Invasive aspergillosis induces complex chemokine and cytokine responses

Subjects: Infectious Diseases

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Invasive aspergillosis is a frequent complication in immunocompromised individuals, and it continues to be an important cause of mortality in patients undergoing hematopoietic stem cell transplantation. In addition to antifungal therapy used for mycoses, immune-modulatory molecules such as cytokines and chemokines can modify the host immune response and exhibit a promising form of antimicrobial therapeutics to combat invasive fungal diseases. Cytokine and chemokine profiles may also be applied as biomarkers during fungal infections and clinical research has demonstrated different activation patterns of cytokines in invasive mycoses such as aspergillosis.

chemokines Aspergillus hematopoietic stem cell transplantation cytokines

1. Background

Fungi are among the most extensively distributed microorganisms and are ubiquitous in the environment. However, a small percentage of these remarkable eukaryotes are also major human pathogens. The frequency of opportunistic fungal infections continues to increase due to the expansion in the numbers of immunocompromised hosts [1]. Aspergillus species (spp.) are one of the most common medically important opportunistic fungi [2]. Invasive infections with Aspergillus spp. are typically considered life-threatening and most frequently occur in immunocompromised individuals such as those receiving chemotherapy, undergoing solid organ transplantation (SOT), or hematopoietic stem cell transplantation (HSCT) [3][4]. Among the human pathogenic species of the genus Aspergillus, Aspergillus fumigatus is the most common causative agent, followed by A. flavus, A. terreus, and A. niger [5]. In compromised hosts, Aspergillus infections most commonly manifest as invasive pulmonary aspergillosis (IPA). The number of patients undergoing transplantation has grown exponentially in recent years, particularly in patients undergoing HSCT for the treatment of hematological malignancy [6]. IPA occurs in 3.6 to 10.3% of allogeneic HSCT recipients leading to a mortality rate of 50 to 80% [6][7].

2. Cytokines and Chemokines Responses in Invasive Aspergillosis after Hematopoietic Stem Cell Transplantation

Cytokines and chemokines are biologically active secreted proteins released by immune cells that play critical roles in cell-to-cell communication. In aspergillosis, they are an important component of host defenses against infection by promoting the initiation, maintenance, and resolution of the host response [8]. Innate immune cells composed of granulocytes, monocytes, AECs, and DCs are the first line of defense against Aspergillus and are the cells that

primarily combat the fungus within the first week after infection [8]. In addition, macrophages phagocytose Aspergillus conidia and inhibit their intracellular germination in the early phase of infection [9], which induces the expression of inflammatory chemokines and cytokines. Furthermore, neutrophils and circulating monocytes damage hyphae by secreting oxidative and non-oxidative microbicidal compounds [10]. Hence, early neutropenia followed by immunosuppressive drugs in HSCT leads to defects in certain immune-related phagocytosis. Thus, these findings indicate that the association between HSCT and the immune system is highly dynamic [11].

The results from in vitro analyses reveal that infection of the immature dendritic cells (iDCs) with small germinating conidia (approximate size, 3–8 μm) significantly increased the secretion of specific cytokines (IL-6, IL-12, TNF-α, and IL-10) and chemokines (IL-8, CCL20, and CXCL10) and the expression of immune receptors (PTX3, CXCR4, CCRL2, and IL2RA) [12]. The significant increase of both the pro-inflammatory cytokine TNF-α and chemoattraction chemokines IL-8, CCL-20, and CXCL10 were also observed with stimulation by the Aspergillus antigen 18-kDa RNase Aspf1 [13], compared to the levels expressed by unstimulated DCs.

Natural killer (NK) cells are lymphoid cells in peripheral blood that play a critical role in the innate host defense and their cell numbers are related to the severity of IPA [11]. NK cells are known for their release of cytokines and play a unique role in the early phase of an immune response against Aspergillus [14]. In vitro infection of human NK cells by A. fumigatus hyphae for 6 h increases the secretion of inflammatory cytokines, such as IFN-γ, TNF-α, and growth factor GM-CSF, as well as several chemokines, including CXCL8/IL-8, CCL3/MIP-1α, CCL4/MIP-1β, and XCL1/lymphotactin [14]. Supporting the results from in vitro studies, a murine intranasal infection model using A. fumigatus conidia suggested that susceptibility to IA is associated with the levels of genes encoding IL-5 (a Th2 cytokine involved in B cell and eosinophil activation) and IL-17a (a Th17 inflammatory cytokine produced by T cells and NK cells). The increased expressions of the genes encoding IFN-γ, high levels of TNF-α and the upregulation of a network of TNF-α–related genes were significantly related to Aspergillus infection [15]. Additionally, the expression of classical Th2 cytokines (IL-4, IL-5, IL-13) was found in bronchiole epithelial lung homogenates of Aspergillus protease-induced murine inhalation model compared to the PBS-treated controls [15].

Several studies have demonstrated an alteration of cytokines and chemokines in patients with hematological malignancy undergoing HSCT who subsequently develop invasive fungal disease [16][17][18] and IA in particular [16][19][20]. For example, in adult hematology patients with proven/probable invasive fungal disease (IFD), increases of serum cytokine levels of IL-15 and IL-2R as well as chemokines levels of CCL2 and MIP-1α were observed, whereas the level of IL-4 was significantly lowered, compared to those with no evidence of IFD [16]. Another study in adult hematology patients with probable/possible IA reported higher levels of cytokine IL-6 and chemokine IL-8 in serum and significant elevations in bronchoalveolar lavage (BAL) fluid levels of IL-8, compared to those with other infections [19]. In support of these findings, Gonçalves et al. demonstrated that the BAL fluid levels of cytokines IL-1β, IL-6, IL-17A, IL-23, TNF-α, and chemokine IL-8 were increased in patients diagnosed with IA, which were also consistent with levels of these cytokines in serum [20]. Notably, although the expression of in vitro and in vivo cytokines/chemokines varies in the different studies, these discrepancies may be explained by differences in cell types responding to Aspergillus stimuli and the different patient populations. However, all these laboratory findings suggest that the elevation of cytokines/chemokines in serum and BAL fluid levels were associated with increased

risk of IA and, thus, may be used as a valuable indicator of the risks associated with development of IA and guide enhanced antifungal prophylaxis and early treatment. These findings are summarized in **Table 1**.

Table 1. Cytokine and chemokine responses in invasive aspergillosis after hematopoietic stem cell transplantation.

Models	Samples	Methods	Major Findings			Interpretation
			Cytokines	Chemokines	Others	
In vitro						
iDC + A. fumigatus-small germinating conidia (6 h of stimulation)	Infected iDCs	qRT-PCR	↑ IL-6 ↑ IL-12 ↑ TNF-α ↑ IL-10	↑ IL-8 ↑ CCL20 ↑ CXCL10	↑ PTX3 ↑ TLR-2 ↓ TLR-4	A. fumigatus germ tubes induced the expression of genes associated with recognition and phagocytosis in iDCs with a time-dependent manner.
iDC + A. fumigatus antigen Aspf1	Infected iDCs	qRT-PCR	↑ TNF-α	↑ L-8 ↑ CXCL10 ↑ CCL20	• –	Aspf1, a member of a family of conserved RNases, induces a pro-inflammatory cytokine response.
NK cells obtained from PBMCs + A. fumigatus hyphae (6 h of stimulation)	Infected NK cells	qRT-PCR	↑ IFN-γ ↑ TNF-α ↑ GM-CSF	↑ CXCL8 /IL-8 ↑ CCL3/MIP-1α ↑ CCL4/MIP-1β ↑ XCL1/ lymphotactin	↓ NKp30 ↑ CD56	NK cells reveal the expression and release of immunomodulatory molecules involved in antifungal immune responses.
In vivo						
Mice CD1 strain infected by intranasal instillation with A. fumigatus conidia (N = 24)	Mouse whole-lung homogenates	• qRT-PCR • ELISA	Immunocompetent mice: Infected vs. Saline controls ↑ IL-17a mRNA ↑ TNF-α protein level			Susceptibility to IA is associated with a high level of TNF-α at the site of infection and the upregulation of a

Models	Samples	Methods	Major Findings			Interpretation
			Cytokines	Chemokines	Others	
			In vitro			
						network of TNF-α–related genes.
			Immunosuppressed mice: Infected vs. Saline controls ↑ IFN-γ mRNA ↑ IL-17a mRNA ↑ TNF-α protein level ↑ IL-5 protein level			
			Immunocompetent vs. Immunosuppressed mice ↓ TNF-α ↓ IFN-γ ↓ IL-4 ↓ IL-12p40			
BALB/c mice infected by intranasal instillation with A. fumigatus proteases, Aspf5 and Aspf13 (N = 20)	Mouse lung homogenates	• ELISA	Infected vs. PBS controls ↑ IL-4 ↑ Serum IgE ↑ IL-5 ↑ IL-13			A. fumigatus secreted allergen proteases, Aspf5 and Aspf13, are important for induction of Th2 cytokines secretion and increased IgE levels, which are fundamental features of allergic asthma and an indication of disease severity.
			Clinical study			
Adult hematology patients with proven/probable IFD (N = 172)	Serum	ELISA	↑ IL-15 ↑ IL-2R	↑ CCL2 ↑ MIP-1α	–	High IL-2R and CCL2 concentrations as indicators for the

Models	Samples	Methods	Major Findings			Interpretation
			Cytokines	Chemokines	Others	
			In vitro			
			↓ IL-4			risk of developing IFD.
Adult hematology patients with probable/possible IA (N = 43)	• BAL • Serum	ELISA	Serum ↑ IL-6	BAL ↑ IL-8 Serum ↑ IL-8	↑ Aspergillus-specific lateral-flow device test	High serum IL-8 levels were highly specific and highly sensitive for the diagnosis of IA.
Patients diagnosed with IA [22] (N = 48)	• BAL • Serum	ELISA	BAL ↑ IL-1β ↑ IL-6 ↑ IL-17A ↑ IL-23 ↑ TNF-α Serum ↑ IL-6 [25] ↑ IL-17A ↑ IL-23	BAL ↑ IL-8 Serum ↑ IL-8	↑ Galactomannan in BAL specimens	Alveolar cytokines might be useful in supporting current diagnostic approaches for IPA [21] biomarkers. IL-8 was the best performing analyte with the most relevant discriminator between cases of IPA and controls.

[23][24]

[26]

factors, pergillus R-2, and uction of CL1 from nocytes, romotion ugh the Dectin-1 l NF-κB defective

cytokine production, leading to an increased susceptibility to IA. Defective production of TNF-α and IL-6 has been found in both PBMCs and BEAS-2B respiratory epithelial cells harboring the Dectin-1 Y238X polymorphism [27][28]. Additionally, Dectin-1 knockout in BALB/c mice have decreased production of IFN-γ, IL-17A, and IL-10, and have a significantly reduced ability to control Aspergillus infection [28]. Conversely, single nucleotide polymorphisms (SNPs) in the intracellular PRR NOD2 can decrease the risk of IA [22]. NOD2 deficiency results in a defective inflammatory response with alterations in the levels of IL-1β, IL-17A, IL-22, and IFN-γ produced by PBMCs from hematological patients undergoing allogeneic HSCT, and IL-6 and TNF levels in Nod2−/− deficient mice [29]. Furthermore, low levels of serum IL-10 and IL-8 have been reported in patients with hematological malignancies undergoing allogeneic HSCT [29]. Thus, targeting assays for alternations in NOD2 may be an attractive method in personalized management strategies for IA. However, at present, these findings fundamentally show that defects in NOD2 potentially reduce Aspergillus-induced cytokine driven inflammation. Importantly, it needs to be elucidated whether cytokine alterations mechanistically protect from fungal infection in HSCT patients with NOD2 variants. Furthermore, polymorphisms in other cytokine genes such as IL-1 and IL-10 have also been implicated as genetic biomarkers of susceptibility to IFD [30][31]. These findings are summarized in **Table 2**.

Abbreviations: BAL, bronchoalveolar lavage; CCL, chemokine (C-C motif) ligand; CD, cluster of differentiation; CXCL, chemokine (CXC motif) ligand; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; h, hour; IA, invasive aspergillosis; iDCs, immature dendritic cells; IFD, invasive fungal disease; IFN, interferon; IgE, immunoglobulin E; IL, interleukin; IPA, invasive pulmonary aspergillosis; MIP, macrophage inflammatory proteins; mRNA, messenger RNA; NK cell, natural killer cell; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PTX, paclitaxel; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TLR, toll-like receptor; TNF, tumor necrosis factor. The black arrows indicate the increase or decrease in cytokines and chemokines.

Table 2. Genetic polymorphisms in hematopoietic stem cell transplantation patients are associated with susceptibility/resistance to invasive aspergillosis.

Models	Polymorphism	Major Findings		Interpretation	Ref.
		Cytokines	Others		
		In vitro			
PBMCs	Dectin-1 Y238X Stop Codon Polymorphism + heat-killed A. fumigatus hyphae + live A. fumigatus conidia	↓ TNF-α ↓ IL-6	↓ binding ability to β-glucan	Dectin-1 Y238X resulted in the reduction of pro-inflammatory cytokines due to the Dectin-1 receptor, which is known to play a role in fungal cell wall β-glucan recognition.	[40]
BEAS-2B (Respiratory epithelial cells)	Dectin-1 blockade by siRNA + Stimuli (β-glucan or Aspergillus conidia)	↓ IL-6 ↓ TNF-α	–	Dectin-1 expressed on epithelial cells contributes to the production of cytokines.	[41]
PBMCs from allogeneic HSCT	NOD2 genetic variation - P268S (TT-genotype) + A. fumigatus conidia - complete NOD deficiency + A. fumigatus conidia	Infected in TT-genotype compared with infected in CC-and CT-genotype ↓ IL-1β ↓ IL-17A Aspergillus infected compared with uninfected ↓ IL-1β ↓ IL-22 ↓ IFN-γ	Aspergillus infected compared with uninfected ↓ IL-17A$^+$, IL-22$^+$, and IFN-γ$^+$ CD4 T-cell populations	Human NOD2 deficiency reduces Aspergillus-induced inflammatory cytokines.	[42]
Human PMBCs from solid-organ transplant recipients	• IL1B rs16944 SNP + A. fumigatus conidia	IL1B rs16944 SNP ↓ IL-1β ↓	• –	Both IL1B rs16944 and IL1RN rs419598 SNPs effect Aspergillus-induced cytokine release.	[43]

Models	Polymorphism	Major Findings		Interpretation	Ref.
		Cytokines	Others		
In vitro					
	• IL1RN rs419598 SNP + A. fumigatus conidia	TNF-α ↓ IL-22 IL1RN rs419598 SNP ↓ IL-1β ↓ TNF-α			
Macrophages from healthy blood donors	IL10 SNP with GG genotype + A. fumigatus conidia	↓ IL-10 ↓ TNF-α ↓ IL-6 ↓ IL-1β ↓ IL-8	↓ fungal clearance	IL-10 overexpression influences IA by suppressing antifungal immunity.	[44]
In vivo					
BALB/c mice with HSCT + Aspergillus (N = 16)	Dectin-1 knockout mice	↓ IFN-γ ↓ IL-17A ↓ IL-10	↑ fungal growth	Dectin-1 modulates immunity and tolerance via IFN-γ / IL-10 production, and both cytokines activate the protection of Th1/Treg antifungal responses.	[41]
Nod2-deficient ($Nod2^{-/-}$) C57BL/6 mice + Aspergillus (lethal dose) (N = 22)	$Nod2^{-/-}$ deficient mice (Splenocytes)	↓ IL-6 ↓ TNF	↑ 14-day survival	NOD2 augments Aspergillus-induced cytokine responses and results in resistance to Aspergillus infection.	[42]
Clinical study					
Patients who developed IA post HSCT (N = 71) Non-HSCT	Y238X Stop Codon Polymorphism	• –	↑ susceptibility to IA	Dectin-1 Y238X heterozygosity had a limited influence on susceptibility to IA.	[45]

Models	Polymorphism	Major Findings				Interpretation	Ref.
		Cytokines		Others			
		In vitro					
patients with IA (N = 21)							
Hematological patients undergoing allogeneic HSCT (N = 310)	NOD2 genetic variation - P268S SNP	↓	serum IL-10	↓	susceptibility to IA	Genetic deficiency of NOD2 results in an alteration of cytokine production in response to Aspergillus infection.	[42]
		↓	serum IL-8				
An allograft with IA (N = 81) or without IA (N = 58)	CXCL10 genetic variation - C+11101T - C+1642G - A1101G	↑	serum CXCL-10	↑	susceptibility to IA	Polymorphisms in CXCL10 altered chemokine secretion and increased the risk of IA after alloSCT.	[40]

Abbreviations: alloSCT, allogeneic stem cell transplantation; CXCL, chemokine (C-X-C motif) ligand; HSCT, hematopoietic stem cell transplant; IA, invasive aspergillosis; IFN, interferon; IL, interleukin; NOD, nucleotide oligomerization domain; PBMCs, peripheral blood mononuclear cells; SNP, single-nucleotide polymorphism; Th cell, T helper cell; TNF, tumor necrosis factor. The black arrows indicate the increase or decrease in cytokines and chemokines. $Nod2^{-/-}$ indicates complete deletion (-) of Nod2 alleles.

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