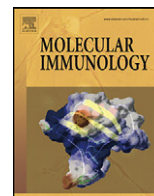




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Susceptibility of mice genetically deficient in SP-A or SP-D gene to Invasive Pulmonary Aspergillosis

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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.

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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.

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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 10⁸ conidia per 50 μ l (Madan et al., 1997). The conidia viability of the challenge inoculum was assessed by plating 10⁶ and 10⁷ 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 amoebocyte lysate system (BioWhittaker, Walkersville, MD, U.S.A.). The amount of endotoxin present in purified SP-A was observed to be 1.6 pg/ μ g of SP-A and for purified SP-D, it was found to be 5.6 pg/ μ g 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 μ g/50 μ l/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

in the rhSP-D preparations was found to be 4 pg/ μ g of purified protein.

2.5. Preparation of AmB

A 4.16 mg/ml solution of AmB (Deoxycholate amphotericin B, Fungizone, E.R. Squibb, Hounslow, UK) was prepared and diluted to 2.692 mg/ml using sterile PBS immediately prior to use.

2.6. Immunosuppression and challenge with *A. fumigatus* conidia

The murine model of IPA was generated as previously described (Madan et al., 2001). The lung cytokine profile of naïve, control (immunosuppressed) and *A. fumigatus*-challenged (IPA) mice of all the three genotypes viz. SP-A^{-/-}, SP-D^{-/-} and WT were evaluated. In order to evaluate the susceptibility of SP-A^{-/-}, SP-D^{-/-} and WT mice to IPA. Effect of various treatments such as SP-A, SP-D, BSA (as a control protein) and AmB (as a positive control) were also assessed. The experimental design has been summarized in Table 1. The study was reviewed and approved by the Institutional review committee. The experiments were conducted in accordance to the rules laid down within appropriate Home Office license. The rationale for the amount of SP-A and SP-D used for therapy, has been reported earlier (Madan et al., 2005). The lung histology and cytokine studies were performed in experiments separate from survival studies. Since various Control groups of mice had shown alterations in cytokine profiles on administration of test or control protein, the study has used the ratios of a test group with their respective control for comparative evaluation between different groups.

2.7. Survival rate analysis

The various mice groups were monitored for the survival for up to 15 days. The survival rate was analyzed by Kaplan Meier test using JMP software 3.2.1 (SAS Institute Inc., North Carolina, U.S.A.).

2.8. Colony forming units (CFU)

Lungs were aseptically removed from the mice sacrificed from each group on day 2, 4 and 8 following the conidia challenge. An equal amount by weight of lung tissue (~10 mg) comprising of four randomly selected representative samples from the four lobes, was homogenized using a mechanical homogenizer for 1 min with a 1:5 dilution (w/v) in sterile PBS, for cytokine studies and CFU analysis. The lung suspension was serially diluted 2-fold and incubated in triplicate on SDA plates at 37 °C. *A. fumigatus* colonies were then counted three times after 24 h incubation. The values represent average $\pm$ standard deviation of 12 data points (3 from each dilution from 2 animals of each group).

2.9. Cytokine levels in lung suspension

A portion of the lung cell suspension obtained above was centrifuged and resuspended in RPMI-1640 culture medium containing 10% (v/v) heat inactivated bovine serum and 10 μ g/ml gentamicin (2×10^6 cells/well). Cells were allowed to proliferate for 72 h in 5% (v/v) CO₂ incubator. The supernatants from the lung suspension were assayed for the cytokines such as IL-4, IL-5, IL-10, IL-12, IL-13, TNF- α and IFN- γ using the kits from Endogen, Cambridge, MA, U.S.A. The values represent average $\pm$ standard deviation of 6 data points (3 from each animal from 2 animals of each group).

2.10. Histological examination of the lung sections

For histopathology, 4 randomly selected representative specimens were taken from all the four lung lobes of the sacrificed mice, fixed in 10% (v/v) formaldehyde, and stored at 4 °C. The tissue sections were made using a microtome, then stained with H&E/GMS, and examined at a magnification of 40 $\times$ and 400 $\times$. Each picture is a representative of 8 sections (4 each from 2 animals of each group).

2.11. Statistical analysis

CFU data were expressed as mean $\pm$ SD and compared by one-population ANOVA test using MicroCal Origin Version 3.0 statistical package (MicroCal Software Inc., Northampton, MA, U.S.A.). Cytokine data were compared using unpaired two-tailed Mann–Whitney (nonparametric) test. Statistical analysis for the

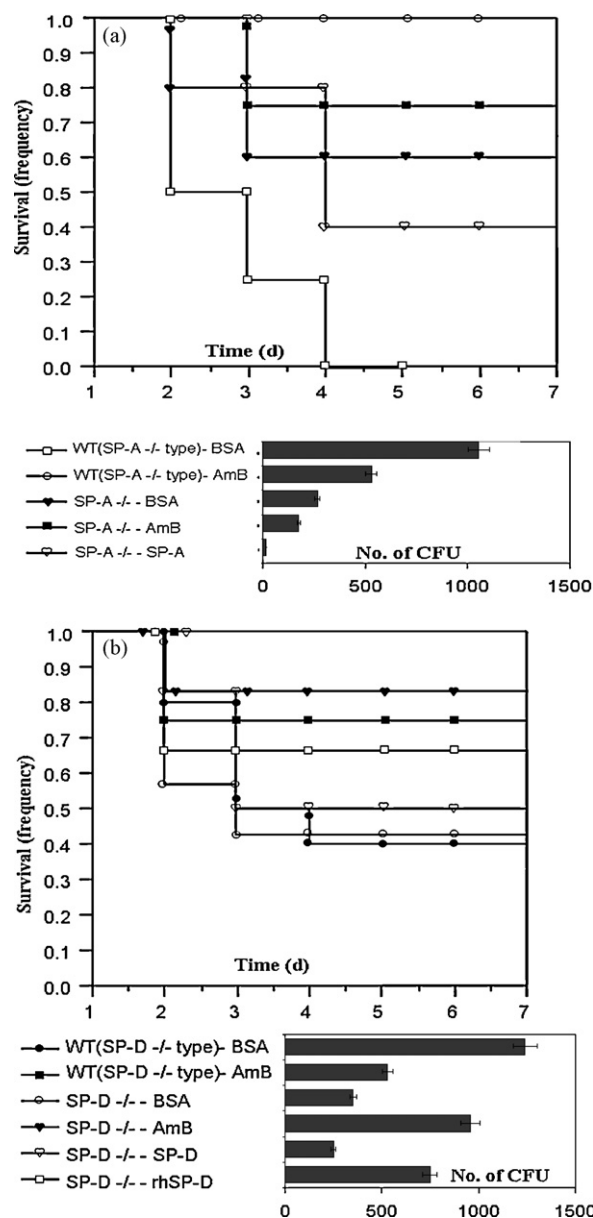


Fig. 1. Mortality in various groups of mice. Mice were immunosuppressed with corticosteroid prior to an intranasal challenge with *A. fumigatus* conidia. (A) SP-A gene deficient mice (SP-A^{-/-}) mice and (B) SP-D gene deficient (SP-D^{-/-}) mice compared with their respective wild-type (WT) mice. The bar graphs depict the lung CFU counts isolated from the above-mentioned groups on day 2 of the study.

Table 2
Ratio of cytokine levels of lung suspensions of *A. fumigatus* challenged, control and naïve SP-A^{-/-} and SP-D^{-/-} mice groups to their respective groups of WT mice on day 0.

	IFN- γ	TNF- α	IL-4	IL-5	IL-10	IL-12	IL-13
SP-A ^{-/-} -IPA-BSA	1.00	1.12	-1.68*	1.87*	-1.21	1.00	2.05*
SP-A ^{-/-} -control-BSA	-1.06	-2.67*	-2.88*	-13.4*	-3.58*	1.29	-1.28
SP-A ^{-/-} -naïve	-1.92*	1.41	1.00	3.93*	1.13	-1.24	13.1*
SP-D ^{-/-} -IPA-BSA	1.52*	1.48	1.00	1.00	1.39	1.00	1.68*
SP-D ^{-/-} -control-BSA	1.12	-1.19	2.04*	1.00	-3.73*	-1.22	1.55*
SP-D ^{-/-} -naïve	-2.7*	-1.23	1.00	4.68*	1.01	-1.26	11.6*

Each value represents a mean of nine readings (triplicate values from three animals of each group). The deviations were calculated for each mean value and were within $\pm 5\%$. The negative sign indicates a decrease in the level of cytokine in the gene deficient mice group with respect to the respective WT mice group. The values for WT mice for SP-A^{-/-} and SP-D^{-/-} mice are from WT (SP-A^{-/-} type) and WT (SP-D^{-/-} type), respectively.

* $p < 0.05$.

IFN- γ and IL-4 ratios were carried out using two by two table (a test for bivariate analysis) The analysis was considered significant only when $p < 0.05$ after Bonferroni correction.

3. Results

3.1. Survival of WT, SP-A^{-/-} and SP-D^{-/-} mice following *A. fumigatus* challenge

The two wild-type groups WT-IPA-BSA (SP-A^{-/-} type) and WT-IPA-BSA (SP-D^{-/-} type) showed mortality rates of 100% and 60%, respectively. The SP-A^{-/-}-IPA-BSA mice had a lower mortality (40%), 3.95-fold less CFU count and a significant increase in IFN- γ to IL-4 ratio (1.69-fold) when subjected to IPA compared to the WT-IPA-BSA (SP-A^{-/-} type) mice (100%) ($p < 0.05$) (Fig. 1A, Table 2).

The SP-D^{-/-}-IPA-BSA mice (57.14%) showed similar mortality ($p = 0.85$) and IFN- γ to IL-4 ratio (1.3-fold increase), but 3.53-

fold lesser CFU count ($p < 0.05$) than WT-IPA-BSA (SP-D^{-/-} type) mice (60%). A statistical comparison of the day of death, however, revealed that more than twice the number of SP-D^{-/-}-IPA-BSA mice died earlier than the WT-IPA-BSA (SP-D^{-/-} type) mice (Fig. 1B). Only 20% of WT-IPA-BSA (SP-D^{-/-} type) mice died on day 2 while SP-D^{-/-}-IPA-BSA mice had a mortality of 42.86% ($p = 0.13$).

Histologically, significant differences were observed in the extent of lung infection and tissue injury between the SP-A^{-/-}-IPA-BSA and WT-IPA-BSA (SP-A^{-/-} type) and between the SP-D^{-/-}-IPA-BSA and WT-IPA-BSA (SP-D^{-/-} type) mice (Fig. 2). All the four groups showed H&E and GMS stained fungal hyphae and inflammatory loci in the lungs. However, there were fewer fungi, inflammatory loci and necrotic lesions in the lungs of SP-A^{-/-}-IPA-BSA mice than WT-IPA-BSA (SP-A^{-/-} type). In contrast, the SP-D^{-/-}-IPA-BSA mice showed severe necrosis of lung tissue, together with an increased fungal burden compared to WT-IPA-BSA (SP-D^{-/-} type).

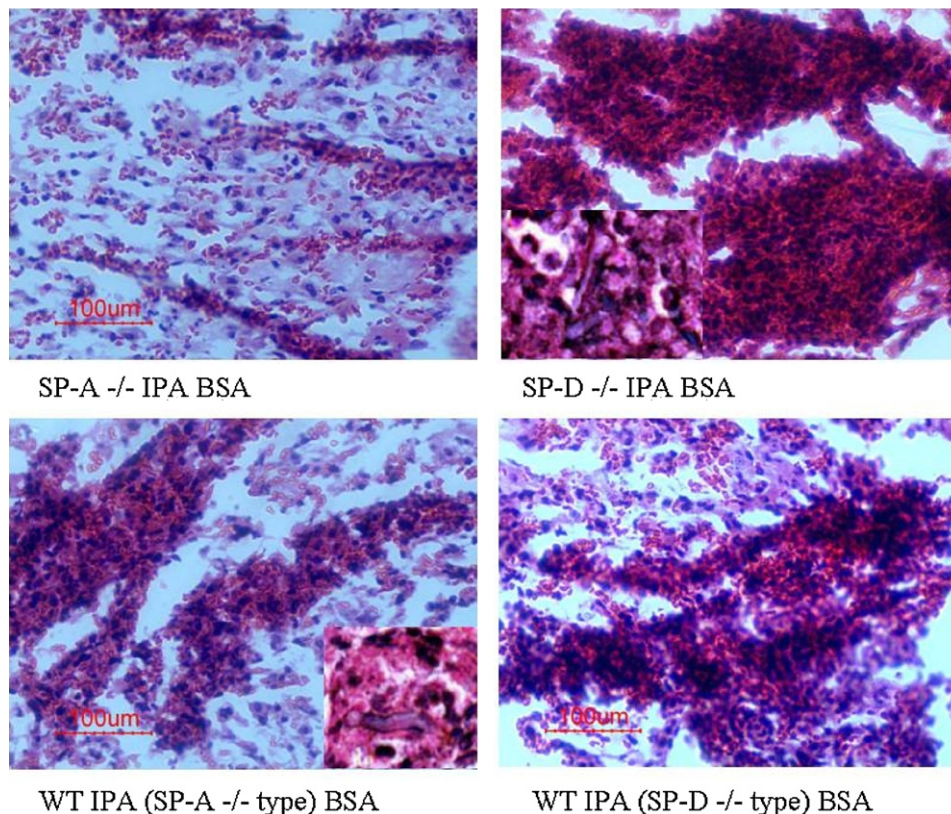


Fig. 2. Histopathological examination of the lung sections stained with H&E stain observed at 40 $\times$ magnification, from the BSA treated SP-A gene deficient (SP-A^{-/-}) mice and SP-D gene deficient (SP-D^{-/-}) mice and their respective wild-type mice, WT (SP-A^{-/-} type) and WT (SP-D^{-/-} type), intranasally challenged with 10^8 conidia of *A. fumigatus* on day 2 of the study. The insets are at 400 $\times$ magnification to show the presence of dichotomously branched hyphae of *A. fumigatus* presence in the infected pulmonary tissue. Each picture is a representative of 6 sections (3 each from 2 animals of each group).

Table 3

Ratio of IFN- γ to IL-4 levels on day 2, 4 and 8 and percent survival in various groups of mice.

Group	IFN- γ /IL-4			% survival
	Day 2	Day 4	Day 8	
WT-IPA (SP-A ^{-/-})-BSA	2.65	4.72	–	0
WT-C (SP-A ^{-/-})-BSA	1.77	4.54	–	
WT-IPA (SP-D ^{-/-})-BSA	1.83	4.8	4.56	40
WT-C (SP-D ^{-/-})-BSA	2.43	5.67	0.086	
WT-IPA (SP-D ^{-/-})-AmB	4.33	6.06	3.84	75
SP-A ^{-/-} -IPA-BSA	4.5	2.62	3.57	60
SP-A ^{-/-} -C-BSA	4.8	^a	0.048	
SP-A ^{-/-} -IPA-SP-A	4.77	2.21	2.5	40
SP-A ^{-/-} -C-SP-A	2.52	3.7	0.081	
SP-A ^{-/-} -IPA-AmB	4.87	4.41	5.43	80
SP-D ^{-/-} -IPA-BSA	2.79	1.93	3.67	42.86
SP-D ^{-/-} -C-BSA	1.34	3.54	3.25	
SP-D ^{-/-} -IPA-SP-D	3.36	3.81	3.73	50
SP-D ^{-/-} -C-SP-D	2.29	4.96	3.11	
SP-D ^{-/-} -IPA-rhSP-D	3.74	7.49	5.67	67
SP-D ^{-/-} -C-rhSP-D	1.9	3.68	3.96	
SP-D ^{-/-} -IPA-AmB	2.77	6.26	4.8	83.33

(–) No mice survived.

^a IFN- γ levels were not in the detectable range in the SP-A^{-/-}-C-BSA mice.

3.2. Treatment of WT, SP-A^{-/-} and SP-D^{-/-} mice following challenge with conidia

3.2.1. Administration of SP-A to SP-A^{-/-}-IPA mice

Intranasal administration of purified SP-A to SP-A^{-/-}-IPA mice led to a 1.5-fold increase in mortality ($p=0.46$), significantly lower IFN- γ to IL-4 ratio (1.42-fold) ($p<0.05$) (Table 3), increased hyphal density but a 19.01-fold lower CFU count on day 2 ($p<0.05$) than

SP-A^{-/-}-IPA-BSA mice (Figs. 1A and 3). The SP-A treatment resulted in a significant decrease in TNF- α (2.3-fold), IL-10 (2.76-fold) and IL-13 (2.56-fold) levels on day 2 ($p<0.05$) (Fig. 4).

3.2.2. Administration of SP-D or rhSP-D to SP-D^{-/-}-IPA mice

Intranasal administration of SP-D to SP-D^{-/-}-IPA mice led to a 7.18% decrease in mortality, higher IFN- γ to IL-4 ratio (significant on day 4, Table 2), 1.4-fold lower CFU count and a lower lung hyphal density than SP-D^{-/-}-IPA-BSA mice ($p=0.85$) (Figs. 1B and 3). Intranasal administration of SP-D resulted in a significant decrease in IL-10 (2.18-fold) on day 2 ($p<0.05$) than SP-D^{-/-}-IPA-BSA mice (Fig. 5).

The SP-D^{-/-}-IPA mice treated with rhSP-D led to a 1.73-fold reduction in the mortality ($p=0.26$), no fungal hyphae with minimal inflammatory foci but, with a 2.13-fold higher CFU count ($p<0.05$) compared to the SP-D^{-/-}-IPA-BSA mice (Figs. 1B, 2 and 3). The IFN- γ to IL-4 ratio in SP-D^{-/-}-IPA-rhSP-D mice was consistently and significantly higher than the SP-D^{-/-}-IPA-BSA mice on day 2, 4 and 8 (Table 2). The rhSP-D treatment resulted in an increase in TNF- α (1.96-fold), IL-10 (5.24-fold) and IL-13 (4.82-fold), but a decrease in IFN- γ (2.1-fold) and IL-4 (2.78-fold) on day 2 ($p<0.05$) compared with SP-D^{-/-}-IPA-BSA mice (Fig. 5).

3.2.3. AmB protects SP-A and SP-D gene deficient mice against IPA

Intranasal administration of AmB to WT (SP-A^{-/-} type) resulted in 100% survival ($p=0.00$) and that in the case of WT (SP-D^{-/-} type) mice resulted in a 2.4-fold increase in survival (75%) ($p=0.21$) in comparison with their respective BSA treated controls (Fig. 1A and B).

The intranasal treatment of AmB to the SP-A^{-/-}-IPA mice (SP-A^{-/-}-IPA-AmB) led to a two-fold increase in the survival and to

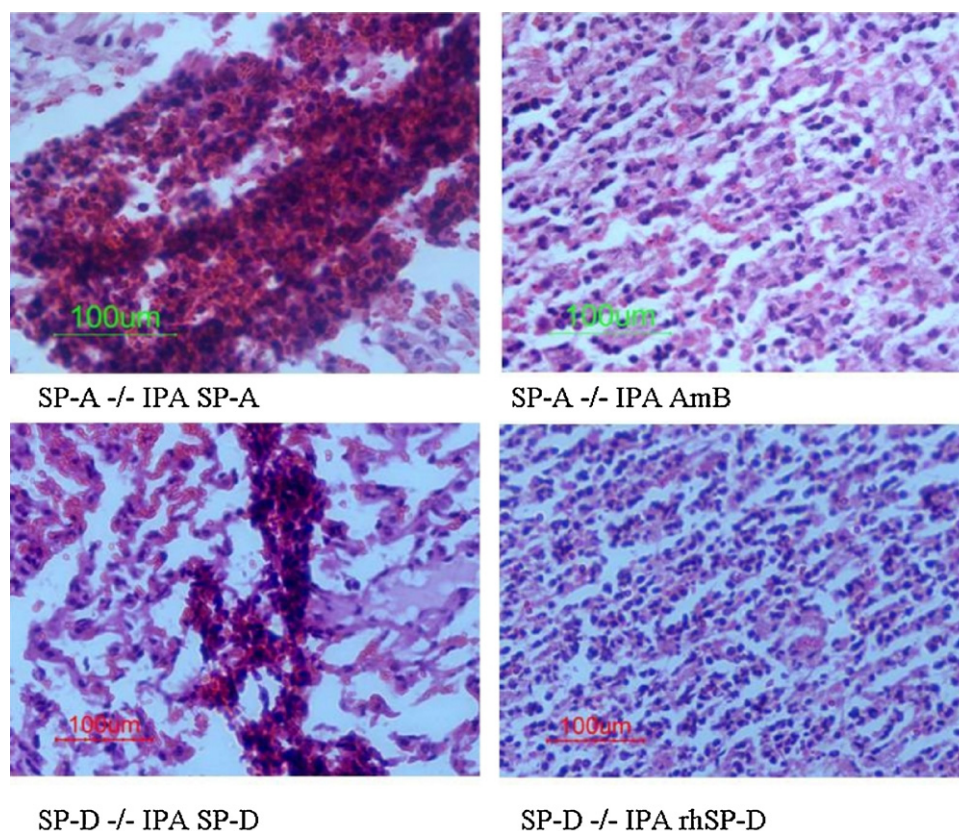


Fig. 3. Histopathological examination of the lung sections stained with H&E stain observed at 40 $\times$ magnification, from the SP-A or AmB-treated SP-A gene deficient (SP-A^{-/-}) mice and SP-D- or rhSP-D-treated SP-D gene deficient (SP-D^{-/-}) mice intranasally challenged with 10^8 conidia of *A. fumigatus* on day 2 of the study. Each picture is a representative of 6 sections (3 each from 2 animals of each group).

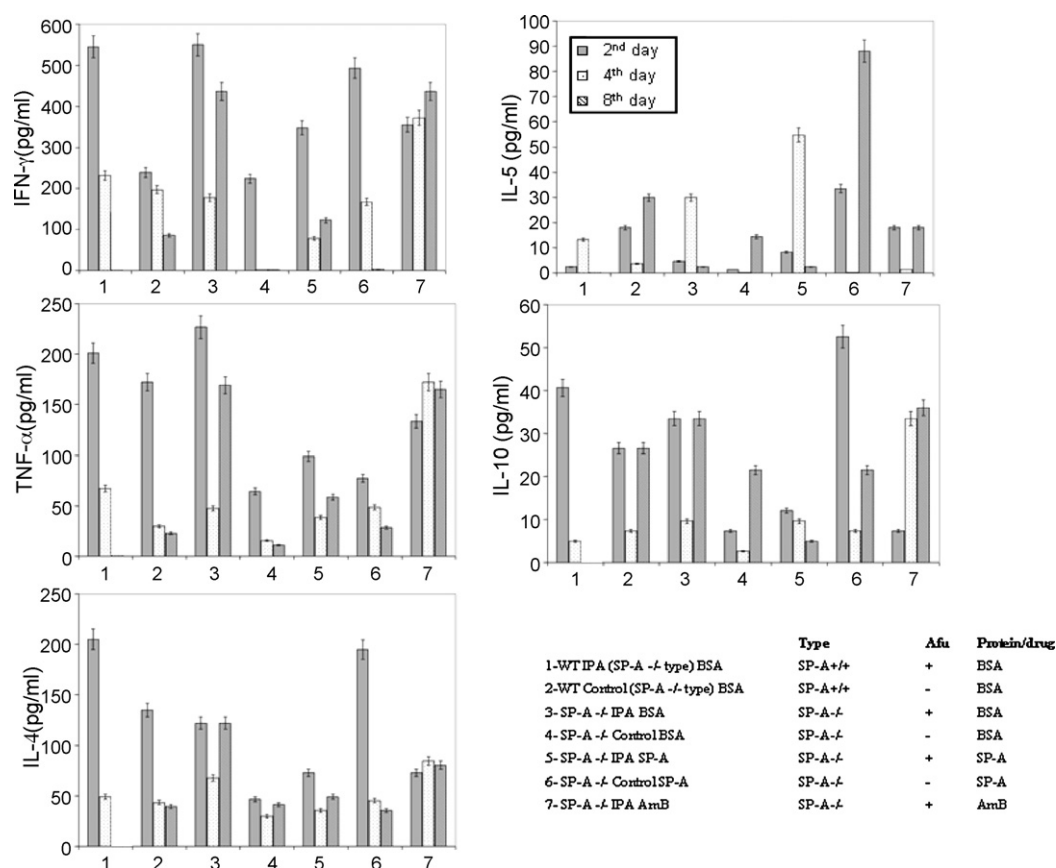


Fig. 4. Concentration of various cytokines in the lung suspensions of *A. fumigatus* challenged and control-SP-A gene deficient and WT (SP-A^{-/-} type) mice groups treated with BSA, SP-A or AmB on day 2 (grey bar), 4 (dotted bar), and 8 (hatched bar). Each value represents a mean of 6 readings (triplicate values from 2 animals of each group).

SP-D^{-/-}-IPA-AmB mice showed a 3.59-fold increase in survival compared to the BSA treated SP-A^{-/-}-IPA mice ($p=0.43$) (Fig. 1A) and SP-D^{-/-}-IPA mice, respectively ($p=0.04$) (Fig. 1B). The lung tissue from the AmB treated WT and SP-A^{-/-} mice did not show any fungal hyphae, whereas the AmB treated SP-D^{-/-} mice showed a few fungal hyphae (Fig. 3) with significant increase in IFN- γ to IL-4 ratio in all the AmB treated groups in comparison with BSA treated respective controls (Table 2).

3.3. Distinct effects of immunosuppression on IFN- γ to IL-4 ratio in SP-A^{-/-} and SP-D^{-/-} mice (without conidia challenge)

The ratio of IFN- γ to IL-4 was significantly higher (2.7-fold) ($p<0.05$) in immunosuppressed SP-A^{-/-} mice (ratio=4.79) than the immunosuppressed WT mice (SP-A^{-/-} type) (ratio=1.77) on day zero. In contrast, immunosuppressed SP-D^{-/-} mice showed 1.8-fold lower ratio of IFN- γ to IL-4 ($p<0.05$) (ratio=1.34) than the immunosuppressed WT (SP-D^{-/-} type) (ratio=2.42) (Table 3).

4. Discussion

4.1. SP-A gene deficient mice are resistant to *A. fumigatus* challenge

A significantly reduced mortality consistent with reduced hyphae and tissue damage in the lungs, of the SP-A^{-/-}-IPA-BSA mice following *A. fumigatus* challenge in comparison with the WT mice suggests that deficiency of the SP-A gene has resulted in the development of resistance to *A. fumigatus* challenge. This observation is further supported by an increased protective Th1 response evident by an increase in IFN- γ to IL-4 ratio and a decrease in IL-4

levels in the lung suspensions of these mice. It is important to note that the immunosuppressed SP-A^{-/-} mice (SP-A^{-/-}-control) also show a significantly higher IFN- γ to IL-4 ratio than the immunosuppressed WT (SP-A^{-/-} type) mice on day zero and an intranasal SP-A treatment results in the lowering of IFN- γ to IL-4 ratio in these mice (SP-A^{-/-}-control-SP-A). That the ability of the SP-A^{-/-} mice to offer resistance to *A. fumigatus* challenge is specifically due to absence of SP-A is evident from an increased mortality and several fold decreased levels of IFN- γ and TNF- α upon administration of SP-A (SP-A^{-/-}-IPA-SP-A). This result is consistent with our earlier study where SP-A administration was found to be ineffective in rescuing IPA mice from fatal *A. fumigatus* challenge (Madan et al., 2001). It is possible that the resistance to *A. fumigatus* challenge in the SP-A^{-/-} mice is partly contributed by a 7-fold increase in the level of SP-D reported in these mice (LeVine et al., 2002). There is also a possibility that SP-A facilitates *A. fumigatus* conidia germination and subsequent infection in vivo.

4.2. SP-D gene deficiency leads to an increase in the severity of IPA pathogenesis

An increased early death rate, hyphal density and tissue injury in lungs suggests that the deficiency of SP-D gene has resulted in compromised defense mechanisms. Previously, the lack of SP-D has been shown to be associated with pulmonary infiltration by foamy alveolar macrophages, increased hydrogen peroxide production, and expression of matrix metalloproteinases (MMPs) such as MMP-2, MMP-9, and MMP-12, resulting in a significantly more severe lung inflammation than that in the SP-A^{-/-} mice (Botas et al., 1998; Wert et al., 2000). This pathophysiological state of lungs in the SP-D^{-/-} mice appears to be aggravated by *A. fumigatus* conidia

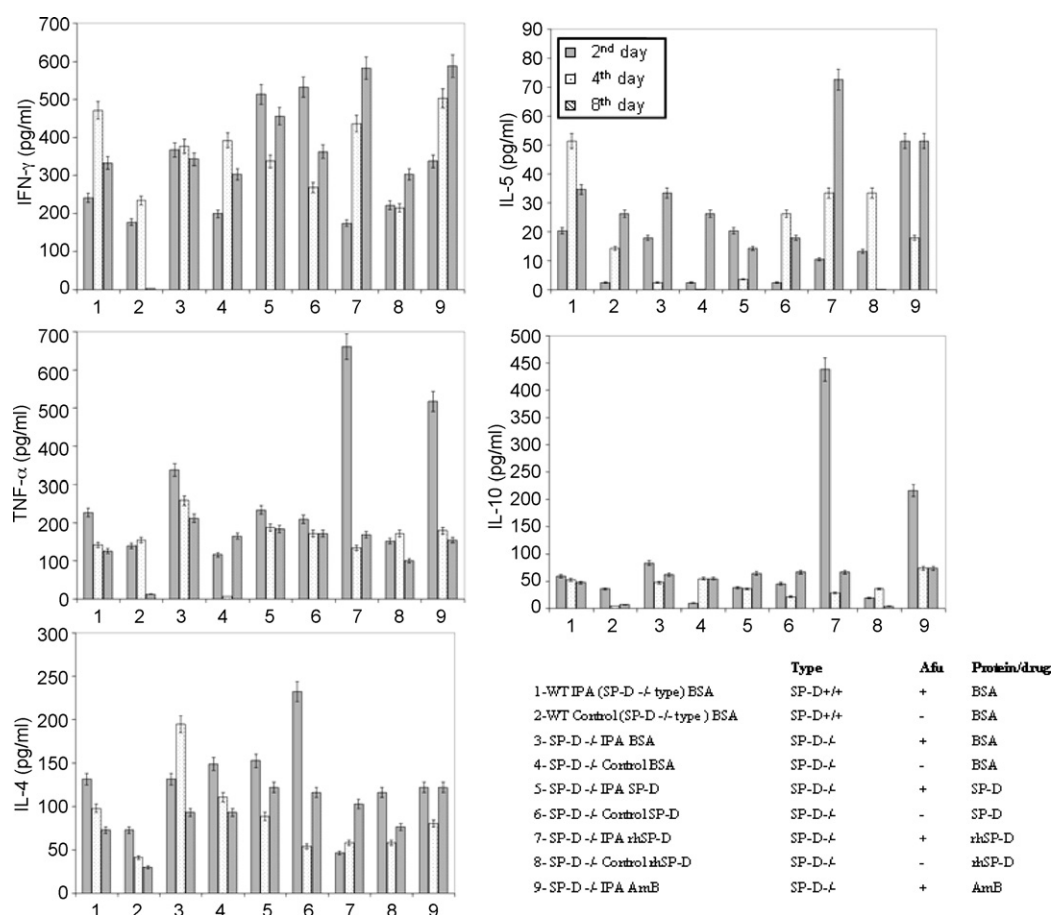


Fig. 5. Concentration of various cytokines in the lung suspensions of *A. fumigatus* challenged and control-SP-D gene deficient (SP-D^{-/-}) and WT (SP-D^{+/+} type) mice groups treated with BSA, SP-D, rhSP-D or AmB on day 2 (grey bar), 4 (dotted bar), and 8 (hatched bar) of the study. Each value represents a mean of 6 readings (triplicate values from 2 animals of each group).

challenge that probably accounts for an increased hyphal density and severe IPA pathogenesis observed in the SP-D^{-/-}-IPA mice. The immunosuppressed SP-D^{-/-} mice showed a significant decrease in protective Th1 response, suggesting that the SP-D^{-/-} mice could be more susceptible to *A. fumigatus* challenge. An increase in the survival of the SP-D^{-/-}-IPA mice following rhSP-D treatment also suggests that the increased susceptibility of the SP-D^{-/-} mice to IPA is specifically due to SP-D deficiency. The observation is adequately supported by an increase in IFN- γ to IL-4 ratios in the lung suspensions of the SP-D^{-/-}-IPA mice treated with either SP-D or rhSP-D. This is consistent with our earlier study where, SP-D or rhSP-D treatment increased the survival of IPA mice by up to 80% as compared to 100% mortality in the untreated IPA group (Madan et al., 2001; Singh et al., 2009). The observed inefficiency of SP-D and rhSP-D in rescuing SP-D^{-/-}-IPA mice in the present study may possibly be due to differences in their molar concentrations used (SP-D, $3 \mu\text{g} - 5.7 \times 10^{-3} \mu\text{M}$ and rhSP-D, $1 \mu\text{g} - 16.7 \times 10^{-3} \mu\text{M}$). Similar to the observations made in the present study, SP-A^{-/-} and SP-D^{-/-} mice respond differently to various microbial challenges (LeVine et al., 2000; Hawgood et al., 2004) and *A. fumigatus* allergens and antigens (Madan et al., 2005). It is also important to note that both SP-A^{-/-} and SP-D^{-/-} mice are different in terms of anatomical and functional abnormalities reported only in the SP-D^{-/-} mice, and not in SP-A^{-/-} mice. This may be one of the underlying issues for their varied response to corticosteroid induced immunosuppression and susceptibility to *A. fumigatus* conidia challenge.

Persistence of Th2 bias in the SP-D^{-/-} mice but not in the SP-A^{-/-} mice following corticosteroid induced immunosuppression, suggests that steroid induced immunosuppression may have dif-

ferential effects on host immunity, when coupled with alterations in levels of SP-A or SP-D. It is worth noting that although immunosuppression in the murine model of IPA can be accomplished via a variety of methods (e.g. glucocorticoids, cyclophosphamide, etc.), these treatments may introduce different possible confounding factors and differently alter both host cell responses and subsequent cytokine synthesis and regulation.

4.3. Increased IFN- γ to IL-4 ratios, TNF- α and IL-10 levels associate with higher survival rates in *A. fumigatus*-challenged SP-A^{-/-} or SP-D^{-/-} mice

A strong correlation between increased IFN- γ to IL-4 ratios and increased levels of TNF- α with an increased survival in various groups of mice observed in the present study is consistent with the view that Th1-dependent immunity and TNF- α play an essential role in host defense against IPA (Cenci et al., 1998; Nagai et al., 1995; Cenci et al., 1997) and indicate that SP-A and SP-D influence TNF- α levels in mice (Sato et al., 2002; Bufler et al., 2004).

IL-10 is considered to have a potent and broad-spectrum anti-inflammatory activity, based on various models of infection, inflammation, and even in cancer. The IL-10 deficient mice have been reported to show an increased survival than the WT mice to lethal systemic aspergillosis (Clemons et al., 2000). Our study, however, indicates a positive correlation of IL-10 levels on day 2 with the survival rate of SP-A^{-/-}-IPA and rhSP-D treated SP-D^{-/-}-IPA mice, suggesting that SP-A and SP-D may have a role in regulating IL-10 levels in the lung. It is likely that the raised levels of IL-10 in the lungs are probably involved in the resolution of inflam-

mation following early episode of lung infiltration in response to *A. fumigatus* challenge. A recent study showed that IL-10 secreting, regulatory T cells are essential to completely suppress airway hyperreactivity in an antigen dependent murine model of asthma (Presser et al., 2008). Our in vitro data using human dendritic cells and T-cells also appear to suggest modulation of IL-10 by SP-A and SP-D (Kishore et al., unpublished).

Nine out of 11 groups of mice in the present study showed a positive correlation of lung CFU counts on day 2 with mortality on day 3, while two groups namely SP-D^{-/-}-IPA-AmB and SP-D^{-/-}-rhSP-D showed a negative correlation with lung CFU counts in comparison with SP-D^{-/-}-IPA-BSA. We are unable to explain the higher CFUs on day 2 in these groups wherein there was no mortality on day 3 or on any subsequent days in comparison with SP-D^{-/-}-IPA-BSA.

In conclusion, the present study reports that deficiency of SP-D may predispose the host to a severe form of invasive aspergillosis with a rapid and fatal outcome. Both SP-A and SP-D have important roles in the regulation of Th1-Th2 homeostasis and thereby host defense status against *A. fumigatus* in the lungs. Their absence leading to varied susceptibility to *A. fumigatus* challenge in corticosteroid immunosuppressed mice highlights their balancing acts in host defense against *A. fumigatus*.

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