

Studies on the interaction of Surfactant protein SP-D with Influenza A Virus, *Aspergillus fumigatus* and Dendritic Cells

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By

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As I get older, I realize more and more how much you have done for me.

This is for you Mom Rest in peace

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ABSTRACT

Surfactant proteins, SP-A and SP-D, are collagen-containing calcium-dependent (C-type) lectins, called, collectins. Their primary structure has four regions: a cysteine-linked N- terminal region involved in multimerization, a collagen region composed of Gly-X-Y repeats, coiled-coil neck region, and the C-terminal carbohydrate recognition domains (CRD) or C-type lectin domain. SP-A looks like a bouquet, while SP-D is a cruciform- like structure, with four arms of equal length. SP-A and SP-D have been shown to act as innate immune molecules at pulmonary as well as extra-pulmonary sites by binding to pathogens, allergens and apoptotic/necrotic cells via their CRD region. SP-A and SP-D can induce pathogen neutralization and enhanced phagocytosis. In addition, SP-A and SP-D can interact via CRDs with allergens and dampen allergic reaction in vitro and in vivo.

This thesis examines in vitro interaction of a recombinant fragment of human SP-D containing neck and CRD regions (rhSP-D) with IAV and *Aspergillus fumigatus*, in addition to characterizing a dichotomy of the effects of SP-A and SP-D on dendritic cells in an attempt to explain how SP-A and SP-D modulate DC functions differentially. Experiments involving interaction of rhSP-D with IAV pandemic strain show that it can be a restrictive factor against the virus, in addition to modulating immune response by a macrophage cell line. The rhSP-D can have anti-*A. fumigatus* effect directly and indirectly in the context of pathogen as well as allergen. A comparison has been made between two recombinant fragments of SP-D that have been expressed with and without 8 Gly-X-Y repeats for their fungistatic properties. The effects of SP-A and SP-D on cultured DC maturation, and effector cytokine and proliferative response of co-cultured cells have also been examined in vitro.

Contents

1. Chapter 1 Introduction.....	16
1.1. Pattern recognition receptors (PRRs) interaction with Pathogen associated molecular patterns (PAMPs).....	17
1.2. Link between innate and adaptive immunity	18
1.3. Complement system	20
1.4. Collectins	22
1.5. The Immunological importance of SP-A and SP-D	28
1.6. Interaction and clearance of viral, bacterial and fungal pathogens.....	28
1.7. Role of SP-A and SP-D in allergy.....	32
1.8 Host-pathogen interaction involving IAV.....	38
1.9 <i>Aspergillus fumigatus</i> , a fungal pathogen.....	41
2. Chapter 2 Materials and Methods	47
2.1. Purification of native of full length SP-A	48
2.2. Purification of native of full length SP-D	48
2.3. Expression and purification of trimeric SP-D.....	48
2.3.1. Competent cells preparation.....	48
2.3.2. Transformation of cells	49
2.3.3. Protein Expression	49
2.3.4. Cell Lysis	49
2.3.5. Dialysis	50
2.3.6. Protein Purification by Affinity chromatography	50
2.3.7. Endotoxin Removal from Recombinant SP-D.....	50
2.3.8. Preparation of D-Maltose-Sepharose 4B Column.....	51
2.4. Activation	51
2.5. Immobilization	51

2.6.	Bis (Sulfosuccinimidyl) substrate (BS3) Cross-Linking.....	51
2.7.	TCA Assay	52
2.8.	Bicinchoninic Acid Protein Assay	52
2.9.	SDS- PAGE: 12% and 15%	52
2.10.	IAV amplification and purification (H3N2, H1N1)	52
2.11.	Purification of IAV by ultra-centrifugation and sucrose cushion	52
2.12.	Haemagglutination assay.....	53
2.13.	Haemagglutination inhibition assay (HAI)	53
2.14.	Binding of H3N2 influenza A virus to rhSP-D.....	53
2.15.	Western blot of rhSP-D bind to H3N2	54
2.16.	rhSP-D binding to H1 and N1 proteins.....	54
2.17.	Infection assay using U937 monocytic cell line.....	54
2.18.	Multiplex cytokine array analysis.....	55
2.19.	Aspergillus strains.....	55
2.20.	Susceptibility testing	56
2.21.	Preparation and labelling of conidia.....	56
2.22.	Binding of rhSP-D to A. fumigatus conidia	57
2.23.	Agglutination assay.....	57
2.24.	Killing assay.....	57
2.25.	Murine models	58
2.26.	Study design summary	58
2.27.	Measurement of cytokine and chemokine levels in the lung cell suspension	58
2.28.	Dendritic cell preparation	59
2.29.	Flow Cytometry	59
2.30.	T-Cell Proliferation Assays.....	60
2.31.	ELISA for supernatant cytokine measurement.....	60

2.32.	Statistical Analysis	60
3.	Chapter 3 Interaction of rhSP-D with Influenza A Virus.....	61
3.1.	Introduction	62
3.2.	Results	64
3.2.1.	Expression in <i>E. coli</i> , purification and characterization of a recombinant fragment of human SP-D (rhSP-D) composed of neck and CRD region.....	64
3.3.	rhSP-D binds IAV in solid-phase direct binding ELISA.....	67
3.4.	Multiplex cytokine array analysis showed downregulation of pro-inflammatory cytokine TNF- α and IL-6 by rhSP-D treated virus.....	71
3.5.	Discussion.....	72
4.	Chapter 4 Interaction of rhSP-D with <i>Aspergillus fumigatus</i>	74
4.1.	Introduction	75
4.2.	Result.....	82
4.2.1.	The rhSP-D can bind <i>A. fumigatus</i> conidia and enhance its phagocytosis and killing by PMNs.....	82
4.2.2.	Binding of rhSP-D to 3wcf allergens/antigens can block IgE-induced histamine release from sensitised basophils.....	84
4.2.3.	The rhSP-D can alter cytokine profile of murine ABPA splenocytes.....	87
4.2.4.	Expression and purification of a recombinant fragment of human SP-D without any collagen extension in the construct (rhSP-D delta)	89
4.2.5.	Fungistatic properties of the two variants of rhSP-D.....	91
4.3.	Discussion.....	94
5.	Chapter 5 Modulation of Dendritic Cell Maturation and Functions by SP-A and SP-D.....	98
5.1.	Introduction	99
5.1.1.	Origin and distribution of dendritic cells	99
5.1.2.	Human Dendritic cells.....	99

5.2. Dendritic cell maturation: The link between Innate and Adaptive Immunity	100
5.3. Role of DCs in Immune- mediated disease	104
5.4. Modulation of DCs by SP-A and SP-D	105
5.5. Results	107
5.5.1. Effect of SP-A and SP-D on LPS induce maturation of DCs.....	107
5.5.2. Effect of SP-A and SP-D on TNF- α induce maturation of DCs	108
5.5.3. Regulation of LPS induced TNF- α secretion By SP-A and SP-D	109
5.5.4. Modulation of biologically active IL-12p70 by mature DCs	110
5.5.5. Modulation of IL-10 production in the presence of SP-A and SP-D	111
5.6. Discussion.....	113
6. General Discussion and perspectives	

List of Abbreviations

- 3wcf: three-week culture filtrate
- ABPA: Allergic bronchopulmonary aspergillosis
- Afu: Aspergillus fumigatus
- AIDS: Acquired Immunodeficiency Syndrome
- AP: Alternative Pathway
- APC: Antigen-presenting cell
- APCS: Antigen-presenting cells
- ARDS: Adult Respiratory Distress Syndrome
- BAL: Bronchoalveolar lavage
- BALF: Bronchoalveolar lavage fluid
- BCA: Bicinchoninic acid assay
- BMDCs: Bone marrow derived dendritic cells
- BMDCs: Bone marrow derived dendritic cells
- BS3: Bissulfosuccinimidyl substrate
- C1q: first subcomponent of the classical pathway
- CD: Cluster Differentiation
- CF: Cystic Fibrosis
- CFU: Colony forming unit
- CL-K1: Collectin kidney 1
- CL-L1: Collectin liver 1
- CL-P1: Collectin placenta 1
- CRD: Carbohydrate-recognition domains
- CRP: Caspase recruitment domain
- CTLD: C-type lectin-like domains
- DC: Dendritic cells
- DCs: Dendritic cells

DPPC: Dipalmitoylphosphatidylcholine
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme linked immunosorbent assay
EPO: Eosinophile peroxidase assay
EPO: Eosinophils peroxidase
FACs: Fluorescence activated cell sorting
GM-CSF: Granulocyte macrophage colony- stimulating factor
HA: Heamagglutinin
HIV: Human immunodeficiency Virus
HLA: Human leukocyte antigens
HMGB-:1 High mobility group box-1
HSV: Herpes Simplex Virus
IFN- α : Interferon- alpha
IFN- γ : Interferon- gamma
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IL: Interleukin
IPA: Invasive Pulmonary Aspergillosis
IPTG: Isopropyl β -D-1-thiogalactopyranoside
JAK/STAT: Janus Kinase/ Transducer and activator of transcription
LCs: Langerhans Cells
LPS: Lipopolysaccharides
LRR: Leucine-rich repeat
MAC: Membrane Attack Complex
MAPK: Mitogen-activated protein kinase
MASPS: MBL associated serine proteases
MBL: Mannose-binding lectin

M-CSF: Macrophage colony-stimulating factor
MHC I: Major histocompatibility complex I
MHC II: Major histocompatibility complex II
MIP-1: Macrophage Inflammatory Protein-1
MLNs: Mediastinal lymph nodes
MMP: Metalloproteinase
NA: Neuraminidase
NOD: Nucleotide-binding oligomerization domain
OD: Optical density
PAF: Platelet activating factor
PAMPs: Pathogen associated molecular patterns
PBMCs: Polymorphonuclear cells
PRRs: Pattern-recognition receptors
RBCs: Red blood cells
RSV: Respiratory Syncytial Virus
SAP: Serum amyloid protein
SARS: Severe acute respiratory syndrome
SDS: Sodium dodecyl sulfate
SP-A: Surfactant protein A
SP-D: Surfactant protein D
TCR: T cell receptor
Th1: type 1 helper cells
Th2: type 2 helper cells
THP-1: Human acute monocytic leukemia cell line
TLRs: Toll-like receptors
TNF- α : Tumor Necrosis Factor- alpha
T-regs: T regulatory

TSP1: Thrombospondin-1

TSR: Thrombospondin-like repeats

WT: Wild type

List of figures

FIGURE 1.1 ANTIGEN CAPTURE AND PRESENTATION BY DENDRITIC CELLS	19
FIGURE 1.2 COMPLEMENT ACTIVATION PATHWAYS	25
FIGURE 1.3 THE STRUCTURES OF COLLECTINS	28
FIGURE 1.4 INNATE IMMUNE MECHANISMS IN THE LUNGS	29
FIGURE 1.5 FUNCTIONS ASSIGNED TO THE DIFFERENT PARTS OF SP-A AND SP-D MOLECULES	30
FIGURE 1.6 X-RAY CRYSTAL STRUCTURES OF RHSP-D	31
FIGURE 1.7 CRYSTAL STRUCTURES OF TRIMERIC RHSP-D AND RHSP-A	32
FIGURE 1.8 SP-A AND SP-D BIND A WIDE RANGE OF PATHOGENS	35
FIGURE 1.9 MULTIPLE FUNCTIONS OF SP-A AND SP-D IN HUMAN HEALTH AND DISEASE	36
FIGURE 1.10 SP-A AND SP-D CAN LINK INNATE AND ADAPTIVE IMMUNE SYSTEMS.....	38
FIGURE 1.11 SENSITISATION AND PRIMING IMMUNE MECHANISMS IN ASTHMA.....	39
FIGURE 1.12 PROTECTIVE MECHANISMS BY SP-A AND SP-D AGAINST LUNG ALLERGY AND INFECTION CAUSED BY ASPERGILLUS FUMIGATUS.....	40
FIGURE 1.13 STRUCTURES AND LIFE CYCLE OF INFLUENZA A VIRUS.....	38
FIGURE 3.1 SDS- PAGE (15%), A RECOMBINANT FRAGMENT OF HUMAN SP-D CONTAINING HOMOTRIMERIC NECK AND CRD REGIONS (RHSP-D).	65
FIGURE 3.2 SDS–PAGE (12% w/v) ANALYSES OF RHSP-D AND RH BOVINE CONGLUTININ AFTER PURIFICATION..	65
FIGURE 3.3 INTERACTION BETWEEN RHSP-D AND H3N2 IN A DIRECT BINDING ELISA	66
FIGURE 3.4 BINDING OF H3N2 VIRUS WITH PROPERDIN AND FACTOR H.	67
FIGURE 3.5 . CELL BINIDING ASSAY	68
FIGURE 3.6 MULTIPLEX CYTOKINE ARRAY ANALYSIS OF SUPERNATANTS OF THP-1 CELLS TREATED WITH RHSP-D.	70
FIGURE 4.1 AGGLUTINATION OF <i>A. FUMIGATUS</i> CONIDIA	76
FIGURE 4.2 KILLING OF <i>A. FUMIGATUS</i> CONIDIA BY ALVEOLAR MACROPHAGES.....	77
FIGURE 4.3 THE SURVIVAL PERCENTAGE OF THE IPA MICE	78
FIGURE 4.4 THE SURVIVAL PERCENTAGE OF THE IPA MICE	79
FIGURE 4.5 MORTALITY IN VARIOUS GROUPS OF MICE.	80
FIGURE 4.6 DIRECT BINDING OF SOLID-PHASE CONIDIA TO RHSP-D.	81
FIGURE 4.7 PERCENTAGE KILLING OF <i>A. FUMIGATUS</i> BY PMNS.	82
FIGURE 4.8 BINDING OF RHSP-D TO 3WCF ALLERGENS/ANTIGENS OF <i>A. FUMIGATUS</i> IN THE PRESENCE OF CALCIUM, SUGARS OR EDTA.	83
FIGURE 4.9 RHSP-D INHIBITS IgE BINDING TO IMMOBILIZED 3WCF ANTIGENS/ALLERGENS	84
FIGURE 4.10 SUPPRESSION OF HISTAMINE RELEASE BY RHSP-D BY SENSITISED BASOPHILS	85

FIGURE 4.11 CYTOKINE LEVELS IN PG/ML OF IL-4, IL-5 AND IFN-GAMMA OF VARIOUS MICE (ABPA)	86
FIGURE 4.12 EXPRESSION AND PURIFICATION OF THE RHSP-D DELTA	88
FIGURE 4.13 SDS-PAGE 15 % (W/V) GEL UNDER REDUCING CONDITIONS IN THE PRESENCE OF BS3	89
FIGURE 4.14 REDUCTION IN GROWTH RATE OF <i>A.FUMIGATUS</i>	92
FIGURE 5.1 DC SUBSETS DERIVED FROM CD34+ MYELOID AND LYMPHOID PROGENITORS	100
FIGURE 5.2 THE MIGRATORY AND MATURATION PATHWAYS OF DCs.....	102
FIGURE 5.3 MATURATION AND DIFFERENTIATION PATHWAYS OF DCs	103
FIGURE 5.4 STIMULATORY AND REGULATORY DENDRITIC CELLS EFFECTS.	104
FIGURE 5.5 CO-STIMULATORY MOLECULES AND THEIR LIGANDS.....	105
FIGURE 5.6 EFFECT OF SP-A AND SP-D ON LPS INDUCE MATURATION OF DCs.....	108
FIGURE 5.7 EFFECT OF SP-A AND SP-D ON TNF-A INDUCE MATURATION OF DCs.....	109
FIGURE 5.8 EFFECT OF TNF-A: REGULATION OF LPS INDUCED TNF-A SECRETION BY SP-A AND SP-D.....	110
FIGURE 5.9 MODULATION OF IL-12 PRODUCTION BY MATURE DCs.....	111
FIGURE 5.10 MODULATION OF IL-10 PRODUCTION IN THE PRESENCE OF SP-A AND SP-D	112
FIGURE 5.11 PROLIFERATIVE ALLOGENIC T CELL RESPONSE BY DC MODULATED BY SP-A AND SP-D	113

List of tables

TABLE 4.1 GLY-X-Y RHSP-D SHOWED REDUCED GROWTH IN A DOSE DEPENDENT FASHION..... 93

TABLE 4.2 GLY-X-Y RHSP-D SHOWED REDUCED GROWTH IN A DOSE DEPENDENT FASHION..... 93

1. Chapter 1

Introduction

Our immune system plays a vital role in our healthy status. Immune system has two main arms, innate and adaptive immune systems. Innate immunity is the first line of defence against any microbial attack (Medzhitov and Janeway, 2002). The average person inhales about 10,000 litres of gas per day, and this gas is laden with a plenty of bacteria, viruses, oxidants, pollutants and allergens (Wright, 2004). The lung help maintain its sterility through several mechanisms. The innate immune response is very crucial in limiting the early replication and spread of pathogens, which may involve opsonins, such as immunoglobulin and complement; and phagocytic cells such as alveolar macrophages, and neutrophils (Wright, 2004). The innate immune system is designed towards recognition of pathogenic non-self.

2.1 1.1. Pattern recognition receptors (PRRs) interaction with Pathogen associated molecular patterns (PAMPs)

The innate immune system recognizes conserved motifs in pathogens called “pathogen-associated molecular patterns” (PAMP) (Barton and Medzhitov, 2002; Medzhitov and Janeway, 2002). Pathogens can be identified as non-self through PAMP by pathogen recognition receptors (PRRs), which can be humoral and cellular receptors (Beutler, 2004). NOD like receptors are intracellular while collectins, and pentraxins are humoral PRRs (Taylor, Gordon and Martinez-Pomares, 2005; Medzhitov and Janeway, 2002). The classical short pentraxins (CRP) and serum amyloid P (SAP) component circulate as pentamers in human plasma. The long pentraxins include PTX3, PTX4 and neuronal pentraxins (Bottazzi *et al*, 2010 ; Deban *et al*, 2011). Toll-like receptors (TLRs) are cell surface sensor PRRs whereas there are also phagocytic/endocytic receptors such as scavenger receptors and lectins (Mukhopadhyay, Pluddemann and Gordon, 2009). TLRs have an essential role in the innate recognition of PAMPs and in triggering acquired immunity (Akira, Takeda and Kaisho, 2001; Aderem and Ulevitch, 2000; Barton and Medzhitov, 2002; Medzhitov and Janeway, 2002). TLR4 recognizes lipopolysaccharide (LPS), whereas TLR2 recognizes various fungal, Gram-positive, and mycobacterial components (Flo *et al*, 2000; Takeuchi, Hoshino and Akira, 2000; Aliprantis *et al*, 1999; Brightbill *et al*, 1999; Hoshino *et al*, 1999; Lien *et al*, 1999; Means *et al*, 1999; Takeuchi *et al*, 1999; Underhill *et al*, 1999a; Underhill *et al*, 1999c).

2.2 1.2. Link between innate and adaptive immunity

The adaptive immune system is a highly complex system found in vertebrates (Takeda, Kaisho and Akira, 2003 ;). TLRs contain a characteristic leucine- rich repeat (LRR) domain in their extracellular domain and a TIR domain in their intracellular domain (Takeda, Kaisho and Akira, 2003). The translation of innate to adaptive immune response requires that antigens be captured and processed prior to the information of ligands for the T cell receptor (Steinman and Hemmi, 2006). The most important player in this story is dendritic cell (DC) as antigen presenting cells (APCs), since they can capture antigens in peripheral tissue and migrate to secondary lymphoid organs where they acquire the ability to stimulate naïve T cells (Cella *et al*, 1997; Banchereau and Steinman, 1998; Mellman *et al*, 2014). DCs load pathogen derived peptides onto MHC class I or class II molecules and present on the cell surface to their cognate T cells which are then induced to clonally proliferate and mount cytotoxic or helper T cell functions (Mellman and Steinman, 2001). DCs can also express ligands such as CD80 and CD86 that bind to costimulatory molecules on T cells (signal 2); aiding MHC-specific-T cell receptors (signal 1). In addition to, DCs produce cytokines such as IL-12, which are essential to stimulate T-cells (Trombetta and Mellman, 2005).

Signal 1 alone promotes naïve T-cell inactivation by anergy, deletion or co-option into a regulatory cell fate, thus to tolerance (Sousa Vde *et al*, 2006). Signal 2 together with signal 1 induces immune response (Keir and Sharpe, 2005) (Domowicz *et al*, 2003). Subsequent cytokine response can determine T cell differentiation into to helper T cells (Th1), or cytotoxic T cells (Th2) (Curtsinger, Lins and Mescher, 2003; Kalinski *et al*, 1999). IL -12, for instance, promotes Th1 cells or CTL development (Trinchieri, 2003).

Interferons are produced rapidly in response to infection. They play a key role in innate immunity and promote adaptive immune responses. IFN- γ , which is secreted by Th1 cells, can modulate both innate and adaptive immunity (Billiau and Matthys, 2009). IFN- γ activates macrophages, stimulates natural killer cells, B cells as well as endothelial cells, and once endothelial cells were activated by IFN- γ it promotes expression of adhesion molecules. Macrophages activated by IFN- γ produce pro-inflammatory cytokines like TNF- α and IL-6, oxygen radicals, and metalloproteinase

(MPPs) (Voloshyna *et al*, 2013) . On the other hand, IFN- α stimulates the expression of MHC molecules (Takaoka and Yanai, 2006; Brassard, Grace and Borden, 2002). IFN- α also enhances expression of IFN- γ in NK, CD4⁺ and CD8⁺ T cells, and upregulates TNF- α (Brassard, Grace and Borden, 2002), IL-18R (Sareneva, Julkunen and Matikainen, 2000; Eriksen *et al*, 2009; Rogge *et al*, 1997; Rogge *et al*, 1997; Dickensheets *et al*, 1999; Pene *et al*, 1988). Finally, IFN- α affects both up- and down regulation of IL-4R signalling in T cells (Eriksen *et al*, 2004; Hansen *et al*., 2011). LPS can induce the production of IL-27(Schnurr *et al*, 2005; Pflanz *et al*, 2002), which signals through activation of STAT1 and STAT3 (Guzzo *et al*, 2010; Pflanz *et al*, 2004; Pflanz *et al*, 2002). In addition, IL-27 functions to promote differentiation of naïve Th cells into Th1 cells (Lucas *et al*, 2003; Pflanz *et al*, 2002). IL-27 also activates monocytic cells, which includes upregulation of pro-inflammatory cytokine and chemokine production (Guzzo *et al*, 2010; Kalliolias and Ivashkiv, 2008).

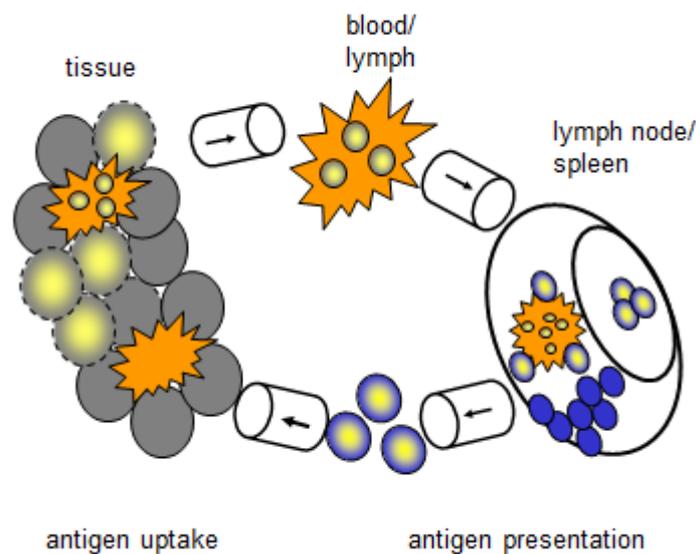


Figure 1.1. Antigen capture and presentation by dendritic cells (DCs). DCs are the most potent antigen capturing and presenting immune cells at the bridging sentinel of the innate and adaptive immunity. Following the interaction of immature DCs with pathogens at the site of infection or foreign non-self, the ingested material is brought to lymph draining by immature DCs where they mature and present the antigenic peptides to the T cells via MHC Class II molecules, triggering the effector adaptive immune response.

2.3 1.3. Complement system

The complement system is made up of a large number of plasma proteins as well as membrane-bound receptors and regulators, which have the ability to react with one another to attack the pathogens and induce a series of inflammatory responses (Shastri, Bonifati and Kishore, 2013). The three pathways of the complement system converge on the generation of C3 convertase, and all ending up with the generation of the same set of effector molecules, membrane attack complex (MAC) (figure 1.2) (Shastri, Bonifati and Kishore, 2013). The three initiating pathways are called (i) classical pathway which is mostly antibody mediated (C1q being the first subcomponent) and is activated by C1 complex (C1q-C1r-C1s). The classical pathway is activated by immune complexes containing IgG and IgM, which bind to microbial surfaces (Kishore *et al*, 1998); (ii) Alternative pathway (AP) which is activated spontaneously involving low-level hydrolysis of C3 to C3 (H₂O). The alternative pathway is dependent upon a spontaneous C3 binding and activation into C3b through thioester activation on charged surface. Properdin stabilizes C3 convertase, thus acting an up-regulator of alternative pathway; (iii) Lectin pathway has its recognition molecule called mannan-binding lectin (MBL) whose binding to carbohydrate PAMPs is followed by binding of MBL-associated serine protease 2 (MASP-2) to MBL (Jensenius *et al*, 2003) Thiel and Gadjeva, 2009). Complement components are mostly synthesized by liver. However, there is plenty of evidence in literature suggestive of local synthesis of complement protein by immune cells at the side of infection, injury, inflammation, and homeostasis (Nayak *et al*, 2010; Nayak *et al*, 2012).

Complement cascade can be activated by any of these three pathways, leading to the following: (1) Activation of C3b, which binds to microbes and opsonizes them for phagocytosis. (2) Generation of C3a and C5a, which are potent anaphylatoxins, capable of cellular infiltration at the site of infection. 3) Activation of C5 through C9, the latter component of which forms membrane attack complex (MAC) that can lyse Gram-negative bacteria, and also inactivate viruses (Kouser *et al*, 2013).

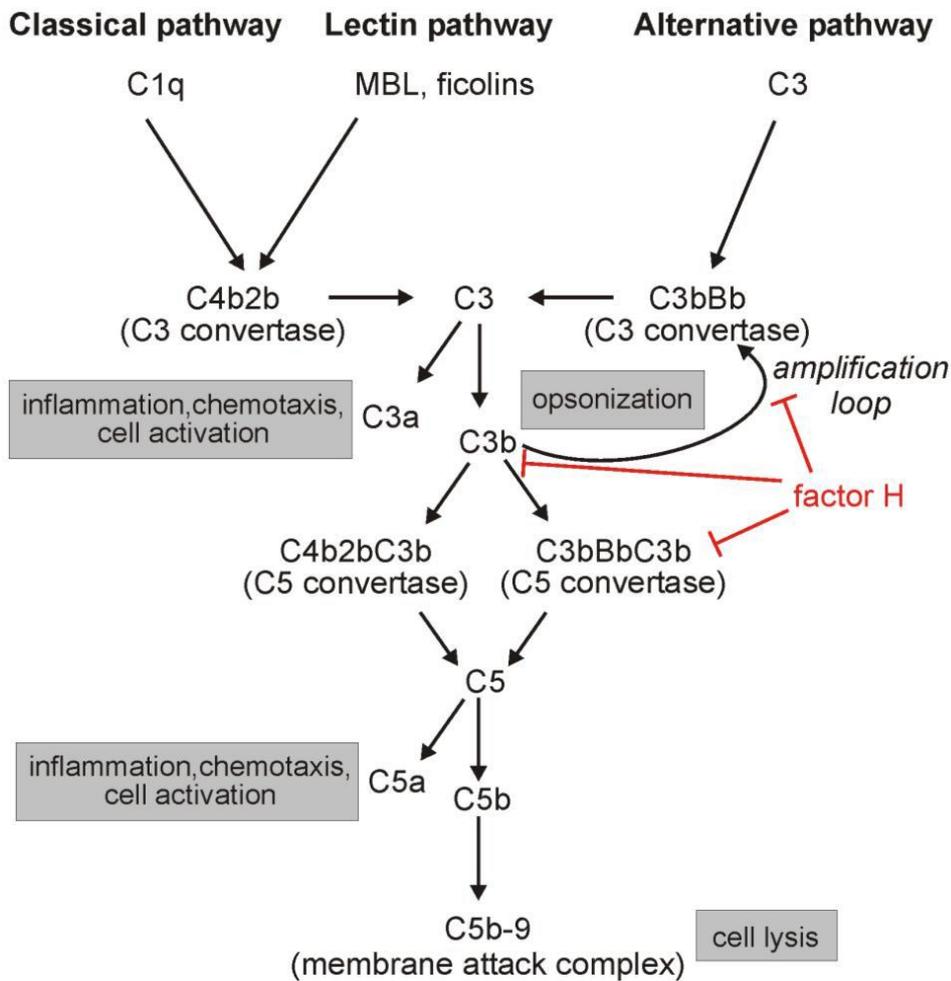


Figure 1.2 Complement pathways. C1q initiates the complement **classical pathway**. In the **lectin pathway**, the recognition proteins include mannan-binding lectin (MBL) that mainly binds to vicinal diols on sugars such as mannose, fucose or glucosamine. After C1q is bound to its targets, proteases C1r and C1s are activated (or for MBL, MBL-associated serine proteases, MASP-1, 2 and 3, are activated). This then activates complement proteins C4 and C2 forming a C3 convertase (C4b2a), which cleaves C3 to form C3b that then binds to the target surface. C3b and its breakdown products, iC3b and C3dg, interact with C3 receptors on phagocytic and other cells. C3b is also a binding site for C5, which is activated by the same protease which cleaves C3, and then forms a complex with C6, C7, C8 and C9 (C5-9), called membrane attack complex (MAC), which disrupts the lipid bilayer of cells. The activation of the complement **alternative pathway** involves a constant slow hydrolysis of C3 in solution, which forms C3(H₂O), and alters the shape of the protein. This conformational change allows the formation of a complex between factor B and C3(H₂O), which allows factor D to cleave the bound factor B into Ba, which is removed and Bb, which remains bound. C3(H₂O)Bb is a protease which cleaves more C3 to form C3b, which can bind to target surfaces, and form a complex with factor B, which is converted to C3bBb, by factor D, as above. This leads to coating of the target particle with C3bBb, which is a homologue of the classical pathway C3 convertase, C4b2a. C3bBb, the alternative pathway C3 convertase, can be further stabilized by properdin (factor P) to C3bBbP. This complex is an enzyme able to generate more C3b to bind to targets. This is an amplification mechanism in order to increase turnover of C3 and coating of targets with C3b. To avoid consuming all available C3, the amplification mechanism needs to be balanced by down regulators: Factor H binds to C3b inhibiting C3 convertase formation, and together with factor I it cleaves C3b to iC3b, which is unable to form C3bBb. (Adapted from Kouser *et al*, 2013)

2.4 1.4. Collectins

Two groups of the C-type lectin family are important components of the innate immune response. The first is collectins (Collagen containing lectins) which are soluble proteins and mediate pathogen neutralization (Crouch and Wright, 2001). The second is the macrophage mannose receptor, which is a cell-surface protein that leads directly to phagocytosis of microorganisms (Weis, Taylor and Drickamer, 1998) and secretion of pro-inflammatory cytokines and chemokines (Ozinsky *et al*, 2000).

Nine types of collectins have been reported so far: MBL, lung surfactant protein A (SP-A) and D (SP-D), bovine conglutinin, collectin-43 (CL-43), and collectin- 46 (CL-46) (Epstein *et al.*, 1996; Holmskov *et al.*, 1994; Hoppe and Reid, 1994). Another set of collectins have also been found, collectin liver -1 (CL- L1), and collectin placenta 1 (CL- P1). CL-43, CL-46 and conglutinin are found in bovine.

The subunit structure of collectins includes an N-terminus linking region, a triple-helical collagen domain, a coiled-coil neck region, and a C-type lectin domain, also called a carbohydrate recognition domain (CRD). Recognition of specific ligands of microorganism is mediated by CRD in presence of calcium (Weis, Drickamer and Hendrickson, 1992; Weis *et al*, 1991) (Lee *et al*, 1991). This interaction often results in enhanced phagocytosis and clearance of the pathogen *in vitro* (Vaandrager and van Golde, 2000).

Pulmonary surfactant is comprised of phospholipids and proteins, including four surfactant proteins (SP): SP-A, SP-B, SP-C, and SP-D (Possmayer, 1988). The most abundant of the surfactant- associated protein is SP-A, while SP-D is present at about tenth the concentration of SP-A (Wright, 1997). SP-A and SP-D, interact with carbohydrate structures on the surface of a pathogen such as virus, bacteria, or fungi through its CRD and then enhancing phagocytosis and killing by myeloid cells (Reid, 1998; Wright, 1997). Surfactant proteins are synthesized and secreted into lung airspaces by type II pneumocytes and Clara cells (Haller *et al.*, 1992; Voorhout *et al*, 1992). SP-A and SP-D comprise of higher oligomers: hexameric (boque like structure) or tetrameric (cruci-form like structure, with four arms of equal length) molecules, having 18 or 12 CRDs, as found in SP-A and SP-D, respectively (Lu, Willis and Reid, 1992; Hoppe and Reid, 1994).

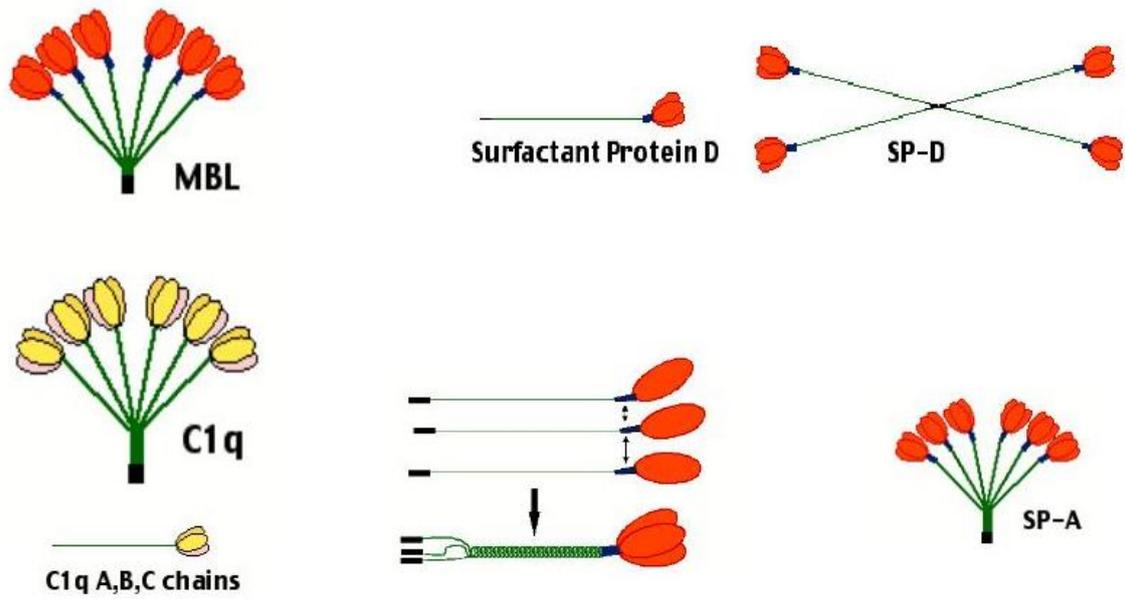


Figure 1.3 The structures of collectins. The primary structure of collectins includes an N-terminal cysteine rich region, a triple-helical collagen region, a coiled-coil neck region, and a C-terminal C-type lectin domain or carbohydrate recognition domain (CRD) which is homotrimeric in organisation. Six of these primary units combine to give the SP-A its classical bouquet shaped 18-mer structures, similar to complement proteins C1q and MBL. Three polypeptide chains combine to form a trimeric structure that combines with another three similar structures to give SP-D its characteristic cruciform structure. (Matsushita *et al.* 1996; Ohashi *et al.* 1997).

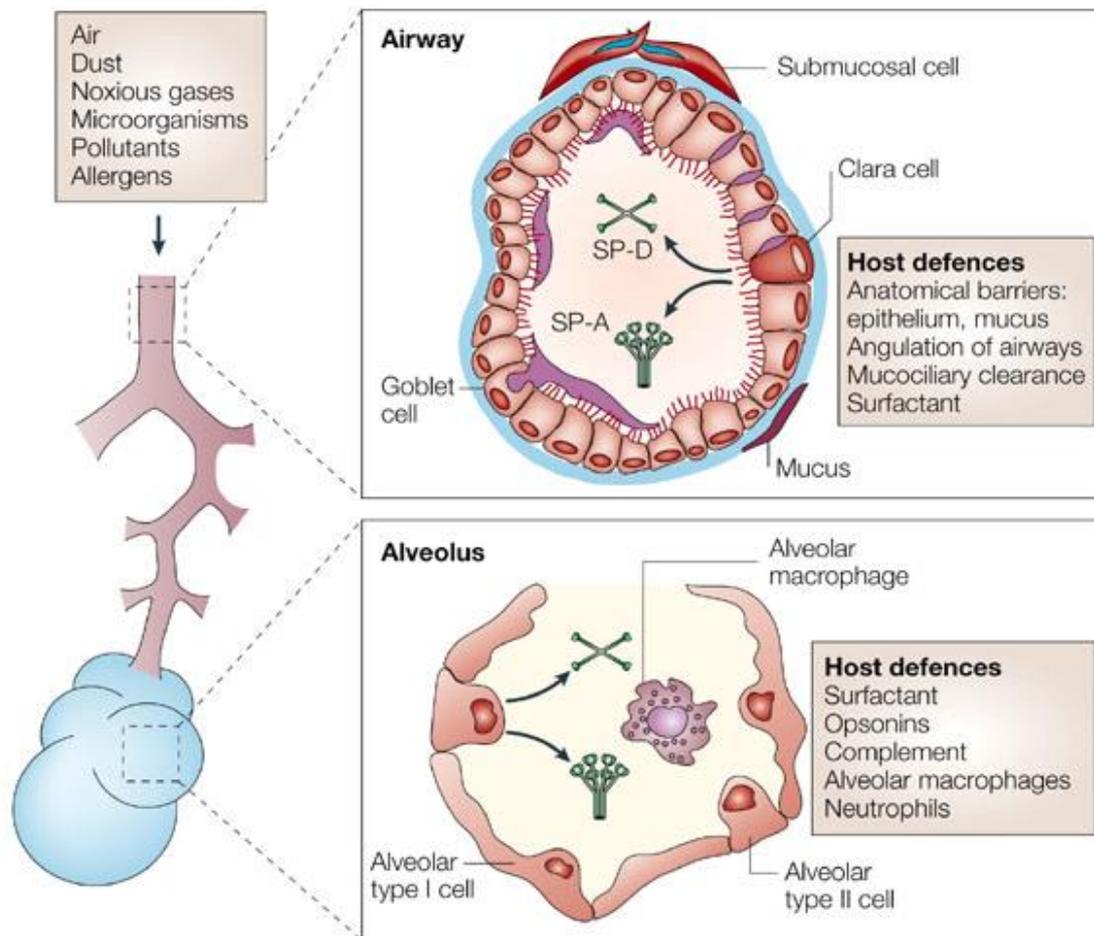


Figure 1.4 Innate immune mechanisms in the lungs. Lung is constantly exposed to a range of toxic materials that include pathogens and allergens, capable of inducing pro-inflammatory immune response. SP-A and SP-D, which are secreted by type II epithelial and Clara cells in the lungs, appear to have a protective and homeostatic function. They can be potent opsonins for pathogens and recruit alveolar macrophages for their enhanced clearance, a function similar to complement proteins following complement activation and deposition on the microbial surface (Wright. 2005).

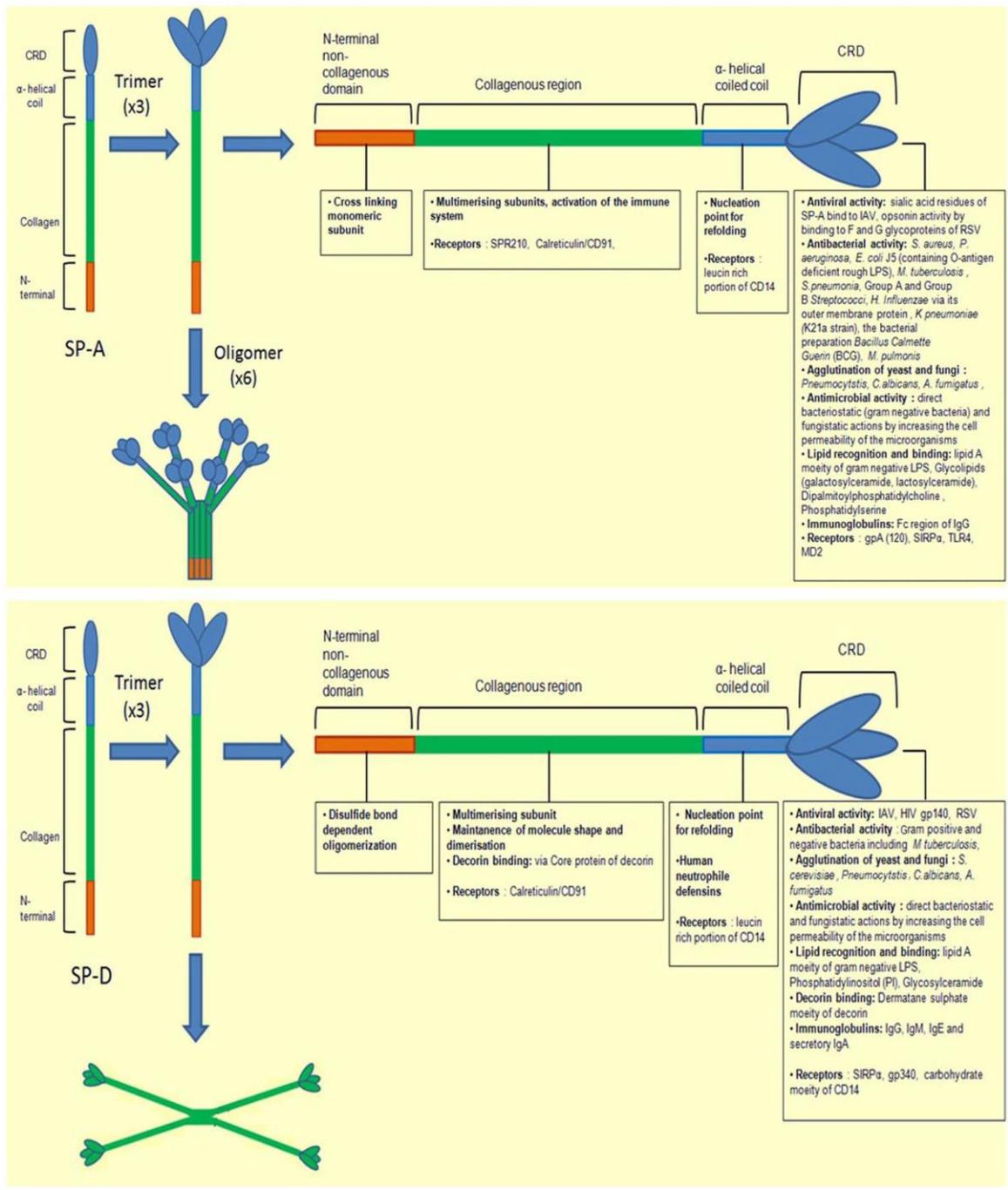


Figure 1.5 Functions assigned to the different parts of the SP-A and SP-D molecules. Different regions of SP-A and SP-D have well-defined functions. The N-terminal cysteine rich region is responsible for cross-linking monomers leading to higher order oligomers. The triple-helical collagen region is the trimerising area that stabilizes the overall structure of collectins. The alpha-helical coiled-coil neck region is considered the nucleation center for the trimerization of the single subunit. The homotrimeric CRD region is the pattern recognition or ligand-binding region of the collectins. Most of the ligands are recognized via the CRD region with few exceptions. The collagen region is also responsible for the protein interaction with their cognate receptor complex of calreticulin-CD91 complex. There are debates about the receptors that bind to the CRD region. (Nayak et al 2012)

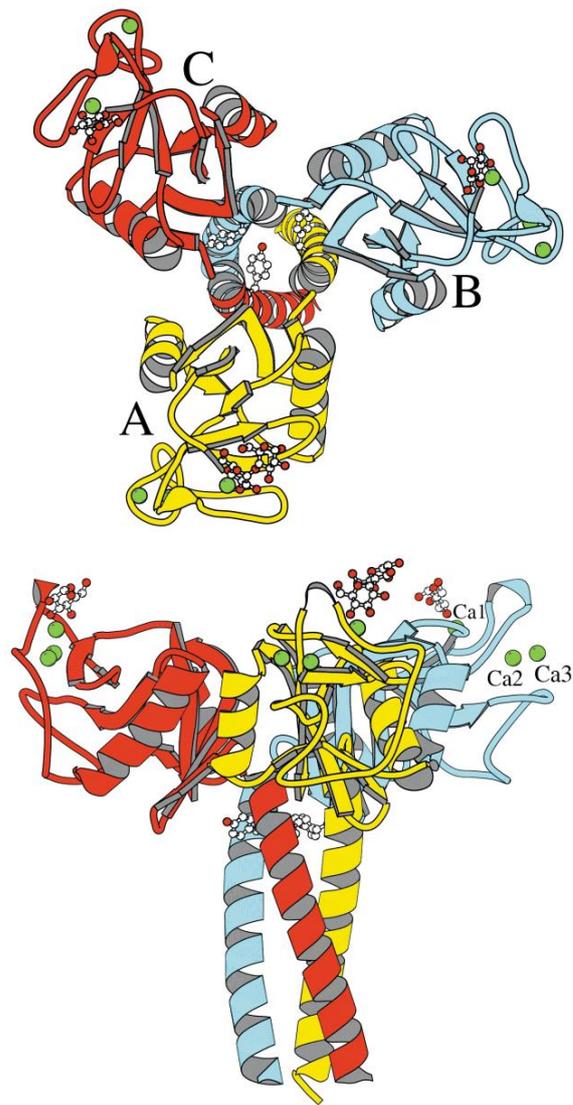


Figure 1.6 X-ray crystal structure of rhSP-D showing three subunits representing CRD domains. The three-dimensional structure of trimeric recombinant neck–CRD fragments of human SP-D (rhSP-D) when viewed perpendicular to the molecular three-fold axis. The three subunits have been designated A, B and C. The maltose-bound rhSP-D trimer shows the bound maltose and the three calcium ions. The tightly packed neck region appears to stabilise the CRD region, consistent with the evidence that when the CRD region is expressed heterologously without neck region, the recombinant protein falls off as a monomer, highlighting the key role played by the neck region (Kishore et al. 2006; Shrive et al, 2009).

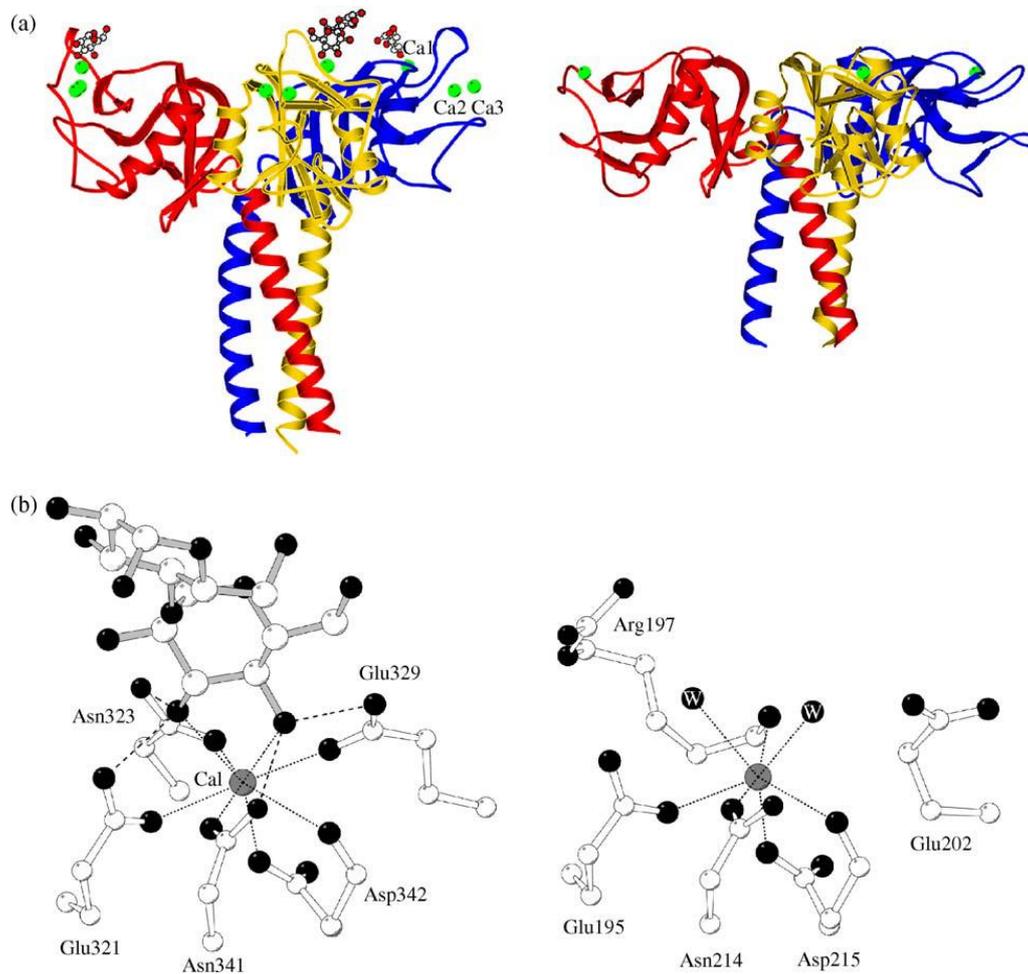


Figure 1.7 Crystal structures of trimeric rhSP-D and rhSP-A showing bound calcium and maltose (Shrive. 2009; Kishore. 2006). Three-dimensional structures of trimeric recombinant neck-CRD fragments of human SP-D (left) and rat SP-A (right). (a) Neck-CRD trimeric fragment viewed perpendicular to the molecular three-fold axis. The maltose-bound rhSP-D trimer shows the bound maltose and the three calcium ions. (b) The coordination of the calcium ion Ca1 and bound maltose in rhSP-D and of Ca1 in rrSP-A.

2.5 1.5. The Immunological importance of SP-A and SP-D

SP-A and SP-D are soluble PRRs which can interact with glycoconjugates and lipids on the microbial surfaces via their CRDs (van Iwaarden *et al*, 1992; Weis, Taylor and Drickamer, 1998). These collectins bind mannose and glucose residues more strongly (Kishore and Reid, 2001 ; Holmskov, Thiel and Jensenius, 2003). SP-A and SP-D interact with a wide range of pathogenic viruses, bacteria and fungi and cause agglutination, enhancement of phagocytosis and killing (Wright, 2005). In addition, SP-A and SP-D can also alter cytokine and chemokine levels at the site of infection.

2.6 1.6. Interaction and clearance of viral, bacterial and fungal pathogens

The interaction of SP-A and SP-D with different strains of Influenza A virus (IAV) involves binding hemagglutinin (HA), neuraminidase (NA), and their levels of glycosylation. The presence of sialic acid residues on the CRD surface of SP-A also helps in targeting IAV (Benne *et al*, 1995). This interaction causes agglutination, and infection inhibition, followed by alveolar macrophage and neutrophil mediated elimination of virus. Co-incubation of IAV with SP-A enhances production of H₂O₂ by neutrophils (Hartshorn *et al*, 1997). SP-D also induces IAV aggregation, inhibits HA and neuraminidase activities, and neutralizes IAV (Hartshorn *et al*, 1996 ; Hawgood *et al.*, 2004; Reading *et al.*, 1997). IAV, pre-incubated with SP-D, also enhances superoxidative burst. SP-A binds respiratory syncytial virus (RSV), and enhances phagocytosis by PBMCs and alveolar macrophage (Ghildyal *et al*, 1999), and leads to TNF- α production (Barr *et al*, 2000; Hickling *et al*, 1999) .

SP-A and SP-D lectin domains bind a variety of Gram-positive and -negative bacteria (Hartshorn *et al.*, 1994; Van Iwaarden *et al.*, 1994; Hartshorn *et al*, 1998), enhancing phagocytosis and killing. SP-A and SP-D bind to *Staphylococcus aureus* (Geertsma *et al*, 1994), *Pseudomonas aeruginosa*, and *Escherichia coli* J5 (containing O-antigen deficient rough LPS) (Van Iwaarden *et al*, 1994), *Mycobacterium tuberculosis* (Pasula *et al*, 1997; Gaynor *et al*, 1995), *Streptococcus pneumoniae*, *Hemophilus influenzae* (McNeely and Coonrod, 1994), *Klebsiella pneumoniae* (Kabha *et al*, 1997), and *Bacillus Calmette Guerin* (BCG) (Weikert *et al*, 1997), causing agglutination and phagocytosis by macrophages (Pikaar *et al*, 1995 ;Sahly *et al*, 2002; Van Iwaarden *et al*, 1994), *Bacillus subtilis*, and *S. aureus* (van de

Wetering et al., 2001), purified LPS from *P. aeruginosa* and *E. coli* (Kishore et al, 1996), *Mycobacterium tuberculosis* (Ferguson et al, 1999; Pasula et al, 1997; Gaynor et al, 1995), and *Mycoplasma pulmonis* (Hickman-Davis et al., 2001).

SP-D interacts with *Saccharomyces cerevisiae* via its CRD, (Allen, Voelker and Mason, 2001) and to the β -glucans of *Pneumocystis*. SP-D binds to *Candida albicans* and inhibits pseudohyphal/hyphal growth (van Rozendaal et al, 2000). SP-A suppresses pro-inflammatory TNF- α , IL-1 β , MIP- α , and MCP-1 production by alveolar macrophage (Rosseau et al, 1999; Rosseau et al, 1997). SP-A and SP-D cluster *Pneumocystis carinii* via gpA (glycoprotein 120) which is present on expressed cysts and trophozoites (Zimmerman et al, 1992). They agglutinate the pathogenic *Cryptococcus neoformans* (Schelenz et al, 1995) as well as *Aspergillus fumigatus* conidia; this enhances their phagocytosis and killing by human alveolar macrophages and circulating neutrophils (Madan et al, 1997a). Intranasal administration of SP-D and rhSP-D in an immunosuppressed murine model of invasive pulmonary aspergillosis (IPA) rescued about 80% of the mice, while SP-A did not have a significant effect on IPA mice survival (Madan et al, 2001). The protective effects were consistent with reduced colony forming units, lack of hyphal growth, and elevated levels of the protective TNF- α and IFN- γ (Singh et al, 2009).

SP-A and SP-D can also have a direct antimicrobial effect on the growth and viability of bacteria (Wu et al, 2003) including *E. coli*, *K. pneumoniae*, and *Enterobacter aerogenes*, and *Histoplasma capsulatum* (McCormack et al, 2003) by making bacterial membranes more permeable. SP-A can attenuate the growth of *M. pneumoniae* via its CRD region (Piboonpocanun et al, 2005).

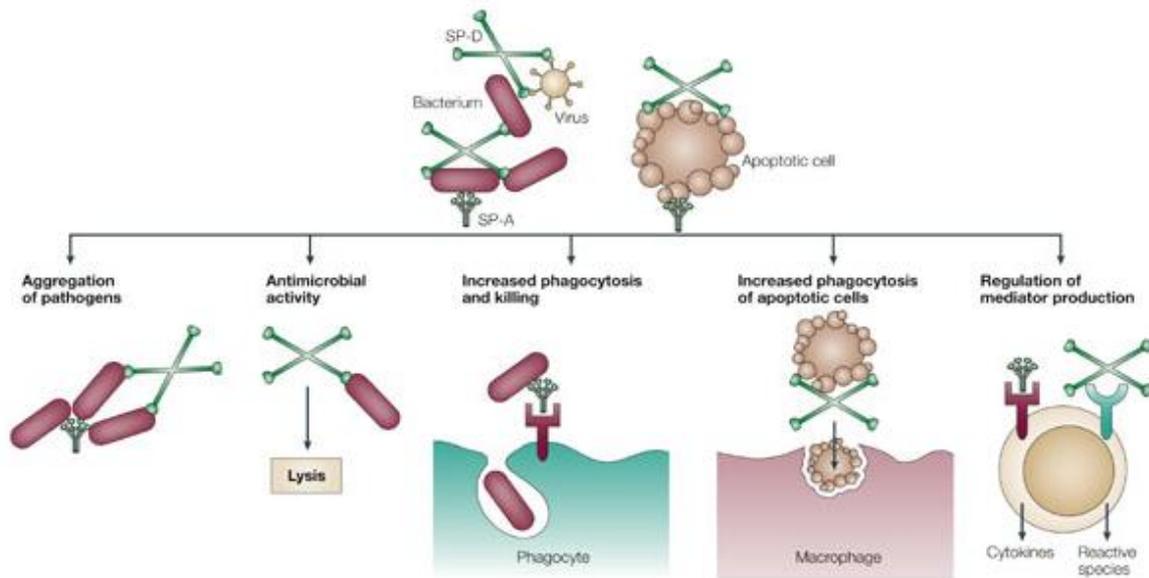


Figure 1.8. Multi-functional SP-A and SP-D. The two hydrophilic pulmonary surfactant proteins bind a wide range of pathogens and act as high affinity opsonins for bacteria, viruses, and apoptotic cells. Their defensive mechanisms include agglutination or aggregation of pathogens, direct growth inhibitory effects, enhancement of phagocytosis, and modulation of cytokine/chemokine response by immune cells. Thus, a robust anti-inflammatory milieu is brought about at the site of infection or injury (Wright, 2005).

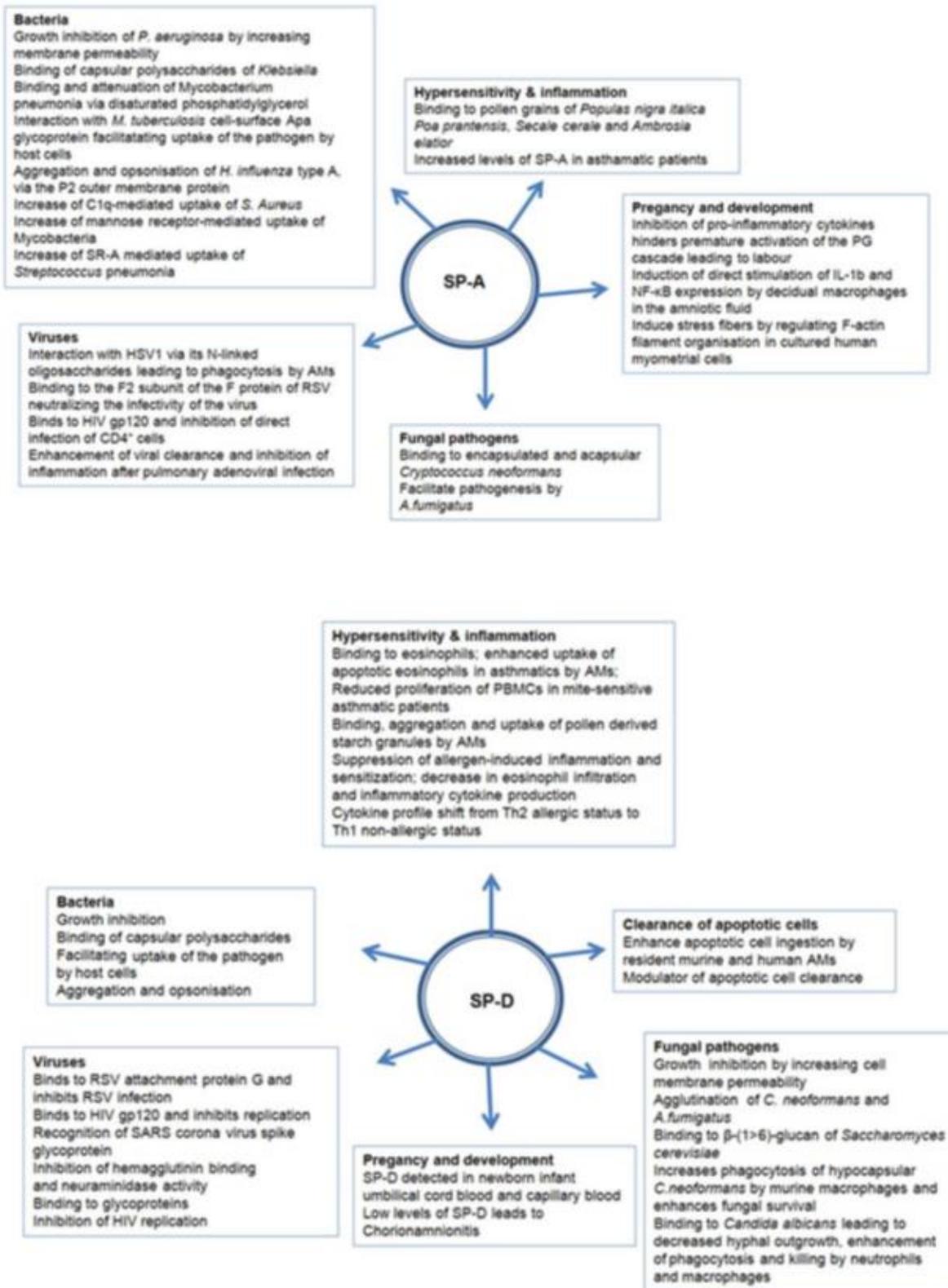


Figure 1.9 Homeostatic functions of SP-A and SP-D in human health and disease. In addition to their defense mechanisms in the lungs, SP-A and SP-D also have extra-pulmonary existence. Their immunomodulatory effects are more evident in pregnancy by virtue of their presence in the amniotic fluid as well as amnion, chorion and decidua (Nayak et al., 2012). Their interaction with glycoprotein allergens that eventually leads to modulation of type I and type III hypersensitivity reactions is another facet of the surfactant proteins SP-A and SP-D.

2.7 1.7. Role of SP-A and SP-D in allergy

SP-A binds to pollen grains derived from Lombardy Poplar, Kentucky blue grass, cultivated rye and short ragweed, and links them with A549 alveolar type II cells (Malhotra *et al*, 1993). SP-A, SP-D and rhSP-D also bind house dust mite extract (Derp) and 3-week culture filtrate (3wcf) of *A. fumigatus*, inhibit specific IgE binding to allergens, and block allergen-induced histamine released from basophils isolated from allergic patients (Wang *et al*, 1998; Wang *et al*, 1996; Madan *et al*, 1997b). SP-D can bind starch granules of grass pollen allergens and enhance phagocytosis by alveolar macrophages (Erpenbeck *et al*, 2004). SP-A and SP-D also reduce the proliferation of PBMC isolated from allergic asthmatic children (Wang *et al.*, 1998), and suppress IL-2 production by PBMC (Borron *et al*, 1998) and IL-8 by activated eosinophils (Cheng *et al*, 1998).

SP-A, SP-D or rhSP-D treatment in a murine model of allergic bronchopulmonary aspergillosis (ABPA) induced by *A. fumigatus* 3wcf alleviates a range of allergic immune responses (Madan *et al*, 2001), as evident by lowered eosinophilia and IgG and IgE antibody levels. This therapeutic effect lasted up to 4 days in the SP-A treated ABPA mice, and up to 16 days in the SP-D or rhSP-D treated ABPA mice. The levels of IL-2, IL-4 and IL-5 were decreased, while that of IFN- γ was raised in supernatants of the cultured spleen cells, indicating a marked pathogenic Th2 to protective Th1 polarization of helper T-cell immune response. Similar protective effects of rhSP-D have been observed in a murine model of lung allergy induced by Derp (Derp mice) (Singh *et al*, 2003 ; Liu, Zhu and Li, 2005; Strong *et al*, 2003; Strong, Reid and Clark, 2002).

SP-A and SP-D appear to offer protection against allergenic challenge at various levels, such as allergen masking, inhibition of allergen-IgE interaction and histamine release, suppression of the activation of sensitized basophils, mast cells or eosinophils, B and T-cell proliferation, modulation of DCs and macrophages, and Th cell polarization (figure 1.11)

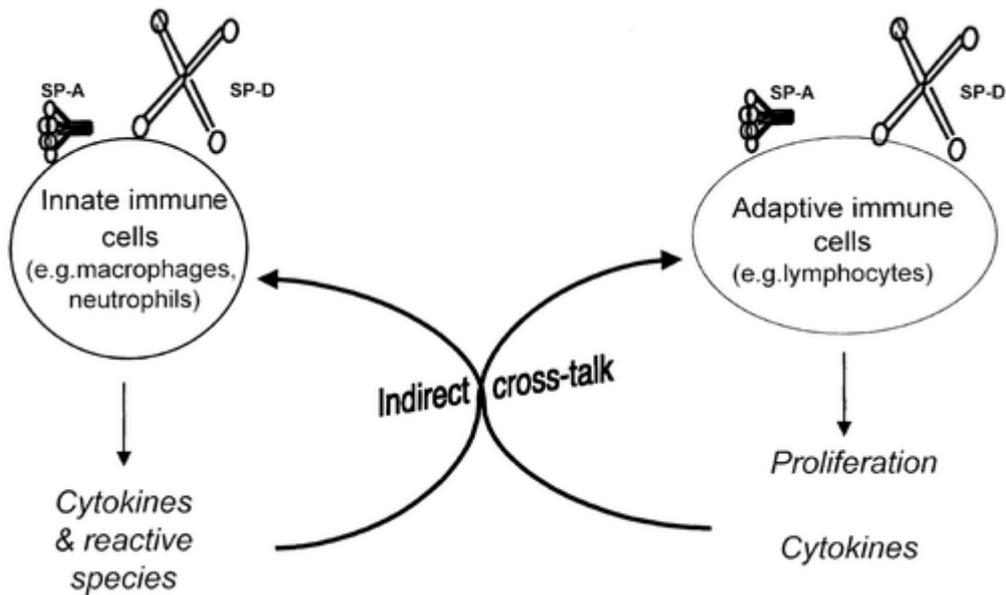


Figure 1.10. SP-A and SP-D can link innate and adaptive immune systems. Due to their ability to modulate cytokine and chemokine production by innate immune cells such as macrophages and dendritic cells, SP-A and SP-D can alter T helper polarization during infection and allergy. In addition, their anti-proliferative effects on T and B cells, coupled with induction of apoptosis in activated T cells can alter the long-term potentiation of adaptive immune response. Thus, SP-D specially is adapted to a general immunosurveillance as it can eliminate dying cells via anti-inflammatory route. The two receptors involved with the two collectins at SIRP-alpha that specifically interacts with CRD domain leading to an anti-inflammatory state. The collagen region, on the other hand, interacts with calreticulin-CD91 complex bringing about a pro-inflammatory response leading to clearance of invading non-self. (Kishore *et al*, 2006)

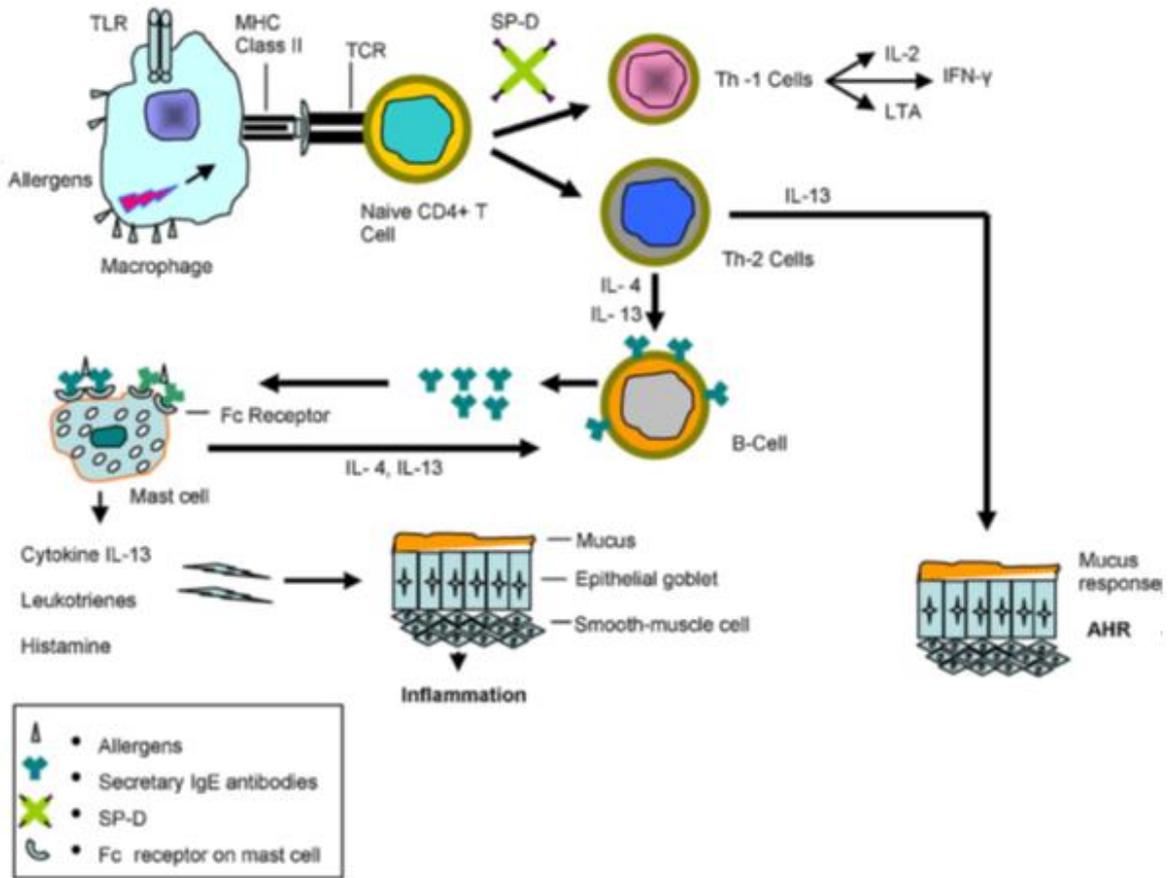


Figure 1.11 Sensitisation and priming immune mechanisms in asthma. SP-D, via its CRD region, can bind allergens and inhibit their interaction with allergen-specific IgE, which in return, can suppress histamine release from sensitized basophils and mast cells. In addition, SP-D can cause T cell polarization from a pathogenic Th2 to a protective Th1 response, thus suppressing IL-4, IL-5 and IL-13 levels and enhancing interferon- γ levels. This appears to have a beneficial effect in the murine models of allergy as reflected in reduced mucus production and lung compliance (Qaseem *et al.* 2004).

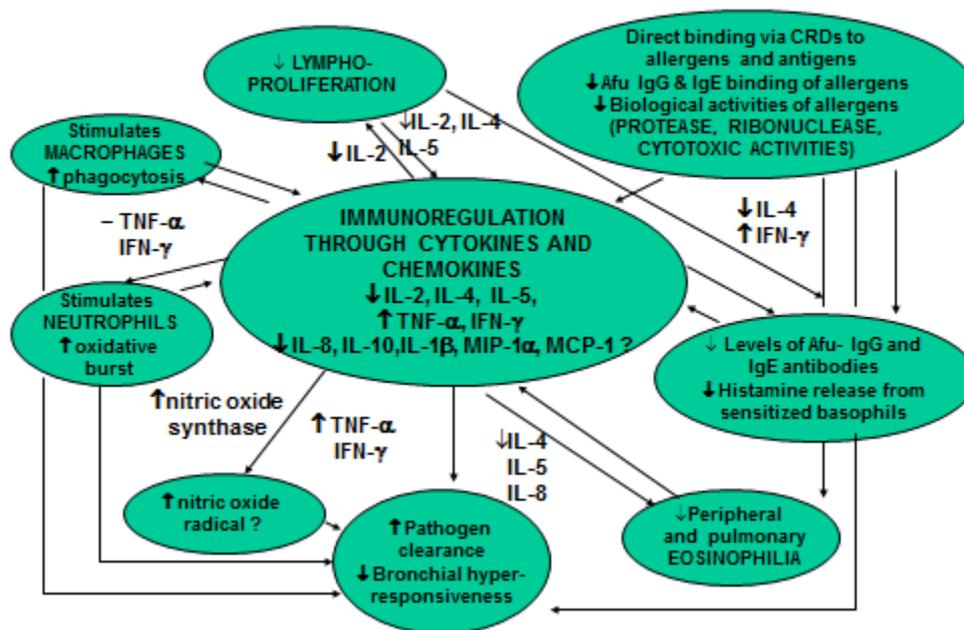


Figure 1.12 Protective mechanisms offered by SP-A and SP-D against lung allergy and infection caused by *Aspergillus fumigatus*. SP-A and SP-D can have protective effects against *A. fumigatus* caused allergy (ABPA) and well as infection. In the immunocompromised hosts, they can reduce the fungal burden via anti-fungal clearance mechanisms that also include raising the levels of TNF- α and IFN- γ . In the immunocompetent hosts, *A. fumigatus* via its secreted allergens and antigens can induce classical type I and type III hypersensitivity reactions. Therapeutic treatment can have protective effects via bringing down IgE and IgG levels and Th2 cytokine response. (Kishore et al, 2002)

The two pathogens that were selected for study in this thesis were Influenza A Virus and *Aspergillus fumigatus*, both well-known respiratory pathogens characterised for their interactions with SP-D. Their life-cycle and pathogenic mechanisms are being described below.

2.8 1.8. Host-pathogen interaction involving IAV

Influenza A virus (IAV) is an orthomyxovirus which causes acute illness of upper and lower respiratory tract in humans. It occurs seasonally 0.25 to 0.5 million mortality worldwide and 5 million cases of severe morbidity per year. IAV infects a variety of avian and mammalian hosts, such as people, birds, pigs, horses, seals, whales, and other animals, although the natural hosts of these viruses are wild birds. Subtypes of IAV are H1N1 and H3N2, are currently in circulation seasonally amongst people. Two membrane-bound surface proteins, hemagglutinin (HA) and neuraminidase (NA) are expressed by IAV on its envelope, which also characterize the IAV nomenclature (Zheng and Tao, 2013). Glycans of HA may be vital for antigenic drift,

thus allowing the IAV to circulate in the human population. Overtime, evolutionary studies suggested that the number of glycosylation increased after the emergence of IAV in humans (Gallagher *et al.*, 1992), suggesting that increasing the number of glycan's may prevent the binding of neutralizing antibodies to antigenic epitopes (Tate *et al.*, 2014).

HA binds to molecules that have sialic acid receptors, thus allowing the virus to infect many cell types due to the sialic acid receptor found ubiquitously. NA is a second surface viral protein, which cleaves sialic acid, allowing the release of virus. However, not all sialic acid-containing molecules bind to IAV with the same efficiency, suggesting that the viral strains have diverse receptor specificity. HA strains of avian influenza virus favour binding to sialic acids linked by α 2-3 linkage, whereas the human influenza viral strains have preferential binding to α 2-6-linked sialic acids. The replication of human IAV occurs in the upper respiratory tract, which has abundance of α 2-6-linked sialic acids in humans (Baum and Paulson, 1990).

1.8.1 Structure and life cycle of influenza virus

The IAV genome contains 8 segments of single-stranded (Palese *et al.*, 1980) negative-sense RNA that is encapsulated as individual rod-shaped ribonucleoprotein complexes (RNPs), giving rise to enveloped icosahedral viruses (Palese *et al.*, 1980). The 8 IAV genes encode for 12 major viral proteins: HA, NA, matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NS2) or nuclear export protein (NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase basic protein 1-F2 (PB1-F2). RNPs have a vital role in the viral RNA replication and transcription, intracellular transport of the viral RNA and gene packaging into progeny particles. A viral RNA, a viral polymerase and many copies of the viral nucleoprotein can be found in each RNP (Zheng and Tao, 2013).

The IAV life cycle begins with entry into the host cell, entry of viral RNPs into the nucleus, RNA replication and transcription, viral RNPs directed from the nucleus, and budding. At the entry stage of the virus, the HA spikes on the viral lipid membrane, which bind to sialic acid of the host cell membrane, and the receptor-mediated endocytosis occurs, allowing the virus to enter the endosome of the host cell. Low pH (5 to 6) of late endosome allows the cleavage of conformation of HA,

followed by the release of hydrophobic peptide that injects into host membrane, thus leads to the fusion of viral and endosome membranes, and opens up the M2 ion channel, acidifying the viral core (Fontana *et al.*, 2012). The acid nature of the viral core releases the RNP from M1, thus allowing the RNP to enter the cytoplasm of the host cell (Figure 5.1). After being released into the host cell, the RNP enters the nucleus, where viral transcription and replication occurs (Shi *et al.*, 2014). The proteins of the RNP (NP, PA, PB1, and PB2) have nuclear localization signals, which can bind to the cellular nuclear import machinery and enter the nucleus. The negative sense strands of RNA need to be converted into positive sense RNA for transcription to occur, by serving as a template for the viral RNA production. Viral RNA dependent RNA polymerase (RdRp) triggers the synthesis of viral RNA. RdRp is formed of three viral proteins: PA, PB1, and PB2 (has endonuclease activity), which binds to the 5' methylated caps of the cellular mRNA cleaved 10 to 15 nucleotides 3' to the cap structure, thus viral transcription takes place (Nayak, Hui and Barman, 2004).

Viral segment 7 encodes M1 and M2, and segment 8 encodes NS1 and NEP. M2 and NEP are spliced products and are in lower amount than NS1 and M1 (Fujii *et al.*, 2003). Influenza hinders the splicing machinery of the host cell for the process of host cell mRNAs, and so uses the host cell splicing machinery to produce viral proteins (Palese *et al.*, 1980). Influenza is able to inhibit the splicing of cellular mRNAs, by the binding of NS1 to small nuclear RNAs (snRNAs), and so re-localising to the nucleus of infected cells (Figure 1.13). Once the viral RNPs leave the nucleus, the enveloped influenza virus uses the host cells plasma membrane forming viral particles to leave and infect other cells, budding of viral particles occurs on the apical side of polarized cells. NA removes the sialic acid residues from glycoproteins and glycolipids, to allow the viral particles to leave from the plasma membrane (Samji, 2009).

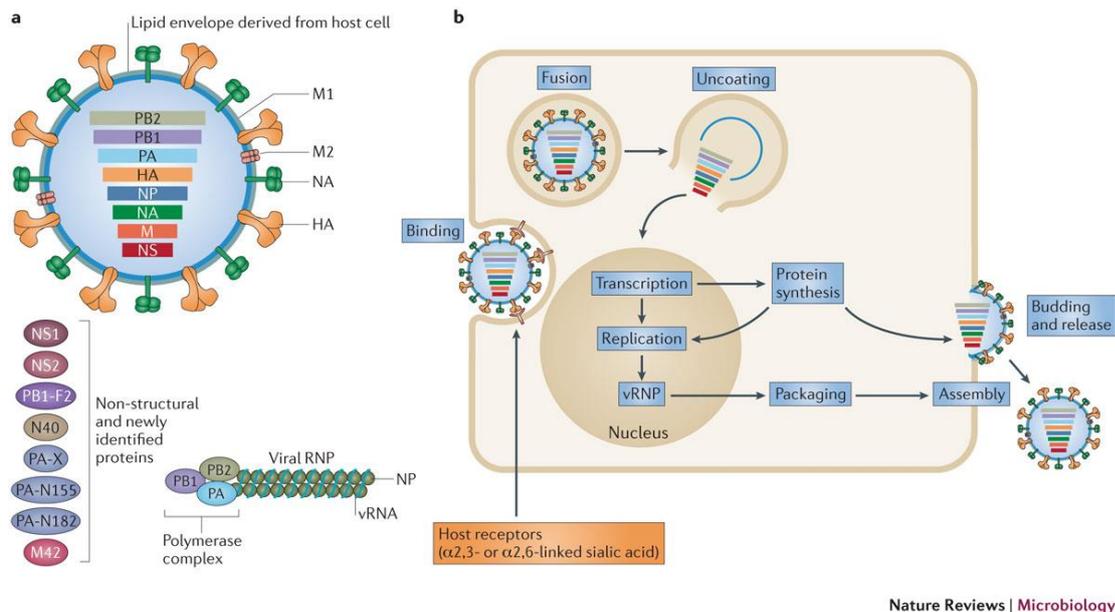


Figure 3.13: Structure and life cycle of influenza A virus: (a) Influenza A virus is a single stranded, encapsulated negative sense RNA, enveloped icosahedral virus. It contains 8 gene segments, which may encode for 16 proteins as follows: nuclear export protein (NS2), host antiviral response antagonist (NS1), matrix protein (M1), ion channel protein (M2), and the M2-related protein M42 (which can functionally replace M2), hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), components of the RNA-dependent RNA polymerase complex (PB1, PB2 and PA), two recently recognised proteins N40 (unknown function) and PA-X, represses the cellular gene expression. Additional two forms of PA (which have amino-terminal truncations) have been identified, which may have vital functions in the replication cycle. Furthermore, PB1-F2 is a pro-apoptotic viral protein, encoded by second ORF in the PB1 segment. (b) Infection begins when the virus binds to the sialic acid of the host cell-surface receptors, and enters by endocytosis. The segmented viral genome is released into the cytoplasm, translocated to the nucleus causing transcription and replication. Within the nucleus the viral proteins are composed into viral ribonucleoproteins (RNPs). M1 and NS2 mediate the export of RNPs. The virus particles are formed at cell membrane and the newly formed viruses bud and infect other cells (Taken from (Shi *et al.*, 2014) .

1.8.2. Pathogenic mechanisms

IAV can be transmitted by inhalation of infectious particles, or by airborne droplet nuclei, and by direct contact. Human IAV infections that caused pandemic are H1N1 (1918, 2009), H2N2 (1957) and H3N2 (1968) (Taubenberger and Morens, 2010). The risks attributed to seasonal or pandemic influenza, causing severe complication and death include age (very young or old), underlying chronic respiratory and cardiovascular conditions, diabetes mellitus, pregnancy and immunosuppression. Although drugs are available for treatment, drug-resistant mutations can emerge. Signs and symptoms include dry cough, chills, sweats, myalgia, shortness of breath, and neurological symptoms (Weycker *et al.*, 2005).

1.8.3. Innate immune involvement with IAV

IAV recognition by PAMPs via PRRs leads to antiviral signalling cascade, causing regulation of interferons, cytokines and chemokines. TLRs, RIG-1 and NOD-like receptor family pyrin domain containing 3 (NLRP3) are the three main PRRs involved in the recognition of IAV. For instance, of TLR 2, 3, 4, and 7, TLR7 recognises single stranded viral RNA (ssRNA) while TLR3 recognises viral RNA in endosomes (van de Sandt, Kreijtz and Rimmelzwaan, 2012). Human respiratory epithelial cells express TLR3, which stimulates the activation of pro-inflammatory cytokines following IAV infection. Thus, TLR3 knock out mice become resistant to lethal IAV challenge as compared to wild type, concomitant with reduced chemokine expression, leukocytes and CD8⁺ T cells. It is apparent that TLR3 induced immune response against IAV challenge is not protective. Although TLR3 expression signals the inhibiting effect on viral replication, it also induces host cell damage (Iwasaki and Pillai, 2014) TLR7 on plasmacytoid DCs recognises viral ssRNA when released into the endosome, activating the NF- κ B and IRF7, and inducing pro-inflammatory cytokines and/or type I IFNs (Iwasaki and Pillai, 2014). TLR2 and TLR4 recognise HA and NA while RIG-1 receptor recognises viral RNAs.

Neutrophils, macrophages and maturation of DCs are activated by IAV infection. During the initial phase of infection monocytes and macrophages are recruited via their CCR2 receptor. Epithelial cells produce CCL2 during IAV infection. Viral spread is reduced as the apoptosing infected cells are phagocytosed by alveolar macrophages. Infected cells can also be lysed by natural killer (NK) cells in a MHC class I dependent manner, may rely on an interferon gamma dependent autocrine/paracrine loop. Infected cells can be directly lysed due to the binding of sialylated NKp44 and NKp46 binding to HA viral protein (Bar-On *et al.*, 2014) . On the other hand, the indirect manner of lysing infected cells is when the NK cells with CD16 receptor bind to the Fc portion of antibodies that are bound to IAV infected cells, causing lysis of infected cells (Klimpel, 1996).

1.8.4. Adaptive immune response against IAV

Adaptive immunity against IAV involves virus-specific humoral as well as cellular immune response (van de Sandt, Kreijtz and Rimmelzwaan, 2012). Antibodies against HA can inhibit the attachment of the virus to host cells, and thus blocking receptor-mediated endocytosis as well as neutralisation of IAV. Since antibodies

against HA are strain-specific, depending on the epitopes involved, they cannot neutralise antigenic drift variants. Neutralizing antibodies offer a long-lasting protection against strains that are similar to the infected strain. In 1950s individuals exposed to H1N1 virus had developed antibodies that cross-reacted with pandemic strain and may develop immunity against infections and disease of present pandemics. High frequency of cross-reacting antibodies against 2009 pandemic H1N1 virus has been reported among the elderly in Finland in 2010. Antibodies against NA are involved in the last phase of viral replication cycle; however they are non-neutralizing antibodies. These antibodies also inhibit the enzymatic activity of NA, thus reducing viral spread (van de Sandt *et al.*, 2012). DCs infected with IAV participate in anti-viral response via MHC class I mediated peptide presentation to CD8⁺ CTLs. Alternatively, viral peptides can also be presented by MHC class II molecules to CD4⁺ T cells (van de Sandt *et al.*, 2012). Over the period it has become apparent that both CD4⁺ and CD8⁺ T cells are important for heterosubtypic immunity and hence viral clearance in vivo. In spite of a lack of protective antibody response B cell deficient mice can still clear IAV infection suggesting a more profound role of T cells in anti IAV defence (Altenburg *et al.*, 2015).

1.9. *Aspergillus fumigatus*, a fungal pathogen

Several species of Aspergilli, such as *A. flavus*, *A. fumigatus*, *A. niger* and *A. terreus* can act as human pathogens (Thiagarajan *et al.*, 2005). *A. fumigatus* spores, 2-3 µm in diameter, are universal in distribution. Given that 0.1-22% of total spores exist in the air, they can easily deposit in the lungs up to the alveolar level (Latge, 1999). However, *A. fumigatus* shows higher pathogenic potential due to production of large numbers of small conidia, presence of number of conidial adhesins, resistance to oxidative stress and thermotolerance (Latge, 1999). In addition, it produces gliotoxin that affects the circulating neutrophils, inhibits phagocytosis and enhances dissemination of the organism (Scharf *et al.*, 2012). Another virulence factor produced by *A. fumigatus* is pigment melanin that inactivates the C3 component of the complement system (Knutson and Slavin, 2012).

A. fumigatus, a major opportunistic fungal pathogen, causes a spectrum of respiratory diseases, depending on the immune status of the host. In the healthy host, the inhaled conidia of *Aspergillus* seldom cause any adverse effects 6. The

innate and adaptive immune responses in a healthy individual result in the mucociliary clearance and phagocytosis of spores (Wannar et al, 1996). In the hypersensitive individuals, the fungal sensitization mostly results in allergic manifestations with allergic bronchopulmonary aspergillosis (ABPA). About 2% of patients with asthma and 1-15% of patients with cystic fibrosis develop ABPA. In the immunocompromised host, the fungus invades various organs leading to systemic form of infection, termed as invasive pulmonary aspergillosis (IPA). The hyphal invasion causes destruction of pulmonary tissue, followed by dissemination of fungus to other organs in approximately 20% cases (Wannar et al, 1996). The number of IPA cases has risen with the increasing incidence of AIDS, organ transplantations and aplastic anemia, etc. The immunity of host is compromised due to pathogens such as HIV or prolonged treatment with corticosteroids or aggressive antineoplastic chemotherapeutic regimens. IPA results in > 80% mortality, and up to 95% among patients of bone marrow transplantation (Patterson, 2002). To understand the role of collectins, murine models of ABPA and IPA in wild type and collectin gene deficient mice have been studied.

Host defense against *Aspergillus* infections is mediated by phagocytic cells including macrophages and neutrophils. Macrophages can bind and phagocytose *A. fumigatus* conidia, and kill conidia as well as hyphae. Innate immune molecules, such as collectins, toll-like receptors and pentraxin-3 are known to be involved in lung resistance to *A. fumigatus* infection (Schaffner et al, 1982). SP-A and SP-D bind and agglutinate *A. fumigatus* conidia in vitro in a sugar and calcium dependent manner and thus, enhance killing of conidia by circulating neutrophils and alveolar macrophages via phagocytosis and superoxidative burst (Madan et al, 1997a). In vitro interactions of SP-A and SP-D with *A. fumigatus* allergens/antigens suggest their hierarchical role at various levels that involves allergen scavenging, inhibition of allergen-IgE cross-linking and histamine release, suppression of the activation of sensitized basophils or mast cells 31-35. SP-A and SP-D can also modulate host immune response by suppression of B- and T-cell proliferation, modulation of dendritic cells and macrophages, and Th cell polarization (Kishore et al, 2002).

1.9.1. Murine models of allergic aspergillosis

Allergic aspergillosis comprises a spectrum of allergic diseases induced by *Aspergillus* species in non-immunocompromised patients and are defined as hypersensitivity disorders. ABPA is the most severe and fatal form of allergic aspergillosis and occurs predominantly in patients predisposed with lung diseases such as cystic fibrosis or bronchial asthma (Burton and Oettgen, 2012). In these lung diseases, the *A. fumigatus* spores are not effectively cleared off. They germinate and grow to hyphae in the mucus plugs. The host suffers from an allergic disorder characterized by Type I and Type III hypersensitivity response to the secreted *A. fumigatus* allergens, antigens toxins and hyphae. The common features of Type I hypersensitivity in ABPA are upregulation of TH2 cytokine pathway that includes IL-4, IL-5, IL-6, IL-10 and IL-13 cytokines (Madan et al, 2001). Presence of IgE antibodies specific to *A. fumigatus* sensitizes and causes mast cell degranulation resulting in bronchoconstriction and increased capillary permeability. Further, immune complexes are formed because of type III reaction and the inflammatory cells get deposited in the mucous membranes leading to necrosis and eosinophilic infiltration of the airways. Briefly, clinical ABPA is characterized by Rosenberg et al.⁴¹ in terms of episodic bronchial hyper-reactivity, reversible airway obstruction, positive immediate skin reactivity, peripheral and pulmonary eosinophilia, central bronchiectasis and history of expectorating brown plugs or flecks, etc.

Experimental murine models of ABPA have been generated in various strains of mice. The development of ABPA has been linked to a number of genetic risks, which include polymorphisms in the genes of HLA-DR and HLA-DQ, surfactant protein A2 (SP-A2), IL-4 receptor alpha chain (IL-4RA), and IL-10-1082GA promoter (Brouard, 2005). Cystic fibrosis transmembrane conductance regulator gene (CFTR) mutations and TLR polymorphisms are also linked to ABPA. The effect of genetic variation is also observable in murine fungal exposure studies. Of the three congenic mouse strains (BALB/c, CBA/J, C57BL/6), BALB/c mice exhibit the strongest inflammatory response. The BALB/c species is more sensitive to hypersensitivity disorders, while C57BL/6 mice are comparatively more resistant (Atochina et al, 2003). An enhanced lung SP-D production has been predicted to attenuate airway hyper-responsiveness to allergic airway sensitization in C57BL/6 mice⁵¹, suggesting an important role of SP-D in regulating pathogenesis in ABPA.

Important features of ABPA model can be developed in mice genetically predisposed to atopy (e.g. BALB/c) by the continual exposure and sensitization of the airways to *A. fumigatus* allergens. Type I and Type III immune responses are present in these models. In case of natural disease, this happens by the growth of *A. fumigatus* spores into the hyphal structures in the lungs. Spores present in central and up to distal parts of the lungs release allergens continuously which in turn lead to clinical symptoms of ABPA. A variety of experimental animal models of ABPA have been studied depending on the pathogen form, inoculum size (conidia/hyphae/allergens) and the frequency and route of exposure in addition to variations on the basis of strain of animals (Kishore et al, 2002).

The BALB/c murine models of ABPA have demonstrated nine-fold increase in SP-D levels on *A. fumigatus*-induced allergic airway inflammation suggestive of its role in pathophysiology. Treatment with SP-A and SP-D drastically decreases the serum *A. fumigatus* IgG and *A. fumigatus* IgE levels in murine models of ABPA (Madan et al, 2001). The decrease in specific IgG and IgE levels persist till 16 days in the SP-D treated ABPA mice, but only 4 days in SP-A treated mice. SP-D treated murine ABPA models show significant decline in peripheral blood eosinophilia and lung eosinophil peroxidase activity, hallmarks of Type I hypersensitivity. The chronic inflammatory infiltrates disappear and the ABPA mice model showed higher levels of *A. fumigatus* specific IgE and IgG levels as well as peripheral blood eosinophilia. Increased ratios of IL-2, IL-4 and IL-5 and levels, while a decrease in IFN- γ were observed in splenic supernatants of the untreated ABPA mice compared to their respective controls, indicating a marked shift from Th2 to Th1 response. Thus, collectins have a major role in modulation of allergic reactions in ABPA.

Murine models of ABPA in SP-A gene deficient (AKO), SP-D gene deficient (DKO), and WT mice in C57BL/6 background were used for such studies (Madan et al, 2005). The studies showed that both AKO and DKO mice exhibit intrinsic hypereosinophilia and several fold increase in levels of IL-5 and IL-13, and decrease in IFN- γ to IL-4 ratio in the lungs. DKO mice develop chronic inflammation, foamy alveolar macrophages secreting 10-fold higher levels of hydrogen peroxide, increased activity of metalloproteinases, emphysema, and fibrosis in the lungs. Administration of SP-A or SP-D to the respective knockout mice decreases

peripheral eosinophil count, EPO activity, IL-13, IL-5, and increases TNF- α and IFN- γ levels. The Th2 bias in AKO or DKO mice was reversible on treatment with SP-A or SP-D, respectively.

The *A. fumigatus* allergen sensitization of AKO and DKO mice further increases the Th2 response, as opposed to the predominantly Th1 profile of the WT C57BL/6 mice. DKO mice are more susceptible than WT mice to pulmonary hypersensitivity induced by *A. fumigatus* allergens. DKO mice also develop 2-fold elevated peripheral eosinophil count with increased eosinophil infiltration around perivascular areas in the lung sections on allergen treatment. A more pronounced Th2 bias of DKO mice is also evident by decrease in IFN- γ levels and increase in IL-13, IL-5, and IL-2. However, IL-4, IL-10, IL-12, and TNF- α levels in both AKO and DKO mice do not show significant changes compared to WT mice challenged with allergens (Madan et al, 2005). The intranasal treatment with SP-D is effective in rescuing the *A. fumigatus* sensitized DKO mice. *A. fumigatus* IgE levels, peripheral eosinophilic count, pulmonary eosinophilia and EPO activity also decrease. SP-D treatment to DKO mice also reduces IL-13 and IL-5 cytokines and increases IFN- γ to IL-4 ratio. SP-A-treated *A. fumigatus*-sensitized AKO mice develop several fold elevated levels of IL-13 and IL-5, resulting in increased pulmonary eosinophilia and lung tissue damage. Hence, SP-A and SP-D may be helpful in treating ABPA, as evident from studies of murine models.

1.9.2. Invasive Pulmonary Aspergillosis

Deficiency of innate and adaptive immune response, induced by infectious agents such as HIV or the organ transplant immunosuppressive regimens, makes the host susceptible to germination and invasion of inhaled fungal conidia. In the patients of IPA, there is low or almost no humoral immunity while the adaptive immune response is of Th2 type. Recovery of these patients on empirical treatment with antifungal drugs is accompanied by revival of innate immunity and a protective Th1 type of response.

To mimic the human IPA, murine model of IPA can be generated by administration of immunosuppressive agents, such as cyclophosphamide, hydrocortisone acetate and FK506, etc., followed by fatal spore challenge(s) (Herbst et al, 2013). The survival

rate and enumeration of pulmonary colony forming unit (CFU) serve as the markers of therapeutic evaluation.

Intranasal administration of SP-D or a recombinant fragment of SP-D composed of trimeric neck and CRD regions (rhSP-D) have protective effect in a murine model of IPA, where mice are immunosuppressed with hydrocortisone and challenged intranasally with *A. fumigatus* conidia (Madan et al, 2001b). Untreated IPA mice have 100% mortality in 7 days, whereas SP-D or rhSP-D treatment rescued 80% of the IPA mice. Interestingly, SP-A does not have significant effect on survival, while SP-D plays an important role in the ability of host to resist *A. fumigatus* challenge and subsequent infection. The rhSP-D treatment lowers IL-4, IL-5 and increases TNF- α and IFN- γ levels in the lung cell suspension, as compared to untreated IPA mice.

The immune status of AKO as well as DKO mice alters distinctly following steroid treatment prior to *A. fumigatus* conidia challenge (Madan et al, 2010). DKO mice have increased susceptibility to the IPA pathogenesis than the WT mice while AKO mice are more resistant than WT mice. Intranasal treatment with SP-D or rhSP-D was effective in ameliorating the pathology in the case of DKO mice, whereas the SP-A treated *A. fumigatus* challenged AKO mice have increased mortality. The survival data also reflected on the CFU counts and hyphal burden in the lung, consistent with the lung cytokine profiles. Thus under immuno-suppressed or immunocompromised conditions, the SP-A or SP-D deficiency may have profound but contrasting effects on host immune response.

The main aims and objectives of the thesis are as follows:

1. To optimise large scale expression and purification of LPS-free rhSP-D, composed of trimeric neck and CRD regions;
2. To test their interaction with IAV and examine the nature and ligands involved in this interaction; to examine the effect on infectivity of IAV by rhSP-D;
3. To assess the in vitro effects of rhSP-D on spleen cells derived from murine *ABPA model*; to examine direct effect of rhSP-D on the conidia of *Aspergillus fumigatus* in the absence of phagocytic cells;
4. To measure the effects of full-length SP-A and SP-D on the maturation, proliferative and cytokine responses by immature and mature human DCs in vitro.

Thus, this thesis examines interaction of a recombinant fragment of human SP-D containing neck and CRD regions (rhSP-D) with IAV and *Aspergillus fumigatus*, in addition to characterizing a dichotomy of the effects of SP-A and SP-D on dendritic cells in an attempt to explain how SP-A and SP-D modulate DC functions differentially. Chapter 3 examines interaction of rhSP-D with IAV pandemic strain showing that it can be a restrictive factor against the virus, in addition to modulating immune response by a macrophage cell line. Chapter 4 looks at how rhSP-D can modulate anti- *A. fumigatus* responses directly and indirectly in the context of pathogen as well as allergen. A comparison has been made between two recombinant fragments of SP-D that have been expressed with and without 8 Gly-X-Y repeats for their fungistatic properties. Finally, Chapter 5 examines effect of SP-A and SP-D on cultured DC maturation, and effector cytokine and proliferative response of co-cultured cells.

2. Chapter 2

Materials and Methods

2.1. Purification of native of full length SP-A

One litre of pooled amniotic fluid was thawed by incubating at 37°C for 6 hours (occasional mixing), amniotic fluid was passed through 3mm Whatman filter paper. Use of a cell strainer further removed fat and other large aggregates. The sample was centrifuged at 14000 g for 30 min at 4°C. 20 ml of 8 M urea in the affinity buffer (50 mM Tris- HCl, pH 7.4, 100 mM NaCl, 5 mM CaCl₂, and 0.02% NaN₃) was added to the pellet for further SP-A extraction. After stirring for 1 hour at 4°C, the suspension was centrifuged at 14,000 x g for 15 min at 4°C. The supernatant was then dialysed against 1 Liter of affinity buffer without urea overnight at 4°C. The dialysate, after another round of high-speed centrifugation, was subjected to affinity chromatography procedure. Maltose-Sepharose was washed with 5 ml bed volumes of distilled water and then equilibration with 4 volumes of affinity buffer. The equilibrated maltose-Sepharose was added to the supernatant containing SP-A and left to stir for 30 min at RT, and then packed into a Bio-Rad column. After few wash with the affinity buffer, SP-A was eluted with elution buffer (50 mM Tris- HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, and 0.02% NaN₃). 1 ml fractions were collected and analysed for protein concentration by Nanodrop and purity by 12% SDS-PAGE.

2.2. Purification of native of full length SP-D

Amniotic fluid was processed first as described above. The amniotic fluid was made 5 mM with CaCl₂ and the supernatant from the first round of centrifugation was purified using maltose-agarose (Sigma) and eluted with MnCl₂ (Strong et al, 1998). 100 µl of each fraction was used for TCA precipitation and this was separated on a 15 % SDS-PAGE to visualize protein concentration and purity.

2.3. Expression and purification of trimeric SP-D

2.3.1. Competent cells preparation

A single colony of E. coli BL21 (λ DE3) pLysS (Invitrogen) in 10 ml of Luria broth (LB) media with 10µl of chloramphenicol (stock: 50 mg/ml dissolved in ethanol) overnight at 37°C shaker. Next day, 500 µl was removed from culture and inoculated into 25 ml LB containing 25µl chloramphenicol and put on a shaker. A₆₀₀ of the culture was measured every hour until it reached 0.3 values. The culture was centrifuged at 2,000 x g for 5 minutes. After centrifugation, the supernatant was

discarded, and 12.5 ml of sterile 0.1 M CaCl₂ was added to the cell pellet. The cells were suspended and placed in the temporary state of competent at ice for an hour. After an hour, the cells were centrifuged for 5 minutes at 2000 x g. The supernatant was discarded, and the pellet was re-suspended in 2 ml of 0.1 M CaCl₂ and was stored in ice.

2.3.2. Transformation of cells

200 µl of cells from the competent cells that prepared earlier were removed and were placed in a 10 ml falcon tube containing 1-2 µl of (pUK- D1) construct. The mixture was placed on ice for an hour. The mixture was exposed to heat shock at 42°C for 90 seconds. Following heat shock, the cells were placed on ice for a further 5 minutes, and then re-suspended in 800 µl of LB. The mixture was placed inside a 37°C incubator for approximately 45 minutes. After incubation, the cells were streaked on an LB agar plate containing Ampicillin/ Chloramphenicol and were left inside a 37°C incubator overnight in order to grow.

2.3.3. Protein Expression

A single colony of transformed (pUK-D1 and D2) cells was grown in 25 ml LB along with 100 µg/ml Ampicillin + 50 µg/ ml chloramphenicol overnight in a shaker at 37° C. On the following morning, the overnight primary culture of 25 ml bacterial culture was inoculated into 1 L LB along with 100 µg/ml Ampicillin + 50 µg/ ml chloramphenicol and was grown inside a 37°C shaker, until the absorbance A₆₀₀ reached 0.6 – 0.8 . After the cells reach log phase (A₆₀₀ at 0.6- 0.8) were induced with 0.4 mM IPTG for 3 h and harvested by centrifugation at 9,000 x g for 15 minutes. Following centrifugation, the supernatant was discarded and the pellet was stored at - 20°C for further processing.

2.3.4. Cell Lysis

The cell pellet that obtained after IPTG induction was resuspended in ice cold lysis buffer containing (50 mM Tris-HCL, 200 mM NaCl, 5 mM EDTA, 0.1 % v/v Triton X-100, 0.1 mM PMSF, pH 7.5, 50 µg/ ml lysozyme) and was vortex for 1 hour in cold room at 4°C and then sonicate the sample (ten cycles at 30 seconds each).

2.3.5. Dialysis

The sonicate was harvested by centrifugation at 9,000 x g for 15 minutes and was solubilized the recovered rh SP-D in the inclusion bodies in 100 ml buffer I (50 mM Tris- HCl pH 7.5, 100 mM NaCl) containing 10 mM 2- Mercaptoethanol and 8 M Urea. Carry out a stepwise dialysis of the resolubilized material against buffer I containing 4M urea, 2 M urea, 1 M urea and no urea, each for 2 hours. The dialysate was clarified by centrifugation at 9,000 x g for 15 minutes, and then dialyzed with calcium buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl₂, 0.05 % sodium azide) for 3 hours to completely remove urea from the dialysate.

2.3.6. Protein Purification by Affinity chromatography

The rhSP-D is purified by affinity chromatography using maltose- sepharose column. The maltose- sepharose column was rinsed with distilled water prior to use. After through rinsing of the column with dH₂O, 50 ml of affinity column buffer (50 mM Tris- HCl pH 7.5, 100 mM NaCl, 5 mM CaCl₂, 0.05 % sodium azide) was passed through the column. After rinsing the column, the protein was passed through the column once, and then the column was washed with affinity column buffer. The bound protein was eluted by using elution buffer containing 5 mM EDTA (25 ml) (50 mM Tris- HCl pH 7.5, 100 mM NaCl, 5 mM EDTA. 0.05% sodium azide. The affinity-eluted protein ran as a trimer on a Superose-12 gel filtration column.

2.3.7. Endotoxin Removal from Recombinant SP-D

5 ml of Polymyxin B agarose gel (sigma) was packed in a 20 ml column. The column was washed with 50 ml of 1% sodium deoxycholate and then further rinsed with 50 ml of autoclaved distilled water to completely removed 1 % sodium deoxycholate. After rinsing the column, the protein was loaded on to the column, and left to be incubated at room temperature for half an hour for the protein to bind completely. After the incubation period, the protein was eluted and collected in 0.1 ml fractions. The LPS-free rhSP-D protein was then checked for purity by running it on 15 %SDS gel to ensure the removal of LPS. The endotoxin level was examined by QCL-1000 Limulus amoebocyte lysate system (Bio Whittaker, Walkersville, MD, USA) and was found to be μg^{-1} of rhSP-D. The protein was furthered quantified by measuring the absorbance at 280 nm of the eluted and the concentration of sample was determined via Spectrophotometry.

2.3.8. Preparation of D-Maltose-Sepharose 4B Column

Sepharose was first activated with the bifunctional reagent divinyl sulfone (DVS, Sigma), and then coupled under basic conditions to the hydroxyl of D- mannose. Reductive amination, epoxy and DVS activation provide three excellent alternatives for the obtaining an optimal affinity support. Activated sepharose 4B (100ml settled gel with DVS), DVS is a bifunctional cross- linking reagent, used to activate agarose and other hydroxyl matrices. DVS introduces reactive vinyl groups into the matrix that will couple to amines, alcohols, sulfhydryls, and phenols. DVS activated gels were more reactive than epoxy-activated gels and therefore coupling proceeds rapidly and completely.

2.4. Activation

100 ml of sepharose 4B gel was washed with 1 litter water and then suspended in 0.5 M sodium carbonate. While stirring, 10 ml of DVS was slowly added drop wise. The gel suspension was stirred for 1 hour at room temperature followed by extensive washing with water.

2.5. Immobilization

D-maltose solution (20 % D-maltose in 0.5 M sodium carbonate) was added to the gel and stirred at room temperature for 24 hours. After filtration and washing with 2 Litre of water and 0.5 M sodium bicarbonate containing 5ml beta-mercaptaethanol at room temperature, the gel was mixed for 2 hours to block the excess vinyl groups. Finally, the gel was washed with 2 L each of water. D-maltose-sepharose 4B can be stored in 0.02% sodium azide at 4°C.

2.6. Bis (Sulfosuccinimidyl) substrate (BS3) Cross-Linking

25 mM solution of BS3 was prepared by dissolving 2 mg BS3 in 140 µl of DMSO. BS3 cross-linker solution was added to the protein sample at the concentration of 0.5 and 5 mM. rhSP-D was incubated with different concentrations (1 mM, 0.1 mM, and 0.01 mM BS3). After 45 minutes incubation, BS3 was quenched with 60 mM Tris-HCl, pH 7.5 for 15 minutes at room temperature. The SDS-PAGE was run for the detection of cross-linking.

2.7. TCA Assay

An equal volume of 20 % TCA (trichloroacetic acid) was used to precipitate the protein.

2.8. Bicinchoninic Acid Protein Assay

BCA assay was used to estimate the protein concentration as per the kit instructions, using BSA (2 mg/ml) as a control protein (Thermo Scientific).

2.9. SDS- PAGE: 12% and 15%

For a standard size gel, 10 ml of resolving gel and 5 ml of stacking gel was used to run a denaturing polyacryamide gel following standard method. Commercial stain and destain solutions based on Coomassie blue R250 were used for the gels.

2.10. IAV amplification and purification (H3N2, H1N1)

Madin-Darby Canine Kidney (MDCK) cells were cultured in complete DMEM (Fisher Scientific) consisting of 10% v/v FBS, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 1mM sodium pyruvate in four 150 cm² flasks (Fisher Scientific), and left to grow in 5% CO₂ at 37°C incubator, until about 80% confluence was reached. Cells were then washed with PBS twice, and infected with 3.3 x 10⁴ pfu/ml virus titre with 10ml of pure DMEM to the flasks. The flasks were left for 1h to allow the virus to adsorb in 5% CO₂ at 37°C incubator, and every 15 min tilted to avoid drying up of the cells. The cells were then washed with 20ml PBS to remove any unabsorbed virus, and 25ml of infection medium (DMEM, 1% penicillin/streptomycin, 1µg/ml TPCK-Trypsin, 0.3% BSA) was added to flasks, left at 5% CO₂ at 37 ° C incubators for 3 days.

2.11. Purification of IAV by ultra-centrifugation and sucrose cushion

After 3 days of infection, infected cells detached, and added to 50ml falcon tubes (Fisher Scientific) and centrifuged at 3000 rpm at 4°C for 15 min. The supernatant was transferred to ultra-clear centrifuge tube (Beckman Coulter), balanced with PBS, and centrifuged at 10,000 rpm for 30 min at 4°C. 8ml of cold 30% w/v sucrose was added to new ultra-clear centrifuge tubes and 26ml of supernatant was transferred carefully into the tubes containing 8ml sucrose, and centrifuged at 28000 rpm at 4°C for 2 h. Then the supernatant in the upper phase was carefully removed and sucrose

phase was removed. The pellets from all tubes were re-suspended in 100µl of PBS, and collected in a screw-capped tube. The virus suspension was briefly spun, then aliquoted in new screw-capped tubes and stored at -80°C.

2.12. Haemagglutination assay

Haemagglutination assay carried out to determine the titration of IAV, which allows the virus to attach to the surface of red blood and may cause them to agglutinate and prevent from settling. Guinea pig red blood cells (RBC) (TCS biosciences) were washed twice with 1 X PBS at 800 g for 5 min. RBCs were made to 10% v/v solution in 1 X PBS, and the final working solution used was 0.75% RBCs in PBS. 50µl of 1 X PBS was added to each well of maxisorp 96 well plate. To the first wells of the plate, 50µl of IAV were added (total volume 100µl) and 50µl was serially diluted until the last well of the plate, discarding the last 50µl. The plate was mixed by tapping and left at room temperature for up to 2 h and photographed. PBS was used as control. Settled cells appeared as halo indicating negative results. Positive results gave a uniform red colour of cells that do not settle. The titre used for the HAI assay was the dilution that the virus did not allow the cells to settle down.

2.13. Haemagglutination inhibition assay (HAI)

In maxisorp 96 well plate, 25µl of 1 X PBS was added to each well. In the first column starting concentration (total volume 50µl) of 5 and 10 µg of rhSP-D, were serially diluted (25µl). Controls used were no protein and PBS only. All strains were diluted accordingly to the haemagglutination assay in PBS and 25µl of respective IAV were added to wells except for PBS control wells. Plate was gently mixed by tapping and left at 37°C for 1 h. 50µl of 0.75% guinea pig RBC working solution was added to each well, plates were gently tapped to mix, and left at room temperature for 1 h to develop. Inhibition of haemagglutination will appeared as halo or circle of settled cells in the centre of round-bottomed plates. Absence of inhibition was evident from a uniform reddish colour across the well (haemagglutination).

2.14. Binding of H3N2 influenza A virus to rhSP-D

Maxisorp 96 well plates were coated with 5 or 10µg of rhSP-D in carbonate bicarbonate buffer and left overnight at 4°C. Microtitre wells were then blocked with 2% w/v BSA for 2 h at 37°C, and washed 3 times with PBS. Different amounts of H3N2 virus were added (10, 5, 2.5 and 1.25µl) and left at 37°C for 2 h. The binding

was probed with anti-Udorn antibody (goat anti-serum against A/Udorn/72 (H3N2) influenza virus was a kind gift from Dr Jeremy Rossman (University of Kent, UK) (1:5000; 100µl/well) in PBS for 1 h at 37°C. Wells were washed again with PBS and incubated with rabbit anti mouse IgG HRP (1:5000) in PBS (100µl/well) for 1 h at 37°C. Colour was developed using TMB and the reaction was stopped using 2N H₂SO₄. Absorbance was read at 450 nm.

2.15. Western blot of rhSP-D bind to H3N2

5µl of H3N2 lysate was loaded onto SDS PAGE, and run at 120 volts until the dye front reached the end of the gel, which was then transferred onto a nitrocellulose membrane by electrophoresis in 1 X transfer buffer (25mM Tris, 192mM Glycine, 20% v/v methanol, pH ~8.3) at 320 mA for 2 h. The nitrocellulose membrane was incubated in blocking solution (5% semi-skimmed milk powder (Tesco, UK) in PBS, pH 7.4) overnight at 4°C. The membrane was then washed in PBST three times, 10 minutes each. The blots were incubated with either anti-H3N2 polyclonal antibodies (1:500) in PBS or with purified rhSP-D (10µg). PA-HRP (1:1000) in PBS was incubated correspondingly to the blot hybridised with anti-H3N2 antibody. Anti-human SP-D (polyclonal) (1:5000) in PBS was added to blot hybridised with properdin. The membranes were incubated for 1 h at 37°C, and washed with PBST three times for 10 min each. The secondary probe used was rabbit protein A HRP for properdin membrane. The membranes were then washed in PBST three times for 10 min each, and developed colour using DAB.

2.16. rhSP-D binding to H1 and N1 proteins

Microtitre wells were coated with different concentrations of H1 or N1 (12.5, 6.25, 3.125, 1.56, 0.78 and 0.39µg) in carbonate bicarbonate buffer. Wells were then blocked with 2% w/v BSA in PBS, and added 10µg/ml of rhSP-D to 100µl to each well. Polyclonal anti-human SP-D (1:5000) in PBS was added to each well, and then probed with PA-HRP (1:5000). Colour was developed using TMB and reaction was stopped using 2N H₂SO₄. Absorbance was read at 450 nm.

2.17. Infection assay using U937 monocytic cell line

U937 cell lines were cultured in complete DMEM medium (Sigma), consisting of 10% v/v FBS, 2mM L-glutamine, 100U/ml penicillin (Sigma-Aldrich), 100µg/ml streptomycin (Sigma-Aldrich) and 1mM sodium pyruvate (Sigma-Aldrich), were left to

grown in 37°C incubator with 5% CO₂, until about 80% confluence of cells were reached. Cells were washed twice in PBS and adjusted to 5 x 10⁵ cells in 12 well plates and left to adhere overnight in AIM serum free medium (Sigma-Aldrich). Cells were washed in PBS before addition of rhSP-D (10µg/ml) in AIM serum free medium containing 10mM HEPES buffer in total volume of 300µl for 1h at room temperature. In parallel, rhSP-D (10µg/ml) in AIM serum free medium containing 10mM HEPES buffer was incubated with MOI 1 of pH1N1 virus for 1 h at room temperature, to allow binding. Properdin incubated with pH1N1 was then added to cells in a circular motion (total volume of 500µl). Pandemic H1N1 was added to cells that were pre-incubated with properdin (total volume 500µl), and then incubated for 1h at room temperature. Cells were washed before the addition of 1 X infection medium (1ml total volume). Supernatants and cells were collected at different time points (15, 30, 45 min, 1 h, 2 h, 6 h, 12 h, 24 h and 48 h) and subjected to multiplex cytokine array analysis.

2.18. Multiplex cytokine array analysis

Cytokines (IL-6, IL-8, IL-10, IL12p40, IL12p70, IL-23, IL-27, IL-1α and IL-1β) and chemokines/growth factors (MIG, I-TAC, MCP-1, G-CSF and M-CSF) concentrations in the supernatants of U937 cells were measured by MagPix Milliplex kit (EMD Millipore). Supernatants collected from the above-mentioned experiments were stored at -80°C until use. Briefly, 25µl of assay buffer was added to each well of a 96-well plate, followed by addition of 25µl of standard, controls or supernatants of (rhSP-D treated) cells, to appropriate wells. 25µl of magnetic beads coupled to analytes were added in each well, and incubated for 18h at 4°C. After washing the plate with assay buffer, 25µl of detection antibodies were incubated with the beads for 1h at RT. 25µl of Streptavidin-Phycoerythrin was then added and incubated for 30'. Following a washing step, 150µl of sheath fluid (BD Biosciences) was added to each well and the plate was read using the Luminex Magpix instrument.

2.19. Aspergillus strains

48 clinical isolates of Aspergillus being 18 strains of fumigatus (AF), 10 strains each of niger (AN), flavus (AFL) and terreus (AT) were used in the study. Susceptibilities were determined using the microdilution procedure of the CLSI M38-A standard. Culture medium was RPMI 1640 + 2% glucose, inoculum 1 x 10⁵/mL, plates

incubated for 24 hours at 37°C; the plates were read both visually and on a spectrophotometer at 490nm (rhSP-D range 0.9273-15mg/l and 12.5-200mg/l for Gly-X-Y and delta rhSP-D, respectively). In a second set of experiments involving 3 isolates of AF and one each of AN, AFL and AT, growth curves were determined in similar conditions with readings taken every 5 minutes for 24 hours (1.5-24mg/l). Susceptibility tests were performed on 48 clinical *Aspergillus* isolates; 18 *A. fumigatus* (AF), 10 isolates each of *A. flavus* (AFL), *A. terreus* (AT) and *niger* (AN). For growth rate assays 3 strains of AF and one strain each of AFL, AT and AN were used.

2.20. Susceptibility testing

Inoculum suspensions were from day 5 to 8 day cultures grown on Sabouraud dextrose agar at 37°C and adjusted to a final inoculum was between 5×10^4 and 2.5×10^5 CFU/ml. Readings were done after 24 h of incubation at 37°C both visually and by spectrophotometer at 490nm. The MIC end-points were recorded as the lowest drug concentration that prevents any discernible growth. In the growth rate assays, microdilution plates were set up in similar conditions but with a dose range of 1.5-24mg/l. Readings taken every 5 minutes for 24 hours. All *Aspergillus* isolates had reduced growth with Gly-X-Y in a dose dependent fashion. MIC values and MIC50 were lower for *A. niger* than any other species. 40/48 strains demonstrated <80% reduction in growth at 15mg/l with MIC50 of 15, 1.875, 15 and 7.5 for AF, AN, AFL and AT, respectively.

2.21. Preparation and labelling of conidia

Spores and culture filtrate were obtained from the 285 strain of *A. fumigatus* isolated from sputum of an allergic bronchopulmonary patient who had been diagnosed based on Rosenberg criteria. Cultures were maintained on Sabouraud dextrose agar slants. Conidia were allowed to swell in phosphate- buffered saline (PBS) containing Ca^{2+} and Mg^{2+} for one hour being used for binding, agglutination, phagocytosis, oxidative burst and killing assay. Conidia were labelled by incubating 10⁸ conidia with a 3 mg/ml stock of fluorescein isothiocyanate (FITC, sigma Chemical co.) in 0.5 M carbonate buffer, pH9.0 for 18 hours at 4°C in the dark and with mild agitation. The labelled conidia were washed twice. The conidia remained viable after labelling. Following 1 h pre-incubation, as previously reported by Schaffner et al. was carried

out to stimulate the natural conditions of conidia since they are wetted by mucous secretions in the respiratory tract.

2.22. Binding of rhSP-D to *A. fumigatus* conidia

A. fumigatus was grown on Sabouraud dextrose agar slants at 37°C. The conidia or spores were harvested after 72 hours, washed twice in pyrogen-free saline, and stored in pyrogen-free saline. For interaction studies, 10⁶ conidia were washed twice in filtered PBS containing 1% bovine serum albumin (BSA) and 5 mM CaCl₂ (PBS-BSA-Ca²⁺) at pH 7.4 and centrifuged at 8,000 x g for 10 minutes at 4°C. The conidia were then suspended in 100 µl of PBS-BSA-Ca²⁺ and incubated with various concentrations of SP-A and SPD for an hour at 37°C. For inhibition studies, conidia were incubated with surfactant proteins in the presence of 10 mM EDTA, 100 mM mannose, and 100 mM maltose. The conidia-surfactant complex were then washed twice with 200 µl of PBS-BSA-Ca²⁺ and incubated with 100 µl of rabbit anti-human SP-D antisera (1:20 dilution) for an hour at 4°C. After further washing as described previously, the conidia were incubated with goat anti-rabbit Ig FITC-labelled antibody (1:50 dilution) for an hour at 4°C and washed again. The conidia were fixed with 2% (w/v) paraformaldehyde in PBS. Conidia-associated fluorescence was analysed by flow cytometry on a Becton Dickinson FACScan with Lysis II software. In control preparations, conidia were incubated with rabbit pre-immune sera and FITC-labelled antibody to assess background fluorescence.

2.23. Agglutination assay

A. fumigatus conidia were washed twice in PBS-BSA-Ca²⁺ buffer and centrifuged at 3,000 x g for 10 minutes at 4°C. The conidia (10⁵ cells) were then re-suspended in 100 µl of buffer containing rhSP-D at concentration between 1 and 5 µg/ml in siliconized tube (to avoid adherence to the tube walls), incubated at 37°C for an hour, and observed by phase-contrast microscopy. A Zeiss MC camera containing Kodak Tri-X Pan 400 ASA black- and white film was used to photograph the agglutination of the spores in size changes due to agglutination by dot plot analysis of cell forward and side scatter by employing a flow cytometer.

2.24. Killing assay

The percentage killing of the *A. fumigatus* conidia in presence of rhSP-D by the PMNs was assessed by the colorimetric MTT assay (Madan et al, 1997). Control

wells containing the PMNs alone were taken as negative controls, while wells containing conidia alone were taken as positive controls.

2.25. Murine models

Male BALB/c mice (National Institute of Nutrition, Hyderabad, India), weighing 20–22 g each, were housed in polycarbonate shoebox cages. Mice fed on a standard laboratory rodent diet and had water ad libitum. Mice were immunosuppressed by three intradermal injections of 2.5 mg/(mouse day) (125 mg/kg body weight) of hydrocortisone acetate (Wycort) 1 day before, on the day, and the day after conidia challenge, as described previously (Madan et al., 2001a).

2.26. Study design summary

Various groups of mice each were selected and randomised. On the day 0, mice were lightly anaesthetised with ether and 10^8 conidia of Afu in 50 μ l sterile PBS were administered intranasal in the IPA group of mice and 50 μ l of PBS alone in the untreated Control mice. The groups of untreated Control and untreated IPA mice received 50 μ l PBS alone intranasal on day 1. Mice were sacrificed from each group on days 2 and 4 following conidia challenge.

2.27. Measurement of cytokine and chemokine levels in the lung cell suspension

In the case of ABPA mice, the model was generated and characterised on various immunological parameters in the laboratory of Dr Taruna Madan, CSIR Institute of Genomics and Integrative Biology, Delhi, India using protocol described by Madan et al, 2001. For the eosinophil peroxidase assay (EPO), lung cell suspension (200 μ l/well) was plated in a 96-well tissue culture plate and incubated in a humidified CO₂ incubator at 37°C for 48 hours. The medium was aspirated and o-phenylene diamine (OPD) was added (100 μ l of 1 mM solution was prepared using sterile PBS containing 0.1% v/v Triton X-100 and 0.0125% vol/vol H₂O₂). After 30-minute incubation at room temperature, the color reaction was terminated by addition of 50 μ l of 4 N H₂SO₄, and the A₄₉₀ was measured. A portion of the lung tissue from sacrificed animals was minced, the cells were suspended in RPMI-1640 culture medium containing 10% (v/v) heat inactivated foetal calf serum and 10 μ g/ml gentamicin (2×10^6 cells/well), and cultured for 72 h. The supernatants from the lung

suspension were assayed for IL-4, IL-5, TNF- α , and IFN- γ , using the kits from Endogen, Cambridge, MA, USA and R&D. Each cytokine assay was performed twice in triplicate and the final values represent average \pm standard deviation of 4 data points (4 animals of each group).

2.28. Dendritic cell preparation

Monocytes were obtained from either buffy coat (National Blood Centre, Oxford), or on few instances, from voluntary blood donors for which ethical approval has been in place at the Brunel University laboratory. Cells were grown in RPMI medium 1640, supplemented with 2 mM glutamine/50 μ g/ml of kanamycin/1% non-essential amino acids (GIBCO/BRL)/1% pooled human AB serum, 50 ng/ml each of IL-4 (specific activity $> 2 \times 10^6$ units/mg) and granulocyte macrophage–colony-stimulating factor (GM-CSF) (specific activity $> 1 \times 10^7$ units/mg, Schering-Plough) for 6 days, or in serum-free XVIVO-15 medium (BioWhittaker) supplemented with 50 μ g/ml kanamycin and the cytokines listed above. At day 6 of culture, non-adherent immature DCs were harvested and depleted of contaminating lymphocytes with the aid of magnetic beads (Milteny biotech) and anti-CD3 and anti-CD19 mAb (DAKO).

For maturation assays, 1×10^6 purified DCs were incubated with or without 25 μ g/ml of isotype-control, test monoclonal antibody, or with SP-A or SP-D, for at least 3 h at 37°C. DCs were then matured with either 100 ng/ml of LPS (*Salmonella typhimurium*, Sigma), 50 ng/ml of TNF- α , 1 μ g/ml of soluble chimeric CD40L (Alexis) for 48 h or left untreated as a control. All maturation assays in the presence of SP-A or SP-D were performed in parallel in RPMI (with serum) and X-VIVO15 medium (without serum), both supplemented with GM-CSF and IL-4.

2.29. Flow Cytometry

The following antibodies directed against the respective human surface markers were used: these markers include CD3, HLA A,B,C, CD14, CD54, CD19 (Dako); CD80, CD40, CD86, HLA-DR (Serotec), CD83 (PharMingen). Staining of DCs was performed and analyzed by using a flow cytometer (Becton Dickinson). Dead cells were excluded from analysis by using PI.

2.30. T-Cell Proliferation Assays

PBMCs were isolated following standard procedure and re-suspended in serum free medium containing RPMI, 2% FCS, Penicillin/Streptomycin and Sodium Pyruvate. Cells were stimulated with Phytohaemagglutinin (PHA) at a concentration of 1µg/ml and 100,000 cells (100µl) and aliquoted per well in a 96 well tissue culture plate. Next, the cells were treated with SP-A or SP-D in their respective wells. At the 72 h time point ³H-thymidine was added and the plate was pulsed for 16hours. Cells were harvested using a semi-automated cell harvester and the amount of ³H-thymidine incorporated into DNA was measured using a liquid scintillation counter. Each condition was carried out.

With experiments involving T cell clones, DCs were added in increasing numbers up to 1×10^5 T cells and incubated for 5 days. We added 0.5 µCi ³H-thymidine/well for the last 18 h of the culture. For clonal T cell responses, 1×10^6 DCs were pulsed for 6 h with known peptide after maturation. In these experiments, increasing numbers of MHC class II-sharing DCs were then incubated with 3×10^4 T cells for 72 h. Proliferation was measured as above.

2.31. ELISA for supernatant cytokine measurement

Supernatants from DC cultures under the conditions described were collected 24 h after addition of LPS, when TNF-α, IL-12, and IL-10 were at plateau levels. Supernatants from proliferation assays of the T-cell clone were collected after 60 h of culture. The concentrations of IL-4, IFN-γ, TNF-α, IL-12p70, and IL-10 were measured according to the manufacturer's specifications (R & D Systems).

2.32. Statistical Analysis

The relative increase in surface marker expression was calculated by dividing the mean fluorescence intensity (MFI) of LPS-exposed DCs by the MFI of immature DCs after subtracting values obtained with isotype-control antibody. Likewise, the cytokine concentrations in the supernatants of LPS-matured DCs were divided by those of immature DCs after subtracting background values with medium alone. In either case, the increases from at least three independent experiments were compared by using a paired-sample Student's t test.

3. Chapter 3

Interaction of rhSP-D with Influenza A Virus

3.1. Introduction

SP-A and SP-D perform innate immune functions in and outside lungs. SP-A and SP-D deal with various pathogens via agglutination/aggregation, enhancement of phagocytosis and killing. SP-A and SP-D are capable of linking innate immunity with adaptive immunity such as modulation of DC maturation and function, and helper T cell polarization. Here, we have examined the nature of interaction between rhSP-D and a common respiratory viral pathogen i.e. Influenza A Virus (IAV), and subsequent immunological consequences/outcomes.

SP-A and SP-D can induce viral neutralization and enhanced uptake by phagocytes. SP-A and SP-D inhibit hemagglutinin (HA) binding activity of IAV (Benne *et al*, 1995; Hartshorn *et al*, 1994a). SP-D also interferes with neuraminidase (NA) activity (Reading *et al*, 1997). SP-D binds via its CRD region to mannosylated NA and HA (Hartshorn *et al*, 2000; Hartshorn *et al*, 1994b). H1N1 IAV strains are resistant to the neutralization by SP-A (Job *et al*, 2010). SP-A can also interact with herpes simplex virus type 1 (HSV-1) via its N-linked oligosaccharides and enhance phagocytosis by alveolar macrophages (van Iwaarden *et al*, 1992; van Iwaarden *et al*, 1991). SP-A binds F protein of RSV and neutralizes the virus (Sano *et al*, 1999 ; Hickling *et al*, 1999 ; Ghildyal *et al*, 1999 ; Sano *et al*, 2003). SP-D binds to the attachment protein G and inhibits RSV infection in vivo and in vitro (Hickling *et al*, 1999). RSV-infected bronchiolar epithelial cell line express reduced SP-A protein levels (Bruce *et al*, 2009). SP-A binds to HIV-1 gp120 and inhibits infection of CD4⁺ cells, but also enhances the transfer of infection to CD4⁺ T cells mediated by DCs (Gaiha *et al*, 2008). SP-D inhibits HIV replication (Meschi *et al*, 2005). SP-D recognizes the SARS coronavirus spike glycoprotein (Leth-Larsen *et al*, 2007) and has also been found to bind bovine strains of the non-enveloped rotavirus (Leth-Larsen *et al*, 2007; Harrod *et al*, 1999).

It is estimated that infection with seasonal strains of IAV results in the death of 250,000 to 500,000 people and cause five million cases of severe illness per year. Young children, pregnant woman and elderly are more susceptible groups. Recently, the world has experienced major zoonotic transmission to humans of two different types of IAV (Galloway *et al*, 2013). Establishment and spread of highly pathogenic avian H5N1 IAV in birds and coincident infections in humans since 2003 have raised

concerns that we may be facing influenza pandemic (Loeffelholz and Chonmaitree, 2010). Recently, a new swine-origin H1N1 IAV identified in April 2009 (Miller *et al*, 2013) spread worldwide and was officially declared pandemic in June 2009. There are concerns that H1N1 or H3N2 viruses reassert with existing H5N1 virus in birds or pig intermediate host giving rise to more pathogenic viruses. The threat is the potential emergence of a highly pathogenic pandemic virus, as it has already happened in 1918 with a deadly IAV of H1N1 subtype, which killed about 50 million people during a two-year period (Miller *et al*, 2013). It is crucial to understand molecular mechanisms of host defense against IAV in order to design novel strategies to fight against this pathogen. As described earlier, collectins SP-A and SP-D play an important anti-viral role during IAV infection (Hartshorn, 2010). These collectins, expressed in the lung and in a variety of mucosal and epithelial surfaces in the body, are involved in a wide range of immune functions including neutralization, agglutination, opsonisation and clearance of pathogens. SP-A and SP-D have been involved in viral inactivation and aggregation (Hartshorn *et al*, 1994b), enhancement of phagocytosis by macrophages (Hartshorn *et al*, 1994), enhancement of neutrophil superoxidative burst (Hartshorn *et al*, 1997; Hartshorn *et al*, 1996). SP-D has been involved in recognition, neutralization and clearance of respiratory viruses (Nayak *et al*, 2012). Mice lacking either SP-A or SP-D show higher IAV replication, inflammation and mortality rates (LeVine *et al*, 2001; Zhang *et al*, 2002) . In these mice instillation or overexpression of rat SP-D restores anti-viral functions.

Multimerization of human SP-A and SP-D is important for their anti-viral activities (Hartshorn *et al*, 1996). Recently, investigators have shown that substitution of arginine343 with alanine amino acid in the CRD region enables a truncated trimeric. Human SP-D has been shown to mediate viral uptake by neutrophils and monocytes and to potentiate neutrophils respiratory burst responses (Hartshorn, 2010). Cross-linking of truncated mutated human SP-D with specific monoclonal antibodies could mimic multimerisation and enhanced viral aggregation (Hartshorn, 2010). N-linked oligosaccharides on the HA and NA envelope glycoproteins of IAV are recognized by CRD region of SP-A and SP-D. Glycans exposed on the globular head of HA, which greatly vary in location and numbers between IAV strains, are responsible for this interaction (Wagner *et al*, 2000 ; Abe *et al*, 2004). Recently, the role of glycosylation

on the head of H1 subtype IAV in modulating sensitivity to SP-D in vitro and virulence in mice has been analyzed (Tate, Brooks and Reading, 2011). For this study, site-directed mutagenesis was used to add or delete specific glycosylation sites on H1HA and produce reassortant viruses. HA glycosylation profile, and mainly HA Asn 144, was shown to be a critical factor in sensitivity to SP-D, neutralization by mouse lung fluids and disease in mice. In addition to agglutination and opsonisation, binding of collectins to HA and NA also inhibit HA-dependent early stage of infection in vitro and NA activity (Teclé *et al*, 2007), respectively. However, the exact mechanism of IAV inhibition by collectins is not yet fully understood. SP-D and mannose binding lectin (MBL) seem to inhibit early phases of IAV infection by blocking viral uptake and/or HA-mediated fusion (Teclé *et al*, 2007 ; Leikina *et al*, 2005). SP-D mediated agglutination may also contribute to reduction of infectious particle numbers (Hartshorn *et al*, 1994a). In this study, we wish to better characterize the molecular mechanisms of neutralization of IAV by rhSP-D.

3.2. Results

3.2.1. Expression in *E. coli*, purification and characterization of a recombinant fragment of human SP-D (rhSP-D) composed of neck and CRD region.

The recombinant human surfactant protein (rhSP-D) was expressed and purified in *E. coli* as described in the methods and materials (section 2.3). Following transformation, a pilot expression was carried out to examine the presence of rhSP-D. Samples were collected before and after induction with IPTG (figure 3.1) for 3 hours and run as described in (methods section) on a SDS-PAGE gel as shown below in (figure 3.1) in order to visualize the presence of rhSP-D, which was confirmed by the presence of rhSP-D band at ~20kD as also previously demonstrated by (Mahajan *et al.*, 2008). After successfully expressing rhSP-D, the time course of IPTG needed to be ascertained in order to find out the optimum time point at which there is the greatest induction with IPTG. This was achieved by removing samples at various time points after induction, varying from 0 hrs to 5 hrs, where the sample was collected after each hour. The samples obtained after each time point were loaded onto an SDS-PAGE gel as shown below in (figure 3.1). From the gel the best protein expression can be observed three hours after induction with

0.4 mM IPTG, where the rhSP-D accumulated as an over-expressed protein of ~20 kDa.

Upon confirmation of rhSP-D protein expression, the protein was subsequently purified as described in (Method section). The refolded protein, obtained after stepwise urea dialysis, was passed through affinity chromatography column (containing Maltose Sepharose) which was also prepared as also described in Chapter 2. The eluted fractions were collected in 1.5ml Eppendorf tubes and an SDS-PAGE gel was run as shown below in (figure 3.1). To show the presence of purified rhSP-D protein as shown below. From SDS-PAGE gel it appears that the highest protein concentrations were eluted into first five fractions.

Bacterial LPS was removed by following the method described, where the purified rhSP-D protein was passed through a column containing, Polymyxin B agarose gel. Following endotoxin removal the LPS free rhSP-D fractions were pooled together and SDS-PAGE (15% w/v) was run as shown below in (figure 3.2) to further confirm the presence of purified rhSP-D after LPS removal.

The BCA Protein Assay was used as described in Chapter 2 (section 2.9.) for the colorimetric detection and quantitation of total protein following the purification of rhSP-D. The amount of protein was also further quantified by measuring the UV absorbance at 280 nm. The highest fractions from each of the purified batches were pooled together separately and then prepared using BCA protein assay reagent, as detailed in (section 2.9.) of materials and methods Chapter 2. The OD values were then determined at 562 nm for both batch 1 and batch 2 pooled samples and were found to be at 1.33 and 1.18, respectively. The protein concentration of each sample was then determined by extrapolation using the plotted standard curve for BSA. From the graph, it was determined that the concentration for batch 1 was approximately 1.3mg/ml, whereas the concentration for batch 2 was found to be approximately 1.1 mg/ml.

The endotoxin level in the protein preparation was assessed by QCL-1000 Limulus amoebocyte lysate system (Lonza). The assay was linear over a range of 0.1–1.0 EU/ml (10 EU=1ng of endotoxin). From the standard graph obtained, a line of best fit was drawn and an equation was derived for this line of best fit. Thus from this

equation, an approximate value for endotoxin content was estimated in the purified rhSP-D preparation, which was found to be in the region of 2.965EU/ml.

Since previous cross-linking studies have indicated that rhSP-D exists predominantly as a trimer in solution, a BS3 Cross-linking Assay, as described under materials and methods, was carried out in order to observe the trimerization of rhSP-D. As it can be seen below in (Fig 3.1) the rhSP-D was incubated with various concentrations of BS3 cross linker in order to determine the best concentration of BS3 cross-linker, which shows the trimerization of rhSP-D.

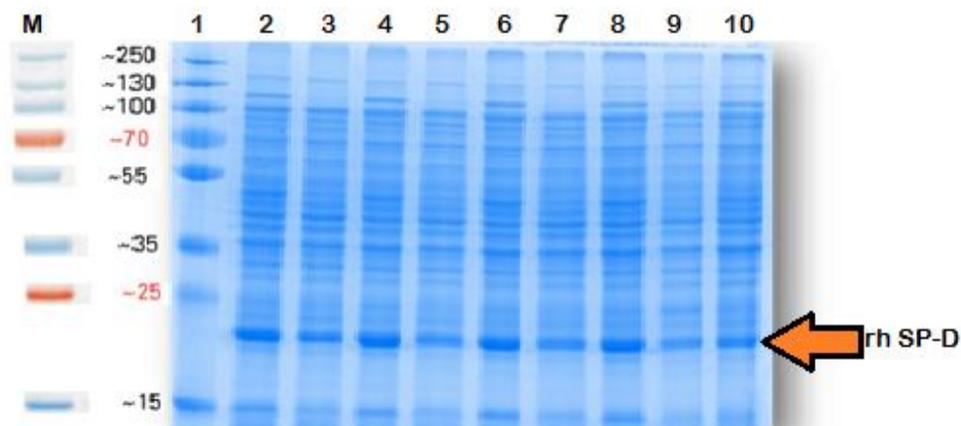


Figure 3.1 SDS- PAGE (15%) showing rhSP-D, which was expressed as inclusion bodies using *Escherichia coli* BL21 (DE3) pLysS. After 3 hours of induction with IPTG, the rhSP-D over-expressed as evident by 20 KDa band. a) Lanes 1,3,5,7,9 b) Lanes 2,4,5 compared with uninduced cells a) Lanes 2,4,6,8 b) Lanes 1,3,6,7,8, Maker used: PageRuler Plus Prestained Protein Ladder.

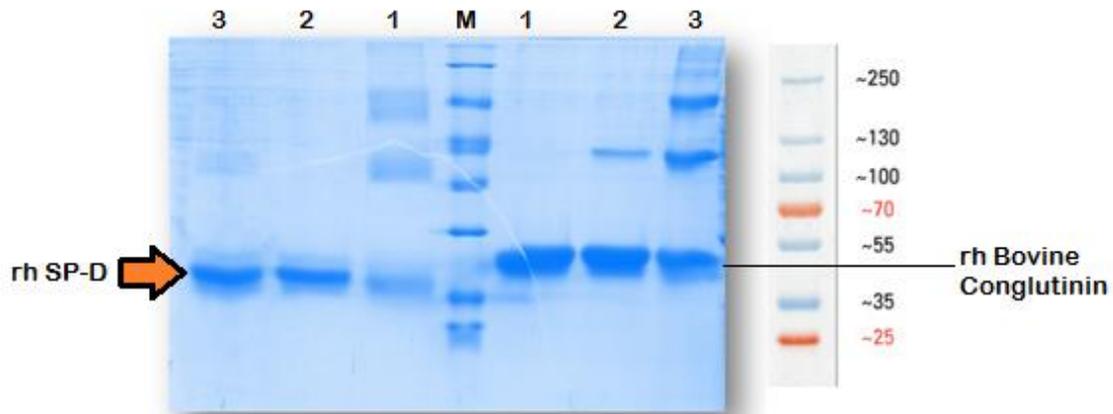


Figure 3.2 SDS-PAGE (12% w/v) analyses of rhSP-D and rh Bovine conglutinin after purification. Cross-linking of protein interactions among themselves to know if they form dimers and trimers to confirm the interactions using the non-cleavable NHS-ester cross-linker bisulfosuccinimidyl suberate (BS3) in dose-dependent manner (0.5, 1 and 10 mM BS3 concentrations respectively).

3.3. rhSP-D binds IAV in solid-phase direct binding ELISA

Since rhSP-D was able to inhibit haemagglutination of IAV, we set out to establish possible interaction of rhSP-D with HA and NA using H3N2 envelope proteins. The recombinant rhSP-D bound envelope comprising hemagglutinin and neuraminidase proteins in a dose- and calcium-dependent manner, suggesting the importance of glycosylation and CRDs in this interaction (figure 3.3). The direct binding ELISA data was validated by far-western blot that established protein-protein interaction between rhSP-D and IAV proteins (figure 3.4). These binding data appear to suggest that direct interaction of rhSP-D with H3 and N2 proteins is likely to either interfere or facilitate viral infection of the target cells.

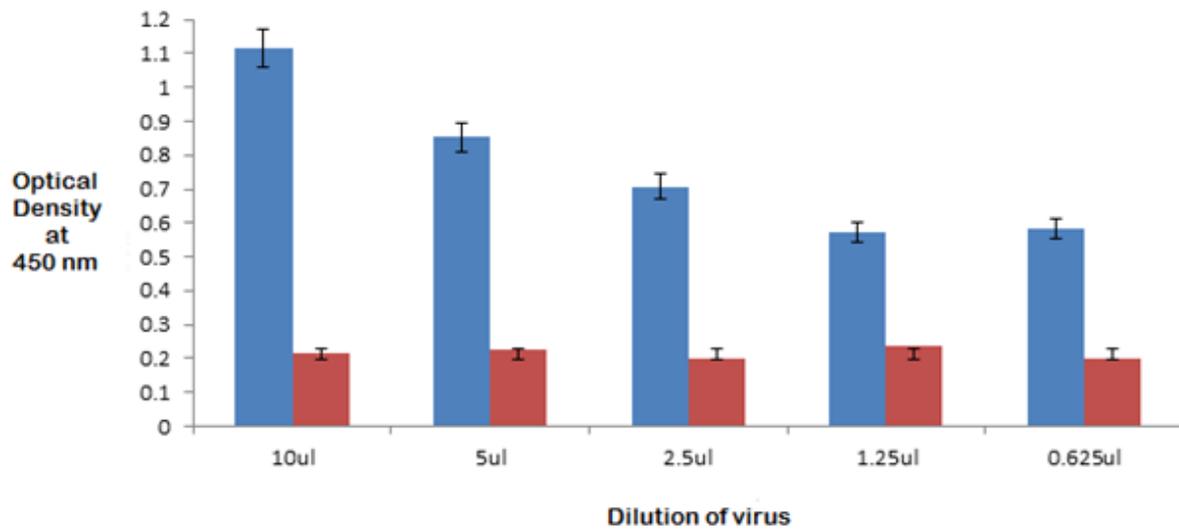


Figure 3.3 Interaction between rhSP-D and H3N2 in a direct binding ELISA. The rhSP-D in constant 5ug/100ul/well dilution was coated overnight at 4 degree C° using multi-sorb plates. The following day, blocking of the non-bound sites with 2% BSA in PBS was performed. Following extensive washing with PBS, H3N2 was added at different dilutions in PBS (10ul, 5ul, 2.5ul, 1.25ul, 0.625ul) per well. The virus was left to incubate with the proteins at 37 degrees for 1.5 hours. The excess unbound virus was washed off using PBS. A polyclonal primary antibody, goat anti-human anti-Udorn antibody, against the virus was used at a dilution of 1:5000 and added to each of the wells. Following incubation for 1 hour at 37 degrees C, excess antibody was washed off with PBS and the secondary antibody (anti-goat protein A conjugated to horseradish peroxidase) was added to the wells. After incubation for one hour at 37 degrees C, the excess secondary antibody was washed off and the colorimetric reaction was developed with OPD.

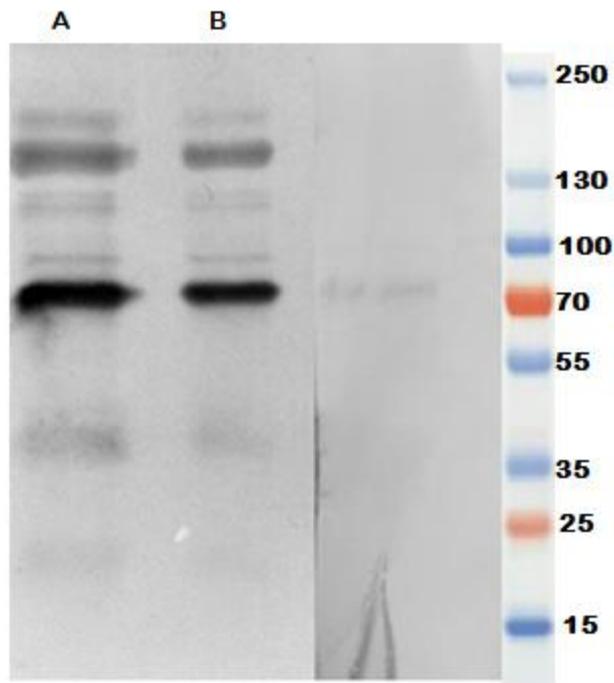


Figure 3.4 Binding of H3N2 virus with rhSP-D. H3N2 virus (2 dilutions) was denatured and viral proteins were separated by SDS-PAGE. The blot was probed with anti-H3N2 polyclonal antibodies, or with rhSP-D (10µg/ml). Purified H3N2 virus was run on SDS-PAGE, and H3N2 viral proteins were detected using anti-Udorn antibodies. rhSP-D binds H3N2 virus proteins, when probed with anti-human SP-D polyclonal antibodies (1:5000). (B) Pure H3N2 was run on gel and probed with Anti-Udorn antibodies, followed by goat anti mouse IgG-HRP conjugate to detect H3N2.

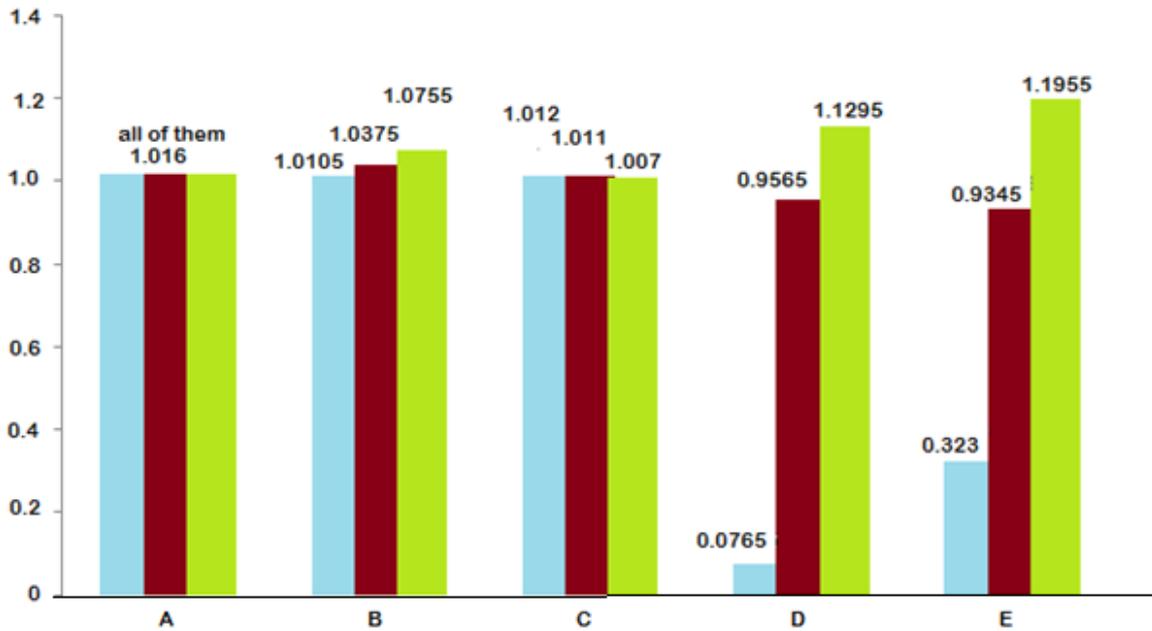


Figure 3.5 Cell binding assay: The rhSP-D inhibits H3N2 interaction with THP-1 cells. Wells were treated with Poly L-Lysine and THP-1 cells (50,000) per well were added in serum free conditions After blocking with 2% w/v BSA for 1 hour at 37 degrees C. A 1/10 dilution of the virus was used as 40 μ l in 400 μ l total (50,000 pfu/ μ l). In one set of experiments, virus was pre-incubated with rhSP-D (20 (grey), 10 (brown) and 5 (green) μ g) for 1 hour at room temperature and added to the THP-1 cells (D). In the second set, cells were pre-treated with rhSP-D for 1 hour at room temperature and then challenged with virus (E). Virus alone (A) and BSA as a control protein (B) and (C) were used as negative control. Excess virus was washed with cold PBS; cells were fixed with Paraformaldehyde, washed with PBS again, and then blocked for 1 hour in PBS/BSA. Primary anti-H3N2 antibody in PBS was added, and then washed with PBS. The secondary antibody IgG HRP in PBS was used as a probe. The y axis represents OD405nm after the development of the colour.

3.4. Multiplex cytokine array analysis showed downregulation of pro-inflammatory cytokine TNF- α and IL-6 by rhSP-D treated virus

To understand the immune response following viral challenge against THP-1 cells, H3N2 (A/Hong Kong/99) virus was incubated with rhSP-D at MOI 1 (THP-1) and supernatant was collected at 24 and 48 h time points (Figure 3.6) and subjected to multiplex cytokine/chemokine array analysis. Cytokines (IL-6, IL-8, IL-10, IL12p40, IL12p70, IL-23, IL-27, IL-1 α and IL-1 β) and chemokines/growth factors (MIG, I-TAC, MCP-1, G-CSF and M-CSF) concentrations in the supernatants of THP-1 cells were measured by MagPix Milliplex kit (EMD Millipore). Cells were incubated with rhSP-D-bound and unbound IAV, spun down at 300g, and supernatants were stored at -80°C until use. Briefly, 25 μ l of assay buffer was added to each well of a 96-well plate, followed by addition of 25 μ l of standard, controls or supernatants of cells, to appropriate wells. 25 μ l of magnetic beads coupled to analytes were added in each well, and incubated for 18h at 4°C. After washing the plate with assay buffer, 25 μ l of detection antibodies were incubated with the beads for 1h at RT. 25 μ l of Streptavidin-Phycoerythrin (PE) was then added and incubated for 30 min. Following a washing step, 150 μ l of sheath fluid (BD Biosciences) was added to each well and the plate was read using the Luminex Magpix instrument.

Most cytokines and chemokines did not show any considerable alteration in their secreted levels. However, there was an important downregulation of TNF- α and IL-6 in the instance where IAV was pre-treated with rhSP-D (figure 3.6), suggesting that the presence of rhSP-D inhibited production and secretion of these two crucial pro-inflammatory factors following viral challenge.

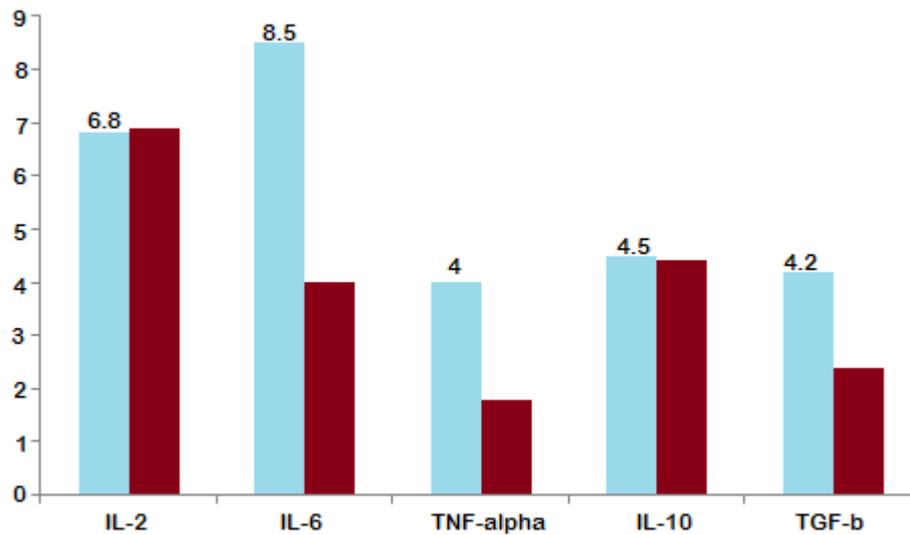


Figure 3.6 Multiplex cytokine array analysis of supernatants of THP-1 cells treated with rhSP-D bound or unbound IAV (following 24hour challenge). Changes shown in fold compared to THP-1 cells alone. The Yaxis represents the amount of cytokines in pg/ml.

3.5. Discussion

It is estimated that infection with seasonal strains of IAV results in the death of up to 0.5 million people and caused 5 million cases of severe morbidity per year. Young children, pregnant woman and elderly are more susceptible groups. Recently, the world has experienced major zoonotic transmission to humans of two different types of IAV (Galloway *et al*, 2013). Establishment and spread of highly pathogenic avian H5N1 IAV in birds and coincident infections in humans since 2003 have raised concerns that we may be facing influenza pandemic (Loeffelholz and Chonmaitree, 2010). Recently, a new swine-origin H1N1 IAV identified in April 2009 (Miller *et al*, 2013) spread worldwide and was officially declared pandemic in June 2009. There are concerns that H1N1 or H3N2 viruses re-assort with existing H5N1 virus in birds or pig intermediate host giving rise to more pathogenic viruses. The threat is the potential emergence of a highly pathogenic pandemic virus, as it has already happened in 1918 with a deadly IAV of H1N1 subtype, which killed about 50 million people during a two-year period (Miller *et al*, 2013). It is crucial to understand molecular mechanisms of host defence against IAV in order to design novel strategies to fight against this pathogen.

Pattern recognition receptors and soluble factors of innate immunity play a very important role in modulating viral infection and subsequent cellular response to viral recognition and intracellular existence. Locally synthesized soluble humoral factors such as SP-D are likely to play a far important role in dealing with IAV in the lungs. Thus, we examined a likely interaction of a truncated SP-D molecule that has previously been well characterized biochemically, biophysically, *in vitro* and *in vivo* using with various strains of IAV.

Initially, a range of IAV strains were selected based on their pathogenesis, epidemic history and pandemic features. These strains were subjected to a simple but powerful hemagglutination assay using guinea pig RBCs which are normally agglutinated by viral incubation in a titre dependent manner. Molecules or factors, which inhibit viral attachment to RBCs reduce the degree of agglutination. We then used direct binding ELISAs using pandemic H3N2 strain to examine if rhSP-D could interact directly with virus via its two most important envelope proteins and virulence factors. In order to identify viral proteins globally that would interact with rhSP-D, protein-protein interaction studies via far western blotting were carried out in which virus was run on an SDS-PAGE and transferred blot was probed with rhSP-D. As a positive control, the same blot was probed with anti-Udorn antibodies that would recognize all viral proteins. We also examined the immune response of THP-1 cells following viral challenge in the presence or absence of rhSP-D. Our results demonstrate a direct interaction between rhSP-D and human IAV viruses of diverse H3N2 subtypes. In the case of H3N2 pre-incubation of rhSP-D with IAV led to downregulation of TNF- α and IL-6, compared to virus alone challenge. This, coupled with inhibition of IAV binding to THP-1 cells, suggests that rhSP-D is able to recognize HA and NA differentially due to varying levels of carbohydrate sialic acid and charge patterns. This is an area that needs further investigation. It is likely that rhSP-D will interfere with TLR IAV interaction given reduced production of TNF- α .

In summary, rh SP-D by virtue of being produced or present locally in the lungs is capable of modifying host-IAV interaction. This interaction between IAV and rhSP-D restrains virus replication in target cells. Currently, we are hoping to identify the exact step of the virus life cycle affected by rhSP-D such as virus binding, endocytosis, and viral RNA uncoating, and consequences for pathogenesis and stimulation of the host inflammatory response.

4. Chapter 4

Interaction of rhSP-D with *Aspergillus fumigatus*

4.1. Introduction

Aspergillus fumigatus (Afu) is a major airborne fungal microorganism with manifestations dependent on the host's immune status. *A. fumigatus* is an opportunistic pathogen, which causes allergic disorders in the immuno-competent host such as allergic rhinitis, allergic sinusitis, hypersensitivity pneumonitis, and allergic bronchopulmonary aspergillosis (ABPA). Invasive pulmonary aspergillosis (IPA) occurs frequently in immunocompromised or immunosuppressed patients, notably in patients with cystic fibrosis, AIDS and asthma, and often proves fatal despite treatment with antifungal drugs. *Aspergillus* spp. is the major fungal pathogens in immunocompromised, and neutropenic patients with mortality rates in excess of 50% in treated patients. Despite advances in antifungal therapy mortality rates following invasive aspergillosis even after treatment remain unacceptably high. Alternative approaches to therapy are therefore required. Infection with *A. fumigatus* is characterized by presence of both type I and type III hypersensitivity reactions leading to increased levels of total IgE, specific IgE (*A. fumigatus*- IgE), specific IgG (*A. fumigatus*-IgG), and blood and pulmonary eosinophilia (Patterson, 1998). In the absence of adequate host immunity, as in severely immunocompromised or neutropenic patients, because of chronic glucocorticoid treatment, AIDS, aplastic anaemia, or chemotherapy-induced neutropenia (Sternberg, 1994), *A. fumigatus* disseminates to other tissue causing systemic and fatal form of disease, called invasive aspergillosis, characterised by hyphal invasion and destruction of pulmonary tissue.

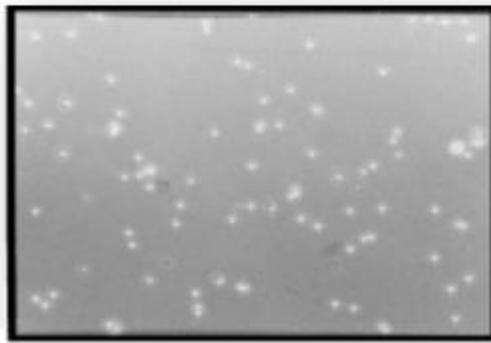
Alveolar macrophages and recruited neutrophils are considered to clear *A. fumigatus* from the lung, which appears to be principal portal of entry of this pathogen (Bardana, E.J., Jr 1982). Neutrophils, various macrophages and polymorphonuclear cells (PMNs) can kill *A. fumigatus* conidia and damage hyphae (Schaffner, 1982; Levitz, 1986; Roilides. 1993). When exposed to *A. fumigatus* conidia *in vitro*, cells of macrophage lineage secrete TNF- α and IL-1 (Taramelli, 1996) as well as cytokines that promote a Th1 response (Grazziutti, 1997). However, *A. fumigatus* conidia can be resistant, to some degree, to reactive oxygen intermediates generated during phagocyte stimulation (Bardana, 1982).

Both SP-A and SP-D bound to carbohydrate structures on *A. fumigatus* conidia in a calcium- dependent manner, causing agglutination of the conidia and enhancing the binding of conidia to alveolar macrophages and neutrophils (Madan, 1997) (figure 4.1). Furthermore, in the presence of SP-A and SP-D, phagocytosis, oxidative burst and killing of conidia were significantly increased by neutrophils (figure 4.2). These findings strongly suggest that SP-A and SP-D may have an important immunological role in the early anti-fungal defense in the lung, through inhibiting infectivity of conidia by agglutination and by enhancing uptake and killing of *A. fumigatus* by phagocytosis. Mice model of IPA intranasally given therapeutic SP-D showed higher survival following a fatal challenge with *A. fumigatus* conidia, as evident from with lower CFU counts, diminished hyphal growth, and elevated lung IFN- γ and TNF- α (figure 4.3) (Madan,T. 2001). Intranasal administration of rhSP-D was protective in a murine model of invasive pulmonary aspergillosis both, in terms of reduced organ burden and improved survival. When a range of full-length and truncated SP-D proteins was given therapeutically to the IPA murine model, 10 μ g was found to be optimal for the observed protective effects (Singh *et al*, 2009).

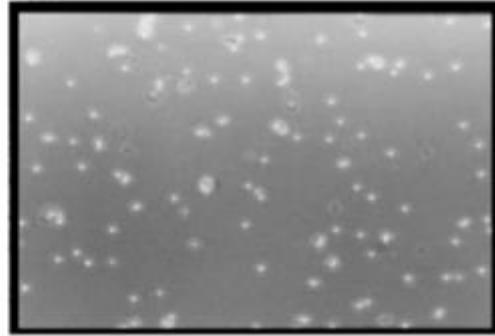
SP-D gene-deficient (SP-D^{-/-}) mice were also more susceptible to IPA, while SP-A gene-deficient (SP-A^{-/-}) mice acquired resistance to IPA (Madan et al., 2010), suggesting that SP-A may facilitate pathogenesis by *A. fumigatus*. *A. fumigatus*-challenged SP-A^{-/-} mice showed less mortality (40%) than the wild-type 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 wild-type 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 (fig 4.5). Treatment with SP-D or rhSP-D fragment reduced the mortality to 50 and 33%, respectively, consistent with higher IFN- γ to IL-4 ratios in treated SP-D^{-/-} mice, compared to untreated control group. The results showed that SP-D^{-/-} mice are more susceptible to IPA while SP-A gene-deficient mice acquire resistance to IPA (Madan et al., 2010).

In addition, SP-D can also interact, via their carbohydrate recognition domains (CRDs), with the carbohydrate residues present on the allergens/antigens of *A.*

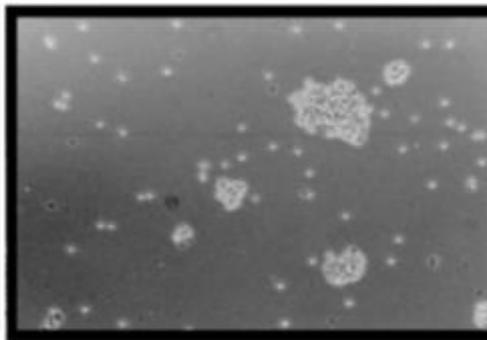
fumigatus and interfere with IgE-mediated release of histamine by sensitised basophils (Madan,1997). In the present study, we have expressed and extensively characterized a recombinant fragment of human SP-D (rhSP-D), composed of trimers of the neck region and carbohydrate recognition domains, in *E. coli* and examined whether this truncated form of human SP-D retains the immunomodulatory properties displayed by full-length SP-D against *A. fumigatus*. The rhSP-D bound *A. fumigatus* conidia and enhanced its phagocytosis by polymorphonuclear cells (PMNCs). The rhSP-D could also inhibit the binding of *A. fumigatus*-IgE to the allergens/ antigens from the three-week culture filtrate (3wcf) of *A. fumigatus* and blocked subsequent histamine release from sensitized basophils, isolated from a murine model of ABPA. The rhSP-D also suppressed the lymph proliferation of mouse splenic cell cultures of ABPA mice. Following incubation with the rhSP-D, the splenic supernatants of the ABPA mice showed a significant decrease in the levels of IL-4 and IL-5 (characteristic of a Th2 response) and an increase in the levels of IFN- γ (Th1 response), whereas an increase in the levels of TNF- α in the case of IPA mice was observed. These results suggest that even a truncated form of rhSP-D, lacking collagen and N-terminal regions, can have a significant immunomodulatory effect against *A. fumigatus* in vitro and implicate trimeric CRDs of human SP-D as a self-sufficient molecule. In addition, we have also compared two variants of rhSP-D: one contains 8 Gly-X-Y repeats of the collagen region (called Gly-X-Y rhSP-D) and the other is only neck and CRD region (called rhSP-D delta). We found that both variants of rhSP-D have the ability to inhibit fungal growth.



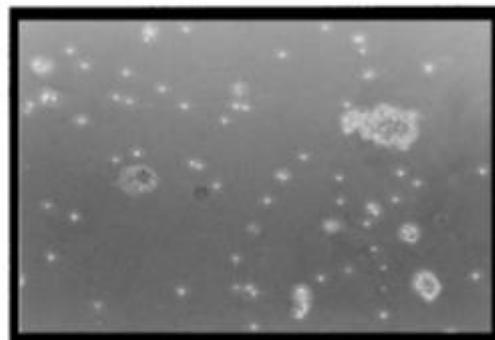
Non- treated



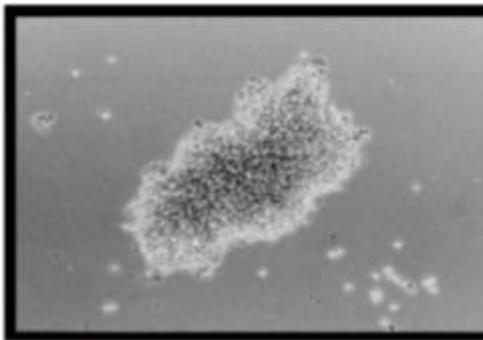
BSA- treated



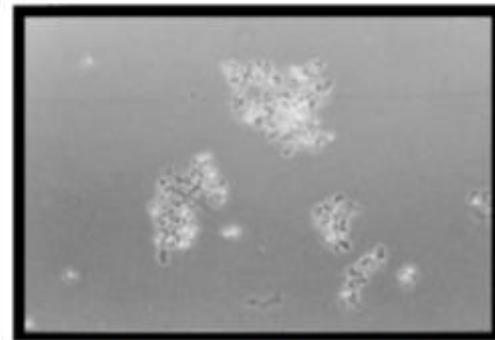
SP-D treated 1micro g/ml



SP-A treated 1 micro g/ml



SP-D treated 10 micro g/ml



SP-A treated 10 micro g/ml

Figure 4.1 Agglutination of *A. fumigatus* conidia before and after incubation with SP-A or SP-D at two concentrations of the proteins. SP-D appear to cause massive agglutinates compared to SP-A, probably due to its cruciform structure as well as ball-like overall multimeric structures. BSA as a negative control protein fails to cause agglutination of *Aspergillus fumigatus* conidia (Madan *et al*, 1997)

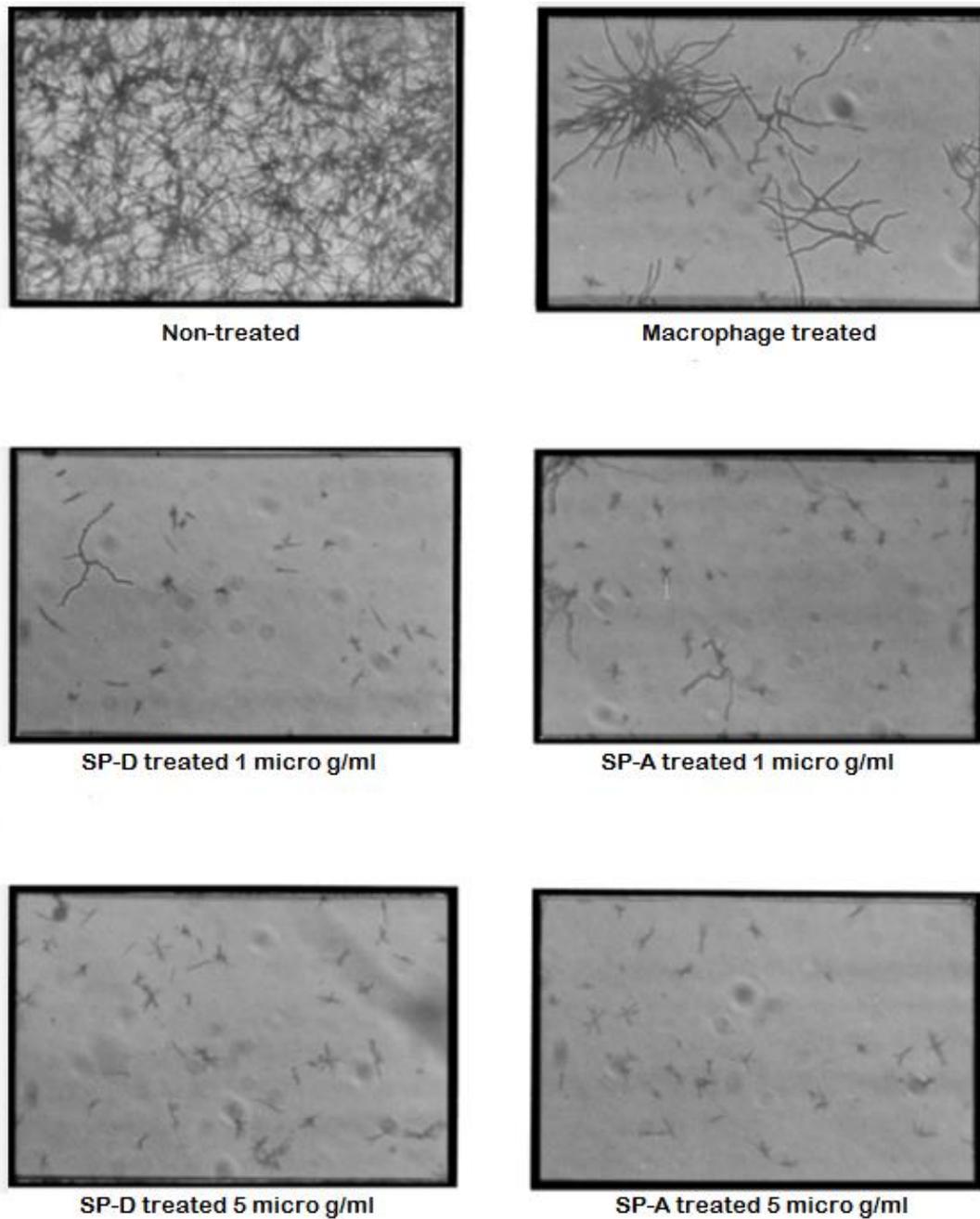


Figure 4.2 Killing of *A. fumigatus* conidia by alveolar macrophages. Germinated hyphae appear resistant when exposed to macrophages alone. However, the presence of SP-A or SP-D in the presence of calcium for 60 minutes appears to fragment the hyphae of *Aspergillus fumigatus*. The untreated hyphae grow on to form mycelial structures (Madan *et al*, 1997).

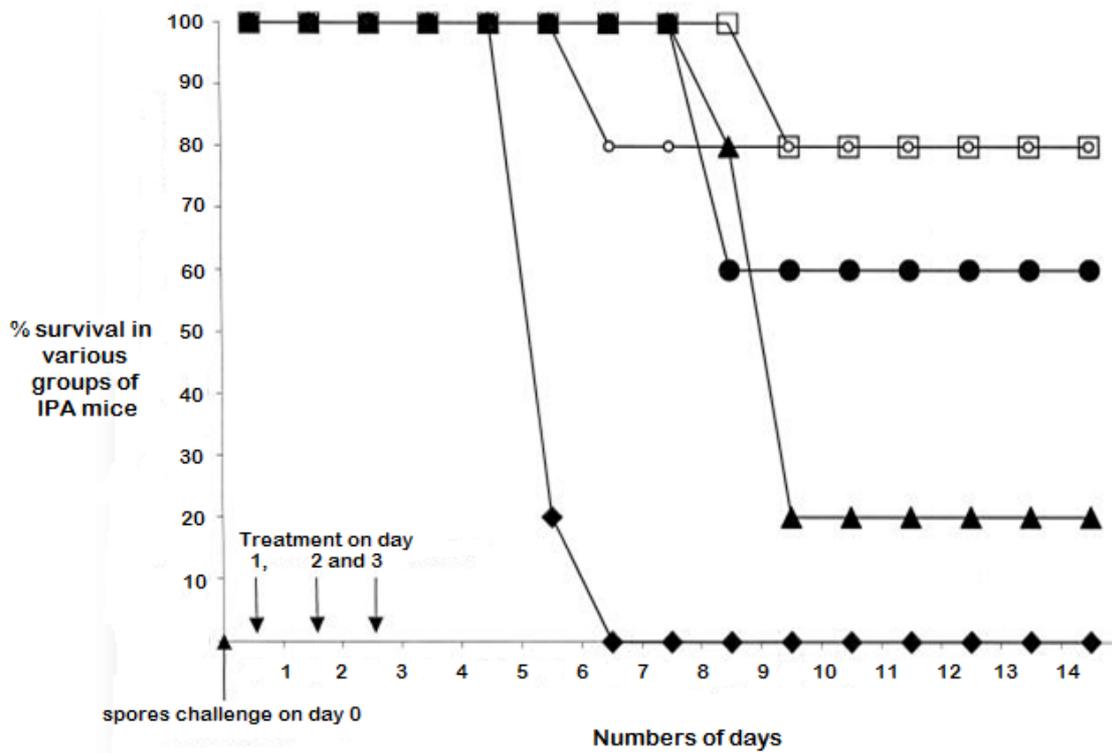


Figure 4.3 The survival percentage of the IPA mice over 15 days after challenge with 10^8 spores of *A. fumigatus* on day 0 and treatment with PBS, Amphotericin B, SP-A, SP-D, and rhSP-D on day 1 (Madan *et al*, 2001). Empty and filled Squares represent Amphotericin B and rhSP-D treatment; empty circles represent SP-D treatment; while triangles and diamonds represent SP-A and untreated groups, respectively.

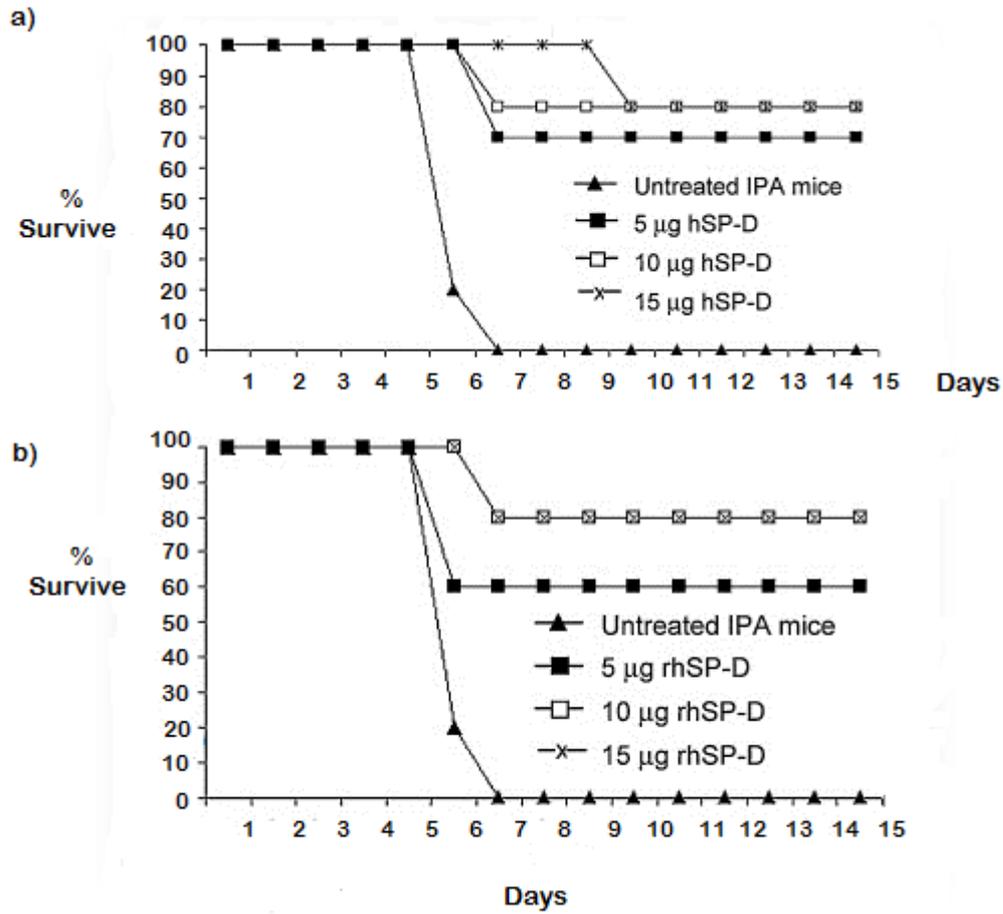


Figure 4.4. The protective effect of various doses of full-length and truncated SP-D in an IPA model. The survival percentage of the IPA mice over 15 days following challenge with (10^8) *A. fumigatus* conidia on day 0, and then treated with PBS; (A) full-length human SP-D and (B) a rhSP-D (5, 10 or 15 g) on day 1 (24 h following conidia challenge) (Singh *et al*, 2009).

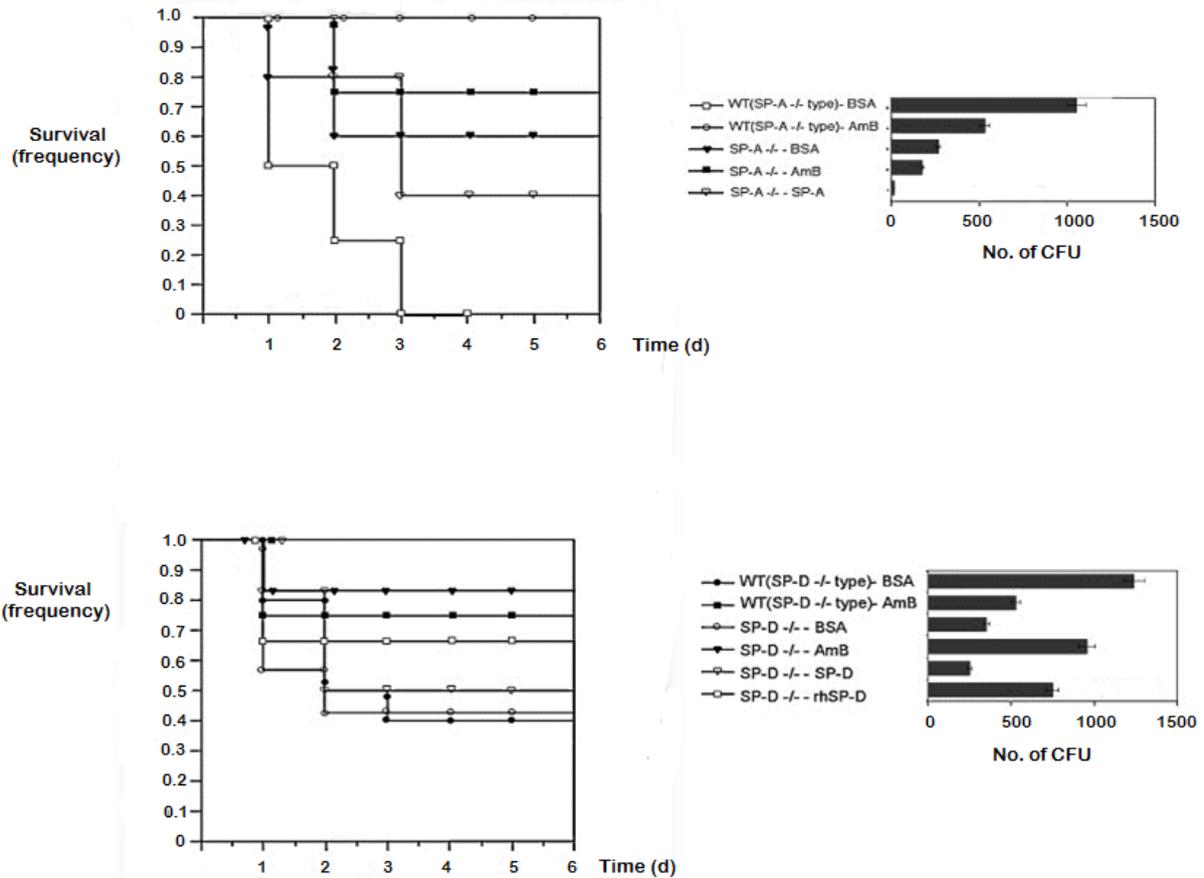


Figure 4.5 Mortality in various groups of mice. Mice were immunosuppressed with corticosteroid prior to an intranasal challenge with *A. fumigatus* conidia. (A) SP-A gene deficient mice (SP-A^{-/-}) mice and (B) SP-D gene deficient (SP-D^{-/-}) mice compared with their respective wild type (WT) mice. The bar graphs depict the lung CFU counts isolated from the above-mentioned groups on day 2 of the study (Madan *et al*, 2010)

4.2. Result

4.2.1. The rhSP-D can bind *A. fumigatus* conidia and enhance its phagocytosis and killing by PMNs

The rhSP-D showed dose-response binding to conidia in the presence of calcium which could be inhibited by EDTA or maltose, indicating involvement of the CRD. When FITC labelled conidia were incubated with leukocytes in the presence of rhSP-D there was a dose responsive increase in uptake of spores with a 3-fold increase observed at 5 µg/ml. A 2.5-fold increase was observed for native human SP-D. The positive control of autologous serum (10%) also resulted in a 2.5 fold increase in mean fluorescence intensity.

The rhSP-D showed a dose responsive increase in killing of conidia by phagocytosis. As expected, phagocytes alone brought about killing (42.36%), but this was increased 2-fold by the addition of 5 $\mu\text{g/ml}$ rhSP-D which was similar to that reported for native human SP-D.

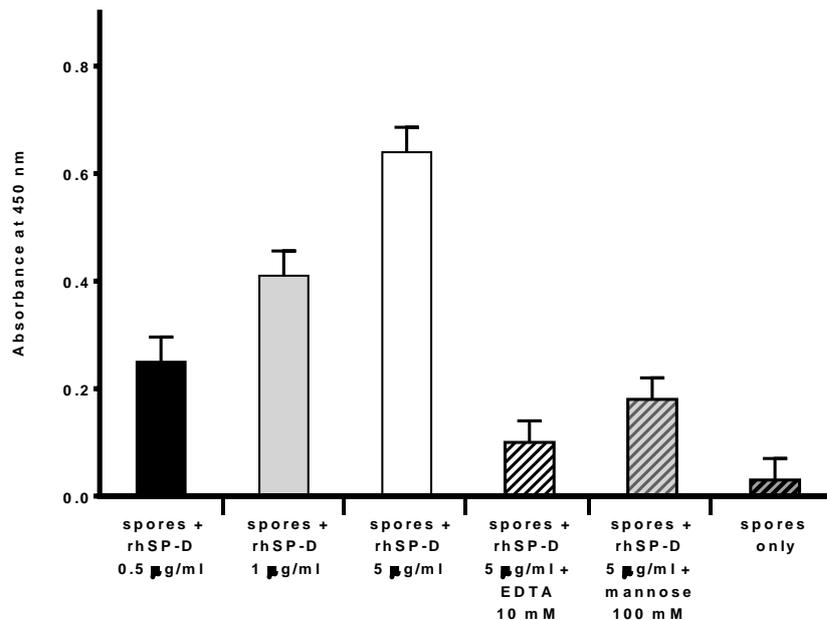


Figure 4.6 Direct binding of solid-phase conidia to rhSP-D with and without sugar and calcium. *A. fumigatus* conidia (10^8) was coated on maxisorb microtitre wells overnight and then blocked with 2% BSA in PBS. rhSP-D was added to the wells for 2 hrs at RT and allowed to bind the spores with and without mannose and EDTA. Anti-SP-D polyclonal antibodies were then probed with protein A-HRP conjugate.

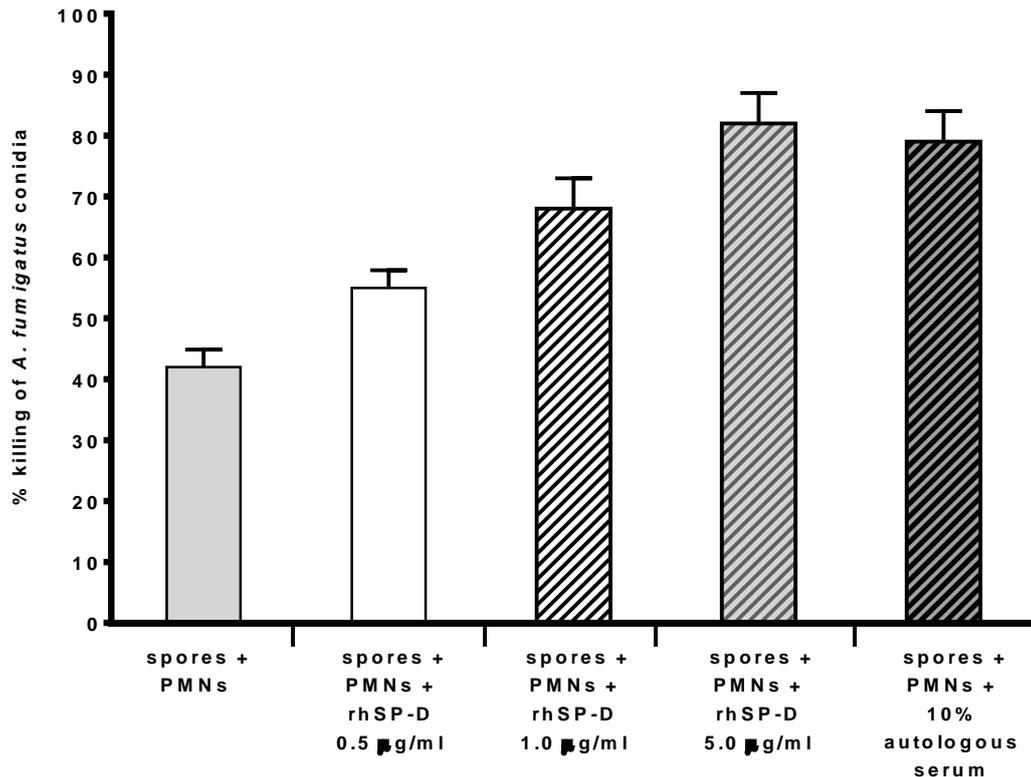


Figure 4.7 Percentage killing of *A. fumigatus* by PMNs at various concentrations of rhSP-D as measured by MTT colorimetric assay, as in Madan et al, 1997a.

4.2.2. Binding of rhSP-D to 3wcf allergens/antigens can block IgE-induced histamine release from sensitised basophils

The allergens/antigens of *A. fumigatus* showed a calcium dependent and dose responsive binding similar to that of native human SP-D. The inhibition of binding with 10 mM EDTA and 100 mM maltose indicated that the CRD region were responsible for binding to the glycosylated allergens and antigens in the 3wcf. The inhibition of the binding of *A. fumigatus* specific IgE to 3wcf allergens by rhSP-D is shown in figure 4.8. The rhSP-D mediated IgE binding inhibition was dose responsive, with over 70% inhibition at 5 µg/ml compared to over 90% inhibition observed with native human SP-D at 5 µg/ml. The sensitised basophils extracted from the whole blood of these mice showed a 3-fold decrease in histamine release in the presence of 5µg/ml rhSP-D, which was as in the same range as seen with native human SP-D.

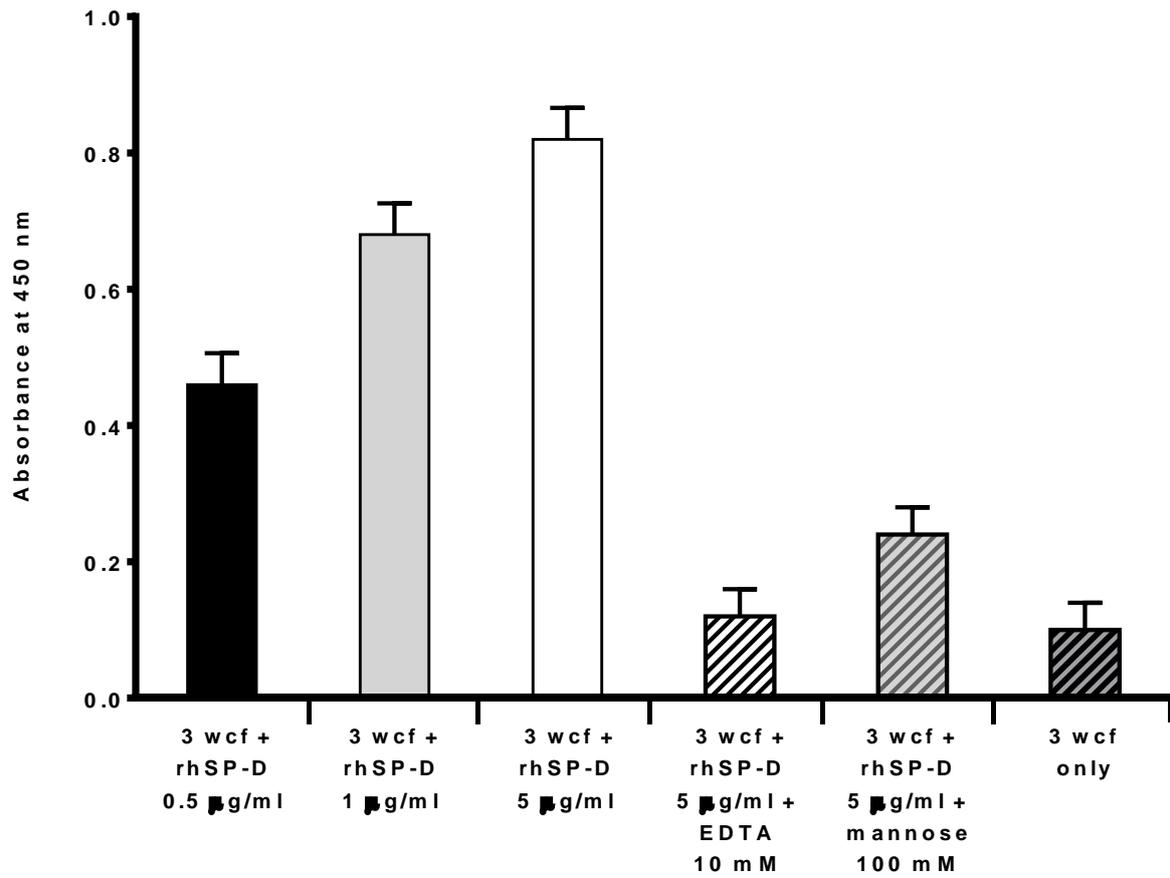


Figure 4.8 Binding of rhSP-D to 3wcf allergens/antigens of *A. fumigatus* in the presence of various concentrations of rhSP-D with and without its inhibitors, EDTA and mannose. rhSP-D bound to allergens/antigens were probed with primary and secondary conjugates as described above.

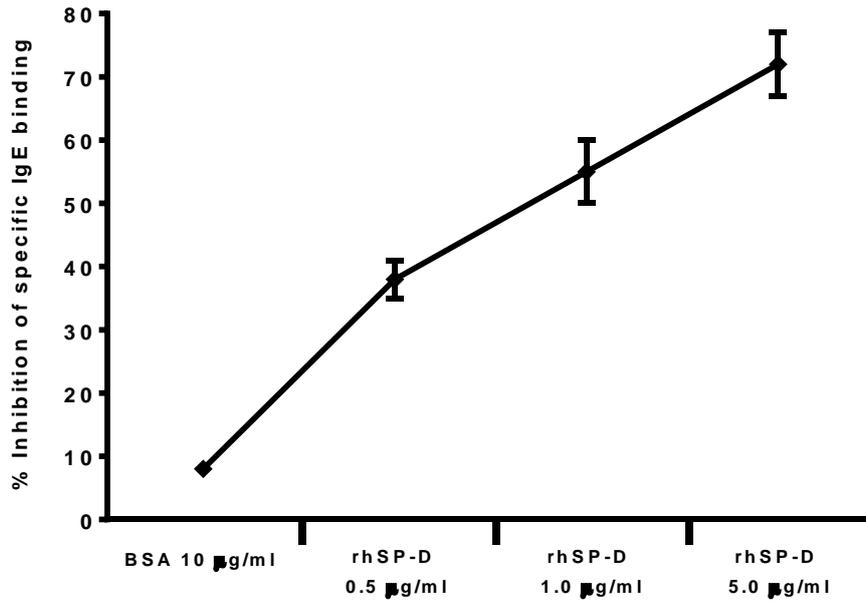


Figure 4.9 rhSP-D inhibits IgE binding to immobilized 3wcf antigens/allergens. *A. fumigatus* 3wcf (5 ug/ml) was coated on maxisorb microtitre wells overnight and then blocked with 2% BSA in PBS. rhSP-D was added to the wells for 2 hrs at RT and allowed to bind the allergens for 2 hr in the presence of 1/10 dilution of ABPA patients' pooled sera. Anti-human IgE conjugated to HRP was then used to assess the level of IgE binding to allergens. The pooled sera derived from ABPA patients were provided by Dr Taruna Madan, CSIR Institute of Genomics and Integrative Biology, Delhi, India.

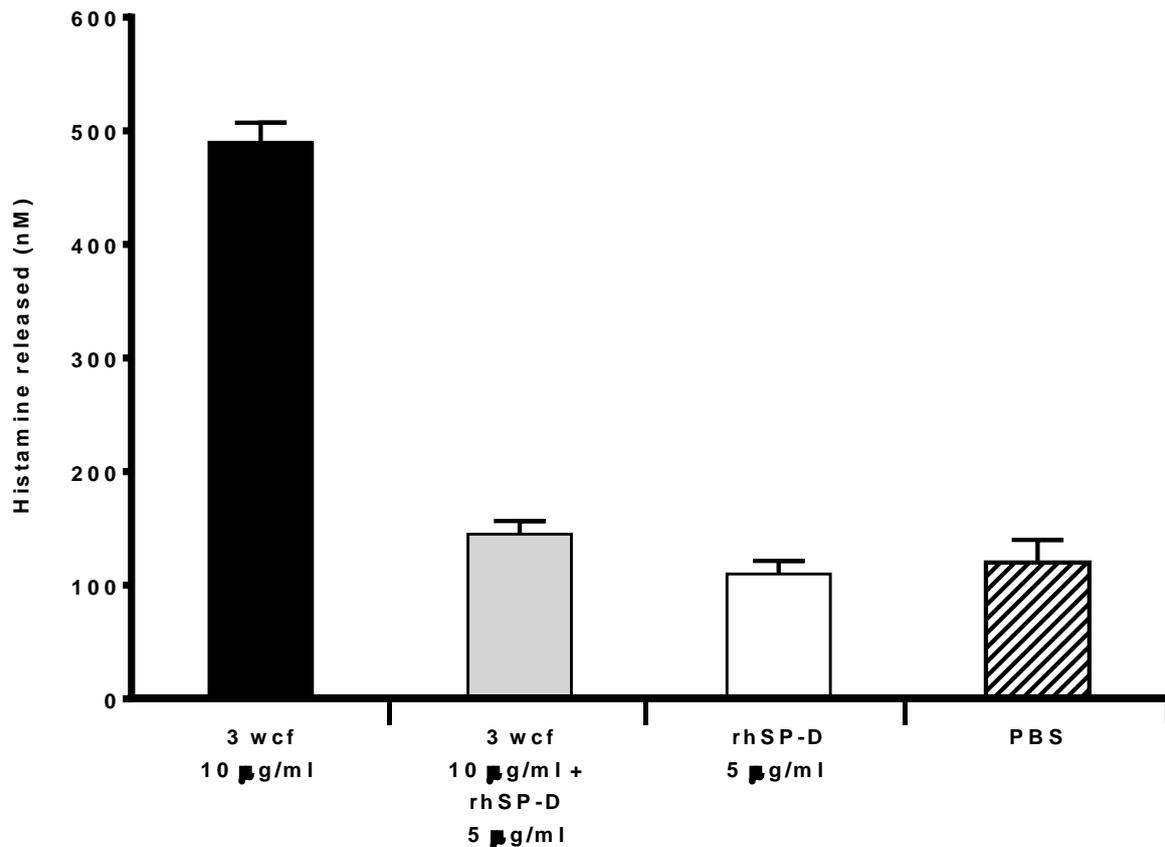


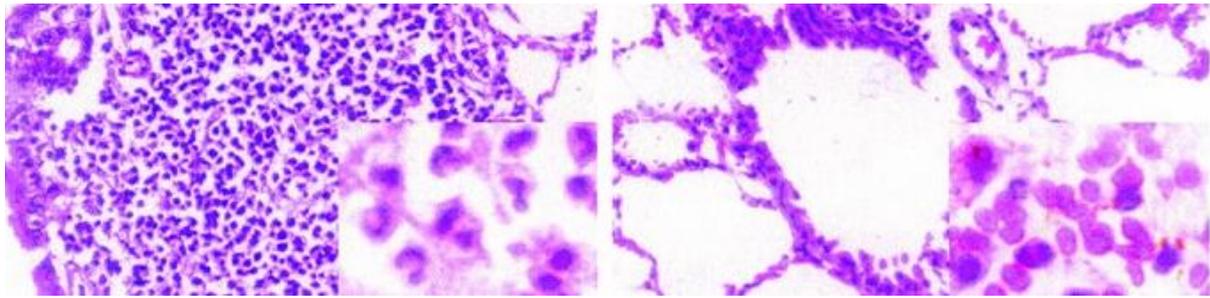
Figure 4.10 Suppression of histamine release by rhSP-D by sensitised basophils.(1)10µg/ml 3wcf. (2) 10µg/ml 3wcf+ 0.5 µg/ml rhSP-D.(3)10µg/ml 3wcf+ 1µg/ml rhSP-D.(4)10µg/ml 3wcf+ 5 µg/ml rhSP-D.

4.2.3. The rhSP-D can alter cytokine profile of murine ABPA splenocytes

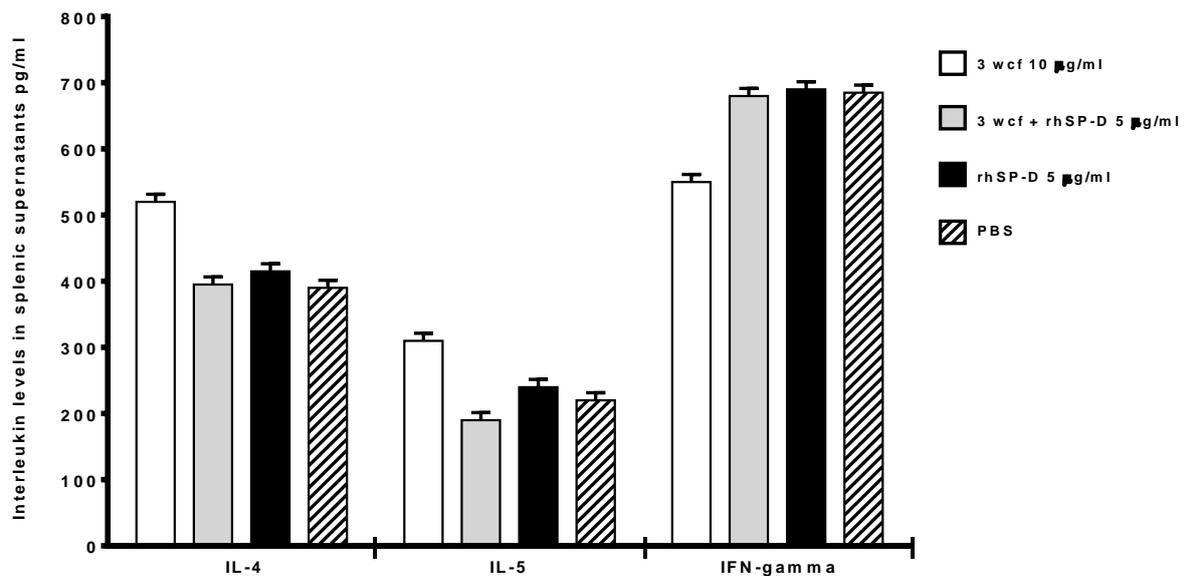
A significant increase in *A. fumigatus*-IgG and *A. fumigatus*-IgE levels was observed in the groups of mice immunised for four weeks with 3wcf, in comparison to those of controls immunised with PBS alone (significantly different at $p > 0.05$). The ratio of peripheral blood eosinophil counts of the sensitised mice was higher compared to the control groups of non-sensitized mice. Pulmonary eosinophilia picture was similar to that of peripheral blood eosinophilia. Histopathological findings revealed that mice in control groups had normal bronchi and parenchyma with a few eosinophils. Animals exposed to 3wcf showed extensive chronic inflammatory

infiltrates, mainly representing lymphocytes, plasma cells, and eosinophils. These inflammatory cells were frequently located around perivascular and peribronchiolar areas. The levels of eosinophile peroxidase assay (EPO) activity in the lung suspensions of mice immunised with 3wcf were elevated markedly in comparison with the mice of control groups on day 0 of the study. The ratio of EPO activity in test versus control mice of group A on day 0 was observed to be 1.752 which gradually came down to 1.220 on 16th day. Ratios of IL-2, IL-4, IL-5 and IFN- γ levels in splenic supernatants of the sensitized mice to controls were observed to be 2.744, 5.469, 1.750 and 0.663, respectively.

The splenocyte cytokine profile produced by 3wcf antigens was characteristic of a Th2 response due to raised levels of IL-4 and IL-5. The addition of 5 μ g/ml rhSP-D brought about a decrease in IL-4 and IL-5 and an increase in IFN- γ in the splenocyte supernatants, which is more consistent with a Th1 response. Addition of rhSP-D without 3wcf antigens did not produce a significant change in the cytokine levels (figure 4.11).



(a)



(b)

Figure 4.11 Cellular infiltration and splenic cytokine profiles in the ABPA mice. (a) The cellular infiltration is quite evident in the three-week culture filtrate treated mice compared to mice that received only intranasal PBS. Most of the infiltrates are neutrophils and eosinophils. (b) Cytokine levels in pg/ml of IL-4, IL-5 and IFN- γ of various mice (ABPA), observed in 72 h splenic supernatants with or without treatment with rhSP-D.

4.2.4. Expression and purification of a recombinant fragment of human SP-D without any collagen extension in the construct (rhSP-D delta)

Two recombinant variants of human SP-D (rhSP-D Gly-X-Y and Delta) composed of homotrimeric neck and carbohydrate recognition domains were expressed in *E. coli* and purified by a procedure involving denaturation-renaturation, ion-exchange, affinity and gel-filtration chromatography, similar to as done for the Gly-X-Y

containing rhSP-D described in Chapter 2. The Gly-X-Y variant contains additional 8 Gly-X-Y triplets from the collagen region of SP-D whereas the Delta variant lacks any additional residue at its N-terminus of the neck region (figure 4.12). A ~18 kDa protein (28 residues of alpha-helical neck region and 125 residues of CRD region of human SP-D) was produced after induction with IPTG and visualised on an SDS-PAGE under reducing conditions. This was found to be the major protein in the insoluble inclusion body pellet after centrifugation of the cell lysate. The pellet readily dissolved in 6 M Urea and initial purification by anion exchange chromatography removed most of the other bacterial proteins. The acetone precipitation step had the surprising effect of increasing the recovery of rhSP-D delta after refolding by 5-fold. After the refolding steps, maltose-agarose affinity chromatography selectively bound functionally active rhSP-D delta which after washing with 1M NaCl was eluted with EDTA. Final purification by ion exchange yielded a pure and homogenous preparation of trimeric homotrimeric rhSP-D delta (figure 4.12). The yield was 10 mg/L of induced bacterial culture.

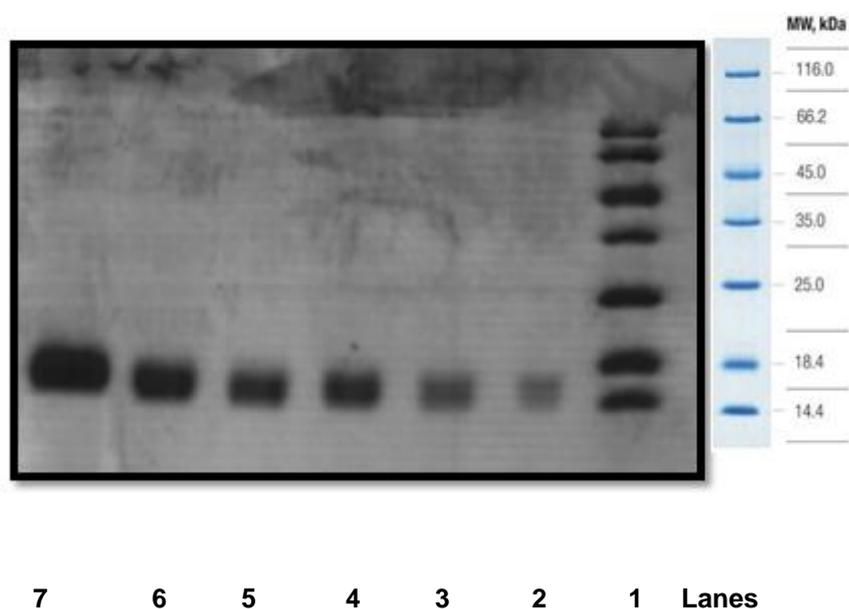


Figure 4.12 Expression and purification of the rhSP-D delta. Gel 1: SDS-PAGE (15% w/v) analysis of Δ rhSP-D after dialysis against HEPES buffer under reducing conditions showing only monomer at 18KDa. Lane 1: Protein marker; Lane 2: 2.5 μ l of dialyzed Δ rhSP-D; Lane 3: 5 μ l of dialyzed Δ rhSP-D; Lane 4: 7.5 μ l of dialyzed Δ rhSP-D; Lane 5: 10 μ l of dialyzed Δ rhSP-D; Lane 6: 15 μ l of dialyzed Δ rhSP-D and Lane 7: 30 μ l of dialyzed Δ rhSP-D.

In order to get whole ranges of spectrum showing monomer, dimer and trimer band using particular concentration of cross linker in SDS-PAGE, the dialyzed Δ rhSP-D was incubated with varying the incubation time with Bissulfosuccinimidyl suberate (BS3). 5 μ l of 0.1mM concentration of BS3 was incubated with 45 μ l of the dialysate for 1 min, 2 min, 4 min, 8 min, 16 min and 18 min at room temperature. The cross-linking reaction was electrophoresed on a 15 % (w/v) SDS-PAGE gel under reduced conditions after adding equal volume of treatment buffer to each time point and stored at room temperature. Later boiled the samples together in the heating block for 7 minutes, then stained and de-stained. Upon reaction with cross-linking agent at various incubation time higher oligomers such as monomer (18 KDa), dimer (~40 KDa), and trimer (~ 60 KDa) were seen (figure 4.13).

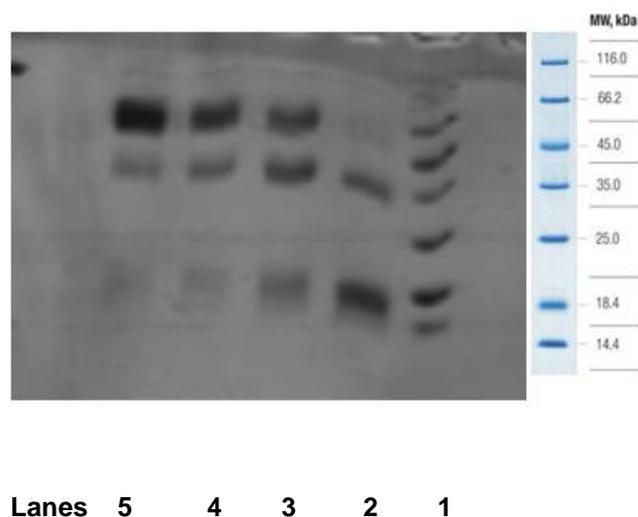


Figure 4.13 SDS-PAGE 15 % (w/v) gel under reducing conditions in the presence of BS3 and stained with Coomassie blue. Lane 1: protein marker Lane 2: 5 μ l of 0.01mM BS3 and sample incubated for 0 min; Lane 3: 5 μ l of 0.01mM and sample incubated for 1min; Lane 4: 5 μ l of 0.01mM and sample incubated for 2min; Lane 5: 5 μ l of 0.01mM BS3 and sample incubated for 4 min I

4.2.5. Fungistatic properties of the two variants of rhSP-D

48 clinical isolates of *Aspergillus* being 18 strains of *A. fumigatus*, 10 strains each of *A. niger*, *A. flavus* and *A. terreus* were used in the study. Susceptibilities were determined using the micro-dilution plate modification of the CLSI (formerly NCCLS) M38-A standard. Culture medium was RPMI 1640 + 2% glucose, inoculum 1 x

105/mL, plates incubated for 24 hours at 37°C; the plates were read both visually and on a spectrophotometer at 490nm (rhSP-D)

Susceptibility tests were performed using the broth micro-dilution modified method of CLSI M38-A using RPMI 1640 medium buffered to pH 7.0 with MOPS and 2% glucose. In brief drug ranges (in mg/L) for rhSP-D were 0.9237, 15mg/L and 12.5mg/L for Gly-XY and Delta rhSP-D, respectively. Inoculum suspensions were from day 5 to 8 day cultures grown on Sabouraud dextrose agar at 37°C and adjusted to a final inoculum was between 5×10^4 and 2.5×10^5 CFU/ml. Readings were made after 24 h of incubation at 37°C both visually and by spectrophotometer at 490nm. The MIC endpoints were recorded as the lowest drug concentration that prevents any discernible growth. In the growth rate assays, micro-dilution plates were set up in similar conditions but with a dose range of 1.5-24mg/L. Readings taken every 5 minutes for 24 hours.

All *Aspergillus* isolates had reduced growth with Gly-X-Y in a dose dependent fashion. MIC values and MIC50 were lower for *A. niger* than any other species. 40/48 strains demonstrated <80% reduction in growth at 15mg/L with MIC50 of 15, 1.875, 15 and 7.5 for AF, AN, AFL and AT respectively.

rhSP-D Gly-X-Y substantially reduces the growth rate of *Aspergillus* spp. *in vitro*. The inhibitory activity of Gly-X-Y is superior to that of the Delta variant. The main antifungal activity can be localized to the lectin domain. This demonstrates an additional mechanism by which this innate immune molecule protects against fungal infection.

With Gly-X-Y rhSP-D, all isolates showed reduced growth in a dose dependent fashion. 40/48 strains demonstrated <80% reduction in growth at 15µg/mL with MIC50s of 15, 1.875, 15 and 7.5 for AF, AN, AFL and AT respectively. With Delta rhSP-D, less inhibition occurred with 35/38 strains showing reduced growth over the dose range. 80% reduction in growth was seen in few isolates (5/48 strains). In the growth rate assays, all strains demonstrated both an overall reduction in growth rate (as measured by increasing OD) in a dose dependent fashion. Reduction in growth rate was most clearly seen by increased lag time (time until the OD increased) which increased by 2h 20mins and 1h 30mins in the Gly-X-Y and Delta rhSP-D assays, respectively.

rhSP-D Gly-X-Y substantially reduces the growth rate of *Aspergillus* spp. in vitro. The inhibitory activity of Gly-X-Y is superior to that of the Delta variant. The main antifungal activity can be localized to the lectin domain. This demonstrates an additional mechanism by which this innate immune molecule SP-D substantially reduces the growth rate of *Aspergillus* in vitro and this fungistatic activity can be localized to the lectin domain. This is an additional mechanism by which SP-D provides protective immunity without involving phagocytes. Thus, the rhSP-D may be used as a potent barrier to fungal infection.

		Gly-X-Y rhSP-D		Delta rhSP-D	
	n	Range	MIC50	Range	MIC50
A fumigatus	18	3.75->15	15	200->200	>200
A niger	10	1.875-3.75	1.875	200->200	>200
A flavus	10	15->15	15	200->200	>200
A terreus	10	3.75-15	7.5	200->200	>200

Table 4.1 Gly-X-Y rhSP-D showed reduced growth in a dose dependent fashion. Delta rhSP-D, less inhibition occurred with 35/38 strains showing reduced growth over the dose range (n= number of samples).

		Gly-X-Y rhSP-D		Delta rhSP-D	
	n	Range	MIC50	Range	MIC50
A fumigatus	18	0.469-15	0.469	12.5- >200	50
A niger	10	0.469-0.938	0.938	12.5- >200	200
A flavus	10	0.469-3.75	0.469	12.5- >200	100
A terreus	10	0.469-1.88	0.468	50 >200	100

Table 4.2 Gly-X-Y rhSP-D showed reduced growth in a dose dependent fashion. Delta rhSP-D, less inhibition occurred with 35/38 strains showing reduced growth over the dose range (n= number of samples).

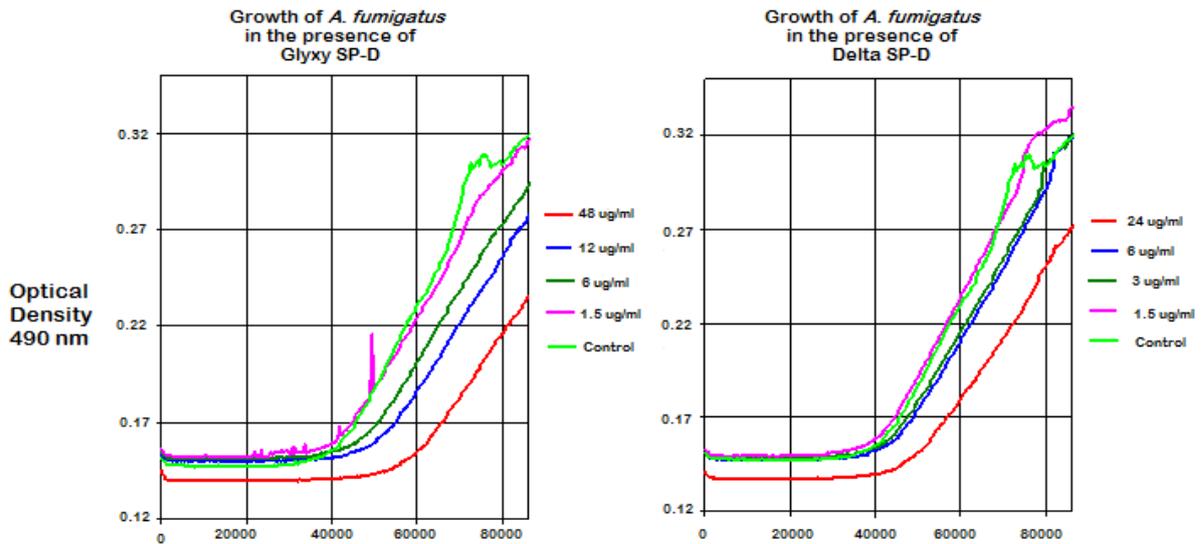


Figure 4.14 Reduction in growth rate was clearly seen by increased lag time (time until the OD increased) which increased by 2h 20mins and 1h 30 min in the Gly-X-Y and Delta rhSP-D assays, respectively.

4.3. Discussion

Aspergillus fumigatus can cause allergic and invasive aspergillosis depending on the host's immune status (Varkey, 1998). In the immunocompetent host, it causes allergic disorders, such as allergic rhinitis, allergic sinusitis, hypersensitivity pneumonitis, and ABPA. In the immunocompromised host, *A. fumigatus* disseminates to other tissue causing systemic and fatal IPA or IA. Bronchiectasis and cystic fibrosis also allow colonization of *A. fumigatus*. Aspergillus infection is rising due to increased chemotherapy due to cancer and organ transplantation. Dissemination of *Aspergillus* infection to other organs occurs in about 20% of the IPA cases (Denning, 1990). In spite of correct diagnosis and treatment, IPA results in mortality of >80% patients. The mortality rate among patients following bone marrow transplantation can be as high as 95% (Sternberg, 1994). Alveolar macrophages and peripheral blood polymorphonuclear and mononuclear phagocytes are considered to form the major host defense against *A. fumigatus*. Macrophages have been shown to participate in the early phase of defense by ingesting inhaled, airborne *Aspergillus* conidia and inhibiting their intracellular germination. In addition, PMN and circulating monocytes also cause damage to escaping hyphae (late phase of defense) by

secreting microbicidal oxidative metabolites and non-oxidative compounds, thus preventing establishment of invasive disease (Schaffner, 1982). Suppression of innate immunity by immunosuppressive drugs results in lowering of essential defence barriers against invasive aspergillosis (Sternberg, 1994). The phagocytic activity of the peripheral blood monocytes-derived macrophages and the anti-fungal activity of neutrophils against *A. fumigatus* from HIV-infected children have been shown to be significantly decreased as compared to normal donors (Roilides, 1993a; Roilides, 1993b). Treatment for the invasive form of aspergillosis, to provide complete cure, is complicated by the chemotherapy of allergic patients with steroids and of chronically infected patients with aggressive anti-fungal drugs, which have highly adverse side effects, such as hepatotoxicity and nephrotoxicity.

Recently, involvement of SP-D in the initial protective immunity against *A. fumigatus* infection has been shown (Madan et al, 1997a). SP-D has an anti-fungal role in the lung, by inhibiting infectivity of conidia via agglutination and enhancing uptake and killing of *A. fumigatus* by recruited phagocytes.

The rhSP-D binds to *A. fumigatus* conidia in calcium-, dose- and carbohydrate-dependent manner. Most excitingly, the phagocytosis and killing of conidia by PMNs were found to be enhanced almost 3-fold by rhSP-D. Nagai et al have reported protective effect of exogenous TNF- α and IFN- γ in a murine IPA. Rollides et al have shown enhancement of phagocytosis of *Aspergillus* conidia by rabbit alveolar macrophages in the presence of 10 ng/ml of TNF- α . Intratracheal challenge of both cyclophosphamide-treated and untreated mice with *A. fumigatus* conidia leads to the increased levels of TNF- α in the lungs. Antibody neutralisation of elevated TNF- α resulted in an increased mortality in both normal as well as cyclophosphamide-treated mice. Depletion of TNF- α also resulted in a reduced lung neutrophil influx, decrease in the lung levels of macrophage inflammatory protein-2 (MCP-2) and macrophage inflammatory protein-1 α (MCP-1 α) (Mehrad, 1999). TNF- α , therefore, appears to be a critical component of innate immunity in anti-*Aspergillus* defense.

The murine model of ABPA, used in the present study to examine the effect of rhSP-D on IgE inhibition, histamine release, lymphoproliferation and cytokine profile, exhibited peripheral blood and lung eosinophilia, elevated *A. fumigatus*-IgE and *A. fumigatus*-IgG levels and Th2 type cytokine profile, as described previously (Kurup,

1992; Wang, 1998). Mast cells release, after cross-linking of bound IgE to *A. fumigatus* allergens, a variety of pro-inflammatory mediators, which induce bronchial smooth muscle contraction and vascular permeability. In ABPA, the *A. fumigatus*-IgG and *A. fumigatus*-IgE bind Fc receptors present on eosinophils, leading to secretion of inflammatory mediators (Varkey, 1998). The rhSP-D bound the antigens/allergens derived from 3wcf, in sugar and calcium specific manner and inhibited the binding of *A. fumigatus*-IgE to these allergens, and blocked histamine release from the sensitised basophils. The binding of allergens/antigens of *A. fumigatus* is likely to reduce their availability to cross-link the IgE present on the surface of eosinophils and mast cells.

Elevation of the levels of *A. fumigatus*- IgG, an important diagnostic criterion for ABPA, is a reflection on the Th2 response of the host to *A. fumigatus* antigens (Varkey, 1998). Th2 lymphocytes can induce IgE synthesis, eosinophil recruitment and activation. We also observed an increase in the levels of IL-4, IL 2 and IL-5 and decrease in the level of IFN-II in the splenic supernatants of ABPA mice, indicating predominance of Th2 cells on prolonged exposure to *A. fumigatus* antigens (Chu, 1996; Kurup, 1992; Kurup, 1994). IL-5 has been implicated in recruitment of eosinophils and maturation of pulmonary eosinophils, since neutralization of IL-5 by anti-IL-5 antibody has been shown to abrogate the peripheral and pulmonary eosinophilia in the murine ABPA (Murali, 1993). An increased IL-4 levels in the splenic supernatants appeared to correlate with IgE levels seen in the serum, suggesting that IL-4 could be a major modulator of IgE production in murine ABPA. Stimulation of T cell proliferation (Knutsen, 1994) and basophil hyper reactivity with increased histamine release has also been reported in ABPA. IL-4 also induces expression of VCAM-1 on vascular endothelial cells and its ligand VL-4 on T cells and eosinophils, in addition to inducing eotaxin and recruits eosinophils to the site of antigen (Bonecchi, 1998). Presence of IL-4 during the priming of naive T cells has been shown to be critical for the development of a Th2 response. In our model, increased levels of IL-2 is likely to support clonal expansion of *A. fumigatus*-specific Th2 cells since IL-2 and its receptor play a crucial role in the growth and differentiation of many cells such as T and B lymphocytes, NK cells, macrophages and monocytes. The lower level of IFN-II in the splenic supernatants of ABPA mice is suggestive of a Th2 CD4+ T cell response (Kurup, 1994) which leads to a hypersensitivity pulmonary

lung disease. When splenocytes were treated with rhSP-D, the splenic supernatant of the sensitized mice showed decrease in the levels of IL-4 and IL-5 (Th2 type) and increase in IFN- γ (Th1 type) levels. IFN- γ , a Th1 type cytokine, promotes the cellular immunity. It is believed that Th1 CD4⁺ T cell response is protective against *A. fumigatus*.

SP-D has been shown to inhibit the PHA and CD3-stimulated lymphoproliferation of PBMCs of normal individuals (Borron, 1998), and also allergen induced proliferations of PBMCs of asthmatic children sensitive to mite allergens (Wang, 1998). The rhSP-D has suppressive effect on the lymphoproliferation of sensitised mouse splenic cells. Since histamine release and lymphocyte proliferation are two essential steps in the development of asthmatic symptoms, this recombinant form of truncated SP-D (rhSP-D) appears to be a potential therapeutics towards the control of allergen-induced disorders. Several recent reports indicate that the CRD regions of SP-D may fulfil other functions besides binding carbohydrate structures. It has been demonstrated that the CRD region of SP-D is chemotactic for neutrophils (Cai, 1999). A putative receptor molecule for SP-D, described as gp-340, has been shown to bind the CRD region (Holmskov, 1997), as opposed to SP-A receptor molecules which appear to bind to collagen region of SP-A. The recombinant form of truncated SP-D (rhSP-D) could potentially be used for the treatment of *A. fumigatus* infections in human patients, in conjunction with standard anti-fungal drugs.

5. Chapter 5

Modulation of Dendritic Cell Maturation and Functions by SP-A and SP-D

5.1. Introduction

A population of adherent cells was initially identified as a requirement for the induction of B and T cell responses *in vitro* and *in vivo* (Steinman and Cohn, 1973; Steinman, Adams and Cohn, 1975; Steinman, Lustig and Cohn, 1974; Steinman and Cohn, 1973).

5.1.1. Origin and distribution of dendritic cells

DCs are found as immature cells in virtually all organs (except the brain) within the interstitial spaces, and mucosal surface, which is highly well developed in the lungs (Karre and Welsh, 1997), in addition to being present in the heart, kidney, dermis, liver, and other organs.

DCs display differences in anatomic localization, cell surface phenotype, and function (Banchereau and Steinman, 1998; Hart, 1997). DCs originate from CD34 bone marrow stem cell; precursor DCs home to tissues where they become immature DCs, which can take up antigen and present via class II MHC II molecules in order to interact with antigen-specific CD4 T cells to initiate immune responses (De Smedt *et al*, 1997; Kalinski *et al*, 1998; Kuroda *et al*, 2000; Liu *et al*, 1997; Staquet *et al*, 1995; van der Pompe *et al*, 1998; Vieira *et al*, 2000). Distinct chemokine receptors occur on immature and mature DCs (Cyster, 1999; Homey and Zlotnik, 1999; Sallusto and Lanzavecchia, 1994; Zlotnik and Yoshie, 2000). IL-12 produced by DCs helps in the activation of naïve CD4 + T cells and their engagement with Th1 cells (Vieira *et al*, 2000). Monocytes from spleen, blood, or bone marrow can potentially generate conventional DCs (Naik *et al*, 2006).

5.1.2. Human Dendritic cells

DCs mature in lymphoid and non-lymphoid tissues (figure 5.1) (Cerio *et al*, 1989). LCs and interstitial DCs evolve from CD34⁺ bone marrow and CD11c⁺ blood precursors in the presence of GM-CSF and IL-4/TNF- α (Caux and Prost, 1999; Romani *et al*, 1994; Sallusto and Lanzavecchia, 1994). Upon activation by CD40L, immature myeloid DCs undergo maturation and produce IL-12 (Cella *et al*, 1996). (Caux and Prost, 1999; Romani *et al*, 1994; Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996). LCs and interstitial DCs share several markers, but LCs uniquely express CD1a, Birbeck granules, langerin, and the adhesion molecule E-cadherin. Interstitial DCs express the coagulation factor XIIIa (Caux *et al*, 1997 ; Caux and

Prost, 1999). Plasmacytoid DCs (pDCs) are found in the T cell zones of lymphoid organs, thymus and blood (Facchetti, Candiago and Vermi, 1999; Grouard *et al*, 1997; Res *et al*, 1999; Sorg, Kogler and Wernet, 1999). pDCs are characterized by a unique phenotype and secrete IFN- α/β upon viral challenge (Cella *et al*, 1999; Grouard *et al*, 1997; Kohrgruber *et al*, 1999; Siegal *et al*, 1999; Grouard *et al*, 1997; Kohrgruber *et al*, 1999). pDCs can also activate CD4 and CD8 naïve T cells and secrete IL-12 (Cella *et al*, 2000 ; Kohrgruber *et al*, 1999).

5.2. Dendritic cells maturation: The link between Innate and Adaptive Immunity

There are endogenous and exogenous factors that affect DCs and their ability to direct T cell phenotypes- anergic, memory, or effector (Lipscomb and Masten, 2002). LPS, peptidoglycans, and microbial DNA enhance the T cell stimulation via DCs through TLRs, which lead to DC maturation (Kaisho and Akira, 2001). TNF- α (Sallusto *et al*, 1995) as well as cellular heat shock proteins (Basu and Srivastava, 2000) also promote DC maturation and T cell effector function (figure 5.2). IL-10, IL-1 α , 25-dihydroxyvitamin D3, ligands to CD36 or CD51, and cAMP-elevating agents, suppress DC function (figure 5.5) (Buelens *et al*, 1995; Sato *et al*, 1999; Penna and Adorini, 2000; Urban, Willcox and Roberts, 2001; Kambayashi, Wallin and Ljunggren, 2001). Thus, the ability of DCs to initiate an appropriate immune defense is critically dependent on signals present in the local microenvironment (figure 5.3) (Brinker, 2001; Kunkel, 1999; Miller and Krangel, 1992; Rollins, 1997 ; Lipscomb and Masten, 2002).

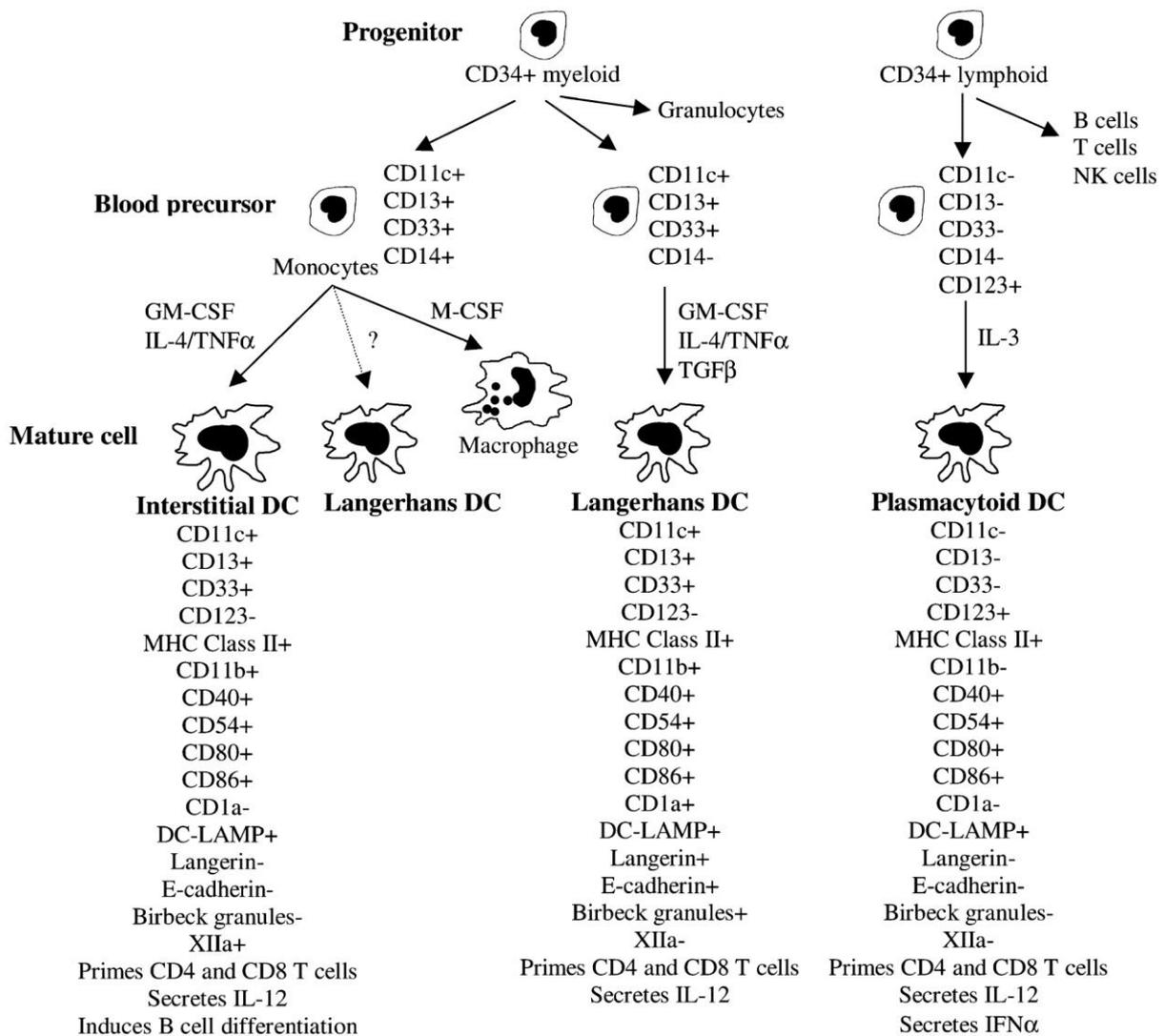
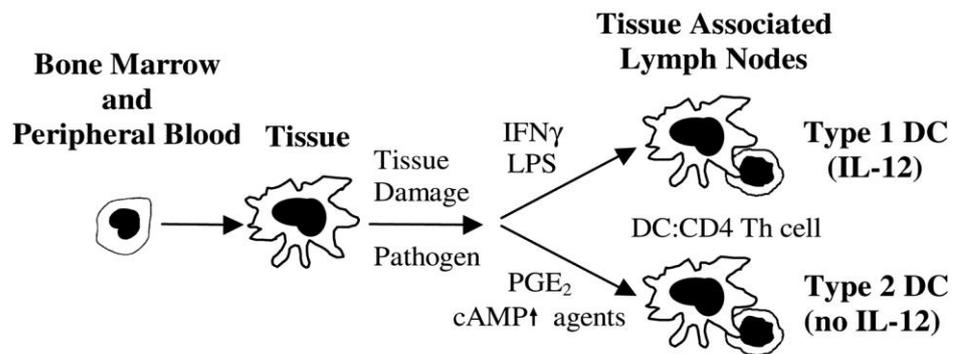


Figure 5.1 DC subsets derived from CD34⁺ myeloid and lymphoid progenitors. GM-CSF, TNF- α , IL-4, TGF- β , and IL-3 are modifiers of this differentiation. The precursor cells are vastly influenced by cytokine and growth factors in the tissue microenvironment. The maturing cells further undergo differentiation based on their residency that includes interstitial DCs, Langerhans DCs, and plasmacytoid DCs. Due to the availability of a number of surface markers and antibodies to characterize them; their tissue phenotyping has been possible in last 10 years (Cella *et al*, 2000).



	<u>pDC</u>	<u>iDC</u>	<u>mDC</u>
Antigen Uptake		+++	+
Antigen Processing		+++	+
MHC Class II		+	+++
Costimulatory Molecules		+	+++
Secreted Chemokines		Inflammatory chemokines (MIP-1 α , MIP-1 β , RANTES, MCP-1, MCP-2, MCP-4, IL-8)	Lymphoid chemokines (ELC, MDC, TARC, PARC, IP-10)
Chemokine Receptors		CCR1, CCR2, CCR5, CCR6 (LC only), CXCR1	CCR4, CCR7, CXCR4

Figure 5.2 The migratory and maturation pathways of DCs. As mentioned above, GM-CSF, TNF- α , IL-4, TGF- β , and IL-3 are modifiers of DC differentiation. The precursor cells are influenced by cytokine and growth factors in the tissue microenvironment. The maturing cells further undergo differentiation based on their residency that includes interstitial DCs, Langerhans DCs, and plamacytoid DCs. Following their relocation to draining lymph nodes, the IL-12 cytokine dictates their differentiation into type I or type II DCs under the influence of PAMPs or prostaglandins. The chemokines subsequently affect their homing and expression of chemokine receptors (Cella *et al*, 2000).

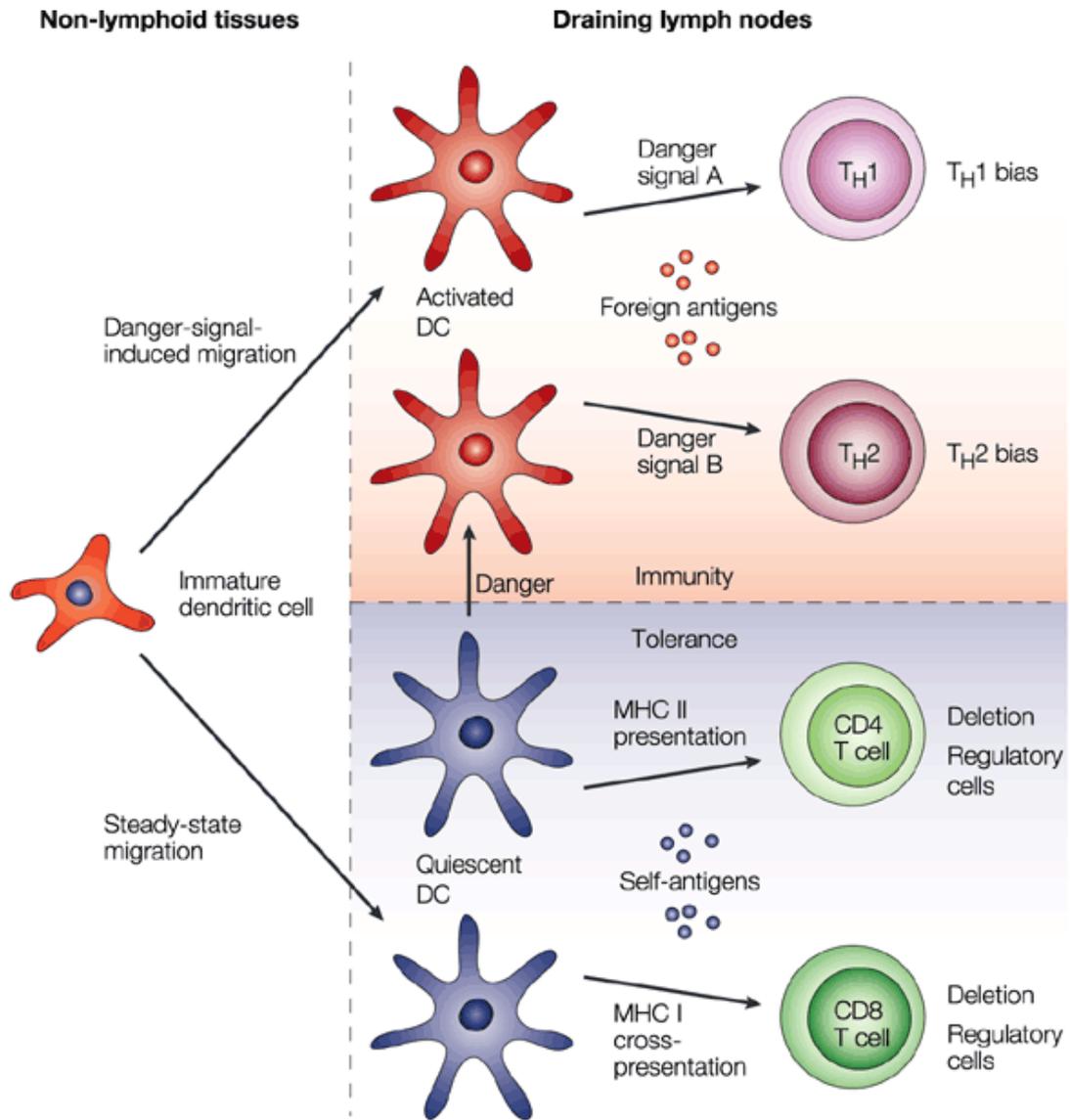


Figure 5.3 Maturation and differentiation pathways of DCs and their involvement in cellular immune response. The danger signals reflecting on the pro-inflammatory stimuli can give rise to effector Th2 or Th1 phenotypes of T cells in the presence of non-self antigens. In the presence of self antigens, a regulatory phenotype is generated that leads to tolerance and non-inflammatory microenvironment (Medzhitov, 2008).

5.3. Role of DCs in Immune- mediated disease

Inflammation is triggered by infection or tissue injury and is characterized by the secretion of inflammatory mediators, including cytokines and chemokines, and the massive recruitment of leukocytes, such as neutrophils and monocytes (figure 5.4) (Medzhitov, 2008). Following inflammation, monocyte derived DCs express HLA-DR, CD11c, BDCA1, CD1a, FcεRI, CD206, CD172a, CD14 and CD11b (Segura et al., 2013 ; Wollenberg et al., 2002 ; Guttman-Yassky et al., 2007), in addition to M-CSFR and ZBTB46 (Segura et al., 2013).

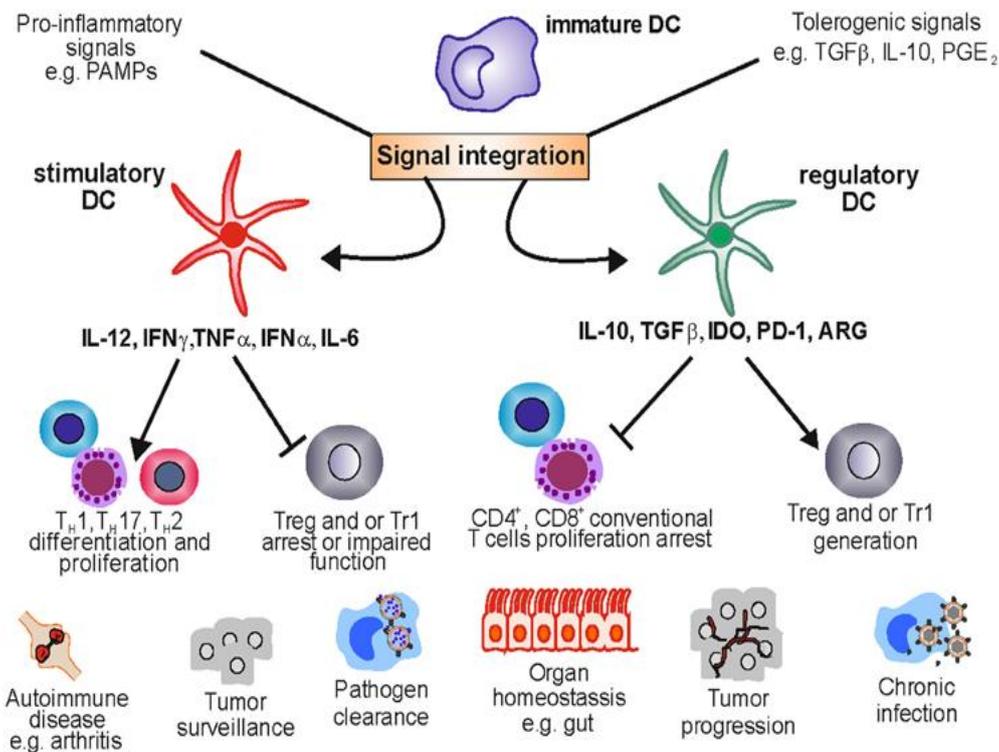


Figure 5.4 Stimulatory and regulatory DCs have a wide range effect on the human cells in both health and disease conditions. The presence of IL-12 or IL-10 by DCs can dictate whether the T cell function will be pro-inflammatory or regulatory. Depending on this, these effector cells can cause pathogenesis. Thus, DC phenotype is of immense importance I the elimination of pathogens as well as tolerance against self-antigens (Medzhitov, 2008). .

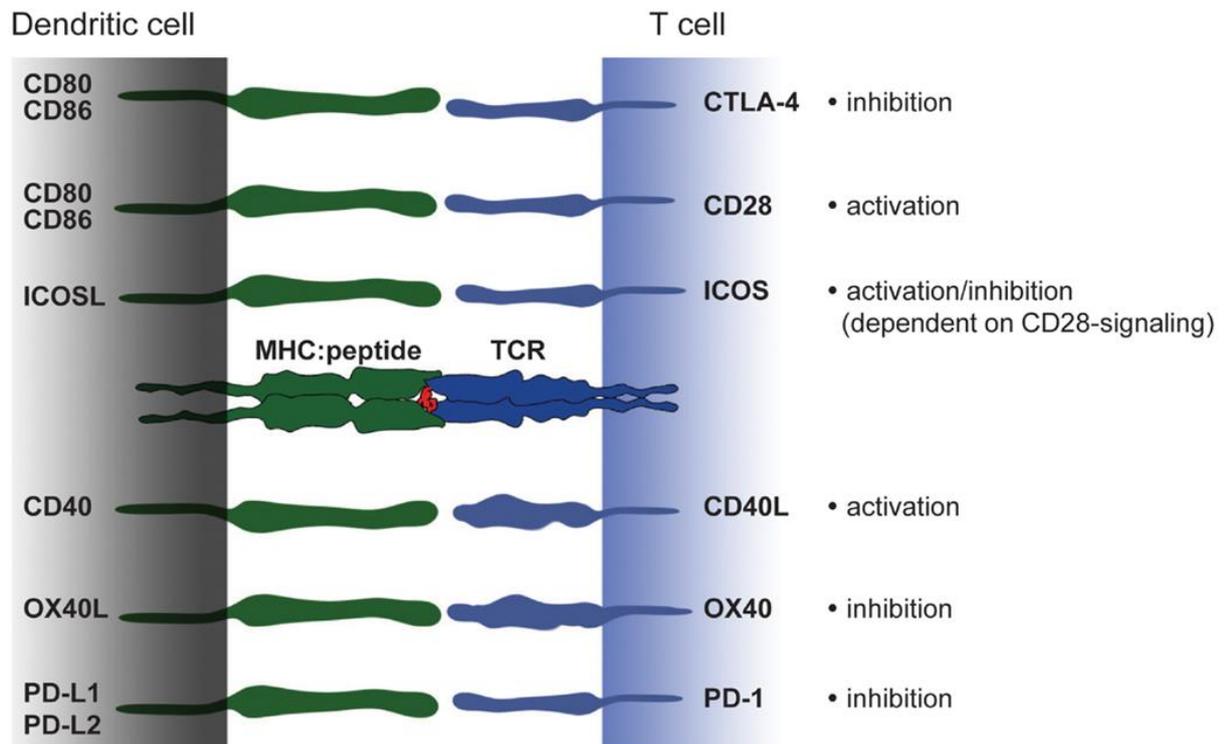


Figure 5.5 Co-stimulatory molecules and their ligands. The interaction between DC and T cells is dependent on signal 1 that originates due to interaction between peptide-loaded MHC class II molecules and T cell receptors. In addition, interaction of PRRs such as TLRs overexpresses co-stimulatory molecules on DCs that will have its cognate partner on T cells, thus yielding signal 2. In the absence of DC expression of co-stimulatory molecules, T cells become either apoptotic or anergic (Medzhitov, 2008).

5.4. Modulation of DCs by SP-A and SP-D

SP-A and SP-D regulate T cell proliferation (Wright and Youmans, 1993; Wright, 2005). SP-A binds to DCs and negatively regulates their maturation *in vitro*, thereby reducing their T cell allo-stimulatory ability (Brinker, Garner and Wright, 2003). Datta et al have used mice deficient in SP-A in order to test the hypothesis that SP-A

regulates recruitment, activation, and maturation of adaptive immune cells in response to *M. pneumoniae* by regulating expression of the endogenous stress factor high-mobility group box-1 (HMGB-1), which, if released in the context of infection, can activate DCs and leads to their maturation (Datta *et al*, 2005). Julie *et al* have reported in their article that *M. Pneumoniae* infection leads to increased numbers of exudative macrophages and DCs in the lung parenchyma, a response that is augmented by the absence of SP-A. Likewise, the total number and activation state of DCs that have migrated to the mediastinal draining lymph nodes during the acute phase of infection are also increased in the absence of SP-A. Additionally, elevated number of activated T and B cells in the lung and mediastinal lymph nodes (MLNs), as well as *M. pneumoniae*, specific IgG in the serum, are observed in mice lacking SP-A after *M. Pneumoniae* infection (Julie *et al*, 2010). More DCs were found in the lung of *M. pneumoniae* infected mice that lacked SP-A, and more functionally mature DCs were detected in MLNs, where they would act as potent T cell stimulators. Additionally, MCP and MIP-1, factors known to be chemotactic for immature DCs (Napoli *et al*, 1996; Sallusto *et al*, 1995), were present in BAL fluid of infected mice at much higher levels in mice lacking SP-A, Suggesting that more DCs migrated into the lung from the blood stream of *M. pneumoniae*- infected mice in the absence of SP-A as a result of greater chemokine production. The amount of GM-CSF, a factor vital to enhancing the differentiation of monocytes into immunostimulatory DCs in the lung vascular, was significantly increased in BAL from wild type and SP-A null *M. pneumoniae* infected mice. Collectively, these findings suggested that, in the absence of SP-A, more DCs infiltrate the airways as a result of increases in chemo-attractant signals (Julie *et al*, 2010)

SP-D also interacts with DCs to enhance uptake and presentation of bacterial antigens. SP-D augments antigen presentation by mouse bone marrow-derived DCs, but binds better to immature than mature DCs (Pfeifer *et al*, 1993; Svensson, Stockinger and Wick, 1997). SP-D enhances the antigen presentation of an ovalbumin fusion protein to ovalbumin- specific MHC II restricted T cell hybridoma (Brinker *et al*, 2001). Similar to SP-D ability to distinguish between immature and mature DCs, mannose receptor is highly expressed on immature DCs for enhancing internalization and antigen presentation of mannosylated antigens, but its expression on mature DCs is reduced (Engering *et al*, 1997; Sallusto *et al*, 1995). TLR-3 and

CD36 are highly expressed on immature DCs (Albert *et al*, 1998; Muzio *et al*, 2000). SP-D increases T cell IL-2 production by 5-fold when DCs are challenged with SP-D opsonized bacterial cells. Similarly, the efficiency of bacterial antigen presentation is increased by SP-D (Brinker, 2001).

In this chapter, the effects of SP-A and SP-D on the maturation and immunologic functions of monocyte derived DCs were examined. The parameters included measurement of surface and co-stimulatory markers, secretion of cytokines and proliferative response of T cell clones, as described in Chapter 2 (Materials and Methods).

5.5. Results

5.5.1. Effect of SP-A and SP-D on LPS induce maturation of DCs

When Salmonella LPS was used at 100 ng/ml concentration to induce maturation of the human monocytes derived immature DCs, the FACS profile of post-treatment DC markers dramatically altered in the presence of native human SP-A and SP-D, using medium alone as a negative control. To achieve this, monoclonal antibodies against DC maturation markers such as those against class II MHC molecules (HLA-DR), CD54, CD40, CD83, and CD86 were used. This range of markers covered for antigen presentation and co-stimulatory molecules. SP-A and SP-D were added three hours post LPS treatment at 10 µg/ml concentration. In medium + LPS, all five markers were up-regulated in response to LPS challenge, while SP-D had no effect (as if it was like medium control) on DC maturation. Curiously, SP-A was down regulating expression of DC maturation markers in the presence of LPS. In the case of HLA-DR and CD54, the down regulation was nearly 50% in the case of CD40, SP-A suppressed surface expression only by 30%. However, in the case of CD83 and CD86, SP-A suppressed surface expression down to approximately 80% (figure 5.6).

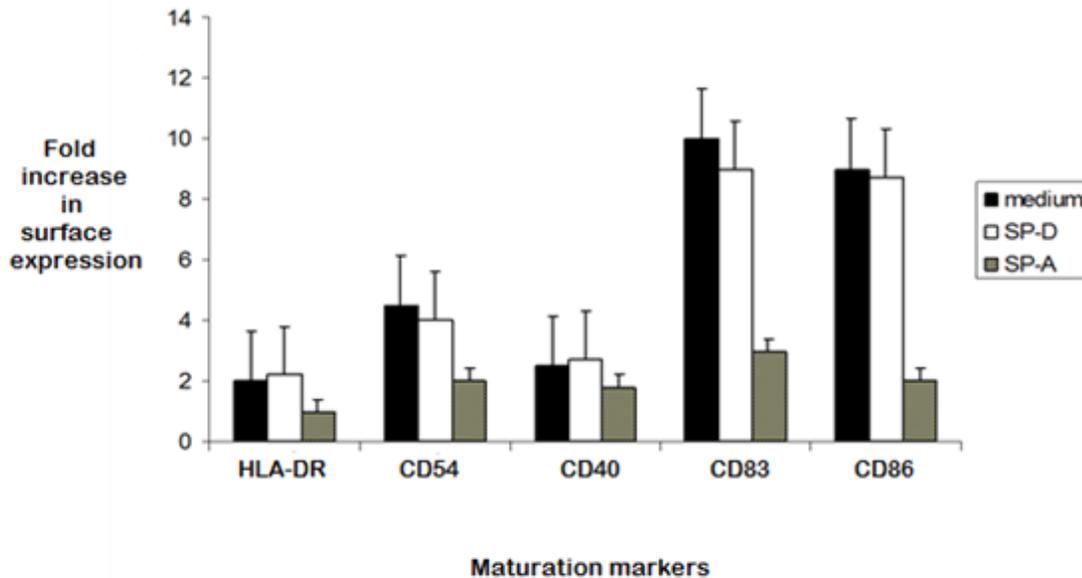


Figure 5.6 Salmonella LPS (100ng/ml): Effect of SP-A and SP-D on LPS induce maturation of DCs. Salmonella LPS (100 ng/ml) was used to against immature DCs. Monoclonal antibodies against DC maturation markers such as those against class II MHC molecules (HLA-DR), CD54, CD40, CD83, and CD86 were used. SP-A and SP-D (10 µg/ml each) were added 3 h after LPS treatment. In medium + LPS, all five markers were up-regulated in response to LPS challenge, while SP-D had no effect (as if it was like medium control) on DC maturation. Curiously, SP-A was down regulating expression of DC maturation markers in the presence of LPS. In the case of HLA-DR and CD54, the down regulation was nearly 50% in the case of CD40, SP-A suppressed surface expression only by 30%. However, in the case of CD83 and CD86, SP-A suppressed surface expression down to approximately 80%.

5.5.2. Effect of SP-A and SP-D on TNF- α induce maturation of DCs

In order to examine if the effect of surfactant proteins (SP-A, SP-D) on DC maturation was only exclusive to LPS, which is a ligand for SP-A and SP-D, as well as TLR, we carried out DC maturation experiment using human TNF- α at 50 ng/ ml concentration. TNF- α was used as pro-inflammatory cytokine, which is produced by macrophages and immature DC in response to stimulation by LPS. Although, TNF- α did not have effect on DC maturation as dramatic as that with Salmonella LPS still all five markers used were up-regulated. In this case the effect SP-D was either comparable to medium + LPS, or even more pronounced. Consistent with data from LPS stimulation in figure number 1, SP-A was able to suppress up regulation of this markers thus acting as inhibitor of DC maturation (figure 5.2). The effect of SP-A on

almost all maturation markers was more than 50% down regulation except in case of CD83, which was down 10 fold (figure 5.7).

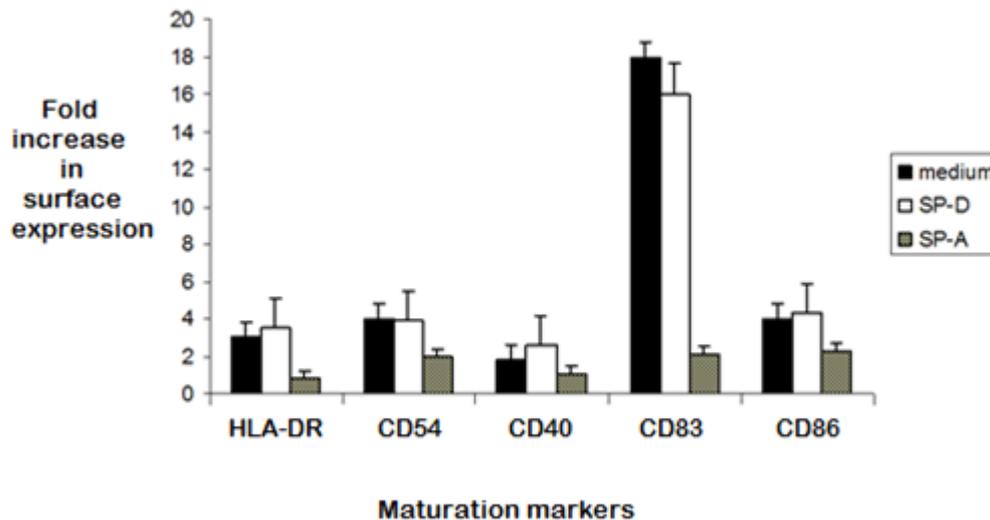


Figure 5.7 TNF- α (50 ng/ml): Effect of SP-A and SP-D on TNF- α induce maturation of DCs. DC maturation experiment was carried out using human TNF- α at 50 ng/ ml concentration. TNF- α did not have effect on DC maturation comparable to Salmonella LPS but most markers used were up-regulated. Similar to the case with LPS, SP-A suppressed up- regulation of surface markers but SP-D had negligible effect.

5.5.3.Regulation of LPS induced TNF- α secretion By SP-A and SP-D

We also examined if immature DC produced TNF- α in response to challenge with Salmonella LPS, and if SP-A and SP-D modulated production of TNF- α secreted by immature DCs. DC alone, DC with SP-D, DC with SP-A secreted basal level of TNF- α , that is less than 50 pg/ml. LPS induced up to 550 pg/ml of TNF- α , which was raised to 800 pg/ml by SP-D. Quite remarkably, SP-A down regulated TNF- α production by 10 fold (figure 5.8).

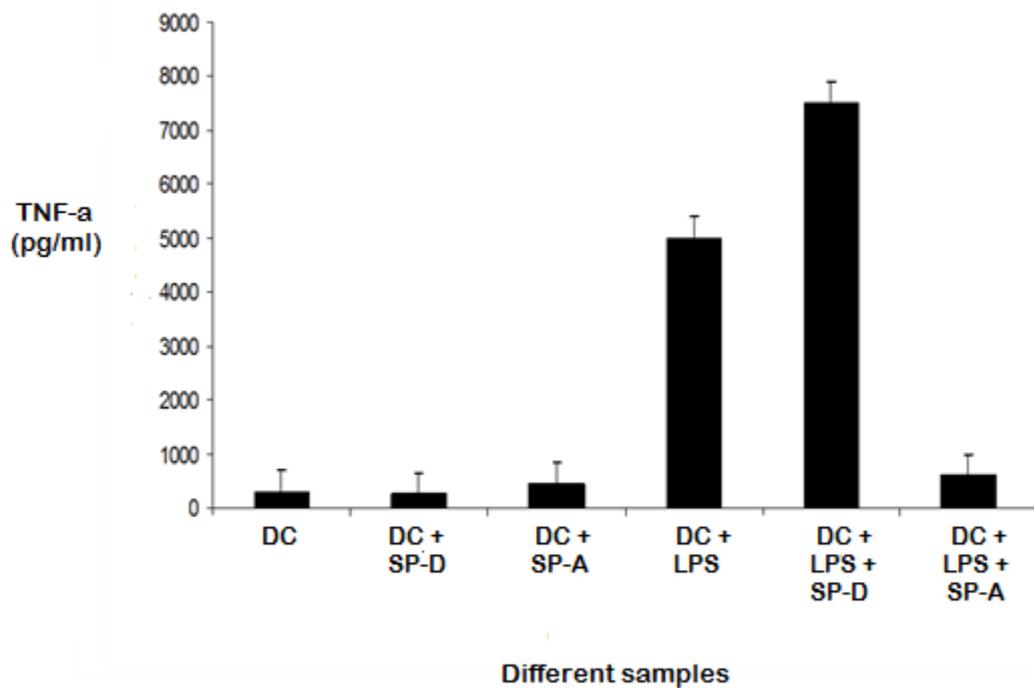


Figure 5.8 Effect of TNF- α : Regulation of LPS induced TNF- α secretion by SP-A and SP-D. Production of TNF- α by immature DC in response to Salmonella LPS was measured by ELISA. DC alone, DC with SP-D, DC with SP-A secreted comparable levels of TNF- α . SP-D raised cytokine production whereas SP-A down-regulated TNF- α production by 10-fold.

5.5.4. Modulation of biologically active IL-12p70 by mature DCs

Given that SP-D was not able to contain DC maturation in response to stimulation by LPS, TNF- α , and CD40L, it was considered that these two proteins may be influencing IL-12 production following DC maturation in a differential way. As clear from figure 5.9, DC on its own and either with SP-D or SP-A, did not produce above basal level secretion of IL-12 as measured by antibodies specific to p70 subunit. In response to LPS induced maturation, DCs produced very good amount of IL-12, that is, nearly 600 pg/ ml, which was further enhanced by SP-D (800 pg/ ml). Not surprisingly, SP-A down regulated production of IL-12 by DCs (figure 5.9).

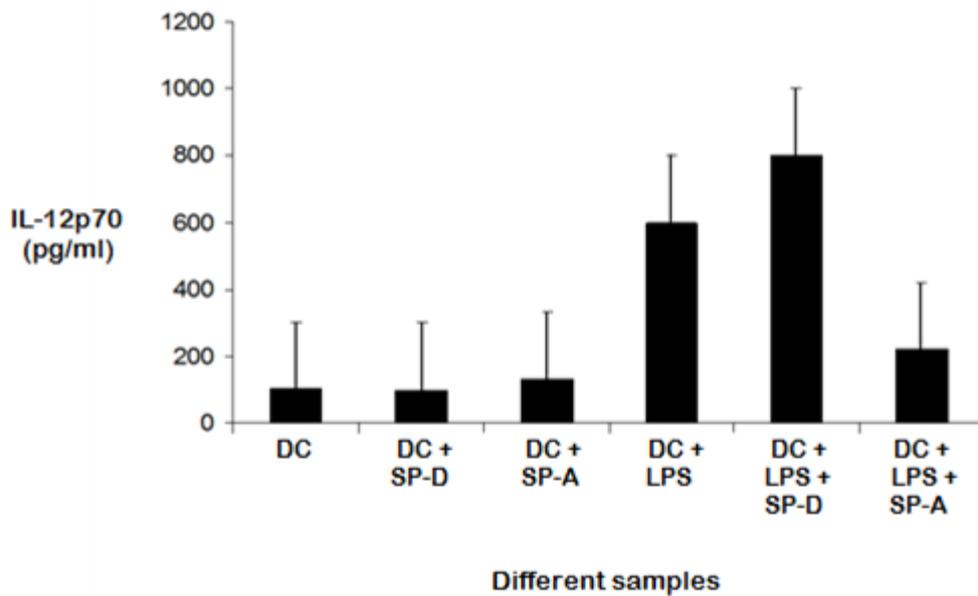


Figure 5.9 Effect of IL-12p70: Modulation of IL-12 production by mature DCs. The effect of SP-A and SP-D on DC mediated production of IL-12 was examined via ELISA (anti-p70 subunit) using the supernatant/culture medium. Maturing DCs produced ~600 pg/ml of IL-12, which was further enhanced by SP-D, whereas SP-A suppressed IL-12 production.

5.5.5. Modulation of IL-10 production in the presence of SP-A and SP-D

Given the ability of SP-A to down regulate DC maturation induced by LPS, TNF- α , and CD40L, and its ability to suppress TNF- α and IL-12 production, we wanted to assess the ability of SP-A and SP-D to modulate IL-10, a potent anti-inflammatory cytokine (figure 5.10). LPS induced near basal level of IL-10 expression with or without SP-D (nearly 200 pg/ml). However, SP-A up-regulated IL-10 production by 7 fold (1400 pg/ml).

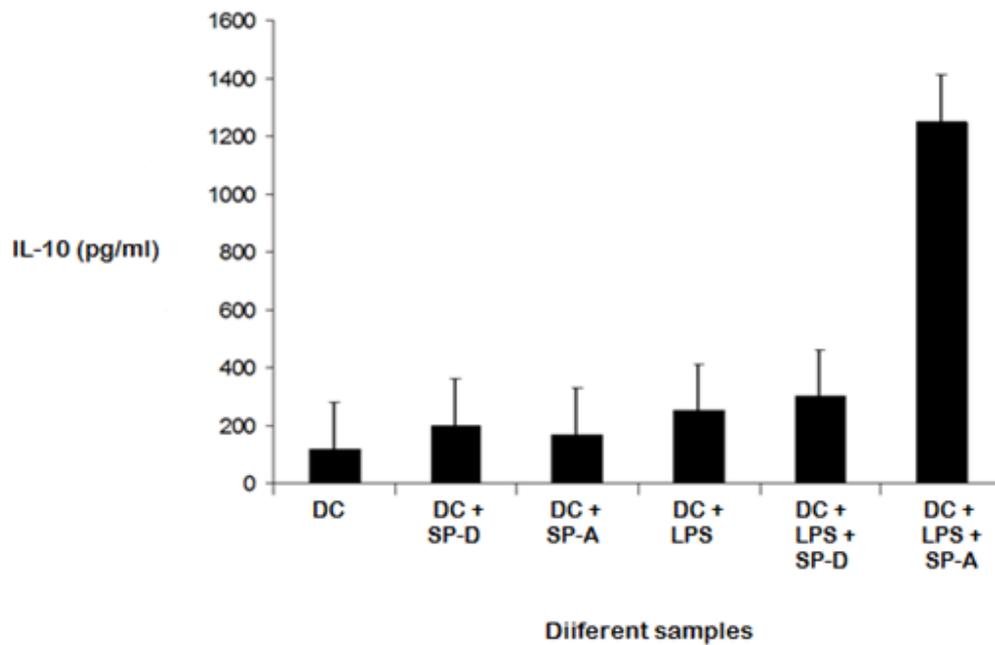


Figure 5.10 Effect of IL-10: Modulation of IL-10 production in the presence of SP-A and SP-D. Given the ability of SP-A to down regulate DC maturation induced by LPS, TNF- α , and CD40L, and its ability to suppress TNF- α and IL-12 production, we wanted to assess the the ability of SP-A and SP-D to modulate IL-10, a potent anti- inflammatory cytokine. LPS induced near basal level of IL-10 expression with or without SP-D (nearly 200 pg/ml). However, SP-A up-regulated IL-10 production by 7 fold (1400 pg/ml).

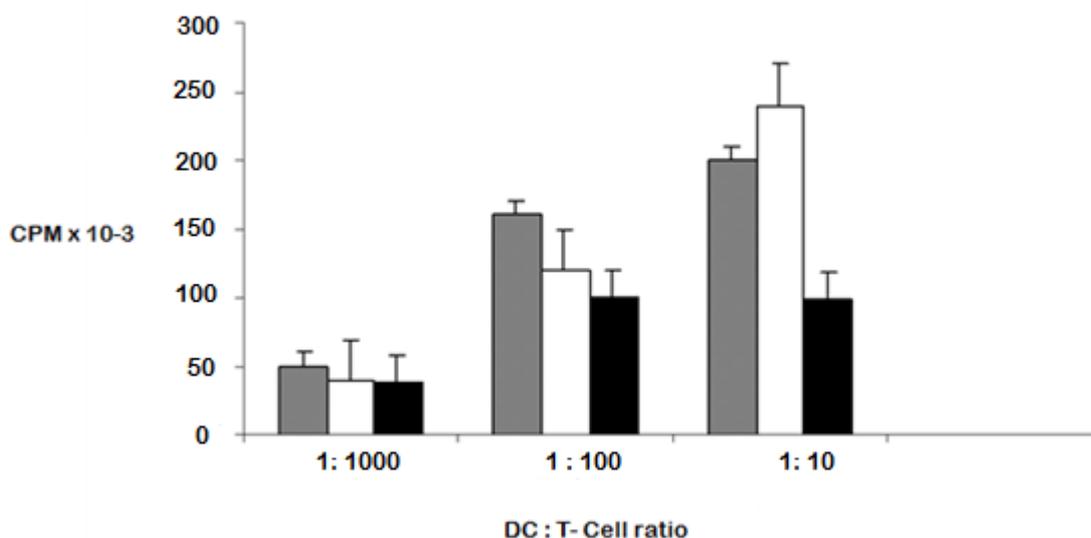


Figure 5.11 Proliferative allogenic T cell response by DC modulated by SP-A and SP-D: To examine if DCs maturation could affect T-cell proliferation in the presence of SP-A and SP-D, DCs were mixed with a varying ratio of T-cell clones (1:1000, 1:100, 1:10 DC: T cell ratio). After co-culturing 1:10 ratio gave the best proliferative response; cells were harvested 72 hours after thymidine pulse. DCs cause T cell proliferation up to 200,000 cpm, which was raised to 250,000 cpm in the presence of SP-D. However, SP-A was able to suppress proliferation of T-cell clones by 50%. The 'y' axis represents cpm x 10³. The grey bar represents medium alone, the white bar represents SP-D, while the black bar represents SP-A exposure to the co-culture system.

5.6. Discussion

SP-A and SP-D by virtue of being a pattern recognition soluble receptor has previously been shown to bind to a range of allergens derived from pollen grains, house mite, and *Aspergillus fumigatus*. Under in vitro conditions SP-A, SP-D, and a recombinant fragment of human SP-D have been shown to bind to mite allergens and purified Derp I in a calcium and maltose dependent manner (Wang *et al*, 1996) this interaction between surfactant proteins and mite allergens also inhibited specific IgE binding to allergens derived from mite allergen sensitive patients. Subsequently, SP-A, SP-D, and rhSP-D were shown to bind *Aspergillus fumigatus* allergens and antigens derived from 3wcf of mycelium, this interaction inhibited specific IgE and IgG to bind to allergens, which leads to suppression of histamine release by sensitized basophils derived from allergic bronchopulmonary aspergillosis patient (ABPA) (Madan *et al*, 1997). A murine model of pulmonary hypersensitivity that mirrored ABPA was then generated by first intranasal challenge with 3wcf followed by repeated dermal injection of the allergens stroke antigens within 4 weeks the

ABPA mice showed high levels of specific IgE and IgG in the serum, peripheral and pulmonary eosinophilia and heightened eosinophils peroxidase activity. Lung sections showed massive infiltration rich in poly-morphonuclear cells suggesting pulmonary inflammation. When the spleen cells were cultured and challenged with 3wcf, supernatant were rich in Th2 cytokines (IL-4 and IL-5). When ABPA mice were given therapeutically SP-A, SP-D, or rhSP-D, only SP-D or rhSP-D treated mice showed a reduced level specific IgG, IgE suppressed bloody eosinophilia, down regulated EPO activity and spleen culture supernatants showed a shift from Th2 cytokine profile to Th1 cytokine (IFN- γ) (Madan *et al*, 2001b). It was curious that SP-A was not very effective at offering resistance to allergenic challenge, subsequently, mice genetically deficient in SP-A or SP-D were examined for the development of ABPA models (Madan *et al*, 2005a). When the gene deficient mice were intra-nasally and systemically treated with 3wcf as described in Madan *et al* 2001, the SP-A knock-out mice showed poor immunological response to allergenic challenge. In other words, mice were resistant to developing ABPA. The SP-D knock-out mice, on the contrary, showed dramatic phenotypes that included exaggerated pulmonary inflammation, hyper eosinophilia, increased IgG and IgE levels, and massive up regulation of IL-13. This was a very novel and exciting development in the field. It was well known that SP-D knock-out mice have characteristics of emphysema, foamy macrophages, hyperlipidaemia, and protienosis (Poulain *et al*, 1998). Curiously, IL-13 over expressing phenotype of mice also include increased phosphor lipid pool, foamy macrophages, type II cell hypertrophy, fibrosis, eosinophilia, inflammation emphysema and AHR. In addition to several, fold increase in levels of IL-5 and IL-13, and lowering of IFN- γ to IL-4 ratio in the lungs. This Th2 bias was reversible by treating knock-out SP-D mice with rhSP-D. Knock-out SP-D mice more susceptible than wild type mice to pulmonary hypersensitivity induced by *A. fumigatus* allergens. Intranasal treatment with rhSP-D rescues *A. fumigatus*-sensitized knock-out mice.

Here, we have investigated the effect of SP-A and SP-D on DC maturation and cytokines profile. SP-A and SP-D seem to have an opposite effect on DC although they belong to the same family (Collectins). SP-A can suppress phenotypic DC maturation induced by LPS and TNF- α . SP-A decreases the secretion of TNF- α and IL-12, and at the same time the production of IL-10 via SP-A modulation suggests an

anti-inflammatory role during allergic response and hence contribution to the eventual resolution of inflammation. IL-10 can also raise the threshold for maturation and antigen presentation by bystander DC at sites of inflammation and tissue injury. Moreover, SP-A discourages IL-12 during T cells activation as well as allogenic T cell proliferative response. Thus, by modulating DC, SP-A may be crucial in balancing pro-inflammatory motives of matured DC and that of SP-D. On the other hand, SP-D has no effect on the progression of DC maturation, and SP-D may have a Th1 bias through increasing the secretion of TNF- α and IL-12. Finally, SP-D can be considered as an excellent inducer of allogenic T cell proliferative response, in addition to its effect to facilitate IFN- γ production.

It is evident that SP-A and SP-D are involved in homeostatic functions via balancing each other's pro-inflammatory and anti-inflammatory properties. By acting at the level of DC via IL-12, a nice balance of helper T cell polarisation can be achieved. The data also suggest that IL-10 production in the presence of SP-A may raise the threshold of inflammatory response and bring about tolerogenic effects. It can be concluded that SP-D has a hierarchical role in protection against allergy and asthma. These stages include binding to allergens, inhibition of IgE binding to allergens, inhibition of histamine release, allergen presentation, IgE synthesis, eosinophil apoptosis and helper T cell polarisation. This study suggests a mechanism through which SP-D can cause Th1 polarisation, as reported previously *in vitro* and *in vivo* (Mahajan *et al*, 2013 ; Madan *et al*, 2001a ; Madan *et al*, 2005b; Singh *et al*, 2003).

6. General Discussion and perspectives

Pulmonary surfactant protein SP-A and SP-D seem to have a range of immune and non-immune functions. Their non-immune functions include maintenance of the surfactant recycling and surface tension in the expiring and inspiring lungs. A number of therapeutic properties have been assigned to a recombinant fragment of human SP-D that is composed of homotrimeric forms of coiled-coil neck region and the CRD (rhSP-D). This includes its protective role murine models of influenza A virus (IAV) infection, invasive pulmonary aspergillosis (IPA) and allergic bronchopulmonary aspergillosis (ABPA) (Kishore et al, 2002). However, how a small fragment of human SP-D brings about these effects is not very clear. This issue becomes more pertinent in the light of existing knowledge about the receptor complex calreticulin-CD91 that is known to bind to the collagen region of human SP-D. A number of receptors and binding proteins have been described for the CRD region but their nature of interaction and pathophysiological significance remains uncertain (Kishore et al, 2006). Thus, this thesis sought to examine in vitro mechanisms via which rhSP-D offers therapeutic effects in the above-described murine models.

The rhSP-D described in the literature contains a fortuitous segment of N-terminal collagen region of eight Gly-X-Y repeats (Kishore et al, 1996). It has been largely debated that this extension provides additional stabilising effect on the trimerising force exerted by the neck region. It is worth noting that the two crystal structures described at high resolutions have been unable to reveal the collagen region (Shrive et al, 2003). It is thus largely believed that the 8 Gly-X-Y regions in the rhSP-D molecule is unstructured. Furthermore, it is also important to recognise that *E. coli* (which is the host strain for expressing rhSP-D) does not have the post-translational machinery to generate hydroxyprolines that would be required for the triple-helical assembly of the collagen-like region of SP-D. Thus, it is not surprising that rhSP-D crystals do not show up collagen extension in the three-dimensional crystallographic symmetry, as revealed by two high quality crystal structures published by Shrive et al (2003; Shrive et al, 2008). However, to address this issue further, we generated a new construct that include only neck and CRD region of human SP-D. Since in this construct, the 8 Gly-X-Y region was removed, we called it delta rhSP-D.

We report here that rhSP-D binds IAV via HA and NA, the two most important viral proteins for pathogenesis. This interaction appeared to calcium dependent. It was also noted that rhSP-D was able to inhibit IAV interaction with the target cells leading to modulation of the pro-inflammatory response triggered by IAV. These results are consistent with those reported by extensive studies carried out by the Hartshorn Group (Hartshorn et al, 1998; 2006). It is clear that rhSP-D can be sufficient to offer protection against IAV challenge in murine models. Thus, a murine model of IAV infection can be generated using a range of IAV strains and the ability of rhSP-D intranasally treated mice can be ascertained. The viral load in the lungs and the pathogenesis including cytokine storm in the lungs are likely to be down-regulated by rhSP-D treatment. Similarly, the delta version can be tested in murine models to establish if the presence of 8 Gly-X-Y region is not important for the potency of the molecule. Further studies can be carried out to examine if virus-like particles conjugated to rhSP-D are likely to be a better candidate for vaccination since rhSP-D is good at polarising Th1 immune response. In this case, rhSP-D can act as an adjuvant for vaccine trials.

In Chapter 5, it is reported that rhSP-D binds to *Aspergillus fumigatus* conidia in a dose-, calcium- and sugar-dependent manner. In addition, it is also able to enhance uptake by PBMCs. This is very intriguing since the current notion is that SP-D binds pathogens via its CRD region while the collagen region engages with calreticulin-CD91 complex in order to trigger phagocytosis. Given the lack of knowledge about a putative CRD receptor that is likely to be a phagocytic receptor, it is possible that SP-D upregulates other bonafide phagocytic receptors on the cell surface. If one examines the crystal structure of maltose-bound rhSP-D trimer (Shrive et al, 2003), it is apparent that only 2 CRD subunits are required for engaging carbohydrate ligand while the third subunit is available for interaction with a yet-to-be-discovered receptor molecule via protein-protein interaction. Thus, rhSP-D is potentially capable of acting as a bridging molecule between the effector immune cell and the target pathogenic ligand that can be virulence factors.

The most striking information that has arisen out of this study is the ability of rhSP-D to have a direct fungistatic effect on the germination of *A. fumigatus* conidia. This is a very novel observation. This observation adds another layer of hierarchical complexity in which SP-D operates against pathogens. Since SP-D is also found in

the body fluids which are unlikely to be the best place for cellular infiltration, a direct effect (Nayak et al, 2013). Therefore, a direct inhibitory effect on the growth of conidia is going to be an excellent strategy to contain the pathogen in a non-inflammatory way. Another advantage of direct fungistatic effect of rhSP-D is a delay in the germination of *Aspergillus* conidia in the lungs would allow infiltrating immune cells to clear the resting conidia that are yet to germinate. It is also evident that rhSP-D is having anti-fungal effect even on drug-resistant strains. A murine model of IPA or IA using drug-resistant strains should be used to ascertain if the in vivo protective effect of rhSP-D persists even for *Aspergillus* that are resistant to Amphotericin B and/or itraconazole. If it works, this will be a remarkable feat for a therapeutic protein.

In order to understand the mechanism of direct fungistatic effect of rhSP-D, a transcriptome analysis of *Aspergillus* using RNA extracted from rhSP-D treated and untreated samples might shed light on the metabolic suppression of the conidia in the presence of rhSP-D. This can be further validated at the protein level via differential proteomics analysis. With respect to the potency of the two variants of rhSP-D, the delta molecule appears to be less effective than the Gly-X-Y containing molecule, suggesting that an N-terminal extension of collagen region is indeed advantageous towards enhancing therapeutic capacity of the rhSP-D molecule. It will be helpful to test the two variants of rhSP-D in murine models of IPA and ABPA in addition to SP-D deficient mice and compare their therapeutic potencies.

Allergic asthma is regarded as a T-cell dependent disorder in which sensitised individuals develop eosinophilic airway inflammation in response to inhaled aeroallergens. Naïve T cells proliferate and differentiate in response to T-cell receptor recognition of peptide-loaded major histocompatibility complex (MHC) only when given co-stimulatory signals (CD80 and CD86) provided on the surface of professional APCs (Kishore et al, 2002). Dendritic cells (DCs) are the most efficient APCs having potent antigen-capturing and presenting capacity, which readily express co-stimulatory ligands for naïve T cells. Depending on the cytokine environment they encounter when maturing and on their degree of maturation, DCs can induce either Th1 or Th2 responses. Being resident within the airway epithelium (and as the only resident cell population expressing surface MHC class II molecules in normal, non-inflamed epithelium), airway DCs capture antigens and migrate to the

paracortical T cell zones of lymph node draining of the lung, where they interact with and sensitise naïve T cells and induce Th2-dependent airway eosinophilia (Medzhitov, 2008). Once allergen-specific Th2 memory cells are drawn into the lung, repeated allergen presentation by lung DCs may drive the persistent stimulation of specific memory Th2 cells and precipitate a state of chronic inflammation that contributes to the remodelled and hyper reactive air-ways that characterise asthma (Qaseem et al, 2013).

Since DCs play a pivotal role in the pathogenesis of allergy, they may also be key sub-jects for therapeutic strategies/interventions. We recently investigated the interaction between DC and SP-D or rhSP-D. Both molecules bind to immature human DC in a Ca²⁺-dependent and carbohydrate-independent manner. Binding of SP-D or rhSP-D is diminished on LPS-matured DC (Binker et al, 2003). In concordance with these observations, immature but not mature DC express the putative SP-D receptor, gp340. A recently published study on bone-marrow derived mouse DC showed that SP-D mediated binding and uptake of Escherichia coli also increased antigen presentation of E. coli expressed proteins to T-cell hybridoma (Binker et al, 2006). Interestingly, full-length native SP-D, but not rhSP-D, enhances the binding and phagocytosis of Staphylococcus aureus by both DC and neutrophils. We are currently investigating if an interaction between SP-D and DC prevent the activation of the existing Th2 response but facilitate the secretion of IL-12 and the induction of Th1 response. These lines of investigation should help understand a possible link between DCs and the SP-A-and SP-D-mediated polarisation of Th response. IL-5 is a differentiation factor for eosinophils, IL-4 (together with IL-13) is an important factor for isotype switching of B lymphocytes (leading to the secretion of IgG1 and IgE); together they mark a characteristic Th2 response in the allergic immune reaction (characterized by secretion of IL-4, IL-5, IL-10 and IL-13 and generation of humoral immune responses) (Qaseem et al, 2013). Shifting of cellular responses from a predominantly Th2 to a Th1 cytokine profile, following treatment with SP-A, SP-D or rhSP-D, appears central to the protective mechanism since IFN- γ , a Th1 cytokine, promotes cellular immunity and normally inhibits Th2 differentiation in response to IL-4.

It appears that SP-A and SP-D are highly specialised, rather than primitive, 'innate' molecules, capable of modulating responses to foreign agents. Among the factors that have been shown to influence the Th1-Th2 balance, IL-12 is dominant in directing the development of Th1 cells that produce high amounts of IFN- γ . DCs function simultaneously as APCs and IL-12-producing cells to induce the development of Th1 cells. Engagement of CD40 ligand (CD40L) by CD40 as well as CD28 by CD80 or CD86 is required for T cell priming by DCs, and IL-12 from DCs is a potent and obligate inducer of differentiation of IFN- γ -producing cells in vivo. On the other hand, it is also considered that Th2 response is a 'default pathway' and Th2 cells would develop spontaneously in the absence of IL-12, or in the presence of PGE₂, a product of the arachidonic acid signalling pathway, induced in allergen-mediated cross-linking of IgE on mast cells (Wang et al, 1996; Wang et al, 1998; Madan et al, 2001).

SP-A and SP-D have been shown to modulate bone marrow-derived mouse dendritic cells (DCs) (Binker et al, 2003). However these studies do not explain very well the in vivo data on murine models of allergy infection and inflammation. They also do not account for the protective effects exerted by SP-D in murine models of ABPA, dust mite allergy, and IPA. This is the first study that has systematically evaluated the modulation of immature as well as mature DCs using human cultured monocytes. Future studies should include testing rhSP-A and rhSP-D in an identical experimental setting. Knock-out mice should be tested for modulation of IL-13 overexpressing phenotype and adoptive transfer experiments.

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