Divergent Effects of Hypoxia on Dendritic Cell Functions

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Running title: Hypoxia inhibits dendritic cell maturation

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Abstract

Dendritic cells (DC) are professional antigen-presenting cells (APC) that patrol tissues to sense ‘danger’ signals and activate specific immune responses. In addition they also play a role in inflammation and tissue repair. Here we show that oxygen availability is necessary to promote full monocyte-derived DC differentiation and maturation. Low oxygen tension (hypoxia) inhibits expression of several differentiation and maturation markers (CD1a, CD40, CD80, CD83, CD86 and MHC class II molecules) in response to lipopolysaccharide (LPS), as well as their stimulatory capacity for T cell functions. These events are paralleled by impaired up-regulation of the chemokine receptor CCR7, an otherwise necessary event for the homing of mature DC to lymph nodes. In contrast, hypoxia strongly up-regulates production of pro-inflammatory cytokines, particularly TNFα and IL-1β, as well as the inflammatory chemokine receptor CCR5. Subcutaneous injection of hypoxic DC into the footpads of mice results in defective DC homing to draining lymph nodes, but enhanced leukocyte recruitment at the site of injection. Thus hypoxia uncouples the promotion of inflammatory and tissue repair from sentinel functions in DC, which we suggest is a safeguard mechanism against immune reactivity to damaged tissues.

Introduction

Low oxygen tension (hypoxia) has been described at virtually every site of extensive inflammation, including necrotic foci and cutaneous sites of infection and wounding ¹. Sites of inflammation are also characterized by extensive infiltration of inflammatory leukocytes, which need to move against oxygen gradients. As a consequence, immune effector cells in hypoxic sites, including dendritic cells (DC), have an acute need to respond to these demanding conditions to maintain their viability and activity. DC are powerful APCs specialized for the activation of resting T cells and the initiation and regulation of many types of immune response ²⁻⁵. Because of this we have
investigated the functional changes that accompanying the metabolic adaptation of DC to hypoxia, as these events are likely to affect the development of both inflammatory and immune functions.

The capacity of DC to activate and regulate T cell responses is acquired during a complex differentiation and maturation programme \(^2\text{--}^5\). DC originate from bone marrow and, at an ‘immature’ stage, they patrol peripheral tissues for the presence of pathogen-associated antigens. In order to perform this function DC express a rich repertoire of pattern recognition receptors (PRR), including Toll-like receptors (TLR), which permit DC to recognise distinct pathogen-associated molecules \(^6\text{,}^7\). The engagement of TLR initiates a cascade of signalling events in DC that leads – in the process of ‘maturation’ – to the secretion of inflammatory and immuno-modulatory factors which mediate protective immunity \(^6\text{,}^7\). For instance stimulation of DC by LPS, through the participation of TLR4, leads to up-regulation of MHC molecules; costimulatory molecules such as CD40, CD80 and CD86; the ‘maturation markers’ CD83 and DC-lysosome-associated membrane protein (LAMP); chemokine receptors CXCR4 and CCR7; a diversity of cytokines and chemokines; and potent APC function \(^4\text{,}^5\). Such inflammatory signals also induce a chemokine receptor switch, with down-regulation of inflammatory receptors (such as CCR1, CCR2, CCR5) being associated with the induction of CCR7 \(^8\). This chemokine receptor switch facilitates the emigration of DC out of peripheral tissues and their localization into the T-cell areas of secondary lymphoid organs where they encounter naive T cells and initiate immune responses \(^8\).

Several studies have provided evidence that the maturation of DC is a highly regulated process that can be influenced by several factors present in the tissue microenvironment. Critical factors include IL-10 \(^9\text{--}^{11}\) and VEGF \(^12\text{,}^{13}\) whose expression is modulated by hypoxia \(^1\). These cytokines inhibit monocyte-derived DC differentiation as well as maturation, and also block the functional activities normally associated with a ‘mature’ state \(^9\text{,}^{10}\text{,}^{12}\). As DC often localize at inflammatory sites characterized by low oxygen tension, such as wounds, tumours and other sites of ischemia \(^1\text{,}^{14}\), we investigated whether low oxygen could modulate the differentiation and maturation of DC. Our results indicate that hypoxia strongly enhances the innate immune functions of DC by inhibiting
their maturation, but increasing both their production of inflammatory cytokines and their chemotactic response towards chemokines selectively expressed at peripheral sites of inflammation. We propose that this modulation represents a safeguard against immune reactivity to damaged tissues.
MATERIAL AND METHODS

Cells and culture conditions. Human monocytes were separated from the peripheral blood of healthy human donors (courtesy of the Ospedale di Desio, Milan) by Percoll gradient, as previously described \(^\text{15}\). Dendritic cells were generated from monocytes by incubation for 6 days at \(1 \times 10^6/ml\) in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, antibiotics, 50 ng/ml GM-CSF and 20 ng/ml IL-13 under normoxic (20% O\(_2\), 5% CO\(_2\), and 75% N\(_2\)) or hypoxic conditions (1% O\(_2\), 5% CO\(_2\), and 94% N\(_2\)). After 6 days of culture, the population consisted of typical immature dendritic cells which generally expressed CD1a (>90% positive cells), low levels of CD80 and CD86, and little or no CD83 (<10%) and CD14 (<10%) \(^\text{16-18}\). To induce terminal maturation, LPS 10 ng/ml was added at day 6 for 18 hrs in normoxic or hypoxic conditions. Hypoxia treatment was performed by placing cells in the \textit{InVivo} 2 400 hypoxic workstation (Ruskinn-Biotrace) maintained at 1% O\(_2\) and 37°C, or in a modular incubator chamber (Billups-Rothemberg Inc., Del Mar, California) flushed with a mixture of 1% O\(_2\), 5% CO\(_2\), and 94% N\(_2\) and placed at 37°C. Highly enriched CD1c\(^+\) DC (>90%) were obtained from PBMC of peripheral blood of healthy donors by immunomagnetic depletion of contaminating CD19\(^-\)CD1c\(^+\) B cells using CD19 mAb-conjugated microbeads (Miltenyi Biotec) followed by immunomagnetic enrichment of CD1c\(^+\) cells.

FACS analysis. Monocytes were stained with FITC-conjugated mouse anti-human monoclonal CD1a (clone HI149, BD Biosciences), APC-conjugated mouse anti-human monoclonal CD14 (clone M5E2, BD Biosciences), R-PE-conjugated mouse anti-human CD16 (clone 3G8, AbD Serotec) and R-PE-conjugated mouse anti-human CD68 (clone Y1/82A, BD Biosciences). Human dendritic cell staining was performed using APC-conjugated mouse anti-human monoclonal CXCR4 (clone 12G5, BD Biosciences), PE-Cy7-conjugated rat anti-human monoclonal CCR7 (clone 3D12, BD biosciences), R-PE-conjugated mouse anti-human monoclonal CD80 (clone L307.4, BD Biosciences), R-PE conjugated mouse anti-human monoclonal CD40 (clone 5C3, BD
Biosciences) and APC-conjugated mouse anti-human monoclonal CD86 (clone 2331(FUN-1), BD Biosciences). For each antibody, the proper isotype controls were used. Furthermore DC were stained with mouse anti-human monoclonal CCR5 (clone CTC5, R&D Systems), mouse anti-human monoclonal CD83 (clone HB15e, BD Biosciences) and MHC class II (hybridoma L243 from ATCC) followed by Alexa 488-conjugated, isotype-matched, affinity-purified, goat anti-mouse antibody (Molecular Probes, Invitrogen). For each antibody, the proper mouse isotype-control antibody was used followed by Alexa 488-conjugated goat anti-mouse antibody.

**Cytokines and reagents.** Human recombinant CXCL12/stromal-derived factor 1 (SDF-1), CCL5/RANTES and CCL19/MIP3β were from PeproTech; LPS was from Sigma-Aldrich (*Escherichia coli* strain 055:B5) or from Alexis Biochemicals (*Salmonella abortus equi* S-form); PAM3CSK4 was from Alexis Biochemicals; poly (I:C) was from GE Healthcare Life Sciences; resiquimod (R-848) was from Alexis Biochemicals; deferoxamine was from Sigma-Aldrich; human recombinant GM-CSF was a kind gift from Novartis; human recombinant IL-13 was from R&D Systems and anti-human VEGF antibody was from R&D Systems.

**Migration assay.** Dendritic cell migration was evaluated using a chemotaxis microchamber technique as described previously. Briefly, 30 μl of chemoattractant solution or control medium (RPMI 1640 with 1% FBS) was added to the lower wells of a chemotaxis chamber (Neuroprobe) and a polycarbonate filter (5μm pore size; Neuroprobe) was placed into the wells and covered with a silicon gasket. 50 μl of cell suspension (1x10^6/ml) were seeded in the upper wells and the chamber was incubated at 37 °C for 90 min. At the end of this period, filters were removed and stained with Diff-Quik (Baxter) and 10 high-power oil immersion fields were counted.
**Enzyme-linked immunosorbent assay (ELISA).** The concentrations of hTNFα, hIL-1β, hIL-10, hCXCL10, hCCL22, mTNFα and mIL-1β levels in DC supernatants were measured using specific Duo-Set kits purchased from R&D Systems, in accordance to manufacturer’s instructions.

**Real-time PCR.** Total RNA was obtained using Trizol (Invitrogen Life Technologies). RT-PCR from 1µg RNA template was performed using a RT-PCR kit (cDNA Achieve kit, Applied Biosystems). Real-time PCR was performed using SyBr Green PCR Master Mix (Applied Biosystems) and detected with a 7900HT Sequence Detection System (Applied Biosystems). The primers used were designed using Beacon Designer5 software (Premier Biosoft International). Data were normalized to the expression of the housekeeping gene, β-actin, in the PCR reactions and results were expressed as fold increase in mRNA expression with respect to the control cells.

**Proliferation assay.** After 18 hrs of culture under normoxic or hypoxic conditions in the presence or absence of LPS 10 ng/ml, DC were exposed to 2000 rad in cell irradiator and then collected and re-distributed in 96-well plates in ratios of 1:10 and 1:20 with CD4+ T cells; CD4+ T cells were isolated from peripheral blood of healthy donors by RosetteSep Human CD4+ T Cell Enrichment Cocktail (StemCell Technologies, USA) in accordance with manufacturer’s instructions. After 3 days of co-culture, the T cell proliferation was assessed using the Cell Proliferation Biotrak ELISA System (Amersham Biosciences, USA). Briefly BrdU was added to the cells and incubated overnight, after which the culture medium was removed, the cells were fixed, and peroxidase-labelled anti-BrdU was added. The immune complexes were detected by the subsequent substrate reaction and read at 450nm.

**Immunoblotting.** Dendritic cells were washed with ice-cold phosphate buffered saline containing 1 mM Na3VO4, then lysed in 50µl of lysis buffer [20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1 mM Na3VO4, 2 mM EDTA, 1 mM PMSF, 20µM
leupeptin and 0.15 U/ml aprotonin] for 20 min at 4°C. The lysates were centrifuged at 13,000 rpm at 4°C for 15 min and the supernatants (containing Triton X-100 soluble proteins) were run on a 10% (w/v) SDS–PAGE (50 µg protein/lane). Separated proteins were transferred onto a nitrocellulose membrane (1 h at 125 mA) and immunoblotted for specific rabbit anti-human HIF-1α antibody (Cell Signalling Technologies Inc, MA) or for specific mouse anti-mouse HIF-1α antibody (Novus Biologicals) according to manufacturer’s instructions. Blocking was done with 5% (w/v) BSA in TBS-0.1% Tween (TBST) for 1 hr at room temperature. Antibody dilutions were prepared in 5% (w/v) BSA-TBST. Primary antibodies were used at 1:1000 or 1:500 dilutions, respectively, overnight at 4 °C. HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Amersham, USA) were used at 1:10000 or 1:2000 dilution for 1 hr at room temperature. Blots were visualized using an ECL kit (Amersham, USA). Immunoblotting for actin was performed using a goat polyclonal anti-actin (c-11) (Santa Cruz Biotechnology), diluted 1:2000 in 5% (w/v) BSA-TBST, and HRP-conjugated anti-goat secondary antibody (Santa Cruz Biotechnology) was used at 1:5000 dilution.

**In vivo migration of murine DC.** CD34⁺-derived myeloid DC (DC) were generated from femurs of C57BL/6 mice as previously described. After 9 days of culture, CD34⁺-derived myeloid DC were collected (>90% CD11c positive) and exposed to LPS 100 ng/ml for 18 hours in normoxia, or in hypoxia, or in the presence of deferoxamine 400 µM (hypoxia mimicker). Afterwards DC were labelled with 0.5 mM of the vital dye 5-(and-6)-carboxyfluorescein diacetate succinimidy ester [mixed isomer (5-(6)-CFDA, SE (CFSE), Molecular Probes Inc., Eugene, OR]. A total of 2x10⁶ labelled cells were injected subcutaneously in the hind leg footpad. Popliteal lymph nodes were recovered 24 hrs later, mechanically disaggregated and treated with collagenase A (1 mg/ml; Boehringer Mannheim, Indianapolis, IN) and DNase (0.4 mg/ml; Roche, Indianapolis, IN) for 30 min, after which the cell suspension was evaluated by FACScount (Becton Dickinson, San Jose, CA).
Histology and Immunohistochemistry. Consecutive frozen sections (8µm) of footpads were cut, mounted on Superfrost slides (Bio-Optica, Milan, Italy) and used for histological and immunohistochemical evaluation. Histological examination was performed on haematoxylin-eosin stained sections. Immunohistochemistry (IHC) was performed on acetone/chloroform fixed slides by using the monoclonal antibody rat anti-mouse Lymphocyte Function Associated-Antigen-1/LFA1 (supernatant, dilution 1:10, raised in our laboratory) to detect lymphocytes, monocytes, granulocytes and some plasma cells). The sections were rehydrated with PBS and then incubated with primary antibody for 2 hrs in a humid chamber. The reactions were revealed by biotinylated anti rat IgG (dilution 1:50, one hour incubation, Vector Laboratories, USA) followed by HRP-Conjugated ZyMax Streptavidin (dilution 1:500, 30 min incubation, Zymed, San Francisco California). The chromogen was 3,3'-diaminobenzidine free base (DAB).

Annexin V staining. Murine tissues were collected and snap frozen by liquid nitrogen; 8 µm sections were cut, mounted on Superfrost slides (Bio-Optica, Milan, Italy) and fixed with 4% paraformaldehyde for 15 min at room temperature. Sections were rehydrated with PBS (pH 7.00) twice for 10 min, incubated for 5 min with PBS- 0.03% H₂O₂ -1% bovine serum albumine (BSA) and with PBS - 1%BSA for 10 min to block endogenous peroxidase and aspecific sites, respectively. A double immunofluorescence was performed with the following antibodies: FITC conjugated anti Annexin V (Immunostep; Salamanca) and rat anti mouse biotinylated CD11c followed by ALEXA fluor streptavidin 594 (Molecular Probes). Nuclei were stained with DAPI (Molecular Probes, Invitrogen). The Fondazione Humanitas per la Salute adheres to the principles set out in the following laws, regulation and policies governing the care and use of laboratory animals: Fondazione Humanitas Regulations and Policies providing internal authorisation for persons conducting animal experiments; the NIH Guide for the Care and Use of Laboratory Animals (1996 edition) Italian Governing Law Legislative decree 116 of Jan. 27, 1992; EU

**Statistical analysis** Data are presented as means with standard deviation. Statistical comparisons between groups were made using Student’s t test and p< 0.05 was considered to be statistically significant.
RESULTS

Hypoxia-induced HIF-1α activation is paralleled by inhibition of monocyte-derived DC differentiation

Hypoxia is a common feature of several inflammatory diseases \(^1,14^\) and strongly affects the expression of specific genes involved in leukocyte activation and recruitment \(^20,21^\). In hypoxia, cells undergo a metabolic adaptation, mainly mediated through the induction and stabilization of the Hypoxia-Inducible Factor-1 (HIF-1), a major regulator of cell adaptation to hypoxic stress \(^22^\). As DC are an important component of the inflammatory infiltrate characterizing inflammatory tissues, we evaluated the activation of HIF-1α in response to hypoxia, during the differentiation and maturation phases of monocyte-derived DC. Human monocytes were cultured for six days under normoxic or hypoxic conditions (1% \(O_2\)), in the presence of GM-CSF and interleukin-13 (IL-13). On day six, cells were treated for 24 hrs with LPS to induce their maturation. HIF-1α expression was evaluated by western blot at different times. As shown in figure 1 (panel A), monocytes activate HIF-1α as part of their adaptation programs to low oxygen conditions (1%) after three days.

CD1a molecules are up-regulated during maturation of DC, coincident with the functions of antigen capture and processing \(^23^\). In contrast, expression of the monocyte marker CD14 is lost during monocyte differentiation to DC. Based on this, it was important to monitor the relative expression of these two markers during various stages of dendritic cell differentiation, in normoxia versus hypoxia. As shown in figure 1 (panel B), monocytes cultured in the presence of the differentiation-inducing cytokines IL-13/GM-CSF showed differential and dynamic changes in the surface expression of CD14 and CD1a, over time. As shown, at day 0, freshly isolated monocytes were 85.5% CD14 positive and CD1a negative. At day three, three different cell populations were identified, both in normoxia and hypoxia. The first population (Q1) displayed a single positivity for
CD14 and cytofluorimetric analysis identified 14.7% of total cells in Q1 in normoxia versus 29.7% in hypoxia. A CD14/CD1a double-positive population (Q2) was present at 43.3% in normoxia and 32.9% in hypoxia. A third population (Q4), CD1a single positive, was 27.3% in normoxia and only 16.4% in hypoxia. This apparent delay of DC differentiation in hypoxia was further observed at day six, when we observed 9.4% of the Q1 population in normoxia versus 14.7% in hypoxia; 17.6% of Q2 in normoxia versus 41.2% in hypoxia; and 61.4% of Q4 in normoxia versus 33.8% in hypoxia. The consistently higher expression of CD14 and lower expression of CD1a, in hypoxia versus normoxia, suggests that low oxygen availability restrains the differentiation process of monocyte-derived DC. To investigate whether hypoxia could promote macrophage- rather than DC-differentiation, we measured the expression of the macrophage markers CD16 and CD68. As shown in panel C, hypoxia promoted a selective up-regulation of CD16 in the CD14 single-positive monocyte population (Q1). Conversely, no changes were observed in CD68 surface expression, suggesting a lack of definite skewing towards macrophage differentiation.

**Hypoxia inhibits the maturation of monocyte-derived DC**

DC mature in response to various microbial compounds, including bacterial wall components such as lipopolysaccharide (LPS). To investigate the effect of hypoxia on DC maturation (as opposed to ‘differentiation’; above), human monocytes were first cultured for six days in normoxia in the presence of GM-CSF and IL-13. On day six, immature DC were subsequently incubated either in normoxia or hypoxia, and then treated with LPS for a further 18 hrs. Hypoxia significantly reduced the expression of CD40, CD80, CD83 and CD86, both in terms of the percentage of positive cells and their mean channel of fluorescence intensity (figure 2, panel A). In addition, despite no differences being observed in the percentage of MHC class II positive DC, their mean fluorescence intensity was consistently reduced in hypoxia. As inhibition of DC maturation by hypoxia could affect their capability to promote adaptive immunity, we next determined the ability of hypoxic DC
to induce proliferation and activation of T cells. Following exposure of DC to LPS in normoxia or hypoxia, the cells were irradiated and subsequently co-cultured in mixed leukocyte reactions with CD4+ T cells obtained from allogeneic donors (figure 2, panel B). Notably, in line with the observed decreased expression of costimulatory molecules by DC in hypoxia, these cells induced lower proliferation of T cells, as well as lower secretion of IFN-γ, than DC cultured in normoxia. These results indicate that oxygen availability is a limiting condition for the expression of costimulatory properties by DC.

**Hypoxia modulates cytokine and chemokine expression by DC**

DC orchestrate adaptive immunity and modulate the inflammatory response by producing inflammatory cytokines and chemokines. Immature monocyte-derived DC were treated for 18 hrs with LPS in normoxia or hypoxia. Following this period, total RNA and supernatants were analyzed respectively for gene expression (figure 3, panel A) and secretion (figure 3, panel B) of selected cytokines and chemokines. As expected, in hypoxic conditions we observed increased mRNA levels of the prototypic hypoxia-inducible genes VEGF and CXCR4 (panel A), as well as increased expression of TNFα, IL-1β and CCL22 mRNAs. In contrast, CXCL10 and IL-10 mRNA expression was diminished. These results indicate that hypoxia can differentially modulate expression of selected cytokine and chemokine genes by DC. In line with these results, we also observed higher secretion of TNFα, IL-1β and CCL22 by hypoxic DC, while CXCL10 and IL-10 secretion was diminished (panel B). Similar results were obtained with hypoxic DC exposed to CD40L rather than LPS (data not shown). Moreover, hypoxia induced enhanced VEGF protein secretion by DC (data not shown) and, because DC maturation itself has been reported to be affected by VEGF, we also tested whether autocrine VEGF could have a role in the increased expression of pro-inflammatory cytokines observed in hypoxia. As shown in panel C, an anti-VEGF antibody significantly decreased the secretion of TNFα and IL-1β in hypoxia, while
significantly restoring CXCL10 production. These results indicate that autocrine hypoxia-induced VEGF plays a role in modulating the inflammatory phenotype of DC. However, the anti-VEGF antibody did not elicit significant changes in the expression of DC maturation markers (eg. CD80, CD40, MHC class II; not shown).

**Maturation of hypoxic DC is refractory to different TLR ligands.**

To test whether maturation of DC in hypoxia was refractory to TLR agonists other than LPS, we first analyzed the level of mRNA expression for different TLR members in immature DC (day 6) cultured for an additional 18 hrs under normoxic or hypoxic conditions (Fig. 4 panel A). As shown, no significant changes in TLR mRNA levels occurred in hypoxia. To evaluate the functional significance of these observations, immature DC were exposed for 18 hrs to several different TLR agonists and then analyzed for the surface expression of selected maturation markers (panel B). In particular we examined responses to an LPS preparation from Sigma which binds both TLR2 and TLR4 and which was used in all other studies (above), a TLR4-specific LPS (provided by Alexis), the TLR2-specific ligand PAM3CSK4, the TLR3-specific ligand Poly (I:C) and the TLR7/8-specific ligand R-848. In all cases hypoxia invariably inhibited the down-regulation of CCR5 and up-regulation of CCR7 that would otherwise accompany DC maturation, while, as expected, CXCR4 expression was induced by low oxygen conditions. Moreover hypoxia inhibited the induction of CD83 surface expression (panel B), as well as CD80 and MHC class II (data not shown). Furthermore, in most cases, hypoxia significantly enhanced TNFα and IL-1β secretion and had little effect on IL-10 secretion in response to the different TLR agonists (panel C). These results suggest that DC ‘maturation’ in hypoxia is impaired in response to a variety of different TLR ligands, thus highlighting the potential relevance of this event in various pathological conditions.
Effects of hypoxia on DC chemotaxis in vitro

Maturing DC migrate from inflamed tissues via the lymphatics into lymph nodes where CCR7 ligands are expressed; here DC initiate and orchestrate adaptive immunity. In contrast, maturing DC are characterized by the loss of responsiveness towards inflammatory chemokines such as CCL5, which acts through CCR5 and CCR1. In addition, up-regulation of CXCR4 by DC is also observed during maturation. To evaluate the effects of hypoxia on chemokine receptor functions, we measured the chemotactic responsiveness of DC matured in hypoxia and normoxia. Hypoxia decreased the chemotactic responsiveness of mature DC towards the CCR7 ligand CCL19, but enhanced DC migration to both the CCR5 and CXCR4 ligands, CCL5 and CXCL12 respectively (figure 5, panel A). Based on this observation, we next examined the surface expression of CCR7, CCR5 and CXCR4 (panel B). In line with the modulation of chemotactic responsiveness, hypoxia significantly prevented the up-regulation of CCR7 and the down-regulation of CCR5 surface expression, and further enhanced the expression of CXCR4, suggesting that low oxygen tension may inhibit an otherwise efficient chemokine receptor switch that occurs during (normoxic) DC maturation. However, the entity of the decrease of CCR7 expression may suggest that hypoxia affects DC responsiveness to CCR7 ligands also at signalling levels.

The effects of hypoxia were also evaluated using human myeloid DC from peripheral blood. After isolation, myeloid DC were exposed to LPS and cultured in normoxia or hypoxia, as indicated (panel C). Similarly to monocyte-derived DC, culture in hypoxia enhanced the secretion of TNFα, reduced IL-10 expression, and partially inhibited CXCL10 secretion. Furthermore, hypoxia promoted a strong decrease in CCR7 surface expression, a partial reduction in levels of expression of CD40, CD83 and MHC class II (in terms of mean fluorescent intensities), along with a significant up-regulation of CXCR4 (panel D). Taken together, these results from in vitro studies of both monocyte-derived DC and primary myeloid DC suggested that low oxygen conditions may alter the tissue distribution of maturing DC in vivo (see below).
Hypoxia enhances inflammatory functions of DC at peripheral sites

Cell re-oxygenation strongly affects various biological functions, including cell migration. Indeed, we observed that LPS-matured hypoxic DC, re-exposed to normoxic conditions for a further 24 hrs, regain levels of expression of both chemokine receptors (CCR5, CCR7 and CXCR4) and maturation markers (CD83, CD80 and MHC class II) equivalent to those expressed by DC matured in normoxia (data not shown). Therefore, to prevent re-oxygenation and assess the in vivo relevance of the effects of hypoxia on DC maturation, mouse CD34+-derived myeloid DC were treated with LPS (100 ng/ml) for 18 hours, in the presence or absence of 400µM of the hypoxia mimicker deferoxamine (DFX)\textsuperscript{25}. Similarly to hypoxia, DFX treatment of DC resulted in HIF-1α activation (figure 6, panel A); up-regulation of TNFα and IL-1β secretion (panel B); decreased CCR7 mRNA expression (panel C); decreased chemotaxis towards CCL19 (panel D) and increased migration towards the CXCR4 ligand, CXCL12 (panel D). The above results indicate that, at the concentration used in our experiments, DFX treatment recapitulates the effects elicited by hypoxia on DC.

The above results enabled us to evaluate the potential in vivo significance of our in vitro findings in a mouse model. DC were labelled with 0.5 mM of the vital dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)\textsuperscript{29} and a total of 2x10^6 labelled cells were injected subcutaneously in the hind leg footpad; 24 hrs later the number of DC recovered from the draining lymph nodes was evaluated by FACS analysis. As shown in figure 6 (panel E), we observed a drastic reduction in the number of DFX-treated DC that had migrated to lymph nodes, as compared to their normoxic counterparts, supporting the in vivo significance of our observations. Next, we evaluated the inflammatory infiltrate induced by subcutaneous injection of hypoxic, DFX-treated or normoxic DC. In agreement with the enhanced expression of TNFα, IL-1β and the inflammatory chemokine receptor CCR5, we observed that hypoxic and DFX-treated DC promoted enhanced
subcutaneous recruitment of leukocytes. As shown in panel F, the inflammation was less intense in footpads of mice injected with normoxic DC and, in this group, it was mainly localized in the deeper zone of the dermis and in the hypodermis. In contrast, in the hypoxic and DFX-treated groups, a higher number of inflammatory cells is present and these also infiltrate the superficial papillary dermis. To rule out possible in vivo toxic effects of DFX on DC, footpad sections of mice injected with normoxic or DFX-treated DC were stained for the DC marker CD11c (red) and the apoptotic marker Annexin (green) (panel G). As shown, only a few CD11c/Annexin double positive cells were present in both groups, and with a similar density. In addition, the viability of normoxic and DFX-treated DC was also tested in vitro and no differences were found at the concentrations used in our experiments (data not shown).
DISCUSSION

Our results indicate that hypoxia prevents full maturation of DC by interfering with specialized functions linked to activation of adaptive immunity. In contrast, hypoxia enhances the capacity of DC to express specialized inflammatory functions at peripheral sites. During the differentiation of monocytes to DC, we observed that low oxygen levels impaired the induction of CD1a surface expression, whilst promoting higher expression of the monocytic marker CD14. Only a minor reduction (20%) of the endocytic activity, a specialized function of immature DC, was observed in hypoxia (data not shown). Further, following stimulation with LPS, hypoxia-matured DC displayed lower expression of the maturation markers CD40, CD80, CD83, CD86 and MHC class II molecules, accompanied by their reduced ability to prime T-cell functions. These observations indicate that oxygen availability is a critical parameter for DC differentiation and maturation. In contrast to the selective impairment of specialized DC functions involved in T cells activation (above), hypoxia induced higher expression of pro-inflammatory cytokines, TNFα and IL-1β in particular, by LPS-exposed DC. This phenotypic adaptation of DC appears instrumental for the early host response against danger signals, such as infections or trauma, during which establishment of strong inflammatory responses may prevent disease progression. As an example, wounds are characterized by local hypoxia due to blood clotting, an event preventing pathogen spread. In these conditions, hypoxia may reinforce local inflammation and antibacterial activities by acting on different cell populations, DC in particular. In support of this contention, we also observed increased expression of pro-inflammatory cytokines (eg. TNFα and IL-1β) in LPS-exposed hypoxic monocytes and macrophages (Rubino L. unpublished results). Interestingly, the proinflammatory action of hypoxia was further strengthen by the increased differentiation of CD14/CD16 double positive proinflammatory monocytes, which are known to play a relevant role in inflammation and infectious disease in man.
As compared to normoxia, the maturation of DC in hypoxia was paralleled by both inhibition of CCR7 upregulation and sustained expression of CCR5 and CXCR4. Our results partially disagree with a previous study by Zhao et al.\textsuperscript{33} who reported a slight upregulation of CCR7 by DC in hypoxia. This discrepancy may be due to different experimental settings, as we used a hypoxia workstation to avoid cell re-oxygenation during the assay. Despite this, we confirmed the decreased migration of hypoxic DC in response to CCR7 ligands, as previously reported\textsuperscript{33,34}. In agreement with our observation on the enhanced inflammatory functions acquired by DC in hypoxia, Ricciardi et al. recently demonstrated that hypoxic DCs express functional CCR3, CCR2, CX3CR1 and CXCR4\textsuperscript{35}. In contrast with DC, it was reported in breast cancer cells that the hypoxia-mimicking compounds deferoxamine and cobalt chloride induce functional CCR7, mainly through a post-transcriptional mechanism\textsuperscript{36}. Our data suggest that, in response to various TLR agonists, hypoxia promotes a "pre-mature DC phenotype", characterized by enhanced inflammatory functions (high TNFα and IL-1β production), selective homing capacity to peripheral tissues (through sustained CCR5 expression) and impaired migration in response to lymphoid-specific chemokines (CCL19). In support of this, hypoxia also elicited similar effects in human myeloid DC isolated from peripheral blood. In line with this scenario, subcutaneous injection of DFX-treated DC in the hind leg footpad of mice resulted in their impaired migration to draining lymph nodes and enhanced an inflammatory infiltrate at the site of injection. The enhanced capability of hypoxic myeloid-derived DC to perform inflammatory activities is in agreement with the observation that metabolic adaptation to hypoxia, through HIF-1α activation, is instrumental in promoting the inflammatory functions of myeloid cells, such as migration and bacterial killing\textsuperscript{37}. In addition, hypoxia-induced CXCR4 expression may play a role in the recruitment and positioning of DC in hypoxic sites, similarly to what has been reported for macrophages\textsuperscript{25}. Of note, we found that inhibition of the biological activity of VEGF in hypoxic DC resulted in decreased IL-1β and TNFα secretion, two cytokines controlled by NF-κB activity\textsuperscript{38}. This result is in line with previous reports showing NF-κB induction by VEGF\textsuperscript{39} and suggests that, along with its inhibitory activity on DC
maturation, hypoxia-induced VEGF may contribute to promote a “pre-mature DC phenotype”, with enhanced inflammatory properties.

The role of the VEGF as key regulator of inflammatory functions is further strengthened by previous observations on VEGF-induced upregulation of functional CXCR4, both in endothelial cells and glioblastoma, where it supports angiogenesis and cancer cell invasion respectively. Thus, the hypoxia/HIF-1/VEGF pathway appears to extend its activity to different components of hypoxic microenvironments, underlying its potential relevance in disease. In this context, inhibition of DC maturation by hypoxia may occur in solid tumours, where the presence of necrotic areas and immature DC is often found, and tumour-derived VEGF has been proposed to impair DC maturation. The signal transducer and activator of transcription STAT3 was shown to enhance HIF-1 activity and to contribute to tumour angiogenesis and growth. However we did not observe phospho-STAT3 upregulation in hypoxic DC, thus ruling out its possible involvement in hypoxia-driven DC differentiation and maturation (data not shown).

In addition to acting as antigen presenting cells, DC have inflammatory activities and promote angiogenesis and tissue repair. Here we show that hypoxia likely dissociates the inflammatory and tissue repair functions of DC from their capacity to act as sentinels for adaptive immunity. It is conceivable that, through this functional uncoupling under hypoxic conditions, DC may contribute to tissue homeostasis. At the same time, inhibition of accessory functions and trafficking to lymph nodes is likely to act as a safeguard mechanism against autoimmunity elicited by tissue damage.

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**Explanation of Author's Contributions**

- Alessandra Mancino performed the majority of the experiments and contributed to the manuscript writing
- Tiziana Schioppa provided technical and scientific support
- Paola Larghi contributed to dendritic cell purification, differentiation and analysis
- Fabio Pasqualini performed the immunoistochemical analysis
- Manuela Nebuloni is a pathologist who supervised the immunoistochemical analysis
- I-Hsuan Chen performed independent, parallel experiments on the effects of hypoxia on DC differentiation and provided data for Fig 1A
- Silvano Sozzani provided critical discussions and contributed to the analysis of myeloid dendritic cells isolated from peripheral blood
- Jonathan Austyn provided critical discussions, contributed to the analysis of HIF-1 and dendritic cell differentiation, and assisted with manuscript preparation
- Alberto Mantovani provided critical discussions
- Antonio Sica supervised the entire work and the manuscript writing

The authors declare no conflict of interest.
REFERENCES

FIGURE LEGENDS

**Figure 1.** Panel A, DC express inducible HIF-1α in response to hypoxia. DC were generated from monocytes cultured in the presence of IL-13 and GM-CSF under normoxic (Norm) and hypoxic (Hyp) conditions. Whole protein extracts were analyzed by western blot at different time points as indicated. P indicates the protein extract from control Hela cells treated with the hypoxia-mimicking compound cobalt chloride (CoCl2). Vertical lines between day 3 and day 6 have been inserted to indicate a repositioned gel lane. Panel B, expression profiles of CD14 and CD1a in DC differentiated in normoxia or hypoxia, as indicated. Freshly isolated monocytes (day 0), and DC differentiating on day 3 and day 6 of culture, were stained with CD14-APC and CD1a-FITC. The results shown are representative of four independent experiments. Q1: single-positive CD14+ cells; Q2: double-positive CD14+/CD1a+ cells; Q4: single-positive CD1a+ cells. Panel C, expression profiles of CD16 and CD68 in the single-positive CD14+ population (Q1), in normoxia and hypoxia. Results are mean ± standard deviation (SD) of three independent experiments.

**Figure 2.** Effect of hypoxia on monocyte-derived DC maturation. Panel A, human monocyte-derived DC were cultured for 18 hrs under normoxic or hypoxic conditions in the presence or absence of LPS and the surface expression of different maturation markers was analyzed by flow cytometry. Cells were stained with CD80-R-PE, CD83-Alexa488, CD86-APC, CD40-R-PE and MHCII-Alexa488 antibodies, or with isotype controls. Left panels: dotted line, normoxia; dashed line, hypoxia; solid line, isotype - results are representative of three independent experiments. Right panels: the average of three independent experiments is shown - data are mean ± standard deviation (SD), (*, P < 0.05, versus DC matured in normoxia). Panel B, effect of hypoxia on co-stimulatory functions of DC. After 3 days of co-culture with DC, CD4+ T cell proliferation (left) and IFN-γ production (right) were evaluated by ELISA - data are mean ± SD of three different experiments (*, P < 0.05, versus normoxic counterparts).
**Figure 3.** Effects of hypoxia on cytokine expression by DC. Panel A, human monocyte-derived DC were cultured under normoxic or hypoxic conditions in the presence or absence of LPS for 18 hrs and analyzed for mRNA expression by Real Time PCR. Results are mean ± SD of three different experiments. (*, $P < 0.05$ versus normoxic counterparts). Panel B, supernatants were collected after 18 hrs of LPS treatment and analyzed for cytokine production by ELISA. Results are mean ± SD of four experiments. (*, $P < 0.05$ versus DC matured in normoxic conditions). Panel C, effects of the inhibition of the biological activity of VEGF on cytokine secretion by normoxic and hypoxic DC. α-VEGF: anti-VEGF antibody (1 μg/ml); iso: isotype-matched antibody. Results are mean ± SD of three experiments. (*, $P < 0.05$ versus DC matured in hypoxia).

**Fig. 4.** Inhibition of DC maturation in response to different TLR ligands. Panel A, human monocyte-derived DC were cultured under normoxic or hypoxic conditions for 18 hrs and analyzed for mRNA expression of TLR members by Real Time PCR. Data are mean ± SD of three independent experiments. TLR mRNA levels in normoxic conditions (dotted line) were set to 1.0 arbitrary unit. Panel B, surface expression (flow cytometric analysis) of selected chemokine receptors and the DC maturation marker CD83 on cells exposed to different TLR agonists. DC were cultured under normoxic or hypoxic conditions for 18 hrs in the presence or absence of different TLRs agonists as indicated: 10 ng/ml LPS (SIGMA, TLR2/4); 10 ng/ml LPS ALEXIS (TLR4); 2 μg/ml PAM3CSK4 (TLR2); 10 μg/ml poly (I:C) (TLR3); or 3 μg/ml R-848 (TLR7/8). Data are mean ± SD of three independent experiments. (*, $P < 0.05$ versus normoxic counterparts). Panel C, supernatants were collected 18 hrs after treatments and analyzed for cytokine production by ELISA after exposure to different TLR agonists. Results are mean ± SD of four experiments. (*, $P < 0.05$, versus DC matured in normoxia).
Figure 5. Modulation of chemokine receptor expression and functions by hypoxia. Human monocyte-derived DC were cultured for 18 hrs under normoxic or hypoxic conditions in the presence or absence of LPS and analyzed for their chemotactic responsiveness and chemokine receptor expression. Panel A, effects of hypoxia on the chemotactic responsiveness of monocyte-derived DC toward CXCR4, CCR5 and CCR7 specific ligands, CXCL12/SDF-1, CCL5/RANTES and CCL19/ MIP-3β respectively. DC were cultured for 18 hrs in the indicated conditions and the migration assay was performed using a chemotaxis microchamber. Chemokines were used at 100 ng/ml. Data are mean ± SD of three independent experiments done in triplicate (*, *P* < 0.05 versus normoxic counterparts). Panel B, flow cytometric analysis of chemokine receptor surface expression of monocyte-derived DC. DC were stained with CXCR4-APC, CCR7-PE-Cy7, CCR5+Alexa488 and isotype-matched antibodies (isotypic control). Results are mean ± SD of three independent experiments (*, *P* < 0.05 versus normoxic counterparts). Panels C and D, effects of hypoxia on human myeloid DC isolated from peripheral blood. CD1c+ myeloid DC were cultured for 18 hrs under normoxic or hypoxic conditions, in the presence or absence of LPS. Panel C, in CD1c+ myeloid DC isolated from blood, LPS-induced TNFα secretion was enhanced in hypoxia, while low oxygen tension reduced IL-10 and CXCL10 secretion. Further (panel D), hypoxia promoted a strong decrease of CCR7 surface expression, partial reduction of CD40, CD83, MHC class II and significant up-regulation of CXCR4. Numbers on top of bars show mean fluorescence intensity.

Fig. 6. Effects of DFX on HIF-1α activation (panel A), production of TNFα and IL-1β (panel B), CCR7 mRNA expression (panel C), and chemotactic responsiveness to CCL19 and CXCL12 (panel D) of LPS-exposed murine DC. Panel E, effects of hypoxia on DC functions in vivo. CD34+-derived myeloid DC were exposed to LPS for 18 hrs in normoxia or in the presence of deferoxamine (DFX; 400µM). Afterwards, 2x10^6 of CFSE-labelled DC were injected subcutaneously in the hind leg footpad. The number of DC that had migrated to the popliteal lymph...
nodes was evaluated by cytofluorimetry. Panel F, histological (left panels) and immunohistochemical (right panels) evaluation of subcutaneous inflammatory infiltrates induced by injection of DC cultured in normoxia (NORM), hypoxia (HYP) or in the presence of deferoxamine (DFX). EE: haematoxylin-eosin; LFA1: anti-LFA1 monoclonal antibody. Ep: epidermis; De: dermis; Sm: skeletal muscle; **: inflammatory infiltrate. Panel G, viability of normoxic and DFX-treated DC after injection. Footpad sections of mice injected with normoxic or DFX-treated DC were stained for the DC marker CD11c (red), the apoptotic marker Annexin (green) and the nuclei marker DAPI (blue), as indicated. Original magnification x100. Bars represent 20µm. Results are representative (A) or average (B-E) of three independent experiments (*, P < 0.05 versus normoxic counterparts).
**Fig. 6**

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Divergent effects of hypoxia on dendritic cell functions
Alessandra Mancino, Tiziana Schioppa, Paola Larghi, Fabio Pasqualini, Manuela Nebuloni, I-Hsuan Chen, Silvano Sozzani, Jonathan M Austyn, Alberto Mantovani and Antonio Sica