mycoses
Diagnosis, Therapy and Prophylaxis of Fungal Diseases

Original article

Hypoxia attenuates anti-*Aspergillus fumigatus* immune responses initiated by human dendritic cells

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Summary

Aspergillus fumigatus is an opportunistic mould that causes invasive pulmonary aspergillosis (IPA), a life-threatening infection in immunocompromised patients. During the course of IPA, localised areas of tissue hypoxia occur. Bacterial infection models revealed that hypoxic microenvironments modulate the function of host immune cells. However, the influence of hypoxia on anti-fungal immunity has been largely unknown. We evaluated the impact of hypoxia on the human anti-*A. fumigatus* immune response. Human monocyte-derived dendritic cells (DCs) were stimulated *in vitro* with germ tubes of *A. fumigatus* under normoxia or hypoxia (1% O_2), followed by analysis of DC viability, maturation and cytokine release. While DC viability was unaffected, hypoxia attenuated cytokine release from DCs and maturation of DCs upon stimulation with *A. fumigatus*. These data suggest that hypoxia at the site of *A. fumigatus* infection inhibits full activation and function of human DCs. Thereby, this study identified hypoxia as a crucial immune-modulating factor in the human anti-fungal immune response that might influence the course and outcome of IPA in immunocompromised patients.

Key words: *Aspergillus fumigatus*, invasive pulmonary aspergillosis, dendritic cells, hypoxia, immune response.

Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen that causes invasive pulmonary aspergillosis (IPA) in immunocompromised individuals, especially in hematopoietic stem cell transplant recipients and in patients suffering from acute leukaemia.[1] During the onset of IPA, inhaled *A. fumigatus* conidia germinate in the lung alveoli, followed by invasive growth of the fungus into lung tissue and blood vessels, leading to tissue necrosis, thrombosis and bleeding.[2] Recent *in vitro* studies on fungal–host interactions have provided a better understanding and characterisation of the human immune response against *A. fumigatus*.[3] However, such studies are usually performed under standard cell culture conditions that poorly mimic the *in vivo* situation and neglect microenvironmental stresses that are encountered by both the pathogen and host immune cells. In the past decade, several reports have provided evidence for a crucial role of hypoxia in regulation of the mammalian immune response, especially during inflammation or bacterial infection, where hypoxia is a common microenvironment.[4] *A. fumigatus* is able to grow at very low oxygen levels ($O_2 \leq 0.5\%$) *in vitro*.[5] Recently, sites of tissue hypoxia ($O_2 \leq 1.5\%$) have been identified *in vivo* at sites of *A. fumigatus* infection in the lungs of mice in three distinct IPA models.[6] Thus, hypoxia is a relevant microenvironmental factor during IPA that may influence the immune response towards *A. fumigatus*.[7]

Immune cells have to operate in areas of decreased oxygen levels during IPA. Dendritic cells (DCs) are professional antigen-presenting, myeloid cells that connect the innate and adaptive immune response. Several

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Submitted for publication 30 December 2015
Revised 25 February 2016
Accepted for publication 25 February 2016

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doi:10.1111/myc.12498

studies have investigated the interaction between human DCs and *A. fumigatus* under standard *in vitro* cell culture conditions.[8–11] DCs express the pattern recognition receptors (PRRs), toll-like receptor (TLR) 2 and TLR 4 as well as the C-type lectin receptor (CLR) Dectin–1 that recognise *A. fumigatus*.[12] Upon uptake of *A. fumigatus* morphologies (including conidia, germ tubes and hyphae) or stimulation with fungal antigens, DCs release inflammatory mediators including various cytokines and chemokines to guide other immune cells to the site of infection. Furthermore, they undergo a maturation process during which they up-regulate co-stimulatory molecules on their surface and migrate to peripheral lymphoid organs to initiate T helper cell responses to the fungus.[13] A number of studies provided evidence of a modulating influence of hypoxia on anti-bacterial DC responses.[14,15] However, the relevance of hypoxia for human anti-*Aspergillus* immunity has been largely unknown. We therefore investigated the effects of hypoxia on human DCs stimulated with *A. fumigatus in vitro*.

Materials and methods

Reagents

RPMI 1640 medium (Invitrogen, Dreieich, Germany) was supplemented with 120 μg $(ml)^{-1}$ gentamicin (Merck, Darmstadt, Germany) and 10% fetal calf serum (FCS, Sigma-Aldrich, Taufkirchen, Germany). Hank's balanced salt solution (HBSS) was supplemented with 2 mmol l^{-1} EDTA and 1% FCS (all Sigma-Aldrich). Premium-grade recombinant human interleukin 4 (IL-4) was obtained from Miltenyi Biotec and granulocyte macrophage colony-stimulating factor (GM-CSF, Leukine sargramostim, Neu-Isenburg, Germany) from Genzyme.

Cell culture

Human monocyte isolation and generation of monocyte-derived DCs were performed as previously described.[9] Germ tubes of the *A. fumigatus* strain ATCC 46645 were grown and ethanol-inactivated as previously described.[10,16] As changes in cell wall composition of *A. fumigatus* grown under hypoxic conditions could potentially alter innate immune activation,[17] inactivated germ tubes grown under normoxic conditions were used to achieve equal fungal stimulation of DCs and to eliminate the variable of fungal growth during DC-*A. fumigatus* interaction. DCs were stimulated with *A. fumigatus* germ tubes at a multiplicity of infection (MOI) of 1 with 1 x 10^6 DCs in a total culture volume of 1 ml. Hypoxic cell culture was performed in a humidified, oxygen-controllable incubator under 1% O_2, 5% CO_2, 94% N_2 and 37°C (Labotect C60).

Flow cytometry

For analysis of cell viability, DCs were stained with FITC-coupled AnnexinV and with propidium iodide (PI) according to manufacturer's instructions (FITC AnnexinV Apoptosis Detection Kit I, BD Bioscience, Heidelberg, Germany). For analysis of surface molecules, DCs were stained with mouse anti-human fluorochrome-coupled antibodies according to manufacturers' instructions (anti-HLA-DR PE, anti-CD40 FITC, anti-CD83 PE and anti-CD86 PE, all BD Bioscience; anti-CCR7 APC and anti-CD80 APC, Miltenyi Biotec, Heidelberg, Germany). Fluorescence was measured by flow cytometry using a FACSCalibur (BD Bioscience). Data were analysed with FlowJo software (Miltenyi Biotec).

Cytokine quantification

Cytokines in the cell culture supernatants were detected using human single-plex sandwich ELISAs according to manufacturers' instructions [CXCL1, IL-6 and IL-10 DuoSet ELISAs, R&D Systems (Freiburg, Germany), IL-1α ELISA MAX Deluxe and IL-12p70 ELISA MAX, BioLegend (London, UK)].

Statistics

Data were analysed by paired *t*-test test when comparing two means or by two-way repeated measures ANOVA followed by Bonferroni's multiple comparison when comparing multiple means using GraphPad Prism 5 Software.

Ethics statement

This study, using whole blood specimens obtained from human healthy volunteer donors, was approved by the Ethical Committee of the University Hospital of Würzburg. Data analysis was conducted anonymously.

Results and discussion

This study investigated the relevance of hypoxic microenvironments during human DC responses against *A. fumigatus*. To evaluate a possible influence

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of hypoxia on DC viability, DCs were stimulated with *A. fumigatus* germ tubes and incubated under normoxic or hypoxic (1% O_2) conditions for 24 h respectively. The percentages of viable, apoptotic and dead DCs among the DC population as determined by flow cytometry are shown in Fig. 1. There was no significant difference comparing control or *A. fumigatus*-stimulated DCs under normoxia to hypoxia respectively. This demonstrated that the viability of both, unstimulated control DCs and *A. fumigatus*-stimulated DCs, was comparable between normoxic and hypoxic culture conditions. A study that investigated the effect of hypoxia (2% O_2) on human monocyte-derived DCs reported reduced viability of unstimulated DCs under hypoxic conditions.[18] However, this effect was no longer observed when the DCs were stimulated with the bacterial ligand lipopolysaccharide (LPS). Similar to our data for a fungal pathogen, the viability of murine bone marrow-derived DCs was reported to be unaffected under hypoxic conditions (1% O_2), either without stimulation or after stimulation with LPS.[14] Taken together, the viability of stimulated DCs seems to be unchanged under hypoxic conditions.

Our previous study analysing the transcription factor hypoxia-inducible factor 1α (HIF-1α) during the interaction of moDCs with *A. fumigatus* indicated that the secretion of IL-6, IL-12p70 and CCL5 were diminished under hypoxic culture conditions.[19] On the other hand, studies that investigated the influence of hypoxia on cytokine release from LPS-stimulated DCs led to varying results. Mancino *et al.* [15] reported an increased release of TNF–α and IL-1β, but a decrease in IL–10 from LPS-stimulated human monocyte-derived DCs cultivated under 1% O_2. Jantsch *et al.* [14] reported increased release of IL-6 and TNF–α from murine bone marrow-derived DCs stimulated with LPS under hypoxia (1% O_2). As cytokine release is a major function of DCs which is crucial to recruit other immune cells to the site of infection and to initiate a functional adaptive immune response, we sought to systematically extend this analysis and quantified the release of key cytokines of moDCs which were challenged with *A. fumigatus*. To give an example of the prominent role of cytokines in the defence against *A. fumigatus*, defects in the early production of CXCL1 rendered mice more susceptible to infection with the fungus.[20] Furthermore, IL-6 knock-out mice are more susceptible to IPA compared to wild-type mice.[21] Therefore, a reduction of cytokines in conditions of reduced oxygen availability may diminish innate immune responses and favour fungal growth at the site of infection. A time-course stimulation experiment was performed to determine a possible influence of hypoxia on the dynamic release of specific cytokines that are of central importance for the anti-*A. fumigatus* immune response. Fig. 2 shows the release pattern of CXCL1, IL-1α, IL-6, IL-10 and IL-12p70 after 3, 6, 9 and 12 h stimulation of DCs with *A. fumigatus* under normoxic and hypoxic conditions respectively. Control DCs that had been cultivated without stimulation under either condition did not release these cytokines (data not shown). All analysed cytokines showed reduced release under hypoxic conditions. Significant differences comparing normoxic to hypoxic conditions were observed after 9 h cultivation for IL-10 and after 12 h for IL-6, IL-10 and IL-12p70. CXCL1 and IL-1α displayed great interexperimental variations, possibly reflecting blood donor-dependent differences, resulting in high

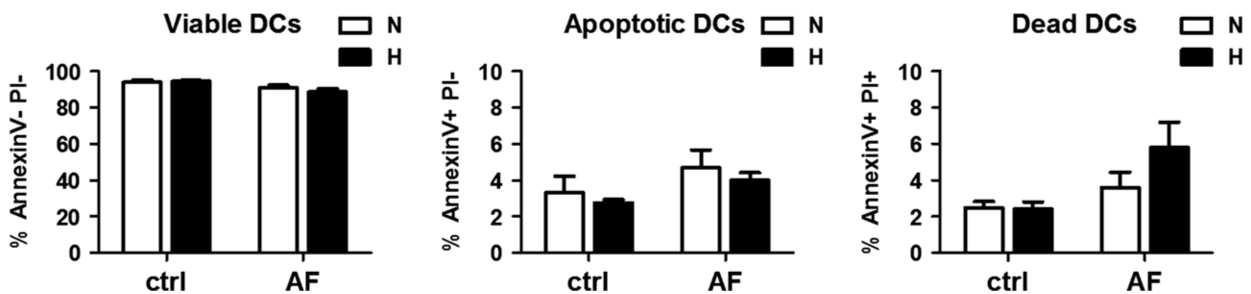

Figure 1 Dendritic Cell (DC) viability remains unchanged under hypoxic culture conditions. DCs were incubated under normoxia (N, white bars) or hypoxia (H, 1% O_2, black bars) without stimulation (ctrl) or stimulated with *A. fumigatus* (AF, MOI = 1). After 24 h, DCs were stained with FITC-conjugated AnnexinV and with propidium iodide (PI) and flow cytometry was performed to determine DC viability. The graphs show percentages of viable DCs (% AnnexinV- PI-), apoptotic DCs (% AnnexinV+ PI-) and dead DCs (% AnnexinV+ PI+). Data are shown as mean + SEM of n = 4 independent experiments. There were no significant differences comparing normoxic to hypoxic conditions (two-tailed, paired t-test).

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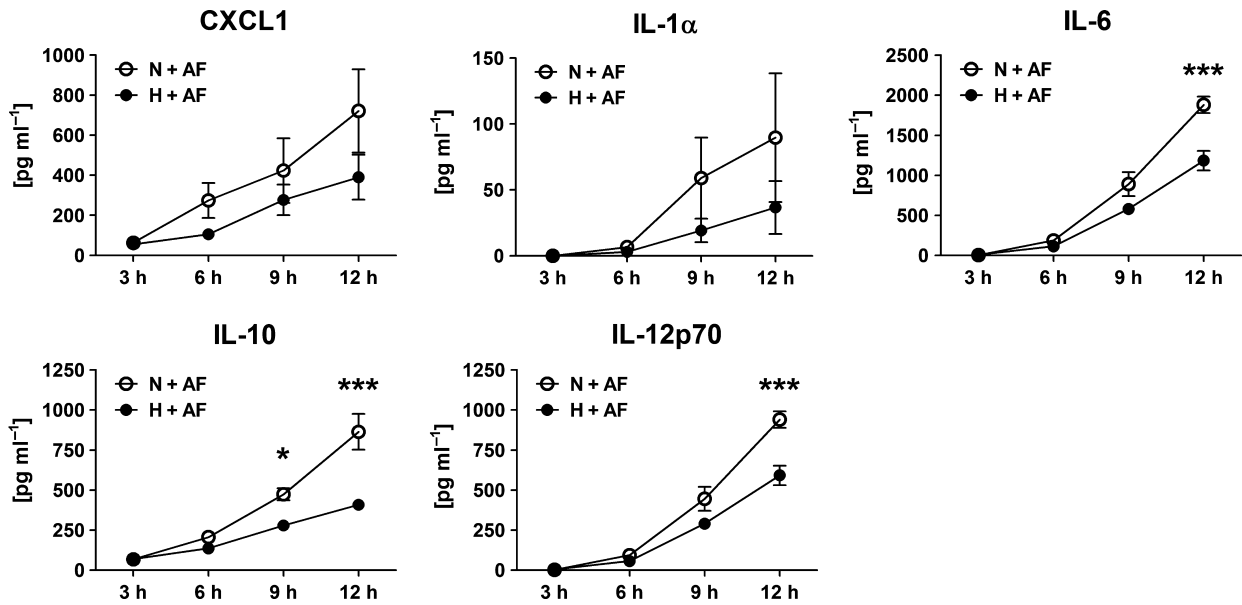

Figure 2 Hypoxia attenuates the inflammatory cytokine response of dendritic cells (DCs) stimulated with *A. fumigatus*. DCs were stimulated with *A. fumigatus* (AF, MOI = 1) and incubated under normoxia (N, white circles) or hypoxia (H, 1% O_2, black circles) for 3, 6, 9 and 12 h. Cytokine concentrations in the cell culture supernatants were quantified using single-plex ELISAs and are shown in pg/ml. Data are shown as mean ± SEM from $n = 3$ independent experiments. Significant differences comparing normoxia to hypoxia are indicated by asterisks (*$P < 0.05$; ***$P < 0.001$; two-way repeated measures ANOVA followed by Bonferroni's multiple comparison test).

sample-to-sample variations. Altogether, the cytokine time-course profiling revealed an overall inhibitory effect of hypoxic culture conditions on the release of cytokines from DCs upon stimulation with *A. fumigatus*.

Besides the release of cytokines, DCs undergo a maturation process that is central for antigen presentation to T cells and the initiation of an adaptive immune response. The influence of hypoxia on the expression of a panel of surface molecules that are up-regulated on mature DCs was measured using flow cytometry. CCR7 is a receptor required for migration of DCs to lymph nodes[22]; the MHC class II molecule HLA-DR presents exogenous antigens to T cells; CD40, CD80 and CD86 are T cell co-stimulatory molecules and CD83 is a marker for mature DCs (Fig. 3). No differences in surface expression of these molecules were observed for unstimulated DCs. This demonstrated that hypoxia alone did not alter the maturation status of DCs. Stimulation with *A. fumigatus* for 24 h under normoxic and hypoxic conditions up-regulated these molecules, confirming that *A. fumigatus* induced DC maturation. However, all markers showed significantly reduced up-regulation on DCs stimulated with *A. fumigatus* under hypoxia compared to normoxia (Fig. 3). Thereby, these data demonstrated an inhibitory effect of hypoxia on DC maturation that is in line with the reduced release of cytokines upon infection with *A. fumigatus*. Interestingly, while Jantsch *et al.* [14] observed increased expression of MHC-II, CD80 and CD86 on the surface of murine DCs stimulated with LPS under hypoxia, Mancino *et al.* [15] reported reduced maturation of LPS-stimulated human DCs under hypoxia. These studies likely reflect differences comparing the human and murine system. Importantly, the data reported for human DCs are in line with this study.

One might speculate that the inhibitory effect of hypoxia on the response of human DCs towards *A. fumigatus* may be due to reduced protein synthesis under hypoxic conditions, as a consequence of reduced metabolic activity and ATP availability. However, unpublished data from our own group demonstrate that the inhibitory effect of hypoxia on the expression of co-stimulatory molecules (CD80, CD83 and CD86) remained when the DCs were further cultured for three additional days under normoxia after the 24 h culture under hypoxia (Fig. 3 and data not published). Intriguingly, changes in cell metabolism are currently believed to govern the phenotype of immune cells, including DCs, by controlling transcriptional and posttranscriptional events that are central to DC activation.[23] In this context, hyoxia is described to act as an activating signal in

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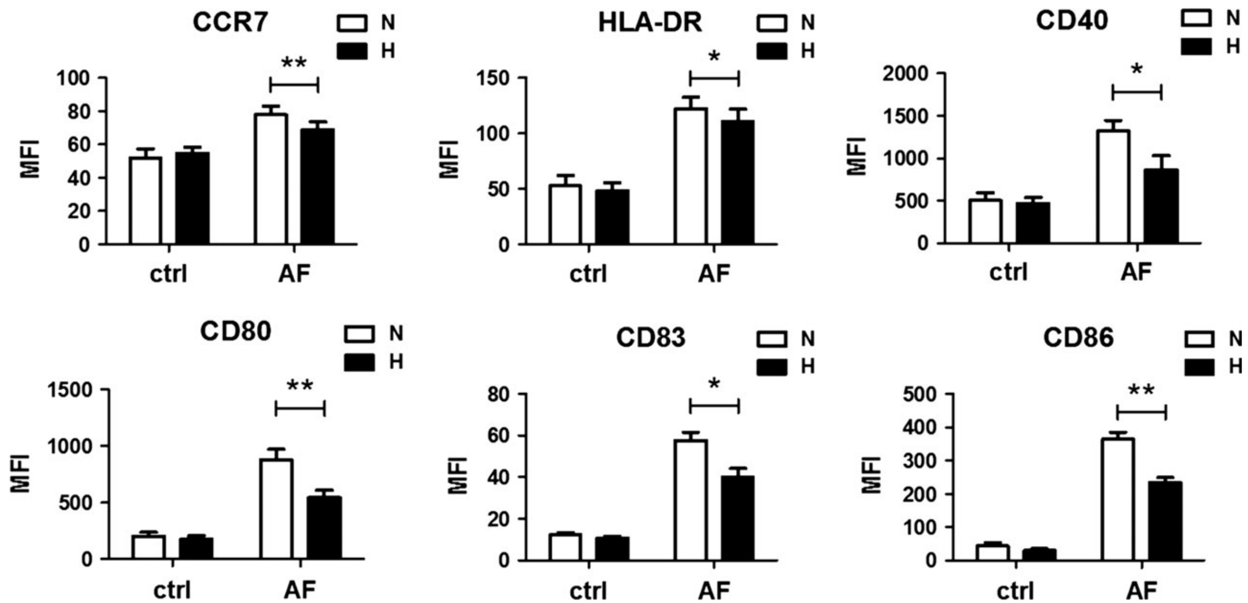

Figure 3 Hypoxia impairs the maturation of *A. fumigatus*-stimulated dendritic cells (DCs). DCs were incubated under normoxia (N, white bars) or hypoxia (H, 1% O_2, black bars) for 24 h without stimulation (ctrl) or stimulated with *A. fumigatus* (AF, MOI = 1). Flow cytometry graphs show CCR7, HLA-DR, CD40, CD80, CD83 and CD86 expression on the surface of DCs. Data are shown as mean + SEM of the mean fluorescence intensity (MFI) of $n = 6$ independent experiments. Significant differences comparing normoxia to hypoxia are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; two-tailed, paired *t*-test).

macrophages and DCs by rapidly enhancing glycolysis to increase ATP levels in the cells.[23] The transcription factor HIF-1α, which enables most of the human body cells to adapt to low oxygen levels, is thought to be involved in regulation of immune cell metabolism and in determining the phenotype of DCs during immune responses.[23]

Various research projects aimed to elucidate the signalling pathways leading to an altered immune response under hypoxic conditions during bacterial, viral or fungal infections. Interestingly, there is accumulating evidence of a central role of HIF-1α for immune responses in various infectious and inflammatory settings. We and others recently published data demonstrating the relevance of HIF–1α for anti-*A. fumigatus* immune responses in the murine[20] and the human[19] system. This included regulation of DC metabolism, which in turn seems to influence the severity and amount of the inflammatory response initiated by these cells upon infection.[14,19] However, our previous study could also demonstrate that HIF-1α had similar functions in human DCs responding to *A. fumigatus* when comparing normoxic to hypoxic conditions.[19] Thus, it is likely that regulatory mechanisms in addition to the HIF-system are responsible for the reduced anti-*A. fumigatus* response of human DCs under hypoxic conditions. The interplay between immune cell activation, hypoxia and HIF-1α signalling seems to be complex and intertwined. HIF-1α contributes to LPS/TLR-induced DC signalling by transcriptional activation which differs from HIF-1α induced gene transcription under hypoxic conditions without TLR ligation.[24]

To the best of our knowledge, this study is the first demonstration of an influence of hypoxia on the response of human DCs towards *A. fumigatus*. In a murine lung transplant infection model, invasion of *A. fumigatus* increases with the extend of hypoxic areas, although the underlying mechanisms remained unclear.[25] Data from this study provides evidence that the anti-fungal immune response is diminished under hypoxia, which may favour fungal growth in areas of reduced oxygen levels. Our *in vitro* cell culture model thereby provides the basis for further investigations to understand the relevance and impact of hypoxic microenvironments in host responses against *A. fumigatus*. Thereby, we contribute to a better understanding of the pathogenesis of *A. fumigatus* infections that is necessary to improve management of IPA.

Acknowledgments

This work was supported by the Wilhelm Sander-Stiftung [Project 2007.102.1] and the Deutsche

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Forschungsgemeinschaft [Collaborative Research Center/Transregio 124 “Pathogenic fungi and their human host: Networks of interaction – FungiNet”, project A2 to HE and JL, project C3 to OK].

References

1 Park SJ, Mehrad B. Innate immunity to Aspergillus species. *Clin Microbiol Rev* 2009; **22**: 535–51.
2 Dagenais TR, Keller NP. Pathogenesis of *Aspergillus fumigatus* in invasive aspergillosis. *Clin Microbiol Rev* 2009; **22**: 447–65.
3 Morton CO, Bouzani M, Loeffler J, Rogers TR. Direct interaction studies between *Aspergillus fumigatus* and human immune cells; what have we learned about pathogenicity and host immunity? *Front Microbiol* 2012; **3**: 413.
4 Eltzschig HK, Carmeliet P. Hypoxia and inflammation. *N Engl J Med* 2011; **364**: 656–65.
5 Hall LA, Denning DW. Oxygen requirements of Aspergillus species. *J Med Microbiol* 1994; **41**: 311–5.
6 Grahl N, Puttikamonkul S, Macdonald JM *et al. In vivo* hypoxia and a fungal alcohol dehydrogenase influence the pathogenesis of invasive pulmonary aspergillosis. *PLoS Pathog* 2011; **7**: e1002145.
7 Schaible B, Schaffer K, Taylor CT. Hypoxia, innate immunity and infection in the lung. *Respir Physiol Neurobiol* 2010; **174**: 235–43.
8 Gafa V, Lande R, Gagliardi MC *et al.* Human dendritic cells following *Aspergillus fumigatus* infection express the CCR7 receptor and a differential pattern of interleukin-12 (IL-12), IL-23, and IL-27 cytokines, which lead to a Th1 response. *Infect Immun* 2006; **74**: 1480–9.
9 Mezger M, Kneitz S, Wozniok I, Kurzai O, Einsele H, Loeffler J. Proinflammatory response of immature human dendritic cells is mediated by dectin-1 after exposure to *Aspergillus fumigatus* germ tubes. *J Infect Dis* 2008; **197**: 924–31.
10 Morton CO, Varga JJ, Hornbach A *et al.* The temporal dynamics of differential gene expression in *Aspergillus fumigatus* interacting with human immature dendritic cells *in vitro*. *PLoS One* 2011; **6**: e16016.
11 Grazziutti M, Przepiorka D, Rex JH, Braunschweig I, Vadhan-Raj S, Savary CA. Dendritic cell-mediated stimulation of the *in vitro* lymphocyte response to Aspergillus. *Bone Marrow Transpl* 2001; **27**: 647–52.
12 Figueiredo RT, Carneiro LA, Bozza MT. Fungal surface and innate immune recognition of filamentous fungi. *Front Microbiol* 2011; **2**: 248.
13 Bozza S, Gaziano R, Spreca A *et al.* Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *J Immunol* 2002; **168**: 1362–71.
14 Jantsch J, Chakravortty D, Turza N *et al.* Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. *J Immunol* 2008; **180**: 4697–705.
15 Mancino A, Schioppa T, Larghi P *et al.* Divergent effects of hypoxia on dendritic cell functions. *Blood* 2008; **112**: 3723–34.
16 Semmlinger A, Fliesser M, Waaga-Gasser AM *et al.* Fever-range temperature modulates activation and function of human dendritic cells stimulated with the pathogenic mould *Aspergillus fumigatus*. *Med Mycol* 2014; **52**: 438–44.
17 Shepardson KM, Ngo LY, Aimanianda V *et al.* Hypoxia enhances innate immune activation to *Aspergillus fumigatus* through cell wall modulation. *Microbes Infect* 2013; **15**: 259–69.
18 Naldini A, Morena E, Pucci A *et al.* Hypoxia affects dendritic cell survival: role of the hypoxia-inducible factor-1alpha and lipopolysaccharide. *J Cell Physiol* 2012; **227**: 587–95.
19 Fliesser M, Morton CO, Bonin M *et al.* Hypoxia-inducible factor 1alpha modulates metabolic activity and cytokine release in anti-*Aspergillus fumigatus* immune responses initiated by human dendritic cells. *Int J Med Microbiol* 2015; **305**: 865–73.
20 Shepardson KM, Jhingran A, Caffrey A *et al.* Myeloid derived hypoxia inducible factor 1-alpha is required for protection against pulmonary *Aspergillus fumigatus* infection. *PLoS Pathog* 2014; **10**: e1004378.
21 Cenci E, Mencacci A, Casagrande A, Mosci P, Bistoni F, Romani L. Impaired antifungal effector activity but not inflammatory cell recruitment in interleukin-6-deficient mice with invasive pulmonary aspergillosis. *J Infect Dis* 2001; **184**: 610–7.
22 Forster R, Schubel A, Breitfeld D *et al.* CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999; **99**: 23–33.
23 O'Neill LA, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. *J Exp Med* 2016; **213**: 15–23.
24 Jantsch J, Wiese M, Schodel J *et al.* Toll-like receptor activation and hypoxia use distinct signaling pathways to stabilize hypoxia-inducible factor 1alpha (HIF1A) and result in differential HIF1A-dependent gene expression. *J Leukoc Biol* 2011; **90**: 551–62.
25 Hsu JL, Khan MA, Sobel RA *et al.* *Aspergillus fumigatus* invasion increases with progressive airway ischemia. *PLoS One* 2013; **8**: e77136.

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