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Susceptibility of mice genetically deficient in SP-A or SP-D gene to Invasive Pulmonary Aspergillosis

Taruna Madan[a,b], Kenneth B.M. Reid[c], Howard Clark[c,d], Mamta Singh[a], Annapurna Nayak[e], P. Usha Sarma[a,f], Samuel Hawgood[g], Uday Kishore[e,*]

[a] Institute of Genomics and Integrative Biology, Council for Scientific and Industrial Research, Delhi University Campus, Mall Road, Delhi 110007, India
[b] Department of Innate Immunity, National Institute for Research in Reproductive Health, Mumbai 400012, India
[c] Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK
[d] University of Southampton, MP 803 Level F, South Block, Southampton General Hospital, Southampton SO16 6YD, UK
[e] Centre for Infection, Immunity & Disease Mechanisms, Biosciences, School of Health Sciences and Social Care, Brunel University, West London UB8 3PH, UK
[f] Department of Plant Pathology, Indian Agricultural Research Institute, Pusa Road, Delhi 110012, India
[g] Department of Pediatrics and Cardiovascular Research Institute, University of California San Francisco, San Francisco, CA 94118-1245, USA

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ABSTRACT

Pulmonary surfactant proteins, SP-A and SP-D, are carbohydrate pattern recognition molecules of innate immunity, which significantly enhance phagocytosis and killing of *Aspergillus fumigatus*, a pathogenic fungus, by neutrophils and macrophages. The present study examined the susceptibility of immunosuppressed SP-A gene deficient (SP-A$^{-/-}$) or SP-D gene deficient (SP-D$^{-/-}$) mice to *A. fumigatus* conidia challenge compared to wild-type (WT) mice. *A. fumigatus*-challenged SP-A$^{-/-}$ (SP-A$^{-/-}$ IPA) mice showed less mortality (40%) than the WT-IPA mice (100%) and increased mortality (60%) following administration of SP-A with decreased TNF-α and IFN-γ to IL-4 ratio than SP-A$^{-/-}$ IPA mice. The SP-D$^{-/-}$ IPA mice (57.14%) showed similar mortality as WT-IPA mice (60%). However, the SP-D $^{-/-}$ IPA mice (42.86% mortality on day 2) died earlier than the WT-IPA mice (20% mortality on day 2), showed a higher hyphal density and tissue injury in lungs. Treatment with SP-D or a recombinant fragment of human SP-D rhSP-D reduced the mortality to 50% and 33%, respectively, concomitant with higher IFN-γ to IL-4 ratios in treated SP-D$^{-/-}$ mice, compared to untreated control group. The results showed that SP-D gene deficient mice are more susceptible to IPA while SP-A gene deficient mice acquire resistance to IPA.

1. Introduction

The hydrophilic lung surfactant proteins, SP-A and SP-D, are carbohydrate pattern recognition molecules of innate immunity. The structure of SP-A and SP-D molecules comprise of an N-terminal triple helical collagen region and homotrimeric ligand-recognition domain called a C-type lectin or carbohydrate recognition domain (CRD). These CRD interact with carbohydrate pattern structures on the surfaces of pathogenic organisms such as viruses, bacteria, and fungi, and cause agglutination and direct inhibition of growth, together with enhanced phagocytosis through neutrophils and macrophages (Kuroki et al., 2007). When challenged with pathogens, allergens, apoptotic cells or necrotic cells, SP-A and SP-D are known to interact with phagocytic cells and enhance their chemotactic, phagocytic, antigen presentation and oxidative properties (Kishore et al., 2005). Thus, the pattern recognition of pathogens via CRDs and subsequent engagement of collagen region with immune cells via collectin receptor enhances phagocytosis and killing of pathogens (Kishore et al., 2006).

Aspergillus species are one of the prominent causes of fungal respiratory infections worldwide. *Aspergillus* infection of immunosuppressed hosts results in Invasive aspergillosis, characterized by fungal invasion and high mortality rates. In earlier studies, both human SP-A and SP-D bound and agglutinated *Aspergillus fumigatus* conidia and these interactions enhanced phagocytosis and killing of germinating conidia by human neutrophils and alveolar macrophages (Madan et al., 1997; Allen et al., 2001). Intranasal administration of an anti-fungal drug—Amphotericin B (AmB), SP-D and rhSP-D (a recombinant fragment of human SP-D comprising neck and carbohydrate recognition domains of human SP-D) to an immunosuppressed murine model of Invasive Pulmonary

Abbreviations: SP-A, human surfactant protein A; SP-D, human surfactant protein D; rhSP-D, a recombinant fragment of human surfactant protein D, composed of homotrimeric neck and C-type lectin domains; *A. fumigatus*, *Aspergillus fumigatus*; ABPA, allergic bronchopulmonary aspergillosis; WT, wild-type; SP-A$^{-/-}$, mice genetically deficient in the SP-A gene; SP-D$^{-/-}$, mice genetically deficient in SP-D gene; HRP, horseradish peroxidase; OPD, o-phenylenediamine; AP, alkaline phosphatase; BALF, bronchoalveolar lavage fluid.

* Corresponding author. Tel.: +44 1895 266362; fax: +44 1895274348.
E-mail addresses: uday.kishore@brunel.ac.uk, ukishore@hotmail.com (U. Kishore).

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Aspergillosis (IPA) challenged intranasally with *A. fumigatus* spores, resulted in increased survival rates of 80%, 60% and 80%, respectively, compared to no survivors in the untreated group (Madan et al., 2001). SP-A treatment did not have significant protective effects. In a recent study, treatment of IPA mice with various doses of SP-D and rhSP-D lowered colony forming unit (CFU) counts and fungal burden in the lung tissues, consistent with raised levels of TNF-α and IFN-γ in the bronchoalveolar lavage fluid (BALF) of treated mice (Singh et al., 2009).

The studies carried out using SP-A$^{-/-}$ or SP-D$^{-/-}$ mice have revealed a key role played by SP-A and SP-D in surfactant homeostasis and pulmonary immunity. Compared to the WT mice, the SP-A$^{-/-}$ mice have been found to have an increased susceptibility to a range of respiratory pathogens, including Group B *Streptococci*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, respiratory syncytial virus, influenza A virus (IAV), *Mycoplasma pneumoniae*, *Pneumocystis carinii* and *Hemophilus influenzae* (Korfhagen et al., 1996; LeVine et al., 1999, 2002; Linke et al., 2001; Li et al., 2002). The SP-D$^{-/-}$ mice show a delayed clearance of an exogenous challenge of pathogens, such as RSV and *P. carinii*, together with an exaggerated lung inflammation that can be restored by an exogenous administration of SP-D (Botas et al., 1998; Wert et al., 2000; Atochina et al., 2004; LeVine et al., 2004). We reported intrinsic hyper-eosinophilia and several fold increase in the levels of IL-5 and IL-13, and lowering of the IFN-γ to IL-4 ratio in the lungs, suggesting a Th2 bias of immune response in both SP-A$^{-/-}$ and SP-D$^{-/-}$ mice (Madan et al., 2005). The SP-D$^{-/-}$ mice were more susceptible than the wild-type while SP-A$^{-/-}$ mice were resistant to pulmonary hypersensitivity induced by *A. fumigatus* allergens.

In the present study, we have examined susceptibility of the SP-A$^{-/-}$ or SP-D$^{-/-}$ mice to *A. fumigatus* conidia challenge under conditions of immunosuppression. Interestingly, the SP-A$^{-/-}$ and SP-D$^{-/-}$ mice responded distinctly to corticosteroid induced immunosuppression as well as to conidia challenge. SP-D$^{-/-}$ mice showed increased susceptibility while SP-A$^{-/-}$ mice were found to be more resistant than WT mice to conidia challenge. Intranasal treatment with SP-D or rhSP-D was effective in ameliorating the pathology and mortality in the case of SP-D$^{-/-}$ mice, whereas the SP-A treated *A. fumigatus*-challenged SP-A$^{-/-}$ mice showed increased mortality.

2. Materials and methods

2.1. Mice

The generation of SP-A$^{-/-}$ (8, 9) and SP-D$^{-/-}$ (Botas et al., 1998) mice via backcrossing in the C57BL/6 background has been reported earlier. Specific-pathogen-free, 6–8-week old, male and female C57BL/6 mice of the strains used for generating SP-A$^{-/-}$ mice, termed as WT (SP-A$^{-/-}$ type), and SP-D$^{-/-}$ mice, termed as WT (SP-D$^{-/-}$ type), were obtained from Harlan-OLAC, Shaw's Farm (Bicester, Oxfordshire, U.K.). Mice were housed in the isolator cages with sterile beddings in a barrier facility of the animal care facility at the Department of Biochemistry, University of Oxford, U.K. Both SP-A$^{-/-}$ and SP-D$^{-/-}$ mice were pathogen free and repeated attempts to culture bacterial and fungal organisms from the lungs of these mice were negative. Mice were randomized before experiments.

2.2. A. fumigatus conidia

Conidia from *A. fumigatus* (strain 285), originally isolated from the sputum of an allergic bronchopulmonary aspergillosis (ABPA) patient, were harvested and suspended in sterile PBS, adjusting the concentration to 10^8 conidia per 50 μl (Madan et al., 1997). The conidia viability of the challenge inocculum was assessed by plating 10^6 and 10^7 dilutions on Sabouraud dextrose agar plates.

2.3. Preparation of native human SP-A and SP-D

Native human SP-A and SP-D were purified from human BALF collected from patients suffering from pulmonary alveolar proteinosis, following the previously described method (Strong et al., 1998). Both SP-A and SP-D preparations were judged to be pure by SDS-PAGE, Western blot and amino acid composition. SP-A preparation was free from any SP-D contamination and vice-versa. Gel filtration confirmed that majority of SP-A and SP-D preparations were octadecamer and dodecamers, respectively. SP-A and SP-D preparations were further evaluated for endotoxin levels by QCL-1000 Limulus amebocyte lysate system (BioWhittaker, Walkersville, MD, U.S.A.). The amount of endotoxin present in purified SP-A was observed to be 1.6 pg/μg of SP-A and for purified SP-D, it was found to be 5.6 pg/μg of SP-D.

2.4. Expression and purification of rhSP-D

A recombinant fragment, composed of the trimeric α-helical coiled-coil neck region and three C-type lectin domains of human SP-D (rhSP-D), was expressed in *E. coli* and purified to homogeneity, as previously described (Singh et al., 2003). The rhSP-D preparation was functionally characterized for its ability to bind simple sugars, phospholipids (Singh et al., 2003), and *A. fumigatus* conidia (Madan et al., 1997). The crystallographic structure of rhSP-D, complexed with maltose in the carbohydrate binding pockets, is also known (Shrive et al., 2003). The amount of endotoxin present

Table 1
Study design.

Groups of mice	Designated groups (number of mice for survival/cytokine study)	Day 0	Day 1	Protein/drug in μg/50 μl/mouse
WT (SP-A$^{-/-}$ type) (test group)	WT-IPA (SP-A$^{-/-}$)-BSA (16/9)	Conidia	BSA	3.0
WT (SP-A$^{-/-}$ type) (control group)	WT-C (SP-A$^{-/-}$)-BSA (6/6)	PBS	BSA	3.0
WT (SP-D$^{-/-}$ type) (test group)	WT-IPA (SP-D$^{-/-}$)-BSA (10/9)	Conidia	BSA	3.0
WT (SP-D$^{-/-}$ type) (control group)	WT-C (SP-D$^{-/-}$)-BSA (6/6)	PBS	BSA	3.0
WT (SP-D$^{-/-}$ type) (test group)	WT-IPA (SP-D$^{-/-}$)-AmB (12/9)	Conidia	AmB	134.6
SP-A$^{-/-}$ (test group)	SP-A$^{-/-}$-IPA-BSA (10/9)	Conidia	BSA	3.0
SP-A$^{-/-}$ (control group)	SP-A$^{-/-}$-C-BSA (6/6)	PBS	BSA	3.0
SP-A$^{-/-}$ (test group)	SP-A$^{-/-}$-IPA-SP-A (10/9)	Conidia	SP-A	3.0
SP-A$^{-/-}$ (control group)	SP-A$^{-/-}$-C-SP-A (6/6)	PBS	SP-A	3.0
SP-A$^{-/-}$ (test group)	SP-A$^{-/-}$-IPA-AmB (10/9)	Conidia	AmB	134.6
SP-D$^{-/-}$ (test group)	SP-D$^{-/-}$-IPA-BSA (14/9)	Conidia	BSA	3.0
SP-D$^{-/-}$ (control group)	SP-D$^{-/-}$-C-BSA (6/6)	PBS	BSA	3.0
SP-D$^{-/-}$ (test group)	SP-D$^{-/-}$-IPA-SP-D (12/9)	Conidia	SP-D	1.0
SP-D$^{-/-}$ (control group)	SP-D$^{-/-}$-C-SP-D (6/6)	PBS	SP-D	1.0
SP-D$^{-/-}$ (test group)	SP-D$^{-/-}$-IPA-rhSP-D (12/9)	Conidia	rhSP-D	1.0
SP-D$^{-/-}$ (control group)	SP-D$^{-/-}$-C-rhSP-D (6/6)	PBS	rhSP-D	1.0
SP-D$^{-/-}$ (test group)	SP-D$^{-/-}$-IPA-AmB (12/9)	Conidia	AmB	134.6