Materials and Methods

Chemicals. Reagents were from Sigma-Aldrich (St Louis, MO), Roche (Basel, CH), Invitrogen (Carlsbad, CA) or Merck/Millipore (Darmstadt, FRG). LPS was from InvivoGen (San Diego, CA). Human and murine cytokines were from PeproTech (Rocky Hill, NJ) or Gentaur (Kampenhout, Belgium). Commercial antibodies were from Santa Cruz Biotech (Santa Cruz, CA), Cell Signaling (Danvers, MA), Abcam (Cambridge, UK), R&D Systems (Minneapolis, MN), Sigma or PeproTech. siRNA were from Ambion/InvivoGen, OriGene (Rockville, MD) or Sigma-Aldrich. Serum and media were from Lonza (Walkersville, MD). Native human LDLs were purified to homogeneity by ultracentrifugation (1.063-1.21 g/dl; 1.066 g cholesterol/dl, and were maintained at 10 mg of protein/ml at -80ºC under N_2 atmosphere).

Treatment of animals and preparation of peritoneal macrophages. C57BL/6 mice were housed and bred in our pathogen-free facility. Experimental procedures for macrophage isolation were approved by the Committee for Research Ethics of our Institute (Madrid) in accordance with Spanish and European guidelines (see Animal Study Section). Animals were used aged 8 to 12-weeks as follows: Mice were i.p. injected 2.5 ml of 3% (weight/vol.) of thioglycollate broth, and after four days elicited peritoneal macrophages were prepared from anesthetized mice (4-6 animals per experiment) injected i.p. 10 ml of sterile RPMI 1640 medium at RT. After 10 min, the peritoneal fluid was aspirated avoiding hemorrhage and kept at 4ºC to prevent cell adhesion to the plastic. The cells were centrifuged at 200***g*** for 10 min at 4ºC and resuspended and centrifuged twice with 25 ml of ice-cold PBS. An aliquot of the cell suspension was used to determine the cell density and the enrichment in the $F4/80^+$ population by flow cytometry. Cells were seeded at 60-75% confluence in RPMI 1640 medium supplemented with 10% of heat inactivated FCS and antibiotics. After incubation for 3h at 37ºC in a 5% CO_2 atmosphere, the remaining non-adherent 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 [1].

Preparation of human monocyte/macrophages. PBMCs were isolated from buffy-coats of blood from healthy donors (under their written informed consent, through the blood bank Cruz Roja, Blood Bank, Madrid, Spain) by centrifugation on Ficoll-Hypaque Plus (GE Biotech., UK) following the manufacturers' protocol. The CD14-enriched fraction was collected after binding to MACS-hCD14-magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were differentiated into macrophages with human CSF-1 (20 ng/ml, PeproTech) for 5 days in RPMI 1640 supplemented with antibiotics and 10% FCS. After this period cells were kept for 48h in medium lacking CSF-1 and treated with the indicated stimuli. Purity of all cultures was verified by $CD14^+$ staining using a flow cytometer; on average >95% of the detached cells were highly positive for this surface marker.

Transfection of macrophages and luciferase reporter assays. Cells (12-well dishes) were transfected with 600 ng/ml of the wild-type (pHREwt-PFKFB3.Luc) or the HRE mutated 29-nt region (pHREmut-PFKFB3.Luc), from -1297 to -1269, corresponding to the HRE domain of the human *pfkfb3* gene promoter cloned in a pGL2-basic vector containing the c-*fos* minimal promoter (for a description see [2]), using Lipofectamine 2000 (Invitrogen) and following the supplier's protocol. A plasmid encoding β-galactosidase was co-transfected (60 ng/ml) to normalize for the efficiency of transfection. Basically, macrophages were transfected in DMEM medium for 4h and the medium was replaced by RPMI 1640 for 12h. After this period, cells were submitted to normoxia or hypoxia (4h) and activated for 6h in the absence or presence of human GM-CSF. Cell extracts were prepared to determine luciferase and β-galactosidase activities (Promega Biotech. Spain). Transfections were performed in triplicate and expressed as the ratio of luciferase to β-galactosidase activities measured in the same cell lysate.

Flow cytometry. Cells were harvested at the indicated times and washed in cold PBS. After centrifugation at 4ºC for 5 min and 200***g***, cells were stained with anti-F4/80 and anti-CD14 mAbs, or with propidium iodide (PI) or annexin-V and analyzed as previously described [1] in a flow cytometer using a FC 500 Becton Dickinson FACScan flow cytometer (Mountain View, CA) with a CXP Software (Beckman Coulter).

PFKFB3 and HIF-1α silencing in macrophages. Cells were transfected overnight with a mixture of Lipofectamine and a pool of at least 3 different Silencer-select predesigned siRNAs (5 nM), following the instructions of the supplier. Controls with the corresponding scrambled (negative) RNAs (scRNA) were used to ensure the specificity of the silencing.

Incubation of macrophages under normoxia and hypoxia. Cell cultures were maintained under normoxia or with 2% to 0.5% O_2. Unless otherwise indicated hypoxia was considered 1% O_2. Media were maintained under these conditions to avoid fluctuations in the O_2 saturation and macrophage treatments were accomplished under these normoxic/hypoxic conditions. Hypoxia and normoxia experiments were run in parallel.

Metabolite assays. NO release was determined spectrophotometrically by the accumulation of nitrite and nitrate in the medium (phenol red-free). Nitrate was reduced to nitrite, and the latter was quantified with Griess reagent by adding 1 mM sulfanilic acid and 100 mM HCl (final concentration) [1]. Lactate was determined enzymatically in the culture medium using a previously described protocol [1, 3]. Fru-2,6-P_2 was extracted from cells (24-well plates) after homogenization in 100 µl of a fresh solution of 50 mM NaOH at 80ºC, transferred to eppendorf tubes and heated at 80ºC for 10 min. The metabolite was measured by the activation of the pyrophosphate-dependent 6-phosphofructo-1-kinase [4].

TNF-α and CCL2 measurements. The accumulation of the cytokines in the culture medium or in tissues was measured per triplicate using commercial kits (PeproTech for TNF-α and Sigma for CCL2), following the indications of the suppliers.

Measurement of oxygen consumption and ROS production. O_2 consumption was determined in a Seahorse device (Seahorse Bioscience) and analyzed using the XF Cell MitoStress test assay. ROS production was measured by the oxidation of DCFH as described [5].

Preparation of modified LDL. Native LDL (100 µg/ml) were incubated for 24h at RT with sterile copper sulfate (3 µM, final concentration). Oxidized LDL (oxLDL) were re-isolated by centrifugation at 125,000***g*** and oxidation was evaluated as previously described [6].

Preparation of macrophage extracts. Cell cultures (6 cm dishes) were washed twice with ice-cold PBS and homogenized in 0.2 ml of buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 1 mM β-mercaptoethanol and 0.1 mM PMSF and a protease/phosphatase inhibitor cocktail (Sigma). The extracts were vortexed for 30 min at 4ºC and centrifuged for 15 min at 13,000***g***. The supernatants were stored at -20ºC. Proteins levels were determined using the Bio-Rad detergent-compatible protein reagent (Richmond, CA). All steps were carried out at 4ºC.

Western blot analysis. Samples of cell extracts containing equal amounts of protein (30 µg per lane) were boiled in 250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and size-separated in 10-15% SDS-PAGE. The gels were blotted onto a PVDF membrane (GE Healthcare, UK) and processed as recommended by the supplier of the antibodies against the murine or human

antigens: phospho-STAT-5(Y694), STAT-5, phospho-ERK1/2, ERK1/2, NOS-2, COX-2, HK-II, PFKFB3, PFKFB1, MMP-9, HO-1, Arg-1, HIF-1α, active caspase 3 and β-actin. 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, BioRad) to ensure the linearity of the band intensities. Bands were normalized for the content of β-actin.

RNA isolation and RT-PCR analysis. 1 μg of total RNA, extracted with Trizol Reagent (Invitrogen) according to the manufacturer's instructions, was reverse transcribed using Transcriptor 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 [1, 3, 7] were 95°C for 10 min, 40 cycles of 95°C for 15s, 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 (FI) was determined in a ΔΔCt based fold-change calculations. Primer sequences are available on request.

Caspase 3 activity assay. Tissue extracts (atheromatous caps) were prepared by homogenization in buffer A supplemented with 2 μg/ml TLCK, 5 mM NaF, 1 mM $NaVO_4$, 10 mM Na_2MoO_4 and 0.5% Nonidet P-40. After centrifugation of the cell lysate the activity of caspase 3 was determined with the fluorogenic substrate N-acetyl-DEVD-7-amino-4-trifluoromethylcoumarin (Merck/Millipore). The linearity of caspase assays was determined over a 30 min reaction period, and was expressed as percentage vs. the activity measured in animals fed a high fat diet.

Animal Model Study.

Animal care and experimental procedures were performed according to the Directive 2010/63/EU of the European Parliament, and the studies were approved by the Institutional Committee on bioethics (authorization 28079-37A to the Instituto de Investigaciones Biomédicas, CISC-UAM).

PFKFB3 and HIF-1α *in vivo* silencing. A mixture of at least 3 different Silencer-select predesigned siRNAs for PFKFB3 and HIF-1α were obtained from different sources (Ambion/InvivoGen, OriGene or Sigma-Aldrich). The transfection mixture was prepared using Invivofectamine 2.0 (InvivoGen) and was administered i.p. at 5 mg/kg per dose, following the instructions of the supplier. Administration of the corresponding scrambled (negative) RNAs (scRNA) was used to ensure the specificity of the silencing.

Atherogenesis in ApoE deficient mice and FDG-PET Image Analysis. 30 male ApoE deficient mice aged 3-4 Mo were fed a high-fat/high cholesterol diet for three weeks and, after anesthesia with isoflurane, FDG (1 mCi/kg; 0.2 ml) was administered *i.p.* and the ^{18}F emission was analyzed in a microCT-microSPECT-microPET (INVEON) system. Images were analyzed and quantified, the first axial slice representing the descending aorta (the first PET/CT slice clear of the aortic arch). The measured maximal standardized uptake values (SUVmax) of the descending aorta for five consecutive slices in intervals of 3 mm was averaged to obtain a mean SUVmax. Measured background SUVs from the paraspinal muscles was used to obtain a corrected TBR. Interobserver variability using this technique was 9%, which is comparable to measured values in similar work by other investigators [8-10]. When PFKFB3 and HIF-1α were silenced in intact animals, the siRNAs were administered at days 3, 7, 10, 14 and 17 after high-fat/high-cholesterol administration. 3PO was resuspended in Solutol and administered i.p. (50 mg/kg; 0.2 ml) at the same time periods. Animals were processed for biochemical analyses at the end of the experiment.

Data analysis

The data shown are the means ± SD of three to five experiments. Statistical significance was estimated with Student's *t* test for unpaired observations or ANOVA followed by the Bonferroni test when appropriate. Differences with values of $P<0.05$ were considered statistically significant.

References

1. Rodriguez-Prados JC, Traves PG, Cuenca J, Rico D, Aragones J, Martin-Sanz P, Cascante M and Bosca L. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. *Journal of immunology*. 2010;185:605-14.
2. Obach M, Navarro-Sabate A, Caro J, Kong X, Duran J, Gomez M, Perales JC, Ventura F, Rosa JL and Bartrons R. 6-Phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. *The Journal of biological chemistry*. 2004;279:53562-70.
3. Traves PG, de Atauri P, Marin S, Pimentel-Santillana M, Rodriguez-Prados JC, Marin de Mas I, Selivanov VA, Martin-Sanz P, Bosca L and Cascante M. Relevance of the MEK/ERK signaling pathway in the metabolism of activated macrophages: a metabolomic approach. *Journal of immunology*. 2012;188:1402-10.
4. Martin-Sanz P, Cascales M and Bosca L. Glucagon-induced changes in fructose 2,6-bisphosphate and 6-phosphofructo-2-kinase in cultured rat foetal hepatocytes. *The Biochemical journal*. 1989;257:795-9.
5. Hortelano S, Traves PG, Zeini M, Alvarez AM and Bosca L. Sustained nitric oxide delivery delays nitric oxide-dependent apoptosis in macrophages: contribution to the physiological function of activated macrophages. *Journal of immunology (Baltimore, Md : 1950)*. 2003;171:6059-64.
6. Sanchez-Quesada JL, Ortega H, Payes-Romero A, Serrat-Serrat J, Gonzalez-Sastre F, Lasuncion MA and Ordonez-Llanos J. LDL from aerobically-trained subjects shows higher resistance to oxidative modification than LDL from sedentary subjects. *Atherosclerosis*. 1997;132:207-13.
7. Traves PG, Hortelano S, Zeini M, Chao TH, Lam T, Neuteboom ST, Theodorakis EA, Palladino MA, Castrillo A and Bosca L. Selective activation of liver X receptors by acanthoic acid-related diterpenes. *Mol Pharmacol*. 2007;71:1545-53.
8. Tahara N, Mukherjee J, de Haas HJ, Petrov AD, Tawakol A, Haider N, Tahara A, Constantinescu CC, Zhou J, Boersma HH, Imaizumi T, Nakano M, Finn A, Fayad Z, Virmani R, Fuster V, Bosca L and Narula J. 2-deoxy-2-[18F]fluoro-D-mannose positron emission tomography imaging in atherosclerosis. *Nat Med*. 2014;20:215-9.
9. Bos R, van Der Hoeven JJ, van Der Wall E, van Der Groep P, van Diest PJ, Comans EF, Joshi U, Semenza GL, Hoekstra OS, Lammertsma AA and Molthoff CF. Biologic correlates of (18)fluorodeoxyglucose uptake in human breast cancer measured by positron emission tomography. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2002;20:379-87.
10. Folco EJ, Sheikine Y, Rocha VZ, Christen T, Shvartz E, Sukhova GK, Di Carli MF and Libby P. Hypoxia but not inflammation augments glucose uptake in human macrophages: Implications for imaging atherosclerosis with 18fluorine-labeled 2-deoxy-D-glucose positron emission tomography. *Journal of the American College of Cardiology*. 2011;58:603-14.