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Hypoxia and Hypoxia-Inducible Factor-1α Modulate Lipopolysaccharide-Induced Dendritic Cell Activation and Function[1]

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Dendritic cells (DC) play a key role in linking innate and adaptive immunity. In inflamed tissues, where DC become activated, oxygen tensions are usually low. Although hypoxia is increasingly recognized as an important determinant of cellular functions, the consequences of hypoxia and the role of one of the key players in hypoxic gene regulation, the transcription factor hypoxia inducible factor 1α (HIF-1α), are largely unknown. Thus, we investigated the effects of hypoxia and HIF-1α on murine DC activation and function in the presence or absence of an exogenous inflammatory stimulus. Hypoxia alone did not activate murine DC, but hypoxia combined with LPS led to marked increases in expression of costimulatory molecules, proinflammatory cytokine synthesis, and induction of allogeneic lymphocyte proliferation compared with LPS alone. This DC activation was accompanied by accumulation of HIF-1α protein levels, induction of glycolytic HIF target genes, and enhanced glycolytic activity. Using RNA interference techniques, knockdown of HIF-1α significantly reduced glucose use in DC, inhibited maturation, and led to an impaired capability to stimulate allogeneic T cells. Alltogether, our data indicate that HIF-1α and hypoxia play a crucial role for DC activation in inflammatory states, which is highly dependent on glycolysis even in the presence of oxygen. ***The Journal of Immunology,*** **2008, 180: 4697–4705.**

Dendritic cells (DC)[4] play a key role in regulating innate and adaptive immune responses and in inducing peripheral tolerance. DC are located in peripheral tissues and continuously scan their environment. Pathogen-derived danger signals, e.g., TLR ligands as well as nonmicrobial factors, convert immature, Ag-capturing DC into immune-stimulating, mature DC. To this end, DC undergo a maturation process enabling them to process and present Ags and to initiate an immune response. This maturation process includes an increased expression of MHC and costimulatory molecules as well as synthesis of cytokines and chemokines. Meanwhile, DC leave the inflamed tissues and migrate to the draining lymph nodes to present processed Ags to naive T cells and induce an adaptive immune response (1, 2). Under steady-state conditions, DC are able to induce tolerance in the absence of proinflammatory factors (3, 4). For instance, it has been demonstrated that immature DC capture apoptotic bodies from cell turnover, migrate to lymph nodes, and subsequently silence potential autoreactive T cells (5).

Many studies have contributed to unravel immunogenic functions of DC to initiate immune responses or to create self-tolerance (6). However, the effects of a potentially important interfering factor for DC biology, the prevailing oxygen tension in inflamed tissues, have not been evaluated, although sites of inflammation are frequently associated with oxygen tensions as low as 5–7 mm Hg (7). Low oxygen tensions in inflamed tissues have been attributed to vascular damage, vascular leakiness, and edema, as well as intense metabolic activity of bacteria and numerous infiltrating cells. The potentially important impact of the local oxygen tension in inflamed tissues on DC regulation is still largely unknown. Two reports (8, 9) suggested a reduced migratory capacity of human monocyte-derived DC differentiated under hypoxia, but further evidence for functional effects of hypoxia on DC biology is lacking to date. In particular, the effect of hypoxia on the capability of DC to induce immune responses has not been analyzed yet.

In many cells, loss of aerobic energy production may lead to functional impairment. It can be hypothesized that hypoxic DC are

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[4] Abbreviations used in this paper: DC, dendritic cell; GLUT-1, glucose transporter-1; HIF, hypoxia inducible factor; LDH, lactate dehydrogenase; ns-siRNA, nonsilencing small interfering RNA; PFA, paraformaldehyde; PGK, phosphoglycerate kinase; PI, propidium iodide; siRNA, small interfering RNA.

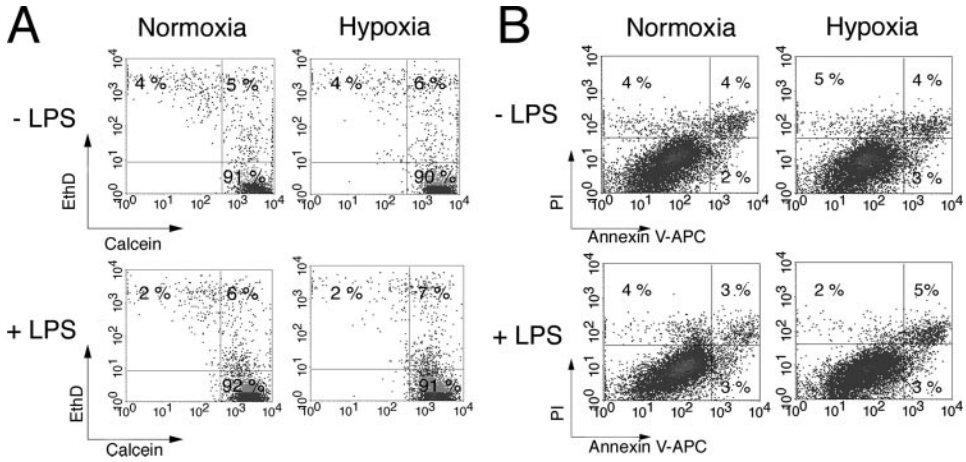

FIGURE 1. Viability of DC under hypoxic conditions. *A*, DC were generated from bone marrow progenitors. Immature DC were subjected to hypoxia (1% oxygen) and normoxia for 24 h and stimulated with 10 ng/ml LPS, if indicated. Viability was determined using a viability/cytotoxicity kit. DC were stained with calcein AM for viable cells and ethidium homodimer 1 for dead cells, and analyzed by flow cytometry. *B*, Apoptotic and necrotic effects were determined by FACS using annexin-allophycocyanin and PI-specific stainings.

less capable of initiating an immune response. However, hypoxia is not only a state of reduced energy supply, but has been demonstrated to actively regulate numerous cellular processes. In particular, the transcription factor hypoxia inducible factor (HIF) has been thoroughly investigated. One of the α-subunits, either HIF-1α or HIF-2α forms a dimer with the aryl receptor nuclear translocator (HIF-β) to enhance transcription of various genes playing a key role in, for example, glycolysis, cell death, cell cycle decisions, angiogenesis, and erythropoiesis (10). The HIF-α subunits are constitutively expressed, but rapidly degrade under normoxic conditions. Degradation is mediated by oxygen representing a substrate for a specific prolyl hydroxylation reaction, which then targets the HIF protein for proteasomal degradation. Bacterial infections are associated with hypoxia (11), and exposure to bacteria leads to HIF protein accumulation in macrophages and granulocytes (12). Importantly, Cramer, and colleagues (13) showed functional importance of the HIF system in myeloid cells using mice with targeted HIF-1α gene disruption. Whereas HIF plays an important role in proinflammatory cascades in myeloid cells (12–14), this protein may have an opposite role in T and B cells (15–17). Recently, Sitkovsky and colleagues (18) reported that HIF-1α inhibits antibacterial capacities of T cells.

In the present study, we investigated how DC cope with reduced oxygen supply. Furthermore, we were interested in whether hypoxia modulates immunological DC functions and whether hypoxia can modulate immunostimulatory effects of DC. Thus, we examined the following: 1) the viability under hypoxic conditions and the HIF-1 expression in DC; 2) the effects of hypoxia on glycolysis and cellular energy synthesis; 3) the consequences of hypoxia on DC activation and DC-induced immunomodulatory functions; and 4) the effect of HIF-1α knockdown for DC activation and ability to induce allogeneic T cell proliferation. We observed that hypoxia and HIF-1α had a marked effect on DC activation, and that this activation was dependent on the HIF-controlled ability for enhanced glycolysis and cellular energy supply.

Materials and Methods

Reagents

All Abs for FACS analysis were purchased from BD Biosciences. Flow cytometry was performed with a FACSCalibur (BD Biosciences). Propidium iodide (PI) and LPS were purchased from Sigma-Aldrich. The viability/cytotoxicity kit was purchased from Molecular Probes and used according to the instructions of the manufacturer. Small interfering RNA (siRNA) oligonucleotides directed against HIF-1α (NM_010431) were purchased from Dharmacon's prevalidated siRNA database: HIF-1α validated siRNA (Catalog L-040638). Nonsilencing siRNA was purchased from Qiagen (Catalog 1027281).

Preparation of DC

DC were prepared from bone marrow, as described previously (19). Between days 7 and 8, cells were harvested, routinely yielding a population of >75% CD11c^{high}-positive cells.

Metabolic quantification: lactate, glucose, lactate dehydrogenase (LDH), and ATP measurements

Lactate and glucose measurements were performed with Blood Gas Analyser, ABL 5 (Radiometer Medical). LDH activity was determined after washing the cells with PBS and lysis with 1% Triton X-100 for 15 min. The LDH activity of the cell extracts was measured with a cell viability assay (Promega). The ATP content of the cells was measured with an ATP bioluminescence assay kit (Roche), according to the instructions of the manufacturer.

Cytokine quantification

Murine TNF and IL-6 OptEIA ELISA (BD Biosciences) were used according to the instructions of the manufacturer. The concentrations of IL-10 and IL-12p40 were measured by specific two-site ELISAs, with reference standard curves obtained from known amounts of the respective murine recombinant cytokine. Matched Ab pairs for the detection of cytokines were purchased from BD Biosciences (anti-mouse IL-10, anti-mouse IL-10 biotin, anti-mouse IL-12p40, and anti-mouse IL-12p40 biotin). They were used according to the supplier's recommendations with a streptavidin/biotin amplification (StreptABComplex/HRP; DakoCytomation) and tetramethylbenzidine (Sigma-Aldrich) as a substrate for HRP.

HIF protein extraction, immunoblotting, and immunohistochemistry

For analysis of HIF protein levels, DC were stimulated as indicated for 24 h. Preparation of cell lysates and immunoblotting were performed, as described previously (20). Equal amounts of proteins were separated by 8% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and probed with HIF-1α (Novus Biochemical) and glucose transporter-1 (GLUT-1; Alpha Diagnostics) Abs. Signals were visualized by chemiluminescence (Pierce). Immunocytochemistry for HIF-1α was performed, as described previously (20).

Quantification of endocytosis in single cells by FACS analysis

DC were seeded and incubated 24 h with the indicated stimulus. OVA (OVA Alexa-Fluor 647; Molecular Probes) at varying concentrations was resuspended in RPMI 1640 and added for 30 min to the DC. The cells were washed three times with PBS and detached with PBS containing 5 mM EDTA for 15 min on ice. DC were analyzed by FACS, and the mean fluorescence intensity was recorded. The background (cells pulsed at 4°C) was subtracted.

Mixed leukocyte reaction

Essentially, the MLR was performed essentially as described by Hill and colleagues. DC from C57BL/6 (H-2^b) mice were incubated for 24 h with the indicated stimuli and subsequently γ irradiated (3600 rad) or treated with 1% paraformaldehyde (PFA). Then single-cell suspensions were prepared from spleens of BALB/c mice (H-2^d) and added to the DC (H-2^b).

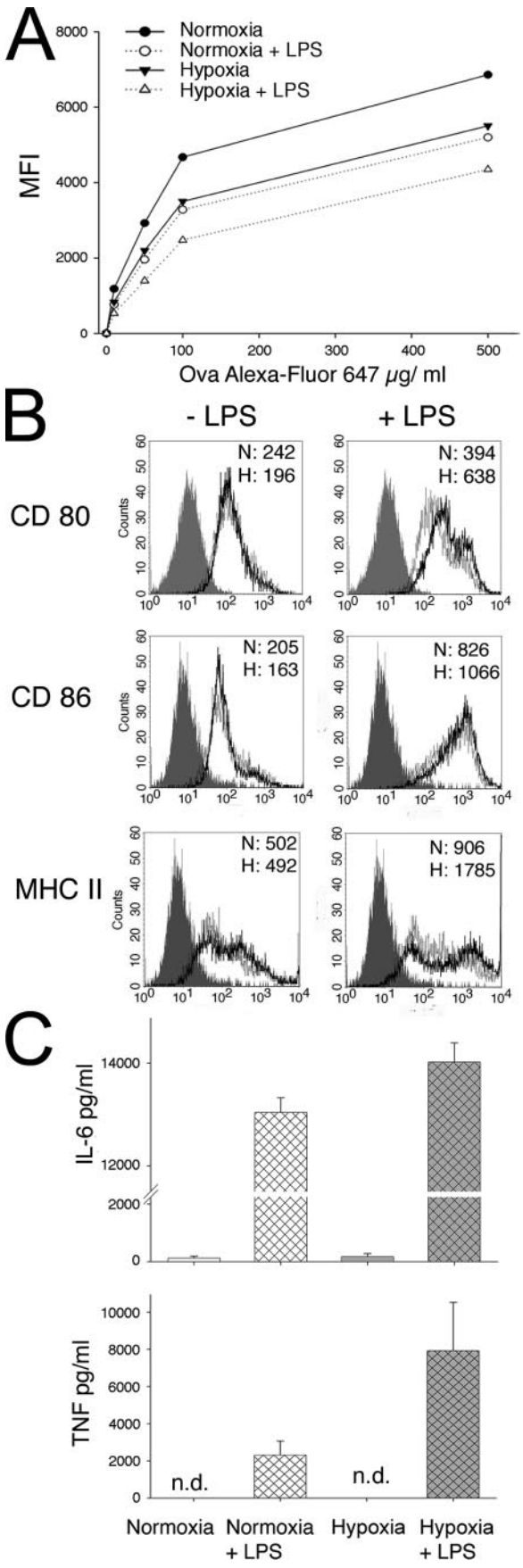

FIGURE 2. Hypoxia modulates DC functions. *A*, Hypoxia reduces the uptake of OVA. Alexa-Fluor 647 OVA was added at various concentrations to DC cultures to investigate the endocytotic capacity of the DC. Twenty-four hours after the exposure of the cells to LPS, hypoxia, or hypoxia and LPS fluorescence labeled OVA was added for 30 min to the DC at 37°C and 4°C. After washing and detachment, the uptake capacity of the differentially treated DC was analyzed by flow cytometry. Mean fluorescence intensity (MFI) is shown. The background (cells pulsed at 4°C) has been subtracted. *B*, LPS in concert with hypoxia led to an increased expression of CD80, CD86, and MHC II on DC compared with DC stimulated with LPS under normoxic conditions. DC were treated as described in *A*, and expression of MHC II, CD80, and CD86 on CD11c high positive cells were analyzed by flow cytometry after hypoxia, LPS, or both. Filled lines show isotype staining, gray lines show expression of the indicated costimulatory molecule under normoxic conditions, and black lines show the expression of the indicated molecule under hypoxic conditions. *Insets*, Show the mean fluorescence intensity of the expression of the indicated molecule on DC stimulated under hypoxic (H) or normoxic conditions (N). Data are representative of at least three independent experiments. *C*, Effect of hypoxia on proinflammatory cytokine secretion. DC were treated as described in *A*, and supernatants were analyzed by sandwich ELISA for TNF and IL-6, as indicated. Data are mean ± SD of duplicates, obtained from at least two independent experiments. N.D. = not detectable.

Two days later, cells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham Biosciences) for an additional 24 h before cells were harvested and thymidine incorporation was quantified using a beta counter. Highly pure (>90%) CD4$^+$ T cells were isolated by depletion of non-CD4$^+$ T cells using MACS technology, according to the manufacturer's instructions (Miltenyi Biotec). Proliferation of CD4$^+$ enriched cells was assessed using the cell-tracking dye CFSE (Molecular Probes) after 3 days or via pulsing the cells with 1 μCi of [^{3}H]thymidine, as described above.

RNA interference studies

DC were harvested, washed four times with Opti-MEM (Invitrogen Life Technologies), and resuspended at a concentration of 4×10^7 cells/ml. Respective amounts of siRNA duplexes were transferred to a 4-mm cuvette (Molecular Bioproducts), and the final volume was adjusted to 50 μl with Opti-MEM. A total of 50 μl of cell suspension (containing 2×10^6 cells) was added and pulsed in a Gene Pulser Xcell apparatus (Bio-Rad). Pulse conditions were 400 V, 150 μF, and 100 Ω. After electroporation, the cells were transferred into RMPI 1640. One hour thereafter, an equal amount of RMPI supplemented with 20% FCS was added. Twenty-four hours later, electroporated DC were exposed to hypoxia or normoxia in the presence or absence of LPS.

RNA isolation and reverse transcription

Cells were harvested and washed with PBS. Total RNA was isolated using the RNeasy mini kit and QIAshredder spin columns (Qiagen). Subsequently, 1 μg of each RNA was reverse transcribed into a single-stranded cDNA using an avian myeloblastosis virus reverse transcriptase, as specified by the manufacturer (Fermentas).

Real-time PCR and relative quantification

Real-time PCR using the LightCycler system (Roche Diagnostics) was performed, as described previously (21). The primers used were as follows: for HIF-1α prevalidated primers were purchased from Qiagen (Catalog QT01039542). For phosphoglycerate kinase (PGK) and GLUT-1, primers were used as described by Cramer et al. (13). Relative quantification was performed using the LightCycler software, according to the manufacturer's instructions.

Results

Viability of DC is maintained under hypoxia alone and simultaneous LPS exposition

To investigate possible effects of low oxygen tensions on the immunobiology of DC, murine bone marrow-derived DC were exposed to an atmosphere of 1% oxygen for 24 h under cell culture conditions in the absence or presence of 10 ng/ml LPS. To test the effects on the viability of these treated cells, DC were first stained with calcein and ethidium homodimer 1 (Fig. 1*A*). There was no significant change in the viability of DC

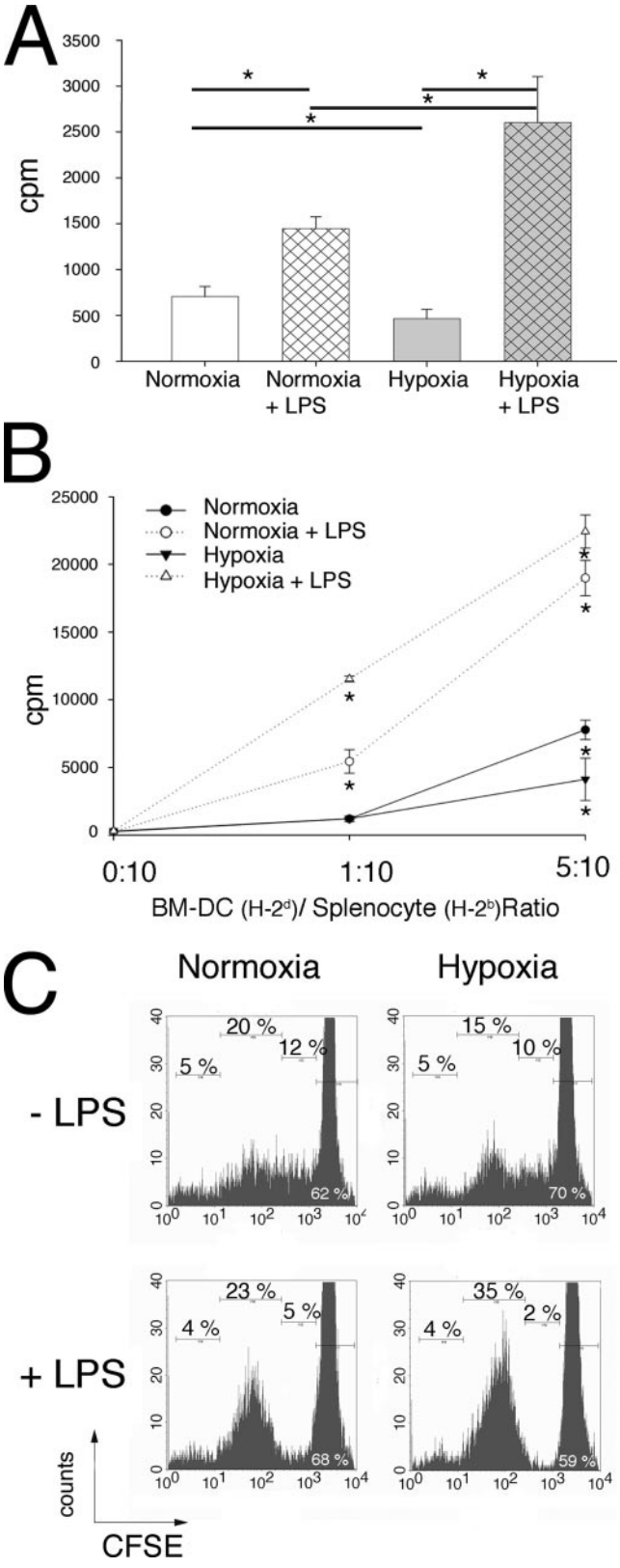

FIGURE 3. Hypoxia modulates the capacity of DC to induce allogeneic lymphocyte proliferation. *A*, In a MLR, DC from C57BL/6 mice (H-2^b) were incubated under hypoxic or normoxic conditions with or without LPS for 24 h. Cells were then fixed with 1% PFA for 15 min and incubated for 30 min with L-lysine before the addition of 1×10^5 splenocytes from BALB/c (H-2^d) for 3 days under normoxic conditions. Proliferation rate was determined using [^{3}H]thymidine incorporation. Statistical analysis was conducted using unpaired Student's *t* test. Statistical significance was defined as follows: $*$, $p < 0.05$. *B*, After the indicated treatment for 24 h, DC (H-2^b) were reoxygenated and γ irradiated (3600 rad), and splenocytes were added from BALB/c (H-2^d) for 3 days under normoxic conditions. Graded numbers of irradiated DC exposed to LPS or LPS and hypoxia, but not hypoxia alone, induced increased allogeneic lymphocyte proliferation under normoxic conditions. *C*, The experiment was performed as described in *B*, but cells enriched for CD4$^+$ T cells from BALB/c (H-2^d) served as responder cells. These cells were labeled with CFSE and added under normoxic conditions to DC from C57BL/6 (H-2^b), which were previously exposed to hypoxia, LPS, or both for 24 h. Viable cells were analyzed by flow cytometry, and histograms for CFSE (FL-1) are shown. Bars indicate percentage of dividing allogeneic T cells.

exposed to LPS and/or hypoxia. These results were in accordance with formazan production rates observed using the MTT assays (data not shown). To further investigate effects of hypoxia on DC viability, stainings with annexin and PI were performed to detect apoptosis and/or necrosis. Hypoxia and the induction of HIF have been associated with protection from apoptosis pathways (22). However, the annexin/PI stainings revealed no significant changes in the proportion of annexin- or PI-positive DC under hypoxia, LPS, or both when compared with controls, indicating that these conditions did not result in increased rates of apoptosis and/or necrosis (Fig. 1*B*). Thus, our data indicate that DC have the ability to adapt to low oxygen tensions. Viability is maintained even under combined inflammatory and hypoxic conditions.

LPS-induced DC maturation is amplified by hypoxia

Maturation of DC is paralleled by reduced Ag uptake rates (23). Accordingly, we found reduced uptake rates of fluorescence-labeled OVA after LPS stimulation (Fig. 2*A*). The capacity of DC to internalize Ags was further reduced after the exposure to LPS and hypoxia, indicating a synergistic effect. Interestingly, hypoxia alone decreased the capacity of DC to engulf Ags to a level that was comparable to normoxic LPS-stimulated cells.

To analyze the effects of hypoxia regarding the DC phenotype, cells were analyzed for the expression of specific cell surface molecules by flow cytometry (Fig. 2*B*). There was an increase of CD80, CD86, and MHC II expression in DC treated with LPS together with hypoxia, compared with solely LPS-treated cells. In contrast, CD80 and CD86 expression slightly decreased under hypoxia alone, whereas MHC II (and CD40; data not shown) expression remained unchanged. Because activation of DC under inflammatory conditions is accompanied by increased cytokine release rates (24), cytokine levels were measured in DC-tissue culture supernatants (Fig. 2*C*). Again, simultaneous stimulation with a combination of LPS and hypoxia significantly increased TNF and IL-6 secretion compared with DC exposed to LPS alone. Hypoxia alone did not alter TNF and IL-6 levels compared with normoxic controls. Additionally, the concentration of IL-10 and IL-12p40 levels was analyzed in the supernatants. LPS induced low levels of IL-10 and high levels of IL-12p40, which were not further increased by additional hypoxia. Very similar to TNF and IL-6, hypoxia alone did not induce IL-10 and IL-12p40 secretion (data not shown).

DC-mediated allogeneic lymphocyte proliferation is increased by simultaneous stimulation with LPS and hypoxia

To investigate whether LPS in concert with hypoxia synergistically enhances DC-mediated lymphocyte proliferation, the effects of hypoxia-treated DC were studied using an allogenic MLR assay (Fig. 3). To exclude reoxygenation effects during the MLR assay in a first step, PFA-fixed DC from C57BL/6 (H-2^b) were used and

incubated with responder splenocytes from BALB/c (H-2^d) mice for 3 days under normoxic conditions. A significant increase in splenocyte proliferation was observed when DC were exposed to LPS in combination with hypoxia when compared with DC activated with LPS alone (Fig. 3*A*). This correlates well with the phenotypic data shown above (Fig. 2*B*), in which stimulation of DC with LPS and hypoxia resulted in a robust induction of CD80, CD86, and MHC II expression. To investigate whether hypoxic conditioning had a sustained effect on DC even after reoxygenation, the capability of γ-irradiated reoxygenated DC to induce an allogeneic lymphocyte proliferation was analyzed (Fig. 3*B*). Similarly, the highest lymphocyte stimulatory capacity was observed when DC were stimulated with LPS under hypoxic conditions. In contrast, hypoxic conditioning of DC alone did not increase the splenocyte proliferation rates. Next, enriched $CD4^+$, CFSE-labeled T cells were used as responder cells and, again, the highest T cell proliferation was observed when DC were previously activated with LPS in the presence of hypoxic conditions. Hypoxia alone did not lead to increased proliferation rates of allogeneic T cells (Fig. 3*C*).

Hypoxia or LPS highly enhances glycolysis and energy generation in DC

Next, we investigated how DC react to low oxygen tensions. It is known that hypoxia leads to a robust glycolytic activation of different cells. To investigate the glycolytic activity, glucose consumption and lactate production were determined in the supernatant as an indicator for glycolytic activity. LPS in combination with hypoxia significantly enhanced glucose consumption (Fig. 4*A*). In addition to glucose consumption also the lactate production by DC was augmented by hypoxia or LPS, and was further increased when LPS and hypoxia were combined (Fig. 4*B*). Consistent with the increased glucose and lactate production, simultaneous DC exposure to LPS and hypoxia led to a significant increase in cellular LDH levels, indicating cellular glycolytic activity (Fig. 4*C*). Finally, total ATP levels in response to hypoxia, LPS, or hypoxia in combination with LPS were investigated. DC exposed to LPS or hypoxia alone showed increased cellular levels of ATP compared with normoxic controls. Cellular ATP concentrations were further increased after simultaneous stimulation with LPS and hypoxia (Fig. 4*D*).

Glycolysis is involved in the LPS-induced DC maturation

Because DC maturation was accompanied by an increase of anaerobic glycolysis even under normoxic conditions, we tested whether or not maturation is dependent on glycolytic energy generation. Addition of 10 mM 2-deoxyglucose to the DC cultures, which acts as an inhibitor of glycolysis, prevented lactate generation under hypoxic and normoxic conditions (Fig. 5*A*). Interestingly, DC were able to sustain hypoxia and did not undergo apoptosis even when challenged with hypoxia after inhibition of glycolysis, as indicated by annexin/PI stainings (Fig. 5*B*). In contrast, under these conditions, DC failed to up-regulate the expression of the costimulatory molecules CD80 (Fig. 5*C*) and CD86 (data not shown). Thus, glycolytic energy seems to be important for the up-regulation of costimulatory molecules during DC maturation.

Upon stimulation with LPS, hypoxia, or both, HIF-1α is detectable and HIF-1α target genes are induced in DC

To analyze the HIF-1α expression in DC on protein level, immunocytochemistry and Western blot analyses were performed. In normoxia, HIF-1α protein expression was not detectable (Fig. 6, *A*

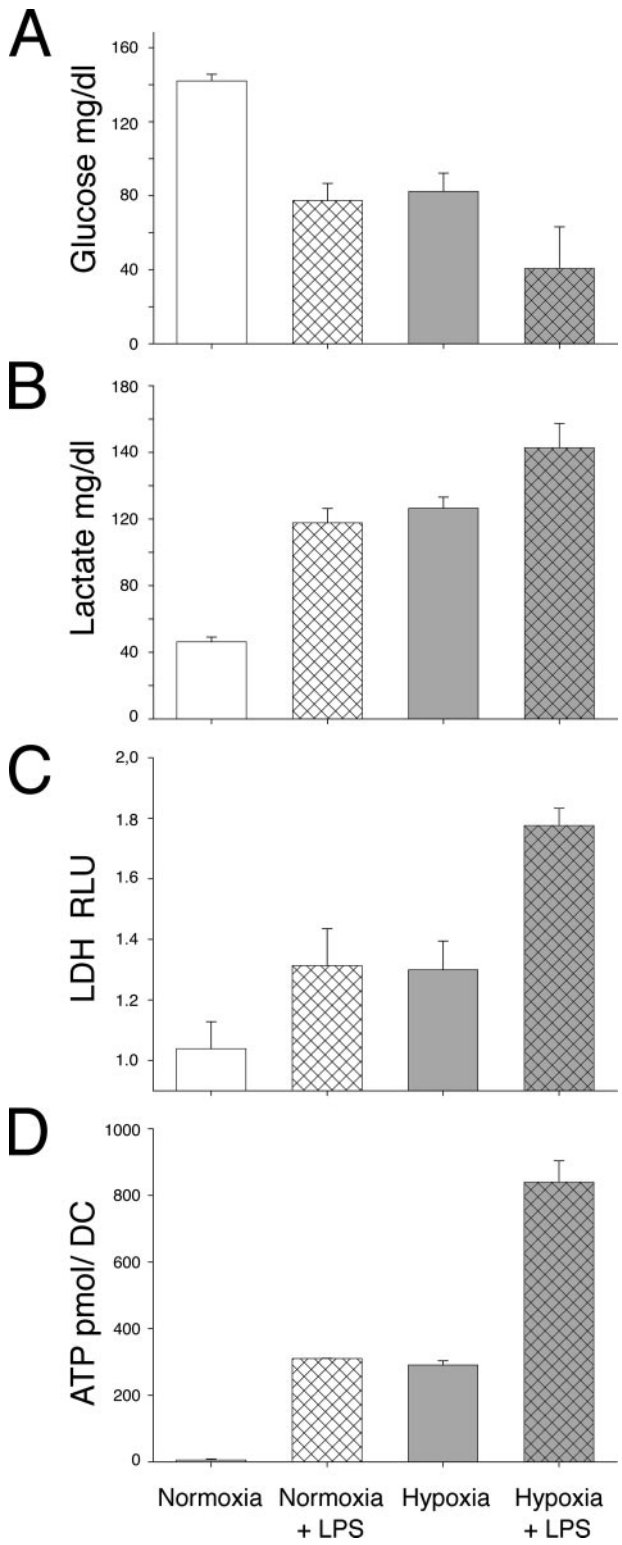

FIGURE 4. Energy metabolism of DC after exposure to normoxia, hypoxia, or LPS. A total of 2×10^6 DC was exposed to LPS, hypoxia, or hypoxia and LPS. Twenty-four hours later, supernatants were analyzed for the concentration of glucose (*A*) and lactate (*B*) as indicators for glycolytic activity. *C*, The experiment was performed as described in *A*, but after 24 h of stimulation, DC were lysed with 1% Triton X-100 and cell lysates were analyzed for LDH activity using the cell viability assay, according to the manufacturer's instructions. *D*, 24 h after treatment with LPS, hypoxia, or hypoxia and LPS, the cellular ATP content of 2×10^6 DC was measured using an ATP bioluminescence assay. Data are representative of three independent experiments performed.

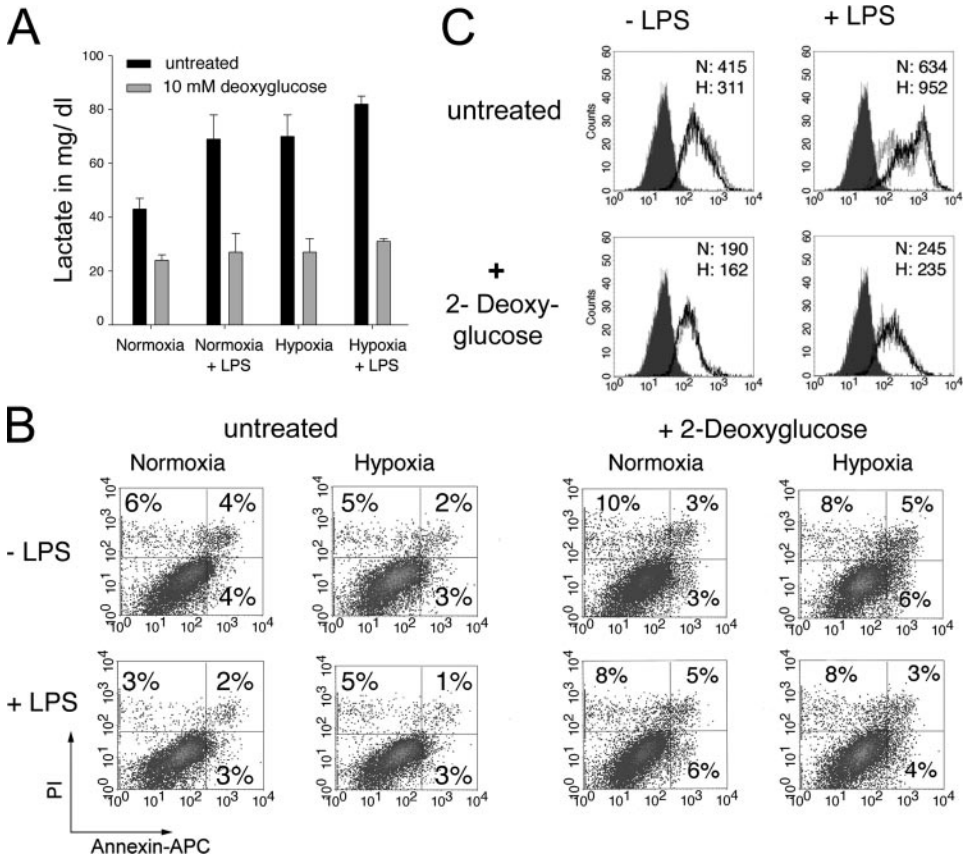

FIGURE 5. LPS-induced up-regulation of costimulatory molecules on DC needs glycolytic energy generation. *A*, Suppression of glycolysis by treatment with 10 mM 2-deoxyglucose. A total of 2×10^6 DC was either left untreated or treated with 10 mM 2-deoxyglucose 1 h prior exposition to LPS, hypoxia, or hypoxia and LPS for 24 h. Lactate concentrations were determined in the supernatants. *B*, The experiment was performed as described in *A*, and after 24 h apoptosis and necrosis signals were analyzed in DC using annexin-allophycocyanin and PI stainings and flow cytometry. *C*, Treatment with 10 mM 2-deoxyglucose reduced the upregulation of the costimulatory molecule CD80 following exposure to LPS on CD11c high positive cells. Filled lines represent the isotype control, gray lines represent the CD80 expression under normoxic conditions, and black lines show the expression of CD80 under hypoxic conditions. *Insets*, Show mean fluorescence intensity of CD80 expression. Data are representative of two independent experiments.

and *B*). However, after exposure to hypoxia and/or LPS, a strong signal was detected using Western blotting and immunocytochemistry (Fig. 6, *A* and *B*; please note the prominent nuclear HIF-1α localization in Fig. 6*B*). Importantly, as shown by immunoblotting, even under normoxic conditions, LPS exposure alone resulted in HIF-1α accumulation comparable to hypoxic controls. Next, HIF-1α mRNA levels were determined after stimulation with LPS, hypoxia, or both. Induction of HIF-1α mRNA was detectable after stimulation with LPS under normoxic or hypoxic conditions (Fig. 6*C*).

Concomitantly with the enhanced HIF-α protein levels, an induction of the HIF-α target GLUT-1 was observed after LPS stimulation, hypoxia, or the combination of both stimuli, as shown by immunoblotting (Fig. 6*D*). To assess the effects of hypoxia, LPS, or both, mRNA levels of HIF-1α target genes were determined. GLUT-1 mRNA levels under hypoxic conditions were comparable to cells stimulated with LPS under normoxic conditions. Furthermore, GLUT-1 mRNA levels were increased after simultaneous application of hypoxia and LPS (Fig. 6*E*). Similar results were obtained for another HIF-1α target gene, namely the PGK (Fig. 6*F*).

HIF-1α knockdown in DC reduces the induction of HIF-1α target genes after stimulation with LPS, hypoxia, or both

To investigate the functional effects of HIF-1α on DC immunobiology and glucose metabolism, a highly efficient method to transfer siRNAs via electroporation into murine DC without affecting their biology has been established (J. Jantsch, N. Turza, M. Volke, K.-U. Eckardt, M. Hensel, A. Steinkasserer, C. William, and A. Prechtel, submitted for publication). DC were electroporated with siRNA against HIF-1α, and 24 h later the cells were exposed to LPS, hypoxia, or both stimuli. A strong knockdown of HIF-1α mRNA was observed under all conditions tested (Fig. 7*A*). To investigate the effect of the knockdown regarding the induction of HIF-1α target genes, the mRNA levels of GLUT-1 were investigated. A clear reduction of GLUT-1 mRNA was observed in HIF-1α-deficient DC stimulated with LPS, hypoxia, or both (especially strong reduction) compared with DC electroporated with an unspecific nonsilencing siRNA (ns-siRNA) or untreated controls (Fig. 7*B*). Similar results were obtained when PGK mRNA levels were investigated (data not shown).

HIF-1α knockdown inhibits expression of costimulatory molecules on DC

Next, the effect of the HIF-1α knockdown regarding the expression of costimulatory molecules (CD80, CD86) was investigated. Twenty-four hours after siRNA transfer, DC were stimulated with LPS, hypoxia, or both and compared with DC, which were transfected with ns-siRNA or untreated controls. CD86-specific FACS analysis revealed that HIF-1α-silenced DC showed a reduced CD86 expression after exposure to LPS under normoxic as well as under hypoxic conditions (Fig. 8*A*). Similar results were obtained when the expression of CD80 was analyzed in HIF-1α-deficient DC (data not shown).

HIF-1α-deficient DC have a reduced potential to stimulate proliferation of allogeneic T cells

Next, the functional consequences of the HIF-1α knockdown in DC were tested using a MLR assay. A clear reduction in allogeneic T cell proliferation could be observed in LPS-stimulated HIF-1α knockdown DC when compared with ns-siRNA-electroporated or untreated control DC (Fig. 8*B*). Thus, we conclude that HIF-1α

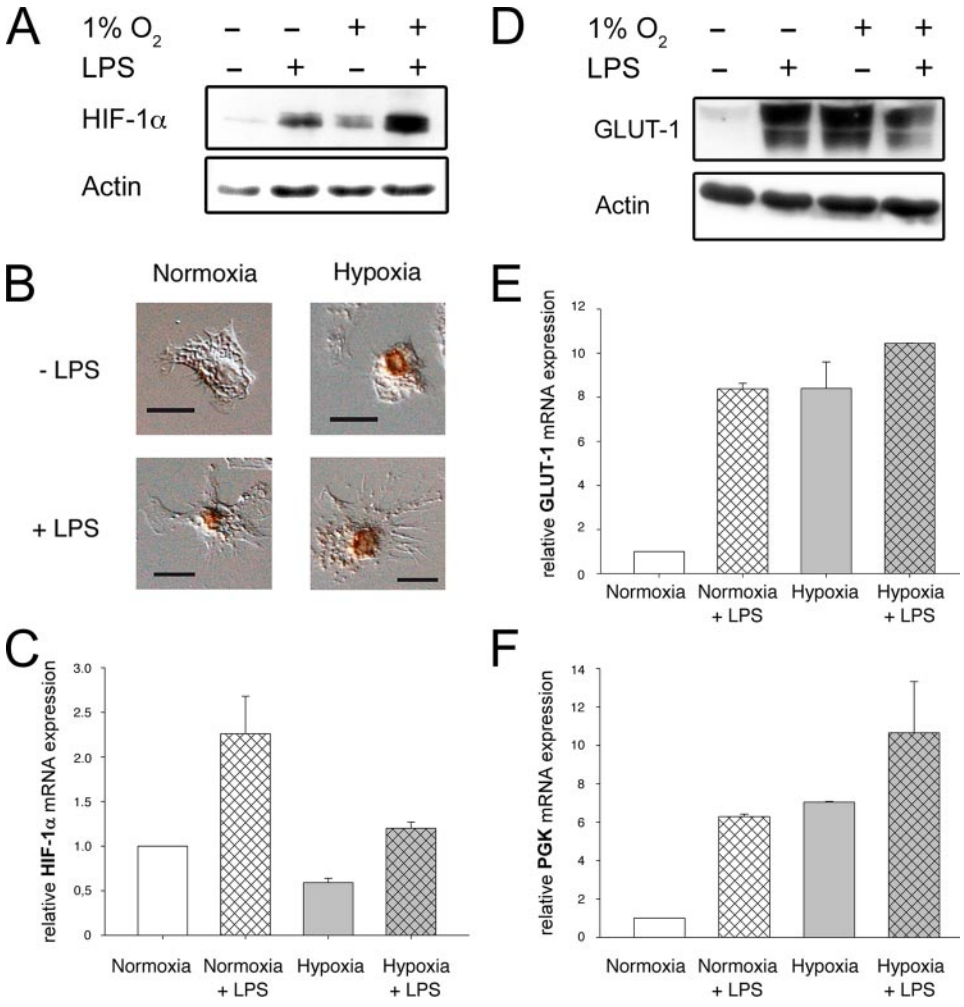

FIGURE 6. Upon stimulation with LPS, hypoxia, or both, HIF-1α is detectable and HIF-1α target genes are induced in DC. *A*, Cellular lysates were prepared from DC that have been exposed to LPS, hypoxia, or hypoxia and LPS for 24 h. Equal amounts of protein were loaded and separated using an 8% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed for HIF-1α and actin. *B*, DC were stimulated for 24 h, as indicated, and immunocytochemistry was performed using a polyclonal anti-HIF-1α Ab. Scale bar corresponds to 14 μm. *C*, 16 h after treatment with the indicated stimuli, total RNA was isolated and reverse transcribed, and real-time PCR was performed with HIF-1α mRNA as target and S14 mRNA as internal control. *D*, The experiments were performed as described in *A*; using Western blot analyses, GLUT-1 and actin expressions were detected. The experiments were performed as in *C*; *E* and *F*, real-time PCRs were used to detect GLUT-1 (*E*) and PGK mRNAs (*F*). S14 mRNA was used as an internal control.

expression in DC is vital to promote T cell proliferation irrespective from an inflammatory activation by LPS.

Discussion

Because inflamed tissues are frequently hypoxic, the effects of hypoxia on DC activation and function in the presence or absence of LPS as a proinflammatory stimulus were investigated. Interestingly, hypoxia together with LPS induced a highly inflammatory state in DC, which exceeded activation levels compared with LPS alone. These synergistic effects of LPS and hypoxia potentially determine a sustained inflammatory activation of DC, resulting in an enhanced immunogenic response. Thus, together with other proinflammatory signals, hypoxia could possibly act as an additional danger signal in concert with LPS according to the proposed terminology by Matzinger (25).

Measurement of glucose uptake and glucose consumption showed that DC activation in inflammatory states was paralleled with enhanced glycolytic activity. Analysis of ATP storage revealed an extensive capability of DC to enhance energy supply, albeit aerobic glycolysis should hypothetically be restricted because of lacking oxygen. Concordantly, the increased lactate levels in culture medium indicated that enhanced glycolysis and ATP storage result from increased anerobic glycolysis, which is independent of the presence of oxygen. On the contrary, application of inhibitory glucose metabolites completely blocked not only glycolysis, but also inhibited expression of certain DC surface proteins, including costimulatory molecules. This demonstrates that an enhanced glycolysis is a prerequisite for inflammatory DC activation. Thus, the enhanced glycolytic activity and energy generation could be an important additional maturation indicator following exposure to an inflammatory stimulus besides the induction of costimulatory molecules or the synthesis of chemokines and cytokines (3).

Glycolysis is known to be tightly controlled by HIF-1α (26–28), and induction of glycolysis presumably adapts the cell to a state of oxygen deprivation. To test for the role of HIF-1α in glycolysis-dependent DC activation, a HIF-1α-specific siRNA-mediated knockdown model has been established using a electroporation protocol, which proved to be highly efficient in transferring siRNAs and did not otherwise interfere with DC biology (J. Jantsch, N. Turza, M. Volke, K.-U. Eckardt, M. Hensel, A. Steinkasserer, C. William, and A. Prechtel, submitted for publication). Knockdown of HIF-1α by siRNA suppressed HIF-1α mRNA levels and subsequently down-modulated the induction of HIF-1α target genes such as GLUT-1 and PGK. Furthermore, HIF-1α knockdown interfered with the expression of costimulatory molecules such as CD80 and CD86, which are typically expressed on mature DC, and hampered the capability of DC to stimulate allogeneic T cell proliferation. These results indicate that HIF-1α is necessary for glucose and energy supply and important for the activation/maturation process of DC. Our observations are in line with previous reports indicating that myeloid cells depend on glycolysis for energy generation (13). Importantly, targeted disruption of HIF-1α expression in macrophages and granulocytes using the lysozyme M promoter led to profound defects in myeloid cell aggregation,

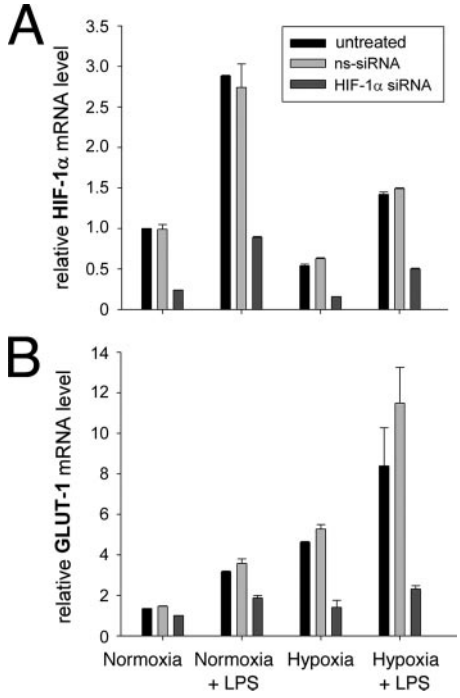

FIGURE 7. HIF-1α knockdown in DC strongly reduces the induction of the HIF-1α target gene GLUT-1 after stimulation with LPS, hypoxia, or both. DC were either electroporated with HIF-1α-specific siRNA duplexes or with a nonsilencing siRNA. After 24 h, cells were exposed with the indicated stimuli, and after another time period of 16 h, total RNAs were isolated and reverse transcribed, and real-time PCRs were performed with HIF-1α mRNA (*A*) as target or GLUT-1 mRNA (*B*) as target. S14 mRNA was used as an internal control. *A*, The normalized ratio of HIF-1α to S14 of untreated DC was set to 1. The specific HIF-1α mRNA levels were reduced up to 80%. *B*, The normalized ratio of GLUT-1 to S14 of untreated DC was set to 1. The specific GLUT-1 mRNA levels were reduced up to 70%.

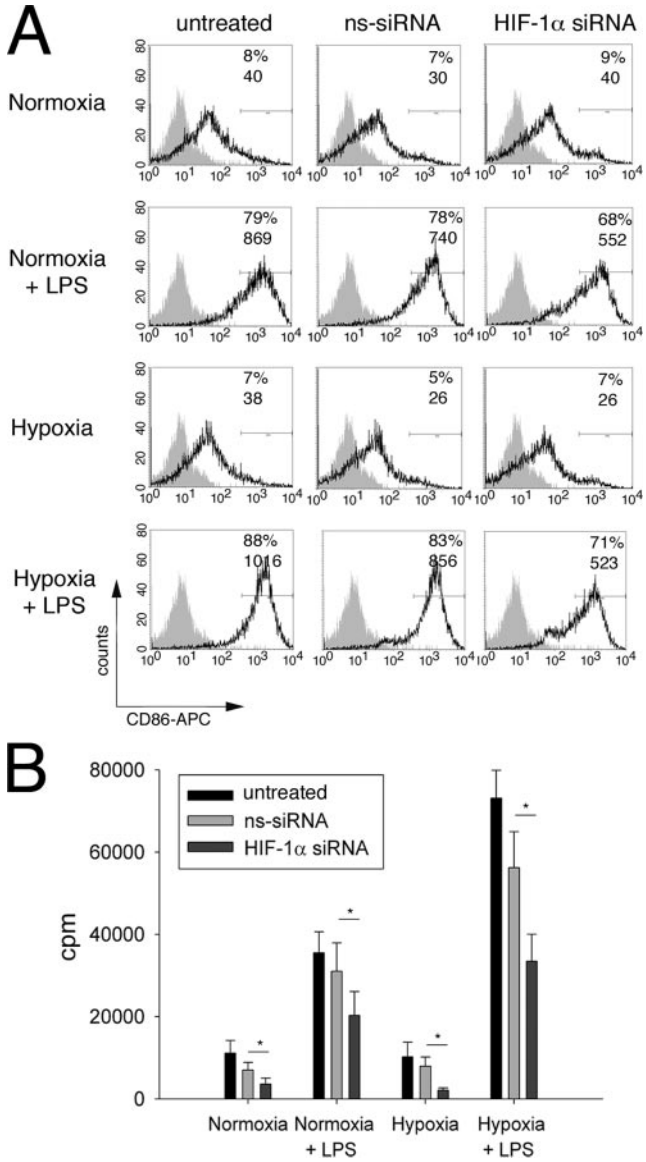

FIGURE 8. HIF-1α knockdown in DC impairs the up-regulation of CD86 and interferes with their capacity to stimulate proliferation of allogeneic T cells. *A*, DC from C57BL/6 (H-2^b) were either electroporated with HIF-1α-specific siRNA duplexes or with a nonsilencing siRNA. After 24 h, DC were stimulated with the indicated stimuli, and after another time period of 24 h, the expression of CD86 on CD11c-positive cells was analyzed (black lines). The gray lines show the isotype control. Data are representative of at least two independent experiments. *B*, HIF-1α knockdown DC were hampered in their capacity to induce an allogeneic T cell proliferation under normoxic and hypoxic conditions. DC were treated as described in *A*; after stimulation with the indicated stimuli for 24 h, the cells were reoxygenated and γ irradiated with 3600 rad. Enriched CD4$^+$ T cells from BALB/c (H-2^d) spleens were added to the DC for 3 days under normoxic conditions. Proliferation was determined using [^{3}H]thymidine incorporation. Data are representative of two experiments. Statistical analyses were performed using unpaired Student's *t* test. Statistical significance has been defined as follows: $*$, $p < 0.05$.

motility, invasiveness, and bacterial killing capacities (13). However, in contrast to our study, Cramer et al. (13) did not investigate the effects of hypoxia and HIF-1α regarding the immunobiology of DC.

Surprisingly, hypoxia alone without a further inflammatory signal did not activate DC. In ischemic tissues, for example, in infarction, DC are frequently confronted with low oxygen tensions without further inflammatory signals or the presence of pathogens. Injured cells may then trigger an immunogenic response by releasing intracellular substances. A missing activation of DC following hypoxia alone could thus hypothetically protect from uncontrolled autoimmune activation in situations of ischemia or apoptosis without concomitant infections. Accordingly, previous studies already described induction of tolerance after uptake of dying cells to DC (29). However, these hypotheses have to be proven by further experimental investigations.

How LPS induces accumulation of HIF-1α protein levels in the presence of oxygen is still in debate. Although this is the first report of HIF-1α induction in DC, previous reports showed induction of HIF-1α by LPS in macrophages (30–32). In this study, we describe the induction of HIF-1α mRNA in DC after stimulation with LPS under normoxic conditions. It has been hypothesized that increased HIF-1α mRNA levels lead to saturation of the prolyl hydroxylase capacity, followed by stabilization of cellular HIF-1α protein (14). But whether the capacity of prolyl hydroxylation is saturated after stimulation with LPS has not been determined yet, and, therefore, other mechanisms of HIF-1α stabilization cannot be excluded.

Furthermore, it cannot be ruled out that additional HIF-1α-regulated, not yet identified genes are directly involved in DC activation/maturation, which do not participate in energy metabolism, but, for example, in generation of inflammatory molecules or

signaling cascades, as follows: 1) Inflammatory cytokines, in particular TNF, are at present not considered to be HIF-1α target genes. However, the presence of hypoxia response elements in the IL-6 and particular TNF gene could possibly implicate a transcriptional response upon HIF-1α (14). 2) Walmsley and colleagues (33) showed in granulocytes that expression of NF-κB is hypoxia regulated in a HIF-1α-dependent manner and promoted survival under hypoxic conditions. This potentially important interaction of the NF-κB and HIF-1α pathway in DC remains to be elucidated. 3) Under hypoxic conditions, reactive oxygen species have been shown to play an important role in hypoxic activation of proinflammatory genes in a macrophage cell line (34). We cannot definitively rule out that alterations in the redox state may influence to some extent proinflammatory signaling pathways in DC.

Altogether, our study indicates that hypoxia significantly amplifies proinflammatory stimuli for DC activation and function, and that these effects depend on enhanced cellular energy supply and on cellular HIF-1α activity. Like other recent studies showed before, the transcription factor HIF-1α turns out to be an important immune modulator. New HIF-1α prolyl-hydroxylase inhibitors (10), which are able to induce the HIF-1α system pharmacologically and to mimic a hypoxic response, may even open new experimental options to modulate DC biology. This may have important implications to promote new concepts to fight against infections or to induce DC-mediated tolerogenic mechanisms.

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Disclosures

The authors have no financial conflict of interest.

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