS), M2a (IL-4) or M2c (IL-10) stimulating conditions were used to achieve macrophage polarization. VEGF-A protein and *Vegfr1* mRNA in the cellular protein or RNA lysates (Cellular, left column), including a 0 hour time point prior to stimulation, and VEGF-A protein in the culture media (right column), including conditioned media (BMM#2, Media) used prior to placing on the cells, were measured at various time points after macrophage polarization. Protein levels were reported for (A) VEGF-A in pg/mL. RNA levels of (B) *Vegfr1* expression with range of raw Ct values 20.9 – 34.3, normalized to *Gapdh* with a range of raw Ct values 15.6 – 21.6, and set relative to 0hr time point was presented as – Ct. Data presented as the mean $\pm$ SD of results derived from four independent cell cultures; # $p < 0.01$ compared to controls at the corresponding time point.