Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression

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Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression

Fernando O. Martinez,*† Siamon Gordon,‡ Massimo Locati,2*† and Alberto Mantovani*†

Comprehensive analysis of the gene expression profiles associated with human monocyte-to-macrophage differentiation and polarization toward M1 or M2 phenotypes led to the following main results: 1) M-CSF-driven monocyte-to-macrophage differentiation is associated with activation of cell cycle genes, substantiating the underestimated proliferation potential of monocytes. 2) M-CSF leads to expression of a substantial part of the M2 transcriptome, suggesting that under homeostatic conditions a default shift toward M2 occurs. 3) Modulation of genes involved in metabolic activities is a prominent feature of macrophage differentiation and polarization. 4) Lipid metabolism is a main category of modulated transcripts, with expected up-regulation of cyclo-oxygenase 2 in M1 cells and unexpected cyclo-oxygenase 1 up-regulation in M2 cells. 5) Each step is characterized by a different repertoire of G protein-coupled receptors, with five nucleotide receptors as novel M2-associated genes. 6) The chemokineome of polarized macrophages is profoundly diverse and new differentially expressed chemokines are reported. Thus, transcriptional profiling reveals novel molecules and signatures associated with human monocyte-to-macrophage differentiation and polarization activation which may represent candidate targets in pathophysiology. The Journal of Immunology, 2006, 177: 7303–7311.

Monocytes and tissue macrophages provide both immediate defense against foreign agents and assist during the setting off and development of the adaptive immune response. Monocytes originally derive from CD34+ myeloid progenitor cells in the bone marrow, circulate in the bloodstream, and enter peripheral tissues where they mature into different types of resident macrophages, characterized by low oxygen consumption, low protein synthesis rate, and modest cytokine production (1, 2). Inflammation due to tissue damage or infection results in resident macrophage activation, which increases the production of cytokines, chemokines, and other inflammatory mediators, as well as monocyte recruitment. In the context of specific immune response, the cytokine milieu compels mononuclear phagocytes to express specialized and polarized functional properties. Mirroring the Th1/Th2 nomenclature, many refer to polarized macrophages as M1 and M2 cells (3–6). Classically polarized activated M1 macrophages have long been known to be induced by IFN-γ alone or in concert with microbial stimuli as LPS, or cytokines as TNF and GM-CSF. M1 cells have an IL-12high, IL-23high, IL-1βlow phenotype, are proficient producers of effector molecules (reactive oxygen and nitrogen intermediates) and inflammatory cytokines (IL-1β, TNF, IL-6), contribute as inducer and effector cells in polarized Th1 responses, and mediate resistance against intracellular parasites and tumors (7–11). In contrast, the alternative M2 form of macrophage activation is a generic name used for various forms of nonclassically activated macrophages resulting from cell exposure to IL-4 or IL-13, immune complexes, IL-10, glucocorticoid, or secosteroid (vitamin D3) hormones (3, 9, 12). The various forms of M2 macrophages share an IL-12low and IL-23low phenotype, generally display high levels of scavenger, mannose (13), and galactose-type receptors (3), and arginine metabolism is shifted to production of ornithine and polyamines via arginase (14).

Previous studies have addressed the issue of profiling gene expression in M1 or M2 macrophage activation in the mouse, leading to the identification of new molecules expressed in polarized murine macrophages (e.g., Ym1, Fizz1, Mrc1) (13, 15, 16). Data on human mononuclear phagocytes on the contrary are scanty and have highlighted important interspecies differences in key molecules, such as arginase and inducible NO synthase, rendering difficult extrapolation (17, 18). In this study, we report for the first time a whole genome transcriptional profile analysis of the human monocyte-to-macrophage differentiation and polarized activation processes, describing distinct molecular signatures which shed new light on these processes and reveal new candidate markers.

Materials and Methods

Reagents

Recombinant human cytokines were obtained from PeproTech. LPS from Escherichia coli (serotype 055:B5) was obtained from Sigma-Aldrich. Abs were purchased from Serotec, unless specified. Human cytokines were measured using commercial ELISA kits purchased from R&D Systems, according to the manufacturer’s instructions. All chemicals were obtained from Sigma-Aldrich, unless specified.

Cell preparation

Human monocytes were obtained from normal blood donor buffy coats by two-step gradient centrifugation followed by an additional step using the

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0022-1767/06/$02.00
Monocyte Isolation kit II (Miltenyi Biotec) as previously described (17). Macrophages were obtained by culturing monocytes (98% CD14+/CD16−) for 7 days in RPMI 1640 (Biochrom) supplemented with 20% FCS (HyClone) and 100 ng/ml M-CSF in FCS-coated dishes at a density of 1.5 × 10^4/cm^2. Macrophage polarization was obtained by removing the culture medium and culturing cells for an additional 18 h in RPMI 1640 supplemented with 5% FCS and 100 ng/ml LPS plus 20 ng/ml IFN-γ (for M1 polarization) or 20 ng/ml IL-4 (for M2 polarization). Five different cell types were generated: freshly isolated monocytes (Mo); ϕ, monocytes after 3 days of culture with M-CSF (Md3); Ͽ, macrophages after 7 days of culture with M-CSF (Mb); △, M1-polarized macrophages (M1); △, M2-polarized macrophages (M2).

Transcriptional profile analysis

The transcriptional profile was evaluated in three independent cell preparations, each derived from a different single donor using the Human Genome U133 A and B arrays (HG-U133, Affymetrix) containing a total of ~39,000 transcripts. RNA purification and labeling, hybridization, and array scanning were conducted as previously described (19). Scanned images and raw data were processed using robust multiarray average (20, 21).

Principal component analysis (PCA)3 was conducted on all genes analyzed to assign the general variability in the data to a reduced set of variables, each derived from a different single donor using the Human Genome U133 A and B arrays (HG-U133, Affymetrix) containing a total of ~39,000 transcripts. RNA purification and labeling, hybridization, and array scanning were conducted as previously described (19). Scanned images and raw data were processed using robust multiarray average (20, 21). Principal component analysis (PCA)3 was conducted on all genes analyzed to assign the general variability in the data to a reduced set of variables, each derived from a different single donor using the Human Genome U133 A and B arrays (HG-U133, Affymetrix) containing a total of ~39,000 transcripts. RNA purification and labeling, hybridization, and array scanning were conducted as previously described (19). Scanned images and raw data were processed using robust multiarray average (20, 21).

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Global transcriptome analysis

The transcriptional events associated with M-CSF-dependent monocyte-to-macrophage differentiation and subsequent M1 or M2 cell polarization induced by LPS plus IFN-γ or IL-4, respectively, were investigated using oligonucleotide microarrays. Repli-plant are available at the Gene Expression Omnibus (GEO) website (www.ncbi.nlm.nih.gov/geo), accession number GSE5099.

Gene expression analysis by real-time PCR

Real-time PCR was performed using gene-specific primers designed using AutoPrime (www.autoprime.de). Primer and probe sequences are available in the public RTPrimerDB database (http://medgen.UGent.be/rtprimerdb/) (gene (RTPrimerDB-ID): GPR105 (3478), GPR87 (3479), P2RY13 (3480), P2RY12 (3481), GPR171 (3482)) (28). Five replicates per each experimental point were performed, and differences were assessed with a two-tailed Student’s t test. Results were normalized using the housekeeping gene GAPDH and the ΔΔ cycle threshold method (19) and are expressed as relative fold of stimulated over control group, used as calibrator.

Western blot

After removing the medium, cells were washed in PBS and lysed in ice-cold lysis buffer (2% Triton X-100, 10 mM Tris-HCl (pH 8), 150 mM NaCl, 2 mM NaN3, 2 mM EDTA) containing protease inhibitors (Roche Molecular Biochemicals) for 45 min at 4°C. Lysates were harvested and centrifuged at 13,400 × g to eliminate nuclei. Protein concentration was determined using the bicinchoninic acid assay (Pierce) and 30 µg of protein was electrophoresed in a 7.5% SDS-PAGE under nonreducing conditions and transferred to nitrocellulose using standard procedures. PTGS1 and PTGS2 were detected using the specific mAbs CX33 and CX229 (Alexis).

Results

Global transcriptome analysis

The transcriptional events associated with M-CSF-dependent monocyte-to-macrophage differentiation and subsequent M1 or M2 cell polarization induced by LPS plus IFN-γ or IL-4, respectively, were investigated using oligonucleotide microarrays. Results demonstrated the existence of a complex network of gene regulation and clearly identified specific gene expression patterns that characterize each phase. PCA analysis was applied to the complete dataset and demonstrated that 98% of the total variance of the
system lies within the first two components. PCA revealed that monocyte maturation was associated with a significant modification of the global transcriptome (~35% of the total variance), with larger changes taking place in the early phase of the process (~24% variance in the first 3 days of differentiation), followed by a smaller overhaul (~11% variance in the last 4 days of differentiation) in the late phase (Fig. 1). Macrophage polarization was also associated with significant changes at the transcriptional level, although the two polarizing conditions were very different, with M1 polarization profoundly affecting the transcriptional profile (~90% variance in the shift from Mφ to M1), and M2 polarization resulting in only subtle adjustments (~8% variance in the shift from Mφ to M2) (Fig. 1).

Differentially expressed genes, selected as described in Materials and Methods, were subjected to figures-of-merit analysis, where K-means clustering performed optimally for 12 clusters, with no increase in the predictive value of the algorithm for additional behavioral categories (data not shown). Squared Pearson correlation was used as similarity measurement, cumulating clusters with mirror performances and generating a total of 6 gene clusters (Fig. 2). To gain insight into the biological processes involved, each cluster was then subjected to hierarchical subclustering (supplemental figures 1–6) and GO analysis as included in the Expression Analysis Systematic Explorer (26) (Fig. 3).

Monocyte-to-macrophage differentiation was associated with modulation of 868 (2.2%) transcripts in total (Fig. 2, A and B). Of

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*The online version of this article contains supplemental material.*
Table I. Genes differentially expressed in M1 vs M2 macrophages

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(Table continues)
Continued

**Table I.**

<table>
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<tr>
<th>Gene Name</th>
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<th>M1</th>
<th>M2</th>
<th>M1-M2 Ratio</th>
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**Extracellular mediators**

- Pentraxin 3
  - FTX3 914 30 30
- Chondroitin sulfate proteoglycan 2
  - CSPG2 1,712 107 16
- Apolipoprotein L3
  - APOL3 4,453 374 12
- Insulin-like growth factor binding protein 4
  - IGFBP4 3,821 349 11
- Apolipoprotein L1
  - APOL1 3,032 465 7
- Platelet-derived growth factor α
  - PDGFA 422 61 7
- Endothelin 1
  - EDN1 539 86 6
- Apolipoprotein L2
  - APOL2 2,027 315 6
- Inhibin β A
  - INHBA 688 129 5
- Apolipoprotein L6
  - APOL6 918 202 5
- Transforming growth factor β-induced protein
  - TGFB1 1,120 8,976 -8
- Selenoprotein P1
  - SEPP1 811 7,641 -9
- Chimerin 2
  - CHN2 76 943 -12
- Fibronectin
  - FN1 330 8,382 -25
- Fibrinogen-like 2
  - FGL2 117 4,569 -39

**DNA-binding factors**

- Homeobox expressed in ES cells 1
  - HESX1 1,238 102 12
- Interferon regulatory factor 1
  - IRF1 4,288 527 8
- Activating transcription factor 3
  - ATF3 4,080 509 8
- Interferon regulatory factor 7
  - IRF7 2,694 394 7
- Growth arrest-specific 7
  - GAS7 257 1,350 -5
- Early growth response 2
  - EGR2 108 1,238 -11
- v-maf musculoaponeurotic fibrosarcoma oncogene homolog
  - MAF 26 1,187 -46

*The table shows a selection of genes strictly associated with macrophage polarization grouped into functional categories. M1 and M2 columns report the mean of the expression values. In each category, genes are ranked according to their fold difference between M1 and M2.*

As indicated by PCA, M1 polarization had a major effect on cell transcription, affecting 2053 (5.2%) transcripts in total (Fig. 2, C and D). Of these, 1108 (2.8%) genes were exclusively associated with classical macrophage activation (Fig. 2C; list of genes in supplemental figure 3SM), while a second cluster of 945 (2.4%) transcripts included a majority of genes associated with classical activation and a minor fraction (20 genes, corresponding to ~2% of the genes) concordantly regulated by IL-4 (Fig. 2D; list of genes in supplemental figure 4SM). GO analysis revealed an overrepresentation of genes related to DNA transcription and protein metabolism, such as ribosomal proteins and eukaryotic translation initiation factors (Fig. 3).

Although less dramatic than M1, M2 polarization exerted a significant effect on macrophage transcriptional profile, modulating a total of 104 (~0.3%) transcripts (Fig. 2E; list of genes in supplemental figure 5SM). GO analysis indicated that this set of genes is particularly rich in immune system-related molecules, including cytokines, chemokines, and G protein-coupled receptors (GPCR) (Fig. 3).

A peculiar cluster included 505 (1.3%) genes with high expression in Mo and M1 cells and opposite regulation in MΦ and M2 cells (Fig. 2, cluster F; list of genes in supplemental figure 6SM). Unexpectedly, genes with high expression in Mo and M1 macrophages included prototypic M1 polarization markers, such as the indoleamine-pyrrole 2,3 dioxygenase (32, 33), the lysosomal-associated membrane protein 3, IL-7R (3, 5), and CCR7, though despite transcript expression, monocytes did not express membrane CCR7 (data not shown). Similarly, genes with high expression in MΦ and M2 cells included classic M2 polarization markers,
such as the mannose receptor 1 (34, 35), the scavenger receptors SR-A and M160 (3, 5, 36). GO analysis revealed that a relevant percentage of these genes is involved in cellular metabolic activities, such as active transport and oxidoreductase activities (Fig. 3).

To identify genes strictly associated with macrophage polarization, genes included in clusters C to F have been ranked according to their fold difference between M1 and M2 profiles and further grouped into functional categories (Table I). The interactome highlights a central role of a restricted panel of molecules, including CXCR4, TRAIL (TNFSF10/TRAIL), insulin-like growth factor I, and fibronectin 1, and clearly shows that macrophage polarization is mainly associated with regulation of membrane receptors and extracellular proteins with a minor contribution of nuclear factors, directly opposite to monocyte differentiation (Fig. 4C).

GO analysis of functional categories: lipid metabolism
GO analysis was used to identify functional categories overrepresented in the panel of genes associated with monocyte differentiation and macrophage polarization. Consistent with the well-recognized capability of macrophages to respond and produce a vast range of lipidic products, one of the most overrepresented categories was lipid metabolism (Fig. 3). In particular, transcriptional analysis revealed a unique regulation profile for different enzymes involved in eicosanoid production (Fig. 5A). Monocyte-to-macrophage maturation was associated with a gradual loss of PG-endoperoxide syntheses (both PTGS1 and PTGS2), as well as the arachidonate 5-lipoxygenase (ALOX5), and leukotriene A4 hydrolase. As expected, classical activation was associated with a marked induction of cyclooxygenase (COX)-2 (37), accompanied by a significant unexpected further down-regulation of COX-1, leukotriene A4 hydrolase, thromboxane A synthase 1, and ALOX5. Conversely, alternative activation resulted in the up-regulation of the M2 marker arachidonate 15-lipoxygenase and unexpectedly COX-1, here confirmed at the protein level (Fig. 5B).

Sphingolipid mediators, such as sphingosine 1-phosphate, ceramide 1-phosphate, and sphingosine are derived by the enzymatic breakdown of sphingomyelin, and display potant effects on multiple organ systems. Within this pathway, the most interesting finding is the opposite regulation of the sphingosine and ceramide kinases, expressed in M1 and M2 macrophages, respectively.

GO analysis of functional categories: GPCRs
A second category highlighted by GO analysis was represented by GPCRs (Fig. 3). From a total of 465 entries of GPCRs represented in the microarray, 53 were detected as differentially expressed during monocyte differentiation and macrophage polarization (Fig. 6A). The hierarchical clustering demonstrates that each stage is characterized by the expression of a specific group of GPCRs. Monocytes are characterized by a cluster of 11 highly expressed genes, 5 of which correspond to chemotactic receptors: CCR2, CCR5, CCR7, CX3CR1, and FPR1. The combination of IFN-γ and LPS has a broad effect with no clear family overrepresentation, while IL-4 activation is characterized by a cluster of 8 genes with high expression, 5 of which are nucleotide receptors: GPR86, GPR105, P2Y8, P2Y11, and P2Y12. Interestingly, GPR86, GPR105, and P2Y12 are positioned together in chromosome 3. Real-time PCR confirmed the up-regulation of this M2-associated gene cluster (P2Y12, GPR105, and GPR86), and revealed minor overexpression for the closest genomic neighbors GPR87 and H963 (Fig. 6B).

GO analysis of functional categories: the chemokinome
Macrophage maturation and polarization are characterized by specific patterns of chemokines as suggested by GO ontology analysis (Fig. 7A). In addition to chemokines already known to be differentially expressed in polarized macrophages (e.g., CXCL10 for M1; CCL17 for M2), we found new chemokine signatures associated with cell polarization (Fig. 7A). The profiling results were confirmed by real-time PCR (data not shown) and by measurements of released proteins (Fig. 7, B and C).

Discussion
The present study was designed to characterize the gene expression profile of human monocytes undergoing differentiation into mature macrophages in the presence of M-CSF and subsequent polarized activation into M1 or M2 cells. These processes were associated with major changes in the global transcriptome.

Macrophage polarization to M1 was associated with the most dramatic change in the transcriptome, whereas stimulation with IL-4 of M-CSF-differentiated macrophages caused a relatively minor alteration in gene expression. This apparently minor effect of IL-4 is due to the fact that M-CSF-driven differentiation leads per se to the acquisition of M2 properties, including expression of mannose receptor 1 and scavenger receptors SR-A. This finding is in agreement with previous data showing divergent M1-M2 properties of macrophages differentiated in GM-CSF compared with M-CSF (38, 39). M-CSF is a homeostatic growth factor circulating in a default pathway in macrophage differentiation.

In particular, monocyte differentiation in the presence of M-CSF was associated with early (day 3) dramatic regulation of cell-cycle genes, including the cyclins A2, B1, B2, D1, D3, E2, and CDC24, and fibronectin 1, and clearly shows that macrophage polarization was associated with major changes in the global transcriptome.

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the culture was also confirmed in the present study (data not shown). Thus, the proliferative potential of human monocytes should not be underestimated and could be exploited and tailored for cell expansion.

Modulation of genes involved in general cellular metabolic activities is a prominent feature of macrophage differentiation and polarization. In addition to providing tools for macrophage function in tissues, these changes may have a more subtle significance. For instance, macrophages are a major component of adipose tissue and play a role in the metabolic syndrome (42).

Macrophages are an active source of pro- and anti-inflammatory lipid mediators, such as arachidonic acid derivatives and phospholipids. COX-2 has long been associated with arachidonic acid metabolism in M1 cells (37). In contrast, the finding that M-CSF-differentiated macrophages retain high levels of COX-1 and that these levels are further augmented by IL-4 is novel and unexpected. This induction is of functional relevance for eicosanoid production (data not shown) and may contribute to pathophysiological reactions, such as toxicity of aspirin and related drugs in asthma (43, 44). The interconvertible ceramide metabolites sphingosine 1-phosphate and ceramide 1-phosphate have emerged as potent bioactive agents which regulate critical cellular functions including cell proliferation, phagocytosis, differentiation, angiogenesis, chemotaxis, and cell survival (45). Our results suggest that these enzymes can aid in distinguishing between polarized forms of activation, being sphingosine and ceramide kinase selectively present in M1 and M2 macrophages, respectively (Table I). M1 polarization is associated with the up-regulation of ABCA1, the primary gatekeeper for eliminating tissue cholesterol, and a set of apolipoproteins clustered on chromosome 22q12.3 including APOL1, APOL2, APOL3, and APOL6, which play a central role in cholesterol transport and atherosclerosis (46).

Different stages of monocyte differentiation and polarization are characterized by different repertoires of GPCRs. In agreement with previous reports, CCR2 is rapidly down-regulated during monocyte differentiation (47). CX3CR1 is also down-regulated, but at a slower
rate, being still expressed on day 3. LPS and IFN-γ up-regulate CCR7 and down-regulate CCR1 in mature macrophages, as they do in dendritic cells (48, 49). Presumably, this reciprocal regulation underlies the trafficking of macrophages to lymph nodes, where their disposal occurs. Strikingly, of the eight GPCRs highly expressed in the M2 cells, five are nucleotide receptors (Fig. 6), the UDP-glucose receptor GPR105 being among the most highly regulated genes in human macrophages in response to IL-4. M2 macrophages are associated with tissue remodeling. High expression of nucleotide receptors endows M2 cells with sensors for tissue damage (50, 51), and preliminary data suggest that these ligands modulate relevant functions in this cell type (data not shown).

The chemokine repertoires of mononuclear phagocytes exposed to polarizing stimuli are profoundly different (4, 5) and the results presented here extend this general view. In addition to well-known polarized chemokines, such as CXCL10 for M1 and CCL17 for M2 cells, we found high levels of CCL8, CCL15, CCL19, CCL20, and CCL13 in M1 cells, and CCL13, CCL14, CCL17, CCL23, and CCL26 in M2 cells. Association of these molecules with polarized macrophage activation may contribute to pathophysiology. For instance, we found that M2 cells do not produce detectable levels of CCL11 but may contribute to the recruitment of CCR3-positive leukocytes such as eosinophils, basophils, and some polarized Th2 cells (4) through the expression of CCL26.

A hallmark of M1 polarization is the synthesis of the proinflammatory cytokines IL-6, IL-12, and IL-15 (5) and receptors for IL-2R α-chain, IL-15R α-chain, and IL-7R as previously described in mice (52, 53). In the other pole, M2 are characterized by the overexpression of several scavenger receptors able to bind a diverse array of endogenous and foreign molecules (54). Our results confirm the up-regulation by IL-4 of the mannose receptor 1 (13), the macrophage scavenger receptor 1(36), the C-type lectin-like receptor Dectin-1 (55) and DC-SIGN (CD209) (56) and report for the first time in mature macrophages the up-regulation of DCIR, also called CLECSF6 (57), thoroughly studied in dendritic cells and the less described C-type lectin DCL-1 (58) and CLECSF13. Alternatively activated macrophages are also characterized by increased expression of fibronectin (59), which is involved in cell adhesion and migration processes during embryogenesis, wound healing, blood coagulation, and metastasis.

The solute carrier family of proteins comprises genes whose primary role is the transport of divalent cations and small organic molecules. They regulate transcription through DNA-binding proteins and metal response elements, the activity of enzymes including metalloproteases, superoxide dismutase, inducible NO synthase, and functions like endosomal fusion, and metabolism. Despite the recognized role of some members in immune disease susceptibility and infection (60), they have not been associated with macrophage polarization. We find that classically activated macrophages are characterized by increased expression of the solute carrier family members SLC2A15 and SLC3A12, while alternatively activated macrophages exhibit increased SLC4A7, SLC38A6 expression (Table I). The role of these molecules remains to be elucidated.

Hitherto, expression data related to macrophage polarization primarily concern the murine system (3). Investigation of selected markers in the human system have previously highlighted interspecies discrepancy (17, 61). This report represents the first comprehensive description of the human mononuclear phagocyte system, and provides further evidence of relevant interspecies variability. For example, IL-4 in this study, as well as IL-13 in our previous expression-profiling experiments (17), did not induce the human homolog of the mouse alternative activation markers arginase 1, Fizz1, MMP1 and Ym1. Similarly, a number of molecules not involved in macrophage polarization in the murine system emerged from this data set as human macrophage alternative activation markers, including fibrinoligase (F13A1) and platelet-derived growth factor C. In contrast, other mouse alternative activation markers such as the GPCR cluster discussed above were confirmed in our system. Collectively, results indicate that ~50% of macrophage polarization markers selectively apply to one species and not to the other, cautioning against direct mouse-to-human translation of polarization markers. A direct comparison based on expression profiling results will be required to fully describe interspecies variability.

Polarization of mononuclear phagocyte function is a useful simplified conceptual framework, describing a continuum of functional states. Different forms of M2 polarization have been described in vitro and ex vivo (3, 5, 9). The study reported here using a global profiling approach, describes new molecules and signatures associated with different stages of the human monocyte-to-macrophage differentiation and polarization, which may represent novel tools and targets in pathophysiology.

Disclosures
The authors have no financial conflict of interest.

References