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AUTHOR MANUSCRIPT
Accepted for publication in a peer-reviewed journal

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Published as: *Blood*. 2009 July 23; 114(4): 844–859.

Hypoxia inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia

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Abstract

Ischemia exists in many diseased tissues including arthritic joints, atherosclerotic plaques and malignant tumors. Macrophages accumulate in these sites and upregulate hypoxia-inducible transcription factors (HIFs) 1 and 2 in response to the hypoxia present. Here we show that the gene expression profile in primary human and murine macrophages changes markedly when they are exposed to hypoxia for 18h. For example, they were seen to upregulate the cell surface receptors, CXCR4 and GLUT1, and the potent, tumor-promoting cytokines, VEGFA, interleukins 1β and 8, adrenomedullin, CXCR4 and angiopoietin-2. Hypoxia also stimulated their expression and/or phosphorylation of various proteins in the NF-κB signalling pathway. We then used both genetic and pharmacological methods to manipulate the levels of HIFs 1α and 2α or NF-κB in primary macrophages in order to elucidate their role in the hypoxic induction of many of these key genes. These studies showed that both HIFs 1 and 2, but not NF-κB, are important transcriptional effectors regulating the responses of macrophages to such a period of hypoxia. Further studies using

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Author contribution: Hsin-Yu Fang designed & performed research. Russell Hughes - designed & performed research. Craig Murdoch - designed & performed research. Seth Coffelt designed & performed research. Subhra K. Biswas - designed & performed research. Adrian L. Harris contributed vital new reagents or analytical tools, analyzed data and wrote the paper. Randall S. Johnson contributed vital new reagents or analytical tools and analyzed data. Hongxia Z. Imityaz performed research. M. Celeste Simon contributed vital new reagents and analytical tools, analyzed data. Erik Fredlund designed & performed research. Florian Greten designed & performed research. Jordi Rius designed & performed research. Claire E. Lewis contributed vital reagents and analytical tools, analyzed data, wrote the paper.

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experimental mouse models are now warranted to investigate the role of such macrophage responses in the progression of various diseased tissues like malignant tumors.

Keywords

macrophage; hypoxia; HIF; NF-κB

INTRODUCTION

Cells experience sustained periods of hypoxia in such diseased tissues as malignant tumors, atherosclerotic plaques and arthritic joints.[1–3] The predominant transcription factors mediating the effects of hypoxia on gene expression are hypoxia-inducible factors (HIFs) 1 and 2.[4,5] These consist of distinct, hypoxia-responsive α subunits and an identical, constitutively expressed β subunit. In the presence of oxygen, the α subunits are hydroxylated by oxygen-sensitive enzymes called prolyl hydroxylases (PDHs), which targets them for degradation by a ubiquitin-proteasomal pathway.[4] In hypoxia, HIFα subunits accumulate and translocate to the nucleus, couple with the HIF-1β subunit and bind to hypoxic response elements (HREs) in the promoters of various genes, activating their transcription.[4,5]

Macrophages accumulate in most ischemic diseased sites including tumors,[6–9] where they accumulate both HIF 1α and 2α,[10,11] and upregulate HIF target genes like the potent proangiogenic growth factor, VEGFA.[12] There are conflicting views of the relative contribution of each HIF to the regulation of hypoxic gene expression in these cells. Some studies suggest that the main form of HIF upregulated by TAMs is HIF-2,[11,13] and over-expression of HIF-2α in normoxic human macrophages upregulates various pro-angiogenic genes.[14] However, human macrophages also markedly upregulate HIF-1α when exposed to hypoxia *in vitro* and in tumors,[10] and HIF-1α-deficient murine macrophages express lower levels of such HIF-regulated genes as VEGF and the glucose receptor, GLUT1 in hypoxia than their wild type counterparts.[15]

Interestingly, the exact contribution of HIFs 1 and 2 to the regulation of hypoxic gene expression appears to vary between different cell types. HIF-1, for example, mediates the induction of virtually all hypoxia-activated genes in mouse embryonic fibroblasts and human breast tumor cells,[16,17] whereas HIF-2 performs this function in renal tumor cells.[17] This depends partly on the cell-type specific expression of other transcription factors like Elk-1 which bind to the promoters of some genes conferring HIF-2 target specificity on them.[18,19]

Hypoxia may also employ another transcription factor, NF-κB, as two major components of canonical NF-κB signaling, κB kinase β (IKKβ) and p65 (RelA) are activated when murine macrophages experience short-term (≤4h) hypoxia. This then upregulates their expression of both HIF-1α and various HIF target genes.[20–22]

In the present study, we show that exposure to hypoxia for 18h markedly upregulates a broad array of tumor-promoting genes in primary macrophages, and then investigated the role of HIFs 1 and 2 and NF-κB in this phenomenon.

MATERIALS & METHODS

Cells

Two forms of primary macrophages were used in this study: macrophages differentiated *in vitro* from human peripheral blood (monocyte-derived macrophages or ‘MDMs’) or bone

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marrow-derived macrophages ('BMDMs') derived from bone marrow progenitors isolated from wt mice or mice bearing deletions in the HIF-1α or HIF-2 α genes.

Isolation and culture of human MDMs—Monocytes were isolated from Buffy coats (National Blood Service, Sheffield, UK) as previously described.[10] 50×10^6 mononuclear cells was seeded in Iscove's Modified Dulbecco's Media (BioWhittaker UK Ltd, Wokingham, UK) with 5% human AB serum (neat AB serum contains ~1 ng/ml human CSF-1) and 2mM L-Glutamine (All from Sigma, Poole, UK) and incubated at 37°C, 5% CO_2. After 2h, adherent cells were washed and cultured for 7 days to allow differentiation into MDMs.

Isolation and culture of murine BMDMs—As previously described, [22] BMDMs were isolated from the bones of wild type mice or mice bearing a targeted deletion of (i) the HIF-1α gene in myeloid cells (2loxP/1loxP, LysM Cre/+ mice[15]) or (ii) the HIF-2α gene in myeloid cells (2loxP/1loxP, LysM Cre/+ mice; HongxiaZ, Simon CS submitted).

Bone marrow aspirates were washed and resuspended in medium with 10% heat-inactivated FCS (BioWhittaker UK Ltd, Wokingham, UK), 2mM L-Glutamine (Sigma), 100IU/ml penicillin and 100μg/ml streptomycin (BioWhittaker UK Ltd, Wokingham, UK), murine macrophage colony stimulating factor (M-CSF) (PeproTech Ltd, London, UK) and cultured at 37°C, 5% CO_2 for 7 days to allow macrophage differentiation. Their purity was assessed after 7 days using an F4/80 antibody. Only BMDMS cultures of >90% purity were used in subsequent experiments.

Successful deletion of HIFs 1 or 2α has been demonstrated previously using Southern and/or immunoblotting assays of extracts from hypoxic BMDMs from the HIF-1α LysM-Cre mice[23] and HIF-2α LysM-Cre (H.Z. Imtiyaz & M.C. Simon, submitted) mice used in this study.

Normoxic and hypoxic cell cultures

Human MDMs or murine BMDMs were subjected to severe hypoxia (< 0.5% O_2) or normoxia (20.9% O_2) in 5% CO_2 humidified multi-gas incubators (Heto, Camberly, UK) for 18h.

siRNA treatment of human MDMs in vitro

siRNA duplexes for HIF-1α or HIF-2α were synthesized by Eurogentec laboratories. A randomly scrambled duplex was synthesized as a negative control. The HIF-1α siRNA duplex sequences were comprised of sense 5-CUGAUGACCAGCAACUUGAdTdT-3 and antisense 5-UCAAGUUGCUGGUCAUCAGdTdT-3. The HIF-2α siRNA duplex sequences were sense 5-CAGCAUCUUUGAUAGCAGUdTdT-3 and antisense 5-ACUGCUAUCAAAGAUGCUGdTdT-3. The scrambled non-specific duplex sequences were sense 5-AGUUCAACGACCAGUAGUCdTdT-3 and antisense 5-GACUACUGGUCGUUGAdTdT-3. Transient siRNA transfections were carried out using RNAifect as described by the manufacturer's instructions (Qiagen, Crawley, West Sussex, UK). Five-day human MDMs were washed and incubated in 100μl, siRNA complex for 48h. Cells were then washed, fresh media added and cells incubated in normoxia or hypoxia for 18h as described earlier.

RNA and protein extraction from human MDMs

Total RNA was prepared using RNeasy kit (Qiagen) according to the manufacturer's instructions and stored at −80°C. For protein extraction, cells were lysed with lysis buffer (50 mM pH 8.0 Tris-HCl, 150 mM NaCl, 1% Triton-X-100 and 1 protease inhibitor tablet (Roche, Mannheim, Germany). Protein levels were measured using the BCA protein assay (Sigma Aldrich Inc, Poole, Dorset, UK).

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RNA and protein extraction from murine BMDMs

Total RNA and protein isolation was prepared using NucleoSpin RNA/Protein kit (Macherey-Nagel, Duren, Germany) and stored at −80°C for RNA and −20°C for protein. For HIF-2α −/− BMDMs, whole cell extracts were prepared using RIPA lysis buffer (50mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.25% deoxycholate, 1mM EDTA) containing phosphotase inhibitors (sodium fluoride 0.1 mM, sodium orthovanadate 1 mM, sodium pyrophosphate 2 mM and β-glycerophosphate 10mM). Again, protein extracts were stored at −20°C until used for immunoblotting.

Transcriptional profile analysis

Human Genome U133A plus 2.0 gene chip arrays (Affymetrix UK, UK) that detect 47,000 transcripts were used. Total RNA was reverse transcribed to generate cDNA libraries using oligo dT and superscript II (Invitrogen, Paisley, UK). cDNA was amplified using MEGscript T7 kit and cleaned using Gene Chip Cleanup (both Affymetrix, High Wycombe, UK). Labelled cRNA was synthesized using Gene Chip IVT kit and then hybridized to the arrays following the manufacturer's instructions (Affymtreix, High Wycombe, UK). Gene chips were processed using an Affymetrix GeneChip scanner 3000.

To verify the results obtained by using Affymetrix arrays, total RNA was extracted from 2 separate experiments, reverse transcribed, amplified and hybridized to Sentrix HumanRef-8_V2 Bead Chip from Illumina (San Diego, CA, USA) according to the manufacturer's protocols. After washing and drying, the Beadarray was scanned using an Illumina Bead Station 500X which employs SentrixScan Application V2.7.2 software. Illumina Bead Studio software was used for quality control assessment and normalization of data using the LOESS normalization method from BioConductor R packages.

Genes that were upregulated in both arrays by > 1.5-fold or downregulated by <0.67-fold in hypoxia relative to normoxia were considered differentially expressed. One Affymetrix and an Illumina microarray were conducted on RNA isolated from separate experiments. Their combined use was considered to be the first level of screening for the most robust hypoxia robust genes in human macrophages. Only mRNA species regulated by hypoxia on *all* arrays were considered to be reproducibly regulated by hypoxia and worthy of further study. Using this criterion, 148 genes were upregulated and 60 genes downregulated by hypoxia. A panel of selected genes were then further analysed using real-time PCR.

Real-time-PCR

cDNAs was prepared from 1μg total RNA using SuperScript Synthesis kit (Invitrogen, Paisley, UK) and amplified with TaqMan gene expression master mix and pre-designed gene probes using a ABI 7900HT Sequence Detection System (Applied Biosystems, Warrington, UK). The human TaqMan gene expression assay probes used were VEGF, IL-1α, IL-1β, IL-6, CXCL8, CXCR4 (chemokine C-X-C receptor 4), adrenomedullin (ADM), STAT4, adenosine receptor 2A (ADORA2A) intercellular adhesion molecule 1 (ICAM1), heme oxygenase 1 (HMOX1), Prolyl Hydroxylase 2 (PHD2), CITED2, Heat shock 70kDa protein 1B (HSPA1B) ADAM metallopeptidase domain 8 (ADAM8) ERO1-like (ERO1L) matrix metalloproteinase 7 (MMP7), glucose transporter 1 (GLUT-1) and β-2-microglobulin as the endogenous control (Applied Biosystems, Warrington, UK). The murine TaqMan probes used for murine homologs of these were also supplied by Applied Biosystems, Warrington, UK. Real-time PCR cycling conditions for both human and murine samples were 2 min at 50°C then 95°C for 10 min followed by 40 cycles of 15 seconds at 95°C followed by 1 minute at 60°C. In addition, the human NF-κB signalling genes were analysed using SyBr green real-time PCR. The primer sequences used were NFKBIA fwd- TCGCAGTGGACCTGCAAAAT rev-TGAGCTGGTAGGGAGAATAGC, IKKα fwd- CACCATCCACACCTACCCTG rev-

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CTTATCGGGGATCAACGCCAG, IKKγ fwd-CGTACTGGGCGAAGAGTCTC rev-GGCTGGCTTGGAAATGCAG, NFKB1 (p50) fwd-TGCCAACAGATGGCCCATAC rev-TGTTCTTTTCACTAGAGGCACCA, and Rel A fwd-TTGAGGTGTATTTCACGGGACC rev-GCACATCAGCTTGCGAAAAGG. Real-time PCR was done using SyBr Green PCR Master Mix, detected by ABI-Prism 5700 Sequence Detector and data processed using Gene Amp software (Applied Biosystems, Warrington, UK) The murine TaqMan probes used for murine homologs of Rel A and IKKβ were also supplied by Applied Biosystems, UK. The threshold cycle (Ct) of all human and murine data was normalised against their respective endogenous controls (unaltered by hypoxia). Real-Time PCR were analysed in RNA extracts generated in 3–5 independent experiments and then fold changes in expression relative to normoxic cells calculated with ΔCt values of the sample and reference gene using the formula $2^{-\Delta\Delta Ct}$.

Immunoblotting studies

Immunoblotting for human HIFs 1α and 2α were conducted as described previously[10,11] using 1:1000 anti-human HIF-1α monoclonal antibody supplied by BD Biosciences, Oxford, UK or 1:1000 anti-human HIF-2α monoclonal antibody from Novus, Soham, UK. Both blots were incubated with HRP-conjugated anti-mouse antibody (Dako, Copenhagen, Denmark) and protein bands visualized using an enhanced chemilluminescence detection system (ECL) (Amersham Biosciences, Buckinghamshire, UK). In all cases expression of β-actin was used as a loading control. For NF-κB immunoblotting assays, an anti-human Phospho-NFκB p65, total NF-κB p65, Phospho-IKKα/IKKβ or total IKKα/β (Cell Signalling Technology, Danvers, MA) was used at a dilution of 1:500 or 1:1000 and incubated overnight at 4°C.

Cytokine release assay

Cell supernatants were centrifuged for 5 min at 400 g and filtered to eliminate cell debris and then stored at −20°C. The levels of VEGF, IL8 and IL-1β in these supernatants were measured using a BD FACS Array bioanalyzer (BD).

Role of NF-κB in hypoxic gene regulation in primary macrophages

This was investigated in two ways. First, human MDMs were exposed to a specific NF-κB inhibitor, 4-Methyl-*N*l-(3-phenyl-propyl)-benzene-1,2-diamine (JSH-23) (Merck Chemicals, Nottingham, UK) which blocks translocation of phophorylated NF-κB (p65) to the nucleus of cells and its subsequent activation of NF-κB gene targets.[24] MDMs were exposed to medium alone or medium containing 40μM JSH-23 (or the equivalent amount of the vehicle for JSH-23, DMSO) for 1.5h, washed and incubated in normoxia or hypoxia for 18h. Normoxic MDMs were also exposed to 10ng/ml rec. human TNF-α (PeproTech, London, UK) for 18h as a positive control for NF-κB activation. RNA and nuclear proteins were then extracted from parallel cultures of MDMS after these treatments for real-time RT-PCR and immunoblot analysis respectively. Some cells were also fixed in 3% formaldehyde in PBS for 15 min, washed and permeabilized with ice cold 100% methanol for 10 min and blocked with 5% goat serum in 0.3% Triton x-100/PBS solution for 1 h. NF-κB p65 was detected using a rabbit anti-mouse antibody (1:25, Cell Signaling Technology, Danvers, USA) followed by addition of goat anti-rabbit Alexa-488 secondary antibody (Invitrogen, Paisley, UK) (1:250 dilution). Cells were counter-stained with 300nM DAPI (Molecular Probes Inc.,) and then photgraphed on a confocal fluorescent microscope (at x400 magnification). Twelve areas of cells were photographed for each treatment group and the degree of nuclear p65 immunofluorescence (ie. Alexa-488-labeled nuclei) in each DAPI-stained nuclei quantified using Analysis D software (Olympus). The proportion of green fluorescence per nuclei was then calculated for all nuclei in 5 fields of view/treatment. The number of all MDMs in each field of view containing Alexa-488-labeled (p65+) nuclei was also counted. To confirm JSH-23 inhibition of NF-κB

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activity in hypoxic MDMs, EMS As for NF-κB binding to an NF-κB DNA consensus site were conducted as described previously by us[25] on lysates from MDMs exposed to nomoxia, hypoxia or hypoxia plus JSH-23 (all in the presence of DMSO as the vehicle for JSH-23). Protein extracts from parallel cultures of MDMs were also immunoblotted for HIFs 1 and 2α (as described above).

The second approach was to infect MDMs with an adenovirus expressing a dominant negative inhibitor of IKKβ to block phosphorylation/activation of p65/RelA. After 4 days in culture, MDMs were exposed to 50ng/ml rec. human M-CSF for 24 h to stimulate upregulation of integrin $\alpha v\beta_5$ (required for adenovirus infection of macrophages[26]). The adv-$IKK\beta^{DN}$ and control adv (Adv-GFP) (a gift from Dr Thorsten Hagemann, London) were E1/E3-deleted, of the Ad5 serotype, and used to transfect MDMS as described previously.[27] MDMS were infected for 2h with 100 multiplicity of infection (MOI) of either adenovirus in serum-free medium. The adenovirus was then removed and fresh medium containing 2% AB serum added. MDMS were maintained for a further 2 days in culture and then exposed to hypoxia or normoxia for 18h. This infection protocol markedly reduces the activity of p65/RelA in human MDMS[46] and human endothelial cells.[28]

Immunofluorescent labelling of IL-1β expressed by TAMs in hypoxic areas of murine 4T1 mammary tumors

Frozen sections of 4T1 murine mammary tumors were generated in a previous study.[29] These had been grown in the mammary fat pads of female BALB/c mice, and removed and snap frozen 2h after injection of mice with the hypoxic cell marker, pimonidazole.[29] Sections (7 μM) were blocked with FcR Blocking Reagent (Miltenyi Biotec, Surrey, UK) in TBS-0.05% Tween 20 (TBST) for 30 min at room temperature and then incubated with rat anti-F4/80-Alexa 488 (1 μg/mL, clone CL:A3–1; AbD Serotec, Oxford, UK), goat anti-mouse IL-1β (15 μg/ml; R&D Systems, Abingdon, UK) and rabbit anti-PIMO (1:4000, a gift from James Raleigh) for 30 min at room temperature. Negative controls included substitution of primary antibodies with species-matched, non-specific antibodies. Sections were then washed twice and incubated in Donkey anti-goat-Alexa 568 (8 μg/mL; Molecular Probes, Eugene, OR, USA) or Alexa 647-conjugated goat anti-rabbit (8 μg/mL; Molecular Probes) secondary antibodies for 30 min at room temperature in the dark and 30nM DAPI (Molecular Probes) for 2 min.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was performed as described previously[30] on gene lists ranked by level of hypoxic gene induction (hypoxia/normoxia fold induction) separately for both the Affymetrix and Illumina gene expression data sets. Correlations to the predefined *Curated and TFT: transcription factor targets* gene set collections were analyzed with the GSEA Pre-ranked tool using 1000 permutations. Further information regarding the gene sets used in these analyses is available in the Molecular Signatures Database (MSigDB) (www.broad.mit.edu/gsea/msigdb).

Statistics

All experiments were repeated 3–6 times. Statistical analyses were performed using the one or two-tailed Student's t test to determine statistical significance after checking the data for normality (as appropriate). P values of <0.05 were considered statistically significant. All data are expressed as means ± SEMs.

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RESULTS

Evidence of distinct transcription signaling in primary human macrophages experiencing hypoxia

Hypoxic MDMs upregulated both HIF-1α and HIF-2α and this was markedly inhibited by prior treatment with siRNA to either HIFα (Fig 1A). As in previous publications,[31,32] genes were defined as being differentially regulated in hypoxia if they exhibited >1.5 fold increase in gene expression (Table 1) or downregulated if they showed <0.67 fold change (Table 2) compared to normoxic cultures. A comparison of our human MDM microarray results (Tables 1 & 2) with those obtained previously for related human myeloid cell types exposed to hypoxia (monocytes and monocyte-derived dendritic cells[31,32]) shows that some genes were seen to be regulated by all three cell types (upregulated: VEGFA, CXCR4, TNFα, TIMP1, PHD3, Aldolases A & C, Enolase 2, TREM1, NCF1; downregulated: Cathepsin C). However, some genes regulated by hypoxia in MDMs are not similarly regulated by hypoxia in these other two cell types such as IL-1β, IL-12p40, Ang-2, endothelin 1, STATS 4 & 6, CCLs 3 & 5, CCR7, HMOX1 & hsp70 (upregulated) and CD36, PECAM1 (CD31), HIF-2α & MHCII DMβ) (downregulated) (Tables 1 and 2). A number of key genes were selected and their upregulation confirmed using qRT-PCR (Table 1). Macrophages were also shown to express abundant IL-1β protein in pimonidazole-stained (hypoxic) areas of murine 4T1 mammary tumors (Fig. 2C).

Genetic manipulation of HIFs 1 and 2α demonstrates the co-regulation of genes in primary human macrophages experiencing hypoxia

The hypoxic accumulation of both HIFs 1 and 2α was ablated following transfection with siRNA for either α subunits. Both VEGFA mRNA and protein were markedly increased by hypoxia and this was significantly inhibited by siRNA for either HIFα subunit (Fig. 1B, left and middle panels). It may appear that the hypoxic induction of VEGF mRNA is higher in hypoxic macrophages treated with the scrambled control siRNA than in the ‘no siRNA’ group. However, this failed to reach statistical significance. This was also the case for these 2 groups in panels E, G, I and K of Fig. 3.

CXCL8 mRNA and protein release were also upregulated in hypoxic MDMS (Fig. 1C) and while both HIFα siRNA treatments reduced hypoxia-induced CXCL8 mRNA, only the effect of HIF-2α siRNA reached significance. However, both HIFα siRNA species significantly reduced CXCL8 *protein* release (Fig. 1C). The inhibitory effect of HIF siRNA on the hypoxic induction of both VEGF and CXCL8 appeared to be slightly greater at the protein than the mRNA level.

Hypoxia also upregulated IL-1β mRNA and protein and this was significantly inhibited by exposure to siRNA for either HIF α subunit (Fig 2A). We then investigated the role of HIFs-1 and 2 in the hypoxic regulation of several other genes listed in Table 1. The hypoxic upregulation of mRNA for CXCR4, GLUT1, adrenomedulin (ADM) and STAT-4 was significantly ($p<0.05$) reduced by HIF-1α or 2α siRNA (Fig. 3A, C, E & G). In contrast to the other genes investigated, the hypoxic induction of adenosine A2a receptor (ADORA2A) and ICAM1 mRNA was significantly ($p<0.05$) inhibited only by HIF-2α siRNA (Fig. 3I & K).

Transcriptional signaling in primary human MDMs experiencing hypoxia for 18h is independent of NF-κB

(i) Gene Set Enrichment Analysis—To assess the likelihood of NF-κB playing a role in hypoxic signal transduction in human macrophages we first searched our data for correlations with several published gene sets relating to hypoxia-regulated regulated genes in other cell types (eg. the *‘HYPOXIA_REVIEW’* gene set[33] - Fig. 4, upper panels). This highlighted a

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significant degree of enrichment of known hypoxia-regulated genes in our array data, showing that hypoxia induced gene expression changes in MDMs follow a consensus hypoxia gene expression profile (Fig. 4 upper panels). This was evident for both the Affymetrix array data (Normalized Enrichment Score (NES): 2.2; False Discovery Rate (FDR) q<0.001) and the Illumina data (NES: 2.24; q<0.001). Table 1 shows that many genes upregulated by hypoxic MDMs have previously been identified as NF-κB target genes. In the GSEA analysis, the hypoxic MDMS array data also correlated significantly with several NFκB-related gene sets (eg. the *V$NFKAPPAB_01* geneset[34] - Fig. 4, lower panels). Again, this was evident for both the Affymetrix data (NES: 1.69, q=0.02) and the Illumina data (NES: 1.67; q=0.12).

(ii) Hypoxic upregulation of NFκB signaling in human macrophages: role of HIFs 1 and 2—The effect of exposure of human MDMs to hypoxia for 18h on NF-κB signalling was then assessed. IKKβ and γ, IκBα, NF-κB1 (p50) and p65/RelA mRNA levels were upregulated (and IKKα mRNA slightly downregulated) in MDMs exposed to 0.1 % O_2 for 18h. This hypoxic regulation (with the exception of IKKs α and γ) was inhibited using siRNA to knock down either HIF-1 or 2α (Fig. 5A). Fig. 5C shows that, while there was a small hypoxic induction of total IKKβ protein, the hypoxic upregulation of p65/RelA mRNA was not mirrored by a similar upregulation of total p65/RelA protein, suggesting a differential effect of hypoxia on mRNA versus protein expression for p65/RelA. By contrast, the *phosphorylation* of both IKKα/β and p65/RelA was upregulated in hypoxic human MDMs (Fig. 5C).

(iii) Role of NF-κB in the transcriptional responses of human macrophages to hypoxia—Fig. 6(A and B) illustrates the effects of the NF-kB inhibitor, JSH-23 on the hypoxic induction of various genes in human MDMs. This shows that immunoreactive p65 was cytoplasmic in normoxic MDMs but transported to the nucleus upon exposure to TNFα or 18h hypoxia. In both cases, this was significantly (P<0.05) inhibited by prior exposure to JSH-23. EMSA assays confirmed the induction of NF-κB DNA binding in hypoxic MDMs, and the inhibition of this by JSH-23 (Fig. 6C). JSH-23-treated cells also exhibited slightly lower levels of HIFs 1 and 2α (particularly HIF-1α) than MDMs exposed to hypoxia alone (Fig. 6C). We then investigated the effect of JSH-23 inhibition of NF-κB activity on the induction of 8 hypoxia-regulated genes listed in Table 1. Exposure to TNFα for 18h significantly (P<0.05) increased the expression of VEGF, CXCL8, G1UT1, STAT4 and ADORA2A in a manner that was inhibited by JSH-23 (Fig. 6). Hypoxia significantly (P<0.05) upregulated all eight genes studied (Fig. 6D) in a manner that was not reduced by prior exposure of cells to JSH-23.

Infection of MDMs with adv-IKKβ^{DN} significantly (P<0.05) inhibited their TNFα–induced expression of CXCL8 mRNA (Fig. 7A) as well as the nuclear accumulation of phospho-p65/RelA by MDMS after 18h of exposure to hypoxia (Fig. 7B). The control adv vector had no such effect. However, adv-IKKβ^{DN} blockade of hypoxia-induced phospho-p65/RelA failed to reduce the hypoxic induction of VEGF, CXCL8, GLUT-1, CXCR4or ADM mRNA.

Hypoxic regulation of genes in primary murine macrophages: role of HIFs 1 and 2

BMDMs from HIF-1α−/− mice were only able to mount partial VEGFA and IL-1β responses to hypoxia (Fig 1B, right panel and Fig. 2B). The hypoxic upregulation of CXCR4 and STAT4 was lost in HIF-1α null BMDMs (Fig. 3B & H). This contrasts with our aforementioned human MDMs data showing that these were regulated by *both* HIFs 1 and 2 (Fig. 3A & G). The fact that GLUT1 and ADM was reduced but not ablated in HIF-1α null BMDMs (Fig. 3D & F) agrees with our finding that these 2 genes are co-regulated by HIFs 1 and 2 in human MDMs (Fig. 3C & E). Also in agreement with the human MDMs data (Fig. 3I & K), the hypoxic upregulation of neither the ADORA2A nor ICAM1 genes was inhibited in hypoxic HIF-1α null BMDMs (Fig. 3J & L).

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Fig. 5B shows that hypoxic upregulation of IKKβ and p65/RelA mRNA levels was lost in HIF-1α−/− BMDMs. Moreover, the hypoxic induction of phosphorylated p65/RelA was lost in HIF-2a−/− BMDMs (Fig. 5D). Similar results were seen for HIF-1α −/− BMDMs (data not shown).

DISCUSSION

Our data show that exposure to hypoxia activates a distinct transcriptional profile in primary human macrophages, including the upregulation of VEGFA, ILs-1α and β, IL-8, STAT4, ADM; the receptors, glucose transporter, GLUT1, CXCR4 and the adenosine receptor 2 A (ADORA2). Some were seen to also be regulated by hypoxia in monocytes and immature dendritic cells (VEGFA, GLUT1 and CXCR4).[31,32] However, others like IL-1β, ADORA2 A and STAT4 were only altered in hypoxic macrophages. These differences could be due to variations in the severity and/or duration of hypoxia applied to cells,[31,32] and/or may reflect differences in the transcription factors employed by these 3 cell types in hypoxia. For example, hypoxic human monocytes exposed to a similar level and duration of hypoxia as in the current study failed to upregulate HIFs 1 and 2α but rather other transcription factors like ATF-4 and Egr-1.[35] Moreover, the ability to regulate hypoxic gene expression via HIFs is maturation-linked in macrophages.[36] Although dendritic cells accumulate HIF-1α in hypoxia,[37] immature forms of this cell type upregulate other hypoxia-responsive genes like CCL20 via upregulated p50/p50 NFκB homodimers rather than HIFs.[38] A study of the responses of such related myeloid cell types to identical hypoxic conditions would be interesting but beyond the remit of this study.

As macrophages are known to express receptors for both VEGF[39] and IL-1[40], it is possible that that during such exposure to hypoxia, their hypoxia-induced release might have then stimulated the expression of other genes in macrophages (making it look as if they are also directly upregulated by hypoxia when, in fact, the effect is indirect). However, hypoxic gene expression by human MDMs is not reduced in the presence of either a neutralising VEGF antibody or an IL-1 receptor antagonist (Fang H-Y, Murdoch C, Hughes R and Lewis CE, unpublished observations).

We also show for the first time that genes encoding the two transcription factors, STATs (signal transducers and activators of transcription) 4 and 6, are upregulated by hypoxia in macrophages. STAT 4 and 6 are known to mediate the marked effects of two central immunomodulatory cytokines, IL-12 and IL-4, respectively.[41,42] It remains to be seen whether their hypoxic induction could 'prime' macrophages to the effects of these cytokines.

Our HIF siRNA studies showed that both HIFs play a part in regulating the hypoxic induction of the known HIF target genes, VEGF, GLUT1, CXCR4, IL-8 and ADM by MDMs. Furthermore, hypoxic induction of these genes was reduced but not lost in murine macrophages from HIF-1α−/− mice. Similar results were obtained for the hypoxic upregulation of VEGF and ADM in murine HIF-2α−/− BMDMs (H.Z. Imtiyaz, M.C. Simon & colleagues, submitted).

The pluripotent cytokine, IL-1β, stimulates many steps in tumor progression[43] and was upregulated by hypoxic MDMs. We show that TAMs express abundant IL-1β in hypoxic areas of murine mammary (4T1) tumors. The IL-1β gene promoter bears multiple HREs and is transactivated by HIF-1[44,45] Our HIF siRNA knock down studies show that HIFs 1 and 2 co-regulate the hypoxic induction of IL-1β in macrophages – a finding confirmed by the hypoxic upregulation of IL-1β being only partially diminished in murine BMDMs from HIF-1α−/− (Fig. 1) and HIF-2α−/− mice (H.Z. Imtiyaz, M.C. Simon and colleagues, submitted).

It remains to be seen whether HIFs 1 and 2 bind to different HREs on the promoters of the above co-regulated genes or whether other, unknown mechanisms underpin the phenomenon

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of dual HIF responsiveness. Furthermore, as mentioned previously, this may vary between cell types as HIF-1 has been shown to be the primary regulator of various genes in some cell types,[16,46] while other cells employ HIF-2 or both HIFs in their regulation.[18,19,47] Interestingly, when just one HIF was inhibited using siRNA the other did not appear to compensate for its loss and maintain maximal hypoxic induction. It is known that many HIF-target genes have multiple HREs. If, once HIFs 1 and 2 have bound to different HREs in a given promoter they then co-operate, both might be required for maximal gene transcription.. This may be similar to the molecular 'co-operation' that takes place between HIF-2 and Elk-1 on some gene promoters.[18,19]

The complete knockdown of both HIFs 1α and 2α in MDMs failed to completely block the hypoxic upregulation of most of the HIF-target genes discussed above. This suggests that other transcription factors may also be involved in regulating their hypoxic induction. The transcription factor, NF-κB, may be one such factor. This has been shown recently to be activated in macrophages by short-term (2–4h) exposure to hypoxia, with the expression and/or phosphorylation of IKKβ, IKBα and p65/RelA in macrophages, as well as the nuclear translocation and DNA-binding activity of p65 being upregulated.[20–22] There also appears to be a close interplay between NF-κB and HIF-1 as p65/p50 heterodimers bind to the HIF-1α gene promoter and drives its expression under hypoxia. Interestingly, HIF-2α is not upregulated by NF-κB in murine macrophages during short-term hypoxia (4h).[22] The present report shows that p65 protein is phosphorylated and binds DNA in the nuclei of MDMs in hypoxia. Furthermore we show that both HIFs 1 and 2 contribute to the maintenance of high levels of p65 expression and phosphorylation in such cells.

As many of the genes we found to be markedly upregulated in human macrophages by hypoxia had previously been identified as potential NF-κB target genes (Table 1 and Fig. 5), we examined the role of p65 in the hypoxic upregulation of the most highly ones. Studies using the synthetic inhibitor of nuclear translocation of p65, JSH-23,[24] or an adenoviral inhibitor of IKKβ showed that NF-κB is not essential for their induction during an 18h exposure to hypoxia. The upregulation of HIF-1α in macrophages exposed to short-term hypoxia (4h) is partially dependent on NF-kB[22] so the fact that both HIFs 1 and 2α continued to be upregulated in JSH-treated MDMs following hypoxia in our study suggests that, either p65 inhibition was incomplete or that the accumulation of these sub units during a more sustained period (18h) of hypoxia is independent of NF-κB.

While both forms of NF-κB inhibition resulted in the marked inhibition of TNFα-induced CXCL8 (as well as other genes examined with JSH-23), it had no detectable effect on the hypoxic expression of any of the genes examined. These data are supported by the recent finding that that the hypoxic induction of several such NF-κB target genes in murine BMDMs does not involve activation of NF-κB (H.Z. Imtiyaz & M.C. Simon, submitted).

Our data indicate that NF-κB signaling may not contribute to the induction of these genes by macrophages in response to an 18h exposure to hypoxia. At first glance, this appears to contrast with the finding that hypoxic induction of HIF-1α and various HIF target genes was diminished in BMDMs from IKKβ−/− mice following exposure to short-term hypoxia (ie. 4h).[22] However, it may be that there is a switch from acute, NF-κB-dependent hypoxic responses in macrophages that are critical for innate immunity (eg. bacterial infection) to an NF-κB-independent, HIF-driven response to the chronic hypoxia present at sites like tumors. Clearly, a detailed investigation of the role of NF-κB in the temporal and gene-specific responses of macrophages to hypoxia is now warranted.

Taken together, our data show that when macrophages experience hypoxia for 18h it elicits a profound change in their expression of various tumor-promoting genes. While this study

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provides invaluable insights into the basic repertoire of such macrophage responses, it should be remembered that macrophages in hypoxic areas of complex tissues like tumors are a heterogenous mix of cells including immature, monocyte-like cells and mature macrophages.[6] Moreover, the responses of these cells to hypoxia will also be influenced by a host of tumor-derived signals like cytokines and enzymes. Further *in vivo* studies are now warranted to investigate the role of hypoxic macrophage responses within the complex milieu of tumors.

Acknowledgments

The authors gratefully acknowledge grant support from the Yorkshire Cancer Research, UK and Breast Cancer Campaign, UK for different parts of this study. The authors also thank Drs Bosco & Varesio (Italy) and Michael Karin (USA) for their helpful comments in the preparation of this manuscript. They also thank Dr Thorsten Hagemann (London) for providing the control and IKKBdn adenoviruses and Dr Sheila Francis (Sheffield) for her help with the adenoviral infections.

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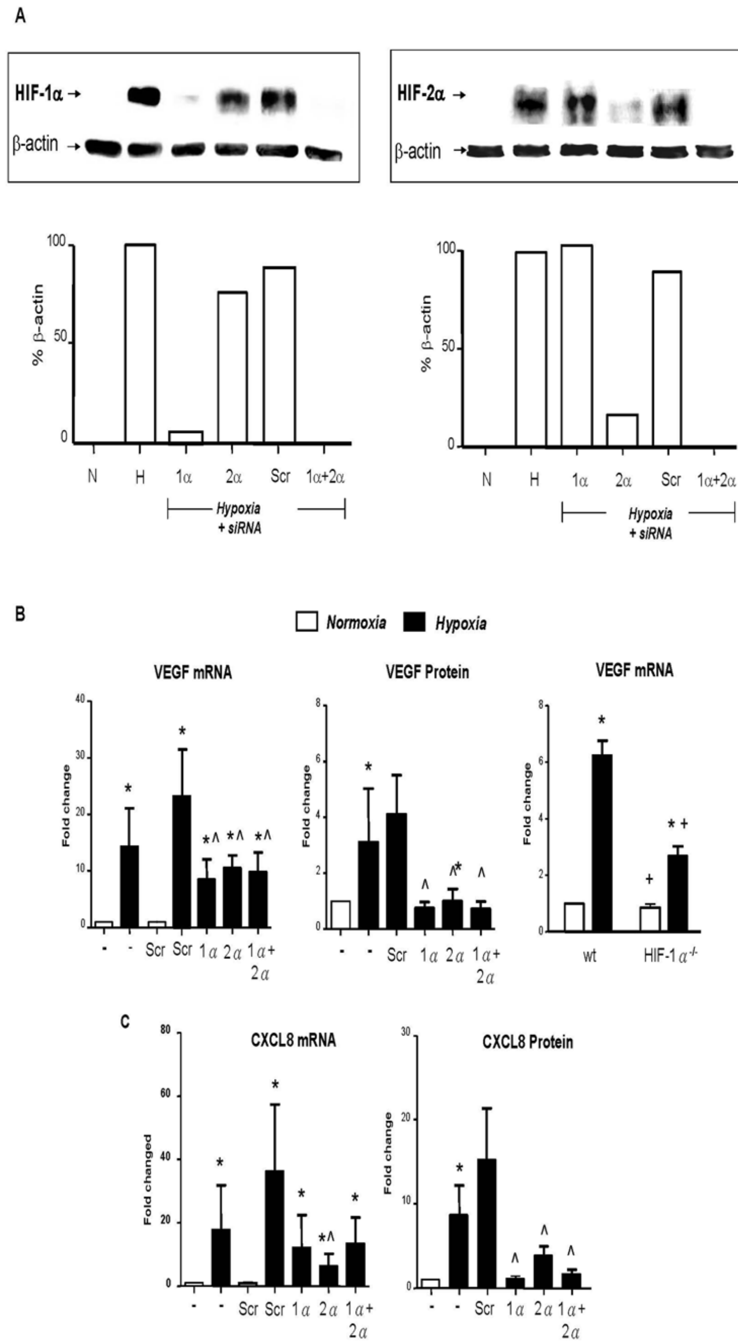

Figure 1. Role of HIFs 1α and 2α in the hypoxic induction of VEGF and CXCL8: insights from siRNA knockdown studies and use of macrophages bearing a deletion in the HIF-1α gene

A: Immunoblots of HIF-1α or HIF-2 α in MDM lysates following their exposure to normoxia (20.9% O_2; 'N') or hypoxia (0.1% O_2; 'H') for 18h, or hypoxia for 18h following exposure to siRNA for HIF-1α ('1 α'), HIF-2 ('2α'), both HIFs 1α and 2α together ('1 α +2 α' or a scrambled control ('Scr'). Loading controls were β-actin. Below each gel picture is the densitometric analysis of HIF expression relative to its β-actin loading control. B & C: Effects of HIF-1α and 2α knockdown on the hypoxic induction of VEGF (B) and CXCL8 (IL-8; C) mRNA and protein. In the case of VEGF, gene expression was also assayed in normoxic and hypoxic BMDMs from conditional knockout HIF-1 $\alpha^{-/-}$ mice *in vitro* by qRT-PCR (right hand panel

in B). It was not possible to do this for CXCL8 as this gene is not expressed in mice. Pooled data from 6 replicate experiments are shown. * $p<0.05$ compared to corresponding normoxic group; ^ $P<0.05$ compared to the scr siRNA/hypoxia group; + $P<0.05$ compared to macrophages from wt mice exposed to hypoxia.

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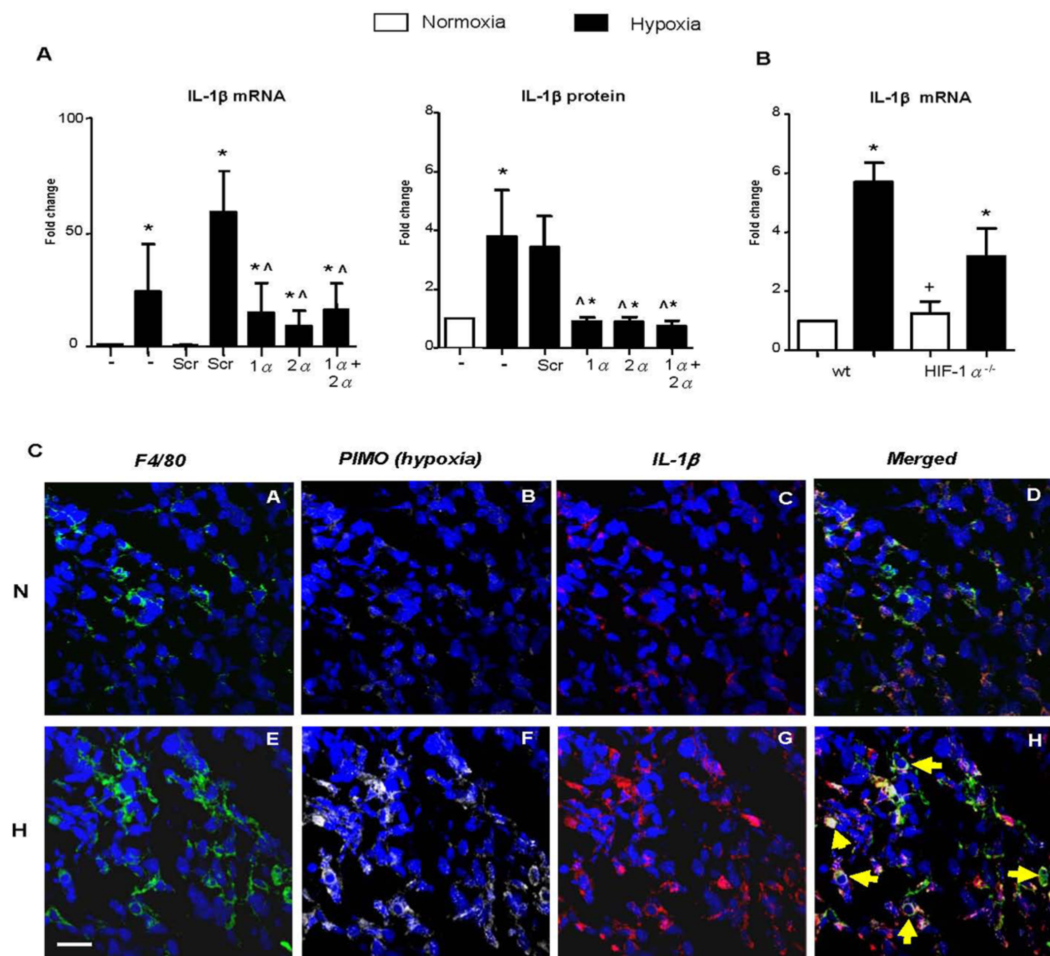

Figure 2. Hypoxic upregulation of IL-1β by human MDMs *in vitro* and by TAMs in hypoxic areas of murine mammary tumors: role of HIFs 1 and 2

A: IL-1β mRNA levels and protein release by human MDMs following their exposure to normoxia (20.9% O_2; 'N') or hypoxia (0.1% O_2; 'H') for 18h, or hypoxia for 18h following exposure to siRNA for HIF-1 α ('1 α'), HIF-2α ('2 α'), both HIFs 1 α and 2 α together ('1 α +2 α') or a scrambled control ('Scr'). *B:* Hypoxic induction of IL-1β mRNA by bone marrow-derived macrophages derived from wt or HIF-1α−/− mice. *C:* Upregulated expression of IL-1β by F4/80+ macrophages in pimonodazole-stained (ie. hypoxic; 'H') compared to pimonodazole-unstained (ie. normoxic; 'N') areas of murine mammary (4T1) tumours (see yellow arrows on the merged 'H' image). Pooled data from 3 replicate experiments are shown. * $p<0.05$ compared to corresponding normoxic group; ^ $P<0.05$ compared to the Scr siRNA/ hypoxia group; + $P<0.05$ compared to macrophages from wt mice exposed to hypoxia.

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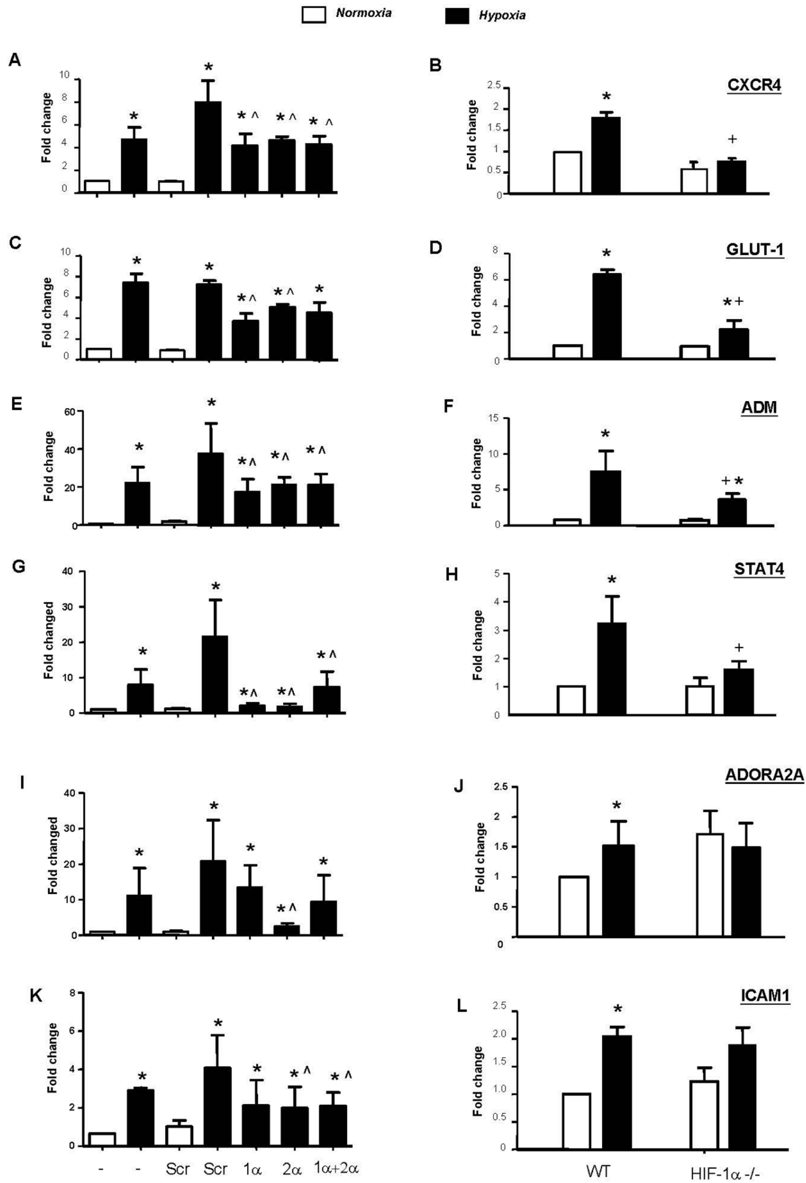

Figure 3. Role of HIFs 1α and 2α in the hypoxic induction of other key genes by macrophages

Panels A, C, E, G, I & K: hypoxic induction of mRNA for CXCR4, GLUT-1, ADM, STAT4, ADORA2A and ICAM1 (as measured by qRT-PCR) following exposure of primary human macrophages to normoxia (20.9% O_2; 'N') or hypoxia (0.1% O_2; 'H') for 18h, or hypoxia following exposure to siRNA for HIF-1 α ('1 α'), HIF-2 α ('2 α'), both HIFs 1 α and 2 α together ('1 α +2 α') or a scrambled control ('Scr'). *Panels B, D, F, H, J & L:* hypoxic induction of the same genes in murine BMDMs from wt and HIF-1α−/− mice. Pooled data from 3 replicate experiments are shown. *p<0.05 compared to corresponding normoxic group; ^P<0.05 compared to the Scr siRNA/hypoxia group; +P<0.05 compared to macrophages from wt mice exposed to hypoxia.

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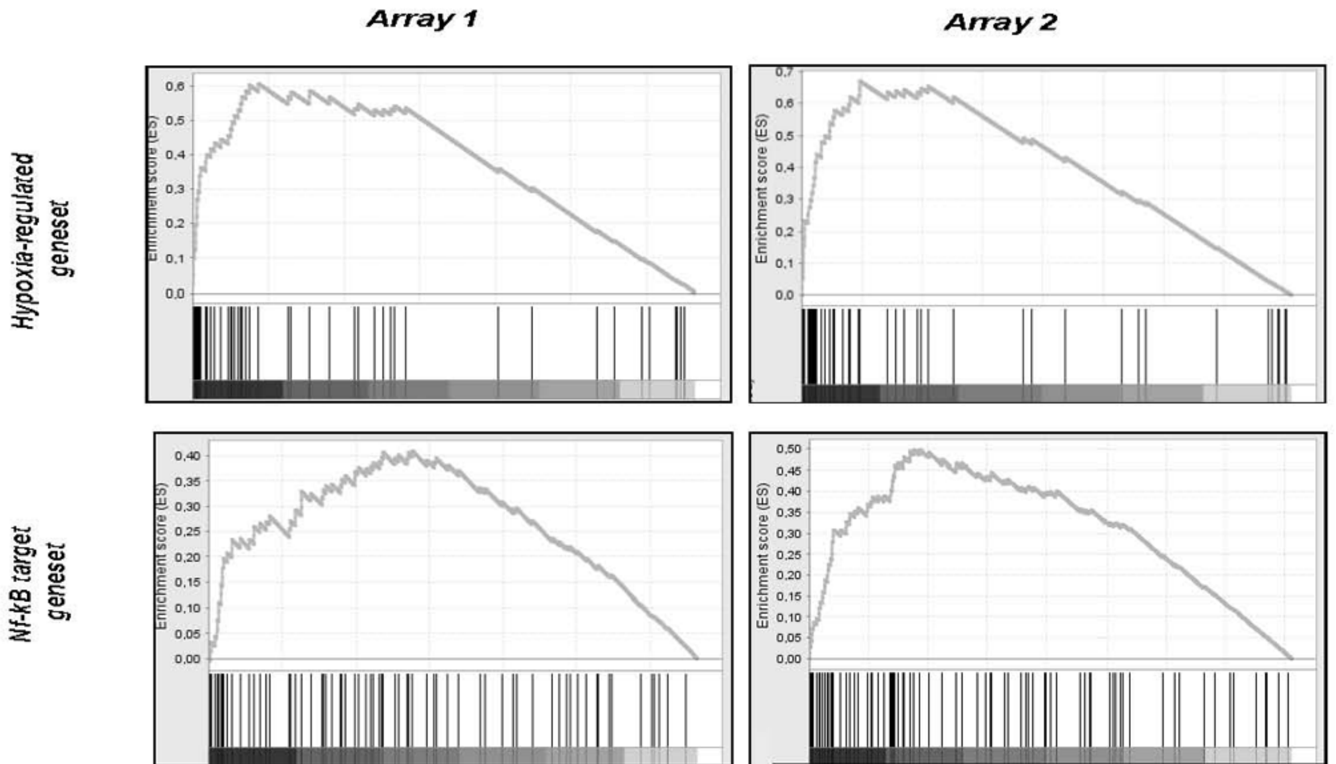

Figure 4. Gene enrichment analysis to compare key genes upregulated by hypoxia in human MDMs and known NF-κB -regulated genes

Hypoxia upregulated genes identified in two separate macrophage cultures using Affymetrix or Illumina microarrays were ranked by level of hypoxia-mediated induction. The ranked gene lists were then compared to both a previously published geneset for hypoxia-regulated genes in tumour cells (the '*hypoxia regulated*' gene set - top row) or genes previously shown to be NFκB target genes (the '*NFκB target*' geneset – bottom row) by geneset enrichment analysis. The '*hypoxia regulated*' gene set (top row) was significantly enriched in the hypoxic macrophage geneset identified on both the Affymetrix ('Array 1'; NES=2.2, q<0.001) and the Illumina ('Array 2'; NES=2.24, q<0.001) arrays. The NFκB target gene set (lower row) was also enriched in the hypoxic macrophage geneset on both Affymetrix (NES=1.69, q=0.02) and Illumina (NES=1.67, q=0.12) arrays.

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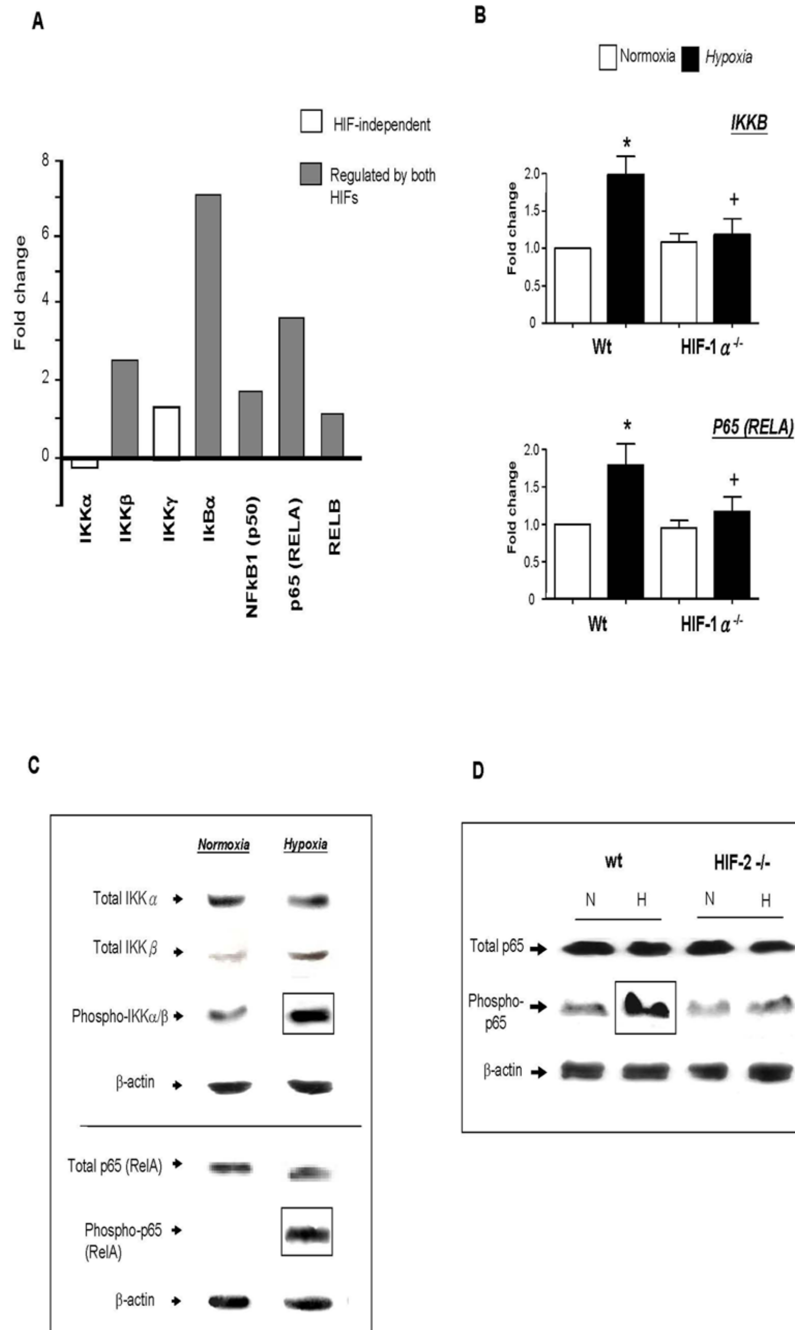

Figure 5. Effect of hypoxia on the expression and/or phosphorylation of components of the canonical NF-κB signaling pathway in MDMs: regulation by HIFs 1α and 2α

Panel A: Fold induction (hypoxia (0.1% O_2)/normoxia (20.9% O_2) of mRNA levels for individual NF-κB signaling proteins in primary human MDMs. The contribution of both HIFs 1 and 2 to the regulation of many of these genes was also assessed using siRNA to knock down the expression of each α subunit in MDMs. *Panel B*: effect of normoxia (N) or hypoxia (0.1% O_2; H) for 18h on the expression of mRNA for IKKβ and p65 in murine bone marrow-derived macrophages from wt or HIF-1 α−/− mice. *p<0.05 compared to corresponding normoxic group; +P<0.05 compared to macrophages from wt mice exposed to hypoxia. *Panel C*: immunoblots showing the effect of exposure to normoxia or hypoxia (0.1% O_2) for 18h on the

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levels of total and phosphorylated IKKβ and p65/RelA in primary human MDMs. *Panel D*: effects of normoxic (N) or hypoxic (0.5% O_2; 'H') culture on the level of total or phosphorylated p65 protein in murine bone marrow-derived macrophages from wt or HIF-2α −/− mice. Similar results were obtained using BMDMs from wt and HIF-1−/− mice (data not shown).

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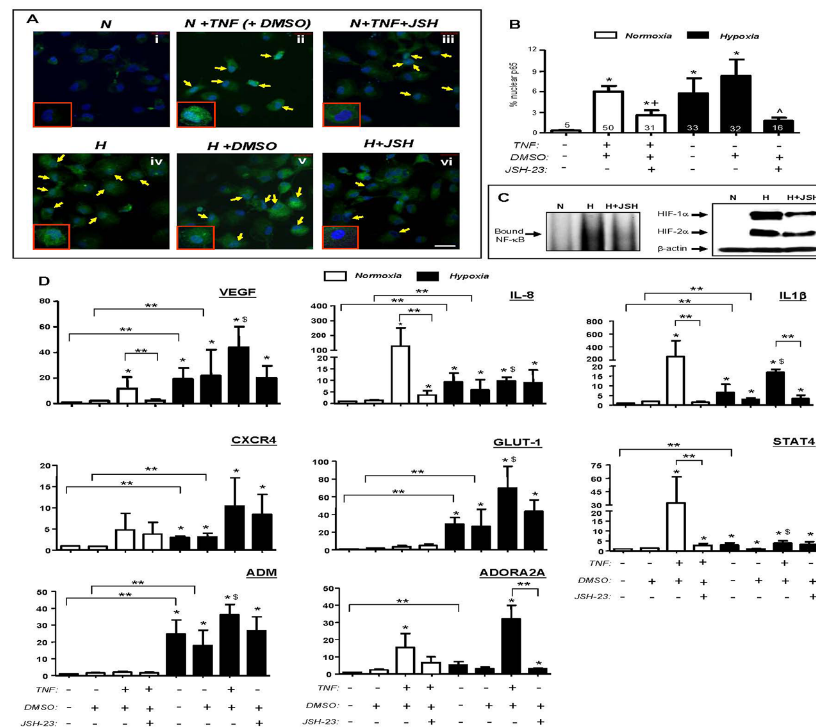

Figure 6. Inhibition of nuclear translocation of p65 has no effect on the hypoxic induction of various genes in human MDMs *in vitro*

Panels A & B: Effect of the p65 inhibitor, JSH-23, (or its vehicle, DMSO) on the nuclear translocation of p65 induced by TNFα or hypoxia by human MDMs. 'N' = normoxia; 'H' = hypoxia (0.1% O_2). The '% nuclear p65 immunofluorescence' = the % of the total, DAPI-stained (blue) area of MDM nuclei that was GFP+ (green). The figures at the base or just above each bar represents the average % of all MDM nuclei immunofluorescent for p65 (*panel B*). * P<0.05 w.r.t. 'normoxia alone' group; + P<0.05 w.r.t TNFα + DMSO group. ^ P<0.05 w.r.t. hypoxia + DMSO group. *Panel C*: effect of JSH-23 on NF-κB binding and accumulation of HIFs 1 and 2α in hypoxic MDMs (N = normoxia; H = hypoxia, H + JSH-23 = hypoxia following JSH.23 treatment. All three groups received the vehicle for JSH-23, DMSO); (i) left panel: electromobility shift assay showing NF-κB binding to a DNA consensus sequence, and (ii) right hand panel: immunoblots for HIFs 1 and 2α. *Panel D*: Effect of JSH-23 blockade of p65 function on the fold induction of VEGF, CXCL8, IL-1β, CXCR4, GLUT-1, STAT4, ADM and ADORA2A by TNFα or hypoxia. *P<0.05 w.r.t. 'normoxia with DMSO' alone; ** P<0.05 w.r.t. group indicated; $ P<0.05 w.r.t. TNF + DMSO group. Pooled data from 3 replicate experiments are shown.

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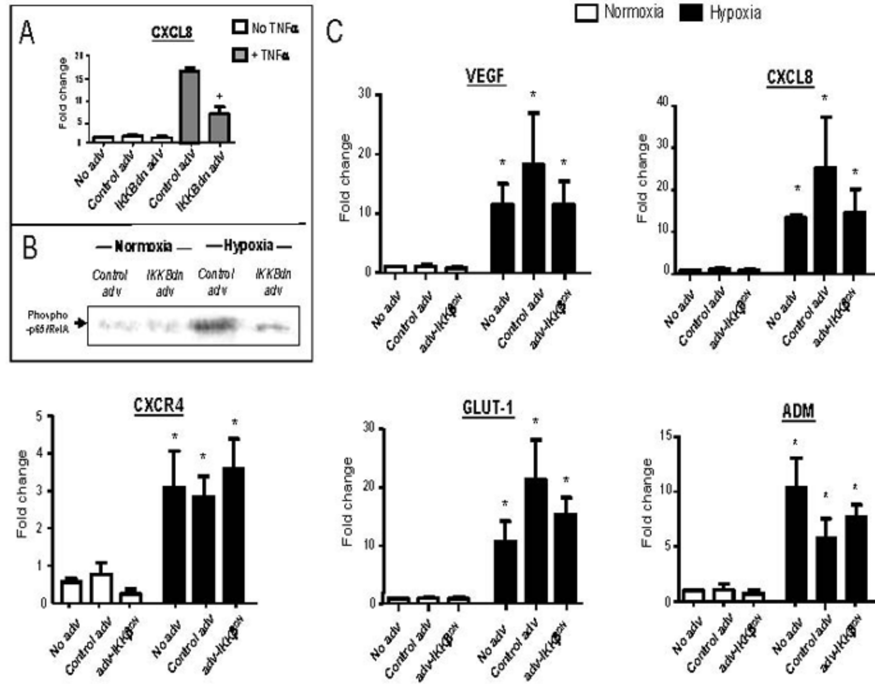

Figure 7. IKKβ inhibition has no effect on the hypoxic induction of various genes in human MDMs *in vitro*: use of a recombinant adenovirus expressing a dominant negative inhibitor of IKKβ (adv-IKKβ^{DN})

MDM infection with adv-IKKβ^{DN} (but not control adv) significantly inhibited both TNFα-induced gene expression of CXCL8 (Panel *A*) and hypoxia-induced nuclear accumulation of phospho-p65/RelA (Panel B) by human MDM. Panel *C*: Hypoxia significantly increased the expression of VEGF, CXCL8, CXCR4, GLUT-1 and ADM mRNA in untreated and adenovirally-infected MDM compared to respective normoxic MDM controls. However, there was no significant difference in the expression of these genes between hypoxic MDM infected with adv-IKKβ^{DN} or the control adenovirus. N=3. * $P<0.05$ w.r.t. respective normoxic group, + $P<0.05$ w.r.t TNFα + control adenovirus group.

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Table 1

Selected genes *upregulated* by hypoxia in human monocyte-derived macrophages *in vitro*.

Gene Symbol	Full Name	Accession No.	Main Functions of Gene Product	Hypoxic Induction (fold change)	
				Array 1 (Affymetrix)	Array 2 (Illumina)
Cytokines, & their receptors					
IL-6*	Interleukin 6 (interferon, beta 2)	NM_000600	Regulates host defence, acute phase reactions, immune responses, inflammation, cell proliferation, hematopoiesis, angiogenesis and metastasis	62	7
IL-23A*	Interleukin 23, alpha (subunit p19)	NM_016584	Subunit of IL-23. Regulates T cells & promotes angiogenesis	31	23
IL-1α*	Interleukin 1 alpha	NM_000575	Multifunctional, pro-inflammatory, proangiogenic cytokine.	26	12
VEGFA*^	Vascular endothelial growth factor A	NM_003376	Stimulates angiogenesis & chemotactic for monocytes & other myeloid cells	14	14
TNFA*	Tumor necrosis factor alpha (TNF superfamily, member 2; cachexin)	NM_000594	Regulates immunity to pathogen, inflammation, apoptosis & proliferation, differentiation, angiogenesis	11	32
WNT5A	Wingless-type MMTV integration site family, member 5A	NM_003392	Binds to receptor, frizzled-5, and regulates tumor cell migration/invasion	11	10
IL-12B*	Interleukin 12B (p40 subunit, natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2)	NM_002187	A subunit of the cytokine, IL-12, which activates T cells. Also anti-angiogenic factor.	9	10
IL-1β*	Interleukin 1 beta	NM_000576	Multifuctional, pro-inflammatory, proangiogenic cytokine.	9	8
ADM*	Adrenomedullin	NM_001124	Vasodilator. Also regulates cell responses to oxidative stress & hypoxic injury. Pro-angiogenic cytokine.	8	17
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	NM_007115	Hyaluronan-binding protein involved in extracellular matrix stability and cell migration	5	17
MIF*	Macrophage migration inhibitory factor	NM_002415	Pleoitrophic cytokine with multiple effects on inflammation	4	4

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Gene Symbol	Full Name	Accession No.	Main Functions of Gene Product	Hypoxic Induction (fold change)	
				Array 1 (Affymetrix)	Array 2 (Illumina)
			including immobilising macrophages		
IL-18*	Interleukin 18 (interferon-gamma-inducing factor)	NM_001562	Pro-inflammatory cytokine that stimulates T and NK cells to secrete interferon-γ (IFN-γ).	3	2
IL-8*	Interleukin 8	NM_000584	Pro-angiogenic & chemoattractant for neutrophils	2	43
EDN1*	Endothelin 1	NM_001955	Vasoconstrictor. Regulates vascular homeostasis. Chemotactic for monocytes	2	4
CLCF1	Cardiotrophin-like cytokine factor 1 (Neurotrophin-1)	NM_013246	IL-6 family protein. Stimulates IL-1 via IL-6R and STAT3. Also stimulates B-cells functions.	2	5
CSF2*	Colony stimulating factor 2 (GM-CSF)	NM_000758	Stimulates stem cells in the bone marrow to produce granulocytes and monocytes.	10	9
ANGPT2*	Angiopoietin 2 (Ang-2)	NM_001147	Pro-angiogenic cytokine. Destabilises blood vessels. Chemoattractant for endothelial cells and Tie2+ monocytes.	2	3
Chemokines & their receptors					
CCL20*	Chemokine (C-C motif) ligand 20 (MIP-3)	NM_004591	Chemotactic for lymphocytes and neutrophils	34	18
CXCL2*	Chemokine (C-X-C motif) 2 (MIP-2α)	NM_002089	Chemotactic for neutrophils and hematopoietic stem cells	23	4
CXCL1*	Chemokine (C-X-C motif) 1 (MSGA-α)	NM_001511	Chemotactic for neutrophils	20	27
CCR7*	Chemokine (C-C motif) receptor 7	NM_001838	Receptor for chemokines, CCL19 and CCL21	10	52
CCL5*	Chemokine (C-C motif) 5 (Rantes)	NM_002985	Chemotactic for T cells, eosinophils, and basophils; recruits leukocytes to inflammatory sites	9	10
CCL3*	Chemokine (C-C motif) 3 (MIP-1a)	NM_002983	Recruitment and activation of neutrophils	3	6
CXCR4*	Chemokine (C-X-C motif) receptor 4	NM_003467	Receptor for SDF-1 (CXCL12) – which regulates hematpoietic stemm and myeloid cell recruitment by tissues. Involved in proliferation and metatasis of	2	6

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Gene Symbol	Full Name	Accession No.	Main Functions of Gene Product	Hypoxic Induction (fold change)	
				Array 1 (Affymetrix)	Array 2 (Illumina)
			tumor cells. Co-receptor for entry of HIV into T cells.		
Intracellular enzymes & metabolism					
EGLN3	HIF prolyl hydroxylase 3 (PHD3)	NM_022073	One of 3 PHD enzymes that hydroxylate HIFs, resulting in their binding to VHL and degradation. Regulates HIF-2α more than HIF-1α.	54	12
CA12	Carbonic anhydrase XII	NM_001218	Enzyme that catalyzes the reversible hydration of carbon dioxide. Acidifies extracellular milieu o tumour cells, stimulating their growth/invasion.	32	81
ALDOC	Aldolase C, fructose-bisphosphate	NM_005165	Glycolytic enzyme. Catalyzes the breakdown of fructose 1,6-bisphosphate.	23	23
SLC2A5*	Solute carrier family 2 (facilitated glucose/fructose transporter), members 5 (GLUT-5)	NM_003039	Transports fructose and glucose into the cell.	12	11
NCF1	Neutrophil cytosolic factor 1	NM_000265	Superoxide production	8	6
SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1 (GLUT-1)	NM_006516	Transports glucose into the cell.	7	23
HMOX1*	Heme oxygenase (decycling) 1	NM_002133	Essential enzyme in heme catabolism -cleaves heme to form biliverdin	7	4
SLC2A6	Solute carrier family 2 (facilitated glucose transporter), member 6 (GLUT-6)	NM_017585	Transports glucose into the cell.	6	3
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3 (GLUT-3)	NM_006931	Transports glucose into the cell.	5	14
PFKFB3	6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3	NM_004566	Synthesis and degradation of fructose 2, 6-bisphosphate	4	7
SLC7A5	Solute carrier family 7 (cationic amino acid transporter, y + system), member 5	NM_003486	Involved in cellular amino acid uptake.	3	3
PFKP	Phosphofructokinase	NM_002627	Glycolytic enzyme	2	3
HK1	Hexokinase 1	NM_000188	Commits glucose to the glycolytic pathway	2	2
ALDOA	Aldolase A	NM_000034	Glycolytic enzyme. Catalyzes the breakdown of fructose 1, 6-bisphosphate	2	2

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Gene Symbol	Full Name	Accession No.	Main Functions of Gene Product	Hypoxic Induction (fold change)	
				Array 1 (Affymetrix)	Array 2 (Illumina)
EGLN1*	HIF prolyl hydroxylase 2 (PHD2)	NM_022051	One of 3 PHD enzymes that hydroxylate HIFs, resulting in their binding to VHL and degradation. Regulates HIF-1α more than HIF-2α.	2	5
Extracellular enzymes/molecules					
SERPINE1	Serpin peptidase inhibitor	NM_000602	Regulation of fibrinolysis	7	5
ADAM 8	ADAM metallopeptidase domain 8	NM_001109	Membrane-anchored protein involved cell-cell and cell-matrix interactions	6	5
CFB	Complement factor B	NM_001710	A component of the alternative pathway of complement activation.	6	5
F3*	Coagulation factor III (Thromboplastin, tissue factor; CD 142)	NM_001993	Cell surface glycoprotein that cleaves prothrombin to thrombin, promoting coagulation.	3	7
TIMP1	TIMP metallopeptidase inhibitor 1	NM_003254	Inhibits activity of most known MMPs. Stimulates proliferation in a wide range of cell types. Also anti-apoptotic.	2	3
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	NM_002423	Enzyme with broad substrate specificity in ECM. Promotes wound healing, angiogenesis, tumour invasion and metastasis.	2	2
Cell viability					
SERPINB2*	Serpin peptidase inhibitor, clade B (ovalbumin), member 2 (PAI-2)	NM_002575	Inhibits serine protease, tissue-type-and urokinase-type plasminogen activator; tPA, uPA. Also regulates gene expression, cell proliferation, differentiation, and apoptosis.	102	4
PTGS2 (COX2)*	Prostaglandin-endoperoxide synthase 2 (cyclo-oxygenase-2)	NM_000963	Pro-inflammatory - stimulates expression of prostanoids	27	8
EN02	Enolase 2	NM_001975	Glycolytic enzyme	8	10
IGFBP6	Insulin-like growth factor binding protein 6	NM_002178	Binds and prolong the half-life of the IGFs.	4	2
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	NM_004331	Pro-apoptotic Bcl family protein.	3	5

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Gene Symbol	Full Name	Accession No.	Main Functions of Gene Product	Hypoxic Induction (fold change)	
				Array 1 (Affymetrix)	Array 2 (Illumina)
IER3*	Immediate early response 3 (IEX-2orDIF-2)	NM_003897	Regulates growth and apoptosis (inhibits NF-kB induced apoptosis)	2	13
NRG1*	Neuroregulin 1	NM_006096	Growth factor which regulates cell apoptosis and proliferation (protects cells in ischemia).	2	4
Receptors & cell adhesion/ signalling					
DDIT4	DNA-damage-inducible transcript 4 (DIG2 or REDD-1)	NM_019058	A stress response gene, an essential regulator of the checkpoint kinase, mTOR.	103	54
HIG2	Hypoxia-inducible protein 2	NM_001098786	Growth factor that stimulates tumour cell growth.	12	21
NFKB2*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NM_002502	Central mediator of non-canonical NFkB signalling pathway in cells	11	3
TRAF1*	TNF receptor-associated factor 1	NM_005658	One of the TNFR members - forms a complex with TRAF2 to activate MAPK8/JNK and NF-kappaB. Mediates the anti-apoptotic signals from TNF receptors	9	8
ADORA2A*	Adenosine A2a receptor	NM_000675	Receptor for adenosine.	8	33
TREM1*	Triggering receptor expressed on myeloid cells 1	NM_018643	Receptor of lg superfamily expressed on human myeloid cells. Regulates their inflammatory functions.	5	6
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	NM_005204	Cell signalling & cycling	4	6
TNS1	Tensin 1	NM_022648	Involved in cell migration and links signal transduction pathways to the cytoskeleton.	3	7
TNIP2	TNFAIP3 interacting protein 2 (ABIN-2)	NM_024309	Inhibits NF-kappa-B activation.	3	4
RelA (p65)	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 3(p65/RelA)	NM_021975	Central mediator of canonical NFkB signalling pathway in cells	3	3
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B (TNF-R2 or CD120P)	NM_001066	One of 2 main TNFRs (I and II) mediating the effects of TNFalpha on cells.	3	2

Gene Symbol	Full Name	Accession No.	Main Functions of Gene Product	Hypoxic Induction (fold change)	
				Array 1 (Affymetrix)	Array 2 (Illumina)
TNFAIP3*	Tumor necrosis factor, alpha-induced protein 3	NM_006290	Interacts with NAF1 & inhibits TNFalpha-induced NF-kappa-B-dependent gene expression	4	5
ANPEP	Aminopeptidase M or N (CD13 or APN)	NM_001150	Zinc-binding metalloprotease.	3	2
STAT4	signal transducer and activator of transcription 4	NM_003151	Signal transduction and activation of transcription. Involved in IL12 signalling	4	4
STAT6	signal transducer and activator of transcription 6	NM_003153	Plays a central role in regulating the alternative (M2) activation of macrophages in response to interleukin 4	4	2
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2)	NM_005239	Transcription factor that regulates MMP-9 expression in macrophages and drives mammary tumor progression	3	3
NFKBIE*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	NM_004556	Inhibits DNA-binding of NF-kappa-B p50-p65 & p50-c-Rel complexes.	2	2
NFKBIA*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM_020529	Inhibits the activity of dim eric NF-kappa-B/REL complexes by trapping REL dimers in the cytoplasm	2	3
IRAK3	Interleukin-1 receptor-associated kinase 3	NM 007199	Inhibits dissociation of IRAK1 & IRAK4 from the Toll-like receptor signalling complex	2	4
RIPK2	Receptor-interacting serine-threonine kinase 2	NM_003821	Apoptosis & activates NFkB.	2	4
Transcription, translation & RNA processing					
ERO1L	Endoplasmic reticulum oxidoreductin-1 (Ero1)-L alpha	NM_014584	Endoplasmic reticular enzyme that helps to maintain VEGF secretion under hypoxia.	3	6
HSPA1B	Heat shock 70kDa protein 1B	NM_005346	Intracellular enzyme. Stabilizes existing proteins against aggregation & mediates the folding of newly translated proteins.	3	3
BTG1	B-cell translocation gene 1	NM_001731	Anti-proliferative protein	3	2
ELL2	Elongation factor, RNA polymerase II, 2	NM_012081	Increase the catalytic rate of RNA polymerase II transcription	2	2

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[*] Genes shown previously to be regulated by NF-κB (or where the gene promoters contain NF-κB binding sites).

^ It is currently debatable as to whether the human VEGFA gene is an NF-kB target gene as there is not a well characterized kB site in its promoter. In mice, there is a putative kB site but it is not clear if it is functional. Gene names that are underlined are ones that were also shown in the present study to be upregulated by hypoxia using qRT-PCR.

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Table 2

Selected genes *downregulated* by hypoxia in human monocyte-derived macrophages in vitro.

Gene Symbol	Full Name	Accession No.	Main Function of Gene Product	*Hypoxic Downregulation (fold change)*	
				Array 1 (Affymetrix)	**Array 2 (Illumina)**
Cell adhesion and cell junction molecules					
CD36	CD36 molecule (thrombospondin receptor)	NM_000072	A multi-functional class B scavenger receptor - binds thrombospondin, apoptotic cells and LDLs	0.16	0.17
VCL	Vinculin	NM_014000	A cytoskeletal protein associated with cell-cell and cell-matrix junctions. Involved in cell adhesion, cell morphology and locomotion	0.42	0.65
PECAM1	Platelet/endothelial cell adhesion molecule (CD31 antigen)	NM_000442	Surface receptor expressed by endothelial cells, platelets and various other cells. Helps macrophages to remove aged neutrophils.	0.62	0.52
Cell Metabolism					
ACAT1	Acetyl-Coenzyme A acetyltransferase 1	NM_000019	Plays a role in lipoprotein assembly and dietary cholesterol absorption	0.25	0.38
NME1	Non-metastatic cells 1 protein (NM23A)	NM_000269	A nucleoside diphosphate kinase linked to metastasis suppression in some cell types.	0.29	0.58
PDHB	Pyruvate dehydrogenase (lipoamide) beta	NM_000925	Catalyzes the overall conversion of pyruvate to acetyl-CoA and CO(2)	0.37	0.49
ST3GAL5	ST3 beta-galactoside alpha-2,3-sialyltransferase 5	NM_003896	Promotes cell differentiation, modulation of cell proliferation, signal transduction, and integrin-mediated cell adhesion.	0.40	0.54
LYPLA3	lysophospholipase 3 (lysosomal phospholipase A2)	NM_012320	Regulates the multifunctional lysophospholipids in cell membranes	0.49	0.48
Intracellular transport					
TOMM22	Translocase of outer mitochondrial membrane 22 homolog (yeast)	NM_020243	Mitochondrial membrane protein. Imports cytosolic preproteins into the mitochondrion.	0.26	0.53
HLA-DMB	Major histocompatibility complex, class II, DM beta	NM_002118	Plays a central role in the peptide loading of MHC class II molecules.	0.29	0.60
SLC17A5	Solute carrier family 17 (anion/sugar transporter), member 5	NM_012434	Primary solute translocator for anionic substances	0.34	0.57

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Gene Symbol	Full Name	Accession No.	Main Function of Gene Product	*Hypoxic Downregulation (fold change)*	
				Array 1 (Affymetrix)	**Array 2 (Illumina)**
ST6GAL1*	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	NM_003032	Transfers sialic acid from the donor of substrate CMP-sialic acid to galactose containing acceptor substrates	0.42	0.31
MRPL3	Mitochondrial ribosomal protein L3	NM_007208	Helps with protein synthesis within the mitochondrion	0.47	0.50
Receptors & cell signalling					
TNFSF13B*	Tumor necrosis factor (ligand) superfamily, member 13b	NM_006573	Promotes cell proliferation	0.39	0.39
RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	NM_015376	Guanine nucleotide exchange factor (GEF) for Ras and Rap1	0.13	0.38
TFRC	Transferrin receptor (p90, CD71)	NM_003234	Regulates iellular uptake of iron and iron metabolism	0.25	0.19
HIF-1A	Hypoxia-inducible factor, alpha subunit (HIF-2α)	NM_001530	Regulates response of cells to hypoxia	0.36	0.21
EPAS1	Endothelial PAS domain protein 1 (HIF-2α)	NM_001430	Regulates response of cells to hypoxia	0.33	0.21
TLR4	Toll-like receptor 4	NM_003266	Cell surface receptor that binds many ligands including bacterial LPS and fibrinogen	0.26	0.63
MAPRE2	Micro tubule-associated protein, RP/EB family, member 2	NM_014268	Involved in microtubule polymerization, cell migration	0.35	0.52
CTSC	Cathepsin C	NM_001814	A lysosomal cysteine proteinase that activates many serine proteinases in immune/inflammatory cells	0.41	0.48
PRCP	Prolylcarboxypeptidase (angiotensinase C)	NM_005040	A lysosomal prolylcarboxypeptidase, which cleaves C-terminal amino acids linked to proline in peptides	0.43	0.48
SPARC	Secreted protein, acidic,	NM_003118	A calcium binding glycoprotein that also	0.46	0.26

* genes shown previously to be regulated by NF-κB (or where the gene promoters contain NF-κB binding sites).