A phased strategy to differentiate human CD14⁺ monocytes into classically and alternatively activated macrophages and dendritic cells

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There are currently several in vitro strategies to differentiate human CD14⁺ monocytes isolated from peripheral blood mononuclear cells (PBMCs) into the M1 or M2 macrophage cell types. Each cell type is then verified using flow cytometric analysis of cell-surface markers. Human CD14⁺ monocytes have the potential to differentiate into M1 and M2 macrophages, both of which demonstrate varying degrees of cell-surface antigen overlap. Using multiple surface markers with current macrophage polarization protocols, our data reveal several limitations of currently used methods, such as highly ambiguous cell types that possess cell-surface marker overlap and functional similarities. Utilizing interleukin-6 (IL-6) and two phases of cytokine exposure, we have developed a protocol to differentiate human monocytes into M1, M2, or dendritic cells (DCs) with greater efficiency and fidelity relative to macrophages and DCs that are produced by commonly used methods. This is achieved via alterations in cytokine composition, dosing, and incubation times, as well as improvements in verification methodology. Our method reliably reproduces human in vitro monocyte-derived DCs and macrophage models that will aid in better defining and understanding innate and adaptive immunity, as well as pathologic states.

The myeloid compartment of the innate immune system consists of a number of cell types whose functions include clearance of damaged and apoptotic cells, antigen presentation, immunosuppression, and protecting the host from foreign microorganisms.

Monocytes represent a highly plastic subset of cells that compose the innate immune system. These cells are capable of traversing the vasculature and, when exposed to specific cytokines, undergo differentiation into different cell types. Circulating monocytes are

classified as either non-classical or classical and can be distinguished by their pattern of cell-surface receptor expression. The classical monocytes within the circulation display CD14⁺⁺⁺HLA-CD16^{-/lo} and are present especially at locations of infection or disease (1,2).

METHOD SUMMARY

Here we present a new 9-day, phased strategy for differentiating human CD14⁺ monocytes into homogenous populations of monocyte-derived dendritic cells (moDCs) and M1 and M2 macrophages. Our macrophage populations are homogenous, and notably, our M2 macrophage populations displayed high mannose uptake as well as cellular fusion. Specific cytokine treatment on day 9 also increased moDC maturation as well as expression of cell-surface markers CD80, CD86, and CD83.

Figure 1. Schematic of treatment strategies for monocyte differentiation into homogenous and functional myeloid cell types. Several polarization strategies were used [granulocyte-macrophage colony-stimulating factor (GM-CSF) only or macrophage colony-stimulating factor (M-CSF) only; continuous or phased]. For polarization to the M1 phenotype, CD14 $^{+}$ peripheral blood mononuclear cells (PBMCs) were given GM-CSF (20 ng/ml) for 6 days, and on Day 6 cells were spiked with GM-CSF in concert with lipopolysaccharide (LPS), interleukin-6 (IL-6), and interferon- γ (IFN- γ) (20 ng/mL) for an additional 4 days. For M2 polarization, cells were treated with M-CSF (20 ng/mL) for 6 days and then on Day 6 cells were then spiked with M-CSF in concert with IL-4, IL-6 and IL-13 (20 ng/mL) for an additional 4 days. By isolating human CD14 $^{+}$ monocytes and differentiating them into multiple myeloid cell types for 9 days, we demonstrated that the right combination of timing, cytokine composition, and dosage yielded homogenous populations of macrophages and dendritic cells (DCs). CD14 $^{+}$ PBMCs under the phased cytokine treatment strategy were driven further than other strategies within the phenotypic continuum to produce homogenous populations that expressed canonical markers of their respective cell types. Under the phased strategy, M2 macrophages also displayed high mannose uptake and cellular fusion. Specific cytokine treatment on Day 9 also increased monocyte-derived DC (moDC maturation) as well as expression of cell-surface markers CD80, CD86, and CD86.

Human non-classical monocytes within the circulation display CD16 $^{+++}$ CD14 $^{+/-}$ surface expression (1). After activation, monocytes undergo several morphological and biochemical functional changes, resulting in monocyte differentiation into new cell types such as macrophages (3).

In a paradigm termed classical activation, human monocytes display phenotypic plasticity that can be initiated by exposure to T $_{H1}$ response-promoting cytokine interferon- γ (IFN- γ) or tumor necrosis factor α (TNF- α), as well as the endotoxin lipopolysaccharide (LPS) (4,5). This mechanism of classical activation generates M1 macrophages, which function to produce pro-inflammatory mediators that provide host protection against bacteria and viruses (6). Human monocytes can also be differentiated

to M2 macrophages by exposure to T $_{H2}$ response-promoting cytokines such as interleukin-10 (IL-10) and transforming growth factor- β (TGF- β) (4,7). M2 macrophages express high levels of CD206 (mannose receptor) and CD163, produce low levels of pro-inflammatory cytokines, and promote wound healing and matrix remodeling.

Dendritic cells (DCs) are another set of monocyte-derived immune cells whose function is to present antigens to T cells to initiate an adaptive T-cell-based immune response. DCs can be categorized largely into three subgroups: classical (cDCs), plasmacytoid (pDCs), and monocyte-derived (moDCs). cDCs are typically found in the lymphoid tissue, spleen, lymph nodes, and bone marrow. pDCs resemble plasma cells and are typically found in the blood circulation (8). Monocytes can

become differentiated into DCs, which are often termed monocyte-derived DCs (moDCs), following exposure to granulocyte macrophage colony stimulation factor (GM-CSF) and IL-4 (9,10). moDCs have been reported to express cell-surface markers CD80, CD83, CD86, and CD1a in vitro (11–13). While moDCs differ from macrophages in cell shape and function, they share expression of several cell-surface antigens, including CD11c, CD206, and CD1a (14).

Several groups have reported successful differentiation of human CD14 $^{+}$ monocytes into macrophages; however, many of these published protocols are dissimilar. A common variation among these methods is the inclusion (15) or omission (16) of IL-6. Another difference between protocols is the treatment timing. This lack of consistency among protocols is problematic.

To address these issues, we examined and compared the inclusion and omission of IL-6 in the cytokine treatment used in common human monocyte differentiation methodologies. We found that in the absence of IL-6, these strategies produce cells with a low degree of homogeneity, which can yield ambiguous experimental results. Secondly, to further address this problem, we developed a novel phased *in vitro* strategy with the inclusion of IL-6. We found that this strategy greatly improved the cellular homogeneity of M1 and M2 macrophages as well as moDCs that were differentiated from human CD14⁺ monocytes. This improvement in methodology will provide researchers with better models for investigating the immune system and disease states.

Materials and methods

Cell preparations

Buffy coats were collected from the whole blood of five or more healthy male donors that had been pooled. Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coats by Ficoll-Paque (1.077 g/mL density) (17–1440–02; GE Healthcare Bio-sciences, Piscataway, NJ) density gradient centrifugation at $400 \times g$ at 18°C for 35 min to separate blood constituents. Cells were washed several times using 1× PBS and counted using a Nexcelom Cellometer Auto T4 plus cell counter (Nexcelom Bioscience, Lawrence, MA). Once counted, CD14⁺ monocytes were isolated from PBMCs by magnetic labeling using MAbs CD14 conjugated microbeads (130–050–201; Miltenyl Biotec, San Diego, CA) followed by separation using magnetic columns (130–042–401 and 130–042–302; Miltenyl Biotec) according to the manufacturer's instructions, with one exception: when collecting CD14⁺ cells from the magnetic column, cells were flushed out initially using 5 mL buffer relying only on gravity (without use of the supplied plunger). Following the initial 5 mL rinse, an additional 5 mL buffer was added and gently flushed using the provided plunger.

Cell culture

Human CD14⁺ monocytes were seeded at a cell density of $2.0\text{--}3.0 \times 10^5$ cells/

Figure 2. Changes in cell-surface marker expression, cell size, and the complexity of dendritic cells (DCs) and M1 and M2 macrophages after phased or constant cytokine treatments. Flow cytometric analysis of M1 and M2 macrophage cell-surface markers expression for CD206 (A), CD36 (B), CD163 (C), E-cadherin (ECad) (D), and CD83 (E) under continuous or phased treatment regimens.

mL in RPMI 1640 medium with 2 mM/L L-glutamine (Life Technologies, Frederick, MD) supplemented with 10% heat inactivated FBS (Sigma, Carlsbad,

CA), 100 U/mL penicillin (Life Technologies), and 100 mg/mL streptomycin (Life Technologies) at 37°C in a humidified 5% CO₂ incubator. To differen-

tiating cells into M1 macrophages, the cytokines used were GM-CSF (20 ng/mL) (PeproTech, Rocky Hill, NJ), IFN- γ (20 ng/mL), IL-6 (20 ng/mL), and endotoxin LPS (20 ng/mL). For M2 macrophage differentiation, the cytokines used were macrophage colony-stimulating factor (M-CSF), IL-4, IL-6, and IL-13 at a concentration of 20 ng/mL (PeproTech). moDCs were generated by the addition of GM-CSF (100 ng/mL) and IL-4 (20 ng/mL). The timing of each strategy is further described in Figure 1.

Flow cytometry

Polarized macrophages and DCs were cultured for 9 days on 10 cm² dishes (BD Falcon, Billerica, MA). On Day 9, media and non-adherent cells were collected; adherent cells were washed using 1 $\times$ PBS, removed using cell dissociation buffer (Life Technologies), and then added to the collected media/non-adherent cells to be washed. Suspended cells were centrifuged and washed twice (1 $\times$ PBS, 0.5% BSA, 2 mM EDTA), counted, and then incubated with fluorophore-conjugated primary antibodies against CD206 (FITC), CD36 (PE), CD163 (PE-Cy7), E-cadherin (AlexaFluor-488), CD86 (PE), CD1a (FITC), CD83 (PE), CD80 (PE), CD124 (PE), and CD64 (FITC) in the dark for 45 min at 4°C. Fluorescence was detected by an S3TM Cell Sorter (Bio-Rad, Hercules, CA).

Endocytosis assay

Polarized human macrophages were plated into 4-well chamber glass slides (MA Nunc Lab-Tek II, Thermo Scientific, Waltham, MA) at a density of 3.0×10^5 cell/mL at Day 7 and continued to be cultured with the appropriate cytokines for the remaining 2 days. Perylene bisimide (PBI-12-Man) (17) was a kind gift from Ke-Rang Wang at Hebei University. Cells were cultured with 10 μ g/mL PBI-12-Man for 30 min at 37°C, then washed with 1 $\times$ PBS. Cells were fixed using 70% ethanol and mounted with DAPI (P36962; Life Technologies). To visualize endocytosis of PBI-12 Man, slides were excited and emitted at 585 nm. Red fluorescence images were quantitated using Metafer slide scanning software (MetaSystems, Boston, MA). Statistical testing was

performed using MATLAB R2015a software (The Mathworks, Inc., Natick, MA). Rank-sum tests were performed to test for univariate statistical significance between samples.

Immunofluorescence

Macrophages were differentiated using previously described methods for 7 days and then transferred to Nunc Lab-Tek II 4-well glass chamber slides with the appropriate cytokines. Cells were left to grow for the next 7 days; 14 days after initial plating, cells were fixed using 70% ethanol and mounted using Prolong Diamond anti-fade mountant with DAPI. Cells were visualized using phase contrast and fluorescence microscopy on the EVOS FL Auto (Life Technologies).

Results and discussion

Strategies and conditions for human CD14⁺ monocyte differentiation

We began by assessing which strategies were most effective in producing homogenous populations of human M1 and M2 macrophages as well as moDCs. Human CD14⁺ monocytes were isolated and seeded onto tissue culture plates to be differentiated into macrophages or moDCs in vitro. We then tested three specific treatment conditions to determine the method that yielded the most uniform macrophage and moDC populations. For the first condition, we cultured cells with the addition of only GM-CSF or M-CSF for 9 days (Figure 1). This condition is termed “only” (Figure 1). The second treatment strategy was to continuously expose cells to the applicable cytokine milieu at Day 0, replenish the media, cytokines and LPS on Day 5 and, analyze the cells on Day 9. This condition is termed “continuous” (Figure 1). The third strategy was to stimulate CD14⁺ monocytes with either GM-CSF or M-CSF for 5 days, followed by the addition of fresh media containing LPS for human M1 macrophages and all applicable cytokines for a given treatment group (GM-CSF, IFN- γ , and IL-6 for M1 macrophages or M-CSF, IL-4, IL-13, and IL-6 for M2 macrophages). This treatment strategy was termed “phased” (Figure 1). Treating CD14⁺ monocytes with 100 ng/mL of GM-CSF and IL-4 for 5 days and

replacing media and all cytokines on Day 5 generated moDCs. The moDCs were then stimulated with IL-6 and LPS for 24 h before analysis (Day 8) to promote DC maturation and activation. This culturing method was termed “stimulated” (Figure 1). The moDCs that never received IL-6 and LPS exposure were termed “un-stimulated” (Figure 1). After 9 days of culture under the specific conditions described above, cell-surface receptor expression and cell functionality were analyzed.

IL-6 exposure increases M2 polarization efficiency in vitro

Previous studies have shown that macrophage exposure to cytokine IL-6 not only skews monocyte differentiation from a dendritic cell to a macrophage phenotype but that IL-6 exposure also increases the mRNA expression profile associated with M2 macrophages (15,18). To confirm these findings and to test the effect of IL-6 on M1 and M2 polarization, human CD14⁺ monocytes under the continuous and phased culturing conditions were treated with or without IL-6. Examination of M2 macrophage surface marker expression in M1 differentiated cells showed no significant changes when cultured with IL-6 (Figure 2), suggesting that IL-6 treatment does not shunt M1 polarized macrophages toward an M2 phenotype.

Conversely, analysis of the continuous and phased M2 macrophage culture groups revealed that IL-6 treatment increased expression of M2 macrophage surface markers. In M2 macrophage populations, both CD206 and CD36 expression remained high and appeared unaltered when exposed to IL-6 (Figure 2, A and B). CD163 and E-cadherin, however, showed significant increases in surface expression levels and population homogeneity when treated with IL-6 (Figure 2, C and D). This is demonstrated by a decrease in the low-expressing CD163 and E-cadherin peaks (red arrows in Figure 2, C and D) and the resulting shift toward a homogeneously high-expressing population.

IL-6 is often used to induce moDC maturation (18,19). In order to determine if IL-6 treatment could result in undesired moDCs within the macro-

Figure 3. Successful polarization leads to increased surface expression of canonical M1, M2, and monocyte-derived dendritic cell (moDC) cell-surface markers. Panels (A-C) are separated into three sections. Histograms depict flow cytometric analysis of labeled cell-surface markers, with dashed lines depicting a representative unstained control. Accompanying tables represent the median fluorescent intensities (MFI) associated with the histograms. Values are shown as MFI relative to the unstained controls for that sample. Phase contrast images were taken of the cells prior to flow cytometric analysis. Analysis of M1 cell-surface markers CD86 and CD80 is shown on the left, next to the M2 markers CD36 and interleukin-4Rα (IL-4R), on the right (A). M2 cell-surface marker expression was determined for M2 markers CD206, CD163, E-cadherin, and IL-4Rα (B). Expression levels for dendritic markers CD86, CD80, CD83, and CD1a were determined for moDCs (C).

phage-polarized populations, mature DC surface marker (CD83) expression was examined. Flow cytometric analysis demonstrated that none of the M1 or M2 culture conditions promoted any significant increase in CD83 expression (Figure 2E). This suggests that there was no moDC contamination within these macrophage populations or that any contaminating moDCs were IL-6 insensitive.

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Figure 4. Polarized macrophages in vitro. M1 and M2 cells were treated with 10 μ g/mL perylene bisimide (PBI-12-Man), a fluorescently tagged mannose, for 30 min at 37°C to detect endocytosis. Both the M1 and M2 cells were then rinsed with 1 $\times$ PBS, fixed, DAPI stained, mounted, and then visualized (A). Red fluorescence intensity per cell was quantified using the Metafer slide scanning software and presented as a box and whisker plot with *** representing P -values < 0.01. (B). Cells were grown in culture under M-CSF-only and phased conditions for 14 days, then fixed, DAPI stained, mounted, and visualized using phase contrast and fluorescence imaging (C). Individual multinucleated cells are outlined in the merged image with a red dashed line.

Phased polarization leads to increased expression of canonical M1, M2, and moDC cell-surface markers

To confirm that human CD14⁺ monocytes were fully differentiated into either macrophages or moDCs by Day 9, flow cytometry was used to analyze cell-surface markers and assess morphological differences (Figure 3, A-C). Human CD14⁺ monocytes were differentiated (Figure 1) to assess which strategy would yield high expression of cell-type appropriate surface markers.

A panel of cell-surface markers was tested for each of the cell types. Our M1 cell-surface marker panel consisted of the canonical markers CD86, and CD80 (Figure 3, A-C). For the M2 macrophage panel, we used CD206, CD163, CD124 (IL-4 receptor- α), E-cadherin, and CD36 (Class B scavenger receptor) (Figure 3, A-C). For moDCs, CD83, CD86, and CD1a were utilized. Surface expression levels for all of the markers tested were determined for all cell types (Supplementary Figure S1). Median fluores-

cence intensity (MFI) was calculated and shown as fold-change relative to the corresponding unstained control (see tables in Figure 3).

Interestingly, analysis of the M2 cell-surface markers, CD36 and IL-4R α , in the M1 GM-CSF-only polarized macrophages revealed dramatic increases (Figure 3A). When comparing GM-CSF-only treated cells to all M1 treatment groups, the GM-CSF-only group had markedly higher CD36 expression. These GM-CSF-

only cells also expressed low levels of the M1-associated markers CD86 and CD80. Similarly, the continuous condition yielded cells that were not only low in CD86 and CD80 but were also high in CD36 and IL-4R α expression. Conversely, when human CD14⁺ monocytes were differentiated under the M1 phased condition, CD86 and CD80 were noticeably up-regulated, while CD36 and IL-4R α expression remained low. Further flow cytometric analysis of cell-surface marker expression revealed that M1 macrophages had lower expression of CD206, CD36, and CD163 relative to M2 macrophages under both phased and continuous exposure (Supplementary Figures S1 and S4). Additionally, these varied culturing conditions generate cells with distinct morphological features and structure (Figure 3A). While the GM-CSF-only treated cells appear to be larger, forward scatter (FSC) data revealed minimal differences in size between the groups (Supplementary Figures S2 and S3). Comparing internal complexity (SSC) between treatment conditions did reveal an increase in granularity in the GM-CSF and phased groups (Supplementary Figure S2). Together, these results show that GM-CSF-only and continuous treatment yield cells that express low levels of M1 markers and high levels of certain M2 markers, as well as exhibiting morphological changes.

M2 macrophage culturing conditions yielded slightly more ambiguous results than those found with the M1 macrophage culturing conditions (Figure 3B). Relative to the M-CSF-only group, the phased and continuous groups showed increased expression levels of CD206. Conversely, IL-4R α surface levels were markedly higher in M-CSF-only conditions, relative to both continuous and phased. E-cadherin expression remained uniform across groups, while CD163 was significantly higher in both the M-CSF-only and phased groups. One observation of note is that the continuous exposure group was consistently far less homogeneous in regards to cell-surface marker expression. This is illustrated by the bimodal distribution and large variance in expression levels found on the histogram. Additionally, dramatic morphological differences can

be seen between the treatment groups. Based on the phase contrast images, the M-CSF-only group produced cells that appear to be smaller and highly granular (Figure 3B). Flow cytometric analysis demonstrates that these cells have similar size (FSC), however, there is an increase in cellular granularity [side scatter (SSC)] in the phased and continuous treatment conditions (Supplementary Figure S2). Lastly, glutamine deprivation altered M2 polarization, demonstrating that metabolic products are necessary under this strategy (Supplementary Figure S5). Altogether, these data suggest the phased conditions yield the greatest efficiency for M2 macrophage polarization.

We then examined surface marker expression of moDCs that were un-stimulated or stimulated with IL-6 and LPS 24 h prior to flow cytometric analysis. Stimulation with IL-6 and LPS resulted in an increase in CD80, CD83, and CD86 expression (Figure 3C). Mature DC marker CD83 cell-surface expression dramatically increased upon activation using IL-6 and LPS on Day 8 relative to the un-stimulated group. This confirmed that IL-6 and LPS were able to promote dendritic cell maturation (Supplementary Figure S1L). CD1a expression remained uniform between the un-stimulated and stimulated groups. Interestingly, the stimulated DCs remained in suspension, however, they did appear to begin to aggregate and adhere to one another (Figure 3C). These results confirm previous findings regarding moDC polarization and provide an additional control for surface marker analysis to identify undesired moDC populations within the various macrophage culturing conditions.

M2 macrophages generated by phased cytokine exposure possess M2-associated functionality

The mannose receptor (CD206) is an endocytic and recycling receptor that is highly expressed in our M2 macrophage populations under phased treatment conditions. We next wanted to assess CD206-mediated endocytic uptake in our macrophages. This was determined using PBI-12-Man, a biocompatible agent that fluoresces

upon binding to the mannose receptor (17). After a 1 h treatment of both M1 and M2 macrophages with PBI-12-Man, the red fluorescence of PBI-12-Man was predominantly intracellular in M2 macrophages under phased and continuous treatment conditions (Figure 4, A and B). This finding suggests that cell-surface binding to CD206 and endocytosis results in vesicular localization. This agrees with the previous data from flow cytometry, which demonstrated that under phased treatment conditions, the M2 macrophages displayed a higher cell-surface expression of CD206 relative to the M1 macrophage population. Interestingly, we found that GM-CSF-only cells displayed high levels of PBI-12-Man endocytosis (Figure 4, A and B). This correlates with our flow cytometric data (Figure 2), which demonstrated that GM-CSF-only culturing conditions produce M1 cells with M2-associated surface marker expression. Altogether, we demonstrate that these cells possessed the normal characteristics of macrophages and that CD206 was functional in the M2 macrophage population.

After 14 days in culture, M2 macrophages also displayed the ability to fuse, reaching over 200 μ m in size and containing multiple nuclei per cell (Figure 4C). These resembled multi-nucleated giant cells (MNGCs) that have been reported to possess phagocytic and other abilities (20). Additionally, MNGCs have been associated with foreign material in the body, tuberculosis, and cancer (20–23). This cellular fusion was found exclusively in our M2 macrophage populations and was far more significant in the M2 phased condition. While the M-CSF-only groups did have some multinucleated cells (Figure 4C), their size and numbers were much lower than the phased group. Given the ability of these cells to perform this macrophage associated function, these results further indicate that the phased culturing conditions produces highly M2-like macrophages.

Here we described our newly developed phased protocol for macrophage polarization. We found that the inclusion of IL-6 and phased polarization treatment timing are absolutely

critical in generating the most M1- and M2-like macrophages in vitro. This finding was tested rigorously when compared with other polarization methods. For that reason, we believe that the phased polarization method provides a substantial step forward. This will give researchers unified experimental standards to produce homogeneous populations of macrophages to use in vitro and the ability to compare their results. While this protocol offers a number of improvements compared to current polarization strategies, we believe that additional research and optimization of exposure times and the cytokine cocktail would be useful. This will help us to further understand the complex roles of M1 and M2 macrophages in various forms of disease progression and advance the development of novel therapeutics.

Author contributions

J.R.H., J.C.Z., J.E.V., C.G.D., and K.J.P. conceptualized the study. J.R.H., J.C.Z., S.P.C., and C.G.D. developed the methodology. J.R.H. and J.C.Z. performed the investigation. J.R.H. and J.C.Z. performed the validation. Formal Analysis was done by J.E.V. The original draft of this article was written by J.C.Z. and J.R.H. The article was reviewed and edited by J.C.Z., J.R.H., J.E.V., K.J.P., and C.G.D. Funding was acquired by K.J.P. and J.C.Z. The study was supervised by J.C.Z. and K.J.P.

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Competing interests

The authors declare no competing interests.

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