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Substrate Fate in Activated Macrophages: A Comparison between Innate, Classic, and Alternative Activation

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Macrophages play a relevant role in innate and adaptive immunity depending on the balance of the stimuli received. From an analytical and functional point of view, macrophage stimulation can be segregated into three main modes, as follows: innate, classic, and alternative pathways. These differential activations result in the expression of specific sets of genes involved in the release of pro- or anti-inflammatory stimuli. In the present work, we have analyzed whether specific metabolic patterns depend on the signaling pathway activated. A [1,2-13C2]glucose tracer-based metabolomics approach has been used to characterize the metabolic flux distributions in macrophages stimulated through the classic, innate, and alternative pathways. Using this methodology combined with mass isotopomer distribution analysis of the new formed metabolites, the data show that activated macrophages are essentially glycolytic cells, and a clear cutoff between the classic/innate activation and the alternative pathway exists. Interestingly, macrophage activation through LPS/IFN-γ or TLR-2, -3, -4, and -9 results in similar flux distribution patterns regardless of the pathway activated. However, stimulation through the alternative pathway has minor metabolic effects. The molecular basis of the differences between these two types of behavior involves a switch in the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) from the liver type-PFK2 to the more active ubiquitous PFK2 isoenzyme, which responds to HIF-1α activation and increases fructose-2,6-bisphosphate concentration and the glycolytic flux. However, using macrophages targeted for HIF-1α, the switch of PFK2 isoenzymes still occurs in LPS/IFN-γ-activated macrophages, suggesting that this pathway regulates ubiquitous PFK2 expression through HIF-1α-independent mechanisms. The Journal of Immunology, 2010, 185: 605–614.

The innate immune system acts as the first line of defense and functions by recognizing highly conserved sets of molecular patterns (pathogen-associated molecular patterns [PAMPs]) through a limited number of germline-encoded receptors called pattern-recognition receptors. TLRs, a class of pattern-recognition receptors, have the ability to recognize pathogens or pathogen-derived products and initiate signaling events leading to activation of innate host defense (1, 2). Macrophages play an essential role in the immune response and normal tissue development by producing proinflammatory mediators and by phagocytic clearance of pathogens and apoptotic cells (3, 4). It has been described that macrophages could undergo different activation processes depending on the stimuli received (5, 6). The classic activation, which can be induced in vitro culture of macrophages with IFN-γ and LPS (inducing TNF-α production), is associated with high microbicidal activity, proinflammatory cytokine, and reactive oxygen species production and cellular immunity; the innate activation, which is mediated in culture by ligation of receptors, such as TLRs, is associated with microbicidal activity and proinflammatory cytokine production; the alternative activation, which can be mimicked in vitro after culture with IL-4, IL-13, glucocorticoids, immune complexes, or IL-10, is associated with tissue repair, tumor progression, and humoral immunity (7). Some authors also distinguish macrophage deactivation, which is induced by cytokines, such as IL-10 or TGF-β, or by ligation of inhibitory receptors, such as CD200 receptor or CD172a, and is related to anti-inflammatory cytokine production and reduced MHC class II expression (8, 9).

Under normal conditions, macrophages are recruited and phagocyte at sites of infection. In addition to playing a crucial role in immunity, some of the mammalian TLRs have been described to regulate bodily energy metabolism, mostly through acting on adipose tissue. This has recently opened new avenues of research on the role of TLRs in pathologies related to metabolism, such as obesity, insulin resistance, metabolic syndrome, or atherosclerosis (10). The accumulation of fatty acids, above all cholesterol in low density lipoprotein form, is the main reason that lipid metabolism has been
studied in macrophages in the atherosclerosis framework. Recent works have built a fatty acid bridge between diet and immune system due to the induction of TLR expression by some fatty acids (11). However, few are known on central metabolism patterns in macrophages since the work of Newsholme and collaborators (12–14) in the 1990s. In this study, we will apply a system biology approach to shed some light in the cross-talk between signal transduction and central metabolism in macrophages. We studied whether the distinct stimulation pathways required different energy demands or have different metabolic patterns. To achieve these goals, tracer-based metabolomics experiments have been used and combined with analysis of the expression of markers of activation. Following the normal activation process of the macrophages, cells were fed with [1,2-13C2]glucose, a nonradioactive isotope of glucose that will incorporate [13C] carbons into metabolite end products (i.e., lactate, glutamate) and the ribose of RNA. This tracer has been broadly used before (15). Our data show that activated macrophages are essentially glycolytic cells, and a clear cutoff between the classic/innate activation and the alternative pathway exists. Interestingly, activation through TLR-2, -4, -9 and -11 resulted in similar patterns of metabolic activation, despite the use of at least in part different signaling pathways and expression of different sets of genes. In the classic and innate activation we observe a shift in the expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) isoforms, from the liver type-PFK2 (L-PFK2), which has a low net activity, to the more active ubiquitous PFK2 (uPFK2) that maintains higher fructose-2,6-bisphosphate (Fru-2,6-P2) concentrations due to a minor bisphosphatase activity and, therefore, potentiates the glycolytic flux (16–18). This isoenzyme is expressed in response to hypoxia through Hif-1α activation (16). Interestingly, LPS challenge results in an increase in Hif-1α levels that might contribute to this uPFK2 expression (19–21). Using macrophages targeted for this transcription factor, we have investigated the contribution of Hif-1α to the enhancement of the glycolytic flux and to the expression of inflammatory molecules in response to classic and alternative activation (22, 23).

Materials and Methods

Chemicals

Reagents were from Sigma-Aldrich (St. Louis, MO), Roche (Basel, Switzerland), Invitrogen (Carlsbad, CA), or Merck (Darmstadt, Germany). TLR ligands were from InvivoGen (San Diego, CA). Cytokines were from PeproTech (Rocky Hill, NJ). Commercial Abs were from Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling Technology (Danvers, MA), Abcam (Cambridge, U.K.), R&D Systems (Minneapolis, MN), Sigma-Aldrich, or PeproTech. Serum and media were from BioWhittaker (Walkersville, MD). Commercial Pckh3 small interfering RNAs (siRNAs) were from Ambion (Austin, TX), and Pckh3 TaqMan gene expression assays were from Applied Biosystems (Carlsbad, CA). [1,2-13C2]glucose (>99% enriched) was purchased from Isotec (Miamisburg, OH).

Treatment of animals and preparation of peritoneal macrophages

Mice were housed and bred in our pathogen-free facility. C57BL/6 mice were from Sigma-Aldrich (St. Louis, MO), Roche (Basel, Switzerland), Invitrogen (Carlsbad, CA), or Merck (Darmstadt, Germany). TLR ligands were from InvivoGen (San Diego, CA). Cytokines were from PeproTech (Rocky Hill, NJ). Commercial Abs were from Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling Technology (Danvers, MA), Abcam (Cambridge, U.K.), R&D Systems (Minneapolis, MN), Sigma-Aldrich, or PeproTech. Serum and media were from BioWhittaker (Walkersville, MD). Commercial Pckh3 small interfering RNAs (siRNAs) were from Ambion (Austin, TX), and Pckh3 TaqMan gene expression assays were from Applied Biosystems (Carlsbad, CA). [1,2-13C2]glucose (>99% enriched) was purchased from Isotec (Miamisburg, OH).

Flow cytometry

Cells were harvested and washed in cold PBS. After centrifugation at 4°C for 5 min and 1000 × g, cells were resuspended in annexin V-binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM CaCl2). Cells were labeled with annexin V FITC solution (BD Pharmingen, San Jose, CA) and/or propidium iodide (PI; 100 µg/ml) for 15 min at room temperature in the dark. PI is impermeable to living and apoptotic cells, but stains necrotic and apoptotic dying cells with impaired membrane integrity, in contrast to annexin V, which stains early apoptotic cells.

Assay of PFK2 activity

Cultured macrophages (6-cm dishes) were homogenized in 1 ml medium containing 20 mM potassium phosphate (pH 7.4, 4°C), 1 mM DTT, 50 mM NaN3, 0.5 mM PMSE, 10 µM leupeptin, and 5% poly(ethylene) glycol. After centrifugation (7000 × g for 15 min), poly(ethylene)glycol was added to the supernatant up to 15% (mass/v) to fully precipitate the PFK2. After resuspension of the pellet in the extraction medium, PFK2 activity was assayed at pH 8.5 with 5 mM MgATP, 5 mM Fru-6-P, and 15 mM Glc-6-P. One unit of PFK2 activity is the amount of enzyme that catalyzes the formation of 1 pmol Fru-2,6-P2 per minute (17).

Metabolite assays

Fru-2,6-P2 was extracted from cells (24–well dishes) after homogenization in 100 µl 50 mM NaOH, followed by heating at 80°C for 10 min. The metabolite was measured by the activation of the pyrophosphate-dependent phosphofructo-1-kinase (17). Lactate and glucose were measured enzymically in the culture medium. NO release was determined spectrophotometrically by the accumulation of nitrite in the medium (phenol red free). Nitrate was reduced to nitrite, and this was determined after reduction of nitrite to nitrate and the latter with Griess reagent (26) by adding 1 mM sulfanilic acid and 100 mM HCl (final concentration).

Cytokine assay

The accumulation of TNF-α, IL-6, and IL-10 in the culture medium was measured per triplicate using commercial kits (Biotak, GE Healthcare, Barcelona, Spain, [TNF-α and IL-6]) and eBioscience, San Diego, CA [IL-10]), following the indications of the supplier.

Preparation of cell extracts

The macrophage cultures (six-well dishes) were washed twice with ice-cold PBS, and the cells were homogenized in 0.2 ml buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 1 mM 2-ME, and 0.1 mM PMSE and a protease inhibitor mixture (Sigma-Aldrich). The extracts were vortexed for 30 min at 4°C and centrifuged for 15 min at 13,000 × g. The supernatants were stored at −20°C. Proteins levels were determined using the Bio-Rad (Richmond, CA) detergent-compatible protein reagent. All steps were carried out at 4°C.

Western blot analysis

Samples of cell extracts containing equal amounts of protein (30 µg/lane) were boiled in 250 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 2% β-mercaptoethanol size separated in 10–15% SDS-PAGE gels. The gels were blotted onto a polyvinylidene difluoride membrane (GE Healthcare) and processed as recommended by the supplier of the Abs against the murine Ags: NO synthase-2 (NOS-2; sc-7271), cyclooxygenase-2 (COX-2; sc-9999), MHC-II (sc-59322), hemeoxygenase-1 (AB-1284), Arg-1 (sc-20510), suppressor of cytokine signaling-3 (2923s), KC/CXCL1 (AF-453-NA), IFN-γ-inducible protein-10 (IP-10)/CXCL10 (500-p129), L-PFK2 (sc-10096), Hif-1α (MAB1536), and β-actin (A-544). For uPFK2, specific peptides of the isoenzyme were used to generate polyclonal Abs by immunization of rabbits (New Zealand White) with multiple intradermal injections with 300 µg Ag in 1 ml CFA, followed by boosters with 100 µg Ag inIFA
for lactate and glutamate, transfer line 250˚C, mass spectrometry source follows: gas chromatography inlet for glucose and ribose 250˚C and 200˚C detector connected to a GC-2010 gas chromatograph. The settings were as spectral data were obtained on the QP2010 Shimadzu mass selective de- carboxylase and pyruvate dehydrogenase to the Krebs cycle (36, 37). Mass in the medium was done to estimate the relative contributions of pyruvate 152 (carbons 2–4 of glutamate, electron impact ionization) were mon- 

m/z monitored for the detection of m1 (lactate with a [13C] in one position) and 

induction was determined in and was normalized to 36B4. The replicates were then averaged, and fold induction was determined in ΔΔCt-based fold-change calculations. Primer sequences are available on request.

Transient transfections

For transient transfections, macrophages were seeded at 80% confluence and transfected using the Cell Line Nucleofector Kit V, following the manu- facturer’s instructions (Amaxa, Cologne, Germany). Forty-eight hours after nucleofection with pkfb3 Silencer Predesigned siRNA or a scrambled RNA (scRNA), macrophages were stimulated for 12 h and mRNA isolated. Trans- fections were performed in triplicate, and expression of a GFP vector was used as a control for transfection efficiency.

Microarray analysis

Normalized expression data were obtained from National Center for Bio- technology Information GEO dataset GDS2429 (27) using GEOquery package from Bioconductor (Berkeley, CA) (28). Differential expression for the following comparisons was tested using limma Bioconductor package (29): 1) naive mature macrophage versus classic activated macrophage, and 2) alternatively activated macrophage. Two gene lists were generated after each comparison, and they were ranked according to the test statistic for sub- traction gene data were submitted to enrichment analysis (29). Enrichment of interest in each list was accomplished using the GSEA method, as de- scribed by Mootha et al. (30). We used “Which genes?” (www.whichgenes- org/) to retrieve the REACTOME (31) pathways as gene sets. The genes from the two lists were also mapped into canonical pathways using In- genuity Pathway Analysis software (Ingenuity Systems, Redwood, CA; see Supplemental Material).

Gas chromatography/mass spectrometry sample preparation and procedure

The macrophage cultures (10-cm dishes) were washed twice with ice-cold PBS, and culture media were replaced by 50% enriched [1,2,13C2]-glucose containing the indicatedTLR ligand or cytokine. After incubation, cells were centrifuged (200 × g for 5 min), and incubation medium and cell pellets were obtained and stored at −80˚C until processing. Glucose, lactate, glutamine, and glutamate incubation medium concentrations were determined, as previously described (32, 33), using a Cobas Mira Plus chemistry analyzer (HORIBA ABX, Montpelier, France). Lactate from the cell culture media was extracted by ethyl acetate after acidification with HCl. Lactate was derivatized to its propylamide-heptafluorobutyric acid form, and the m/z 328 (carbons 1–3 of lactate, chemical ionization) was monitored for the detection of m1 (lactate with a [13C] in one position) and m/z 326 (neutral loss-labeled m/z) for the extraction of m2 (enrichment of mole- cules versus anaerobic glycolysis (34). RNA/Ribose was isolated by acid hydrol- ysis of cellular RNA after TRIzol purification of cell extractions. Ribose isolated from RNA was derivatized to its aldonitrile acetate form using hydroxylamine in pyridine and acetic anhydride. We monitored the ion cluster around the m/z 256 (carbons 1–5 of ribose, chemical ionization) to find the molar enrichment of [13C] labels in ribose (34). Glutamate was separated from the medium using ion-exchange chromatography (35). Glu- tamate was converted to its n-trifluoroacetyl-n-butyln derivative, and the ion clusters m/z 198 (carbons 2–5 of glutamate, electron impact ionization) and m/z 152 (carbons 2–4 of glutamate, electron impact ionization) were mon- itored. Isotopomic analysis of C2–C5 and C2–C4 fragments of glutamate in the medium was done to estimate the relative contributions of pyruvate carboxylase and pyruvate dehydrogenase to the Krebs cycle (36, 37). Mass spectrometry data were submitted on the QP2010 Shimadzu mass selective de- tector connected to a GC-2010 gas chromatograph. The settings were as follows: gas chromatography inlet for glucose and ribose 250˚C and 200˚C for lactate and glutamate, transfer line 250˚C, mass spectrometry source 200˚C. A Varian VF-5 capillary column (30-m length, 250-mm diameter, 0.25-mm film thickness) was used to analyze all of the compounds studied.

Statistical analysis

The data shown are the means ± SD of three or five experiments. Statistical significance was estimated with Student t test for unpaired observations. A p value of <0.05 was considered significant.

Results

Distribution of substrate fluxes in activated macrophages

Elicited peritoneal macrophages were isolated, maintained in culture overnight, and activated for the indicated periods of time through TLRs involved in innate immunity (TLR4, LPS; TLR2, lipoteichoic acid; TLR3, polyriboinosinic-polyribocytidylic acid; and TLR9, CpG), classic activation (LPS/IFN-γ), and alternative activation (IL-4/IL-13; IL-10) (5, 6). Fig. 1A shows the expression of a panel of representative markers of macrophage activation. NOS-2 and COX-2 were expressed in cells activated by the in- native and classic response, except after CpG stimulation. MHC-II upregulation was very sensitive to LPS signaling and to the classic activation pathway, and Arg-1 was notably upregulated in re- sponse to IL-4/IL-13. Hemeoxygenase-1 was moderately induced by innate and alternative pathways of activation. Suppressor of cytokine signaling-3 was upregulated in response to LPS or LPS/IFN-γ. The chemokine CXCL1/KC was expressed in cells activated through classic activation pathway, and innate immunity mediated by TLR2 and TLR4 and CXCL10/IP-10 was upregu- lated through the classic and the innate response. In addition to these parameters related to macrophage activation, cell viability was determined; apoptosis was evaluated by annexin V exposure of the cells and by positivity for PI labeling, the later characteristic of dying cells. As Fig. 1B shows, an increase of annexin V was observed in cells treated with LPS/IFN-γ, and to a lesser extent with LPS; however, the percentage of PI-positive cells at this time (12 h) was modest, suggesting that the integrity of the plasma membrane remained preserved. These data were reminiscent of the expression of NOS-2, indicating that NO plays an important role in the induction of apoptosis in these cells (38). Staurosporine was used as a reference for apoptosis induction in macrophages (39). Moreover, the accumulation in the culture medium of nitrite/ nitrate, IL-6, TNF-α, and IL-10 contributed to establish the profile of activation elicited by the different stimuli used; for example, Cpg failed to promote NOS-2 expression, but notably increased IL-6 and TNF-α synthesis (Fig. 1C).

Previous data suggested the relevance of carbohydrate metabo- lism in the commitment for activation of macrophages (40–43). In this regard, we investigated the glucose consumption and fate in macrophages activated through the innate, classic, and alternative pathways. As Fig. 2A shows, two clear profiles were observed in terms of glucose consumption and lactate release. TLR activation promoted an enhancement in the glycolytic flux to lactate that can be divided into two time-dependent steps: one from 0 to 4 h, and a second flux rate from 4 to 12 h. Indeed, when the same experi- ment was performed with [1,2,13C2]-glucose, the incorporation of [13C] atoms from glucose in different metabolites, according to the scheme depicted in Fig. 2B, allowed the measurement of the pre- cise contribution of these time-dependent fluxes in activated macro- phages. From 0 to 4 h, the glycolytic activity was much lower than from 4 to 12 h (Fig. 2C). Interestingly, alternative stimulation exhibited a metabolic profile that essentially matched that of control cells, despite the influence of the expression of a specific set of genes (vide infra). Also, macrophages displayed a metabolic flux that involved the conversion of glucose into lactate by more than 95%, regardless of the activation pathway considered (Fig. 3A).
The metabolic features revealed by glucose consumption and lactate release are followed by a basal consumption of glutamine and a release of glutamate (Figs. 2A, 3A). Besides, low enrichment in glutamate and RNA (Fig. 3A) evidenced the high glycolytic rate in peritoneal macrophages. Peritoneal macrophages are quiescent cells; however, when the macrophage cell line RAW 264.7 was used and kept overnight with 1% FCS, stimulation for 12 h under these conditions revealed that the basal proliferation rate of these cells did not influence the metabolic profile associated with the macrophage activation process. As Fig. 3B shows, RAW 264.7 cells followed a similar m0 (nonlabeled lactate) and m2 (double-labeled lactate produced from [1,2-13C2]glucose through glycolysis) mass isotope distribution than peritoneal macrophages, without differences in the distribution among the different pathways of stimulation, and with ~80% of the glucose label converted into lactate. However, proliferation induces higher metabolic fluxes and [13C] enrichment in ribose and glutamate (enrichment is the weighted sum of all the labeled species; Fig. 3C).

**Gene profiling in stimulated macrophages**

The previous experiments showed that the carbohydrate metabolism in macrophages is fundamentally glycolytic, and that the rate of glucose consumption is lower in alternatively activated macrophages than in those activated through the classic pathway. Therefore, the expression levels of genes involved in energetic metabolism may differ between the two types of macrophage activation. To test this hypothesis, we compared the functional enrichment (in up- or downregulated genes) of the following REACTOME (31) pathways: glycolysis, pyruvate metabolism and TCA cycle, and electron transport chain. Glycolysis was significantly enriched in upregulated genes from the glycolytic pathway and the repression of genes encoding for proteins that participate in oxidative phosphorylation.

**PFK2 isoenzyme changes in activated macrophages**

The aforementioned differences in transcription of the glycolysis gene set between classic and alternative activation of macrophages allowed us to hypothesize that PFK2, one of the key enzymes of the pathway, could be regulated at the protein and/or enzymatic level. Accordingly, the expression of the isoforms of PFK2 and the levels of Fru-2,6-P2 were determined under these conditions as an indication of the capacity of these cells to metabolize glucose (17, 44). As Fig. 4A shows, resting macrophages expressed the L-type isoenzyme of PFK2, but not the uPFK2 isoenzyme, resulting in low steady state levels of Fru-2,6-P2. However, as result of the activation through the classic and innate pathway, but not the alternative pathway, a robust expression of the uPFK2 isoenzyme occurred, concomitant with a 9-fold rise in the PFK2 activity and a 5-fold increase in the levels of Fru-2,6-P2. Interestingly, the levels of expression of uPFK2, and more importantly, the enzyme activity and intracellular concentration of Fru-2,6-P2 exhibited parallel profiles for each activation condition. Moreover, neither IL-4/IL-13 nor IL-10 was able to change the expression pattern of PFK2 or to increase Fru-2,6-P2 levels. To evaluate the capacity of these ILs to influence the response to LPS costimulation, studies were done. As Fig. 4C shows, when combining IL-10 and IL-4/IL-13 with LPS, the intracellular levels of Fru-2,6-P2 were only minimally influenced with respect to the LPS condition, and an important expression of uPFK2 occurred, despite maintaining a certain level of L-PFK2 expression. The time course of the changes in Fru-2,6-P2 (Fig. 5A) was compared with that of uPFK2 (Fig. 5B) and exhibited a parallel pattern. Because uPFK2 expression has been described to be regulated by Hif-1α, the levels of this protein were determined and only the classic pathway was able to promote its expression (Fig. 5B); COX-2 and NOS-2 levels exhibited the expected profile. In addition to these protein levels, the time course of the expression of Hif-1α, uPFK2, IL-10, and Glut-1 and Glut-4 was determined, showing a kinetics compatible with the changes in Fru-2,6-P2 concentration (Fig. 5C). Interestingly, when the mRNA levels...
of the four PFK2 isoenzymes were determined at 12 h in LPS/IFN-γ-treated macrophages, minimal differences in L-PFK2 expression were observed, whereas uPFK2 and testis-PFK2 were upregulated by classic activation and downregulated in alternatively activated cells (Fig. 5D).

Macrophage activation in the absence of Hif-1α

uPFK2 has been described to be a gene regulated by Hif-1α (18, 45) and, therefore, the differences in transcription of the glycolysis gene set between classic and alternative activation of macrophages might be explained through the changes in Hif-1α promoted after LPS activation (23). However, as Fig. 6A shows, targeting of Hif-1α in macrophages failed to induce changes in the levels of Fru-2,6-P2 (cells treated with IL-10 exhibited a similar behavior as with IL-4/IL-13), or in the glycolytic flux measured as lactate release (Fig. 6B), or in the expression of uPFK2 in these cells (Fig. 6C). Moreover, the mRNA levels of the Hif-1α– and the Hif-1α–targets PHD3, Glut-1, and, to a lesser extent, Glut-4 exhibited the expected drop in
Hif-1α−/− macrophages (Fig. 6D); however, those of the pro-inflammatory cytokines IL-6 and IP-10, and the inflammatory markers NOS-2 and Arg-1, showed a similar expression in Hif-1α+/- and Hif-1α−/− macrophages activated with LPS/IFN-γ or IL-4/IL-13, respectively (Fig. 6E). In agreement with these data, the protein levels of COX-2, NOS-2, KC, IP-10, uPFK2, and L-PFK2 did not

FIGURE 3. Metabolic fluxes using tracer-based distribution of [13C] in stimulated macrophages. Peritoneal macrophages were maintained in culture and stimulated for 12 h in the presence of [1,2-13C2]glucose, as indicated in Fig. 1. Samples of culture medium were collected at 4, 8, and 12 h, and the [13C] label distribution in lactate (m0, nonlabeled [13C0]lactate; m1, one label [13C1]lactate; and m2, two labels [13C2]lactate) was determined by GCMS to establish the metabolic fluxes (A). The macrophage cell line RAW 264.7 was stimulated in the presence of [1,2-13C2]glucose as indicated for peritoneal macrophages, samples of culture medium were collected at 6 and 12 h, and the distribution of the [13C] label was determined by GCMS to establish the metabolic fluxes (B). The comparison between peritoneal macrophages and the RAW 264.7 cell line of the main metabolic fluxes and [13C] enrichment in ribose and glutamate is shown in C. Results show the mean ± SD of four experiments. *p < 0.001 versus peritoneal macrophages.

FIGURE 4. GSEA of energy metabolism pathways and PFK2 isoenzyme switch in activated macrophages. The normalized enriched score calculated by GSEA was shown in the bars, and numbers indicated the FDR for the enrichment. Positive NES indicates enrichment in upregulated genes, whereas negative normalized enriched score corresponds to enrichment in down-regulated genes; see Supplemental Material for more details (A). Peritoneal macrophages were maintained in culture and stimulated for 12 h, as indicated in Fig. 1. The levels of the L-PFK2 and uPFK2 isoenzymes were determined by Western blot using specific Abs. Cell extracts were prepared, and the activity of PFK2 was determined in vitro. The levels of Fru-2,6-P2 were determined in cell extracts after collection of the cell pellets in 50 mM NaOH at 80˚C (B). Cotreatment of cells with LPS and IL-10 or IL-4 results in a decrease in Fru-2,6-P2 content (C). Results show the mean ± SD of five experiments. *p < 0.05; **p < 0.01; ***p < 0.001 versus resting macrophages (A). *p < 0.01 versus untreated cells; #p < 0.05 versus the LPS condition (C).
exhibit differences between Hif-1α+/+ and Hif-1α−/− macrophages; Arg-1 protein levels were slightly higher in Hif-1α−/− cells activated with IL-4/IL-13 (Fig. 6F).

uPFK2 interference attenuates macrophage activation

In view of the minimal effects observed on metabolic fluxes after Hif-1α targeting, the interference of uPFK2 expression was investigated. Using a GFP expression vector as a tracer for the estimation of the percentage of transfected cells, the maximal efficiency of PI-positive cells upon stimulation with LPS/IFN-γ was determined at 18 h (Fig. 7A). The time-dependent changes in uPFK2 mRNA or scRNA resulted in a loss of macrophage viability (Fig. 7A). Fig. 7B shows the analysis of PI-positive cells upon stimulation with LPS/IFN-γ or IL-4/IL-13. Interference of uPFK2 with 100 nM siRNA increased significantly the apoptosis of LPS/IFN-γ–activated cells at 18 h. For this reason, further analyses were carried out in cells after 10 h of stimulation. As Fig. 7C and 7D shows, treatment with uPFK2 siRNA decreased the protein levels of uPFK2, NOS-2, and COX-2, as well as those of the corresponding mRNAs in macrophages stimulated with LPS/IFN-γ. These data suggest that impairment of the expression of uPFK2 results in attenuation of the classic activation of macrophages. However, the loss of viability observed after transfection with siRNA and the moderate efficiency of the process required further approaches to validate the effects due to a reduction in the expression (or activity) of uPFK2. An additional way to circumvent this metabolic aspect is to modulate glucose availability, from 5 to 100 mM, and therefore to modify the glycolytic flux. As Fig. 7E shows, the intracellular levels of Fru-2,6-P2 and the synthesis of lactate and nitrite in LPS/IFN-γ–activated macrophages were modulated by glucose concentration. However, the protein levels of COX-2, NOS-2, uPFK2, and Arg-1 apparently did not show significant differences among the range of glucose concentrations assayed (Fig. 7F). At the mRNA level, small, but reproducible differences in NOS-2 and Arg-1 at 5 mM glucose versus 25–100 mM glucose were observed in macrophages activated for 8 h with LPS/IFN-γ or IL-4/IL-13, respectively (Fig. 7G). Taken together, these data suggest a role for uPFK2 expression and activity in the metabolic activation elicited after LPS/IFN-γ treatment of macrophages.

Discussion

Previous work established that peritoneal macrophages are essentially glycolytic cells (13, 14, 41, 42, 46, 47). Compared with other cell types, macrophages use mainly anaerobic glycolysis in the metabolization of glucose, and the flux in isolated cells is ~10% of the actual capacity through 6-phosphofructo-1-kinase, one of the enzymes that controls the glycolytic pathway (42). In addition to this, glutamine is converted into glutamate and
aspartate, and less than 10% is being oxidized, at the time that fatty acids appear to be the main substrates for oxidative metabolism (41). In the present work, we have assessed the metabolic profiles associated with stimulation of macrophages through three well-defined activation pathways, using a metabolomic approach. The data obtained show that regardless of the stimulation pathway involved, macrophages remain glycolytic cells and accelerate notably the conversion of glucose into lactate when challenged through the classic/innate activation pathways; however, the activation through the alternative pathway exerted minimal effects on the basal consumption of glucose. One of the relevant regulators of glucose metabolism is the rise in the levels of Fru-2,6-P2, which in turn activates the flux through 6-phosphofructo-1-kinase (43, 48, 49).

Fru-2,6-P2 is synthesized/degraded by the bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB1). This enzyme has a balanced kinase/bisphosphatase ratio and, unless the bisphosphatase activity is inhibited, it maintains low levels of Fru-2,6-P2 through a futile cycle of synthesis and degradation of the metabolite (16, 48–51). The PFKFB3 encoded by the PFKFB3 gene has a higher kinase activity (~10:1 kinase/bisphosphatase), is induced by hypoxia, and can be regulated by phosphorylation, playing a role in the high glycolytic rate of various cell types, such as cancer cells (18, 43, 45, 49, 52). A relevant finding of this work is the observation that concomitant to the classic/innate activation, there is a significant change in the expression of the PFK2 from the L type to the uPFK2 isoenzyme. Interestingly, uPFK2 exhibits a higher capacity to accumulate Fru-2,6-P2 in macrophages, due to its lower bisphosphatase activity compared with L-PFK2, which results in an increase in the enzymatic activity determined in vitro, and in the glycolytic flux. Moreover, the substitution between the two isoenzymes is remarkable, and, in fact, the protein levels of L-PFK2 almost disappear and are substituted by the uPFK2 form in the course of TLR-2, -3, -4, or -9 activation, despite the use of non-redundant signaling pathways (i.e., MyD88-dependent and independent pathways). One likely mechanism to explain this switch between PFK2 isoenzymes after classic activation is the rise in the levels of Hif-1α because a functional relationship has been described between the hypoxia-responsive element motifs present in the uPFK2 promoter and the expression of this isoenzyme (16, 18, 19, 22, 23, 53). Accordingly, experiments in macrophages lacking Hif-1α were carried out with the idea to impair uPFK2 expression. However, our data clearly show that classic activation retains almost intact the ability to promote uPFK2 expression, and, indeed, the protein levels of L-PFK2 almost disappear and are substituted by the uPFK2 form in the course of TLR-2, -3, -4, or -9 activation. These results were unexpected in view of the decreased glycolysis observed in B cells lacking Hif-1α (54). Moreover, a cross-talk between Hif-1α and LPS signaling has been reported in macrophages, and it appears to be involved in the development of immune tolerance to LPS and in the sepsis (21, 55, 56). More recently, a specific polarization of classic and alternative activation of macrophages has been attributed to Hif-1α and Hif-2α, with a predominant role of Hif-1α in classic
activation, in particular when comparing bone marrow-derived macrophages versus thioglycolate-induced peritoneal macrophages (22). In this regard, a drop in the expression of NOS-2 in bone marrow-derived macrophages from Hif-1α knockout mice challenged with pathogens was previously described (57); however, this is not the case when peritoneal cells were used, probably because of the expression of Hif-2α, as recently reported (22). The question remains on the identification of the regulatory elements in the uPFK2 promoter that are responsible for this induction upon classic activation in the absence of Hif-1α. In addition to other mechanisms, the possibility of the involvement of Hif-2α in the induction of uPFK2 cannot be disregarded, as described in tumor-associated macrophages (23, 58). Next, the expression of uPFK2 was interfered with a specific siRNA. However, primary cultures of macrophages (and also bone marrow-derived macrophages; data not shown) were difficult to transfect and, using concentrations up to 300 nM siRNA, the maximal efficiency was ~35% of the cells with a clear toxicity and loss of viability. Using this approach, it was clearly observed that upon classic activation interfered macrophages exhibited a high apoptotic rate, not observed in the scRNA counterparts nor in the IL-4/IL-13–activated cells, suggesting that the siRNA was decreasing uPFK2 expression and promoting an apoptotic response. In this regard, the levels of expression of some inflammatory markers, such as COX-2 or NOS-2, were significantly attenuated in the absence of uPFK2. Alternatively to this approach, attempts to decrease the concentration of Fru-2,6-P2 via modulation of glucose availability showed that the metabolic activity of enzymes involved in inflammation, such as NOS-2, exhibited a decreased activity at 5 mM versus the cells maintained at 25 and 100 mM glucose. Interestingly, a certain modulation by glucose of NOS-2 mRNA was observed in macrophages activated through the classic pathway.

Regarding the mass isotopomer distribution in both resting and activated macrophages, the distribution of the label is essentially identical, confirming the minor impact of cell activation among switching between glucose-fueling pathways. Although the three activation pathways studied involve changes in the expression of a large number of genes, the present work shows that only classic and innate activation through TLRs result in an enhanced expression of PFKFB3 corresponding to a higher activation of PFK2 activity and glycolytic flux. These results correlate with model flux predictions in LPS-stimulated RAW 264.7 cells (data not shown). This model could estimate the central metabolism flux distribution from [13C] labeling data and metabolites consumption and production rates. According to our data and those deduced from published arrays, ATP generation is mainly due to anaerobic glycolysis after innate and classic activation, whereas only minimal differences on glucose metabolism are observed between alternative versus unstimulated macrophages. However, an increase in the expression of genes involved in oxidative phosphorylation has been observed upon alternative activation (Fig. 4A).

This metabolic effect upon macrophage challenge is observed both in proliferating (the RAW 264.7 cells) and nonproliferating cells (elicited peritoneal macrophages). However, our results highlight that proliferation has a higher effect on metabolism than that induced by the activation process. Therefore, metabolic changes strictly associated with macrophage activation correspond to results observed in experiments with peritoneal macrophages. Metabolic differences induced by proliferation can be deduced from the experiments reported in Fig. 3. RAW 264.7 cells have higher metabolic fluxes than peritoneal macrophages. Besides, [13C] enrichment in ribose and glutamate was higher in RAW 264.7 to cover the demand in metabolites to proliferate. Moreover, the higher flux through pentose phosphate pathway (PPP) to synthesize nucleotides results in a higher [13C]1 lactate abundance (m1) due to the recycling through the nonoxidative branch of PPP. Finally, the lower glycolytic flux in RAW 264.7 indicates that proliferation promotes the recruitment of other carbon source like glutamine in an anaplerotic flux, corroborating the role of glutamine metabolism in macrophage activation already described (12).

In conclusion, the data show that the stimulation of macrophages through the classic, innate, and alternative pathways exhibits, as expected, a distinct expression of activation markers due to different signaling events involved in each pathway. However, the innate and classic activation show a higher glycolytic rate versus alternative activation, due to a switch in the expression of PFK2 isoenzymes that appears to be a common event after LPS/IFN-γ or TLR-2, -3, -4, and -9 stimulation. Despite the differences in glucose consumption and lactate release, label distribution analysis shows that there is a common anaerobic pattern followed regardless of the activation pathway. Further studies on the molecular mechanisms that govern the switch between the PFK2 isoenzymes might contribute to unravel metabolic aspects related to accommodation of macrophages to biological microenvironments, such as sepsis, endotoxin tolerance, or tumor growth.

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Disclosures

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References


