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 effects. The molecular basis of the differences between these two types of behavior involves a switch in the expression of 6-phosphofructo-2-

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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 \([1,2-^{13}C]_{2}\text{glucose}\), a nonradioactive isotope of glucose that will incorporate \([^{13}C]_{2}\) 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, and -9 results in similar patterns of metabolic activation, despite the use of at least in part distinct 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-bisphosphatase (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 Pfkfb3 small interfering RNAs (siRNAs) were from Ambion (Austin, TX), and Pfkfb3 TaqMan gene expression assays were from Applied Biosystems (Carlsbad, CA). [1,2-\(^{13}\)C]\text{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 anesthetized with light-ether anesthetized mice (four to six animals aged 8–12 wk, as follows: 4 d prior to the assay, mice were i.p. injected 2.5 ml 3% (w/v) of thioglycolate broth (26). Elicited peritoneal macrophages were prepared from light-ether anesthetized mice (four to six animals per condition), killed by cervical dislocation, and injected i.p. 10 ml sterile RPMI 1640 medium. The peritoneal fluid was carefully aspirated, avoiding hemorrhage, and kept at 4°C to prevent the adhesion of the macrophages to the plastic. An aliquot of the cell suspension was used to determine the cell density in the peritoneal fluid. The cells were centrifuged at 200 × g for 10 min at 4°C, and the pellet was washed twice with 25 ml ice-cold PBS. Cells were seeded at 1 × 10^6/cm² in RPMI 1640 medium supplemented with 10% of heat-inactivated FCS and antibiotics. After incubation for 3 h at 37°C in a 5% CO2 atmosphere, nonadherent cells were removed by extensive washing with PBS. Experiments were carried out in phenol-red free RPMI 1640 medium and 1% of heat-inactivated FCS plus antibiotics (26). When glucose concentration was changed, this was added to glucose-free RPMI 1640 medium. Prior to stimulation, the medium was aspirated and replaced by warm medium containing the indicated TLR ligand or cytokine. When the murine macrophage RAW 264.7 cells were used, they were processed as indicated for the peritoneal macrophages.

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 CaCl\text{2}). 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 NaF, 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 Fr2-6-P, and 15 mM Glc-6-P. One unit of PFK2 activity is the amount of enzyme that catalyzes the formation of 1 pmol Fr2-6-P per minute (17).

Metabolite assays

Fr2-6-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 and nitrate 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 (Biotek, 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 MgCl\text{2}, 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. 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-20150), iNOS (sc-7271), cyclooxygenase-2 (COX-2; sc-1999), 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. Protein levels were determined using the Bio-Rad (Richmond, CA) detergent-compatible protein reagent. All steps were carried out at 4°C.
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(a gift of R. Barrtron, University of Barcelona, Barcelona, Spain). The blots were developed by ECL protocol (GE Healthcare), and different exposition times were performed for each blot with a charged coupling device camera in a luminescent image analyzer (Molecular Imager, Bio-Rad) to ensure the linearity of the band intensities.

**RNA isolation and RT-PCR analysis**

A total of 1 μg total RNA, extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, was reverse transcribed using Transcripter First Strand CDNA Synthesis Kit for RT-PCR following the indications of the manufacturer (Roche). Real-time PCR was conducted with SYBR Green on a MyiQ Real-Time PCR System (Bio-Rad) using the SYBR Green method. PCR thermocycling parameters were 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. All samples were analyzed for 36B4 expression in parallel. Each sample was run in duplicate and was normalized to 36B4. The replicates were then averaged, and fold induction was determined in ∆∆Ct-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 manufacturer’s instructions (Amaza, Cologne, Germany). Forty-eight hours after nucleofection with pklf313 Silencer Predesigned siRNA or a scrambled RNA (scRNA), macrophages were stimulated with 12 h and mRNA isolated. Transfections 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 Biotechnology 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 subtable gene data were obtained through analysis (107) of the analysis of Enrichment of interest in each list was accomplished using the GSEA method, as described 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 Ingenuity 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 indicated TLR 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 anhydride 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/IPN−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). Stauorosporine was used as a reference for apoptosis induction in macrophages (39). Moreover, the accumulation in the culture medium of nitrite/nitrateric, IL-6, TNF-α, and IL-10 contributed to the establishment of 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 metabolism 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 experiment 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 precise contribution of these time-dependent fluxes in activated macrophages. 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) pattern chain. Glycolysis was significantly enriched in upregulated genes (downregulated genes) of the following REACTOME (31) pathways: showing a kinetics compatible with the changes in Fru-2,6-P2 (Fig. 5C). These data suggest that classic activation has a stronger effect on the expression of genes related with the energetic metabolism, favoring the upregulation of 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. 4B 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 downregulated 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).
PFK2 pathways were compared at 12 h induction (FI) of Hif-1α normalization. Results show the mean ± SD of four experiments.

**DISCUSSION**

Previous work established that peritoneal macrophages are essentially glycolytic cells. Compared with other cell types, macrophages use mainly anaerobic glycolysis in 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.
Fru-2,6-P₂ is synthesized/degraded by the bifunctional enzyme. It activates the flux through 6-phosphofructo-1-kinase (43, 48, 49). 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 that encoded by the PFKFB3 gene has a higher kinase/bisphosphatase ratio and, unless the bisphosphatase activity is inhibited, it maintains low levels of Fru-2,6-P₂ and the accumulation of lactate and nitrite in the culture medium were determined (E). The protein levels at 18 h of genes related to inflammation and uPFK2 were measured by immunoblot (F). The mRNA fold inductions of these genes were determined at 8 h (G). Results show the mean ± SD of four experiments. *p < 0.01 versus the vehicle condition in nonstimulated cells; †p < 0.05 versus the corresponding vehicle condition (B, D); ‡p < 0.01 versus the corresponding 5 mM glucose condition (E).

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. Four genes encode the PFK2 in higher mammals. The L type is encoded by the PFKFB1 gene and is mainly expressed in liver, and a splicing variant is expressed in muscle. 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 uPFK2 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, despite the use of non-redundant signaling pathways (i.e., MyD88-dependent and independent pathways).
activation, in particular when comparing bone marrow-derived macrophages versus thioglycollate-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 transfected 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 siRNA 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 [13C1] 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 [13C1] 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

The authors have no financial conflicts of interest.

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