Short Communication

Fever-range temperature modulates activation and function of human dendritic cells stimulated with the pathogenic mould Aspergillus fumigatus

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Abstract

In immunocompromised patients, invasive aspergillosis (IA) is the most frequent disease caused by the pathogenic mould Aspergillus fumigatus. Fever is one of the most common yet nonspecific clinical symptoms of IA. To evaluate the role of hyperthermia in the innate immune response to A. fumigatus in vitro, human monocyte-derived dendritic cells (DCs) were stimulated with germ tubes of A. fumigatus or the fungal cell wall component zymosan at 37°C or 40°C, followed by characterization of specific DC functions. While maturation of DCs was enhanced and DC phagocytic capacity was reduced at 40°C, we observed that DC viability and cytokine release were unaffected. Thus, our results suggest that hyperthermia has substantial impacts on DC function in vitro, which might also influence the course and outcome of IA in immunocompromised patients.

Key words: Aspergillus fumigatus, invasive aspergillosis, hyperthermia, dendritic cells.

Introduction

Invasive aspergillosis (IA) is a life-threatening fungal infection most commonly caused by the pathogenic mould Aspergillus fumigatus. IA mainly occurs in immunocompromised patients and is a rapidly progressive, often fatal disease [1]. For hematopoietic stem cell transplant recipients, mortality rates due to IA range from 60% to 80% [2], and the long-term prognosis for survival is poor [3]. The pathogenesis of IA usually starts in the alveoli of the lung with the germination of inhaled A. fumigatus conidia. The fungus then grows invasively into lung tissue and blood vessels, leading to tissue necrosis, thrombosis, and bleeding [4].

Clinical signs of pulmonary IA are rather nonspecific and include fever (the core temperature ranging from 37.9°C to 41°C), cough, and dyspnea [1]. In a study that included neutropenic and nonneutropenic IA patients, fever was the most common clinical symptom and occurred in 85% of all cases [5]. Corticosteroid-treated patients may not have fever due to the antipyretic effects of these drugs [6]. However, in
patients with neutropenia, persistent or recurrent fever of unknown origin that does not respond to broad-spectrum antibiotics may be the only sign of IA [1].

Dendritic cells (DCs) are antigen-presenting cells that act as a bridge between the innate and adaptive immune systems [7]. They recognize specific cell-wall and secreted molecules from A. fumigatus via pattern-recognition receptors, including Toll-like receptor (TLR) 2 and TLR 4 [8] and the C-type lectin receptor Dectin-1 [9]. Upon pathogen encounter, DCs mature and upregulate costimulatory molecules on their surface. In parallel, they release important inflammatory mediators to guide other immune effector cells to the site of infection. DCs have been shown to phagocytose inhaled A. fumigatus conidia and transport them to draining lymph nodes in order to initiate a T-helper cell response against the fungus [10].

Several studies have investigated the interaction between human DCs and A. fumigatus in vitro [9,11–13]; however, physiological aspects such as febrile body temperature were never taken into account. Thus, we investigated the effect of hyperthermia on human DCs confronted with A. fumigatus in vitro. We cocultured DCs with the fungus at 37°C or 40°C and analyzed DC viability, cytokine release, phagocytosis, and maturation. Our data suggest that hyperthermia enhances DC maturation and decreases DC phagocytic capacity. Both observations might be relevant factors that contribute to the course and outcome of IA in immunocompromised patients.

Material and methods

Reagents

Roswell Park Memorial Institute 1640 medium (Life Technologies, Darmstadt, Germany) was supplemented with 120 µg/ml gentamicin (Merck, Darmstadt, Germany) and 10% fetal calf serum (FCS; Sigma-Aldrich, Schnelldorf, Germany). Hank’s balanced salt solution (HBSS) was supplemented with 2 mM ethylenediaminetetraacetic acid and 1% FCS (all Sigma-Aldrich). Premium-grade recombinant human interleukin 4 (IL-4) was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany), granulocyte macrophage colony-stimulating factor (GM-CSF; leukine sargramostim) from Genzyme (Neu-Isenburg, Germany), and zymosan from InvivoGen (Toulouse, France).

Fungal preparation

Conidia of the A. fumigatus isolate American Type Culture Collection 46645 were prepared as previously described [12]. Germ tubes were grown by incubating conidia in medium without FCS at 200 rpm and room temperature (RT) overnight followed by incubation at 37°C until germination. For 24 h cocultures, germ tubes were inactivated by incubation with 100% ethanol for 30 min.

Differentiation of DCs

Monocyte isolation and DC generation were performed as previously described [14].

Analysis of viability and surface markers

DCs (1 × 10⁶/ml) were incubated at 37°C or 40°C for 24 h with zymosan (10 µg/ml) or inactivated germ tubes of A. fumigatus with a multiplicity of infection (MOI) of 1 or were left untreated. To analyze cell death, DCs were stained with a fluorescein isothiocyanate–coupled antibody directed against annexin V and with propidium iodide (PI; FITC Annexin V Apoptosis Detection Kit I; BD Bioscience, Heidelberg, Germany).

For analysis of surface molecules, 2 × 10⁵ DCs were diluted in 100 µl HBSS and incubated with mouse anti-human fluorochrome-coupled antibodies for 15 min at 4°C (anti-cluster of differentiation (CD) 1a allophycocyanin (APC), anti-CD83 phycoerythrin (PE), anti-CD86 PE, anti–human leukocyte antigen DR (HLA-DR) PE, all BD Bioscience, Heidelberg, Germany; anti-CD80 APC, Miltenyi Biotec, Bergisch Gladbach, Germany), followed by flow cytometry in a FACSCalibur (BD Bioscience, Heidelberg, Germany). Data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cytokine quantification

DCs (1 × 10⁶/ml) were incubated at 37°C or 40°C with A. fumigatus germ tubes (MOI = 1) or left untreated. Supernatants were harvested after 6, 9, and 12 h and stored at −20°C. Cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, tumor necrosis factor-alpha [TNF-α], GM-CSF, and interferon-gamma [IFN-γ]) were quantified with the Cytokine Human 10-Plex Panel (Life Technologies, Darmstadt, Germany) according to the manufacturer’s instructions. Because DCs were generated in the presence of IL-4 and GM-CSF, these cytokines were excluded from analysis.

Phagocytosis assay

DCs were preincubated at 37°C or 40°C for 24 h. Each preincubated DC sample was then separated into two samples, one (1 × 10⁵ DCs) was incubated at 37°C while the other one was incubated at 40°C for 1 h. Then, green fluorescent protein (GFP)-expressing A. fumigatus conidia were...
Figure 1. Viability of dendritic cells (DCs) maintained at fever-range culture temperature. Graphs show DC populations as defined. DCs were incubated at 37°C (white bars) or 40°C (black bars) for 24 h with inactivated germ tubes of Aspergillus fumigatus (multiplicity of infection = 1) or zymosan (Zym, 10 µg/ml) or left untreated (ctrl). (A) The DC population was defined by light scatter characteristics to exclude debris; percentage of DCs among all events is shown. (B–D) To determine apoptotic and/or dead cells in the DC population, DCs were stained with an antibody directed against annexinV and with propidium iodide (PI). Percentages shown are (B) viable DCs (annexinV−/PI−), (C) early apoptotic DCs (annexinV+/PI−), and (D) dead DCs (annexinV+/PI+). Mean and standard deviation of seven independent experiments are shown. Significant differences between temperatures are indicated by an asterisk (*; P < 0.05; Wilcoxon matched-pairs signed-rank test).

Statistics

Data were analyzed with the Wilcoxon matched-pairs signed-rank test using GraphPad Prism 5 software. A value of $P < 0.05$ was considered statistically significant.

Ethics statement

The University Hospital of Würzburg Ethical Committee approved this study, which used whole blood specimens obtained from human healthy volunteer donors. Data analysis was conducted anonymously.

Results

Viability of DCs maintained at fever-range culture temperature

DCs were incubated at 37°C or 40°C for 24 h to determine if they were able to maintain viability at fever-range temperature. DC viability was measured using flow cytometry. The total numbers of DCs among all events were compared (Fig. 1A). Percentages of viable (annexinV−/PI−; Fig. 1B), early apoptotic (annexinV+/PI−; Fig. 1C), and dead (annexinV+/PI+; Fig. 1D) DCs were defined within the DC gate, respectively.

Increased numbers of dead DCs were only observed in unstimulated control vials, which were cultured at 40°C in comparison to control cells incubated at 37°C (Fig. 1A and 1D). However, most importantly, there was no significant
Cytokine secretion is similar comparing DCs stimulated with *Aspergillus fumigatus* at 37°C and 40°C

Upon fungal encounter, DCs release a finely tuned spectrum of both pro- and anti-inflammatory cytokines to orchestrate innate as well as adaptive immune cell functions. Neither incubation time nor increased temperature induced cytokine secretion that differed significantly from unstimulated control DCs (Fig. 2), demonstrating that fever-range temperature alone did not induce cytokine secretion. However, when control DCs were compared with DCs stimulated with *A. fumigatus*, secretion of IL-1ß, IL-6, IL-8, IL-10, and TNF-α was induced after 6 h of coincubation and concentrations further increased over time at 37°C as well as 40°C (Fig. 2). In contrast, IL-2, IL-5, and IFN-γ were not released after 6 h and exhibited only minor induction after 9 h and 12 h of coincubation at 37°C and 40°C (data not shown).

Overall, no significant differences in cytokine release from DCs cocultured with *A. fumigatus* were observed between samples incubated at 37°C and 40°C (Fig. 2). In summary, a fever-range temperature of 40°C did not significantly alter cytokine release of DCs cocultivated with *A. fumigatus* compared with those cocultivated at 37°C, suggesting DCs maintain this important function when encountering *A. fumigatus* at fever-range temperature.

DC phagocytic capacity reduced after 24 h incubation at fever-range temperature

We investigated phagocytosis of *A. fumigatus* conidia under fever-range temperature as one of the most essential functions of immature DCs. Uptake of *A. fumigatus* conidia by DCs was not affected by temperature during phagocytosis (Fig. 3; 0.5 h), even after 1 h and 1.5 h of phagocytosis (data not shown). In contrast, phagocytosis was clearly affected by the 24 h preincubation temperature in that DCs preincubated at 40°C showed significantly reduced phagocytosis compared with those preincubated at 37°C (Fig. 3). These results point to a more mature phenotype of DCs after 24 h of preincubation at fever-range temperatures as phagocytic capacity of DCs diminishes during maturation and DC function changes from antigen capture to antigen presentation [7].
Figure 3. Dendritic cell (DC) phagocytic capacity reduced after 24 h incubation at fever-range temperature. Graph shows percentage of DCs that phagocytosed Aspergillus fumigatus conidia. DCs were preincubated at 37°C (white bars) or 40°C (black bars) for 24 h. Then, one portion was cultured at 37°C and one at 40°C; after 1 h of adaption time, GFP-expressing A. fumigatus conidia were added for 30 min (multiplicity of infection = 10). Phagocytosing (green fluorescent protein [GFP]-positive) and non-phagocytosing (GFP-negative) DCs were determined by flow cytometry. Mean and standard deviation of six independent experiments are shown. Significant differences between temperatures are indicated by an asterisk (*; P < 0.05; Wilcoxon matched-pairs signed-rank test).

Fever-range temperature shifts marker expression on DCs toward a mature phenotype

To further investigate the influence of fever-range temperature on DC maturation, we analyzed the expression of the maturation markers CD80 and CD86 and the MHC (major histocompatibility complex) class II molecule HLA-DR; all are typically upregulated on mature DCs [7]. All three markers were upregulated on DCs when stimulated with A. fumigatus or zymosan after 24 h at 37°C and 40°C (Fig. 4). HLA-DR expression was not affected by temperature on control DCs as well as on DCs stimulated with zymosan or A. fumigatus (Fig. 4). However, we observed significantly enhanced surface expression of CD80 and CD86 after incubation at 40°C compared with 37°C; this effect was observed both with and without stimulation by zymosan or A. fumigatus.

In addition, we found reduced expression of CD1a on those DCs cultivated for 24 h at 40°C compared with DCs incubated at 37°C. This effect was observed with and without zymosan or A. fumigatus stimulation (Fig. 4). Similar to our findings with fungal maturation stimuli, Cao et al. reported decreased CD1a expression on TNF-α–maturated

Figure 4. Fever-range temperature shifts marker expression on dendritic cells (DCs) toward a mature phenotype. Graphs show CD80, CD86, CD1a, and HLA-DR expression. DCs were incubated with Aspergillus fumigatus germ tubes (multiplicity of infection = 1) or zymosan (Zym, 10 µg/ml) or left untreated (ctrl) at either 37°C (white bars) or 40°C (black bars) for 24 h. Mean fluorescence intensity (MFI) and standard deviation of seven independent experiments are shown. Significant differences between temperatures are indicated by an asterisk (*; P < 0.05; Wilcoxon matched-pairs signed-rank test).
Discussion

Fever has been linked to improved survival from different kinds of infection and to enhanced function of immune cells, including DCs [16]. Additional studies have shown in vitro effects of fever-range culture temperature on DC maturation and function [17–19]. Furthermore, hyperthermia has been widely investigated as a therapeutic support for cancer patients [20]. However, to our knowledge, there are no data available on the influence of hyperthermia during fungal infections, especially in IA where fever is frequently the only clear sign of disease (5). In addition, *A. fumigatus* is highly thermotolerant and survives temperatures that approach the upper viability limit for all eukaryotes. Factors that convey fungal thermoresistance at fever-range temperatures may also contribute to *A. fumigatus* virulence [21]. Thus, our study aimed to investigate the effect of fever-range culture temperature on DCs challenged with *A. fumigatus* and the fungal antigen zymosan.

Hyperthermic conditions vary widely among different studies, with temperatures ranging from 39°C [18] to 42°C [19]. Our in vitro experiments were performed at 40°C, which represents a fever-range temperature regularly found in febrile IA patients. Previous studies have shown that DCs are able to tolerate elevated culture temperatures without losing viability [16, 17]. Our in vitro experiments demonstrated that DCs maintained viability when cultivated for as long as 24 h at 40°C.

Synthesis and release of inflammatory cytokines are important functions of DCs, leading to migration and activation of additional immune cell populations to the area of infection. We demonstrated that hyperthermia itself does not induce cytokine secretion by human DCs. This observation is in accordance with previously published studies that used murine bone marrow–derived DCs (BMDCs) [18, 19] and human DCs [17]. In addition, fever-range temperature did not significantly alter cytokine release from DCs cocultivated with *A. fumigatus* compared with those kept at 37°C. However, this observation is in contrast with DCs challenged with other stimuli such as the bacterial endotoxin lipopolysaccharide (LPS) [18, 19] or DCs matured with a cytokine cocktail [17]. While LPS in combination with temperatures up to 40°C leads to decreased release of IL-10 and TNF-α from murine BMDCs [18], human DCs release larger amounts of IL-10 and TNF-α but lower amounts of IL-8 when cultivated in the presence of a maturation cocktail at 40°C compared with 37°C [17]. We observed a strong influence of *A. fumigatus* on cytokine release from DCs that was not significantly altered at fever-range temperature. This indicates that in vitro, hyperthermia might not have any unfavorable effect on this essential DC function during IA.

In addition, we determined the antifungal activity of DCs at both temperatures by cocultivating DCs with *A. fumigatus* for 5 h and quantified the remaining metabolically active fungi with XTT assay (2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt). However, we could not detect any differences in the ability of DCs to inhibit fungal growth at 37°C compared with 40°C (data not shown).

In murine resident peritoneal macrophages, short-term hyperthermia (2 h with culture temperature up to 40°C) enhanced phagocytosis [22, 23]. This is in contrast to our findings, which showed no changes in phagocytosis after short-term (up to 1.5 h) hyperthermia. However, we revealed that the phagocytic capacity of DCs was diminished after 24 h of preincubation at fever-range temperature. We postulate that this reduced efficacy might be related to maturation of DCs after incubation at 40°C compared with DCs maintained at 37°C, as reduced phagocytosis is a typical feature of mature DCs [7].

In combination with reduced antigen uptake, DCs upregulate costimulatory molecules such as CD80 and CD86 during maturation [7]. We demonstrated that hyperthermia is linked to increased expression of both CD80 and CD86 even without fungal stimulation. Compared with DCs stimulated at 37°C, hyperthermia further enhanced upregulation of these costimulatory molecules after encounter with the whole fungus or with the fungal antigen zymosan. Our results are in accordance with previous studies that showed the ability of hyperthermia to increase levels of CD80 and CD86 on murine BMDCs [19] and on human DCs [17] with and without stimuli that induce maturation.

Despite major advances in diagnosis and treatment of fungal infections, IA remains a challenging problem in immunocompromised patients, especially patients undergoing allogeneic stem cell transplantation. A better understanding of factors that influence the pathogenesis and immune response during IA may help to better manage this disease and to develop new treatment strategies. Our data demonstrate that fever-range body temperature influences DC function in vitro. This includes augmented maturation of DCs under hyperthermia, as indicated by reduced phagocytic capacity and higher expression of the costimulatory molecules CD80 and CD86. Mature DCs are no longer conducting
surveillance and have reduced capability to uptake antigen. Consequently, hyperthermic DC maturation in the presence of A. fumigatus antigens might be beneficial for antigen-specific T-cell stimulation in patients. However, future studies to analyze a potential correlation between fever and the course and outcome of IA should be performed.

The potential of hyperthermia for novel vaccination strategies has already been widely recognized [16–19]. Our data suggest that in the future, hyperthermia might also serve as an additional adjuvant for in vitro–generated DCs that are directed against A. fumigatus.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References