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Macrophage Migration Inhibitory Factor Counterregulates Dexamethasone-Mediated Suppression of Hypoxia-Inducible Factor-1α Function and Differentially Influences Human CD4$^+$ T Cell Proliferation under Hypoxia

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Hypoxia, a feature of inflammation and tumors, is a potent inducer of the proinflammatory cytokine macrophage migration inhibitory factor (MIF). In transformed cells, MIF was shown to modulate and to be modulated via the oxygen-sensitive transcription factor hypoxia-inducible factor (HIF)-1. Furthermore, anti-inflammatory glucocorticoids (GCs) were described to regulate MIF action. However, in-depth studies of the interaction between MIF and HIF-1 and GC action in nontransformed primary human CD4$^+$ T cells under hypoxia are missing. Therefore, we investigated the functional relationship between MIF and HIF and the impact of the GC dexamethasone (DEX) on these key players of inflammation in human CD4$^+$ T cells. In this article, we show that hypoxia, and specifically HIF-1, is a potent and rapid inducer of MIF expression in primary human CD4$^+$ T cells, as well as in Jurkat T cells. MIF signaling via CD74, in turn, is essential for hypoxia-mediated HIF-1α expression and HIF-1 target gene induction involving ERK/mammalian target of rapamycin activity complemented by PI3K activation upon mitogen stimulation. Furthermore, MIF signaling enhances T cell proliferation under normoxia but not hypoxia. MIF also counterregulates DEX-mediated suppression of MIF and HIF-1α expression. Based on these data, we suggest that hypoxia significantly affects the expression of HIF-1α in a MIF-dependent manner leading to a positive-feedback loop in primary human CD4$^+$ T cells, thus influencing the lymphoproliferative response and DEX action via the GC receptor. Therefore, we suggest that HIF and/or MIF could be useful targets to optimize GC therapy when treating inflammation. *The Journal of Immunology*, **2011, 186: 764–774.**

A common feature of injured tissues, tumors, and inflammation is the change in oxygen tension toward hypoxia: oxygen supply is unable to meet the cellular demand as a result of an injured vascular network, tumor cell progression, and/or distinct influx of metabolically active inflammatory cells (1). In response to hypoxia, major transcriptional changes occur leading to cellular adaptation processes that are controlled by the transcription factor hypoxia-inducible factor (HIF)-1. HIF-1 is a heterodimeric protein that is composed of an oxygen-sensitive α subunit and a constitutively expressed β subunit. In nonhypoxic cells, HIF-1α is tagged by O_2-dependent hydroxylation via prolyl hydroxylases (PHDs)1–3, prior to binding of the von Hippel–Lindau tumor-suppressor protein (pVHL), which targets HIF-1α for proteasomal degradation. Another mechanism of inhibiting HIF-1α function is mediated by factor inhibiting HIF (FIH), which prevents the transcriptional activation of HIF-1α by blocking interaction with the coactivators p300 and CREB binding protein (2). However, under hypoxia, HIF-1 ensures cellular adaptation and functional integrity by inducing/enhancing the synthesis of proteins, such as erythropoietin, glucose transporter type 1, glycolytic enzymes (PGK1 and LDHA), vascular endothelial growth factor (VEGF), and matrix metalloproteinases, which are implicated in angiogenesis, energy metabolism, apoptosis, metastasis, and invasion (2). Thus, HIF-1 links cancer and inflammation.

Macrophage migration inhibitory factor (MIF) is a multifunctional protein involved in a broad range of inflammatory activities. MIF counteracts the anti-inflammatory effects of glucocorticoids (GCs), participates in the regulation of cell proliferation and differentiation, and plays a role in the progress of septic shock, chronic inflammation, tissue damage, and autoimmune diseases (3). Because inflammation is a critical component of tumor progression,

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The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; DEX, dexamethasone; DRFZ, German Rheumatism Research Center; FIH, factor inhibiting hypoxia-inducible factor; GC, glucocorticoid; GCR, glucocorticoid receptor; GIF, glycosylation-inhibiting factor; HIF, hypoxia-inducible factor; HRE, hypoxia response element; MIF, macrophage migration inhibitory factor; mTOR, mammalian target of rapamycin; PBA, PBS/BSA/azide; PHA-L, phytohemagglutinin-L; PHD, prolyl hydroxylase; pVHL, von Hippel–Lindau tumor-suppressor protein; qPCR, quantitative real-time PCR; rh, recombinant human; shRNA, short hairpin RNA; VEGF, vascular endothelial growth factor.

MIF represents an important link between inflammation and tumor biology, similar to HIF.

The functional relationship between HIF-1 and MIF has been investigated in human cancer cell lines and in murine cancer models, indicating an HIF-dependent, as well as an HIF-independent, induction of MIF (4, 5) and an indirect protein interaction of HIF and MIF (6, 7). In our study, we investigated the HIF/MIF interaction, focusing on nontransformed primary human cells of the adaptive immune response; additionally, we addressed the effect of GCs under hypoxic conditions on this relationship.

GCs represent a very important and frequently used class of anti-inflammatory and immunosuppressive drugs. Our understanding of the action of GCs has advanced in the last few years with regard to recent insights into signaling, regulation of transcription processes, and gene expression (genomic GC effects); dosage–plasma level–effect relationships; nongenomic GC effects; and new GC receptor (GCR) ligands (8, 9). However, there is only scattered information available on their effects under conditions of restricted oxygen availability. This is surprising because this topic is of clinical importance for the reasons given above. Controversial results obtained from tumor cell lines with known defects in cell-signaling transduction and transcriptional regulation point to a cell type-specific GC action on HIF-1–mediated function under hypoxia (10–12).

To our knowledge, this is the first report of a functional relationship between MIF and HIF-1 in primary human Th cells, with focus on the interplay with the synthetic GC dexamethasone (DEX). The results obtained increase our knowledge about immune cell adaptation toward hypoxic areas (e.g., tumors, inflamed and injured tissues), which may be important with respect to impaired tissue regeneration, such as wound healing after GC therapy (13).

Based on our findings, we suggest that hypoxia affects $CD4^+$ Th cell function and regulation of HIF-1α expression in a MIF-dependent manner, thus influencing the IL-2–mediated lymphoproliferative response and DEX action via the GCR.

Materials and Methods

Abs and reagents

DEX, the GCR antagonist RU486, and phytohemagglutinin-L (PHA-L) were purchased from Sigma-Aldrich. For immunoblotting, mouse anti–HIF-1α mAb and anti–β-actin were purchased from BD Transduction Laboratories and Sigma-Aldrich. Anti-MIF and anti–HIF-2α were purchased from R&D Systems. Mouse anti-Jun B and anti–NF-κB(p65) mAbs and polyclonal goat anti-lamin B Ab were purchased from Santa Cruz Biotechnologies. Secondary HRP-labeled Abs, goat anti-mouse IgG, and donkey anti-goat IgG were obtained from Promega. For chromatin immunoprecipitation (ChIP), rabbit polyclonal anti-HIF-1α Ab was purchased from Abcam. For in vitro assays, recombinant human (rh)MIF was purchased from R&D Systems, and anti-CD74 blocking Ab was obtained from Santa Cruz Biotechnologies. For surface staining of CD3, CD4, CD25, and CD74, anti-human CD4-FITC and anti-human CD3-PE were obtained from the German Rheumatism Research Center (DRFZ), and anti-human CD25-allophycocyanin and anti-human CD74-Cy5 were purchased from ImmunoTools and Santa Cruz Biotechnologies; the latter was labeled at DRFZ. For intracellular staining of IL-2, FITC-conjugated anti-human IL-2 was obtained from BD Pharmingen.

Cell isolation and cell culture

Human $CD4^+$ T cells (>99% purity and >95% viability) were prepared as described previously (14). Cells were resuspended in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS (Sigma-Aldrich), 100 U/ml penicillin G, 100 μg/ml streptomycin (both from PAA Laboratories), and 50 μM 2-ME (Sigma-Aldrich).

Induction of hypoxia and stimulation

T cells were incubated in a hypoxic chamber (Binder) at 5% CO_2 and <1% O_2, balanced with N_2. Normoxic controls were incubated at 5% CO_2 in a humidified atmosphere with 18% O_2. Mitogen stimulation was done using 5 μg/ml PHA-L (Sigma-Aldrich).

For the analysis of rapid GC effects, $CD4^+$ T cells were incubated in a water-jacket chamber sealed with a Clark-type oxygen electrode (Strathkelvin Instruments, North Lanarkshire, U.K.), which continuously monitored cellular oxygen consumption as a decrease of the oxygen concentration. The Clark-type oxygen electrode has a small slot for introduction of a 22-gauge needle, which enabled us to apply drugs and obtain samples without influencing the oxygen concentration of the analyzed cell suspension. Detailed information is given in Supplemental Fig. 1.

RNA isolation and quantitative real-time PCR

After cell lysis, total RNA was extracted (RNeasy Mini Kit; Qiagen), and the quality was assessed on a Bioanalyzer (Agilent). The cDNA was synthesized by reverse transcription using TaqMan Reverse Transcription Reagents (Applied Biosystems). Quantitative real-time PCR (qPCR) was carried out using the LightCycler Fast-Start DNA Master SYBR Green I Kit (Roche). Data were normalized to the expression of β-actin (*ACTB*). As a second "so-called" housekeeping gene, *HPRT* was tested to verify the accuracy of normalization. All primers used were obtained from TIB Molbiol (Table I).

Immunoblot of HIF-1α, MIF, lamin B, and β-actin

Cell lysis. For whole-cell extracts of T cells, 10^6 cells were lysed in 20 μl Laemmli buffer. For the preparation of nuclei, the Nuclear Extract Kit from Active Motif was used on T cells, according to the manufacturer's instructions.

Immunodetection of proteins. Twenty microliters of whole-cell extract or 10 μg nuclear/cytoplasmic fraction was separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Millipore). Blotted proteins were analyzed, as indicated, and visualized by enzymatic chemiluminescence (Amersham Biosciences).

Quantification of cytokine production

Intracellular cytokine staining. Intracellular cytokine staining was performed based on the paraformaldehyde-saponin procedure (15). After PHA stimulation of $CD4^+$ T cells and 66 h of hypoxic incubation, the cells were restimulated for an additional 6 h with 10 ng/ml PMA and 1 μg/ml ionomycin. Finally, protein transport was blocked by adding brefeldin A at 10 μg/ml for the final 3 h to obtain intracellular cytokine accumulation. Cells were washed with PBS and fixed for 20 min at room temperature using 2% (v/v) formaldehyde (Merck KGaA). Cells were stained at room temperature for 15 min in PBS/BSA/azide (PBA) with 0.5% (w/v) saponin (Sigma-Aldrich). After blocking nonspecific binding with 5 mg/ml polyclonal human IgG (Grifols), we stained the cells for 15 min using 2.5 μg/ml FITC-conjugated anti-human IL-2 (clone MQ1-17H12, isotype $rIgG_{2a}$; BD Pharmingen). The cells were washed with PBA/saponin and then stored in PBA until FACS analysis.

Secreted MIF. Culture supernatants of $CD4^+$ T cells (10^6 cells/ml), incubated under normoxic or hypoxic conditions, were immediately frozen and stored at −70°C. Secreted MIF was quantified by multiplex suspension array (Bio-Rad).

Quantification of proliferation

CFSE (Molecular Probes Europe, Leiden, The Netherlands) was used to measure the quantitative extent of proliferation in the PHA-L–activated $CD4^+$ T cells. The cells were labeled before activation in a 1.5-μM solution for 3.5 min at room temperature. Cell activation was performed using PHA-L (5 μg/ml).

Lentiviral-based short hairpin RNA-mediated knockdown of HIF-1α

Based on pLentiLox 3.7 (Addgene plasmid 11795), short hairpin RNA (shRNA) constructs were generated by subcloning short hairpin oligonucleotides (Table II), as previously described (16). Lentiviral stocks were obtained by calcium phosphate cotransfection of HEK293 cells with the lentiviral packaging plasmids pVSVG and pPAX2. The medium was replaced after 4 h, and viral supernatants were collected 48–72 h later. Jurkat T cells were infected by a 90-min centrifugation at 700 × *g* at 37°C with viral supernatants and 8 μg/ml polybrene (Sigma-Aldrich), followed by replacement of the viral supernatant with fresh, fully supplemented culture medium after 4 h. shRNA construct-containing cells were enriched via coexpressed GFP using flow cytometry (>95% GFP^+). Enriched cells were used for 20-h hypoxic/normoxic incubation using the hypoxic incubator (Binder).

HIF-1 promoter activity

shRNA construct-containing Jurkat T cells were transfected with 1 μg/transfection Cignal HIF reporter plasmid mixture (Cignal HIF Reporter

[luc] Kit: CCS-007L; SA Biosciences). The HIF reporter plasmid mixture contains an HIF-responsive luciferase construct premixed in a 40:1 ratio with a constitutively expressing *Renilla* luciferase construct. The HIF-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and an optimized number of tandem repeats of the hypoxia response element (HRE). The *Renilla* luciferase construct serves as an internal control for normalizing transfection efficiencies and monitoring cell viability.

Transfection was performed using nucleofection (Amaxa), according to the manufacturer's instructions for Jurkat T cells (transfection reagent C; program C-016; Nucleoporator II). After 48 h of transfection, cells were incubated under hypoxia (1% O_2) for 20 h. Dual Luciferase assay (Promega) was performed, according to the manufacturer's instructions. Promoter activity values are expressed as arbitrary units using a *Renilla* reporter for internal normalization.

ChIP assay

$CD4^+$ T cells were incubated under hypoxic conditions for 20 h. ChIP assay was performed, according to the manufacturer's instructions (Upstate). The HIF-1–DNA fragments were precipitated by polyclonal HIF-1α rabbit Ab (Abcam). After removal of cross-linking, PCRs were performed with 50 ng DNA by initial denaturation of DNA at 95˚C for 3 min, followed by 40 amplification cycles (95˚C for 20 s, 63.7˚C for 15 s, and 72˚C for 30 s) and final elongation at 72˚C for 10 min. The primer sets used were MIF forward: 5′-GCGGTGACTTCCCCACTC-3′ and MIF reverse: 5′-ATGGCAGAAG-GACCAGGAG-3′ (chr22: 22,566,483–22,566,660).

Statistical analysis

Data are reported as the mean ± SD of at least three experiments. Differences between normally distributed groups were compared using the Student *t* test, and those in nonnormally distributed groups were compared with the Mann–Whitney *U* test for independent groups and with the Wilcoxon *t* test for dependent samples. Multiple comparisons were analyzed by one- or two-way ANOVA, as indicated, with the Bonferroni multiple-comparison post hoc test. The *p* values <0.05 were considered statistically significant.

Results

HIF-1α and HIF-1 target genes are induced under hypoxia in primary human $CD4^+$ T cells and Jurkat T cells

To clarify the relationship between HIF and MIF in T lymphocytes, we first analyzed whether Th cells are capable of expressing HIF-1α, the master regulator of the hypoxic response, under hypoxia alone or only upon hypoxia plus mitogenic stimulation. Therefore, we cultured isolated peripheral blood $CD3^+CD4^+$ T cells, untreated or treated with PHA, under normoxic or hypoxic conditions. At the transcriptional level, we found no significant differences in the amounts of HIF-1α mRNA (*HIF1A*) after 6 h or even after 48 h under hypoxia compared with normoxia (Fig. 1*B*, Supplemental Fig. 2). At the protein level, we observed an induction of HIF-1α in quiescent cells and a translocation of HIF-1α to the nucleus, as shown by immunoblot (Fig. 1*A*). Upon PHA stimulation under hypoxia, HIF-1α expression strongly increased in the nucleus and in the cytoplasmic fraction (Fig. 1*A*). We also observed that this increase in HIF-1α expression in the nucleus under hypoxia resulted in a significant induction of HIF-1 target gene transcription, such as shown for *GLUT1*, *LDHA*, *PGK1*, and *VEGF* (Fig. 1*B*). In contrast, HIF-2α protein was not expressed in unstimulated or PHA-stimulated primary human $CD4^+$ T cells under normoxic or hypoxic incubation conditions (Supplemental Fig. 3).

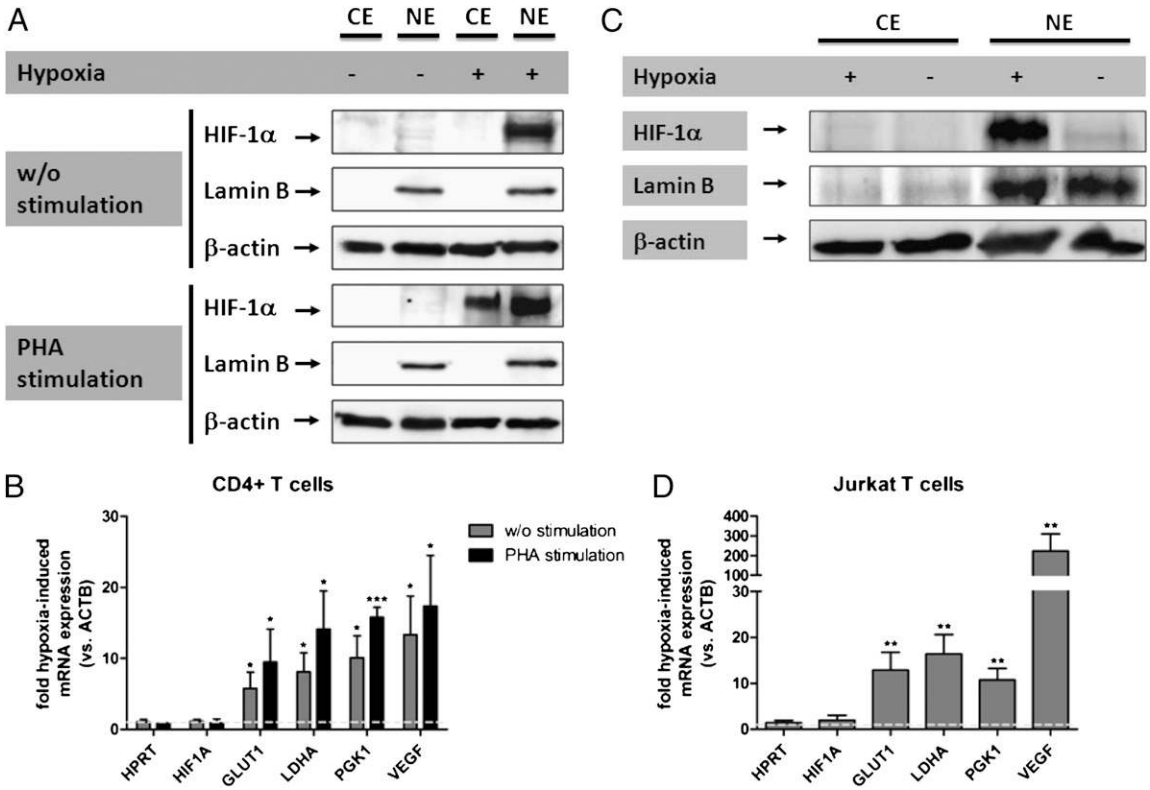

FIGURE 1. HIF-1α and HIF-1 target genes are induced under hypoxia in primary human $CD4^+$ T cells and Jurkat T cells. *A*, Primary human $CD4^+$ T cells derived from peripheral blood were incubated for 6 h under normoxic or hypoxic conditions and stimulated with PHA-L (5 μg/ml) or left untreated. Nuclear extracts (NE) and cytosolic extracts (CE) were analyzed by immunodetection for HIF-1α, lamin B, and β-actin (one of three independently performed experiments). *B*, As in *A*, mRNA was obtained and analyzed for *HPRT* and *HIF1A*, as well as HIF-1 targets *GLUT1*, *LDHA*, *PGK1*, and *VEGF* by qPCR normalized to *ACTB* and normoxia (mean ± SD; normoxia = 1 as indicated by the dashed line; $n = 4$). *C*, Jurkat T cells were incubated for 20 h under normoxic or hypoxic conditions. Nuclear extracts (NE) and cytosolic extracts (CE) obtained were blotted and analyzed by immunodetection for HIF-1α and β-actin (one of two independently performed experiments). *D*, As in *C*, mRNA was obtained and analyzed for *HPRT* and *HIF1A*, as well as HIF-1 targets *GLUT1*, *LDHA*, *PGK1*, and *VEGF* by qPCR normalized to *ACTB* and normoxia (mean ± SD; normoxia = 1 as indicated by the dashed line; $n = 4$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-sample *t* test.

Table I. Primer sets for qPCR

Gene Symbol	Gene Name	Forward Primer	Reverse Primer
ACTB	β-actin	5′-gACAggATgCAgAAggAgATCACT-3′	5′-TgATCCACATCTgCTggAAggT-3′
GLUT1	Glucose transporter-1	5′-ACgCTCTgATCCCTCTCAgT-3′	5′-gCAgTACACACCgATgATgAAg-3′
HIF1A	Hypoxia-inducible factor-1α	5′-CCATTAgAAAgCAgTTCCgC-3′	5′-TgggTAggAgATggAgATgC-3′
HPRT	Hypoxanthine-guanine phosphoribosyl-transferase	5′-gCCAgACTTTgTTggATTTg-3′	5′-CTCTCATCTTAggCTTTgTATTTTg-3′
LDHA	Lactate dehydrogenase A	5′-ACCCAgTTTCCACCATgATT-3′	5′-CCCAAAATgCAAggAACACT-3′
MIF	Macrophage migration inhibitory factor	5′-CAgTggTgTCCgAgAAgTCA-3′	5′-TAggCgAAggTggAgTTgTT-3′
PGK1	Phosphoglycerate kinase 1	5′-ATggATgAggTggTgAAAgC-3′	5′-CAgTgCTCACATggCTgACT-3′
VEGF	Vascular endothelial growth factor	5′-AgCCTTgCCTTgCTgCTCTA-3′	5′-gTgCTggCCTTggTgAgg-3′
FIH	Factor inhibiting hypoxia-inducible factor	5′-TTCCCgACTAggCCCATTC-3′	5′-CAgggCAggATACACAAgATTT-3′
pVHL	von Hippel–Lindau tumor-suppressor	5′-TTgTgCCATCTCTCAATgTTgAC-3′	5′-TCTCAggCTTgACTAggCTCC-3′
GCR	GC receptor α	5′-ATgAACCTggAAgCTCgAAA-3′	5′-gATCCTCCAAgTTgAgTCTgg-3′
PHD1	HIF prolyl hydroxylase 1	5′-GCTgggCAgCTATgTCATCAA-3′	5′-AgAgTggCTCgATgTTggCTA-3′
PHD2	HIF prolyl hydroxylase 2	5′-gAAggCgAACCTgTACCCC-3′	5′-CATgCACggCACgATgTACT-3′
PHD3	HIF prolyl hydroxylase 3	5′-ACAggCTggTCCTCTACTg-3′	5′-gATCCCACCATgTAgCTTggC-3′

Furthermore, we demonstrated that Jurkat T cells expressed HIF-1α protein (Fig. 1*C*) and HIF-1 target genes *GLUT1*, *LDHA*, *PGK1*, and *VEGF* after 20 h under hypoxia (Fig. 1*D*). The latter finding enabled us to use Jurkat T cells as a model for in-depth analysis of the HIF-1 response in some of the experiments. Furthermore, *VEGF* was chosen as the representative HIF-1 target gene (primer sets used are given in Table I).

MIF induction under hypoxia in primary human CD4$^+$ T cells and Jurkat T cells is mediated via HIF-1α

Next, we analyzed whether this induction of HIF under hypoxia was accompanied by an induction of MIF, as already shown in tumor cells (4, 6, 17). Under hypoxia, Th cells significantly (up to 2-fold) upregulated *MIF* mRNA levels after 6 h when left unstimulated and up to 4-fold with TCR engagement compared with normoxic controls ($p = 0.0057$ and $p < 0.001$, respectively; Fig. 2*A*). Hypoxia-mediated upregulation of MIF mRNA increased up to 4-fold without stimulation and up to 10-fold with PHA stimulation, in a time-dependent manner, after 48 h (Supplemental Fig. 4). In addition, hypoxia upregulated *MIF* mRNA levels after 20 h in Jurkat T cells compared with normoxia ($p = 0.0053$; Fig. 2*B*). With regard to the protein level, we observed a pronounced increase in secreted MIF after 24 h (Fig. 2*C*). Focusing on the HIF-1–MIF relationship, we analyzed whether HIF-1 induced MIF in human T cells. Using shRNA-mediated RNA interference (Table II) in a T cell model (Jurkat T cells), we efficiently knocked down HIF-1α on the mRNA ($p < 0.0001$) and protein levels (Fig. 2*D*, 2*F*). The loss of HIF-1α efficiently blocked HRE-mediated reporter gene activity (Fig. 2*G*). Furthermore, loss of HIF-1α resulted in a significantly reduced HIF target gene expression of *GLUT1*, *LDHA*, *PGK1* (data not shown), and *VEGF* ($p < 0.0001$; Fig. 2*H*), as well as transcript expression of *MIF*, after 20 h ($p < 0.0001$; Fig. 2*E*). To verify that HIF-1 is responsible for the induction of *MIF* in primary Th cells, we performed a ChIP assay to demonstrate that HIF-1 binds to the proximal promoter region of *MIF* (Fig. 2*I*). In addition, HIF-1α knockdown led to a reduction in MIF at the protein level (Fig. 2*F*).

MIF is a key regulator of HIF-1α and HIF-1 target gene expression in primary human CD4$^+$ T cells and Jurkat T cells

Because MIF is a cellular factor released from cells, we next investigated the impact of rhMIF on primary human CD4$^+$ T cells. Addition of rhMIF (100 ng/ml) led to an increase in HIF-1α expression after 20 h of incubation under hypoxia (Fig. 3*A*, 3*B*). In contrast, inhibition of MIF action, by the addition of the small-molecule inhibitor of MIF (ISO-1; 10 μM), or blocking MIF by adding anti–MIF-IgG (2 μg/ml) resulted in reduced HIF-1α protein expression, but it did not influence HIF-1α transcript abundance (Supplemental Fig. 2). In addition, we knocked down MIF expression by shRNA-mediated RNA interference (Table II) in Jurkat T cells at the mRNA (Fig. 3*C*) and protein levels (Fig. 3*G*). MIF knockdown did not result in a downregulation of HIF-1α mRNA (Fig. 3*D*), but it significantly reduced HIF-1α protein expression (Fig. 3*G*), HIF-1 reporter gene assay activity (Fig. 3E), and HIF-1 target gene expression of *VEGF* (Fig. 3*F*). Adding rhMIF back to Jurkat T cells with knocked down MIF expression re-established HIF-1α protein expression (Fig. 3*G*).

MIF stimulates HIF-1α expression via CD74 under hypoxia involving ERK/mammalian target of rapamycin activity complemented by PI3K upon mitogen stimulation

MIF was demonstrated in cell lines (THP-1, Raji) and murine and human monocytes/macrophages to act via binding to the MHC class II invariant chain CD74 (18). Therefore, we first confirmed the expression of CD74 on primary human CD4$^+$ T cells (Fig. 3*H*). Second, by blocking CD74 using an anti–CD74-IgG during hypoxia (20 h) before hypoxic treatment, we reduced the amount of HIF-1α protein expression in CD4$^+$ T cells in vitro (Fig. 3*I*). The reduction in HIF-1α protein expression was not due to a reduced mRNA level (data not shown). Furthermore, addition of rhMIF (100 ng/ml) did not abolish this effect.

It was shown that HIF-1α protein synthesis is upregulated mainly via the PI3K/mammalian target of rapamycin (mTOR) pathway (19) and that MIF induces angiogenesis and HIF-1α in a MAPK-dependent manner in the MCF-7 cell line (6, 20). Therefore, we analyzed these pathways by using specific inhibitors (Fig. 3*J*). We first examined the effect of the specific mTOR inhibitor rapamycin on HIF-1α accumulation in peripheral blood CD4$^+$ T cells. Rapamycin completely inhibited the induction of HIF-1α protein expression in quiescent and mitogen-stimulated T cells under hypoxic conditions. The same effect was observed using the MEK inhibitor U0126. In contrast, the PI3K inhibitor Ly294002 inhibited the hypoxic induction of HIF-1α protein expression in mitogen-stimulated T cells only. The observed effects on HIF-1α protein expression were not due to

Table II. Sequences of cloned short hairpin oligo nucleotides (backbone: pLL)

Construct Name	Short Hairpin Oligonucleotides
pLL-scr	5′-T gCTATCgAgAAgATCAgCC TTCAAgAgA ggCTgATCTTCTTTAgC TTTTTTC-3′
pLL-HIFsh1	5′-T CCgCTggAgACACAATCATAT TTCAAgAgA ATATgATTgTgTCTCCAgCgg TTTTTTC-3′
pLL-HIFsh2	5′-T CCAgTTATgATTgTgAAgTTA TTCAAgAgA TAACTTCACAATCATAACTgg TTTTTTC-3′
pLL-MIFsh1	5′-T CCGATGTTCATCGTAAACA TTCAAGAGA TGTTTACGATGAACATCGG TTTTTTC-3′
pLL-MIFsh2	5′-T AACAACTCCACCTTCGCCTAAGA TTCAAGAGA TCTTAGGCGAAGGTGGAGTTGTT TTTTTTC-3′

Target (nontarget in case of pLL-scr) sequences are underlined.

increased cell death or apoptosis after application of the respective pathway inhibitors (Supplemental Fig. 5), and they likely did not result from modifying the expression of short-lived proteins, which was controlled for by NFκBp65 or Jun B (Supplemental Fig. 6).

Therefore, we assumed that ERK/mTOR is the major pathway regulating HIF-1α protein synthesis in T cells, independent of activation state, whereas the PI3K pathway complements ERK/mTOR signaling in PHA-engaged T cells.

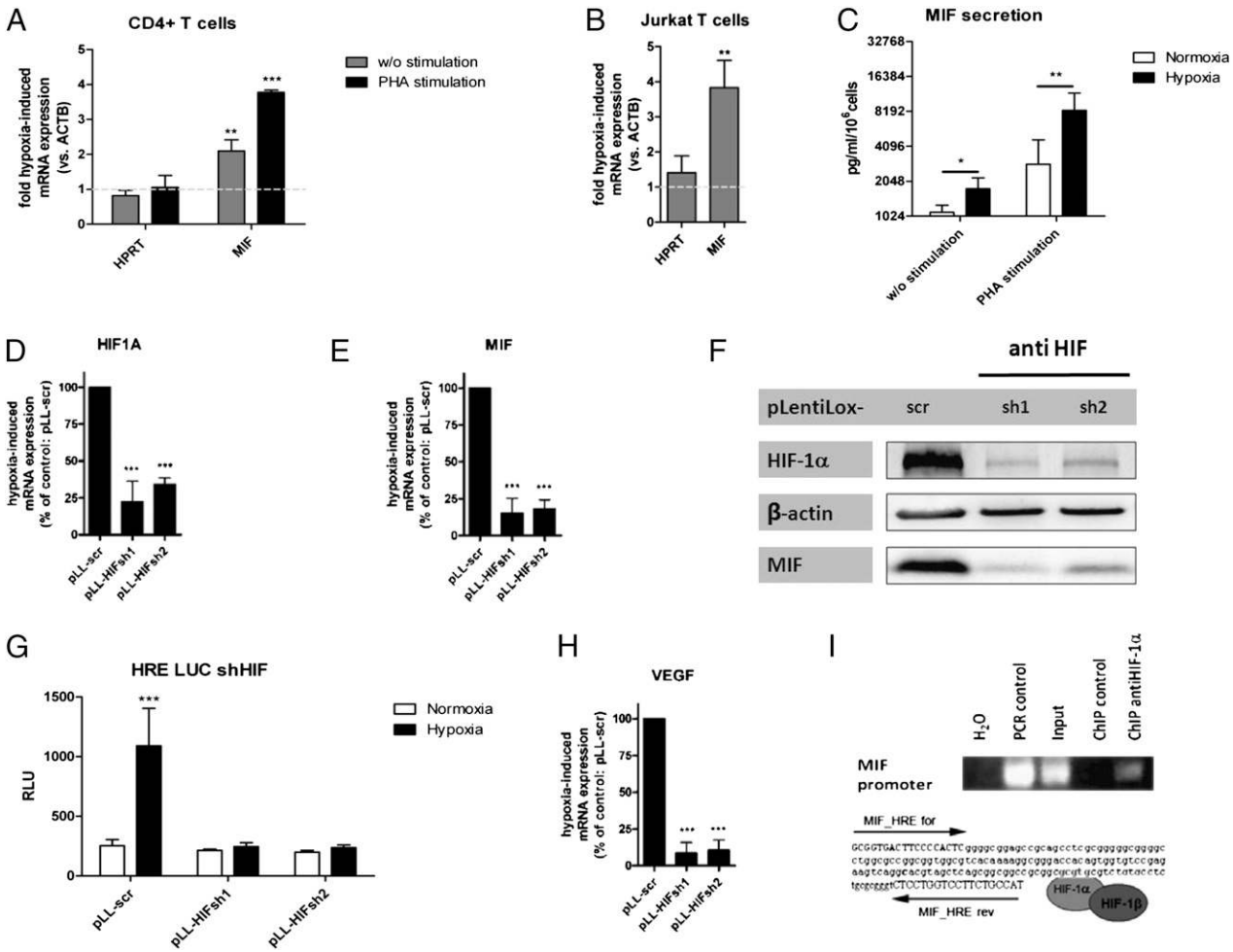

FIGURE 2. MIF induction under hypoxia is mediated via HIF-1α in primary human CD4$^+$ T cells and Jurkat T cells. Primary human CD4$^+$ T cells were incubated for 6 h under normoxic or hypoxic conditions and stimulated with PHA-L (5 μg/ml) or left untreated (*A*), and Jurkat T cells were incubated for 20 h under normoxic or hypoxic conditions (*B*). mRNA levels of *MIF* and *HPRT*, as a second housekeeping gene, were analyzed by qPCR and normalized to *ACTB* and normoxia (mean ± SD; normoxia = 1 as indicated by the dashed line). $^{**}p < 0.01$, $^{***}p < 0.001$; one-sample *t* test. *C*, MIF secreted from primary human CD4$^+$ T cells incubated for 24 h under normoxic or hypoxic conditions and stimulated as in *A* ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$; two-way ANOVA with the Bonferroni multiple-comparison post hoc test. mRNA obtained from anti–HIF-1α–shRNA-vector(pLL)–transfected Jurkat T cells was analyzed by qPCR for HIF-1α (*D*) and MIF transcript abundance normalized to mRNA obtained from control (scr)-shRNA-vector-transfected Jurkat T cells (*E*) (mean ± SD; $n = 4$). $^{***}p < 0.001$; one-way ANOVA with Bonferroni multiple-comparison post hoc test. *F*, Whole-cell extracts obtained from anti–HIF-1α– and control (scr)-shRNA–vector-transfected Jurkat T cells were analyzed by immunodetection for HIF-1α, β-actin, and MIF after 20 h of incubation under hypoxia (one of three independently performed experiments). *G*, Analysis of HRE-driven reporter gene activity (normalized to constitutively expressed *Renilla* luciferase) in anti–HIF-1α/control-shRNA–vector-transfected Jurkat T cells (mean ± SD). $^{***}p < 0.001$; two-way ANOVA with the Bonferroni multiple-comparison post hoc test. *H*, qPCR analysis of mRNA obtained as in *D* and *E* for HIF-1 target gene expression of *VEGF* (mean ± SD). $^{***}p < 0.001$; one-way ANOVA with Bonferroni multiple-comparison post hoc test. *I*, ChIP of HIF-1α of cell extracts from primary human Th cells was analyzed by PCR for the proximal promoter region of MIF on chromosome 22 region 22,566,483–22,566,660. H_2O, no template control; PCR control, human genomic DNA; ChIP control, unspecific rabbit IgG (all after 20 h of hypoxic incubation without stimulation; one of three independently performed experiments).

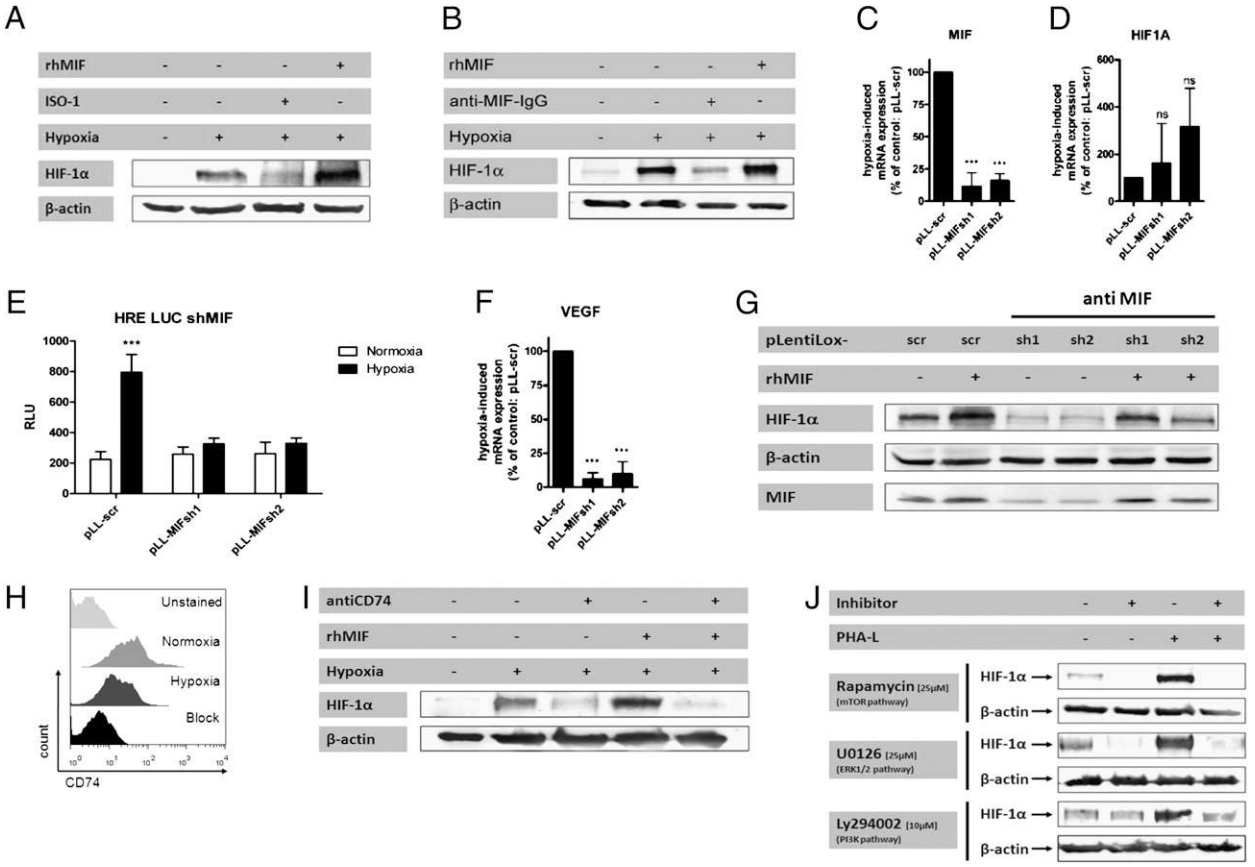

FIGURE 3. MIF acts via CD74 and is a key regulator of HIF-1α stabilization and HIF-1 target gene expression. *A* and *B*, Whole-cell extracts that were obtained from primary human Th cells that were treated with the MIF inhibitor ISO-1 (10 μM), anti–MIF-IgG, or rhMIF (100 ng/ml) and incubated for 20 h under hypoxic conditions were analyzed by immunodetection for HIF-1α and β-actin (one of at least three independently performed experiments). mRNA obtained from anti–MIF-shRNA–vector(pLL)-transfected Jurkat T cells was analyzed by qPCR for MIF (*C*) and HIF-1α (*D*) transcript abundance normalized to mRNA obtained from control (scr)-shRNA–vector-transfected Jurkat T cells (mean ± SD; $n = 4$). ***$p < 0.001$; one-way ANOVA with Bonferroni multiple-comparison post hoc test. *E*, Analysis of HRE-driven reporter gene activity (normalized to constitutive expressed *Renilla* luciferase) in anti-MIF/control-shRNA–vector-transfected Jurkat T cells (mean ± SD). ***$p < 0.001$; two-way ANOVA with Bonferroni multiple-comparison post hoc test. *F*, qPCR analysis of mRNA obtained as in *C* and *D* for HIF-1 target gene expression of VEGF. **$p < 0.01$, ***$p < 0.001$; one-way ANOVA with Bonferroni multiple-comparison post hoc test. *G*, Whole-cell extracts obtained from anti–MIF-shRNA–vector(pLL)-transfected Jurkat T cells were analyzed by immunodetection for HIF-1α, β-actin, and MIF after a 20-h incubation under hypoxia treated previously with rhMIF (100 ng/ml) as indicated (one of at least three independently performed experiments). *H*, Analysis of CD74 expression on primary human $CD4^+$ T cells after a 20-h hypoxic or normoxic incubation without stimulation by flow cytometry (unstained and block control from normoxic $CD4^+$ T cells; one of at least three independently performed experiments). *I*, Whole-cell extracts obtained from primary human Th cells pretreated with anti–CD74-IgG (2 μg/ml) or rhMIF (100 ng/ml) after a 20-h incubation under hypoxia were analyzed by immunodetection for HIF-1α and β-actin (one of at least three independently performed experiments). *J*, Whole-cell extracts obtained from primary human Th cells pretreated with rapamycin (25 μM), U0126 (25 μM), or Ly294002 (10 μM) after a 20-h incubation under hypoxia were analyzed as described in *I*.

MIF impact on $CD4^+$ T cell proliferation depends on oxygen availability

We then asked what functional effects extracellular MIF has on human $CD4^+$ T cells under hypoxic conditions. Therefore, we analyzed activation-induced proliferation and CD25 expression under normoxic and hypoxic conditions. Under normoxic conditions, $CD4^+$ T cell proliferation was significantly reduced when MIF signaling was blocked via CD74 ($p < 0.001$), whereas the addition of rhMIF did not influence T cell proliferation (Fig. 4*A*, 4*B*). In contrast, under hypoxia, PHA-stimulated $CD4^+$ T cell proliferation was significantly reduced compared with normoxia ($p < 0.05$), and it remained almost unaffected when MIF signaling was blocked via CD74 (compared with PHA-stimulated hypoxic $CD4^+$ T cells). Furthermore, the addition of rhMIF significantly reduced T cell proliferation compared with PHA-stimulated $CD4^+$ T cells under hypoxia (Fig. 4*B*). These findings correlated with an altered frequency of CD25-expressing $CD4^+$ T cells and CD25 surface expression (Fig. 4*C*, 4*D*). Under normoxia, the proportion of CD25-expressing $CD4^+$ T cells upon PHA stimulation was significantly enhanced when CD74 was blocked, whereas the addition of rhMIF had no effect. In contrast, under hypoxia, PHA stimulation resulted in a significant increase in CD25-expressing $CD4^+$ T cells. Blocking CD74 and treatment with rhMIF did not significantly influence CD25 expression under hypoxia.

Interestingly, when blocking CD74 under normoxia, surface expression of CD25 by PHA-activated $CD4^+$ T cells increased significantly (Fig. 4*D*). Hypoxia alone also increased the surface expression of CD25 by PHA-stimulated $CD4^+$ T cells, which was decreased when CD74 was blocked and increased when rhMIF was added. With respect to IL-2 production by activated Th cells, we observed a significant reduction in IL-2–producing PHA-activated $CD4^+$ T cells when CD74 was blocked under normoxia (Fig. 4*E*, 4*F*). Hypoxia alone significantly decreased the frequency of IL-2–producing PHA-stimulated $CD4^+$ T cells, which was further reduced when rhMIF was added. In contrast to normoxia, the frequency of

IL-2–producing PHA-stimulated CD4$^+$ T cells did not decrease when CD74 was blocked compared with the corresponding control.

These data suggested that MIF may play a dual role when influencing Th cell proliferation via CD74 by maintaining/enhancing T cell proliferation via IL-2 under normoxia but not under hypoxia. In addition, we assumed that the increase in CD25-expressing Th cells and CD25 expression per cell may be due to a feedback loop to overcome inhibition of IL-2–producing T cells and T cell proliferation.

DEX dose dependently abrogates hypoxia-induced HIF-1α expression

Keeping in mind that MIF acts in concert with GCs to control T cell activation [e.g., MIF antagonizes GC-mediated inhibition of T cell proliferation, as shown by Bacher et al. (21)], we studied the effects of the GC DEX on the expression of HIF-1α. In untreated and PHA-stimulated Th cells, DEX dose dependently abrogated HIF-1α expression under hypoxic conditions at the protein level but not at the mRNA level (Fig. 5*A*, 5*B*). This inhibition of the HIF-1α protein expression was blocked by the GCR antagonist RU486. Thus, the inhibitory effect of DEX on HIF-1α protein expression is mediated through the GCR. Furthermore, antagonizing DEX in PHA-activated Th cells abolished the inhibition of HIF-1α protein expression (Fig. 5*A*). Suppression of HIF-1α by DEX resulted in a significant downregulation of HIF-1 target gene induction (e.g., VEGF) under hypoxia in a GCR-dependent manner (Fig. 5*C*).

Abrogation of HIF-1α by DEX, as well as its induction by the combination of DEX and RU486, in PHA-treated Th cells did not result from the induction or repression of inhibitors of HIF-1α, such as pVHL, FIH, and PHD1-3. Transcript expression of the molecules increased slightly as a result of hypoxia but remained independent of GC treatment, with the exception of mitogen-stimulated hypoxia-induced PHD1 expression (Fig. 5*E–G*). However, stimulated hypoxia-induced PHD1 expression decreased after DEX treatment, which was blocked by the combination of DEX and RU486 (Fig. 5*G*). Therefore, the dose-dependent regulation of PHD1 by DEX did not contribute to the abrogation of HIF-1α by DEX or its induction by the combination of DEX and RU486. Moreover, PHD1 is regulated in a HIF-1 target gene fashion, such as observed for VEGF (Fig. 5*C*).

Hypoxia also increased the expression of GCR mRNA, whereas GC treatment, as well as antagonizing GCR binding by DEX, increased GCR mRNA in quiescent cells but not in PHA-stimulated Th cells (Fig. 5*D*). Therefore, the reduction in HIF-1α expression might be a rapid event.

To analyze the rapid effects of DEX on established HIF-1α expression in primary CD4$^+$ T cells under a stable hypoxic environment, we used a Clark-type electrode within a sealed chamber; this enabled us to induce hypoxia in the T cell suspension, continuously monitor the oxygen concentration of the T cell suspension (including a hypoxia phase), and add drugs and remove samples via a fine slot for the introduction of a 22-gauge needle. Under these controlled, stable, hypoxic conditions that were achieved after cellular respiration had removed dissolved oxygen, we found a very rapid reduction in HIF-1α expression 10 and 20 min postapplication of DEX, which was not observed

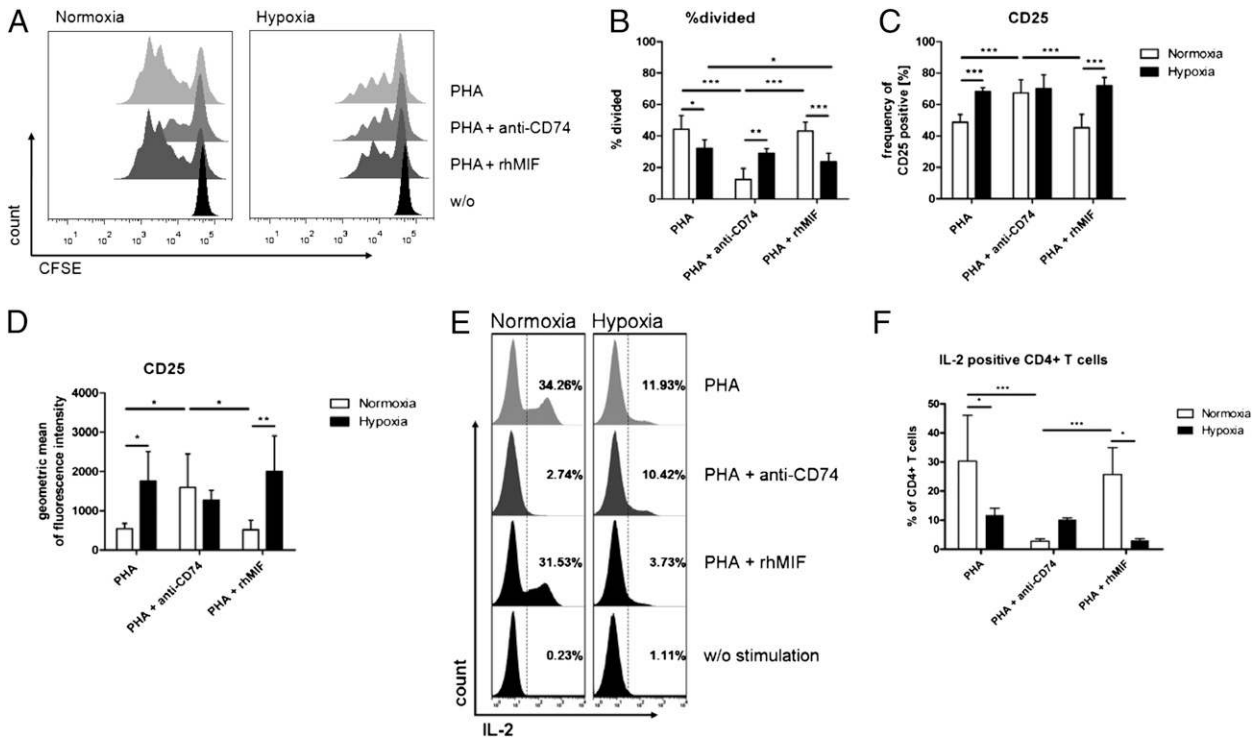

FIGURE 4. rMIF acts via CD74 and differentially influences T cell proliferation under normoxia and hypoxia. *A* and *B*, CFSE staining of CD4$^+$ T cells was analyzed by flow cytometry after a 96-h normoxic or hypoxic incubation with and without PHA stimulation and initial treatment with anti–CD74-IgG (2 μg/ml) or rhMIF (100 ng), as indicated. CD4$^+$ T cell proliferation is demonstrated as representative CFSE staining of a single donor after exclusion of dead cells via propidium iodide staining (*A*) and the percentage of divided cells (*B*) (mean ± SD; $n = 6$). Analysis of the frequency of CD25$^+$CD4$^+$ T cell population (*C*) and the amount of CD25 surface expression by flow cytometry (*D*) after a 96-h normoxic or hypoxic incubation with and without PHA stimulation and initial treatment with anti–CD74-IgG (2 μg/ml) or rhMIF (100 ng), as indicated (mean ± SD; $n = 3$). *E* and *F*, Analysis of the frequency of IL-2$^+$CD4$^+$ T cell population by flow cytometry after a 72-h normoxic or hypoxic incubation with and without PHA stimulation and initial treatment with anti–CD74-IgG (2 μg/ml) or rhMIF (100 ng), as indicated. IL-2$^+$CD4$^+$ T cells are shown as a representative graph of IL-2 staining of a single donor (*E*) and as a bar graph of four 4 experiments (*F*) (mean ± SD). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; two-way ANOVA with Bonferroni multiple-comparison post hoc test.

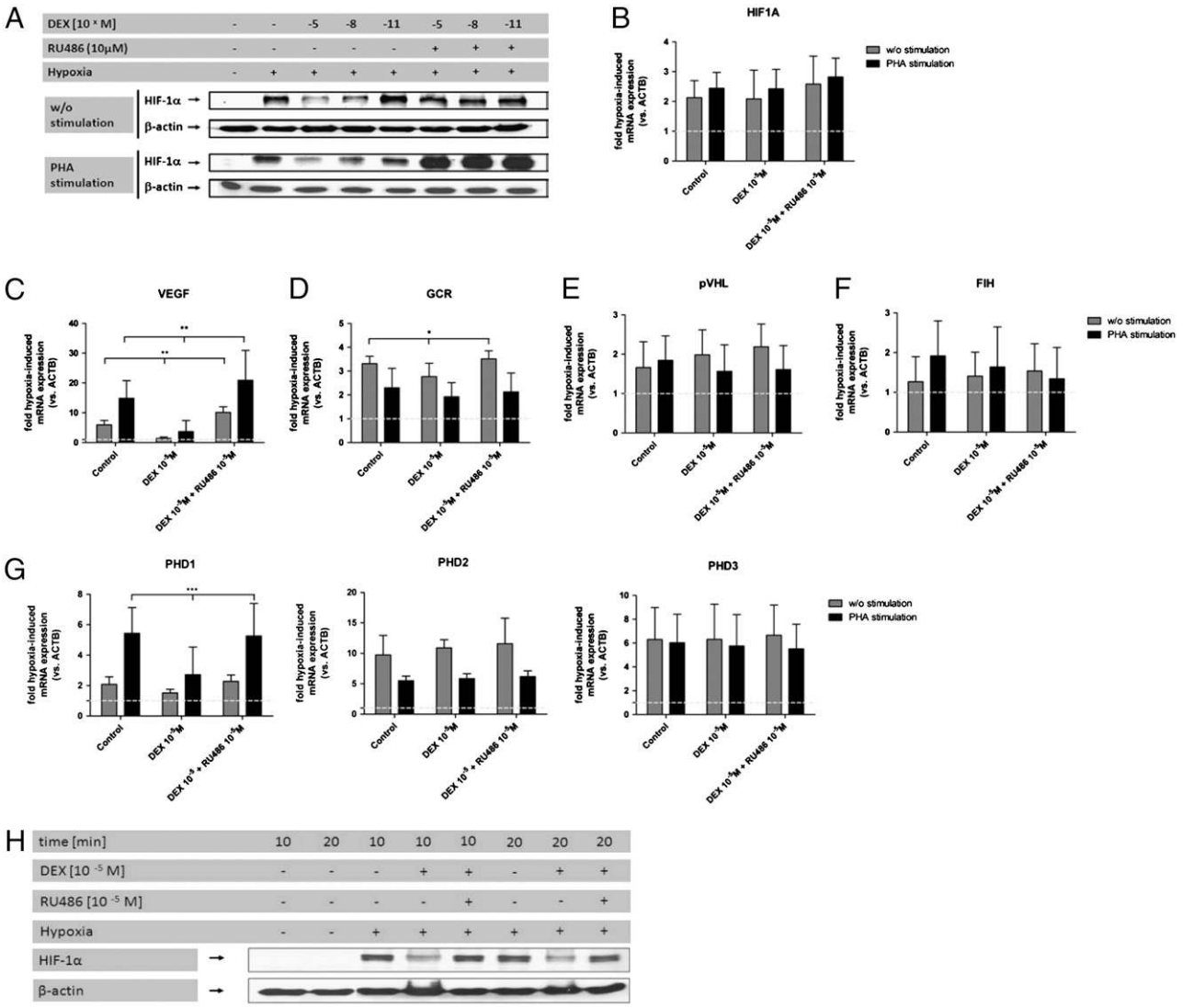

FIGURE 5. DEX dose dependently abrogates hypoxia-induced HIF-1α. *A*, Whole-cell extracts obtained from quiescent and PHA (5 μg/ml)-stimulated primary human Th cells treated with 10^{-5}, 10^{-8}, or 10^{-11} M DEX alone, in combination with the GCR antagonist RU486 (10 μM), or those left untreated and incubated for 6 h under normoxic or hypoxic conditions were analyzed by immunodetection for HIF-1α and β-actin (one of at least three independently performed experiments). *B–G*, Quiescent and PHA (5 μg/ml)-stimulated primary human $CD4^+$ T cells were incubated for 20 h under normoxic or hypoxic conditions and treated with 10^{-5} M DEX alone or DEX in combination with RU486 (10 μM) or were left untreated. mRNA expression of *HIF1A* (*B*), HIF-1 target gene *VEGF* (*C*), *GCR* (*D*), or suppressors of HIF-1α stabilization, including *pVHL* (*E*), *FIH* (*F*), and *PHD1–3* (*G*) were analyzed by qPCR (mean ± SD; $n = 4$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; two-way ANOVA with the Bonferroni multiple-comparison post hoc test. *H*, Quiescent primary human Th cells incubated for 6 h under hypoxic conditions were treated with 10^{-5} M DEX alone or DEX in combination with the GCR antagonist RU486 (10 μM) or were left untreated. Whole-cell extracts were obtained after 10 and 20 min of drug treatment and analyzed by immunodetection for HIF-1α and β-actin (one of two independently performed experiments).

with the vehicle control (DMSO) or the mixture of DEX and RU486 (Fig. 5*H*). These results suggested that MIF may overcome the DEX-mediated reduction in HIF-1α expression.

MIF overcomes DEX-mediated reduction in HIF-1α expression

We analyzed the effects of DEX at very high, but clinically relevant, concentrations on MIF expression itself. DEX significantly downregulated the expression of MIF at mRNA and protein levels under hypoxia. This effect was efficiently blocked by the GC antagonist RU486, suggesting a GCR-mediated signaling (Fig. 6*A*, 6*B*). When adding increasing amounts of MIF to primary human $CD4^+$ T cells prior to the addition of DEX at 10^{-5} M, we observed a MIF-mediated counterregulation of the inhibitory effect of DEX on HIF-1α expression (Fig. 6*C*) and on HIF-1 target gene induction, such as shown for VEGF (Fig. 6*D*). Blocking CD74 resulted in a similar inhibitory effect on hypoxia-induced VEGF expression, which was not reversible by the addition of rhMIF (Fig. 6*D*). Summarizing the data obtained, we propose a model of MIF-mediated induction and DEX-mediated suppression of HIF-1α expression as shown in Fig. 7.

Discussion

Inflammation, autoimmune disorders, and tumorigenesis share common features, such as hypoxia and the pathogenetic involvement of HIF-1 and MIF. In this study, we comprehensively investigated the MIF/HIF relationship and the effect of GCs in human primary nontumor $CD4^+$ Th cells and Jurkat T cells. The following major findings emerged: Th cells are capable of inducing HIF-1α and HIF-1 target genes under hypoxia with and without PHA stimulation; the induction of MIF under hypoxia is

part of the HIF-1 activity; MIF, in turn, is a key regulator of the hypoxia-induced HIF-1α protein expression involving the MIF receptor CD74, thus forming an autocrine positive-feedback loop (Fig. 7); hypoxia-induced HIF-1α expression in resting T cells involves ERK/mTOR activity, complemented by the PI3K pathway upon mitogen stimulation; MIF influences IL-2 production/signaling and Th cell proliferation via CD74: it maintains/enhances T cell proliferation under normoxia but not under hypoxia, where it instead seems to inhibit proliferation; and DEX is able to abolish MIF and HIF expression rapidly in a dose-dependent and GCR-dependent manner, which can be reversed by extracellularly administered MIF.

In contrast to previous studies by Makino et al. (22) and Nakamura et al. (19), we demonstrated that HIF-1α expression and nuclear translocation are inducible under hypoxia, even without mitogen stimulation or TCR engagement. This resulted in a pronounced impact on HIF target gene expression in primary human CD4$^+$ Th cells. In addition, a further increase in HIF-1α at the protein level under PHA stimulation in primary Th cells and Jurkat T cells confirmed our previous studies (23) (Fig. 1). We also demonstrated that hypoxia alone is able to induce MIF expression and secretion in primary nontransformed human Th cells, which is further upregulated by mitogen stimulation (Fig. 2). This is of clinical importance in several autoimmune diseases and in cancer biology where MIF is highly expressed and was shown to trigger the innate immune response and the adaptive immune response (reviewed in Ref. 3). Furthermore, we provided evidence that HIF-1 is responsible for the induction of MIF under hypoxia in human T cells, as shown by HIF-1α knockdown in Jurkat T cells (Fig. 2). This finding is supported by the study of Baugh et al. (4), who showed that hypoxia-induced MIF expression is driven by HIF-1 in three tumor cell lines. However, hypoxia-induced HIF-1–dependent MIF expression seems to be a cell type-specific feature and remains controversial (5, 6).

To test the functional impact of MIF under hypoxia on HIF-1α expression in primary Th cells, we blocked MIF signaling using the MIF inhibitor ISO-1, as well as MIF-neutralizing Abs (Fig. 3*A*, 3*B*). We used shRNA-mediated RNA interference in Jurkat T cells. Indeed, loss of MIF resulted in a clear inhibition of HIF-1α protein expression, whereas rhMIF increased HIF-1α protein expression in primary Th cells. Inhibition of MIF in Jurkat T cells using RNA interference also efficiently reduced HIF-1α protein production (Fig. 3*C–G*). Interestingly, we did not find any reduction in HIF-1α mRNA expression, but rather found a slight but

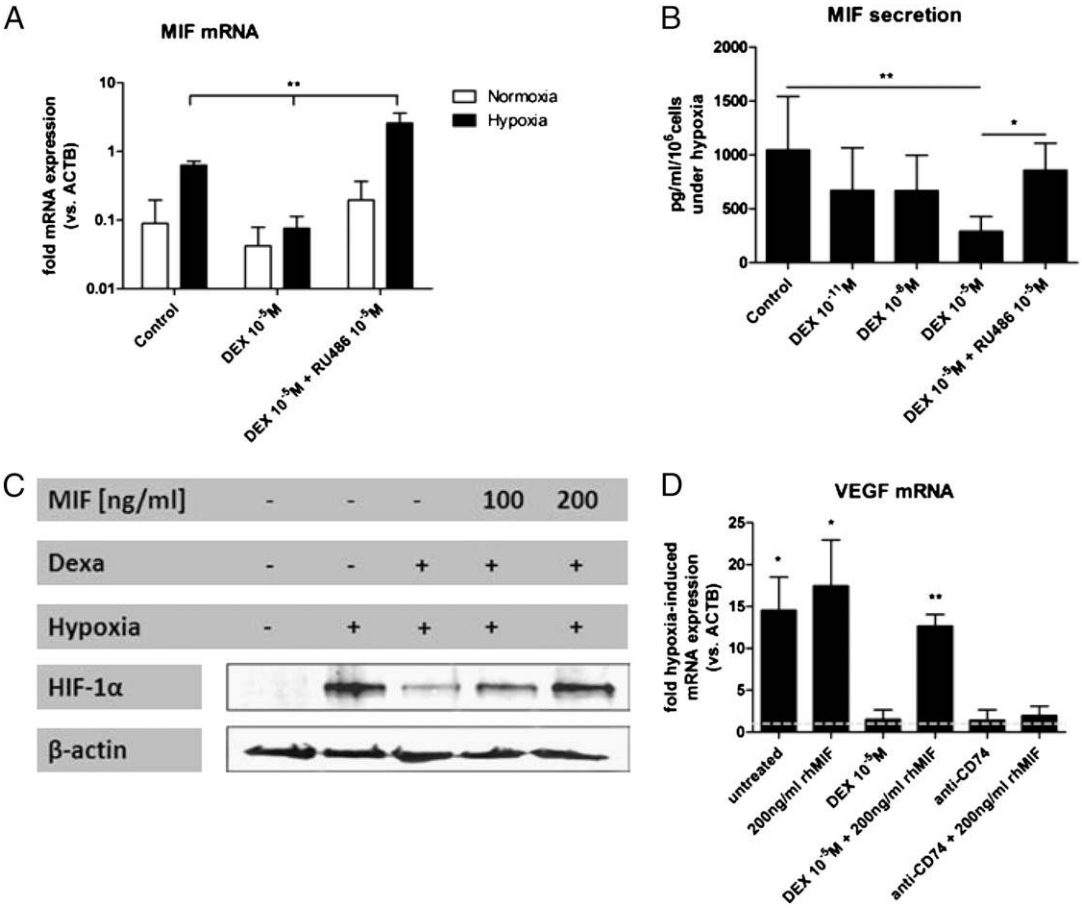

FIGURE 6. DEX abrogates the hypoxia-induced expression of the HIF-1 target MIF, which, inversely, can counteract DEX-mediated inhibition of HIF-1α. *A* and *B*, Primary human CD4$^+$ T cells were incubated for 20 h under normoxic or hypoxic conditions and stimulated with PHA (5 μg/ml). Then, the cells were treated with 10^{-5}, 10^{-8}, or 10^{-11} M DEX alone or DEX in combination with the GCR antagonist RU486 (10 μM) or were left untreated. mRNA was analyzed by qPCR (*A*), and secreted MIF was determined by multiplex suspension array (*B*) (mean ± SD; $n = 4$). *$p < 0.05$, **$p < 0.01$; one-way ANOVA with Bonferroni multiple-comparison post hoc test. *C*, Whole-cell extracts obtained from Th cells, cultured as described above and treated with 10^{-5} M DEX alone or DEX in combination with the indicated amounts of rhMIF or left untreated, were analyzed by immunodetection for HIF-1α and β-actin (one of at least three independently performed experiments). *D*, Primary human CD4$^+$ T cells were incubated for 20 h under normoxic or hypoxic conditions. Th cells were left untreated or were treated with 200 ng/ml rhMIF, 2 μg/ml anti-CD74-IgG, or 10^{-5} M DEX alone or in combination, as indicated. HIF-1 target gene *VEGF* was analyzed by qPCR (mean ± SD; normoxia = 1 as indicated by the dashed line; $n = 3$). *$p < 0.05$, **$p < 0.01$; one-sample *t* test.

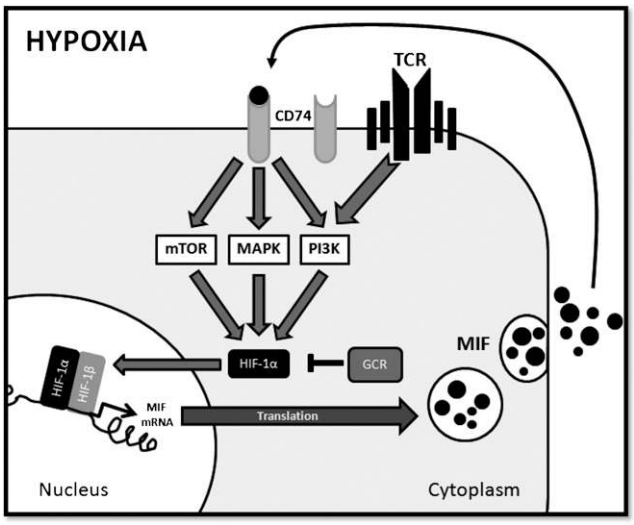

FIGURE 7. Model of MIF-mediated induction and DEX-mediated suppression of HIF-1α expression.

nonsignificant increase, indicating a MIF-dependent posttranslational regulation of HIF-1α protein expression (Fig. 3). However, the reduction in HIF-1α protein expression could be reversed by extracellularly applied MIF. The underlying mechanism seems to be similar to that reported for the breast cancer cell line MCF-7 by Oda et al. (6). Nevertheless, to our knowledge, the results represent the first evidence of MIF-mediated HIF-1α regulation in nontransformed primary Th cells.

In line with the notion that primary T cells behave like oncogenic transformed cells in terms of MIF-dependent HIF-1α regulation, we demonstrated that MIF acts through CD74 under hypoxia. Hypoxia-mediated HIF-1α protein expression involves ERK/mTOR activity in resting T cells, and it is complemented by PI3K activity upon mitogen stimulation (Fig. 3*H*, 3*J*). These findings confirm the observations of Nakamura et al. (19), who reported a TCR-driven stimulation of HIF-1α protein synthesis via PI3K/mTOR. In addition, our results extend previous studies by showing the involvement of the hypoxia-induced protein synthesis of HIF-1α via the MAPK/mTOR pathway in primary human Th cells.

With regard to the influence of extracellular MIF on specific functions of human $CD4^+$ T cells under hypoxic conditions, we observed a dual role for MIF, which strongly depends on oxygen availability. In this study, we showed that MIF is necessary for optimal T cell proliferation under normoxia but not under hypoxia, where it instead inhibits proliferation (Fig. 4*A*, 4*B*). In addition, inhibition of T cell proliferation by blocking the MIF receptor CD74 under normoxia was accompanied by enhanced expression of the IL-2R CD25 (Fig. 4*C*, 4*D*), suggesting an adaptive process to overcome the reduction in the frequency of IL-2–producing Th cells and Th cell proliferation (Fig. 4*A*, 4*B*, 4*E*, 4*F*). CD25 expression was enhanced under hypoxia, as reported previously (24). However, in this study, CD25 upregulation was almost independent of the addition of MIF or blocking CD74, presumably reflecting adaptations to the reduced proportion of IL-2–producing Th cells and the decreased proliferation under limiting oxygen availability.

Several reports described a suppressive activity for a cysteinylated MIF-related protein called glycosylation-inhibiting factor (GIF) (25–28). MIF and GIF share an identical gene, but the respective proteins vary in structure and function as the result of different posttranslational modifications. It was demonstrated that the cysteinylated factor GIF (Cys-60), but not the noncysteinylated MIF, exhibited immunosuppressive effects, such as suppression of Th2 responses by inhibiting the initiation of IL-4 production (26, 27). Protein cysteinylation is a type of oxidation that results from the toxicity of reactive oxygen species (29). Hypoxia was found to increase intracellular reactive oxygen species in stimulated human T cells (24). Therefore, we suggest that the hypoxic microenvironment found in tumors and severely inflamed tissue may increase the amount of secreted suppressive cysteinylated GIF (C60MIF/GIF), which decreases the T cell proliferation rate, whereas normoxia increases the ratio of MIF/(C60MIF/GIF), which supports T cell proliferation, as demonstrated by Bacher et al. (21). This notion is supported by the finding that tumor-derived MIF inhibits T lymphocyte activation (30).

These considerations led to the anti-inflammatory hypoxia hypotheses by Sitkovsky et al., focusing on the anti-inflammatory role of HIF-1α in T cells, such as shown for murine HIF-1α($^{-/-}$) T cells (31). Supporting the latter idea, our data indicated that MIF reduces T cell proliferation under hypoxia.

Our findings are supported by MIF($^{-/-}$) tumors, which showed pronounced infiltration of $CD8^+$ and $CD4^+$ T cells (32). The HIF-1α–mediated inhibition may be additive or synergistic with immunosuppression caused by hypoxia-induced extracellular adenosine, which is protecting tumors by inhibiting the incoming antitumor T cells via their A_{2A} adenosine receptors (33).

In the next step, we focused on the impact of GC on HIF-1α, which was shown to induce MIF, which is capable of antagonizing GC (21). Therefore, we studied the effects of the GC DEX on the expression of HIF-1α. DEX treatment led to a clear dose-dependent and rapid inhibition of HIF-1α expression; this inhibition was GCR dependent and impacted HIF-1α–mediated target gene expression.

We did not find an effect of DEX treatment on the gene expression of HIF-1α or suppressors of HIF-1α, such as PHD1–3, FIH, and pVHL, or on GCR expression itself, which could explain the reduction in HIF-1α protein expression (Fig. 5). Therefore, we suggested a rapid DEX-mediated induction of activity of HIF-1α suppressors or a rapid DEX-mediated inhibition of hypoxia-induced signaling, because it has been reported for the immunosuppressive effects of GC, which are mediated through rapid inhibition of TCR downstream kinases Lck and Fyn (34).

The first evidence for an interaction between HIF and GCR was provided by Kodama et al. (11), who observed an induction of HIF expression and target gene induction in HeLa cells. In contrast, Wagner et al. (12) demonstrated a DEX-mediated inhibition of induced HIF-1 target gene expression under hypoxia in HEPG2 cells. Furthermore, they described a retention of HIF-1α in the cytoplasm, suggesting a block of nuclear import. However, we found a clear inhibition of HIF-1α protein expression, which resulted in reduced HIF-1 target gene expression, such as for VEGF (Fig. 5). Interestingly, we also found that PHD1 was regulated in a similar manner as VEGF. del Peso et al. (35) showed that PHD1 was upregulated by hypoxia in HeLa cells. Fig. 5*G* also shows that PHD2 and PHD3 are not regulated in primary human $CD4^+$ T cells. This is somewhat in contrast to the data published by del Peso et al. (35), but these investigators showed a respective regulation in transformed cells. In summary, the results obtained in this study are important with regard to impaired tissue regeneration, such as wound healing, after GC therapy (13).

In addition, we found a clear inhibition of the HIF-1 target MIF at the transcriptional and protein levels by DEX in a dose- and GCR-dependent manner (Fig. 6). We did not observe an induction of MIF by low-dose GC exposure. In contrast, Leng et al. (36) recently reported that low concentrations of DEX induced MIF secretion from GC-sensitive CEM-C7 T cells but not from GC-insensitive CEM-C1 T cells by a bell-shaped dose response, thereby confirming the observations of Calandra et al. (37) with regard to the

release of MIF by GC-exposed monocytes/macrophages. The impact of the hypoxic environment applied in our experimental setting might explain the divergence of the results. In contrast to the studies by Leng et al. (36) and Calandra et al. (37), Elsby et al. (17) demonstrated a repression of MIF-promoter activity in GC-exposed CEM-C7A T cells, which is in line with our results. Moreover, we provide evidence for MIF signaling to overcome GC-suppressed HIF-1α expression (Fig. 6). The MIF-mediated induction and DEX-mediated suppression of HIF-1α expression may be deduced from the fine-tuning of phosphorylation events during CD74 signaling, as described in the model shown in Fig. 7. Under hypoxia, MIF is released from intracellular stores and induces signaling cascades via CD74 to promote hypoxia-induced expression/stabilization of HIF-1α. HIF-1α is imported into the nucleus and dimerizes with its partner HIF-1β to induce HIF-1 target genes, such as MIF itself. This autoamplifying feedback loop is interrupted by high doses of GCs via the GCR or the inhibition of HIF-1α expression/stabilization under normoxia (Fig. 7).

These findings are of clinical importance because they reflect the function of therapeutically administered high doses of GCs during treatment of inflammatory processes in which hypoxia is a critical part of pathogenesis. Furthermore, our findings suggest that targeting HIF or MIF (C60MIF/GIF) may be useful to promote antitumor immune responses, optimize GC therapy, and control inflammation.

Acknowledgments

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Disclosure

The authors have no financial conflicts of interest.

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