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*J Immunol* 2015; 195:2442-2451; Prepublished online 24 July 2015;
doi: 10.4049/jimmunol.1403045
http://www.jimmunol.org/content/195/5/2442

Supplementary Material

http://www.jimmunol.org/content/suppl/2015/07/23/jimmunol.1403045.DCSupplemental

References

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Reshaping of Human Macrophage Polarization through Modulation of Glucose Catabolic Pathways

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Macrophages integrate information from the tissue microenvironment and adjust their effector functions according to the prevalent extracellular stimuli. Therefore, macrophages can acquire a variety of activation (polarization) states, and this functional plasticity allows the adequate induction, regulation, and resolution of inflammatory responses. Modulation of the glucose metabolism contributes to the macrophage adaptation to the surrounding cytokine milieu, as exemplified by the distinct glucose catabolism of macrophages exposed to LPS/IFN-γ or IL-4. To dissect the acquisition of macrophage effector functions in the absence of activating cytokines, we assessed the bioenergetic profile of macrophages generated in the presence of GM-CSF (GM-MØ) or M-CSF (M-MØ), which do not release pro- or anti-inflammatory cytokines unless subjected to additional activating stimuli. Compared to M-MØ, GM-MØ displayed higher oxygen consumption rate and aerobic glycolysis (extracellular acidification rate [ECAR]), as well as higher expression of genes encoding glycolytic enzymes. However, M-MØ exhibited a significantly higher oxygen consumption rate/ECAR ratio. Surprisingly, whereas aerobic glycolysis positively regulated IL1B, TNF, and INHBA mRNA expression in both macrophage subtypes, mitochondrial respiration negatively affected IL6, IL1B, TNF, and CXCL10 mRNA expression in M-MØ. The physiological significance of these results became evident under low oxygen tensions, as hypoxia enhanced ECAR in M-MØ via HIF-1α and HIF-2α, increased expression of glycolytic enzymes and GM-MØ–specific genes, and diminished expression of M-MØ–associated genes. Therefore, our data indicate that GM-MØ and M-MØ display distinct bioenergetic profiles, and that hypoxia triggers a transcriptomic switch in macrophages by promoting a HIF-1α/HIF-2α-dependent increase in ECAR. The Journal of Immunology, 2015, 195: 2442–2451.

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Received for publication December 8, 2014. Accepted for publication July 1, 2015.

This work was supported by Ministerio de Economía y Competitividad Grants SAF2011-23801 and SAF2014-52423-R and by grants from the Instituto de Salud Carlos III (Red de Investigación en Enfermedades Reumáticas) and the Comunidad Autónoma de Madrid/Fonds Européen de Développement Régional (RAPHYME Program) to A.L.C. E.I. was supported by a “Juan de la Cierva” Contract ICI-2011-09836 from the Ministerio de Economía y Competitividad. E.O.-Z. was funded by an “Estancia Postdoctoral al Extranjero para la Consolidación de Grupos de Investigación” scholarship (reference 237152, Consejo Nacional de Ciencia y Tecnología, CONACYT, Mexico). M.M.E. was supported by Sara Borrell Postdoctoral Contract CD09/00386 from the Instituto de Salud Carlos III. V.D.C. was supported by an FPI predoctoral fellowship from the Ministerio de Economía y Competitividad.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ECAR, extracellular acidification rate; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; GM-MØ, macrophage generated in the presence of GM-CSF; M-MØ, macrophage generated in the presence of M-CSF; OCR, oxygen consumption rate; qRT-PCR, quantitative RT-PCR; shHIF-1α, HIF-1α-specific small interfering RNA; shHIF-2α, HIF-2α-specific small interfering RNA; siRNA, small interfering RNA; TBP, TATA box-binding protein.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1403045
where low tensions of oxygen are a common feature (13), as in atherosclerosis (14), cancer (15), and rheumatoid arthritis (16). In fact, macrophage functions acquired in response to LPS/IFN-γ or IL-4 have been shown to be dependent on the hypoxia-inducible transcription factors HIF-1- or HIF-2, respectively (17).

At present, the influence of glucose metabolism on macrophage polarization has been analyzed mostly in the murine system and exclusively in the presence of potent activating stimuli (e.g., LPS, IFN-γ, IL-4) (11). However, no information is available on the metabolic status of macrophages generated in the presence of GM-CSF (GM-MØ) or M-CSF (M-MØ), factors that do not trigger an overt polarization but just prime macrophages for proinflammatory or anti-inflammatory responses upon exposure to a subsequent stimulus (18). Thus, we have compared the bioenergetic profiles of GM-MØ and M-MØ and analyzed the influence of glucose catabolism on their respective transcriptional programs and pro- or anti-inflammatory functions. Our results have revealed that the GM-MØ– and M-MØ–specific expression profiles and effector functions are determined by their distinct bioenergetic profiles, and that hypoxia triggers a transcriptomic switch through an HIF-1α/HIF-2α–dependent increase in anaerobic glycolysis in macrophages.

Materials and Methods

**Generation of human monocyte-derived macrophages**

Human PBMCs were isolated from buffy coats from normal donors over Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient according to standard procedures. Monocytes were purified from PBMCs by magnetic cell sorting using CD14+ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (0.5 × 106 cells/ml; ≥95% CD14+ cells) were cultured in RPMI 1640 supplemented with 10% FBS and 2% L-glutamine, 1 mM pyruvate, and 2% FBS (Sigma-Aldrich) or HEPES/F12 (111 mM NaHCO3, 2 mM L-glutamine, 1 mM pyruvate, and 2% FBS (Sigma-Aldrich) and allowed to recover for 24 h. Cells were then incubated in bicarbonate-free DMEM (Sigma-Aldrich) supplemented with 11.11 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, and 2% FBS (Sigma-Aldrich) in a CO2-free incubator for 1 h. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), a proxy for lactate production, were recorded to assess the mitochondrial respiratory activity and glycolytic activity, respectively. After four measurements under basal conditions, cells were treated sequentially with 1 mM sodium oxamate, 1 μM oligomycin, 10 μg/ml LPS (Sigma-Aldrich, St. Louis, MO), or 300 μM CoCl2 for the indicated periods of time. Cells were routinely cultured in 21% O2 and 5% CO2 (normoxic conditions). Hypoxic conditions (1% O2) were generated in a cell incubator under controlled anaerobic atmosphere with 5% CO2 and 94% N2.

**Measurement of cellular respiration and extracellular acidification (bioenergetic profile)**

The XF24 extracellular flux analyzer (Seahorse Biosciences, North Billerica, MA) was used to determine the bioenergetic profile of intact cells. Briefly, cells were seeded (200,000 cells/well) in XF24 plates (Seahorse Biosciences) and allowed to recover for 24 h. Cells were then incubated in bicarbonate-free DMEM (Sigma-Aldrich) supplemented with 11.11 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, and 2% FBS (Sigma-Aldrich) in a CO2-free incubator for 1 h. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), a proxy for lactate production, were recorded to assess the mitochondrial respiratory activity and glycolytic activity, respectively. After four measurements under basal conditions, cells were treated sequentially with 1 μM oligomycin, 0.6 μM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), 0.4 μM FCCP, and 0.5 μM rotenone plus 0.5 μM antimycin A (Sigma-Aldrich), with three consecutive determinations under each condition that were subsequently averaged. Nonmitochondrial respiration (OCR value after rotenone plus antimycin A addition) was subtracted from all OCR measurements. ATP turnover was estimated from the difference between the basal and the oligomycin-inhibited respiration, and the maximal respiratory capacity was the rate in the presence of the uncoupler FCCP (19). Nine independent replicates of each analysis were done, and results were normalized according to protein concentrations.

**Sample preparation for metabolomics analysis**

Cell pellets were resuspended in 20 μl methanol and lyzed with three cycles of frozen/unfrozen in liquid N2 and sonicated with three cycles of 30 s. Then, samples were centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant was aliquoted in a microfial for analysis with gas chromatography coupled to a quadrupole time-of-flight mass spectrometer with an electronic impact source, which was carried out in a 7890A gas chromatograph coupled with an electronic impact source to a 7200 quadrupole time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA). Helium was used as a carrier gas at a flow rate of 1.5 ml/min in constant-flow mode. The oven program was set at an initial temperature of 70°C that was increased to 190°C at a rate of 12°C/min followed by an increase to 325°C at a rate of 20°C/min and a final hold at 325°C for 3.25 min.

**Quantitative real-time RT-PCR**

Total RNA was extracted using the NucleoSpin RNA/protein kit (Macherey-Nagel, Düren, Germany), retrotranscribed, and amplified using the Universal ProbeLibrary (Roche Diagnostics, Mannheim, Germany). Oligonucleotides for selected genes were designed according to the Roche software for quantitative real-time PCR (Roche Diagnostics). Assays were made in triplicate and results normalized according to the expression levels of TATA box-binding protein (TBP) mRNA. Results were expressed using the ΔΔCT method for quantification.

**ELISA**

Macrophage supernatants were assayed for the presence of cytokines using commercial ELISA kits for TNF-α, IL-12p40, CCL2 (BD Biosciences, San Jose, CA) and IL-10 (BioLegend, San Diego, CA) according to the protocols supplied by the manufacturers.

**Western blot**

Cell lysates were obtained in 10 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40 lysis buffer containing 2 mM Pefabloc, 2 mg/ml aprotinin/antipain/leupeptin/pepsstatin, 10 mM NaF, and 1 mM Na3VO4. Cell lysate (10 μg) was subjected to SDS-PAGE and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore). Protein detection was carried out using an anti-human MAFB polyclonal Ab (Abcam, Cambridge, U.K.), an mAb against GAPDH (sc-32233; Santa Cruz Biotechnology, Santa Cruz, CA), an anti-human HIF-1α Ab (BD Pharmingen), an anti-human HIF-2α Ab (Novus Biologicals), and a monoclonal anti-β tubulin Ab (Sigma-Aldrich).

**Cell proliferation assays**

Cell proliferation was assessed using an MTT assay. Briefly, 5 × 103 KM12c cells (four replicates) were seeded into flat-bottom 96-well plates and allowed to adhere overnight. They were then treated with macrophage-conditioned media for 72 h at 37 °C in 5% CO2. MTT (Sigma-Aldrich) was added to a final concentration of 1 mg/ml and the cells were incubated for an additional hour under the same conditions. Percent DMSO (Sigma-Aldrich) was added and the absorbance (570 nm) was measured with a microplate reader (Bio-Rad Laboratories). Percent control refers to the percentage of viable cells in treated wells compared with that in control (untreated) wells.

**Cell transfection**

M-MØ (106 cell/ml) were transfected with 50 nM HIF-1α–specific (siHIF-1α) or HIF-2α–specific (siHIF-2α) small interfering RNA (siRNA; Ambion; Applied Biosciences) using Hiperfect (Qiagen). As a control, macrophages were transfected with siScramble (Ambion/Applied Biosciences). After transfection, cells were cultured for 18–20 h in complete medium containing M-CSF (10 ng/ml). When indicated, cells were treated for 4 h with CoCl2 (300 μM) alone or in the presence of oxamate (30 mM). Protein and RNA were isolated for further analysis using RNAeasy (Qiagen).

**Statistical analysis**

Statistical analysis was performed using a Student t test, and a p value < 0.05 was considered significant.

**Results**

Human GM-MØ and M-MØ display a different bioenergetic profile

GM-MØ and M-MØ exhibit distinct phenotypes (3) and gene signatures (5), and they exert opposite effector functions on tumor cells and T lymphocytes (3–5, 20). To determine whether glucose metabolism lies at the basis of these phenotypic and functional differences, the mitochondrial respiration and glycolytic metabolism
of both macrophage subtypes were initially assessed through the measurement of their respective OCR and ECAR (a proxy for the rate of lactate formation). The determination of bioenergetic parameters was accomplished by the combined use of oligomycin (mitochondrial ATP synthase inhibitor), FCCP (protonophore that uncouples mitochondrial respiration from ATP synthesis), and rotenone plus antimycin A (blocks mitochondrial respiration by inhibiting complexes I and III, respectively), as previously described (19). GM-MØ exhibited a significantly higher basal OCR with a concomitant higher level of oligomycin-sensitive respiratory than would be associated to an increased mitochondria ATP production (Fig. 1A, 1B). The respiratory capacity was also greater in GM-MØ than in M-MØ (Fig. 1B). The rate of glycolysis, estimated from the ECAR values, was also significantly higher in GM-MØ under basal conditions, as was the glycolytic capacity, determined from the stimulation of lactate formation after the inhibition of mitochondrial ATP synthesis (Fig. 1C, 1D).

All these parameters are indicative of a greatly increased metabolic activity and capacity in GM-MØ. Under basal conditions, the ratio between respiration and glycolysis (OCR/ECAR ratio) in M-MØ is higher than in GM-MØ, suggesting that proinflammatory primed macrophages exhibit a proportionally higher aerobic glycolysis (Fig. 1E).

The higher reliance on the glycolytic metabolism shown by GM-MØ prompted us to measure the levels of glucose catabolites and the expression of genes encoding glycolytic enzymes in both macrophage subsets. In line with the above findings, the concentration of glycolysis catabolites (phosphoenolpyruvate, pyruvate, lactate), as well as TCA metabolites (α-ketoglutarate, succinate, fumarate, malate), were significantly higher in GM-MØ (Fig. 1F).

Moreover, the expression of SLC2A1, HK3, FBP1, PFKF, LDHA, PDK1, and PKM2 mRNA was also significantly higher in GM-MØ than in M-MØ, as evidenced by both microarray data (Gene Expression Omnibus GSE27792; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE27792) (5) and quantitative RT-PCR (qRT-PCR) on independent samples (Fig. 1G). Conversely, M-MØ expressed significantly higher levels of amino acids (aspartate, glutamate, isoleucine, leucine, serine, valine) as well as a higher expression of PFKFB2 and PFKFB3 mRNA (Fig. 1F, 1G).

Altogether, these results demonstrate that GM-MØ and M-MØ exhibit a different bioenergetic profile, and that GM-MØ display a more robust glucose catabolism than do M-MØ under basal conditions, a property that correlates with their higher expression of glycolytic enzyme-encoding genes. Notably, this differential bioenergetic profile is reversible and dependent on the exogenous priming cytokine, because exposure to M-CSF (4 h) significantly decreased OCR in GM-MØ, whereas GM-CSF (4 h) was able to increase ECAR in M-MØ (Fig. 1H–J). Therefore, macrophages rapidly modify glucose catabolism in response to the surrounding cytokines.

**Glucose catabolism determines polarization-associated cytokine expression in the steady-state**

As a means to determine whether the differences in glucose catabolism underlie the establishment and/or maintenance of the distinct phenotypic and functional properties of GM-MØ and M-MØ, we exposed both macrophage subsets to agents that inhibit either the aerobic glycolytic pathway (oxamate, a lactate dehydrogenase inhibitor) or mitochondrial respiration (oligomycin, an ATP synthase inhibitor). Oxamate treatment (30 mM, 4 h) of GM-MØ led to a reduction of ECAR, left OCR unaffected (Fig. 2A), and significantly reduced the mRNA levels of the proinflammatory cytokines IL6, TNF, and IL1B and that of the prototypic GM-MØ-specific gene INHBA, but it did not alter the mRNA levels of other GM-MØ-associated markers (EGLN3, MMP12, SERPINE1), IL23, or CXCL10 (Fig. 2B and not shown). Conversely, oligomycin (1 μM, 4 h) reduced OCR and led to a compensatory increase in ECAR (Fig. 2C), but it did not affect proinflammatory gene expression (Fig. 2D). Therefore, aerobic glycolysis contributes to the expression of IL6, TNF, and IL1B mRNA under basal conditions in GM-MØ. In the case of M-MØ, oxamate-mediated reduction of ECAR (Fig. 3A) enhanced IL6 gene expression (3-fold, Fig. 3B) and downregulated GM-MØ-associated marker INHBA and SERPINE1 mRNA (Fig. 3B), without influencing TNF and IL1B (Fig. 3B) or the M-MØ-associated marker CD163 mRNA (Supplemental Fig. 1). Conversely, unlike in GM-MØ, blockade of mitochondrial respiration by oligomycin (Fig. 3C) dramatically enhanced the mRNA expression level of the proinflammatory genes IL6, TNF, and IL1B in M-MØ (between 18- and 80-fold, Fig. 3D), increased CXCL10 mRNA to a lower extent, and left unaffected the levels of INHBA and SERPINE1 (Fig. 3D). Therefore, the expression of IL6, TNF, and IL1B mRNA is negatively regulated by oligomycin-sensitive mechanisms in M-MØ (Fig. 3D), as their enhanced expression is concomitant with the oligomycin-mediated OCR decrease. Because blockade of mitochondrial respiration also results in a subsequent ECAR enhancement (Fig. 3C), we next assessed whether oligomycin-mediated changes are solely caused by ATPase synthase inhibition or due to the secondary enhancement in ECAR. To this end, M-MØ were exposed to the ATP synthase inhibitor in the presence or absence of oxamate. As shown in Fig. 3E, inhibition of lactate dehydrogenase prevented the oligomycin-induced increase in TNF, IL1B, and CXCL10 mRNA, whereas it had no effect on IL6 mRNA levels. Therefore, it can be concluded that enhanced ECAR contributes to the oligomycin-triggered expression of proinflammatory cytokine mRNA.

As a whole, the above results indicate that expression of proinflammatory cytokine IL6, TNF, and IL1B mRNA is positively regulated by aerobic glycolysis in GM-CSF–primed macrophages and negatively regulated by mitochondrial respiration in macrophages primed by M-CSF. These results support the link between the metabolic profile and the production of IL6, TNF, and IL1B under non–activating conditions, and they demonstrate that the relationship of OCR/ECAR inversely correlates with the expression of TNF and IL1B in both GM-MØ and M-MØ. Moreover, note that although to a much lower extent, the expression of the anti-inflammatory cytokine gene IL10 is also sensitive to modulation by the bioenergetics profile, because oligomycin treatment weakly (<2-fold) upregulated IL10 expression, whereas oxamate diminished by 50% the levels of IL10 mRNA in M-MØ (Supplemental Fig. 1). Therefore, IL10 mRNA expression increases upon OCR inhibition (similar to proinflammatory cytokine mRNA) but is reduced upon ECAR inhibition by oxamate, thus suggesting that glucose catabolism differentially affects pro- and anti-inflammatory cytokines.

Along the same line, the link between glucose catabolism and the expression of polarization-associated markers in GM-MØ and M-MØ is not so straightforward. In the case of GM-MØ–associated markers, INHBA expression was reduced by oxamate in both macrophage subtypes, SERPINE1 was only dependent on aerobic glycolysis in M-MØ (Figs. 2B, 3B), and the expression of EGLN3 and MMP12 was not altered by oxamate (data not shown). Regarding M-MØ–associated markers, no significant effect of either oligomycin or oxamate on the expression of CD163, FOLR2, or HTR2B was observed (Supplemental Fig. 1 and data not shown).

**Hypoxia triggers a polarization shift in human macrophages via modulation of the glycolytic metabolism**

To clarify all of the above results in a more “physiological” context, we next sought to assess the influence of hypoxia (which
FIGURE 1. Characterization of the energy metabolism of GM-MØ and M-MØ macrophages. (A) OCR profile of human GM-MØ and M-MØ monitored using the Seahorse Biosciences extracellular flux analyzer. Cells were treated sequentially, as indicated, with 1 μM oligomycin (Oligo), 0.6 plus 0.4 μM FCCP, and 1 μM rotenone plus 1 μM antimycin A (Rot/AA). (B) Metabolic parameters obtained from the OCR profiling after subtraction of the rotenone/antimycin-insensitive respiration. Basal OCR is the oxygen consumption rate in the absence of effectors, ATP turnover is considered as the oligomycin-sensitive respiration, and maximal respiration is the OCR value in the presence of the uncoupler FCCP. Results are normalized according to protein concentrations and presented as mean ± SD of eight independent samples. (C) ECAR, a proxy for the rate of lactate production, measured in human GM-MØ and M-MØ under basal conditions and after the stimulation with 1 μM oligomycin. (D) Metabolic parameters obtained from ECAR profiling. Basal indicates the rate of glycolysis in the absence of effectors, and glycolytic capacity is the ECAR value after the inhibition with oligomycin of the mitochondrial ATP synthesis. Results are normalized according to protein concentrations and presented as mean ± SD of eight independent samples. (E) The bioenergetics profile of the cells is defined as the ratio between the basal OCR and basal ECAR. Results are presented as mean ± SD of eight independent samples. (F) Abundance of metabolites in GM-MØ and M-MØ, relative to GM-MØ. Data are presented as mean ± SD of eight independent samples. (G) Gene expression levels of SLC2A1, HK3, H6PD, FBP1, PFKP, LDHA, PDK1, PKM2, PFKFB2, and PFKFB3 in GM-MØ and M-MØ determined by microarray analysis of three independent samples (filled bars) or by qRT-PCR (open bars). Data are presented as mean ± SD of three independent samples. (H–J) Bioenergetic profiling of GM-MØ and M-MØ under basal conditions (−) and after stimulation for 4 h with GM-CSF or M-CSF, respectively: (H) basal OCR, (I) basal ECAR, (J) OCR/ECAR ratio. Data are presented as mean ± SD of four independent samples. *p < 0.05, **p < 0.01, ***p < 0.001.
increases glycolysis and limits oxidative phosphorylation) on the expression of proinflammatory cytokines and polarization-associated genes. The positive correlation between an active glycolytic pathway and the expression of proinflammatory cytokine genes raised the possibility that the oligomycin-enhanced gene expression in M-MØ was caused by an augmented glycolysis (ECAR) secondary to the inhibition of mitochondrial respiration (see Fig. 3C). If so, and because glycolysis is greatly potentiated in response to low oxygen tension (21), GM-MØ and M-MØ should differ in their sensitivity to hypoxia: M-MØ would exhibit a higher hypoxia sensitivity, whereas GM-MØ would show a weaker hypoxia responsiveness, as expected from their higher content of glycolytic enzymes. In line with our prediction, exposure to the chemical hypoxia-mimicking agent CoCl₂ (4 h) caused a significant increase in ECAR in M-MØ but did not modify the bioenergetic profile of GM-MØ (Fig. 4A). Moreover, CoCl₂-induced hypoxia led to a significant enhancement in the expression of glycolytic enzyme-coding genes (SLC2A1, LDHA, PDK1, PKM2, PFKFB2, and PFKFB3) exclusively in M-MØ (Fig. 4B). Therefore, CoCl₂-induced hypoxia induces the acquisition of a GM-MØ–like glycolytic profile in M-MØ.

The relevance of the above findings was substantiated by assessing whether actual hypoxia (1% O₂, 24 h) also modified the basal phenotype of GM-MØ and M-MØ. In M-MØ, hypoxia (1% O₂, 24 h) induced the acquisition of GM-MØ–associated polarization genes SERPINE1, CCR2A, and INHBA (Fig. 5A), drastically reduced the expression of the M-MØ–associated markers SLC40A1, FOLR2, and MAFB (Fig. 5B), and led to a strong reduction in MAFB protein levels (Fig. 5C). Additionally, low oxygen tension induced M-MØ to acquire tumor cell growth inhibitory activity (Fig. 5D), a property exclusively displayed by GM-MØ (5). Therefore, unlike GM-MØ, M-MØ are extremely
sensitive to hypoxia (1% O\textsubscript{2}) and respond to low oxygen levels by increasing GM-MØ–specific genes, losing M-MØ–specific markers and acquiring functions that are characteristic of proinflammatory GM-MØ.

Because all of the above parameters were evaluated in the absence of macrophage-activating cytokines or TLR ligands, we next assessed whether hypoxia differentially affected the response of GM-MØ and M-MØ to an activating stimulus. To this end, both macrophage subtypes were exposed to LPS under hypoxic conditions. At 1% O\textsubscript{2} (24 h), M-MØ responded to LPS by producing significantly higher levels of GM-MØ–related cytokines IL-12p40 and TNF-σ, but lower levels of anti-inflammatory CCL2 and IL-10 (9) (Fig. 5E). In contrast, the LPS-stimulated cytokine secretion from GM-MØ was not significantly modified by hypoxia (Fig. 5E). Essentially similar effects were observed on macrophages subjected to CoCl\textsubscript{2}-simulated hypoxia (Fig. 5F). Therefore, hypoxia modifies the bioenergetic profile of M-MØ and induces the acquisition of GM-MØ–associated transcriptomic and functional properties in M-MØ.

To evaluate whether a causal relationship exists between the transcriptomic switch and the changes in glucose catabolism initiated by hypoxia in M-MØ, these macrophages were exposed to hypoxia in the presence of oxamate. As shown in Fig. 6A, CoCl\textsubscript{2}–induced hypoxia triggered a significant increase in the expression of proinflammatory cytokine genes, whereas oxamate inhibited the hypoxia-enhanced mRNA expression of TNF, IL-1B, and the GM-MØ–specific genes INHBA and SERPINE1. Additionally, oxamate reduced the hypoxia-augmented production of LPS-induced TNF-α in M-MØ (Fig. 6B). These results further illustrate the link between the bioenergetic profile and the macrophage polarization state, and they demonstrate that the hypoxia-triggered shift in the transcriptomic and functional polarization of M-MØ is dependent, at least partly, on hypoxia-triggered changes in the macrophage bioenergetic profile.

**HIF-1α and HIF-2α contribute to the polarization shift induced by hypoxia in human macrophages**

Given the link between hypoxia-driven glycolytic changes and the macrophage polarization shift, and taking into account that HIF-1α and HIF-2α mediate most cellular responses to hypoxia, we sought to evaluate the role of both factors in the change of the bioenergetics profile that underlies the hypoxia-driven polarization switch in M-MØ (Fig. 6). No HIF-1α, and a low level of HIF-2α, was detected in M-MØ under normoxia, whereas CoCl\textsubscript{2}–induced hypoxia led to a great accumulation of HIF-1α and a weak increase in HIF-2α levels (Supplemental Fig. 2). Because similar results were observed in GM-MØ, it can be hypothesized that the distinct bioenergetics profile of GM-MØ and M-MØ is not secondary to a differential content of HIF factors.

To definitively assess whether HIF factors mediate the change in the bioenergetic profile that leads to a proinflammatory polarization of M-MØ during hypoxia, the expression of HIF-1α and/or HIF-2α was knockdown in M-MØ before exposure to CoCl\textsubscript{2}. siRNA-mediated knockdown resulted in a considerable decrease of the expression of each factor in CoCl\textsubscript{2}-exposed M-MØ (Fig. 7A). The simultaneous siRNA-mediated knockdown of HIF-1α and HIF-2α significantly reduced the CoCl\textsubscript{2}–augmented ECAR in M-MØ (Fig. 7B), a reduction that was not accomplished when each factor was silenced alone (Fig. 7B). Along the same line, knockdown of both factors diminished the expression of the glycolytic enzyme-encoding genes SLC2A1, LDHA, and PDK1, when each factor was silenced alone (Fig. 7B). Along the same line, knockdown of both factors diminished the expression of the glycolytic enzyme-encoding genes SLC2A1, LDHA, and PDK1. Additionally, oxamate reduced the hypoxia-augmented production of LPS-induced TNF-α in M-MØ (Fig. 6B). These results further illustrate the link between the bioenergetic profile and the macrophage polarization state, and they demonstrate that the hypoxia-triggered shift in the transcriptomic and functional polarization of M-MØ is dependent, at least partly, on hypoxia-triggered changes in the macrophage bioenergetic profile.

**Discussion**

Glucose metabolism has been already shown to differ between macrophages exposed to Th1- or Th2-derived cytokines or TLR ligands (11). However, the glucose metabolism status in human macrophages exposed to either GM-CSF or M-CSF, which prime macrophages for proinflammatory (GM-MØ) or anti-inflammatory (M-MØ) responses, respectively (18), has not been previously addressed. GM-MØ and M-MØ greatly differ in their phenotypic, transcriptomic, and functional status (2–6) and, therefore, constitute a valid cellular system to address the contribution of glucose metabolism to the acquisition and maintenance of macrophage functions in the absence of TLR stimulation. In the present study we provide evidence that GM-MØ and M-MØ exhibit distinct bioenergetic profiles, that their distinct glucose
catabolism determines their different transcriptional and functional programs, and that hypoxia triggers an HIF-1α/HIF-2α-dependent transcriptomic switch in macrophages through an increase in anaerobic glycolysis. As a whole, our results illustrate that the acquisition of polarization-specific features of human macrophages rely, at least partly, on glucose catabolism,
and that modulation of the glucose metabolism alters transcriptomic and functional features in macrophages.

Our findings indicate that, in terms of glucose catabolism, GM-MØ and M-MØ significantly differ in their preferential pathway, as GM-MØ exhibit a significantly higher level of aerobic glycolysis (OCR) and mitochondrial respiration (ECAR), as well as a lower OCR/ECAR ratio, than M-MØ. These results are in an apparent contradiction with those reported on murine macrophages, which were shown to be essentially glycolytic cells (11). This discrepancy might derive from the high levels of NO found in M1 murine macrophages, because NO inhibits mitochondrial respiration (22, 23). In contrast, NOS2 mRNA expression could not be detected in human GM-MØ (data not shown), in agreement with findings in human alveolar macrophages stimulated with IFN-γ plus LPS, where epigenetic methylation-dependent gene silencing explains the lack of NOS2 mRNA and protein expression (24). Thus, the differences between previous results and ours might be explained by the lack of NO production by GM-MØ (25, 26).

Our findings have another important implication because GM-CSF or M-CSF just prime macrophages toward a pro- or anti-inflammatory state, but a secondary stimulus is required for triggering pro- or anti-inflammatory cytokine production. Therefore, and given the distinct bioenergetics profiles of GM-MØ and M-MØ, our results predict that both macrophage subtypes would respond differently to cytokines promoting overt polarization (IFN-γ, IL-4), further contributing to the plethora of macrophage polarization states. In support of this hypothesis, we have already observed that the signaling pathways and gene expression initiated by LPS on these macrophage subtypes differ both qualitatively and quantitatively (V.D. Cuevas and A.L. Corbi, unpublished results). GM-MØ display a high expression of glycolytic enzymes and glycolysis-related metabolites, thus resembling the metabolic...
profile of glycolytic tumor cells and activated inflammatory immune cells (10). This shift toward aerobic glycolysis, known as the Warburg effect, rapidly provides immune cells with ATP and metabolic intermediates for the synthesis of immune and inflammatory proteins (27, 28). In the case of human macrophages, note that our results indicate that GM-MØ exhibit higher levels of PKM2 mRNA than M-MØ, which might contribute to the enhanced glycolysis observed in GM-MØ. Besides, higher levels of succinate are also seen in GM-MØ, further contributing to the similarity of the glucose metabolic profile between GM-CSF–primed human macrophages and tumor cells.

The glycolytic metabolism is known to regulate cytokine expression in activated immune cells (29–32), as IL-6 expression in murine monocyte cell lines is lowered upon downregulation of the glycolytic pathway by 2-deoxyglucose (33), TNF-α is downregulated by lactate (34), and IL-1β expression is enhanced by a TLR-dependent stabilization of HIF-1α (35), a process further enhanced by succinate accumulation (36). Our data show that IL6, IL1B, and TNF mRNA are positively regulated by the glycolytic pathway in proinflammatory GM-MØ; whereas a drop of GM-MØ ECAR levels correlates with reduced IL6, IL1B, and TNF mRNA levels, inhibition of mitochondrial respiration leaves the expression of these cytokine genes unaffected. In the case of M-MØ, IL1B and TNF and IL6 mRNA are regulated by an increase in ECAR levels secondary to the inhibition of oxidative phosphorylation by oligomycin treatment. Therefore, the expression of proinflammatory cytokine mRNA is also under the control of the glucose metabolism in human macrophages primed by either GM-CSF or M-CSF. Interestingly, the expression of IL10 mRNA also increases upon inhibition of oxidative phosphorylation (similar to proinflammatory cytokine mRNA) but is reduced by ECAR inhibition, suggesting that glucose catabolism differentially affects pro- and anti-inflammatory cytokines. The positive regulatory effect of the glycolytic pathway on the proinflammatory cytokine expression in M-MØ is also observed under hypoxic conditions, which promote the acquisition of GM-MØ–like transcriptional and functional features in M-CSF–dependent macrophages. Therefore, it can be hypothesized that GM-CSF, similar to hypoxia, primes macrophages for a more rapid and robust response to pathogens by enhancing glycolytic metabolism and, thus, proinflammatory cytokine expression. This “priming” action might therefore resemble the HIF-1α–mediated “training” that has been recently reported in monocytes exposed to β-galactos (37, 38).

Although low oxygen concentrations are found in certain tissues under physiological conditions, exacerbated tissue hypoxia is common to many inflammatory pathologies (16, 39, 40). One of the main features of the cellular response to hypoxia is a significant increase in glucose catabolism (41, 42). In the case of murine macrophages, the expression of hypoxia-inducible factors HIF-1α and HIF-2α appears to be dependent on the stimulatory agents: whereas M1-promoting factors induce the expression of HIF-1α, IL-4 primarily induces HIF-2α that regulates the expression of M2 marker genes (17). In human monocyte-derived macrophages, we have observed that both GM-MØ and M-MØ express HIF-1α and HIF-2α in response to hypoxia. M-MØ respond to hypoxia by changing their metabolic profile and acquiring a GM-MØ–like transcriptomic and functional profile, with both changes dependent on both HIF-1α and HIF-2α. This hypoxia-driven response could explain the elevated number of proinflammatory macrophages found in the synovium of rheumatoid arthritis patients (43), where the severe hypoxic milieu might promote or contribute to the activin A–dependent M-MØ to GM MØ switch (5, 6, 43). In contrast, hypoxia does not modify GM-MØ at the transcriptional (expression of polarization-associated genes) or functional (cytokine production) level (Fig. 5). We hypothesize that the lack of response to hypoxia of GM-MØ might be explained by their high glycolytic state, which resembles that of cells under hypoxic conditions. If so, the elevated ECAR level and the high expression of key enzymes of the glycolytic pathway seen in GM-MØ would impair a further increase in their glycolytic mechanisms, thus preventing the hypoxia-driven transcriptomic and functional effects seen in M-MØ.

In summary, our study provides new insights into the regulation of the macrophage transcriptomic and functional features by the glucose catabolism, thus suggesting that modulation of glucose metabolism represents a potential strategy to modify macrophage polarization under pathological conditions such as cancer or inflammatory diseases. In this regard, because high PDK1 expression correlates with increased aerobic glycolytic rate (44–46) and PKM2, the major isoform expressed in cancer cells (47, 48), promotes rapid glycolytic energy production through upregulation of HIF-1α target genes (GLUT1, EGLN3, PDK1, LDHA) (49, 50), it seems plausible that both enzymes (PDK1 and PKM2) might be appropriate targets to tackle the link between the glucose catabolism and the inflammatory response in macrophages.

Disclosures

The authors have no financial interests of conflict.

References


