GM-CSF differentiation of human monocytes stabilizes macrophage state via oxidative signaling

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Abstract

Macrophages are central mediators of immunity that integrate diverse signals derived from differentiation cues, tissue location, and disease. Controlling macrophage state and function is an appealing therapeutic objective across many diseases including cancer, atherosclerosis, and tuberculosis. Despite the growing appreciation for the *in vivo* complexity of macrophage state, existing *in vitro* models of human monocyte-derived macrophages have used a limited number of individual perturbations to explore the complex phenotypic space that macrophages can occupy. Here, we leverage a tiered differentiation, activation, and stimulation strategy to generate libraries of *in vitro* monocyte-derived macrophages and examine the *in vitro* state space of macrophage function using high-dimensional technologies. Our tiered experimental approach further revealed a striking relationship between GM-CSF differentiation and IL-10 production. Cells that were differentiated with GM-CSF produced very low or undetectable levels of IL-10 independent of activation or stimulation condition. To nominate candidate regulators of this IL-10 response, we leverage unbiased single-cell mRNA sequencing to identify transcriptional networks associated with GM-CSF-derived cells. Using these data, we identify oxidative signaling pathways as upregulated in GM-CSF derived cells and demonstrate that scavenging of oxidative radicals can enhance IL-10 production in these cells. Collectively, these data underscore the complexity of monocyte-derived macrophage state over time and highlight a dominant role for GM-CSF in tuning macrophage inflammatory phenotype, metabolic state, and plasticity.

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Introduction

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Understanding how macrophages interpret diverse signal inputs to dictate their state and
function is an actively evolving field. While previous nomenclature to describe
macrophage state adopted an M1 or M2 naming convention to parallel elements of Th1
and Th2 immunity, new naming conventions integrate additional dimensions of
macrophage diversity including differentiation or activation cytokine and signal duration
(Murray et al., 2014). This naming convention recognizes that distinct cues can drive
specific cytokine-associated programs in addition to generic inflammatory or anti-
inflammatory programs that are conserved across diverse inputs. As we continue to
identify additional cues that redirect macrophage function, it is important to understand
how these signals reinforce existing macrophage states or perturb them. Due to this
evolving nomenclature, determining macrophage state using standard tools such as flow
cytometry with canonical macrophage markers remains challenging as these markers are
often expressed independent of macrophage state.

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Recent murine studies highlight the complex and exquisite trajectories monocytes
traverse as they differentiate into tissue-resident macrophages. In fact, in many tissues,
monocyte-derived macrophages serve as the dominant contributor to the steady-state
macrophage pool (Liu et al., 2019). These *in vivo* studies have been utilized to highlight
features of macrophage plasticity and identify the signals that drive tissue-resident
macrophage identity. For example, Lavin and colleagues demonstrated that tissue-
resident macrophages can be transferred to new tissues and adopt features of the new
microenvironment (Lavin et al., 2014). They demonstrated that peritoneal macrophages
could be transferred intratracheally to the alveolar cavity of recipient mice and adopt
features of the alveolar macrophages as evidenced by transcriptional profiling. These
studies and countless others have been utilized to demonstrate features of macrophage
phenotypic plasticity.

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In contrast to the study of experimentally available murine tissue-resident macrophages,
studies of human macrophages rely mostly on monocyte-derived macrophages
differentiated from peripheral blood mononuclear cells of human donors. To differentiate
these monocytes into macrophages, common protocols supplement culture media with
either macrophage colony-stimulating factor (M-CSF) or granulocyte macrophage colony-
stimulating factor (GM-CSF). Sander and colleagues utilized transcriptional profiling to
demonstrate that human monocytes differentiated with M-CSF or GM-CSF resembled
macrophages isolated directly from human ascites thus underscoring their experimental
utility (Sander et al., 2017). Additionally, the authors demonstrated that monocytes
integrate simultaneous signals from IL-4 and GM-CSF distinguishing them from
monocytes differentiated with GM-CSF alone and suggest that varying the exposure time
of IL-4 tunes the functional identity of the cells. Taken together, these previous studies
highlight the complexity of macrophage behavior and their modes of signal integration.

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To build upon these previous studies and examine how signal identity and duration
influence human macrophage functional state, we established an *in vitro* experimental

platform to create libraries of monocyte-derived macrophages. Using a combination of
flow cytometry, cytokine release assays, and single-cell RNA sequencing (scRNAseq),
we demonstrate that GM-CSF differentiation of monocytes abolishes the capacity of these
cells to produce IL-10 in response to TLR ligands. Moreover, we were unable to identify
a cytokine or small molecule that was capable of restoring IL-10 production in these cells
following differentiation. This finding provides an important update to the long-held model
of macrophage plasticity. Using scRNAseq, we overcome existing challenges in
macrophage characterization and identify novel markers of macrophage state that permit
discrimination of macrophages on the basis of differentiation cytokine. We further
leverage these scRNAseq results and nominate oxidative signaling as a driver of reduced
IL-10 production in macrophages differentiated by GM-CSF which we then confirmed in
our *in vitro* system. Taken together, these data emphasize the complexity of macrophage
functional properties and suggest a dominant role for GM-CSF in modulating macrophage
state.
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108 **Results:**
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110 **Generation of libraries of *in vitro* monocyte-derived macrophages provides flexible
111 platform for interrogating macrophage state and function**
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Human monocytes differentiated with M-CSF or GM-CSF are widely used as *in vitro*
models for studying macrophage behavior (Lacey et al., 2012; Sander et al., 2017; Xue
et al., 2014). To examine the contribution of these diverse axes, we generated libraries
of *in vitro* monocyte-derived macrophages from CD14+ monocytes isolated from healthy
human donors using a systematic framework for perturbation and characterization.
CD14+ monocytes were first differentiated for 3 days with either GM-CSF or M-CSF to
generate MO-GMCSF or MO-MCSF. To generate additional diversity in our *in vitro*
cultures, on day 3 of differentiation, we supplemented our differentiation media with an
additional activation stimulus (vehicle, LPS, IFN-γ, IL-4, IL-6, and IL-10) ultimately
generating a total of 12 macrophage populations (**Figure 1A**) (Roussel et al., 2017).
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We first utilized flow cytometry to characterize macrophage state by selecting commonly
used macrophage surface markers from previous studies (Roussel et al., 2017). We
compared surface expression of CD32, CD36, CD64, CD86, CD206, CD274, and HLA-
DR focusing first on the differences in MO-MCSF and MO-GMCSF libraries separately.
We used two metrics to characterize surface expression of these markers across our
samples: (1) percentage of cells expressing any given marker relative to an isotype
control and (2) the mean fluorescence intensity (MFI) of the surface markers. We sought
to examine if any of the markers in our panel demonstrated digital expression among the
macrophages generated in our *in vitro* libraries. In contrast to lymphocytes where several
markers exist to distinguish subpopulations using a binary gating strategy, none of the
macrophage markers used in this panel were capable of discriminating between the
different macrophage populations (**Figure 1B, Supplemental Figure 1)** (DuPage and
Bluestone, 2016; Murray et al., 2014)**.** As previously observed by others, our results
displayed differences in the level of expression of various markers across all macrophage
types (**Figure 1C, Supplemental Figure 2**) (Schulz et al., 2019). Specifically, we

observed CD274 consistently upregulated in cells stimulated with IFN-γ or LPS in both
MO-MCSF and MO-GMCSF cells. **(Figure 1D (MO-MCSF), Supplemental Figure 3**
(MO-GMCSF)).
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As a complementary approach to flow cytometry, we next characterized our macrophage
library by examining cytokine release in response to purified TLR ligands. We initially
conducted a multiplex cytokine screen focusing on the differences between MO-MCSF
and MO-GMCSF in response to lipopolysaccharide (LPS), a TLR4 agonist and
Pam3CSK4 (PAM), a TLR1/2 agonist, for 24 hours prior to cytokine analysis. We
measured the release of several cytokines, including IL-1β, IL-6, and IL-10. Comparing
MO-MCSF and MO-GMCSF cells, we observed a marked reduction in the production of
IL-10 and CCL-2 in MO-GMCSF cells in response to both TLR ligands, consistent with
previous reports comparing these cells (**Figure 1E**) (Lacey et al., 2012; Sander et al.,
2017). We next extended these measurements to all twelve populations of MO-MCSF
and MO-GMCSF cells. We focused on the production of TNFα, IL-6, and IL-10 given the
dynamic range of their response to TLR stimulation. By enzyme-linked immunosorbent
assay (ELISA), we were able to validate our observation of decreased production of IL-
10 in GM-CSF derived cells. Notably, we observed levels of IL-10 production that were
near or below the ELISA limit of detection across all MO-GMCSF cells independent of
TLR stimulation or activation cue (**Figure 1F**). This was in contrast to M-CSF derived
macrophages which retained their ability to produce IL-10. MO-GMCSF cells, despite
lacking an IL-10 response, retained their ability to respond to TLR ligands and produce
cytokines as indicated by production of TNFα (**Figure 1G**). We sought to recapitulate
these findings using intracellular cytokine staining; however, we were unable to find a
antibody clone that demonstrated robust differences between unstimulated and
stimulated MO-MCSF cells (**Supplemental Figure 4**).
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Since our cytokine secretion results pointed to an altered IL-10 signaling network in MO-
GMCSF cells, we next sought to determine if MO-GMCSF cells were muted in both their
IL-10 production and responsiveness or production alone. To assess the capacity of MO-
GMCSF cells to respond to IL-10, we monitored the phosphorylation status of STAT3, a
well-characterized protein downstream of IL10R, following stimulation with IL-10
(Niemand et al., 2003). We observed a dose-dependent increase in phosphorylated
STAT3 relative to total STAT3 in MO-GMCSF cells indicating that GM-CSF differentiation
does not abrogate IL10R signaling (**Figure 1H)**. Collectively, these data demonstrate how
macrophage differentiation cues can precisely fine-tune macrophage state and illustrate
the utility of diverse modalities to characterize macrophage phenotypes.
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177   **scRNAseq reveals novel markers of the MO-GMCSF state**
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Given the phenotypic differences observed between MO-MCSF and MO-GMCSF cells,
we employed scRNAseq to identify additional markers capable of distinguishing between
these two macrophage states. To robustly interrogate the transcriptomes of thousands of
cells we used Seq-Well, a nanowell platform for high-throughput scRNAseq (Gierahn et
al., 2017). This produced 1,742 high-quality MO-GMCSF and 4,803 high-quality MO-
MCSF cellular transcriptomes from 2 donors (**Supplementary Figure 5**). We first

examined the expression of canonical markers of macrophage state to characterize their
ability to discriminate between MO-MCSF and MO-GMCSF cells. A subset of canonical
marker genes achieved statistically significant differential expression (CD68, CD86,
MRC1 (CD206); however, the magnitude of their transcriptional difference was minimal
(**Figure 2A**, **Supplementary Table 1**). Other canonical markers either displayed no
significant differences or had low capture rates. We next examined the expression of all
genes measured by scRNAseq. Using a Wilcoxon rank sums test, we identified 178
significantly upregulated and 137 significantly downregulated genes in MO-GMCSF cells
relative to MO-MCSF cells (**Figure 2B**). We identified several novel marker genes of MO-
MCSF and MO-GMCSF cells. We identified CHI3L1 and MGLL as markers of MO-MCSF
and TXN and ALOX5AP as markers of MO-GMCSF (**Figure 2C**). These genes included
CHI3L1 and MGLL as markers of MO-MCSF and TXN and ALOX5AP as markers of MO-
GMCSF. To gain an unbiased view of the transcriptional programs differentially active in
MO-MCSF and MO-GMCSF cells, we next identified Gene Ontology Molecular Function
(GO MF) terms enriched in the set of marker genes for each state. This analysis revealed
MO-GMCSF cells were marked by transcriptional programs related to redox signaling, as
well as antigen presentation (**Figure 2D, Supplementary Table 2**).
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Using scRNAseq, we found several novel markers that distinguished MO-MCSF and MO-
GMCSF cells. The gene encoding thioredoxin (*TXN*) was a robust marker of MO-GMCSF
cells by an AUC classifier (AUC = 0.85). Thioredoxin is an antioxidant that is involved in
many different signaling pathways via redox control (Lee et al., 2013). Previous studies
in human macrophage-like cell lines have demonstrated that thioredoxin is highly
expressed in response to the combination of LPS and IFN-γ, a well-characterized inducer
of an inflammatory phenotype (Plugis et al., 2018). Other studies have suggested that
MO-GMCSF cells may serve as another model of inflammatory macrophages (Fleetwood
et al., 2007; Hamilton, 2019). We hypothesized that the induction of thioredoxin may be
conserved in both MO-MCSF treated with LPS+IFN-γ and MO-GMCSF cells. Consistent
with the scRNAseq data and our hypothesis, thioredoxin protein was highly expressed in
MO-GMCSF, as well as M(LPS+IFN-γ), compared to MO-MCSF cells (**Fig 2E**). We next
sought to validate an additional marker of MO-GMCSF, 5-lipoxygenase accessory protein
(ALOX5AP), which is essential for the production of inflammatory lipids such as
prostaglandin E2. Again, consistent with our scRNAseq data, ALOX5AP was highly
expressed in MO-GMCSF cells relative to MO-MCSF cells; however, ALOX5AP was not
highly expressed in M(LPS+IFN-γ), suggesting distinct regulatory axes contributing to
expression of these marker genes (**Figure 2F**). These results are consistent with previous
work demonstrating altered expression of ALOX5AP and dichotomous production of
inflammatory and pro-resolving lipids (Werz et al., 2018). Further supporting our
scRNAseq results, we also observed enhanced expression of CD36 on MO-MCSF cells
and increased expression of CD206 on MO-GMCSF cells by flow cytometry (**Figure 2G,**
Figure 2H). Taken together, these results identify novel markers that can more definitively
characterize macrophage state and distinguish common transcriptional programs that are
active across macrophage culture systems.
228
229 **GM-CSF mediates a durable switch in IL-10 production**
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We reasoned that the immunophenotyping analyses had provided us with markers that
we could leverage to interrogate phenotypic transitions between macrophage state.
Macrophages have long been described as cells that demonstrate exquisite phenotypic
plasticity; however, our IL-10 secretion results suggested that following GM-CSF
differentiation, several cytokines were unable to enhance TLR-mediated IL-10 production
to levels above the detection limit (Biswas and Mantovani, 2010; Liu et al., 2020; Locati
et al., 2020).

Using IL-10 production in response to TLR stimulation as a measure of macrophage state,
we sought to interrogate the stability of the MO-MCSF and MO-GMCSF phenotype. We
first sought to examine the expression of M-CSF and GM-CSF receptors on the surface
of MO-MCSF and MO-GMCSF cells reasoning that we might be able to utilize the
opposing cytokine to shift macrophage state. We performed flow cytometry on MO-MCSF
or MO-GMCSF cells following 6 days of differentiation. We detected surface expression
of GM-CSF (CD116) and M-CSF (CD115) receptors on the surface of MO-MCSF and
MO-GMCSF cells (**Figure 3A**). We next sought to examine how treatment of MO-MCSF
and MO-GMCSF cells with the opposing cytokine would modulate IL-10 production.
Monocytes were differentiated in the presence of M-CSF or GM-CSF for 6 days, washed,
and then cultured in media containing 50 ng/mL M-CSF, media containing 50 ng/mL GM-
CSF, or media alone for an additional 3 days. After this second phase of culture, cells
were washed and then stimulated with LPS for 24 hours prior to analysis of IL-10 secretion
(**Figure 3B**). As expected from our previous observations, MO-MCSF cells that received
no cytokine or M-CSF during the second media phase produced IL-10 in response to
LPS. In contrast, MO-MCSF cells treated with GM-CSF during the second media phase
produced less IL-10 in response to LPS (**Figure 3C**). Strikingly, MO-GMCSF cells
independent of their second media phase did not produce IL-10 at levels above the limit
of detection in response to LPS stimulation similar to our results observed with IFN-γ, IL-
4, IL-6, IL-10, and LPS.

While macrophages may encounter asynchronous stimuli as modeled by temporally
staggering the addition of cytokines, we reasoned that monocytes upon recruitment to
tissues may simultaneously encounter combinations of cytokines such as M-CSF and
GM-CSF that may act synergistically to drive the functional state of the resulting
macrophages. We next sought to examine whether the monocyte response to GM-CSF
or M-CSF differentiation could be modeled as an additive response. To examine these
dynamics, we generated a matrix of combinations of M-CSF and GM-CSF ranging from
0 ng/mL to 50 ng/mL in ten-fold increments (**Figure 3D, Figure 3E**). Cytokines were
premixed and arrayed prior to the addition of monocytes to eliminate any temporal
asynchrony in cytokine exposure. Monocytes were differentiated for 6 days, washed, and
then stimulated with LPS. IL-10 production was monitored by ELISA. Monocytes cultured
for 6 days in the absence of exogenous cytokines on tissue-culture treated plasticware
did not survive. In the absence of GM-CSF, we observed a dose-dependent increase in
LPS-mediated IL-10 release as we increased the concentration of M-CSF in the
differentiation media. Holding the M-CSF concentration at 0 ng/mL, we observed no
increase in LPS-mediated IL-10 production as the concentration of GM-CSF increased.
Intriguingly, nearly all combinations of M-CSF and GM-CSF resulted in low or
undetectable levels of IL-10 in response to LPS suggesting a non-additive hypersensitivity

to GM-CSF in driving macrophage state as previously observed at the transcriptional level
(Lacey et al., 2012). Taken together, these data highlight the potency of GM-CSF in
modulating macrophage state and underscore the importance of understanding the
additive and non-additive gene and protein expression relationships in myeloid cells.
281
282 **Disruption of elements of the canonical GM-CSF signaling network does not**
283 **enhance IL-10 production in MO-GMCSF cells but scavenging of ROS does.**
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285 Our tiered macrophage differentiation strategy did not identify a cytokine capable of
286 restoring IL-10 production in MO-GMCSF cells. As an alternative strategy to
287 mechanistically dissect the drivers of muted IL-10 production in MO-GMCSF cells, we
288 turned to previously established models for modifying either macrophage phenotypic
289 stability or IL-10 production.
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Previous studies in murine and human macrophages have demonstrated that
macrophages adopt semi-stable phenotypes as demonstrated by surface marker and
marker gene expression (Van den Bossche et al., 2016). In these studies, the authors
implicate mitochondrial dysfunction mediated by inducible nitric oxide synthase (iNOS) as
a driver of limited M1 to M2 repolarization in murine macrophages. We sought to test the
hypothesis that iNOS may play a role in the limited plasticity observed in MO-GMCSF
cells recognizing that expression of iNOS in human macrophages has been widely
debated (Bogdan, 2001; Schneemann and Schoedon, 2002). Nevertheless, we sought to
reproduce the experimental model employed previously to examine functional stability in
MO-GMCSF macrophages. Human monocytes were pre-treated with 1400W, an iNOS
inhibitor, or DMSO for one hour prior to the addition of M-CSF or GM-CSF. After 6 days,
the cells were washed and stimulated with LPS. Production of IL-10 was observed in all
MO-MCSF cells; however, IL-10 remained undetectable in MO-GMCSF cells across all
conditions (**Figure 4A**).
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Salt inducible kinase (SIK) inhibitors were previously identified as potentiators of IL-10
production in macrophages following their differentiation into macrophages (Lombardi et
al., 2016; MacKenzie et al., 2013). We examined the capacity of salt inducible kinase
inhibitor, HG-9-91-01, to enhance IL-10 production in MO-MCSF and MO-GMCSF cells.
MO-MCSF and MO-GMCSF cells were incubated with HG-9-91-01 for 2 hours prior to
the addition of LPS; however, we observed no increase in the production of IL-10 in SIK-
inhibitor treated cells relative to the vehicle control (**Figure 4B**). To test the hypothesis
that the absence of robust IL-10 production in MO-GMCSF cells was mediated by the
activity of epigenetic regulators, we next examined the contribution of Class I and Class
II histone deacetylases by pre-treating the cells with trichostatin A (TSA), an inhibitor of
these enzymes, prior to the addition of GM-CSF. Pre-treatment with 100 nM TSA had no
impact on the capacity of MO-GMCSF cells to produce IL-10 (**Figure 4C**). In parallel, we
sought to interrogate the effects of M-CSF and GM-CSF treatment on chromatin
accessibility through the lens of ATAC-sequencing with a specific focus on the IL-10
locus. IL-10 did not achieve a statistically significant p-value after Bonferroni hypothesis
correction (**Supplemental Table 3**).
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323 Interfering with previously described networks involved in IL-10 production or phenotypic
324 stability in macrophages had minimal impact in enhancing IL-10 production in MO-
325 GMCSF cells.
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We next sought to review our unbiased transcriptomic data for additional insight and
hypotheses for programs that we could perturb in MO-GMCSF cells to restore IL-10
production. In MO-GMCSF cells, we observed statistically significant enrichment of
pathways associated with oxidoreductase signaling including TXN, SOD2, and GLRX
(**Figure 4D**). We next sought to examine the contribution of oxidative signaling in MO-
GMCSF cell function by first pre-treating monocytes with N-acetylcysteine (NAC) for one
hour prior to the addition of M-CSF or GM-CSF. After 6 days, the cells were washed and
stimulated with LPS. As expected, production of IL-10 was observed in all MO-MCSF
cells. Consistent with a model whereby oxidative signaling contributes to the suppression
of IL-10 release in MO-GMCSF cells, pre-treatment of monocytes with NAC prior to the
addition of differentiation cytokines mediated an increase in IL-10 release following LPS
treatment, though not to levels of MO-MCSF cells (**Figure 4E**). Together, these data
demonstrate the utility of unbiased approaches to augment hypothesis generation as well
as underscore the contribution of immunometabolic circuits to macrophage state.
341
342 **Discussion**
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Macrophage states, mostly studied through the lens of M1 and M2 activation, have been
studied extensively both *in vivo* and *in vitro.* Many models have been proposed to
describe the mechanisms that underlie macrophage state in a diverse array of settings
(Lawrence and Natoli, 2011; Murray, 2017). Traditionally, macrophages have long been
considered to be plastic cells that can polarize to distinct states as needed. However,
challenges exist when determining macrophage state and examining phenotypic
transitions in tissues in a cell type such as primary human macrophages. Unlike
lymphocytes, few, if any, robust binary markers exist for cell state determination in primary
human macrophages. Here, we studied a widely utilized model of human macrophages
using high-resolution tools to define novel markers of cell state and examine macrophage
functional stability. Using our systematic approach to examine macrophage state, we
show that MO-GMCSF cells adopt a stable phenotype with respect to IL-10 production
and that this phenotype can be disrupted by the addition of scavengers of reactive oxygen
species.
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We show that conventional surface markers are of limited utility to discern macrophage
state using a binary gating strategy. These results are consistent with countless previous
studies that utilize analog differences in surface protein expression instead of digital
expression as done with lymphocytes (Becher et al., 2014; Roussel et al., 2017; Schulz
et al., 2019). Cytokine profiling in response to TLR ligands helped discriminate LPS-
tolerized macrophages in both MO-MCSF and MO-GMCSF states. Moreover, cytokine
profiling aided in discriminating MO-MCSF and MO-GMCSF cells through the lens of IL-
10 production, consistent with previous studies of MO-MCSF and MO-GMCSF cells
(Lacey et al., 2012; Sander et al., 2017). Here, our work extends upon these previous

studies by examining the temporal dimension of macrophage state and reveals the
striking stability in macrophage state that can be achieved.
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To address challenges in characterizing macrophage state using protein expression, we
conducted scRNAseq of MO-MCSF and MO-GMCSF cells and identified numerous novel
markers of macrophage state. Namely, in MO-GMCSF cells, we identified strong
enrichment of pathways associated with oxidative signaling consistent with emerging
models relating metabolism in driving macrophage state (Cameron et al., 2019; West et
al., 2011). We speculate that this interplay between oxidative signaling and metabolic
state influence a multitude of macrophage functions, such as bacterial clearance and
atherosclerosis, *in vivo* (Di Gioia et al., 2020; Huang et al., 2018; Wang et al., 2020). We
identified and validated several novel markers of macrophage state. We also highlight
shared programs that are active across multiple models of inflammatory macrophages
such as thioredoxin signaling, as well as programs such as ALOX5AP that are specific to
differentiation cue. Previous studies in human macrophages are consistent with our
unbiased study of macrophage gene expression. Studies by Werz and colleagues
similarly observed digital expression of ALOX5AP in MO-GMCSF cells and demonstrate
that this digital protein expression is correlated with digital production of the inflammatory
lipid PGE2 (Werz et al., 2018). Taken together, these data identify novel markers of
macrophage state as well as regulatory axes that should be considered when selecting
in vitro culture models. PGE2 has been previously demonstrated to modulate
macrophage state through the lens of metabolic function and cytokine release highlighting
a critical role for this lipid in immunoregulation (MacKenzie et al., 2013; Sanin et al., 2018).
It is appealing to hypothesize that high levels of PGE2 production and low IL-10
production may contribute to amplification of an inflammatory macrophage state.
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Using IL-10 release as a tool to interrogate functional stability of MO-MCSF and MO-
GMCSF cells. Surprisingly, we observed that MO-MCSF cells retained functional
plasticity with respect to IL-10 production while MO-GMCSF cells did not. Previous
studies in primary human macrophages demonstrate that classical M(LPS+IFN-γ)
macrophages do not upregulate CD206 and CD200R markers upon treatment with IL-4
whereas M-CSF macrophages do upregulate CD206 and CD200R when treated with IL-
4 (Van den Bossche et al., 2016). IL-10 production has traditionally been associated with
anti-inflammatory macrophage states. Our *in vitro* results demonstrate a shared property
among inflammatory macrophages where limited phenotypic transitions from
inflammatory to anti-inflammatory states are possible. Previous studies in M(LPS+IFN-γ)
human macrophages did not identify a strategy to reverse the inflammatory state. Here,
using an unbiased systems-level analysis, we identified oxidative signaling as a driver of
IL-10 suppression. Our results demonstrating an association between GM-CSF signaling
and reactive oxygen species is consistent with several other studies demonstrating a role
for GM-CSF in modulating ROS production in diverse myeloid cell types (Kasahara et al.,
2016; Spath et al., 2017; Subramanian Vignesh et al., 2013). Our protein expression
analysis revealed that thioredoxin was highly expressed in both MO-GMCSF and
M(LPS+IFN-γ) cells suggesting a potentially conserved mechanism whereby oxidative
signaling contributes to inflammatory macrophage phenotypic stability. Previous work has
highlighted the transcriptional regulator BHLHE40 as an essential repressor of IL-10

production in murine cells treated with GM-CSF; however, regulation of BHLHE40
expression in M(LPS+IFN-γ) has not been explored (Huynh et al., 2018). Upregulation of
BHLHE40 has been observed in response to DNA damaging agents, and recent studies
demonstrate that DNA damage mediated by reactive oxygen species is observed in
murine macrophages following the addition of inflammatory agents such as LPS and IFN-
γ (Cameron et al., 2019; Kim et al., 2014). Examining the regulation of BHLHE40
expression and the contribution of BHLHE40 in inflammatory macrophage stability should
be the subject of future inquiry.
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While many *in vitro* macrophage models focus on driving a particular state in a digital
fashion providing a single cytokine in isolation, here, we began to investigate the role of
combinations of cytokines in mediating cell state. Again, even when M-CSF is provided
in excess, we observed that GM-CSF is incredibly potent in mediating IL-10 suppression.
In tissues where GM-CSF is constitutively present like the lungs, it is highly plausible that
GM-CSF may play a dominant role in modulating macrophage function independent of
ontogeny. In fact, previous studies of isolated murine alveolar macrophages show
undetectable or very low levels of IL-10 production in response to TLR ligands (Sajti et
al., 2020; Salez et al., 2000). These data raise the possibility that MO-GMCSF cells may
provide a functionally similar macrophage to those isolated from tissues consistent with
transcriptional analyses performed by Sander; however, additional functional studies are
necessary to more fully characterize axes of *in vitro* and tissue-resident macrophage
state. These data suggest that complex immune environments may contain dominant
soluble mediators of cell state, similar to how GM-CSF drives a low-IL-10 producing state
even at low concentrations. How these relationships scale for other cytokine pairs and
other responses has been partially investigated in the past; here we build on the
experimental designs employed in those studies with a cellular reprogramming model
involving staggered addition of cytokines or TLR ligands and suggest future work to
explore combinatorial signal perception and gene expression manifolds more
exhaustively in macrophages (Natarajan et al., 2006; Xue et al., 2014).
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Since human monocyte-derived macrophages have limited to non-existent replicative
capacity, leveraging the markers identified in our study may allow recovery of cellular
history in a manner akin to fate-mapping experiments conducted in genetically
engineered mice. Future studies should further define the relationship between signal
inputs and the expression of markers identified in our study. We found that GM-CSF was
a driver of ALOX5AP expression; however, it is not known if there are other cytokines that
induce ALOX5AP expression. Similarly, we identified TXN as a marker of MO-GMCSF
cells as well as MO(LPS+IFN-γ) cells. Shared expression of TXN in MO(LPS+IFN-γ) and
MO-GMCSF cells suggests a shared response network, possibly oxidative signaling, and
may serve as an equally valuable marker to inform metabolic history of immune cells.
Lastly, as we continue to decipher the relationship between marker expression and
cytokine stimulation, it will be essential to understand the relationship between signal
duration and magnitude and marker expression to fully exploit the potential of these novel
markers.
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In conclusion, our study demonstrates the phenotypic stability of MO-GMCSF cells by exploring the temporal dimension of macrophage state. Here, we demonstrate using a combination of immune profiling assays with scRNAseq that oxidative signaling contributes to limited IL-10 release in human macrophages. For future work, we propose deeper study of metabolic and oxidative signaling in both MO-GMCSF and other models of inflammatory macrophages to identify shared mechanisms of phenotypic stability.

Methods

PBMC Isolation and Monocyte Differentiation

Deidentified buffy coats from healthy human donors were obtained from Massachusetts General Hospital. PBMCs were isolated from buffy coats by density-based centrifugation using Ficoll (GE Healthcare). CD14+ monocytes were isolated from PBMCs using a CD14 positive-selection kit (Stemcell). Isolated monocytes were differentiated to MO-MCSF or MO-GMCSF in RPMI 1640 (ThermoFisher Scientific) supplemented with 10% heat-inactivated FBS (ThermoFisher Scientific), 1% HEPES, and 1% L-glutamine. Media was further supplemented with either 50 ng/mL M-CSF or 50 ng/mL GM-CSF unless otherwise specified (Biolegend, MCSF: 572902 and GM-CSF: 574802). Monocytes were cultured on low-adhesion tissue culture plates (Corning) for 6 days. Where specified, additional polarization stimuli were added on day 4 (10 ng/mL IFN-γ, 10 ng/mL IL-4, 10 ng/mL IL-6, 10 ng/mL IL-10, and 10 ng/mL LPS) for a total of 6 days in culture. For 6 well dishes, cells were seeded at a density of 0.5e6 cells/mL. For 12- and 96-well dishes, cells were seeded at a density of 0.25e6 cells/mL.

Surface Epitope Staining and Flow Cytometry

Macrophages were detached from low-adhesion tissue culture plates using pre-warmed 1X PBS (Ca2+ and Mg2+ free) (Corning, 21-040-CV) supplemented with 2mM EDTA. Detached macrophages were counted, pelleted at 500xg, and blocked using Human TruStain FcX (Biolegend: 422301) at 2x for 5 min at room temperature. Cells were then stained directly using a pre-mixed antibody panel cocktail for 15 min in the dark at 4° C. Cells were then washed twice with FACS buffer (1X Ca2+ and Mg2+ free PBS, 2% heat-inactivated FBS, and 1mM EDTA) prior to fixation in 2% paraformaldehyde for 15 minutes at room temperature. Following fixation, cells were washed twice with FACS buffer and then analyzed by flow cytometry.

Antibody panels are as follows:
Panel 1: CD11b-BV421 (Biolegend, 301323), CD32-PECy7 (Biolegend, 303213), CD36-APC (Biolegend, 336207), HLA-DR-BV510 (Biolegend, 307645)
Panel 2: CD11b-BV421 (Biolegend, 301323), CD274-PE (Biolegend, 329705), CD86-APC (Biolegend, 305411), CD64-BV510 (Biolegend, 305027)

Additional antibodies utilized in this study included CD36-PE (Biolegend, 336205), CD206-PE (Biolegend, 321105), CD123 (Biolegend, 306005), CD125 (R&D Systems, FAB253P), CD115-BV421 (Biolegend, 347321), CD116-PE (Biolegend, 305908).

505
506 Samples were analyzed on a BD LSR Fortessa.
507
508 **Intracellular Cytokine Staining and Flow Cytometry**
509
MO-MCSF cells were simultaneously treated with Brefeldin A (Biolegend, 420601) and
stimulated with 100 ng/mL LPS for 12 hours. After this incubation, cells were detached
from low-adhesion tissue culture plates using pre-warmed 1X PBS (Ca2+ and Mg2+ free)
(Corning, 21-040-CV) supplemented with 2mM EDTA. Detached macrophages were
counted, pelleted at 500xg, and resuspended in Fixation Buffer (Biolegend, 420801) and
allowed to fix for 20 minutes at room temperature in the dark. Following fixation, cells
were pelleted at 500xg and resuspended in Intracellular Staining Perm Wash Buffer
(Biolegend, 421002). Three total washes in Intracellular Staining Perm Wash Buffer were
performed. Following permeabilization, cells were pelleted for 5 minutes at 500xg and
resuspended in 50 µL Intracellular Staining Perm Wash Buffer supplemented with 5 µL
of TruStain FcX (Biolegend, 422302) for 10 minutes at room temperature. Then, 50 µL
Intracellular Staining Perm Wash Buffer supplemented with 5 µL of IL-10-BV421 antibody
(Biolegend, 501421) was added for an additional 20 minutes at room temperature.
Following antibody incubation, cells were pelleted for 5 minutes at 500xg and washed
twice with Intracellular Staining Perm Wash Buffer prior to resuspension in FACS buffer
(1X Ca2+ and Mg2+ free PBS, 2% heat inactivated FBS, and 1mM EDTA) prior to
analysis on a BD LSR Fortessa.
527
528 **Luminex**
529
MO-GMCSF or MO-MCSF cells were treated for 16 hours with either 100 ng/mL LPS or
100 ng/mL Pam3CSK4 prior to analysis of cytokine release. Multiplexed cytokine
measurements on macrophage cell culture supernatants were performed using a custom
Bio-Plex Express Human Cytokine kit (Bio-Rad 17004073) according to manufacturer's
instructions, using a Bio-Plex 3D Suspension Array System (Bio-Rad). Measured
cytokines included IL-6, IFN-γ, GM-CSF, TNFα, IL-1β, IP-10 (CXCL10), MCP-1 (CCL-2),
IL-8, IL-10, and IL-12p70. Supernatants were stored at -80°C until analysis.
537
538 **Enzyme-linked Immunosorbent Assays (ELISA)**
539
540 0.25e6 cells were plated per well of a 12 well dish for cytokine release assays. Cells were
541 stimulated with either 100 ng/mL LPS or 100 ng/mL Pam3Csk4 for 16 hours.
542 Supernatants were analyzed fresh or stored at -80°C until analysis. Cytokine release was
543 measured by ELISA using TNFα, IL-6, and IL-10 ELISA kits (Biolegend: TNFα 430201,
544 IL-6 430501, and IL-10 430601).
545
546 **Single Cell RNA Sequencing**
547
548 We performed SeqWell analysis as previously described (Gierahn et al., 2017). Briefly,
549 beads were loaded into the PDMS array followed by subsequent addition of ~12,000 cells.

550 After periodic gentle rocking and washing, a polycarbonate membrane was attached to
551 the array surface.
552
553 After membrane attachment, the array was transferred to a dish containing cell lysis
554 buffer.   After lysis, buffer was exchanged for hybridization buffer to enable RNA
555 hybridization to barcoded beads. Beads were removed from microwell arrays by scraping
556 the array with a microscope slide.
557
558 Recovered beads were transferred to a 1.5 mL tube and reverse transcription was
559 performed using Maxima H- reverse transcriptase (Invitrogen). Beads were then treated
560 with ExoI to digest unoccupied primer sites (NEB).
561
562 cDNA was amplified and the pooled PCR library was purified, and tagmentation was
563 subsequently performed. Libraries were sequenced on an Illumina NextSeq500. 20 bases
564 were allocated (12 bp cell barcode and 8 bp UMI) for Read 1, which was primed using
565 Custom Read 1 Primer.
566
567 **Transcriptome Alignment**
568
Read alignment was performed as in described previously (Macosko et al., 2015). Briefly,
for each NextSeq sequencing run, raw sequencing data was converted to FASTQ files
using bcl2fastq2 that were demultiplexed by Nextera N700 indices corresponding to
individual samples. Reads were first aligned to HgRC19 and individual reads were tagged
according to the 12-bp barcode sequence and the 8-bp UMI contained in read 1 of each
fragment. Following alignment reads were binned and collapsed onto 12-bp cell barcodes
that   correspond   to   individual   beads   using   DropSeq   tools
(http://mccarrolllab.com/dropseq/). Barcodes were collapsed with a single-base error
tolerance (Hamming distance =1) with additional provision for single insertions or
deletions. An identical collapsing scheme (Hamming distance = 1) was then applied to
unique molecular identifiers to obtain quantitative counts of individual mRNA molecules.
580
581 **Single Cell RNAseq Data Pre-Processing**
582
We identified a total of 1,742 good-quality GM-CSF-macrophages (donor 1: 399, donor
2: 1,343) and 4,803 good-quality M-CSF-treated cells (donor 1: 1,941, donor 2: 2,862)
with total transcripts >2,500 and <75,000 and mitochondrial read percentage less than
20%. After filtering based on quality control metrics, raw count matrices were normalized
by library size. For each cell, UMI-collapsed gene expression counts were divided by total
transcript counts for that cell, multiplied by a scale factor of 10,000, then natural logarithm-
normalized using Seurat v3.0.2. Log-transformed expression levels were standard scaled
using Seurat's ScaleData function. During scaling, technical covariates (percentage
mitochondrial reads and number of UMIs) were regressed out using a generalized linear
model, as implemented in Seurat, and the residuals of the generalized linear model were
used as 'corrected' gene expression values for downstream scaling.
594
Differential Expression Analysis

Differential gene expression between MO-MCSF and MO-GMCSF macrophages was
assessed by Wilcoxon and AUC tests using Seurat's FindMarkers function and log-
transformed expression levels [ln(TPM+1)]. Genes with absolute log2 fold change > 0.25
and Bonferroni-corrected p-values < 0.05 were considered differentially expressed genes
(DEGs). DEGs with AUC < 0.7 or AUC > 0.3 were selected as markers of MO-GMCSF
and MO-MCSF macrophages, respectively. Differential expression analyses were
conducted on individual donors separately, as well as on all cells together.
603
Gene Ontology Enrichment Analysis
Gene symbols were mapped to Entrez gene ids using org.Hs.eg.db annotations. GO
enrichment was analyzed by hypergeometric test using the clusterProfiler package's
enrichGO function against a background set of genes captured in all sequencing runs
with > 100 total counts across runs. GO terms with Benjamini-Hochberg-correct p-values
< 0.05 were considered significantly enriched. Significant GO terms were collapsed into
representative GO terms using clusterProfiler's simplify function and a similarity threshold
of 0.7.
612
613 **Data availability**
614
615 All RNA-seq data are available in GEO under accession number XXXX. Code needed to
616 complete the RNA-seq analysis can be found on github.com/cgunnars/mcsf-gmcsf-sc
617
618 **Western Blotting**
619
Macrophages were lysed using RIPA buffer with Proteinase Inhibitor. Protein
concentration was estimated using a BCA assay. 15ug of protein were loaded per sample
in a Bolt 4-12% Bis-Tris Plus Gel. PageRuler Plus was used as a molecular weight ladder.
The gel was transferred to a nitrocellulose membrane using the iBlot 2 dry blotting system
(Invitrogen). Membranes were blocked for 1 hour using Odyssey blocking buffer at room
temperature. Membranes were next incubated in primary antibody shaking overnight at
4C. TRX (Santa Cruz, sc-166393), β-actin (Biolegend, Poly6221), ALOX5AP (GeneTex,
GTX106689), and β-actin (Santa Cruz, sc-47778) were diluted with TRIS buffered saline
containing Tween-20 (TBST). After incubation with the primary antibody, membranes
were washed three times with TBST and then incubated with a fluorescent secondary
antibody (LiCor IRDye 680 or LiCor IRDye 800) at a dilution of 1:10,000 in TBST for 1
hour. The membranes were then washed three times with TBST. The western blots were
imaged by Odyssey CLx.
633
634 **STAT3 Phosphorylation Analysis**
635
MO-MCSF or MO-GMCSF cells were washed once with PBS following 6 days of
differentiation, and then stimulated with media containing vehicle or media containing 10
or 50 ng/mL IL-10 for 15 minutes. Following incubation with IL-10, cells were washed
once with cold PBS and lysed immediately on ice. Western blotting was performed as
described above. Membranes were incubated with either a total STAT3 antibody (Cell

Signaling Technology, Clone: 79D7) or a phosphorylated STAT3 antibody (Cell Signaling
Technology, Clone: D3A7).
643
644 **Macrophage Durability Assay**
645
To evaluate the stability of TLR-mediated IL-10 release by macrophages, following 6 days
of differentiation with M-CSF or GM-CSF, MO-MCSF cells or MO-GMCSF cells were
washed with pre-warmed media without cytokines and then incubated with media
supplemented with 50 ng/mL M-CSF, 50 ng/mL GM-CSF or no cytokine. Following 3 days
of incubation, cells were washed with PBS and then stimulated with 100 ng/mL LPS for
16 hours prior to quantification of IL-10 release by ELISA.
652
653 **GM-CSF + M-CSF Checkerboard Assay**
654
For the GM-CSF/M-CSF checkerboard assay, a range of concentrations of GM-CSF and
M-CSF (0, 0.5 ng/mL, 5 ng/mL, and 50 ng/mL) were premixed in a pairwise fashion prior
to the addition of monocytes. Monocytes were then added to the individual wells to
commence differentiation. Monocytes were allowed to differentiate for 6 days. After 6
days, cells were washed twice with PBS and incubated with maintenance media with or
without 100 ng/mL LPS.
661
662 **GM-CSF and Drug Perturbation Experiments**
663
To evaluate the impact of inhibition of iNOS on IL-10 production in MO-MCSF and MO-
GMCSF cells, monocytes were pretreated with 1400W, an iNOS inhibitor, at a final
concentration of 100 µM for 1 hour prior to the addition of 50 ng/mL GM-CSF or 50 ng/mL
M-CSF. 1400W was dissolved in DMSO. After 6 days of differentiation, cells were washed
and then treated with 100 ng/mL LPS for 16 hours. Cytokine release was compared to
cells treated with DMSO alone (0.1% v/v final concentration). Supernatants were
analyzed fresh or stored at -80°C until analysis.
671
To evaluate the capacity of salt-inducible kinase inhibitor or PGE2 treatment on
production of IL-10 by MO-MCSF cells, cells were pre-treated with DMSO (0.1% v/v), 250
or 500 nM HG-9-91-01 (VWR, dissolved in DMSO, final concentration 0.1% v/v DMSO),
or 5 or 10 µM PGE2 (Santa Cruz Biotechnology) for two hours prior to treatment with
LPS. Without washing, cells were then treated with 100 ng/mL LPS for 16 hours prior to
analysis by ELISA.
678
To evaluate the impact of inhibiting class I and II histone deacetylases, monocytes were
pretreated with 100 nM trichostatin A (TSA, Sigma Aldrich). Monocytes were pre-treated
for 1 hour prior to the addition of 50 ng/mL GM-CSF or 50 ng/mL M-CSF to induce
differentiation. After 6 days of differentiation, cells were washed and then treated with 100
ng/mL LPS for 16 hours. Supernatants were analyzed fresh or stored at -80°C until
analysis.
685

To evaluate the impact of scavenging oxidative radicals prior to differentiation, monocytes
were pretreated with 5 mM N-acetylcysteine (NAC) which was dissolved directly in cell
culture media and filtered through 0.2 µm filter. Monocytes were pre-treated for 1 hour
prior to the addition of 50 ng/mL GM-CSF or 50 ng/mL M-CSF to induce differentiation.
After 6 days of differentiation, cells were washed and then treated with 100 ng/mL LPS
for 16 hours. Supernatants were analyzed fresh or stored at -80°C until analysis.
692
693   **ATAC Sequencing and Analysis**
694
MO-GMCSF and MO-MCSF cells were prepared for ATAC sequencing as previously
described (Buenrostro et al., 2015). Briefly, cells were detached and washed twice in PBS
and diluted to a final concentration of 1e6 cells/mL. 5,000 cells were then mixed with TD
buffer, 10% NP-40, 20x Illumina Tn5, and water. The reaction was allowed to proceed for
30 mins with shaking at 37°C with mixing. Transposed DNA is isolated using a PCR
purification kit (Qiagen) and eluted. 5 cycles of PCR were performed on the transposed
product with indexing primers and qPCR was performed on 5 µL of the PCR product to
identify the optimal number of total cycles for each sample. The remaining PCR mix was
stored on ice until the optimal number of additional cycles was identified. The amplified
PCR product was purified using a PCR purification kit (Qiagen), and DNA concentration
was determined using a PhiX standard curve. DNA from each sample was pooled at
approximately equal input and sequenced on an Illumina NextSeq 500.
707
708   Raw sequencing data was converted to FASTQ files using bcl2fastq2 that were
709   demultiplexed according to ATACseq sample indices corresponding to individual
710   samples. Reads were aligned to GRCh38.
711
Chromatin accessibility near transcriptional start sites was analyzed following the
standards set forth by the Encode Consortium (Buenrostro et al., 2015). Briefly, scores
were obtained by aligning ATAC reads to genome and performing peak calling and next
examining peak summit heights at +/- 1kb around the transcriptional start site (TSS) of
each gene, then averaging peak scores with a linearly decreasing weight with increasing
distance to TSS.
718
719

References

Becher, B., Schlitzer, A., Chen, J., Mair, F., Sumatoh, H.R., Teng, K.W.W., Low, D., Ruedl, C., Riccardi-Castagnoli, P., Poidinger, M., et al. (2014). High-dimensional analysis of the murine myeloid cell system. Nat. Immunol. *15*, 1181–1189.

Biswas, S.K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat. Immunol. *11*, 889–896.

Bogdan, C. (2001). Nitric oxide and the immune response. Nat. Immunol. *2*, 907–916.

Buenrostro, J., Wu, B., Chang, H., and Greenleaf, W. (2015). ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. Curr. Protoc. Mol. Biol. Ed. Frederick M Ausubel Al *109*, 21.29.1-21.29.9.

Cameron, A.M., Castoldi, A., Sanin, D.E., Flachsmann, L.J., Field, C.S., Puleston, D.J., Kyle, R.L., Patterson, A.E., Hässler, F., Buescher, J.M., et al. (2019). Inflammatory macrophage dependence on NAD + salvage is a consequence of reactive oxygen species–mediated DNA damage. Nat. Immunol. *20*, 420.

Di Gioia, M., Spreafico, R., Springstead, J.R., Mendelson, M.M., Joehanes, R., Levy, D., and Zanoni, I. (2020). Endogenous oxidized phospholipids reprogram cellular metabolism and boost hyperinflammation. Nat. Immunol. *21*, 42–53.

DuPage, M., and Bluestone, J.A. (2016). Harnessing the plasticity of CD4 + T cells to treat immune-mediated disease. Nat. Rev. Immunol. *16*, 149–163.

Fleetwood, A.J., Lawrence, T., Hamilton, J.A., and Cook, A.D. (2007). Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. J. Immunol. Baltim. Md 1950 *178*, 5245–5252.

Gierahn, T.M., Wadsworth Ii, M.H., Hughes, T.K., Bryson, B.D., Butler, A., Satija, R., Fortune, S., Love, J.C., and Shalek, A.K. (2017). Seq-Well: portable, low-cost RNA sequencing of single cells at high throughput. Nat. Methods *14*, 395–398.

Hamilton, J.A. (2019). GM-CSF-Dependent Inflammatory Pathways. Front. Immunol. *10*.

Huang, L., Nazarova, E.V., Tan, S., Liu, Y., and Russell, D.G. (2018). Growth of Mycobacterium tuberculosis in vivo segregates with host macrophage metabolism and ontogeny. J. Exp. Med. *215*, 1135–1152.

Huynh, J.P., Lin, C.-C., Kimmey, J.M., Jarjour, N.N., Schwarzkopf, E.A., Bradstreet, T.R., Shchukina, I., Shpynov, O., Weaver, C.T., Taneja, R., et al. (2018). Bhlhe40 is an essential repressor of IL-10 during Mycobacterium tuberculosis infection. J. Exp. Med. *215*, 1823–1838.

Kasahara, S., Jhingran, A., Dhingra, S., Salem, A., Cramer, R.A., and Hohl, T.M. (2016). Role of Granulocyte-Macrophage Colony-Stimulating Factor Signaling in Regulating Neutrophil Antifungal Activity and the Oxidative Burst During Respiratory Fungal Challenge. J. Infect. Dis. *213*, 1289–1298.

Kim, J., D'Annibale, S., Magliozzi, R., Low, T.Y., Jansen, P., Shaltiel, I.A., Mohammed, S., Heck, A.J.R., Medema, R.H., and Guardavaccaro, D. (2014). USP17- and SCFβTrCP-Regulated Degradation of DEC1 Controls the DNA Damage Response. Mol. Cell. Biol. *34*, 4177–4185.

Lacey, D.C., Achuthan, A., Fleetwood, A.J., Dinh, H., Roiniotis, J., Scholz, G.M., Chang, M.W., Beckman, S.K., Cook, A.D., and Hamilton, J.A. (2012). Defining GM-CSF– and Macrophage-CSF–Dependent Macrophage Responses by In Vitro Models. J. Immunol. *188*, 5752–5765.

Lavin, Y., Winter, D., Blecher-Gonen, R., David, E., Keren-Shaul, H., Merad, M., Jung, S., and Amit, I. (2014). Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment. Cell *159*, 1312–1326.

Lawrence, T., and Natoli, G. (2011). Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat. Rev. Immunol. *11*, 750–761.

Lee, S., Kim, S.M., and Lee, R.T. (2013). Thioredoxin and thioredoxin target proteins: from molecular mechanisms to functional significance. Antioxid. Redox Signal. *18*, 1165–1207.

Liu, S.X., Gustafson, H.H., Jackson, D.L., Pun, S.H., and Trapnell, C. (2020). Trajectory analysis quantifies transcriptional plasticity during macrophage polarization. Sci. Rep. *10*.

Liu, Z., Gu, Y., Chakarov, S., Bleriot, C., Kwok, I., Chen, X., Shin, A., Huang, W., Dress, R.J., Dutertre, C.-A., et al. (2019). Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells. Cell *178*, 1509-1525.e19.

Locati, M., Curtale, G., and Mantovani, A. (2020). Diversity, Mechanisms, and Significance of Macrophage Plasticity. Annu. Rev. Pathol. Mech. Dis. *15*, 123–147.

Lombardi, M.S., Gilliéron, C., Dietrich, D., and Gabay, C. (2016). SIK inhibition in human myeloid cells modulates TLR and IL-1R signaling and induces an anti-inflammatory phenotype. J. Leukoc. Biol. *99*, 711–721.

MacKenzie, K.F., Clark, K., Naqvi, S., McGuire, V.A., Nöehren, G., Kristariyanto, Y., Bosch, M. van den, Mudaliar, M., McCarthy, P.C., Pattison, M.J., et al. (2013). PGE2 Induces Macrophage IL-10 Production and a Regulatory-like Phenotype via a Protein Kinase A–SIK–CRTC3 Pathway. J. Immunol. *190*, 565–577.

Macosko, E.Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I.,
Bialas, A.R., Kamitaki, N., Martersteck, E.M., et al. (2015). Highly Parallel Genome-wide
Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell *161*, 1202–1214.

Murray, P.J. (2017). Macrophage Polarization. Annu. Rev. Physiol. *79*, 541–566.

Murray, P.J., Allen, J.E., Biswas, S.K., Fisher, E.A., Gilroy, D.W., Goerdt, S., Gordon,
S., Hamilton, J.A., Ivashkiv, L.B., Lawrence, T., et al. (2014). Macrophage Activation
and Polarization: Nomenclature and Experimental Guidelines. Immunity *41*, 14–20.

Natarajan, M., Lin, K.-M., Hsueh, R.C., Sternweis, P.C., and Ranganathan, R. (2006). A
global analysis of cross-talk in a mammalian cellular signalling network. Nat. Cell Biol. *8*,
571–580.

Niemand, C., Nimmesgern, A., Haan, S., Fischer, P., Schaper, F., Rossaint, R.,
Heinrich, P.C., and Müller-Newen, G. (2003). Activation of STAT3 by IL-6 and IL-10 in
Primary Human Macrophages Is Differentially Modulated by Suppressor of Cytokine
Signaling 3. J. Immunol. *170*, 3263–3272.

Plugis, N.M., Weng, N., Zhao, Q., Palanski, B.A., Maecker, H.T., Habtezion, A., and
Khosla, C. (2018). Interleukin 4 is inactivated via selective disulfide-bond reduction by
extracellular thioredoxin. Proc. Natl. Acad. Sci. 201805288.

Roussel, M., Ferrell, P.B., Greenplate, A.R., Lhomme, F., Gallou, S.L., Diggins, K.E.,
Johnson, D.B., and Irish, J.M. (2017). Mass cytometry deep phenotyping of human
mononuclear phagocytes and myeloid-derived suppressor cells from human blood and
bone marrow. J. Leukoc. Biol. *102*, 437–447.

Sajti, E., Link, V.M., Ouyang, Z., Spann, N.J., Westin, E., Romanoski, C.E., Fonseca,
G.J., Prince, L.S., and Glass, C.K. (2020). Transcriptomic and epigenetic mechanisms
underlying myeloid diversity in the lung. Nat. Immunol. *21*, 221–231.

Salez, L., Singer, M., Balloy, V., Créminon, C., and Chignard, M. (2000). Lack of IL-10
synthesis by murine alveolar macrophages upon lipopolysaccharide exposure.
Comparison with peritoneal macrophages. J. Leukoc. Biol. *67*, 545–552.

Sander, J., Schmidt, S.V., Cirovic, B., McGovern, N., Papantonopoulou, O., Hardt, A.-L.,
Aschenbrenner, A.C., Kreer, C., Quast, T., Xu, A.M., et al. (2017). Cellular
Differentiation of Human Monocytes Is Regulated by Time-Dependent Interleukin-4
Signaling and the Transcriptional Regulator NCOR2. Immunity *47*, 1051-1066.e12.

Sanin, D.E., Matsushita, M., Klein Geltink, R.I., Grzes, K.M., van Teijlingen Bakker, N.,
Corrado, M., Kabat, A.M., Buck, M.D., Qiu, J., Lawless, S.J., et al. (2018). Mitochondrial
Membrane Potential Regulates Nuclear Gene Expression in Macrophages Exposed to
Prostaglandin E2. Immunity *49*, 1021-1033.e6.

Schneemann, M., and Schoedon, G. (2002). Species differences in macrophage NO
production are important. Nat. Immunol. *3*, 102–102.

Schulz, D., Severin, Y., Zanotelli, V.R.T., and Bodenmiller, B. (2019). In-Depth
Characterization of Monocyte-Derived Macrophages using a Mass Cytometry-Based
Phagocytosis Assay. Sci. Rep. *9*, 1–12.

Spath, S., Komuczki, J., Hermann, M., Pelczar, P., Mair, F., Schreiner, B., and Becher,
B. (2017). Dysregulation of the Cytokine GM-CSF Induces Spontaneous Phagocyte
Invasion and Immunopathology in the Central Nervous System. Immunity *46*, 245–260.

Subramanian Vignesh, K., Landero Figueroa, J.A., Porollo, A., Caruso, J.A., and
Deepe, G.S. (2013). Granulocyte Macrophage-Colony Stimulating Factor Induced Zn
Sequestration Enhances Macrophage Superoxide and Limits Intracellular Pathogen
Survival. Immunity *39*, 697–710.

Van den Bossche, J., Baardman, J., Otto, N.A., van der Velden, S., Neele, A.E.,
van den Berg, S.M., Luque-Martin, R., Chen, H.-J., Boshuizen, M.C.S., Ahmed, M., et
al. (2016). Mitochondrial Dysfunction Prevents Repolarization of Inflammatory
Macrophages. Cell Rep. *17*, 684–696.

Wang, Y., Ji, N., Gong, X., Ni, S., Xu, L., and Zhang, H. (2020). Thioredoxin-1
attenuates atherosclerosis development through inhibiting NLRP3 inflammasome.
Endocrine.

Werz, O., Gerstmeier, J., Libreros, S., Rosa, X.D. la, Werner, M., Norris, P.C., Chiang,
N., and Serhan, C.N. (2018). Human macrophages differentially produce specific
resolvin or leukotriene signals that depend on bacterial pathogenicity. Nat. Commun. *9*,
1–12.

West, A.P., Brodsky, I.E., Rahner, C., Woo, D.K., Erdjument-Bromage, H., Tempst, P.,
Walsh, M.C., Choi, Y., Shadel, G.S., and Ghosh, S. (2011). TLR signalling augments
macrophage bactericidal activity through mitochondrial ROS. Nature *472*, 476–480.

Xue, J., Schmidt, S.V., Sander, J., Draffehn, A., Krebs, W., Quester, I., De Nardo, D.,
Gohel, T.D., Emde, M., Schmidleithner, L., et al. (2014). Transcriptome-Based Network
Analysis Reveals a Spectrum Model of Human Macrophage Activation. Immunity *40*,
274–288.

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Author Information

Contributions

CYI, CG, GHB, AN, NAA, MHW, TKH, SLS, BH, BB, AKS, SMF, and BDB conceived and performed experiments. CYI, CG, GHB, AN, NAA, MHW, TKH, SLS, BH, BB, AKS, SMF, and BDB all performed data analysis. All authors contributed to writing of the manuscript and analysis of experimental results.

Competing Interests

A.K.S. reports compensation for consulting and/or SAB membership from Merck, Honeycomb Biotechnologies, Cellarity, Repertoire Immune Medicines, Orche Bio, and Dahlia Biosciences.

Figure Legends

Figure 1: Multi-dimensional profiling of human monocyte-derived macrophages identifies differentiation and activation-mediated phenotypes.

(A) Methodological workflow for generation of macrophage libraries from CD14+ monocytes from healthy donors. Monocytes were differentiated with M-CSF or GM-CSF for 3 days (differentiation only phase) prior to the addition of additional ligands (PBS, LPS, IFN-γ, IL-4, IL-6, and IL-10) for an additional 3 days (activation phase). After 3 days in the activation phase, macrophages were washed with PBS, detached, counted, and re-plated equally to characterize macrophage phenotype.

(B) Analysis of macrophage surface marker expression using a digital gating strategy to identify the percentage of macrophages expressing a given marker (MO-MCSF cells). Representative of 3 donors.

(C) Analysis of macrophage surface marker expression using an analog quantification strategy (mean fluorescence intensity, MFI) to quantify the abundance of a given marker on the macrophage surface (MO-MCSF cells). Representative of 3 donors.

(D) Quantification of CD274 across multiple donors reveals upregulation of CD274 following IFN-γ or LPS stimulation (MO-MCSF cells). Each colored line is an individual donor. Comparisons made using a ratio paired t test. (*, $p < 0.05$).

(E) Z-score of cytokine secretion screen in MO-MCSF or MO-GMCSF cells stimulated with media, 100 ng/mL LPS or 100 ng/mL Pam3Csk4 (P3C).

(F) Secretion of IL-10 across all 12 macrophage populations following 100 ng/mL LPS or 100 ng/mL P3C stimulation. (****, $p < 0.0001$, ***, $p < 0.001$, **, $p < 0.01$, ns = not significant). Representative of 3 donors.

(G) Secretion of TNFα across all 6 MO-GMCSF populations following 100 ng/L LPS or 100 ng/mL P3C stimulation. (****, $p < 0.0001$, ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$, ns = not significant). Representative of 3 donors.

(H) Phosphorylation of STAT3 in MO-GMCSF cells following IL-10 stimulation.

Figure 2: Single cell RNA sequencing reveals characteristics of macrophage state.

(A) Log-normalized expression of canonical marker genes in 1,742 MO-GMCSF and 4,803 MO-MCSF from 2 donors (N = 399 MO-GMCSF- and 1,941 MO-M-CSF donor 1; 1,343 MO-GMCSF- and 2,862 MO-MCSF-treated cells, donor 2). Center line, median; box limits, first and third quartile; whiskers, +/- 1.5 interquartile range; dots, individual cells. G = MO-GMCSF-treated cells; M = MO-MCSF-treated cells.

933 (B) Standard scaled expression of marker genes identified in 250 randomly subsampled
934 cells from each donor and condition. M_A = M-CSF-treated, donor A; M_B = M-CSF-treated,
935 donor 2; G_A = GM-CSF-treated, donor A; G_B = GM-CSF-treated, donor B.
936
937 (C) Log-normalized expression of top marker genes identified in (B) across all cells. (AUC,
938 TXN: 0.845, ALOX5AP: 0.808, CHI3L1: 0.222, MGLL: 0.225)
939
(D) Significantly enriched gene ontology terms in marker gene set for MO-GMCSF- cells
(left) and M-CSF-treated cells (right), determined by hypergeometric test after filtering by
semantic similarity (Benjamini-Hochberg-corrected p-value < 0.05; semantic similarity
threshold = 0.5). GO term enrichment in the marker set was calculated relative to
enrichment in the background set of genes captured in all sequencing runs with >100
total counts across runs.
946
947 Western blotting of cell lysates treated with 50 ng/mL M-CSF, 50 ng/mL GM-CSF, or 10
948 ng/mL each of IFN-γ and LPS (Y+L) for 24 h for thioredoxin (E) and 5-lipoxygenase (F).
949 Beta-actin was used as a loading control. Representative of 4 donors.
950
951 (G) Analysis of CD36 expression by flow cytometry in MO-MCSF or MO-GMCSF cells.
952 Representative of 3 donors.
953
954 (H) Analysis of CD206 expression by flow cytometry in MO-MCSF or MO-GMCSF cells.
955 Representative of 3 donors.
956
957 **Figure 3: Combinatorial treatment of monocytes or MO-MCSF or MO-GMCSF cells**
958 **with GM-CSF or M-CSF reveals unexpected features of the GM-CSF-IL-10 circuit.**
959
960 (A) Analysis of GM-CSF (CD116) or M-CSF (CD115) receptor surface expression by flow
961 cytometry in MO-MCSF or MO-GMCSF cells. Representative of 3 donors.
962
963 (B) Design of IL-10 phenotypic stability experiment in MO-MCSF or MO-GMCSF cells.
964
965 (C) Secretion of IL-10 across polarized or repolarized MO-MCSF and MO-GMCSF cells
966 following 100 ng/mL LPS stimulation. (****, p < 0.0001, ***, p < 0.001, **, p < 0.01, ns =
967 not significant). Representative of 3 donors.
968
969 (D) Design of GM-CSF/M-CSF titration checkerboard experiment.
970
971 (E) Secretion of IL-10 across monocytes differentiated with various combinations of GM-
972 CSF or M-CSF following 100 ng/mL LPS stimulation. Representative of 3 donors.
973
974 **Figure 4: Interference with inducible nitric oxide synthase or salt-inducible kinase**
975 **activity does not enhance IL-10 production while scavenging oxidative radicals**
976 **with N-acetylcysteine in MO-GMCSF cells does.**
977

978   (A) Secretion of IL-10 in MO-GMCSF or MO-MCSF cells following incubation with 1400W,
979   an iNOS inhibitor, and treatment with 100 ng/mL LPS stimulation. (****, $p < 0.0001$, ns =
980   not significant). Representative of 3 donors.
981
982   (B) Secretion of IL-10 in MO-MCSF cells following incubation with HG-9-91-01, a salt-
983   inducible kinase inhibitor, and treatment with 100 ng/mL LPS stimulation. (****, $p <$
984   0.0001, ***, $p < 0.001$, **, $p < 0.01$). Representative of 3 donors.
985
986   (C) Secretion of IL-10 in MO-GMCSF cells that were pre-treated with 100 nM trichostatin-
987   A (TSA) or vehicle control prior to 6-day differentiation and next stimulated with 100 ng/mL
988   LPS for 16 hours. (****, $p < 0.0001$, ns = not significant). Representative of 2 donors.
989
990   (D) Log-normalized expression of genes associated with oxidative signaling in MO-MCSF
991   or MO-GMCSF cells.
992
993   (E) Secretion of IL-10 in MO-GMCSF or MO-MCSF cells that were pre-treated with 5 mM
994   N-acetylcysteine prior to 6-day differentiation and next stimulated 100 ng/mL LPS for 16
995   hours. (***, $p < 0.001$, *, $p < 0.05$, ns = not significant). Representative of 3 donors.

Supplemental Figure Legends

Supplemental Figure 1: Analysis of macrophage surface marker expression using a digital gating strategy to identify the percentage of macrophages expressing a given marker (MO-GMCSF cells). Representative of 2 donors.

Supplemental Figure 2: Analysis of macrophage surface marker expression using an analog quantification strategy (mean fluorescence intensity, MFI) to quantify the abundance of a given marker on the macrophage surface (MO-GMCSF cells). Representative of 2 donors.

Supplemental Figure 3: Quantification of CD274 across multiple donors reveals upregulation of CD274 following IFN-γ or LPS stimulation (MO-GMCSF cells). Each colored line is an individual donor.

Supplemental Figure 4: Analysis of IL-10 production in MO-MCSF cells following LPS stimulation using intracellular cytokine staining and flow cytometry. Production of IL-10 following 100 ng/mL LPS stimulation was monitored using intracellular cytokine staining.

Supplemental Figure 5: Read mapping quality in samples. Violin plots depict number of transcripts (A), genes (B), and percentage of reads aligning to mitochondrial genes (C), separated by GM-CSF and M-CSF treatment and donor.

Supplemental Table 1: Differential expression of all genes captured in scRNAseq analysis of MO-GMCSF and MO-MCSF cells. The AUC column presents the results of the area under the curve analysis used to identify marker genes for MO-GMCSF and MO-MCSF cells. The avg_log2FC column presents the log2(fold change) in gene expression for MO-GMCSF cells relative to MO-MCSF cells. The power column represents the classification power of a particular gene. The pct.g column indicates the percentage of MO-GMCSF cells expressing a particular gene. The pct.m column indicates the percentage of MO-MCSF cells expressing a particular gene. The p_val column represents the p-value of differential gene expression between MO-GMCSF and MO-MCSF cells as determined by Wilcoxon rank sums test. The p_val_adj column represents the p-value of differential gene expression between MO-GMCSF and MO-MCSF cells following Benjamini-Hochberg correction.

Supplemental Table 2: Gene ontology analysis of marker genes from analysis of MO-GMCSF and MO-MCSF cells by scRNAseq. The ID column represents the ID of the gene ontology term. The Description column describes and summarizes the gene ontology term. The GeneRatio column captures the number of significant marker genes belonging to a particular gene ontology category out of all the significant marker genes for MO-GMCSF or MO-MCSF cells. The BgRatio column captures the number of captured genes belonging to a particular gene ontology category out of all captured genes for MO-GMCSF or MO-MCSF cells. The background set of genes was constructed by identifying genes captured in all sequencing runs with >100 total counts across runs. The

pvalue column captures the results of the hypergeometric test. The p.adjust column is
after filtering gene ontology terms by semantic similarity (Benjamini-Hochberg-corrected
p-value < 0.05; semantic similarity threshold = 0.5). The qvalue column was determined
using a false-discovery rate approach. The geneID column captures the gene names of
those genes belonging to the particular Gene Ontology term. The Count column
enumerates the number of significant marker genes falling into a particular Gene
Ontology category.
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Supplemental Table 3: Chromatin accessibility scores of MO-MCSF and MO-
GMCSF cells. The Gene column represents the gene ID. The GM-CSF Score column
summarizes the chromatin accessibility scores for all 6 MO-GMCSF ATACseq samples.
The M-CSF Score column summarizes the chromatin accessibility scores for all 6 MO-
MCSF ATACseq samples. The Mean GM-CSF Score column presents the mean
chromatin accessibility score for all 6 MO-GMCSF samples. The Mean GM-CSF Score
column presents the mean chromatin accessibility score for all 6 MO-GMCSF samples.
The Mean M-CSF Score column presents the mean chromatin accessibility score for all
6 MO-MCSF samples. The Uncorrected p-value column is the result of a t-test for each
gene.
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Figure 1

Figure 2

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Figure 4