IMMUNE SURVEILLANCE OF ACTIVATED IMMUNE AND TUMOUR CELLS BY SURFACTANT PROTEIN D

A thesis submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

Centre for Infection, Immunity and Disease Mechanisms

Biosciences

Brunel University, London

By

Eswari Marri

May 2014

=

Acknowledgements

The completion of this thesis would not have been possible without the help and support of many important people in my life. I would like to thank each and every one of them for their contributions and encouragement. In particular, I would like to thank my parents, Shri. Dodagatta Prabhakar, Smt. Dodagatta Saraswathi and my Husband Mr. Marri Chandra Sekhar, without whom it would have been impossible. Mr. D Pavan Kumar and Mr. D Maruthi Prasad, the best brothers that I could ask for.

I am in dilemma if I have to put my supervisor in my family list or in the nest paragraph, I whole-heartedly thank Dr. Uday Kishore for welcoming me into his laboratory, accepting me as a PhD student and more importantly I would like to thank him for his encouragement, support, and guidance. Words cannot accurately portray the support, guidance, education, and friendship he has provided and especially in the stressful and depressed times. In short, he has been a great pillar and support without which I couldn't have reached this stage and I am indebted to him for everything he had showered on me including his blessings. His corrections of publications, thesis and mistakes are incredibly appreciated. His humour and insight will stay with me for years to come.

Dr. Anthony Tsolaki for his valuable help, ideas, support, lessons, encouragement, faith, smiles and just being my second supervisor, another gem of a person, who is being a great support. He, being an expert molecular biologist guided me in molecular biology aspects of my work and had been a great pleasure working under his guidance.

Dr. Ansar Pathan for all of his help, ideas and guidance without which I couldn't have learnt how to do Science patiently, he being a very good teacher, always reminded me of my School and College days and at the same time being available whenever I needed him, during weekends, late nights and giving me advice and help. I will be thankful to him for my microscopy data. I cannot stop thanking him and my teachers who have been a major cause of what I am today.

Dr. Dinakar Salunke, Dr. Kanwaljeet Kaur and Ms. Sushma Nagpal from NII, India who gave me all the liberty to learn everything in the lab and treated me like their own kid which ingrained in me great research values and love for science more (I used to be called as Baby of the lab and it continued so for all the five years of my stay). I would like to thank each and every teacher and guide from my School and college days.

I would like to dedicate my thesis to my father-in-law late. Shri. Marri Narsaiah. He would have been one of the happiest people to look at my thesis if he was with us. He always had the trust that I would make him proud one day! I would like to thank him for treating me like his own daughter, equivalently or may be more!

I would like to thank my uncle Shri. Ajmer Dhillon, without whom I would have almost lost trust in Humanity and Shri. Desabandu Manivannan, for just being there when I needed them.

Last but not least! I would like to thank all my friends at CIIDM Janez, Asif, Naj, Lina (being a great friend and coolest), Aline, Abhi (treating me like his own sister), Suhair (for being a great moral support, an elder sister), Lubna (Lubbi, cutest friend that I could

ever have), Suha (being a good friend, always helpful), George (one of the person's I enjoy working with and being a good friend), Agnes, Kirsten, Syreeta, Chrissi, Basu, Anu and Anna.

I would especially thank Lubbi and Basu for helping me with my thesis corrections, going through the pain equivalently and at the same time without complaining, proud to have juniors like them.

Abstract

Surfactant protein D (SP-D) is a carbohydrate/charged pattern recognition molecule of the innate immune system. By virtue of its ability to recognize an array of carbohydrate patterns on the surface of a range of pathogens, SP-D can bring about opsonisation, enhanced phagocytosis and killing of a diverse range of viruses, bacteria and fungi. In addition to antimicrobial functions, which also includes bacteriostatic and fungistatic properties SP-D has also been shown to bind allergens derived from a number of sources including house dust mite, *Aspergilllus fumigatus* and pollen grains. SP-D allergen interaction leads to inhibition of specific IgE binding and subsequent downregulation of histamine release from sensitized basophils and mast cells. Thus, a number of murine models of pulmonary hypersensitivity and allergic asthma induced by ovalbumin, house dust mite and Aspergillus fumigatus allergens/antigens have been tested for the ability of SP-D to dampen allergic symptoms on the immunological parameters. In general, treatment of allergic models with a recombinant fragment of human SP-D (rh SP-D; composed of trimeric, neck and carbohydrate recognition domain) has been shown to cause downregualtion of specific IgE synthesis, pulmonary and peripheral eosinophilia and airway hyper reactivity, and Th2→Th1 polarisation. However, therapeutic alleviation of eosinophilia by rh–SP-D treatment became evident when SP-D gene deficient mice were found to be hypereosinophilic In fact, rhSP-D binds well to eosinophils derived from allergic patients and induces apoptosis without affecting eosinophils derived from healthy individuals or non-activated/non-sensitized eosinophils. Proteomic analysis of rh–SP-D treated eosinophillic cell line that revealed that apoptosis induction takes place via p53 pathway. In this thesis, proteomic signatures were replicated using a leukemic cell line AML14.3D10 via qPCR analysis by identifying targets from a spectrum of genes, which were either upregulated or downregulated. It appears that in spite of induction of apoptosis by rh-SP-D, different cells respond differentially at molecular levels (Chapter 3). Sensing that SP-D can induce apoptosis in altered or transformed cells; the effect of SP-D gene expression within pancreatic cancer cells was also investigated. The experiments confirmed p53 pathway dependence for suppression of cancer. Interestingly, factors responsible for metastasis for cancer are also downregulated by endogenous overexpression of SP-D, as validated by wound healing assay. We conclude that SP-D is a general immunosurveillance molecule, which is involved in the clearance of altered and transformed cells (Chapter 4). Chapter 5 shows a direct interaction between DC-SIGN and rh-SP-D that inhibits DC-SIGN interaction of allergens and HIV-1, tow common ligands for SP-D and DC-SIGN. Using transfected human embryonic kidney (HEK) cells expressing surface DC-SIGN, we found that pre-treatment of these cells with rhSP-D suppressed DC-SIGN mediated transmission of HIV-1to co-cultured PBMCs. The effect of rhSP-D-DC-SIGN In conclusion, this

thesis highlights a broader immune role of SP-D in homeostasis and probably assigns potential functions of extrapulmonary and/or locally synthesized SP-D within non-lung tissues and blood.

Table of Contents

Abbreviations

ABC1- ATP-binding cassette transporter

ACAMPs- apoptotic-cell-associated molecular patterns

AF- amniotic fluid

Afu- Aspergillus fumigatus

APC- antigen-presenting cell

APCS- antigen-presenting cells

BAL- Bronchoalveolar lavage

CAP- contraction-associated protein

CARD- caspase recruitment domain

CB-composite bodies

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

CV- clathrin-coated vesicles

CysLT1R- cysteinyl leukotriene receptors

DC- dendritic cells

DC- dendritic cells

DC-SIGN- dendritic cell-specific intercellular adhesion molecule 3-grabbing non integrin

ECP-eosinophil cationic protein

EDN-eosinophil-derived neurotoxin

EE-early endosomes

EPO-eosinophils peroxidase

ER-endoplasmic reticulum

FBG- fibrogen like domain

GA-Golgi apparatus

GLCnac- N-acetyl-glucosamine

HDLS- high-density lipoproteins

HDLs-high-density lipoproteins

HMGA1: High mobility group A1

ICAM-1- intercellular adhesion molecule-1

IFN-γ, TNF-α, NGF, GM-CSF, SCF, TGF-α, Rantes (CCL5), eotaxin (CCL11), GRO-α and ENA/78/CXCL5

ITAM- immunoreceptor tyrosine-based activation motif

ITIM- immunoreceptor tyrosine-based inhibition motif

KIR2DL3-, killer cell immunoglobulin-like receptor 2DL3

LB- lamellar body

LBP-lipopolysaccharide-binding protein

LDLs- low-density lipoproteins

LDLS- low-density lipoproteins

LOX1- oxidised low-density lipoprotein receptor 1

LTP- lipid-transfer protein

Lys-lysosomes

MANnac- acetyl-mannosamine

MAPK- mitogen-activated protein kinase

MASPS- MBL associated serine proteases

MBL- mannose-binding lectin

MBP-major basic protein

MFGE8- milk-fat globule epidermal growth factor 8

MMP- metalloproteinase

MØ- macrophages

MVB- multivesicular bodies

NBS-nucleotide-binding site

NF-kappaB- nuclear factor-kappaB

NITR-novel immune-type receptor

NK- natural killer

NKGA-natural killer glycoprotein C-lectin receptor

NOD- nucleotide-binding oligomerization domain

oxLDL- oxidised low-density lipoprotein

PCD- programmed cell death

PCD-Programmed cell death

PRRs- pattern-recognition receptors

PSGL- P-selectin glycoprotein ligand-1

PMF: Peptide mass fingerprinting

PtdSer- phosphatidylserine

SAP-serum amyloid protein

SFTPD- Human SP-D gene

SNP- single nucleotide polymorphisms

SP-A- surfactant protein A

SP-D-

SRA- class-A scavenger receptor

TGN-trans-Golgi network

TICAM1-TIR domain-containing adapter molecule 1

TIRAP-Toll/interleukin-1 receptor domain-containing adapter protein.

TLRS- toll-like receptors

TSP1- thrombospondin-1

WT- wild type

β-Hex-β-hexosaminidase

β_2-GPI- protein β_2-glycoprotein I

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

CHAPTER 5

2 List of Tables

Chapter 1

Introduction

1.1 Collectins

Innate immune defense mechanism includes epithelial surfaces with mechanical barriers and digestive enzymes. When non-self microbes get inside the barriers, they are recognized because of their pattern by the immune system to engineer the downstream mechanisms for pathogen clearance. Toll-like receptors (TLRs) are the primordial structures which recognize the pathogen-associated molecular patterns, PAMPs (Hallman, Ramet and Ezekowitz, 2001) . The interaction of these receptors leads to the activation of other defense mechanisms involving cells and molecules for pathogen clearance (Zasloff, 2002) (Figure 1.1)

Collectins are a group of C-type lectins which bind selectively to mannose, glucose, L-fucose, acetyl-mannosamine (ManNAc) and N-acetyl-glucosamine (GlcNAc) and not to galactose, which binds selectively via its C-terminal carbohydrate recognition domain (CRD). The sugar moieties should be presented at the terminal nonreducing position in a clustered manner for higher avidity interactions to take place. High avidity binding relates to biological activity depending on the oligomerization of the protein and hence the higher availability of CRDs clustering and also depending on the sugar molecules, e.g., One monosaccharide binding to CRD avidity is 10^{-3} M and for glycosylated albumin, it is 10^{-9} M(Kawasaki, 1983). Carbohydrate Pattern recognition on the pathogen surface varies from the mammalian cells and hence collectins differentiate between self and non-self. Collectins (see the table and figure below) do not recognize sialic acid, galactose at the same time these proteins bind to aberrantly glycosylated cancer cells and apoptotic cells. Affinity of the proteins may vary to different sugars, like SP-D has higher bonding with maltose, a disaccharide and in mouse, MBL-A binds to glucose more strongly than MBL-C (Types of MBL) (Hansen, 2000) .

Lectins from most of the animal species have been known to play a major role in the immune defense, subsequently shown to be very crucial role in the way they recognize the sugar moieties at the different levels of defense mechanisms in humans. The first animal lectin known was Conglutinin by Bordet and Streng and was termed due to the ability of agglutination, the protein agglutinates erythrocytes coated with antibody and complement (Bordet, 1906), which was later shown to be a calcium-dependent carbohydrate recognizing protein (Leon and Yokohari, 1964)

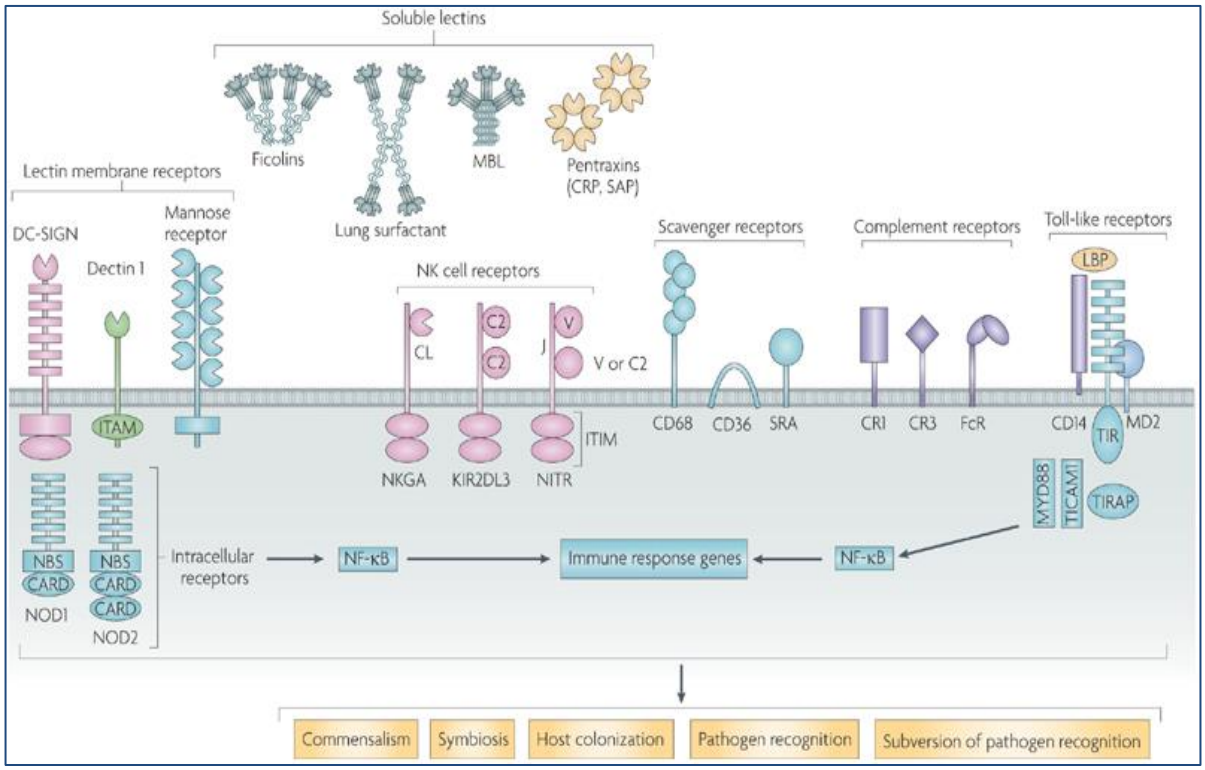


Figure 1.1 Extracellular pattern-recognition receptors (PRRs) include the soluble lectins, such as collectins (for example, mannose-binding lectins and lung surfactants), ficolins and pentraxins (for example, C-reactive proteins and serum amyloid-P), and integral membrane C-type lectins, including dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), dectin 1 and the macrophage mannose receptor. Other extracellular receptors shown are the natural killer (NK) cell receptors, scavenger receptors and complement receptors. The extracellular Toll-like receptors and the intracellular nucleotide-binding oligomerization domain (NOD) receptors, both of which are rich in leucine rich repeats, activate immune genes through nuclear factor-kappaB (NF-kappaB). Soluble and membrane-associated lectins mediate interactions with microorganisms that may lead to mutualistic interactions (commensalism or symbiosis), host colonization, immune recognition by the host or 'subversion' of the non-self recognition functions of the receptors by the microorganisms for attachment to the vector or host invasion. CARD, caspase recruitment domain; CRP, C-reactive protein; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; KIR2DL3, killer cell immunoglobulin-like receptor 2DL3; LBP, lipopolysaccharide-binding protein; MBL, mannan-binding lectin; NBS, nucleotide-binding site; NKGA, natural killer glycoprotein C-lectin receptor; NITR, novel immune-type receptor; SAP, serum amyloid protein; TICAM1, TIR domain-containing adapter molecule 1; TIRAP, Toll/interleukin-1 receptor domain-containing adapter protein. (Vasta, 2009).

.

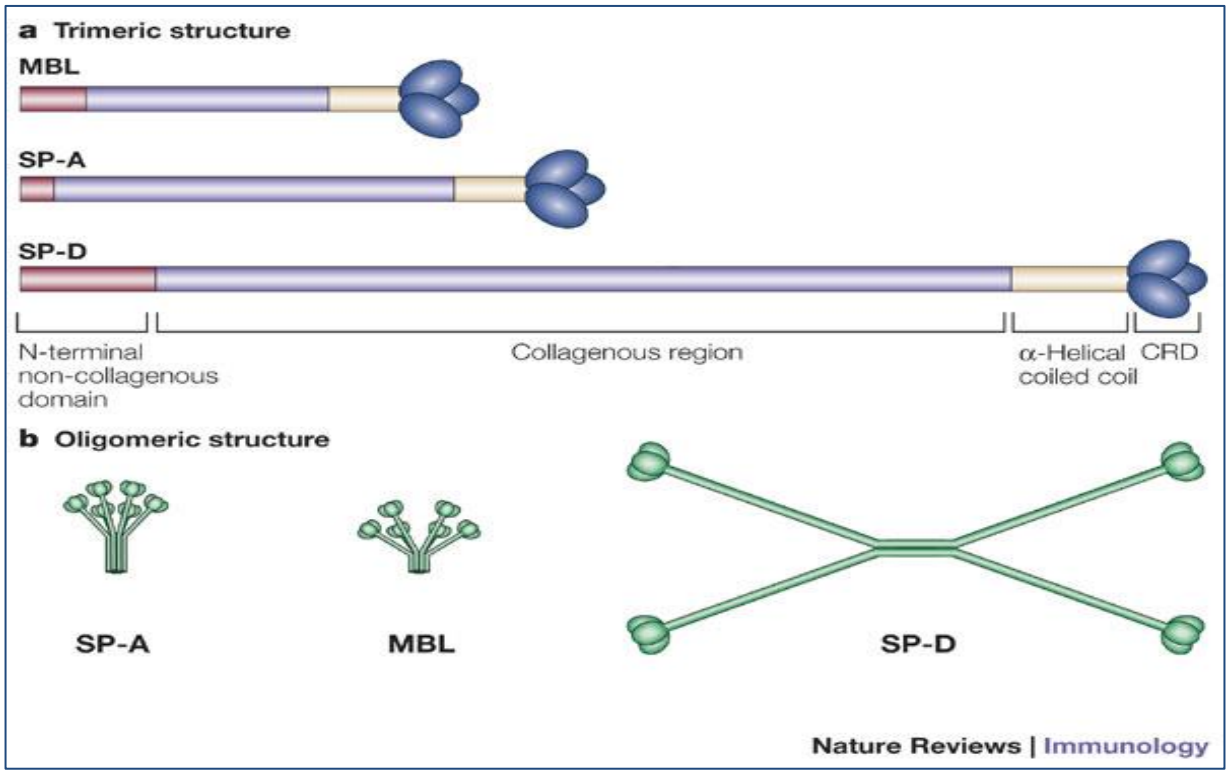


Figure 1.2 Surfactant protein A (SP-A) and SP-D are members of a family of proteins known as collectins. a | Collectins have collagen-like amino (N)-terminal regions and C-type (calcium dependent) carbohydrate-recognition domains (CRDs). Collectins consist of structural subunits that are composed of trimeric polypeptide chains, which are identical except for human SP-A. The trimers are assembled into oligomers. b | SP-A and mannose-binding lectin (MBL) are octadecamers (18-mers), consisting of six trimeric subunits. SP-D is a dodecamer (12-mer), consisting of four trimeric subunits. Although C1q is structurally homologous to SP-A and MBL, it is not a collectin as it does not have a lectin domain (CRD). Note that these models are not drawn to scale(Wright, 2005).

The nomenclature “collectin” means a collagenous structure with C-type carbohydrate-recognizing domains (CRDs) (Holmskov *et al.*, 1994) . Collectins are innate immune molecules in all the vertebrates; Mannan binding lectin (MBL) was first found in Rabbit (Kawasaki, Etoh and Yamashina, 1978) and Surfactant protein A (SP-A) (White *et al.*, 1985) and Surfactant protein D (SP-D) were discovered later in human lungs (shown in figure 1.2) (Persson *et al.*, 1988) .

Major subgroup of calcium-dependent lectins is collectins, which belong to superfamily of calcium-dependent lectins (C-type lectins). Collectins are characterized by a collagen-like sequence and a carbohydrate recognition domain and are members of the vertebrate C-type lectin superfamily. Conglutinin, CL-43 and CL-46 are found only in the family Bovidae (Hansen, 2003). “Novel collectins”, different from “classical collectins” consisting of MBL and surfactant proteins A and D (SP-A and SP-D), have been found by reverse genetics. The “novel collectins” consist of collectin liver 1 (CL-L1) (Ohtani *et al.*, 1999) , collectin kidney 1 (CL-K1), and collectin placenta 1 (CL-P1) (Ohtani, 2001) which are encoded by three separate genes. Structurally and functionally related protein family to collectins are “Ficolins”, whose CRD is a fibrogen like domain (fbg) and lectin domains attached to collagenous regions (Le *et al.*, 1997; Ichijo, 1993) . L-ficolin, M-ficolin and H-ficolin are the three human proteins known (Matsushita and Fujita, 2001).

Nine different members have been identified so far in the collectin family;

1. Mannose binding lectin (MBL)
2. Conglutinin
3. Surfactant protein A (SP-A)
4. SP-D
5. Collectin (Cl)-43 [43K Da]
6. CL-46 [46 KDa]
7. CL-P1
8. CL-L1 (Liver)
9. CL-K1

Secretory Type

Bovine

Non-secretory

Secretory Type

10. Collectin 11 also a unique class found recently in Humans and *Xenopus laevis*. Recently its chicken homologue cCL-2 was also discovered.

Both novel and classical collectins play an important role in innate immunity, in addition to embryonic morphogenesis and development. Also play a role in the embryonic morphogenesis and development.

Innate immune system has evolved in such a way that it contains as well as eliminates pathogens while curbing attack on self (autoimmune response) and excessive inflammation. The primary structure of collectins consists of three similar polypeptide chains with the trimerization of the CRDs at the C-terminal that is initiated by an α-helical coiled-coil neck region, continueing through the collagenous triple-helical region at the N-terminal. Glycine residues present at every third position are packed inside the collagen region helping in the tightly twisting of this region. Collagen region of the collectins varies from protein to protein; MBL has 19 Gly-Xaa-Yaa triplets and SP-D has 59 triplets. N-terminal region has 7-28 amino acids with 1-3 cysteines, which help in the covalent bonding of the three-polypeptide chains and multimerisation (Figure 1.3) of unto 6 subunits. The collagen region of SP-A and MBL has the kink. SP-A has two forms of similar polypeptides named as SP-A1 and SP-A2; in case of SP-A1, the protein sequence has extra cysteine in-between the collagen and α-helical neck region allowing it to participate in the cross-linking of the polypeptide of the same subunit. It is not known if SP-A1 and SP-A2 form homo- or heteromers in vivo. Electron microscopy shows a central knob at the N-terminal linking regions showing the subunits radiating out to form a sertiform (sertula-small umbel) (Holmskov *et al.*, 1994) . SP-D forms a cruciform or even cart wheel like structure when it oligomerizes with other cruciform structures and MBL forms dimers to hexamers. Structure of collectins and ficolins resembles with the structure of C1q, which is the first component of the classical pathway of complement activation. C1q is formed of three different polypeptide chains and does not belong to the family collectins. Collectins are large molecules upto 90nm (SP-D) thus help in the agglutination of the targets with repulsive electrostatic charges. CL-P1 is the only collectin, which belongs to type II membrane protein with short cytoplasmic tail that contains the endocytosis motif(Ohtani, 2001), coiled-coil region like the macrophage scavenger receptor (extracellular), which is polycharged, has a C-type CRD with 6 conserved cysteines instead of 4 (other collectins) acting as a scavenger and galactose affinity. This protein evolved independently to other collectins as a chimeric molecule

resembling MARCO and SR-A1. Bovine collectins Conglutinin and CL-46 resemble SP-D, forming cruciform like structures and CL-43 has a homologous subunit resembling to human Cl-1(Hansen, 2003). Genes for SP-A, SP-D and MBL is located on the long arm of chromosome 10 in humans (Holmskov, 2000). CL-P1 gene is located on the long arm of chromosome 8 (Ohtani *et al.*, 1999) and CL-L1 gene on the short arm of the chromosome 18 (Ohtani, 2001).

MBL activates complement through associated serine proteases (MASPs). Three proteases MASP-1(Matsushita and Fujita, 1992), MASP2 (Thiel *et al.*, 1997) and MASP3 (Dahl *et al.*, 2001) and a non-protease MAp19(Stover, 1999)that are associated with MBL are known. MASP-1 and MASP-3 are encoded by MASP1/3 gene located on chromosome 3 (Dahl *et al.*, 2001) and MASP-2 and MAp19 are encoded by MASP-2/MAp19 gene located on chromosome 1 (Stover, 1999). Binding of MBL to the MASPs and MAp19 which resembles the proposed structure of the C1 complex (C1q with the tetramer C1s-C1r-C1r-C1s), but MASPs can form only homodimers (Chen, 2001; Thielens, 2001)and bind to distinct MBL oligomers (Dahl *et al.*, 2001) (but do not bind to C1q even though MASPs resemble C1r/s(Thiel, 2000)) no sequential activation is seen. Other collectins have not been shown to be binding to proteases (figure 1.4).

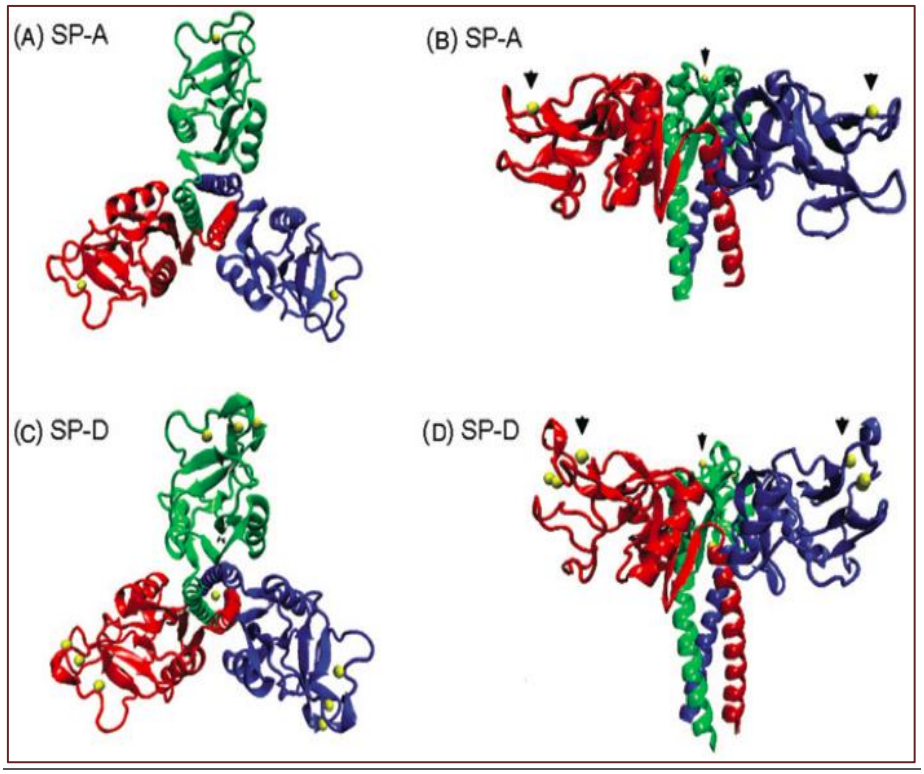


Figure 1.3 Ribbon diagrams of the neck and CRD domains of SP-A (A,B) and SP-D (C,D). Figures B and D are rotated 90 from the figures A and c respectively. The individual chains are coloured red, green and blue with the calcium ions, one in SP-A and 4 in SP-D coloured yellow. The sugar binding sites are highlighted with arrow-heads (Waters *et al.*, 2009)

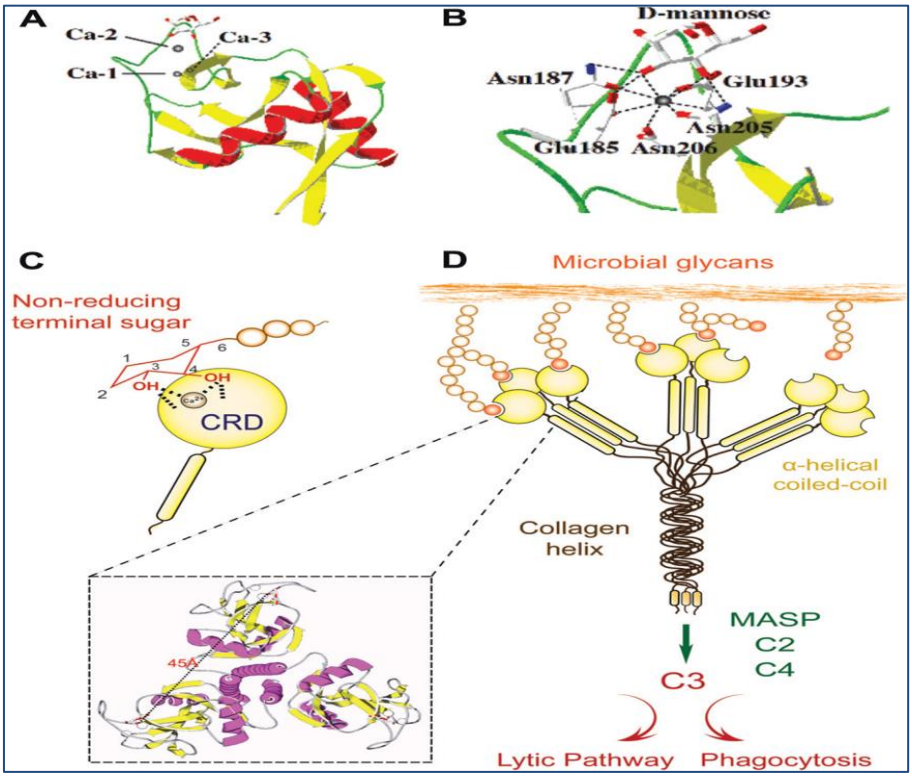


Figure 1.4 Recognition and effector activities of the mannose-binding lectin (MBL). (A) A schematic representation of CTLD organization; (B) Crystallographic model of CTLD; (C) Ca2+-dependent carbohydrate binding of CTLD. The CRD recognizes equatorial hydroxyls on C3 and C4 of non-reducing terminal mannose with participation of the Ca2+ atom. (D) The MBL trimer binds to ligands that are displayed about 45 Å apart on the microbial surface, and via association with the MASP) may activate the complement cascade, leading to opsonization or lysis of the microbe (Vasta *et al.*, 2012)

The Liver produces MBL and the MASPs that are released during immune response and inflammation. Lung synthesizes majority of SP-A and SP-D by the alveolar type II cells and non-ciliated bronchial epithelial cells, which are secreted on to the epithelial surfaces. Epithelial cells of gastrointestinal tract and exocrine glands also secrete SP-D (Stahlman *et al.*, 2002; Madsen, 2000) SP-A has been shown in the rat gastric mucosa(Lacaze-Masmonteil, 1995)

Respiratory tract is covered with pulmonary surfactant in order to keep the alveoli from collapsing by reducing the surface tension. Pulmonary surfactant is a mixture of 90% lipids and 5-10% of proteins produced by alveolar type II cells, which forms a liquid phase covering the alveolar epithelium. Surfactants produced by the alveolar type II cells are stored in the "lamellar bodies" as intracellular inclusion organelles and secreted to form a lattice known as "tubular myelin" into the alveolar space (forming a monolayer lipid layer). Major lipids in pulmonary surfactant are phospholipids that include phosphatidylcholine and phosphotidylglycerol, which help is lowering surface tension (e.g., dipalmitoyl phosphatidylcholine-DPPC). Phosphotidylglycerol and phosphatidylinositol help in the regulation of inflammatory responses(Haagsman and van Golde, 1991; Wright and Dobbs, 1991).

Four surfactant proteins are known as SP-A, SP-B, SP-C and SP-D. SP-A and SP-D are hydrophilic where as SP-B and SP-C extremely hydrophobic and small. SP-B is required for the reduction in surface tension in the lungs and SP-C binds to lipopolysaccharides (LPS). In the absence of the surfactants, surface tension is extremely high at end expiration and tends to collapse. SP-A is most abundantly found (5.3%) out of the four proteins, SP-C least of around 0.4% and with SP-B and SP-D of 0.7 %and 0.6 % respectively(Kishore and Reid, 2001; Weaver and Whitsett, 1991). SP-B (14 kDa) and SP-C (6 kDa) are hydrophobic proteins with smaller molecular weights that are known to have major role in adsorption to air-liquid interface (lowering surface tension) in the peripheral air space following expiration, surfactant organization, phospholipid packaging. SP-B interacts with DPPC stabilizing the phospholipid monolayer and SP-C stabilizes the phospholipid layers, which are formed during the film compression at low lung volumes (Figure 1.6).

SP-A and SP-D are large oligomeric hydrophilic (not part of the classic surfactant system), and does not readily bind to phospholipids on cell surfaces and as

The organs due to the inflammation or other stimuli synthesize MBL, SP-D and SP-A. SP-A and SP-D are also found in the amniotic fluid from the 16 to 20 weeks of gestation in lower levels and the concentration increases during gestation. Other surfactant components increase with the glucocorticoids treatment(Ballard, 2000). MBL levels increase to the optimal level after a few weeks of delivery(Thiel, 1995). CL-L1 is produced in liver (Ohtani *et al.*, 1999) and CL-P1 by many tissues, displayed by endothelial cells isolated from placenta(Ohtani, 2001).

Collectins bind to various micro-organisms ranging from viruses, bacteria, fungi to protozoa (Figure 1.5) (Jack, Klein and Turner, 2001; Crouch and Wright, 2001; Holmskov, 2000) . Binding of the collectins is modulated by the plasma and surfactants when they interact with these microorganisms. The structure of the collectins help in the mechanism of defense and its binding. MBL and ficolins opsonize the microorganisms via complement activation and C3 deposition. SP-D and SP-A aggregate and opsonize the microorganisms directly, leading to phagocytosis and killing and clearing from the lungs modulating the cytokine and oxidant responses (Tino, 1996; Pikaar *et al.*, 1995) .

Collectins can bind to bacteria via Lipoteichoic acid (gram-positive bacteria) and LPS (gram-negative bacteria) in their cells walls(Polotsky, 1996), including the other complex arrays of surface glycoconjugates which act as ligands. MBL binds to *Escherichia coli,* which leads to the activation of complement activation and then killing of the bacteria(Kawasaki, 1989). Initially MBL was known to activate complement pathway(Ikeda, 1987), later it was shown to bind with various bacteria (Jack, Klein and Turner, 2001) and microbial species cultured from patients and with varied strains (Townsend *et al.*, 2001; Neth *et al.*, 2000) . Noncapsulated strains of Haemophilus influenza and Neisseria meningitidis isolated from meningitis patients bind better to capsulated ones, thus escaping from the opsonization (van Emmerik *et al.*, 1994) . SP-A and SP-D bind to both gram positive and gram-negative bacteria(McNeely and Coonrod, 1993). The ligand on *H.influenzae* type A is the P2 outer membrane protein for SP-A, but doesn't bind to more virulent type b bacteria(McNeely, 1994).

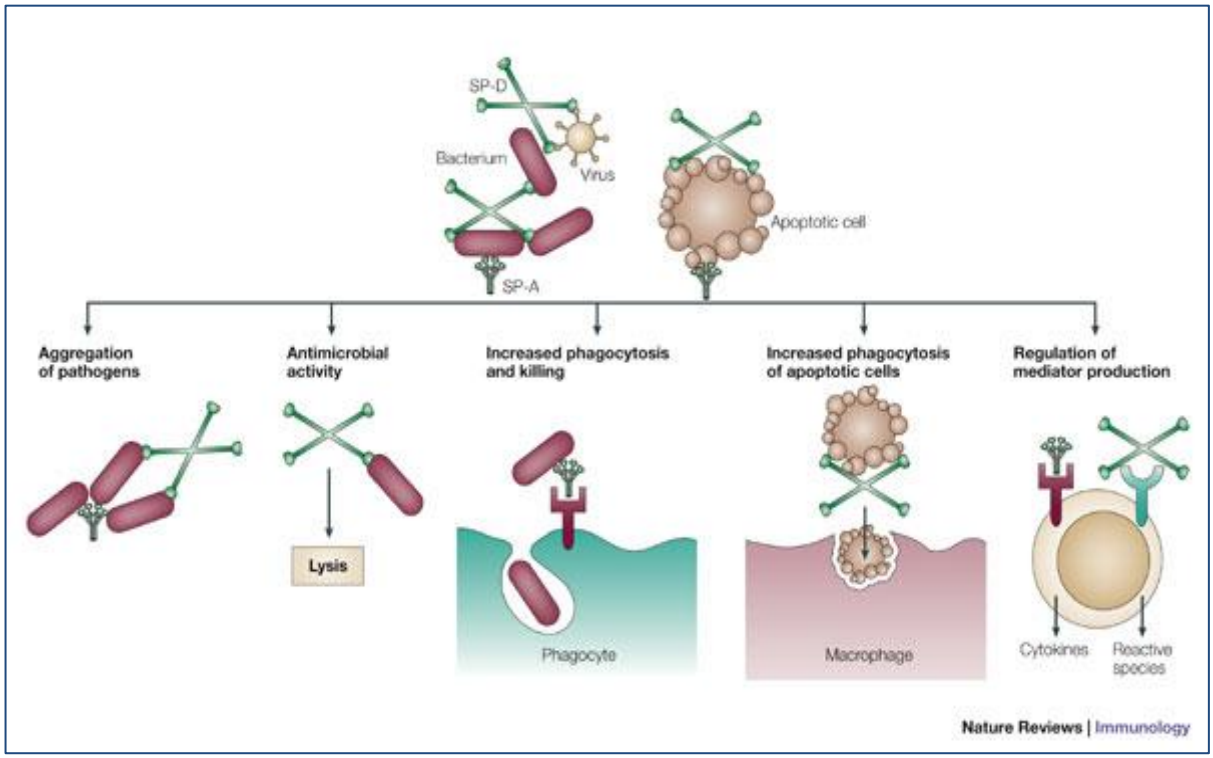


Figure 1.5 Surfactant protein A (SP-A) and SP-D bind to a variety of bacteria, viruses, allergens and apoptotic cells and thereby function as opsonins to enhance the uptake of these cells and particles. Binding of the collectins to pathogens occurs by various mechanisms. Some pathogens are aggregated by SP-A and/or SP-D. SP-A and SP-D also have direct effects on immune cells and modulate the production of cytokines and inflammatory mediators.(Wright, 2005)

SP-D binds to the lipids in the cell membrane of Mycoplasma pneumonia(Chiba, 2002). SP-A and SP-D modulate the binding of M.tuberculosis with macrophages by direct interaction with the alveoalar macrophages and upregulating the mannose receptor activity on the macrophages(Ferguson, 2002). SP-D inhibits uptake of M.tuberculosis is independent of bacterial agglutination(Ferguson, 2002). *Staphylococcus aureus*, *E.coli* and *Saccharomyces cerevisiae*, oxidized low-density lipoprotein bind to CL-P1, which mediates phagocytosis(Ohtani, 2001)LPS-induced cytokine production is modulated by the collectins, which bind to LPS. Intratracheal LPS delivery leads to the increase in the levels of SP-A and SP-D (McIntosh *et al.*, 1996) and bind to lipid A aggregates and rough LPS and not to smooth LPS directly but might interact with CD14 and thus indirectly inhibits LPS binding, thus cellular responses (Sano, 1999; Sano *et al.*, 1999) SP-D binds to core saccharides of LPS and modulate alveolar macrophages to uptake and degrade LPS. SP-A knockout mice produced TNF-α and administration of SP-A exogenously reduced the cytokine production (Borron, 2000; Borron *et al.*, 2000) .

Collectins bind to viruses through the glycoproteins on enveloped viruses like influenza virus, human immunodeficiency virus, herpes simplex virus and to the non-enveloped rotavirus. Influenza virus and collectins binding has been shown *in vitro* and *in vivo* (Crouch, Hartshorn and Ofek, 2000) and different sensitivity of the different strains of viruses is related to the level of glycosylation of the globular heads (Hartley *et al.*, 1997; Reading *et al.*, 1995) . Influenza A virus hemagglutination and infectivity is inhibited by MBL (Hartshorn *et al.*, 1997; Hartshorn *et al.*, 1993) , SP-D (Hartshorn *et al.*, 1997; Hartshorn *et al.*, 1996) and SP-A (Hartshorn *et al.*, 1997; Benne *et al.*, 1995) . MBL activates complement activation and lysis of influenza virus infected cells (Reading *et al.*, 1995) and also binds to HIV-1 and HIV-2 to gp120 (Haurum *et al.*, 1993) SP-D agglutinates viruses and also enhances the uptake by the neutrophil granulocytes and protecting them against the virus (Hartshorn *et al.*, 1997; Hartshorn *et al.*, 1996) whereas SP-A binds to the virus, but doesn't protect the neutrophils against virus (Hartshorn *et al.*, 1997) and opsonizes influenza A virus for the phagocytosis by alveolar macrophages but not observed in case of SP-D (Benne *et al.*, 1997)

SP-A and SP-D binds to *Pneumocystis carinii* glycoprotein gpA, and enhance the alveolar macrophages to interact with (O'Riordan *et al.*, 1995; Zimmerman *et al.*, 1992) . SP-A, SP-D

(agglutinate the bacteria) and MBL bind to non-capsulated *Cryptococcus neoformans* (Schelenz *et al.*, 1995) SP-A and SP-D bind, phagocytise and kill the *Aspergillus fumigatus* conidia with the help of human alveolar macrophages and neutrophils (Madan *et al.*, 1997a) . 80% murine survival showed that intranasal administration of SP-D is as efficient as amphotericin in the treatment of invasive pulmonary aspergillosis when compared to control (Madan *et al.*, 2001) .

MBL binds to protozoans like *Leishmania* major and *Lieshmania mexicana* promastigotes (Lipophosphoglycans on the cell surface) (Green *et al.*, 1994) , promastigotes (mannose-containing glycoinositol phospholipids) and the intracellular amastigotes and not to trypomastigotes or epimastigotes of *Trypanosoma cruzi*. Opsonization of amastigotes with the help of MBL leads to the clearance of the protozoan from the circulation (Kahn *et al.*, 1996)

1.2 Surfactant Protein SP-A and SP-D

SP-D binds in a calcium-dependent manner to complex carbohydrates and lipids that serve as pattern-recognition molecule. Unlike SP-A, it does not bind to the lipid A, but to the contiguous core oligosaccharide of LPS. It is known to preferentially bind to Inositol, maltose, mannose and glucose.

The primary structure is made of cysteine-containing N-terminal region, a triple helical collagen region with Gly-X-Y repeats, an α-helical coiled-coil region and C-terminus C-type lectin (Figure 1.7) (Kishore *et al.*, 2006) . The lining of the lung epithelium of alveoli and distal airways play a major role in the neutralization and clearance of inhaled microorganisms and other inhaled toxic particles. SP-A and SP-D are potent innate immune molecules, which are secreted by the airway epithelial cells with a range of pulmonary and extra pulmonary sites (Table 1.1); interact with microorganisms, leukocytes modulating the function of phagocytic cells in vitro and in vivo using immune mechanisms such as agglutination/aggregation, enhancement of phagocytosis (Eggleton and Reid, 1999; Reid, 1998; Haagsman, 1998; Korfhagen, LeVine and Whitsett, 1998; Wright, 1997) and also modulate the cellular effects of inhaled pollens and house dust mites (Wang *et al.*, 1996; Malhotra *et al.*, 1993)

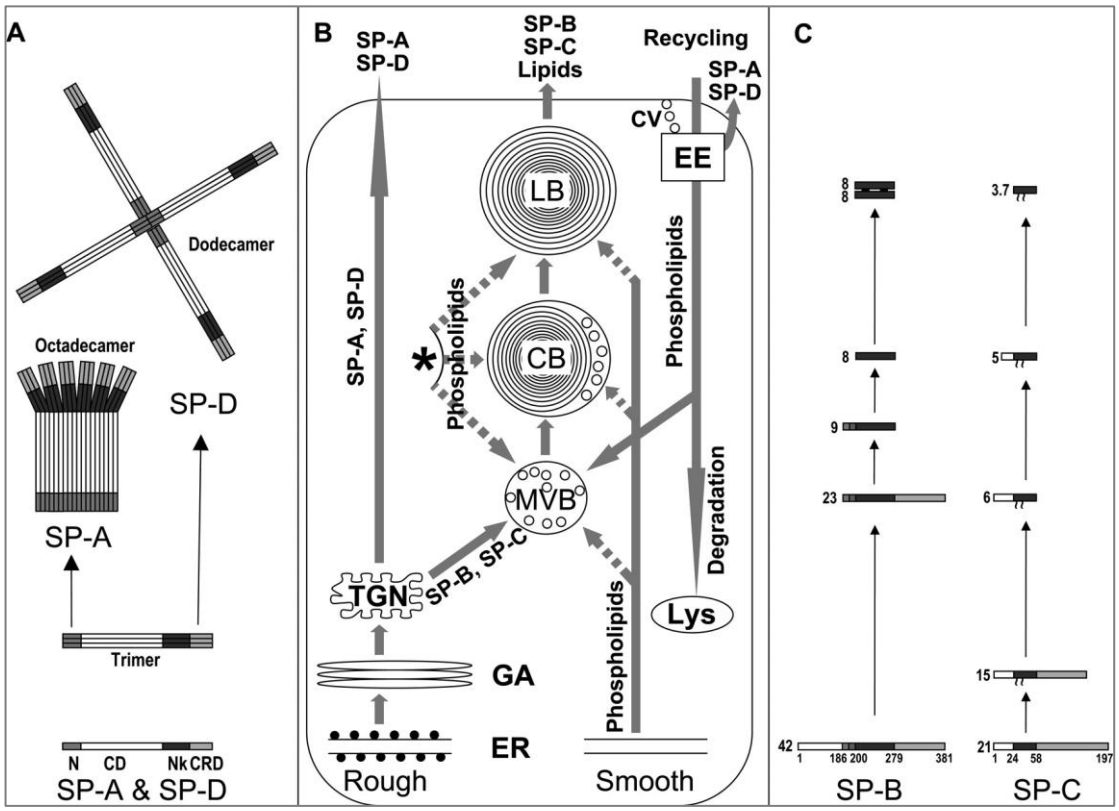


Figure 1.6 Processing and secretion of surfactant components. *A*: domain structure and assembly of surfactant protein (SP)-A and SP-D. SP-A/SP-D domain organization: N, NH2-terminal domain; CD, collagenous domain; Nk, neck domain; CRD, carbohydrate recognition domain. *B*: lamellar body (LB)-independent (SP-A and SP-D) and LB-dependent (SP-B, SP-C, and phospholipids) secretion. Recycling of surfactant components via clathrin-coated vesicles (CV) is also shown, followed by either degradation or recycling to multivesicular bodies (MVB) and eventually resecretion. ER, endoplasmic reticulum; GA, Golgi apparatus; TGN, trans-Golgi network; EE, early endosomes; Lys, lysosomes; CB, composite bodies. *Phospholipids synthesized in the cytoplasm, e.g., in the glycogen pool (see main text). *C*: domain structure of SP-B and SP-C preproteins and their processing. Processing products are shown at the level of their presumed locations in the organelles of secretory pathways shown (*middle*) (Andreeva, Kutuzov and Voyno-Yasenetskaya, 2007) .

These proteins bind to target ligands on pathogens, allergens, and apoptotic cells through their CRD region and collagen region might bind to the cell receptors bringing about the effector functions. SP-A and SP-D are known to be playing a major role in the control of pulmonary inflammation in diseases like asthma and allergy.

These proteins link the innate and adaptive immunity by the modulation of dendritic cell function and helper T cell polarization. SP-A (over 99% in lavage fluid) binds to surfactant phospholipids like DPPC strongly forming a tubular myelin (Kuroki and Voelker, 1994; Van Iwaarden *et al.*, 1994) . 180 µg/ml is the concentration of SP-A in the normal human (van de Graaf *et al.*, 1992) and helps in the adsorption of surface-active material to air-liquid interface and prevents the inactivation of surface-active material by serum proteins which is crucial in acute lung injury (Schurch *et al.*, 1992; Cockshutt, Weitz and Possmayer, 1990) . It increases uptake of phospholipids into type II cells for recycling of surfactant and stops secretion of surface-active material by alveolar type II cells (Wright, Borchelt and Hawgood, 1989; Wright *et al.*, 1987) . SP-A is thus important for surfactant homeostasis in the lungs, also inhibits calcium-independent phospholipase A2 and decrease degradation of internalized phospholipids during recycling (Fisher, Dodia and Chander, 1994) . SP-A is seen in lamellar inclusions of type II cells and the tubular myelin lattice, in secretory granules of non-ciliated bronchiolar cells along conducting airways, small airways, few cuboidal epithelial cells at alveolar terminal airway junction, tracheal glands, etc. SP-A has hexameric structure of 6 structural subunits, which get associated to form a molecule of 630 kDa of 18 chains.

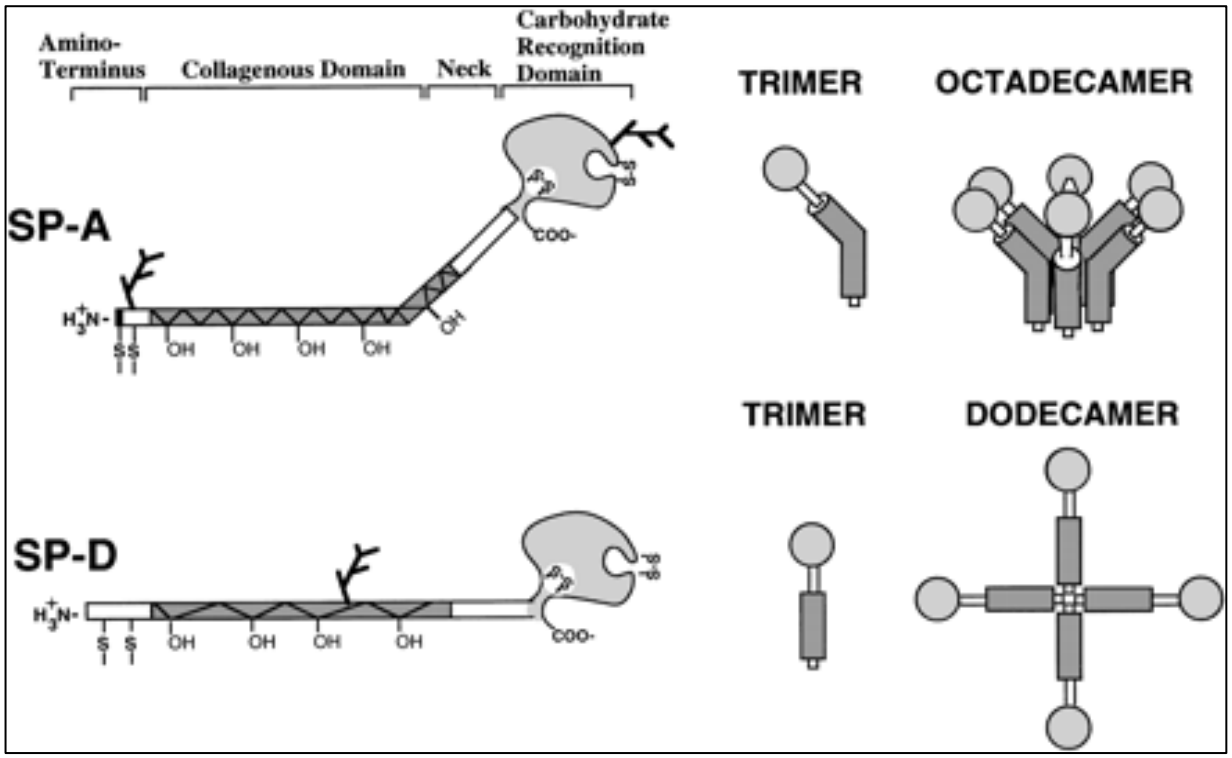


Figure 1.7 Structural organization of surfactant protein (SP) A and SP-D. Monomeric units of SP-A and SP-D can be conceptually divided into an amino-terminal position, a collagen-like region, a neck, and a carbohydrate recognition domain. Monomers form trimers that, in turn, form higher ordered oligomers of 18 units for SP-A and 12 units for SP-D (Mason, Greene and Voelker, 1998)

Table 1.1 SP-D secretion in the pulmonary and extra pulmonary sites

System	Organ	Type of cells
Respiratory Tract/Orthopharynx/Naso Pharynx	Lung	Alveolar Type II cells, non-ciliated clara cells, bronchiolar cells
	Trachea	Sub mucosal glands and respiratory epithelial cells
	Lacrimal glands	Ductal epithelial cells
	Salivary glands	Ductal epithelial cells
	Eustachian tube	Squamous epithelium
Gastrointestinal Tract	Oesophagus	Squamous epithelium
	Stomach	Glandular epithelial cells
	Small Intestine	Mucosal epithelial cells
	Pancreas (Exocrine)	Ductal epithelial cells and Islets
	Biliary tract	Intrahepatic ductal epithelial cells
Genitourinary Tract	Prostrate	Glandular epithelium
	Bladder	Transitional epithelium
	Kidney	Collecting duct epithelium
	Uterus	Endometrium (endocervical epithelium)
	Fetal membranes	-
Skin and soft tissue	Cutaneous squamous epithelium	Squamous epithelium
	Sweat glands	Ductal epithelium
	Sebaceous glands	Glandular epithelium
	Breast	Mammary epithelium
	Mesentery	-
Other	Brain	-
	Testes	Leydig cells
	Pancreas (endocrine)	Islets
	Placenta	-

SP-D is a 43 K Da protein of three polypeptide chains, predominantly assembled as dodecamers, which consists of homotrimers. Each trimeric subunit has four major domains (Holmskov, Thiel and Jensenius, 2003; Kishore and Reid, 2001) :

1. N-terminal domain, cysteine-containing NH2-terminal crosslinking.
2. Collagen domain with a triple helical collagen domain of variable length.
3. Neck region with a trimeric coiled-coil linking domain and
4. A carboxy-terminal domain, C-type lectin carbohydrate recognition domain (CRD).

Six of the trimeric subunits make up the overall structure of SP-A, while SP-D is composed of a cruciform-like structure, with four arms of equal length (Figure 1.8). Predominant collagens in the trimeric complex are hydroxylysine and hydroxylysyl glycosides.

SP-D dodecamers can bond at their amino-termini to form multimers with complex arrays of up to 32 or more trimeric CRDs (Kishore *et al.*, 2006; Holmskov, Thiel and Jensenius, 2003) . Natural SP-D from human alveolar proteinosis and bovine lavage and recombinant human SP-D contain a high proportion of these multimers. 130 kDa subunit comprises of three identical polypeptide chains of 43 kDa each containing N-linked oligosaccharide structure at Asn (LeVine *et al.*, 1999) . Human SP-D is gets assembled to 520 kDA tetrameric structure with 4x130 kDa homotrimeric subunits linked by their N-terminal regions, also get assembled to trimmers (3x130 kDa), dimers (2x130 kDa) and monomer (1x130 kDa) in SP-D preparations. 8 of the 520 kDa tetrameric structures can form multimers of a large molecule of 96 (8x12) CRDs (Figure 1.8). EDTA does not dissociate them or competing sugars, and is cross-linked by disulphide and non-disulphide bonds. SP-D multimers show higher apparent binding affinity to a variety of ligands and are considerably more potent on a molar or weight basis in mediating microbial aggregation and aggregation-dependent interactions with leukocytes (Holmskov, Thiel and Jensenius, 2003) . In most cases, the trimeric structures are further modified into a complex quaternary cruciate structure, consisting of four trimeric subunits. CRD regions of these collectins can recognize pulmonary pathogen by binding terminal monosaccharide residues, which are characteristic of cell surfaces of a range of pathogens such as viruses, bacteria and fungi and collagen region can recruit and activate the immune cells for the clearance of pathogens and apoptotic/necrotic cells (Kishore *et al.*, 2006) .

SP-D has different functions and properties when compared to SP-A and other surfactant system proteins, as it doesn't bind to phospholipids of surface-active materials and is mostly soluble in alveolar fluid, hence extracted easily by centrifugation of the surfactant. SP-D binds to the lipids with carbohydrate motifs namely phosphatidylinositol and glucocylceramide and not phosphotidylcholine (Figure 1.9) (Ogasawara, Kuroki and Akino, 1992; Persson *et al.*, 1992)

SP-D is constitutively synthesized and secreted by alveolar type II cells in the endoplasmic reticulum, more proximally in the lung, SP-D secreted by a subset of bronchiolar epithelial cells, the non-ciliated "Clara Cells" bronchial epithelial of the terminal bronchioles and conducting airways (Crouch *et al.*, 1994; Voorhout *et al.*, 1992; Crouch *et al.*, 1992; Crouch *et al.*, 1991; Persson *et al.*, 1988) . Is found predominantly in the endoplasmic reticulum of type 2 pneumocytes and in the secretory granules of Clara or non-ciliated bronchiolar cells. Proteins are packed into intracellular organelles called lamellar bodies and tubular myelin and secreted into alveolar lining layer.

As SP-D is stored within the secretory granules of Clara cells, it seems likely that SP-D is subject to regulated secretion via granule exocytosis at this level of the respiratory tract. Non-pulmonary expression is restricted to cells lining epithelial surfaces or ducts in the serous glands of proximal human trachea, endocytic compartment of macrophages, rat intestinal epithelia, human and rat mesentery, human inner ear, normal human serum (low levels of material antigenically similar), gastric mucosa, tracheobronchial, lachrymal and salivary glands. Possible role in mucosal immunity of SP-D is seen due to its presence in tear and saliva epithelial lining defining its scavenging defense role (Listed in Table 1.1). The highest expression of SP-D in human tissues is observed in the distal airways and alveoli.

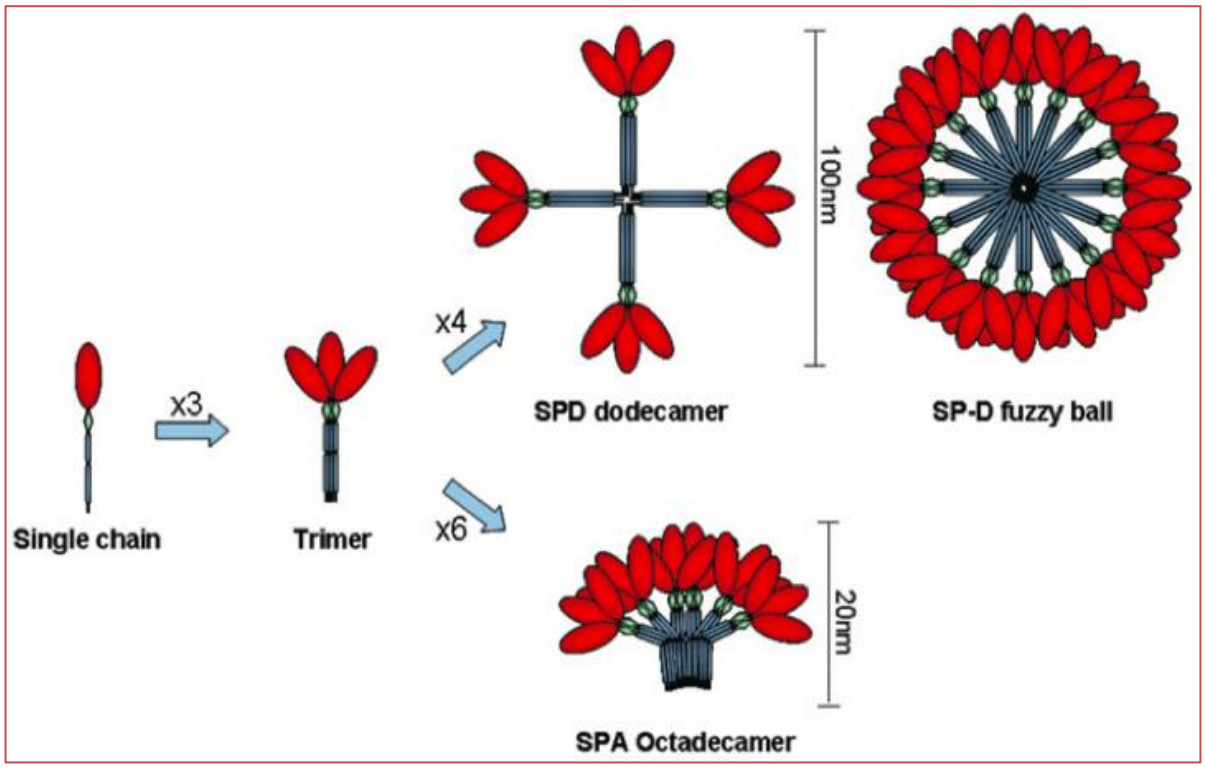


Figure 1.8 Oligomers of SP-A and SP-D. The single chain architecture of the collectins is composed of a cysteine containing N-terminus, a collagen-like region, a trimerising neck domain and spherical calcium dependent sugar-binding domain. All collectins can form trimers. SP-D can further multimerize into dodecamers and fuzzy balls, while SP-A has a sertiform shape (Waters *et al.*, 2009) .

.

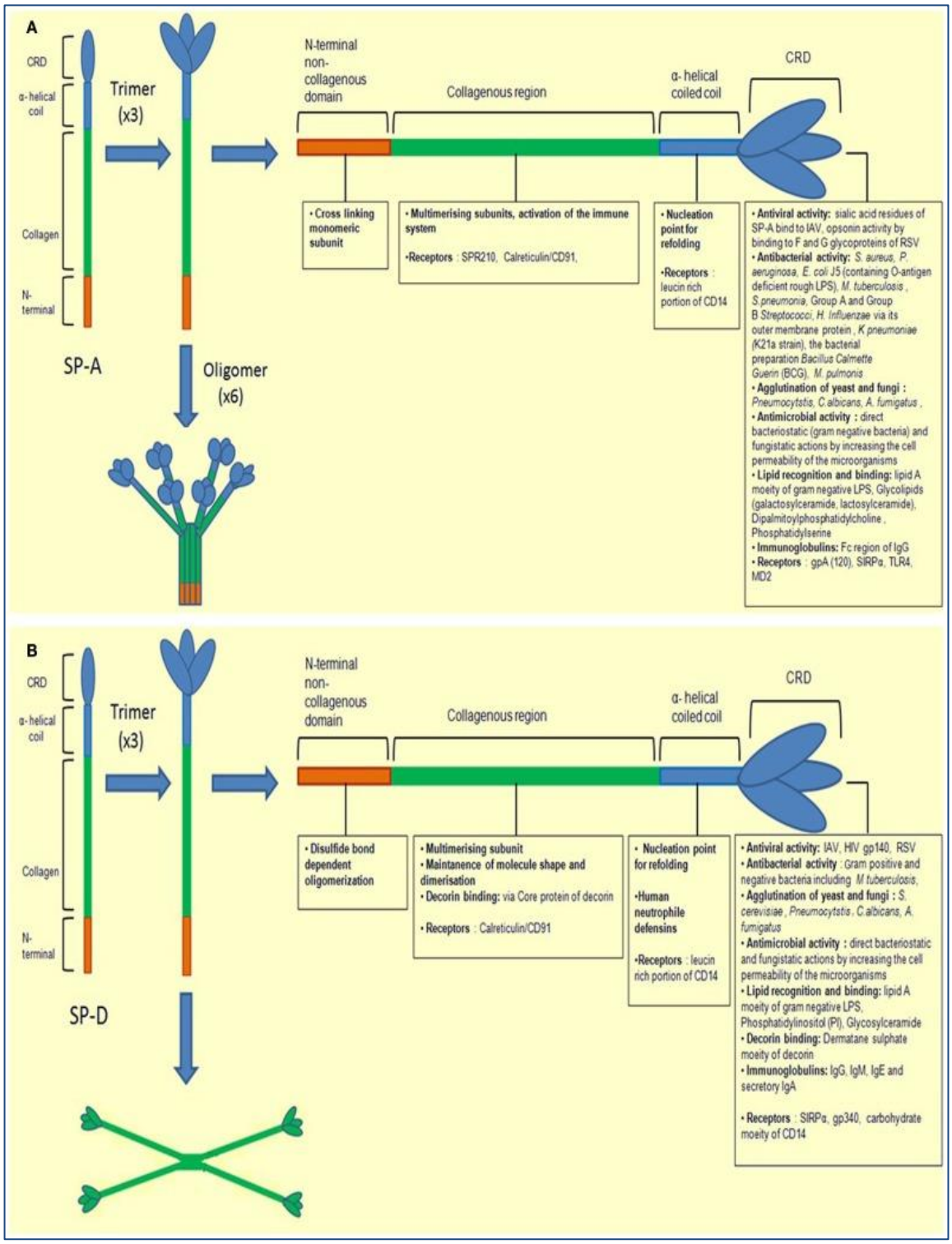


Figure 1.9 Illustration of a molecule of (A) SP-A and (B) SP-D depicting different regions. The molecules are first shown as monomers and trimers. They are divided into four subunits, the N-terminal non-collagenous domain, collagenous region, helical neck, and C-terminal carbohydrate recognition domain. Each subunit has different ligand binding affinities (Nayak *et al.*, 2012) .

In addition SP-D is known to interact with phagocyte via putative receptor molecules and enhance their phagocytic and super oxidative properties, which eventually lead to killing and clearance of pulmonary pathogens (Figure 1.10 and Table 1.2).

In certain diseased states (e.g., pulmonary alveolar proteinosis) there is a high prevalence of very high-ordered multimeric forms of SP-D that contain more than 12 monomeric chains (Table 1.3).

Absence of SP-D plays a major role in the pathogenesis of COPD. Targeted SP-D knockout mice survive and breed normally. Major abnormalities were found at 3 weeks of age in the lungs with enlarged air spaces and increased accumulation of atypical foamy macrophages. Increase in oxidative molecules (H_2O_2 by 10 fold), metalloproteinase (MMP-2, 9 and 12). Macrophages also become necrotic or apoptotic at an accelerated rate, which is reversed by the administration of exogenous SP-D. Emphysematous changes at seven months were observed with increase in collagen deposition and fibrosis (subpleural region of the lungs). Increase in peribronchial and perivascular accumulation of lymphocytes and upregulation of IL-6 and IL-12.

SP-D appears to play a distinct role in protecting murine lungs from the development of emphysematous changes by reducing inflammation and oxidative stress in the lungs.

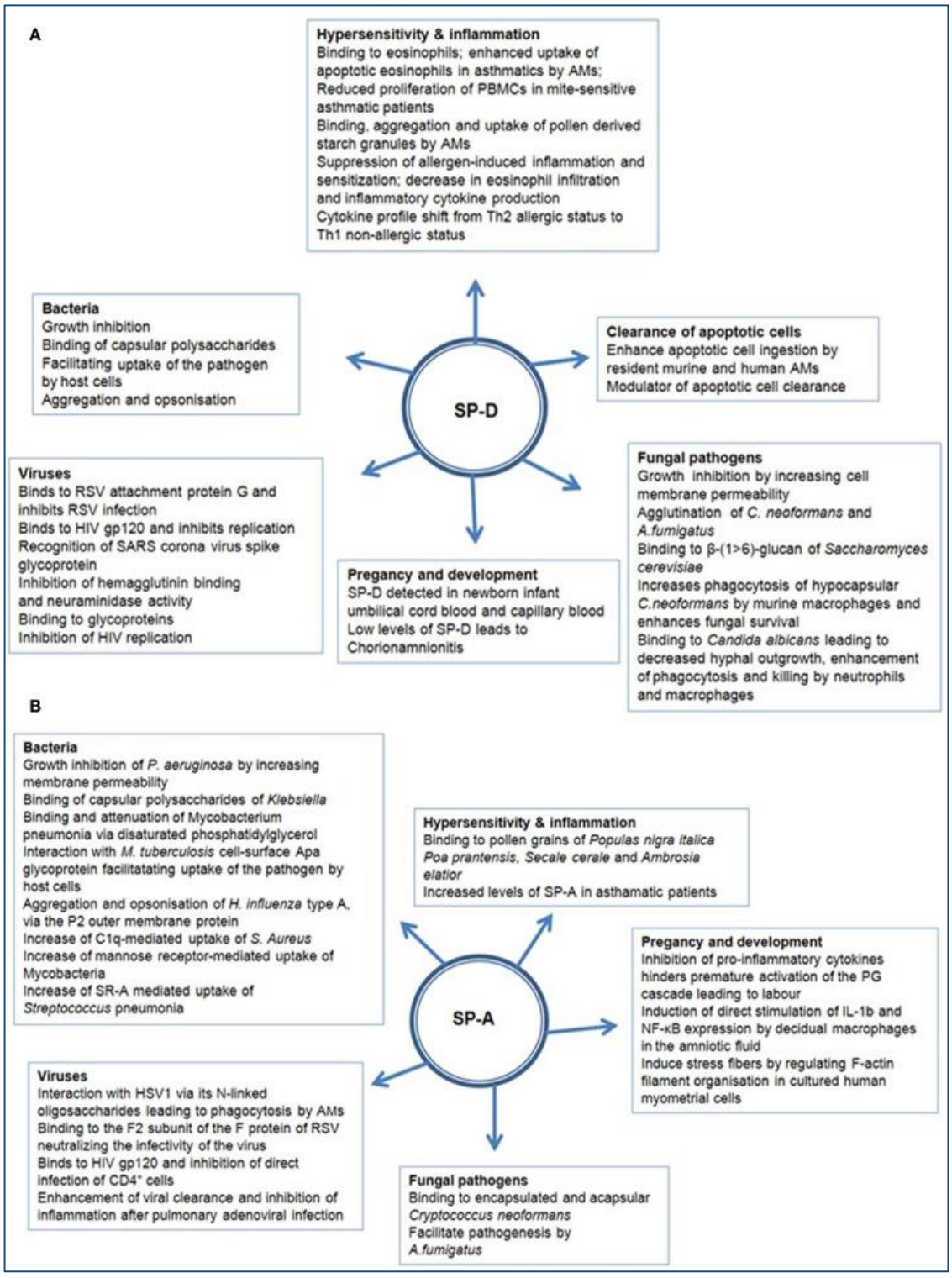


Figure 1.10 Multiple functions of (A) SP-D and (B) SP-A in human health and disease (Nayak *et al.*, 2012)

Table 1.2 Interactions with SP-D and various microbes.

Microbe	Binds	Target	Implications	Reference
GRAM-NEGATIVE BACTERIA				
Escherichia coli	+	LPS	Agglutination, enhanced uptake and growth inhibition[b]	(Wu *et al.*, 2003; Hartshorn *et al.*, 1998; Kuan, Rust and Crouch, 1992)
Enterobacter aerogenes	+	LPS	Inhibits growth[a]	(Wu *et al.*, 2003)
Haemophilus influenzae	+	nd	nd	(Restrepo *et al.*, 1999)
Klebsiella pneumoniae	+	LPS	Inhibits growth[a]	(Wu *et al.*, 2003; Lim *et al.*, 1994)
Legionella pneumophila	+	LPS	Inhibits growth	(Sawada *et al.*, 2010)
Pseudomo_nas aeruginosa	+	LPS	Enhanced uptake by phagocytes	(Restrepo *et al.*, 1999; Lim *et al.*, 1994)
GRAM-POS_ITIVE BACTERIA				
Bacillus subtilis	+	Lipoteichoic acid	nd	(van de Wetering *et al.*, 2001)
Staphylococcus aureus	+	peptidoglycan	Enhanced uptake	(van de Wetering *et al.*, 2001; Hartshorn *et al.*, 1998)
Group A *Streptococcus*	nd	nd	nd	(Shepherd, 2002)
Group B *Streptococcus*	+	nd	nd	(Shepherd, 2002)
Streptococcus pneumoniae	+	nd	Agglutination[b] and enhanced uptake[b]	(Jounblat *et al.*, 2004; Hartshorn *et al.*, 1998)
Mycobacterium avium	+	Lipoarabinomannan	Enhances uptake by macrophages	(Kudo *et al.*, 2004)
Mycobacterium tuberculosis	+	Lipoarabinomannan	Reduces uptake by macrophages	(Ferguson *et al.*, 1999)
Mycoplasma pneumoniae	+	nd	nd	(Chiba, 2002)
VIRUS				
Cytomegalovirus	+	nd	Neutralization	(Shepherd, 2002)
Influenza A virus	+	Hemagglutinin, neuraminidase	Agglutination, neutralization, enhanced phagocytosis	(Hartshorn *et al.*, 2000; Hartshorn *et al.*, 1994)
Human Immunodeficiency virus	+	Glycoprotein 120 (gp120)	Neutralization	(Meschi *et al.*, 2005)
Rotavirus (bovine)	+	VP7 glycoprotein	Agglutination, neutralization	(Reading, Holmskov and Anders, 1998)
Respiratory syncitial virus	+	G protein	Neutralization	(Hickling *et al.*, 1999)
SARS coronavirus	+	Spike glycoprotein (S-protein)	nd	(Leth-Larsen *et al.*, 2007)
FUNGI				
Aspergillus fumigatus	+	Mannose, maltose, 45 and 55 kDa glycoproteins	Binds to condia forms. agglutination, attachment to phagocytes, and enhanced uptake	(Madan *et al.*, 1997b; Madan *et al.*, 1997a)
Blastomyces dermatitidis	+	1,3-β-glucan	Binds to yeast form	(Lekkala *et al.*, 2006)
Candida albicans	+	Mannose, maltose	Agglutination, growth inhibition, and inhibition of phagocytosis	(van Rozendaal *et al.*, 2000)
Coccidioides posadasii	+	nd	Disrupts levels of pulmonary surfactant	(Awasthi, Magee and Coalson, 2004)
Cryptococcus neoformans	+	Glucuronoxylomannan and mannoprotein 1	Agglutination	(van de Wetering *et al.*, 2004; Schelenz *et al.*, 1995)
Histoplasma capsuiatum				
Pneumocystis jiroveci (carinii)	+	gpA(msg, gp120)	Binds to cyst and trophic forms. Attachment to alveolar macrophages. Agglutination[c]	(Yong *et al.*, 2003; Vuk-Pavlovic *et al.*, 2001; O'Riordan *et al.*, 1995; Limper *et al.*, 1995)
Saccharomyces cerevisiae	+	1,6-β-glucan	Agglutination	(Allen, Voelker and Mason, 2001)
PROTOZOA_				

Schistosoma mansoni	**+**	**nd**	**nd**	(van de Wetering *et al.*, 2004)

[a]Strain dependent; [b]rough/smooth LPS – strain dependent; [c]decreased uptake by phagocytes; nd, not determined.

Table 1.3 Levels of SP-D and SP-A in various diseases.

Condition/Disorder	Serum SP-D levels (ng/ml)	Serum SP-A levels (ng/ml)	Comments	References
Normal levels	**~48.7**	**~24.9**		
Sarcoidosis	**96.67**	**23.7**	**Sarcoidosis is a systemic disorder that affects a range of organs ranging from the lungs, eyes, cardiac tissues along with altered functioning of the CNS, hepatic and renal system. SP-D levels change according to the organ affected. For example, uveitis patients with Sarcoidosis has a serum level of 57.0 ng/ml as compared to stage III lung sarcoidosis that shows 96.67 ng/ml. SP-A levels do not seem to change drastically.**	(Kitaichi *et al.*, 2010; Kucejko *et al.*, 2009)
Hypersen_sitivity pneumonitis				
Idiopathic pulmonary fibrosis	**307- 817**	**80-205**		(Barlo *et al.*, 2009; Kinder *et al.*, 2009; Kondo *et al.*, 1998; Kuroki *et al.*, 1993)
Pulmonary alveolar proteinosis	**230**	**285**	**This disorder is a rare condition characterised by the accumulation of surfactant within the alveoli and the terminal airways. The levels of both SP-D and SP-A increase in the serum. SP-D content in the BALF even increased to a high of 2363 ng/ml. This immune protein increase along with upregulation of secreted pro-inflammatory cytokines suggests the possibility of this disorder being an inflammatory lung disease.**	(Lin, Chen and Chang, 2008; Kuroki *et al.*, 1993)
Pulmonary tuberculosis	**140.6**	**37 – 49**	**Increase in serum levels of SP-D depends on the intensity of pulmonary TB. Thus SP-D serves as a good marker of disease progression along with a disease biomarker.**	(Kondo *et al.*, 1998; Kuroki *et al.*, 1993)
Acute lung injury/Acute respiratory distress syndrome	**73**	**30**	**ALI/ARDS is a disorder in which the lung reacts severely to various forms of injuries to the lungs ranging from trauma to drug abuse. SP-D level in alive subjects was 73 ng/ml which is ~40% increase to the control serum level. However increased SP-D levels (101 ng/ml) in post-mortem subjects, demonstrates that the relationship between the substantial increase in lung SP-D and a greater risk of death.**	(Eisner *et al.*, 2003)
SARS	**453**	**N/A**	**The upregulation of SP-D levels in serum of SARS patients is inversely proportional to the IgG levels in the serum with post-mortem IgG levels plunging to low. Thus in this case, the SP-D levels serve as a good marker of disease progression and IgG measurement can be a good prognostic marker.**	(Wu *et al.*, 2009)
Smoking	**N/A**	**29.8**	**SP-A levels in smoker's serum is comparatively higher than that of non-smokers. Although the serum levels are upregulated, the SP-A levels within the BAL are severely downregulated. This is facilitated by the increased alveolar permeability that leads to leakage of SP-A into the serum and hence drainage of the localised SP-A within the alveoli.**	(Mazur *et al.*, 2011; Nomori *et al.*, 1998)
COPD	**150-230**	**Increased levels in serum**	**SP-D levels in serum have been observed to change during acute exacerbations of COPD. Patients who experienced the acute exacerbation had a higher level of SP-D (~227ng/ml) when compared to stable disease patients (~151ng/ml). SP-A too is a good biomarker for COPD, with increased levels found in lungs, serum and sputum of COPD patients.**	(Ishikawa *et al.*, 2011; Shakoori *et al.*, 2009; Lomas *et al.*, 2009; Ohlmeier *et al.*, 2008)
Cystic fibrosis	**No change in serum**	**No change in serum but other fluids**	**SP-A1, SP-A2 and SP-D are significantly increased (82-. 100-, 47-fold respectively) in sinus mucosa of CF patients.**	(Woodworth *et al.*, 2007; Alcorn and

	but other fluids		Interestingly, SP-D and SP-A have been reported to be decreased in the BALF of CF patients having bacterial infection and consequent inflammation. This might be due to the proteolytic degradation of the surfactant proteins or impaired production of surfactant proteins by the epithelial cells. The other possibility of this decrease might also be due to the increased utilisation of SP-A and SP-D in phagocytosis during chronic infection.	Wright, 2004; Noah *et al.*, 2003)
Atopic dermatitis and psoriasis	No change in serum levels	N/A	Levels of SP-D is seen upregulated in the stratum spinosum layer of the skin in psoriatic and atopic dermatitis lesions. Function of SP-D in skin diseases is still unclear.	(Hohwy *et al.*, 2006)
Acute eosinophiliac pneumonia	1025	178	The increase of serum SP-D and SP-A in AEP was observed to normalize following steroid therapy in AEP patients. The hypothesis for the increased levels of the surfactant protein is based on the increase of alveolar permeability, similar to that observed in ARDS.	(Akihiro *et al.*, 2004)
Thoracic radiotherapy	68-74% increase in serum SP-D	21-26% increase in serum SP-A	Thoracic radiation leads to development of radiation pneumonitis in a number of cases. This side effect leads to upregulation of both the SPs in the serum depending upon the intensity of radiation administered.	(Sasaki *et al.*, 2001)
Systemic sclerosis	98.8	N/A	Systemic sclerosis is a systemic autoimmune connective tissue disease that also involves pulmonary fibrosis as a part of the disease progression. SP-D levels in serum are seen to be upregulated, irrespective of the presence of pulmonary fibrosis.	(Yanaba *et al.*, 2004; Asano *et al.*, 2001)
Interstitial pneumonia with collagen disease				

1.3 Eosinophils

Eosinophils play crucial role in host defence to parasites, allergic responses, immunoregulation and tissue remodelling. Eosinophilia (450 cells per microliter) in peripheral blood is observed in allergies, drug reactions, helminth infections, Churg-Strauss syndrome, metabolic disorders, eosinophillic gastrointestinal disorders and hypereosinophillic syndrome.

Biologically active proteins, cationic proteins like major basic protein (MBP), eosinophils peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), cytokines, chemokines and growth factors [IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNF-α, NGF, GM-CSF, SCF, TGF-α, Rantes (CCL5), eotaxin (CCL11), GRO-α and ENA/78/CXCL5] and cytoplasmic granules are present in the eosinophils which makes these cells very special and important (Figure 1.11). Few Cytoplasmic granules, which are morphologically unique known as secondary, specific or crystalloid granules are membrane bound organelles that contain a crystalloid core that is in turn surrounded by a matrix (Muniz, Baptista-Dos-Reis and Neves, 2013) .

At the site of inflammation, intact membrane-bound granules, which are secreted by eosinophils, were observed which release cationic proteins and cytokines (Muniz, Weller and Neves, 2012; Neves, Radke and Weller, 2010; Neves and Weller, 2009; Neves *et al.*, 2009; Neves *et al.*, 2008) . Human extracellular eosinophil granules are secretion-competent granules and cell-free eosinophil granules isolated by subcellular fractionation from disrupted human peripheral blood eosinophils express different receptors like CCR3, IFN-γR α chain (Neves *et al.*, 2008) , two cysteinyl leukotriene receptors (CysLT1R, CysLT2R) and the purinergic receptor P2Y12 which are topologically oriented to engage their ligands (Neves, Radke and Weller, 2010) . Intragranular signal transduction can be observed in purified cell-free granules, which causes differential secretion of granule-stored cytokines and other proteins upon stimulation (Neves, Radke and Weller, 2010; Neves *et al.*, 2008) . IFN-γ stimulation of eosinophils leads to the dose dependent secretion of ECP, EPO, hydrolytic enzymes like β-hexosaminidase (β-Hex) and cytokines IL-4 and IL-6 and not IL-13 (Neves *et al.*, 2008) . Intracellular kinase inhibitors like tyrosine kinases; protein kinase C and p38 mitogen-activated protein kinase (MAPK) prevent the IFN-γ dependent release of the effector proteins from the granules. Leukotriene (LT) C4 stimulated, extracellular generated LTD4 and LTE4 stimulated granules secreted ECP and not cytokines (Neves, Radke and Weller, 2010) . Intragranular membranous network plays an

impressive role in cell-free granule protein mobilization and secretion; even though the mechanism is still unclear (Neves *et al.*, 2008) . Eosinophils, which release their intact cytolytic granules “cluster bombs” which in turn gets activated to the extracellular responses, make us understand the persistent inflammatory response during the modulation of host-pathogen interactions.

Asthma is caused due to inflammation of the airway wall rich in eosinophils, which in turn leads to bronchial hyper-reactivity and airways obstruction. One of the major symptoms of asthma is airway eosinophilia, mucus hyper-production and airway remodelling. Recruitment of the eosinophils from the circulation blood needs to be activated, which makes them to stop at the endothelium and extravasation. Circulating eosinophils cannot have preactivated or “primed”, or fully activated forms, as they are marginated at the activated endothelium or extravagated into the tissue. Eosinophil-surface proteins, including CD69, L-selectin, intercellular adhesion molecule-1 (ICAM-1, CD54), CD44, P-selectin glycoprotein ligand-1 (PSGL-1, CD162), cytokine receptors, Fc receptors, integrins including αM integrin (CD11b), and activated conformations of Fc receptors and integrins have been proposed to report cell activation. Variation in eosinophil activation states may be associated with asthma activity. Cytokines like IL-4, IL-5, IL-13 and IL-17 are released which are produced due to the Th2 and Th17 lymphocytes of the adaptive immune system. Innate immune cells; mast cells, basophils, neutrophils, eosinophils, dendritic cells (DCs) and innate lymphoid cells also play a crucial role in initiation and maintenance of the disease. Airway epithelial cells are the most exposed cells to the pathogenesis, which take the help of PRRs (Pattern recognition receptors) to talk to DCs and activate them (Johansson, 2014; Schuijs *et al.*, 2013) .

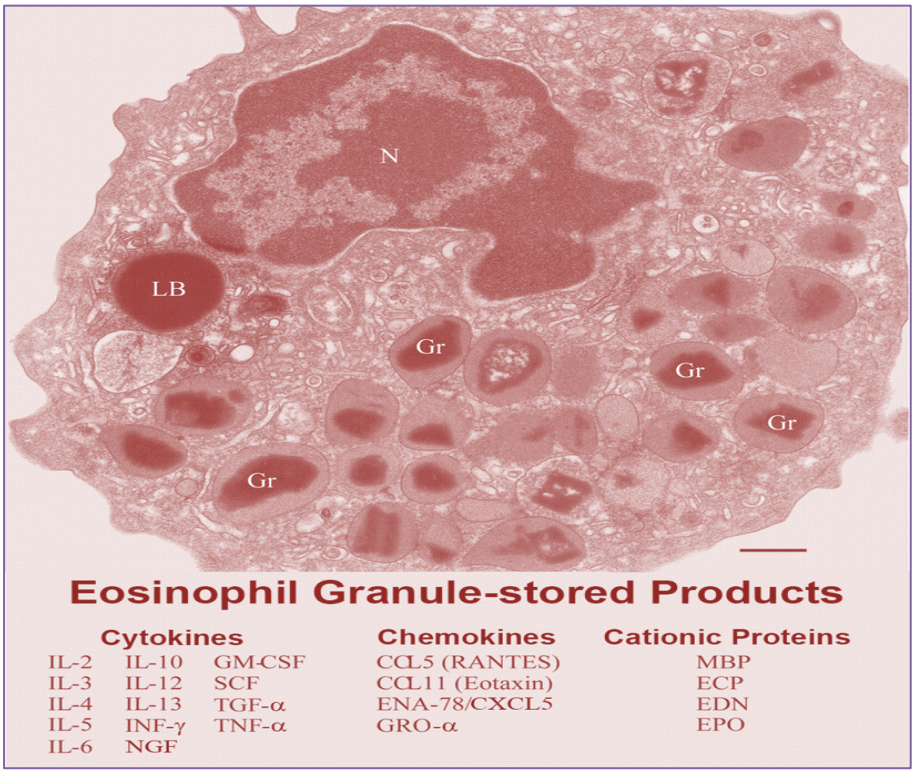


Figure 1.11 Multifunctional granule-stored products within human eosinophils. Ultrastructural image of a human eosinophil shows the cytoplasm packed with specific granules containing an internal, often electron-dense crystalline core, and an electron-lucent matrix surrounds cores. In response to a variety of stimuli, eosinophils secrete cytotoxic cationic proteins and an array of cytokines and chemokines. Eosinophil proteins documented within specific granules are listed. Original bar, 480 nm. Gr, Specific granules; N, nucleus; LB, lipid body. CCL5 (RANTES) (Moqbel and Coughlin, 2006) ; CCL11 (eotaxin) (Lee and Lee, 2005) ; ECP, eosinophil cationic protein (Ueki *et al.*, 2013) ; EDN, eosinophil-derived neurotoxin (Simon, Simon and Yousefi, 2013) ; ENA-78, epithelial cell-derived neutrophil-activating peptide/CXCL5 (Palić *et al.*, 2007) ; EPO, eosinophil peroxidase (Ueki *et al.*, 2013) ; GM-CSF (Chuammitri *et al.*, 2009; Grinberg *et al.*, 2008) ; GRO-α, growth-related oncogene-α (Wardini *et al.*, 2010) ; IL-2 (Ermert *et al.*, 2009) ; IL-3 (Chuammitri *et al.*, 2009) ; IL-4 (von Kockritz-Blickwede *et al.*, 2008; Brinkmann *et al.*, 2004) ; IL-5 (Lin *et al.*, 2011; Chuammitri *et al.*, 2009) ; IL-6 (Chow *et al.*, 2010) ; IL-10 (L. A. Spencer, P. F. Weller, unpublished data); IL-12 (L. A. Spencer, P. F. Weller, unpublished data); IL-13 (Brinkmann and Zychlinsky, 2012); INF-γ, (L. A. Spencer, P. F. Weller, unpublished data); MBP, major basic protein (Remijsen *et al.*, 2011; Fuchs *et al.*, 2007) ; NGF, nerve growth factor (Urban *et al.*, 2006) ; SCF, stem cell factor (Brinkmann and Zychlinsky, 2007); TGF-α (Yousefi *et al.*, 2009) ; TNF-α (Pilsczek *et al.*, 2010) .

Bronchial asthma, characterised by airway inflammation and infiltration of eosinophils, neutrophils and T lymphocytes becomes severe with (Remijsen *et al.*, 2011; Brinkmann and Zychlinsky, 2007; Urban *et al.*, 2006) , eosinophil accumulation and activation. (Pilsczek *et al.*, 2010; Yousefi *et al.*, 2009; Brinkmann and Zychlinsky, 2007) . As mentioned, eosinophils release various products like major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase in addition to oxygen and nitrogen metabolites, when activated which in turn cause mucosal injury and creates disturbances in the lung physiology. Eosinophils also generate lipid mediators like platelet-activating factor, leukotrienes B4 and C4, cytokines and chemokines which activate eosinophils, other leukocytes and structural cells and in the series of the events it triggers the structural cells to release more chemotactic factors (chemokines and leukotriene B4) creating more intense inflammatory response (Oliveira and Lukacs, 2003).

1.4 Role in allergy

Animal models and human studies have shown that eosinophils play a pivotal role in the pathogenesis of several allergic diseases (Akuthota, Xenakis and Weller, 2011) . Asthma models of partially and completely eosinophil-deficient mice (Δdbl GATA) when challenged with ovalbumin showed lower airway remodeling and embryonic lethality with erythrocyte and megakaryocyte deficiency and lower IL-4 production respectively when compared to the wild types. PHIL mice (murine strain-lacking eosinophils) also had decreased response to allergic airway challenge when experimented by Lee at al. PHIL mice are selectively deficient in eosinophils due to the expression of diphtheria toxin A in the promoter region for eosinophil peroxidase. Lowered mucous production and decreased airway hyper-responsiveness were observed. Jacobson et al has shown that eosinophils are not only effector cells, but also active immunoregulators in allergic pathway. Ovalbumin-primed and –challenged PHIL mice had decreased Th2 cytokine production in the lungs, lower accumulated lymphocytes in the airways to the pulmonary parenchyma and to the draining lymph nodes. Not only T-cell receptor specific to ovalbumin (OT-II T cells) intravenous administration, but intratracheal administration of eosinophils was important for the restoration of lymphocyte accumulation in the respiratory system and for the production of Th2 cytokines IL-5 and IL-13 in the lungs was shown elegantly by the group. Blocking of eosinophil recruitment to the lung by eotaxin receptor CCR3, eotaxin-1 and eotaxin-2 deficiency in the knockout mice by Fulkerson et al showed reduced mucous

production when challenged with intranasal *Aspergillus fumigatus* extract. CCR3-deficient mice showed decreased TH2 cytokines IL-4 and IL-13. IL-33 has also been shown to be an important key factor in the allergic airway inflammation indirectly showing the crucial role of eosinophils (Oboki *et al.*, 2011)

Induced airway hyperesponsiveness and induced inflammatory cell accumulation to the airways were observed in the intratracheal adoptive transfer of activated eosinophils to wild-type recipient mice by Kanda et al (Kanda *et al.*, 2009) SP-A was first shown to bind to pollen grains of *Populas nigraitalica* (Lombardy poplar), *Poa prantensis* (Kentucky blue grass), *Secale cerale* (cultivated rye), and *Ambrosia elatior* (short ragweed) in a calcium and carbohydrate (mannose) dependant manner (Malhotra *et al.*, 1993) SP-A, SP-D, and rhSP-D bind to 3-week culture filtrate (3wcf) and two immunodominant glycoprotein allergens, gp45 and gp55, of *A. fumigatus* in a calcium, dose- dependent and sugar-specific manner (Madan *et al.*, 1997a) as well as allergens derived from *Dermatophagoides pteronyssinus* (house dust mite) (Wang *et al.*, 1998; Wang *et al.*, 1996) SP-A, SP-D, and rhSP-D can inhibit specific IgE binding to the allergens of *A. fumigatus* and block allergen-induced histamine release by basophils (Madan *et al.*, 1997a) Consistent with binding of SP-A, SP-D, and rhSP-D binding to mite allergens and competitive inhibition of IgE-allergen interaction, SP-D reduces the proliferation of peripheral blood mononuclear cells (PBMC) isolated from mite- sensitive asthmatic children (Wang *et al.*, 1998) . Dodecameric form of SP-D has been shown to induce aggregation of pollen derived starch granules (PSG) derived from *Dactylis glomerata* and *Phleum pratense* and increase their binding and uptake by AMs. Pre-treatment of these PSG with SP-D resulted in inhibiting the release of β-hexosaminidase in PSG induced IgE dependent mast cell degranulation (Erpenbeck *et al.*, 2005) In view of the ability of SP-A and SP-D to inhibit IgE-mediated histamine release by basophils, a number of allergic murine models have been used to examine *in vivo* effects of SP-A and SP-D following allergenic challenge. Intranasal administration of SP- D and rhSP-D in a murine model of pulmonary hypersensitivity induced by *A. fumigatus* allergens and antigens suppressed specific IgG and IgE levels in serum, reduced peripheral and pulmonary eosinophilia, and caused helper T cell (Th) polarization from aller- gic Th2 to a protective Th1 phenotype (i.e., lowering the levels of IL-4 and IL-5 and elevating IFN-γ level in the spleen supernatant (Madan *et al.*, 2001) . The rhSP-D, when given intranasal to Derp mice (a murine model of lung allergy induced by house dust mite allergens) decreased Derp specific IgE levels,

periph- eral blood eosinophilia and pulmonary infiltration, in addition to causing Th1 polarization (Singh *et al.*, 2003)

1.5 Homeostatic functions of SP-D

Foamy alveolar macrophages, alveolar lipoproteinosis and pulmonary emphysema were majorly observed in the SP-D knockout mice. Coding sequence of the Human SP-D gene (SFTPD) has been known of three single nucleotide polymorphisms (SNPs). Studies have shown that SNP SFTPD with Methionine to Threonine (at position 11) mutation in the nucleotide Adenine to Cytosine has been shown to be a very crucial in the activity of the gene. Mice expressing Met (11) and Thr (11) under pROSA26 promoter in C57BI/6 SP-D deficient mice (SP-$D^{-/-}$) mice lines expressed human SP-D along with the wild type. BAL pellet cytospins showed foamy alveoalar macrophages when compared to wild type (Knudsen *et al.*, 2013)

SP-A and SP-D gene deficient mice (SP-$A^{-/-}$) and SP-$D^{-/-}$) when challenged with *A. fumigatusconidia* when compared to wild type (WT) mice, showed less mortality (40%) than the WT-IPA mice (100%). Mortality was increased to 60% when exogenous SP-A was administered with the decreased levels of TNF-α and IFN-γ to IL-4 ratio when compared to SP-$A^{-/-}$ IPA mice. SP-$D^{-/-}$ IPA mice mortality increased to 57.14%, day 2 mortality to 42.86% and the mice died earlier than the WT-IPA mice (20% on day 2) showing higher hyphal density and tissue injury in the lungs. Exogenous treatment with rh SP-D or SP-D lead to reduced mortality (50% and 33%, respectively) and higher IFN-γ to IL-4 ratios in treated SP-D. SP-D gene deficient mice are more susceptible to IPA when compared to SP-A gene deficient, which acquire resistance to IPA later (Madan *et al.*, 2010) .

SP-A expression plays a crucial role in the initiation of term labour signalling by the fetal lung and secretion into the amniotic fluid (AF) with the help of AF macrophages (Mφ), Toll-like receptors (TLR). WT C57BL/6 mice when compared to homozygous null for TLR, SP-A, SP-D or doubly deficient mice in SP-A and SP-D. TLR-/- females delay in the timing of labour ($P<0.001$) than WT with reduced myometrial contraction-associated protein (CAP) gene, connexion-43, Mφ marker, F4/80 at 18,5 d postcoitum (dpc). Second pregnancies in SP-A-/-, SP-D-/- and SP-A/D-/- mice showed 12 h delay in parturition. Myometrium of these gene deficient mice

showed low levels of IL-1β, IL-6 and CAP genes, connexion-43 and oxytocin receptor at 18.5 dpc compared to WT. Lower levels of pro and anti-inflammatory activation markers were observed in F4/80+AF Mφ from TLR-/- and SP-A/D-/- mice showing that pulmonary collectins through TLRs modulate the timing of labour (Montalbano, Hawgood and Mendelson, 2013; Madsen, Mollenhauer and Holmskov, 2010) .

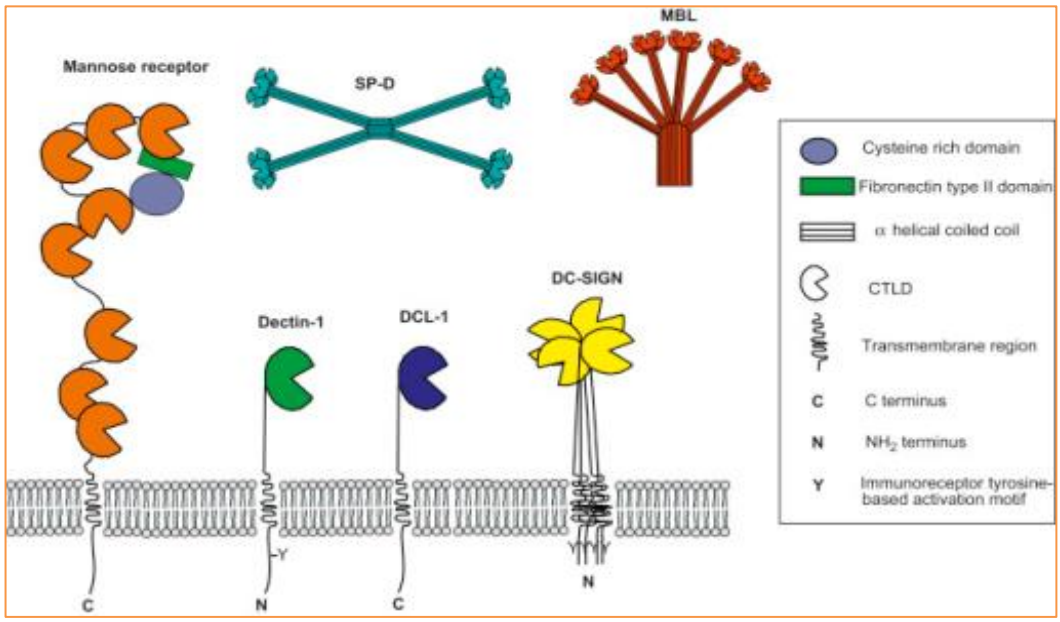


Figure 1.12 Structural representation of C-type lectin proteins. Mannose receptor ("bent" conformation), Dectin-1, DCL-1 and oligomers of DC-SIGN, mannose binding lectin and surfactant protein-D are shown. Not drawn to scale. C-type lectins were originally understood to be Ca2 + Ca2+-dependent carbohydrate binding proteins containing a conserved carbohydrate recognition domain. It has since been determined that other proteins contain the same domain, yet do not necessarily bind carbohydrates or Ca2 + Ca2+. The C-type lectin superfamily is a large group of proteins that are characterised by the presence of one or more C-type lectin-like domains (CTLDs). The superfamily is divided into 17 groups based on their phylogeny and domain organization. Despite the presence of a highly conserved domain, C-type lectins are functionally diverse and have been implicated in various processes including cell adhesion, tissue integration and remodelling, platelet activation, complement activation, pathogen recognition, endocytosis, and phagocytosis. Here, we will review the phagocytic potential of C-type lectins(Kerrigan and Brown, 2009)

1.6 Hypereosinophilia in SP-D knock out and other hypereosinophilic syndromes

Pulmonary proteins SP-D and SP-A have been shown to modulate the cytokine and chemokine profiles during the hypersensitivity responses. Therapeutic effects have also been shown when SP-A and SP-D were given to the murine model of lung hypersensitivity to Aspergillus fumigatus (Afu allergens). When SP-A and SP-D knock out mice (SP-A $^{-/-}$ and SP-D$^{-/-}$ mice) were challenged with Afu allergens had intrinsic hypereosinopilia and high levels of IL-5 and IL-13 and low IFN to IL-4 ratio in the lungs showing Th2 bias immune response. Exogenous administration of SP-A and SP-D to the SP-A $^{-/-}$ and SP-D$^{-/-}$ mice respectively reversed the Th2 bias, suggesting that they play a major role in the immune modulation. Different responses were seen for the SP-A $^{-/-}$ and SP-D$^{-/-}$ mice to Afu sensitization, where SP-D$^{-/-}$ mice showed more susceptibility to pulmonary hypersensitivity induced by the Afu allergens. Intranasal treatment of SP-D$^{-/-}$ mice with SP-D or rh SP-D was more effective in the treatment when compared to SP-A $^{-/-}$ mice treated with SP-A, which showed high IL-13, and IL-5 levels with pulmonary eosinophilia and damaged lung tissue.

When SP-D$^{-/-}$ out mice and wild type mice when exposed to ozone (0.8 parts per million, 3h), increased BAL proteins and nitrogen oxides were observed in the Bronchoalveolar lavage (BAL) fluid and tissue after 72hrs. Prolonged ozone exposure resulted in the lung injury and oxidative stress and increased number of macrophages in the BAL fluid of SP-D$^{-/-}$ mice when compared to wild type. The cells of the knock out mice were enlarged, foamy which confirm the BAL chemoactivity and increased expression of the inducible nitric oxide synthase in lung macrophages. This shows the importance of SP-D in the lung injury, oxidative stress, and macrophage accumulation and activation in response to ozone and functional changes consistent with the loss of parenchymal integrity (Groves *et al.*, 2012) .

1.7 Apoptosis and pathways

Programmed cell death (PCD) is the process of cell death under pathological conditions, which occurs through intercellular and intracellular programs and with the pathways involved. Autophagy and programmed necrosis are also forms of programmed cell deaths, which decide the fate of the malignant neoplasms leading to the cell death (Figure 1.13). Autophagy has pro-survival/death role depending on the situation. Studies show the importance of the programmed cell death in the understanding the cancer initiation and progression. Studying of the pathways involved in the PCD will help in the anti-cancer therapeutic strategies. Complex interplay between apoptosis, autophagy and programmed necrosis may ultimately allow the treatment of cancer more specific and more targeted. PCD plays a very crucial role in the cell death and cell survival and its relevance in the equilibrium of the cells and in the fate of deciding the cancer. Apoptosis, autophagy and programmed necrosis are distinguished on their morphological differences. Cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing and loss of adhesion to extracellular matrix or to neighbouring cell are the characteristic features of Apoptosis (Kerr et al). Chromosomal DNA cleavage into internucleosomal fragments, phosphatidylserine externalization, intracellular substrate cleavages by specific proteolysis are the biochemical changes observed in apoptosis (Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B, Bao JK., 2012 Dec).

An inflammatory immune response may occur to reduce the accumulation of these dying cells (Palaniyar *et al.*, 2003; Clark *et al.*, 2002) . SP-A and SP-D have been shown to enhance apoptotic cell ingestion by resident murine and human AMs *in vitro* (Vandivier *et al.*, 2002) . SP-D is a potent modulator of apoptotic cell clearance in a naïve lung when com- pared to SP-A. SP-A and SP-D bind to apoptotic cells and enhance apoptotic cell uptake by phagocytes through a mechanism dependent on calreticulin and CD91, similar to that of C1q, suggesting that the entire collectin family of innate immune proteins (including C1q) works through a common receptor complex to enhance removal of apoptotic cells, and that collectins are integral, organ-specific components of the clearance machinery (Vandivier *et al.*, 2002) . In resting, non-inflamed lung, lung collectins suppress AM phagocytic function through interacting with SIRPα and enhance apoptotic cell removal by opsonizing apoptotic cells and helping their removal through CD91 (Janssen *et al.*, 2008; Gardai *et al.*, 2003).

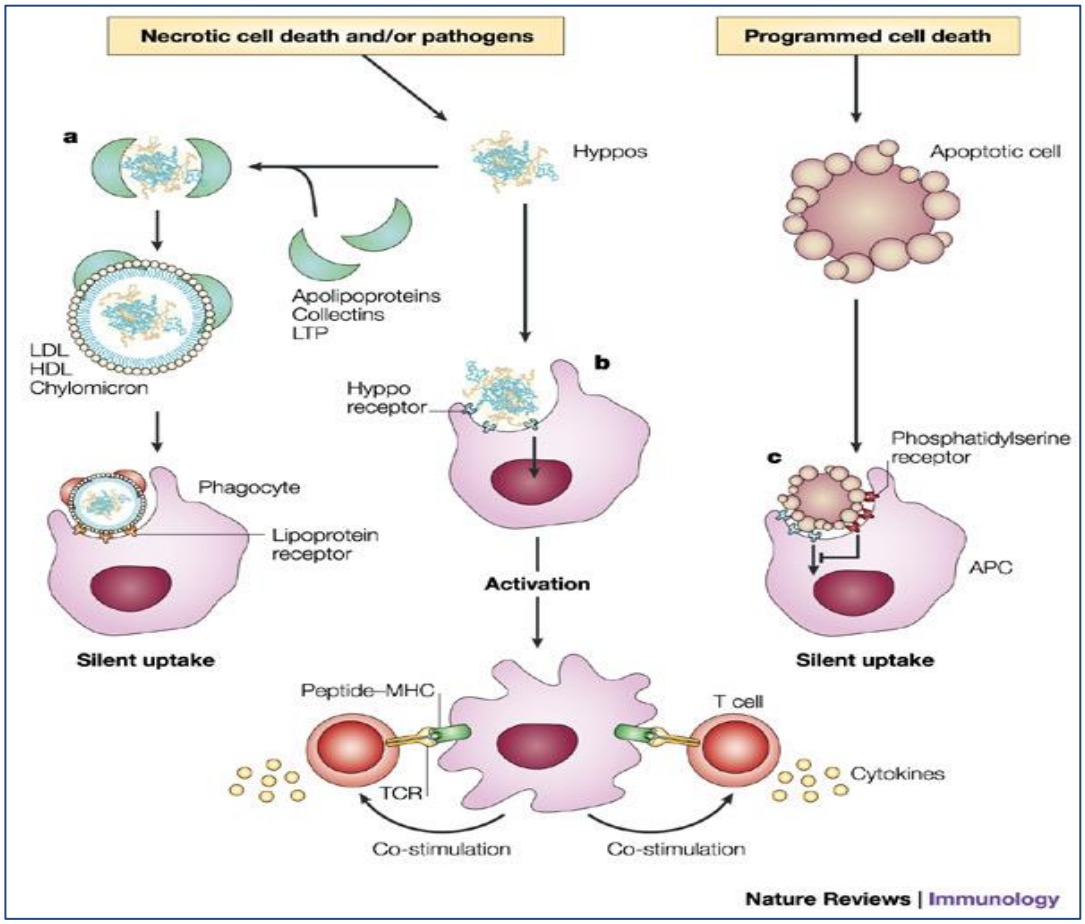


Figure 1.13 At normal physiological concentrations, free hydrophobic portions (hyppos) are bound by hyppo quenchers, such as apolipoproteins, collectins and lipid-transfer protein (LTP), which transfer hyppos to hyppo carriers, such as low-density lipoproteins (LDLs), high-density lipoproteins (HDLs) and chylomicron. These complexes are cleared by cellular uptake through specific receptors for the quenchers or carriers that do not send stimulatory signals. b | In pathological conditions, in which the concentration of free hyppos might overload the local scavenging capacity, unquenched hyppos can activate the coagulation system, complement systems and cell-surface hyppo receptors, such as the Toll-like receptors (TLRs). Signalling through TLRs activates antigen-presenting cells (APCs), which provide co-stimulatory signals and cytokines for T-cell activation. c | A combination of negative and positive signals might result in silence. For example, during normal programmed cell death, various hyppos might be exposed on apoptotic cells, however, the exposed phosphatidylserine binds to the phosphatidylserine receptor, which sends negative signals115 and prevents the activation of APCs. TCR, T-cell receptor

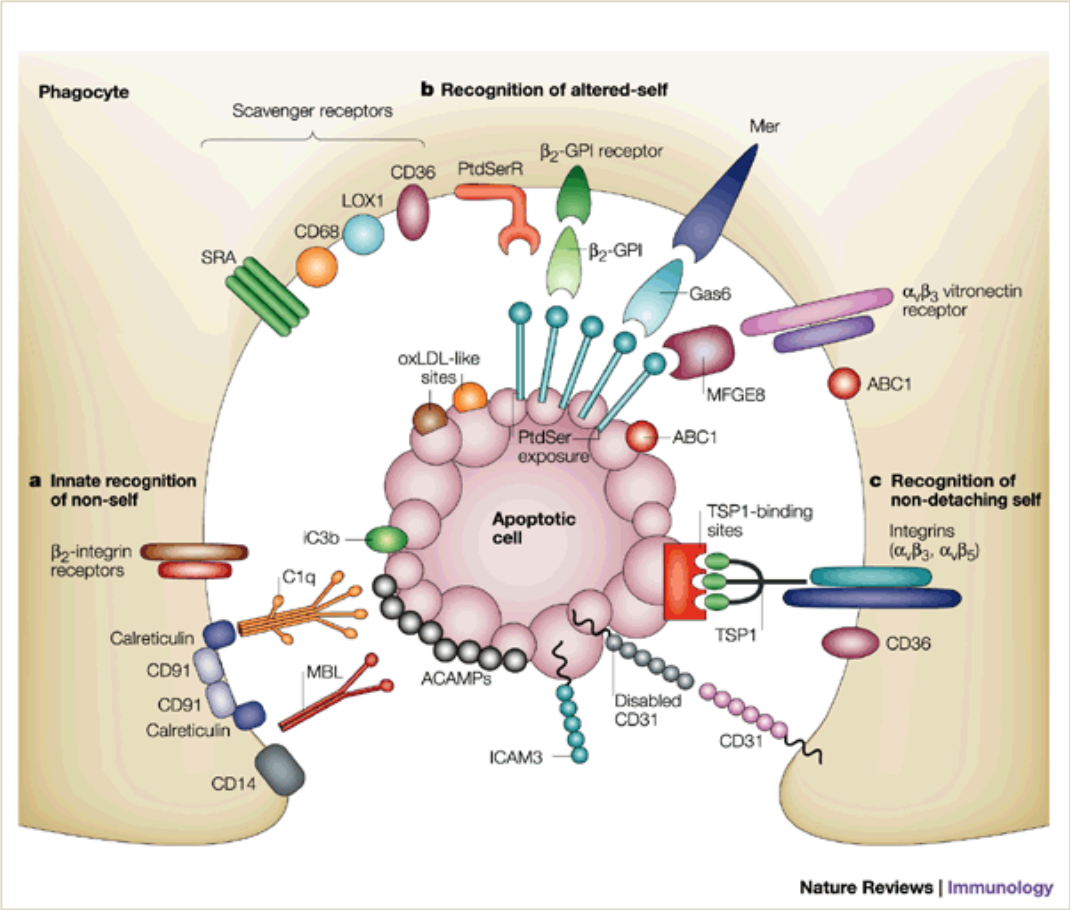


Figure 1.14 Phagocytic receptors for apoptotic cell phagocytosis. a | Innate recognition of non-self involves phagocyte CD14, β_2-integrins (which bind the opsonic complement fragment inactivated C3b, iC3b) and the CD91–calreticulin complex (which can bind the first component of complement, C1q, and mannose-binding lectin, MBL, which recognizes pathogen-like apoptotic-cell-associated molecular patterns, ACAMPs). **b** | Recognition of altered-self involves an array of scavenger receptors, including the class-A scavenger receptor (SRA), CD68, LOX1 (oxidised low-density lipoprotein receptor 1) and CD36, which recognize oxidised sites on apoptotic cells that mimic oxidised low-density lipoprotein (oxLDL). Exposure of phosphatidylserine (PtdSer) on the surface of apoptotic cells is a key 'eat-me' flag. It is detected by phagocyte phosphatidylserine receptor (PtdSerR), receptors for the bridging plasma-protein β_2-glycoprotein I (β_2-GPI), the Mer kinase receptor for the bridging protein Gas6, and $\alpha_v\beta_3$ integrin (vitronectin receptor), which binds the bridging protein milk-fat globule epidermal growth factor 8 (MFGE8). Rearrangement of plasma-membrane lipids in both the dying cell and the phagocyte by the ATP-binding cassette transporter ABC1 can contribute to this type of recognition. **c** | Recognition of non-detaching self involves disabling the detachment signals that are conferred by apoptotic-cell CD31 and, possibly, similar alterations in another immunoglobulin-superfamily member, intercellular adhesion molecule 3 (ICAM3). Disabled apoptotic-cell CD31 binds tightly to phagocyte CD31, which may promote binding of the bridging protein thrombospondin-1 (TSP1) by phagocyte integrins (Savill *et al.*, 2002) .

Murine models with SP-D knockout have shown allergy and Hypereosinophilia rhSP-D with human eosinophils from allergic patients and healthy donors showed sugar and calcium-dependent binding showing its binding through the CRD region. Eosinophils from allergic patients have been shown to undergo apoptosis in the presence of SP-D with oxidative burst and CD-69 expression when compared to healthy patients eosinophils. Healthy patients when primed with IL-5 increase in apoptosis on treatment with rhSP-D and were not affected by the antioxidant, N-acetyl-L-cysteine. Apoptotic eosinophil uptake by the J774A.1 macrophage cells was shown in the presence of SP-D; illustrating that rh SP-D mediated increase of apoptosis in primed eosinophils, while not normal eosinophils and increased phagocytosis of apoptotic eosinophils and thus might play an important role in resolution of allergic eosinophillic inflammation (Mahajan *et al.*, 2008) .

In this thesis,

Chapter 3 examines the interaction of the SP-D with an eosinophillic cell line. Proteomic an real time quantitative PCR analysis have identified upregulation of p53 and downgegulation of antiapoptotis markers. However, rh SP-D interaction and subsequent apoptosis induction of Raji and Jurkat cells, although p53 dependent, appeared to have a differential molecular signature.

Chpater 4 examines the effect of endogenous over expression of full length SP-D in cancer cell lines with intact as well as mutated p53 genes. It shoes that p53 wild type cell lines are susceptible to rh SP-D mediated apoptosis induction. In addition, SP-D also appears to downregulate markers/proteins which are involved in cancer metastasis suggesting that SP-D may be involved in resisting cancer metastasis.

Chapter 5 examines interaction between recombinant forms of DC-SIGN and grass pollen allergens, which has recently been shown to induce Th2 response, since rhSP-D also binds to these allergens, the demonstration here that rhSP-D can inhibit allergen interaction with DC-SIGN is likely to effect the Th2 polarization

Chapter 2

General methods and materials

2.1 Competent Cells

A single colony of *E. coli* BL21 (λDE3) pLysS (Invitrogen) was inoculated in 10 ml of Luria Broth (LB) media with 10 µl of chloramphenicol (stock: 50 mg/ml dissolved in ethanol) overnight at 37^0C shaker. 500 µl of BL21 (λDE3) pLysS culture was inoculated into 25 ml of LB medium with 25 µl of Chloramphenicol. Incubated at 37^0C on a shaker (200 rpm) and measured optical density (O.D 600nm) until it reached a value of 0.3-0.4 (early log Phase). Centrifuged the culture at 2000 xg for 5 min, discarded the supernatant, and added 12.5ml of sterile 0.1M $CaCl_2$ to the cell pellet. Resuspended the cells and placed them in the temporary state of competence on ice for 1 hour. Cells were then centrifuged at 2000 xg for 5 min. Discarded the supernatant, and resuspended the pellet in 2ml of 0.1M $CaCl_2$ and stored on ice, the cells are now ready to be transformed.

2.2 Transformation of cells

200 µl of the competent cells prepared was added to the tube containing 1-2 µl of (pUK-D1) construct for the expression of the rhSP-D protein. Placed the cells on Ice for 1 hour, followed by heat shock at 42^0C for 90 sec and the cells were immediately placed on ice for five minutes and then 800 µl of LB was added to the tube containing competent cells and the plasmid. The tube was left inside a 37^0C incubator for approximately 45 minutes. After incubation, cells were spread plated on an LB agar plate containing (100 µg/ml of ampicillin and 50 µg/ml of chloramphenicol) and left inside a 37^0C incubator to grow overnight.

2.3 Protein Expression

A single colony of transformed (pUK-D1) cells was picked and grown in 25 ml LB along with 100 µg/ml ampicillin + 50 µg/ml chloramphenicol overnight in a shaker at 37^oC. The overnight primary culture of 25 ml bacterial culture was inoculated into 1 litre LB along with 100 µg/ml ampicillin + 50µg/ml chloramphenicol and grown at 37^0C shaker, until the absorbance A600 reaches 0.6-0.8. After the cells reached log phase (A600 at 0.6-0.8) induced with 0.4 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) for 3 hours and harvested the cells by centrifugation at 9000 xg for 15 min. Following centrifugation discarded the supernatant and stored the pellet at -20^0C for further processing.

2.4 Cell Lysis

Resuspended the cell pellet obtained after IPTG induction 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 left spinning for 1 hour in cold room at 4^0C, followed by sonication of the sample (10 cycles at 30 s each).

2.5 Dialysis

Harvested the sonicated sample by centrifugation at 9000 xg for 15 min and solubilised the recovered rhSP-D in the inclusion bodies in 100ml buffer I (50 mM Tris-HCl pH 7.5, 100 mM NaCl) containing 10 mM 2-mercaptoethanol and 8 M Urea. Carried out a step wise dialysis of the resolubilzed material against buffer I containing 4 M urea, 2 M urea, 1 M urea and no urea, each for 2 hrs. Centrifuged the dialysate at 9000 xg for 15 min to get the clear supernatant, and then dialysed with calcium buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Sodium Azide) for 3 h to completely remove urea from the dialysate.

2.6 Protein purification by Affininty Chromotography

The rhSP-D was purified by affinity chromatography using maltose-sepharose column. The maltose sepharose column was rinsed with distilled water prior to use. After thorough rinsing of the column with d.H_2O, 50 ml of affinity column buffer containing (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$, sodium azide-0.05%) was passed through the column. After rinsing the column the protein sample was applied to the column. The column was washed with affinity column buffer, followed by elution of the bound protein 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).

2.7 Expression and Purification of SP-D

2.7.1 Transformation of Recombinant SP-D-Gly-x-y (pUK-D1) in BL21 (λDE3) pLysS

200 µl of competent cells were taken and added to a 15 ml Falcon tube. Added 2 µl of plasmid directly to the competent cells and incubated on ice for 1 h. Heat shock was given to the cells to take up the plasmid at 42°C for 90 s and transferred the culture on to ice for 5 min. Added 800 µl of LB to the sample to make 1 ml total volume, and incubated at 37°C for approximately 45 min to allowed one full cell cycle to take place to increase transformed cells. Added 100 µl of transformed cultured to an LB agar

containing ampicillin/chloramphenicol plate and incubate overnight at 37°C.

2.7.2 Pilot-Scale Expression

Inoculated four separate colonies in 5 ml LB containing 5 µl of ampicillin and chloramphenicol to grow overnight in shaking incubator at 37°C. 500µl of primary culture was added to 10 ml LB with 10 µl ampicillin and chloramphenicol and incubated in the 37°C shaking incubator until A_{600} of 0.6-0.8 was reached (log phase). 1 ml of un-induced sample was taken and added 5µl of 1 M IPTG to make a final concentration of 0.5 µM to the 10 ml culture. Both induced and un-induced samples were left at 37°C incubator shaking for further 3h to allow induction to take place and to over-express protein of interest. After 3 h, took 1 ml of un-induced and induced samples and centrifuge at 13,800 ×g for 15 min to pellet cells and discarded supernatant. Added 100 µl of 2× treatment buffer and denatured proteins to separate 10 µl of each sample on a 15 % SDS-PAGE. After observing gel, the colony giving best expression was streaked on a LB agar plate containing ampicillin and chloramphenicol using the primary inoculum. This plate of colonies was used to make multiple large-scale batches before reseeding the plate of colonies the following month.

2.7.3 Large-Scale Expression

Large-scale expression was carried out after looking at the positive results from the pilot scale. A single colony was picked and inoculated from SP-D Gxy culture plate into 25 ml LB medium with 25 µl ampicillin and chloramphenicol, incubated at 37°C shaking for 16 h. Transferred 25 ml of the primary culture into 1 litre LB medium with 1,000 µl ampicillin and chloramphenicol. This secondary culture was left at 37°C shaker for approximately 3 h until the OD reached between 0.6 and 0.8. 1 ml sample from culture was taken for negative control (“un- induced sample”). To the rest of the sample, “induced” added 500 µl (0.5 mM) IPTG from 1 M stock and re-incubated in the 37°C shaker for 3 h with “un-induced” sample. After 3 h, took 1 ml from “induced” sample and centrifuge along with 1 ml “un-induced” sample at high speed 13,800 ×g for 15 min. Discarded supernatant and added 100 µl of 2× treatment buffer to the pellet. Denatured at 95°C and separated on a 15 % SDS- PAGE to determine protein expression. Centrifuged the entire 1 litre “induced” culture at 13,800 ×g at 4°C, for 15 min to collect pellet containing proteins in inclusion bodies. The pellet was stored at −20°C or used

straight away for lysing and sonication.

2.7.4 Lysing Inclusion Bodies to Extract Protein

Pellet stored at −20°C was thawed on ice and resuspended in 50 ml of lysis buffer and left stirring at 4°C, for ~2 h (until there were no visible lumps). Whilst stirring, 1 mM PMSF and 0.5 mg/ml lysozyme was added to the lysis buffer. Suspension was kept on ice and sonicated at 4 kHz for 30 s with 2 min interval between each cycle to allow sonicator to cool down, repeated for 15 cycles. The sample was centrifuged to collect the protein rich pellet out of inclusion bodies at 13,800 ×g for 15 min at 4°C. 50 µl sample of supernatant and pellet were collected and diluted in 2 x Laemmli sample buffer (950 µl of 2 x Laemmli sample buffer (Bio-Rad; # 161-0737) containing 50 µl of ß-ME (Bio-Rad; # 161-0710), to verify protein on 12% SDS-PAGE.

2.7.5 Endotoxin removal from recombinant SP-D

5 ml of Polymyxin B agarose gel (Sigma) I was packed in a 20 ml column and washed with 50 ml of 1% sodium deoxycholate and then further washed with 50 ml of autoclaved distilled water to completely remove 1% sodium deoxycholate. After rinsing the column, the protein was loaded onto the column, and left to incubate at room temperature for half an hour for the protein to bind completely. After the incubation period the protein was eluted and collected in 1.0-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 and performing the LAL (Limulus amebocyte lysate) assay. The endotoxin level was examined by QCL-1000 Limulus amebocyte lysate system (BioWhittaker, Walkersville, MD, USA) and was found to be ~4 pg µg−1 of rhSP-D (acceptable levels). The protein was further quantified by measuring the absorbance at 280 nm of the eluted and the concentration of the sample was determined via NanoDrop**.**

2.8 Expression and Purification of DC-SIGN Tetramer, R Tetramer, monomer and R monomer

Transformation of pT5T plasmid containing the DC-SIGN proteins (Inserted at the Bam HI restriction site) was done into *E.coli* strain BL21 DE3.

DC-SIGN Tetramer, DC-SIGN R Tetramer and DC-SIGN R monomer transformed colonies were plated onto agar containing ampicillin (50 µg/ml) and DC-SIGN monomer were plated onto Kanamycin (50 µg/ml) agar plate and left overnight at 37°C. Single colonies of the respective DC-SIGN proteins were picked and inoculated for pilot scale

for each DC-SIGN protein. Following the pilot scale, based on the expression, again single colony was picked and inoculated from culture plate into 25 ml LB medium with 25 µl ampicillin and 25 µl kanamycin accordingly and left in a 37°C incubator, shaking for 16 h. Transferred 25 ml of the primary culture into 1 L LB medium with 1,000 µl ampicillin or kanamycin. This secondary culture was incubated at 37°C shaker for approximately 3 h until the OD reached 0.7. 1 ml sample was taken from culture for negative control ("un- induced sample"). To the rest of the sample, "induced" added 500 µl (0.5 mM) IPTG from 1 M stock and re-incubated in the 37 °C shaker for 3 h with "un-induced" sample. After 3 h, took 1 ml from "induced" sample and centrifuge along with 1 ml "un-induced" sample at high speed 13,800 × g for 15 min. Discarded supernatant and added 100 µl of 2 x Laemmli sample buffer to the pellet. Denatured at 95°C and separated on a 15 % SDS- PAGE to determine protein expression. Centrifuged the entire 1 L "induced" culture at 13,800 ×g at 4°C, for 15 min to collect pellet containing proteins in inclusion bodies. The pellet was stored at −20°C or processed for lysing and sonication.

Inclusion body preparation for DC-SIGN proteins were carried out by taking out the pellet and adding 20 ml 6M Urea in 100 mM Tris pH-7.5 and leaving the pellet stirring in cold room for 1 h. Following the lysis and sonication, centrifugation was done 12,000 rpm for 30 min, and the supernatant containing the denatured DC-SIGN proteins were refolded by adding 80 ml of loading buffer (1 M NaCl in 25 mM Tris (pH-8) and 5 mM CaCl2) drop by drop. The mixture was dialysed against loading buffer with three changes of buffer after every 6 hours. The dialysate was centrifuged for 30 minutes at 12,000 rpm and the supernatant was passed through the mannan-agarose (Sigma-Aldrich) column and the peak pure fractions were collected.

2.9 Preparation of Maltose-sepharose column

Preparation of D-maltose-//-sepharose 4B by Divinylsulphone activation method: (Immumobilizing hydroxyl-containing compounds). Sepharose was first activated with the bifunctional reagent divinyl sulphone (DVS) (Sigma) and then coupled under basic conditions to the hydroxyls of D-mannose. Reductive amination, epoxy and DVS activation provide three excellent alternatives for the obtaining an optimal affinity support.

2.9.1 Immobilization of Sepharose B: (DVS to activate sepharose) (Since DVS is highly toxic, all operations involved in activation and coupling should be done in the fume hood) Sepharose 4B was activated (100 ml settled gel with DVS); DVS is a bifunctional cross-linking reagent, used to activate agarose and other hydroxyl matrices. (DVS activated gels are very useful for immobilizing sugars through hydroxyl groups). DVS introduces reactive vinyl groups into the matrix that will couple to amines, alcohols, sulfhydryls and phenols. Immobilized ligands prepared by the DVS method are unstable at alkaline pH (not stable above pH-8.0-amine linked gels and above pH-9.0- or 10.0-hydroxyl-linked gels) DVS activated gels are more reactive than epoxy-activated gels, therefore coupling proceeds rapidly and completely. (Primary stability of this procedure is caused by the liability of the ether bond formed between DVS and the matrix at the time of activation).

2.9.2 Activation of Sepharose: Washed sepharose 4B (100 ml) settled gel with 1 litre water in a sintered glass funnel, suction dried to a wet cake and transfer to a 500 ml beaker. Suspended the gel in 100 ml 0.5 M sodium carbonate and stir the suspension. Slowly added drop wise, DVS (10 ml) with constant stirring over a 15 min period. After addition was completed, stirred gel suspension for 1 h at room temperature. The activated gel was extensively washed with water until the filtrate was no longer acidic. (Gel can be used to couple to ligands or stored for further use. For storage, extensively wash the activated gel with acetone and keep as suspension in acetone at 4˚C).

2.9.3 Immobilization of Sepharose: Washed the DVS-activated sepharose 4B with 1 litre water, suction dried to a moist cake and added to a D-maltose solution (20% D-maltose in 0.5M sodium carbonate). The reaction mixture was left at room temperature for 24 hours stirring on a stirrer. Filtered and washed the gel successively with 2 litres each of water and 0.5 M sodium bicarbonate containing 5 ml β-merceptoethanol.

Mix at room temperature for 2 hours to block the excess vinyl groups.

Finally wash the gel with 2 litres of water.

D-maltose-//-sepharose 4B can be stored in 0.02% sodium azide at 4˚C.

2.10 bis(sulfosuccinimidyl)suberate (BS_3)cross-linking

Prepared a 25 mM stock solution of BS_3, by dissolving 2 mg of BS_3 in 140 µL of DMSO. Using a 20-fold excess approach (20:1 Crosslinker: Protein), added BS_3 crosslinker

solution to the protein sample so that the final crosslinker concentration was between 0.5 to 5 mM. Incubated rhSP-D with different concentrations (1 mM, 0.1 mM and 0.01 mM BS_3), and allowed the samples to react at room temperature for 45 min to 1 h or slightly longer if reaction was done on ice (this reaction rate was only slightly slower at low temperatures). Quenched the unreacted BS_3 with 25 mM to 60 mM Tris-HCl (pH-7.5) and allowed reaction for 10-15 min at room temperature. SDS-PAGE was carried out for the detection of cross-linking.

2.11 TCA (Tri acetic Acid) Precipitation assay

200 µl of protein was taken and mixed with equal volumes of 20% TCA in a 2 ml eppendorf tube. Kept on ice for 20 min and the concentrations were low then kept 5 min more on ice and 15mins at -20°C. Centrifuged for 15mins at 13000 rpm at 4°C. Decanted the supernatant and added 200 µl of 70% ethanol. Flicked and mixed the contents and centrifuged for 15mins at 13000 rpm at 4°C. Decanted the supernatant and allowed excess ethanol to air dry for 5-10 min. Excessive drying was avoided as this makes the pellet difficult to dissolve. Added 30 µl of 2 x Laemmli sample buffer and pipetted up and down for equal mixing. Protein was denatured on heating block for 10 min at 95°C and loaded each sample to a well of SDS-PAGE.

2.12 BCA (Bicinchoninic Acid Protein Assay)

BCA assay was carried out according to manufacturer's instructions. See below for protocol using BCA (Thermo Scientific- 23225).

To carry out protein estimation it was important to perform a standard curve by preparing protein standards using the BSA (Bovine Serum Albumin) ampule of 2 mg/ml provided in the kit. 9 vials were labelled A-I and BSA was diluted to range from 20-20,000 µg/ml according to table 1 using PBS as a diluent.

Table 2.1 Dilution of protein standards to perform standard curve for protein estimation

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000 µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0= Blank

Prepared working reagent by mixing Reagent A and Reagent B at a 1:50 dilution. 2 ml of working reagent was required per sample including for the protein standards which was repeated twice. The following formula can be used to calculate the total working reagent required

(no. of standards + no. of unknown samples) x (2 replicates) x (2ml volume of working reagent per sample)

Labelled 2 x test tubes for each sample containing 100 µl of sample + 2 ml working reagent and was incubated at 37°C for 30min. The reagent will turn from purple to green, depending on the protein concentration. The higher the concentration the greener the solution. The test tubes were then cooled down to room temperature, however measuring the absorbance within the first 10min. Using a spectrophotometer at 562 nm, the optical density of each sample was detected and the average of the duplicate sample was taken for a more accurate measurement of protein. The working

reagent was used as a reference. A standard curve was made using the standard protein samples and the optical density detected for the unknown samples can be used to detect the approximate protein content in the sample.

2.13 SDS (Sodium dodecyl sulfate)-PAGE – 12% and 15%

Two separate 50 ml beakers were labelled one 'resolving gel' and the other 'stacking gel'. For a standard size gel, 10 ml of resolving gel and 5 ml of stacking gel was sufficient. Assembled the clean glass plates onto the glass holder and stand and checked for no leakages. Prepared the following for either 12% or 15% resolving gel then the standard stacking gel. Required TEMED (Tetramethylethylenediamine) was added just before adding to the glass plates.

Table 2.2 Components and volume for preparing 12% and 15% SDS-PAGE

Resolving gel	**12%**	**15%**	**Stacking gel**	
Components	**Volume (ml)**		**Components**	**Volume (ml)**
dH2O	3.3	2.3	dH2O	3.4
30% Bis-Acrylamide mix	4	5	30% Bis-Acrylamide mix	0.83
1.5M Tris-HCl, pH8.8	2.5	2.5	1.0M Tris-HCl, pH6.8	0.63
10% SDS (Sodium dodecyl sulphate)	0.1	0.1	10% SDS	0.05
10% APS (Ammonium Persulfate)	0.1	0.1	10% APS	0.05
TEMED (Tetramethylethylenediamine)	0.015	0.015	TEMED	0.014

Once the TEMED has been added, the polymerisation reaction will have begun, therefore added the resolving gel immediately onto the glass plates. After ~20 min the gel polymerised and then 10% APS (Ammonium Persulfate) was added to the stacking gel and loaded on top of the resolving gel with the comb inserted for the gel to polymerise and form wells. The gel with the glass plates could be assembled into the gel holder with the anode and cathode conductors and a buffer dam can be added to the opposite side to allow current to flow. The protein samples for separation were mixed with equal amounts of 2 x Laemmli sample buffer and heated at 95°C for 10 min for denaturation. The sample was cooled to room temperature and microfuged at 10,000 rpm for 1 min before loading one sample per well. Protein standard marker was

added to the first well to determine unknown protein sample size. 1x running buffer was added to the tank as instructed by manufacturer. Set the power pack connected to the gel apparatus to 100V for 90min to allow the proteins to be separated according to size, with the smaller molecules passing through the gel matrix quickly with the larger molecules trailing behind. The gel was then placed in staining solution overnight and destained the next day for the protein bands to be visible.

2.14 Western blot

Western blot is a technique used to separate proteins through an SDS-PAGE and then probed by specific antibodies to detect protein and binding specificity to the protein by the antibody. Initially an SDS-PAGE was prepared as mentioned above for protein separation. After the dye front had reached the end of the gel, the gel was equilibrated in 1x transfer buffer (containing Tris-HCl and Glycine) along with fibro pads, nitrocellulose membrane and filter paper for 10 min. The sandwich was then assembled in the following order: fibro pads, 2x filter paper, gel, membrane, 2x filter paper and fibro pads. The sandwich was then enclosed into the cassette and placed inside the holder with the electrodes as by manufacturer's instructions. For transfer to take place, 1x transfer buffer was added to the tank as required and set to 340 amps for 90 min for proteins to be transferred to the membrane. The membrane was blocked with 5% non-fat milk powder/PBS buffer overnight at room temperature. The next day the blocking buffer was washed with 20 ml 1x PBS. 10 ml of 1:10000 dilution of primary antibody was added to the membrane. This was left on the membrane for 2 h at room temperature on a rotating rocker. The membrane was washed thrice with PBS tween 20, 0.02% for 10min each. 10 ml of 1:10000 dilution of secondary antibody was prepared fresh and this was poured over the membrane and left for 1 h incubation at room temperature on a rotating rocker. Again the membrane was washed with PBS tween 20, 0.02% thrice for 10 min each. 2 tablets of DAB (sigma-Fast) were dissolved in dH2O as recommended by manufacturer and poured over the membrane. The solution was left on the membrane to incubate for 2-10 min until sufficient colour developed for bands to be visible.

2.15 Analysis of Cell viability by flourescent microscopy

Cell lines (AML14.3D10, Raji and Jurkat) were treated with rh SP-D along with a

positive control (500ngs Ionomycin) and negative control treated with affinity buffer for 24 and 48 h. Cells were washed twice with cold BioLegend's Cell Staining Buffer, and then resuspended cells in Annexin V Binding Buffer (provided by BioLegend) at a concentration of 0.25-1.0 x 10^7 cells/ml. Transferred 100 µl of cell suspension in a 5 ml test tube and added 5 µl of FITC Annexin V, 5 µl of 7-AAD Viability Staining Solution and 5 µl of DAPI. The cells were incubated for 15 min at room temperature (25°C) in the dark. After which, 400 µl of Annexin V Binding Buffer was added to each tube. Then analyzed the cells by taking 40 µl onto the slide and covering and sealing the cells with the coverslip by fluorescence microscopy, which had required machine settings.

2.16. Cloning

2.16.1 Primer handling

Primers were typically delivered de-salted and lyophilised after they were designed and ordered from Sigma. As DNA is prone to self-degradation in acidic pH, they were resolubilized in Hypo clone quality or TE buffer (10 mM Tris-Hcl, 0.1 mM EDTA, pH 8.0). Resuspended the DNA to final concentration of 100-ng/ml.

2.16.2 PCR

The PCR was performed in small 200 µl tubes with very thin walls that allow quick heat transfer.

In these tubes pipetted:

2.16.3 PCR Mixture

Buffer HF (X5)	**10µls**	**30µls**
dNTPs (10µM)	1µl	3µl
Forward primer	2.5µl	7.5µl
Reverse Primer	2.5µl	7.5µl
Template (1:10)	1µl	2.0µl
Phusion Polmerase	0.5µl	1.5µl

DMSO	1.5ul	4.5µl
Water (Hypoclone)	31.5µl	94.0µl
Total Volume	50µl	150µl

2.16.4 PCR Reaction

98°C	**2'**
98°C	10"
58°C	30"
72°C	30"
72°C	10'
4°C	forever

Loop had 35 cycles.

2.16.5 Analysing the PCR products

While the PCR reaction was running, poured a 1% agarose gel. An agarose gel was cast as follows: Weight (0.5gm) required amount of agarose into a 250 ml bottle or flask. 50 ml of Tris-borate-EDTA (TBE) buffer was added and boiled in a microwave until agarose was completely dissolved. The solution was cooled to reach 50 C under running water and added 5µl of 1 % ethidium bromide (this is carcinogen and was handled carefully with gloves on). Disposed off any material that has been in touch with EtBr correctly into the EtBr waste. Casted the gel into the gel chamber, which has been fitted with correct combs— for analytical gels, Let the solution to cool and agarose to solidify.

Removed the stoppers and the combs from the gel and poured enough of TBE buffer to cover the wells. Once the PCR was finished, removed the tube(s) from the machine and placed on ice. 5 µl of the reaction mixture was add into it 1 µl of 10x DNA loading buffer (98 % glycerol, 0.1 % SDS and bromphenol blue and ran molecular weight (MW) markers along in the gel. Loaded sample(s) and MW marker next to each other in the gel and ran the gel at 80 volts until the dye was almost at the edge of the gel. After the

gel has been finished, poured the buffer into appropriate waste bottle. The gel was taken to the UV transilluminator and checked for the DNA fragment of PCR sample of correct molecular weight so as to continue to the next step.

2.16.6 Restriction digestion and Purification of the PCR fragment

The PCR product (SP-D gene) after the restriction digest (Double digest with HindIII/Bam HI) was carried by following the catalogue of the enzyme manufacturer to see which buffer gives optimum activity for both enzymes and purified the DNA after the PCR using simple spin columns. Protocol for Qiagen's PCR Quickspin purification kit was as follows: 5 times the PCR reaction volume was added (PB buffer) from Qiagen's Qiaquick PCR purification kit. Placed the into 50 C heat-block until the agarose has dissolved completely. Mixed frequently to speed the melting and added 100 µl of isopropanol per 100 mg of gel and pipette the sample into a spin-column. Pipetted the sample into a spin-column. Spun the sample for 30"-60" max. Rpm on a minifuge. Discarded the flow-through. 750 µl of PE buffer was added (made sure ethanol has been added to it). Spun as above and discard the flow-through. Spun one more time to dry the column. Column was placed into a new eppendorf and added 45 µl of EB (elution) buffer on the filter of the column. After one minute, this was spun for 60" and kept the flow-through with DNA. DNA fragment purified from the oligos, polymerase, buffer, free nucleotides etc. was ready for restriction digestion.

2.17 Transfecting of plasmid DNA into HEK 293 cells in a 24-well

The transfection was carried out in 24-well initially to check for transfection and accordingly scaling up was carried out. The day before transfection, trypsinization and counting of the HEK cells was done. Plated 0.5 -1.25x105 cells per well in 0.5 ml of complete (FBS and antibiotics) growth medium. Cell density should be 50-80% confluent on the day of transfection. On the day of transfection growth medium from cells was removed and replaced with 0.5 ml of complete growth medium. For each well of cells to be transfected, diluted 0.5 µg to 5 µg of DNA in 100 µl of Opti-MEM® I Reduced Serum Media without serum (Table 2.3). For each well of cells, added 0.75-1.75 µl of Lipofectamine LTX® Reagent into the above diluted Opti-MEM®:DNA solution, mixed gently and incubated for 30 minutes at room temperature to form DNA- Lipofectamine LTX® Reagent complexes.

After 30 minutes of incubation, added 100 µl of the DNA- Lipofectamine LTX® Reagent complexes directly to each well containing cells and mix gently by rocking the plate back and forth. Complexes do not have to be removed following transfection. Incubated the cells at 37oC in a CO2 incubator for 18-24 hours post-transfection before assaying for transgene expression. Supernatants were collected and checked for the transfection after passing the supernatants through the maltose agarose column. The purified fractions collected were analysed using the western blot for the full length SP-D against anti-SP-D and developed the blot with protein A HRP and OPD respectively.

Table 2.3 Scaling up of Transfection IN HEK 293 cells

Culture vessel	Surface area per well	Volume plating medium	Cells per well	Volume dilution medium	DNA	Lipofectamine® LTX Reagent
96-well	0.3 cm2	100 µl	2.5 x 104	20 µl	100 ng	0.15 - 0.35 µl
48-well	1 cm2	200 µl	5 x 104	40 µl	200 ng	0.30 - 0.7 µl
24-well	2 cm2	500 µl	1.25 x 105	100 µl	500 ng	0.75 - 1.75 µl
12-well	4 cm2	1 ml	2.5 x 105	200 µl	1 µg	1.5 - 3.5 µl
6-well	10 cm2	2 ml	6.25 x 105	500 µl	2.5 µg	3.75 - 8.75 µl

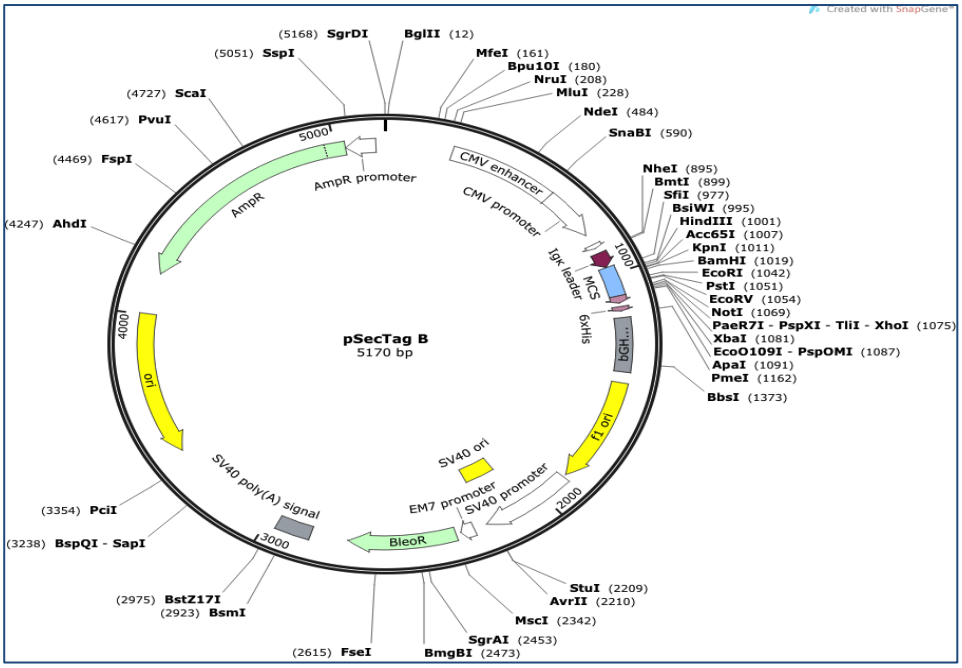


Figure 2.1 pSecTagB Vector Map

2.18 Measurement of gene expression using quantitative PCR analysis

In a 24 well cell culture plate, 10µg of SP-D in PBS were added to each well containing 5 x 10^5 AML14.3D10 cells (Baumann, Paul 1998) and incubated for 15, 30, 45, 1, 2, 6, 12, 24 and 48 hrs; rhSP-D were also incubated with 5 x 10^5 Raji and Jurkat cells. Control samples were incubated with PBS only for 30 min. Cells were harvested and spun down (3000 g, 5 min) and stored at -80°C. Total RNA was extracted from frozen cell pellets using the GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich). Samples were treated with DNase I to remo§ve any contaminating DNA. To inactivate both DNase I and RNase, samples were heated at 70^0C for 10 min, and subsequently chilled on ice. The amount of total RNA was determined at 260nm using the NanoDrop 2000/2000c (Thermo-Fisher Scientific), and the purity using the ratio of absorbance at 260nm and 280 nm. The cDNA was synthesized using High Capacity RNA to cDNA Kit (Applied Biosystems) from a quantity of 2µg of total RNA extract.

Primer sequences were designed and analysed for specificity using the nucleotide BLAST and Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The following primers were used:

18S forward (5'-ATGGCCGTTCTTAGTTGGTG-3'), 18S reverse (5'-CGCTGAGCCAGTCAGTGTAG-3'), MTCBP-1 forward (5'- TACTGGAAGCTGGATGCTGAC -3'), MTCBP-1 reverse (5'-TCGAAGTACCCACTGCCATC-3'), PRDX3 forward (5'- CACCCAGCATGCACCCTATT -3'), PRDX3 reverse (5'- CCACTGAGACTGCGACAACT -3'), SOD2 forward (5'- CTAACGGTGGTGGAGAACCC -3'), SOD2 reverse (5'- GCCTGTTGTTCCTTGCAGTG -3'), UQCRFS1 forward (5'-CAGCGAGGCTAGGAAAGGTT -3'), UQCRFS1 reverse (5'- AAACAGGGGTTTGCCTCTCC -3'), HMGA1 forward (5'- GAGCTCGAAGTCCAGCCAG -3'), HMGA1 reverse (5'- GTGCTGTGTAGTGTGGTGGT -3'), MDM2 forward (5'- ATGTCTGTACCTACTGATGGTGC -3') and MDM2 reverse (5'-TCACAGAGAAGCTTGGCACG -3'), PKCI-1 forward (5'- ACATTTCCCCTCAAGCACCA -3') and PKCI-1 reverse (5'- ACAGACTGTCCACCATCTGAAC -3'), AIP forward (5'- TCAAGGCCTACTTCAAGCGG -3') and AIP reverse (5'- CAATGGGAGAAGATCCCCCG -3'), S100A11P forward (5'-TGGCAAAAATCTCCAGCCCT -3') and S100A11P reverse (5'- ATCATGCGGTCAAGGACACC -3'), CBFB forward (5'- AGGCTCCCATGATTCTGAATGG -3') and CBFB reverse (5'-GCCTCCATTTCCTCCCGATG -3'), p53 forward (5'- AGCACTGTCCAACAACACCA -3') and p53 reverse (5'- CTTCAGGTGGCTGGAGTGAG -3'), Bax forward (5'- TGCTTCAGGGTTTCATCCAGG -3') and Bax reverse (5'- GGAAAAAGACCTCTCGGGGG-3'), Fas forward (5'- ACACTCACCAGCAACACCAA -3') and Fas reverse (5'- TGCCACTGTTTCAGGATTTAA-3').

The qPCR reaction consisted of 5µl Power SYBR Green MasterMix (Applied Biosystems), 75nM of forward and reverse primer and 500ng template cDNA in a 10µl final reaction volume. PCR was performed in a StepOne PCR System (Applied Biosystems). The initiation steps involved 2 min at 50°C followed by 10 min at 95°C. The template was then amplified for 40 cycles, each cycle comprised of 15 sec at 95°C and 1 min at 60°C. Samples were normalized using the expression of human 18S rRNA. Data was analysed using ExpressionSuite software v1.0.3 (Applied Biosystems). Ct (cycle threshold) values for each cytokine target gene were calculated and the relative expression of each cytokine target gene was calculated using the Relative Quantification (RQ) value, using the equation: $RQ = 2^{-\Delta\Delta Ct}$ for each cytokine target gene, and comparing relative expression with that of the 18S rRNA constitutive gene product. Assays were conducted in triplicate.

2.19 Cell culture

Human pancreatic cancer L3.6 cells were cultured in 10% foetal bovine serum containing MEM medium (Life Technologies), Panc-1 and PaTu8988t cells were cultured in DMEM containing 10% foetal bovine serum with 2 mM L-glutamine medium.

2.20 Quantitative Real-time Polymerase Chain Reaction Analysis

Total RNAs were extracted from cells by using RNeasy kit (Qiagen). RNAs were reverse-transcribed by using Superscript II kit (Life Technologies) and experiments were performed in triplicate. Real-time PCR analysis was performed using an ABI-7500 instrument (Applied Biosystem/ Life Technologies) and the quantification of RNA levels was normalized to XS13. Primer sequences were used in this experiment is listed below.

2.21 Real-time PCR primers (human) used in these experiments

Name	Sequence "Forward"
Xs13	5'-gtcggaggagtcggacgag-3'
Twist1	5'- actccaagatggcaagctg-3'
Snai1	5'- ttcacatgtcccagcactac-3'
Zeb1	5'- gcatacacctactcaactacgg-3'

E-cadherin	5'-acggaggagaacggtggtca-3'
Vimentin	5'-cggaaagtggaatccttgca-3'

2.22 Plasmids Transfection

The SP-D expression construct cloned in pSecTagB was used. The transient transfection of SP-D expression constructs was performed 24 h after seeding using Lipofectamine 2000 (Invitrogen) as transfection reagents following the manufacturer's instructions.

2.23 Western Blotting

Western blotting was performed using standard methods on protein lysates from cultured cell lines, with antibodies detected against SP-D, ZEB1 (Santa Cruz), Snai1 (Cell Signaling), Twist1 (Abcam), Vimentin (BD Biosciences), E-cadherin (BD Biosciences) and ß-actin (Sigma).

2.24 Wound healing and time laps experiments

For wound healing experiments, PaTu8988t cells were seeded at 5×10^4 cells/well and cultured in 6 well plates and grown to a 80% confluent monolayer. Subsequently, cells were transfected with SP-D or control vector for 24 h. The confluent cell layers were scratched with a p200 pipette tip. Wound width and relative wound density were measured by time laps analyzer software as described previously (Huth et al, 2010).

2.25 AML14.3D10 and other leukemia cell lines

Dr. Michael Baumann and Dr. Cassandra Paul, Wright State University, Dayton, Ohio provided the AML14.3D10 cell line. The cell line exhibited advanced eosinophilic differentiation and was grown in RPMI-1640 (Sigma) containing 10% fetal calf serum (FCS, Gibco BRL), 50µM 2-mercaptoethanol (Sigma), 1 mM sodium pyruvate (Sigma), and Gentamicin (50 µg/ml) (Gibco BRL). The leukemia cells lines such as THP-1: acute monocytic leukemia cell line, Jurkat: T lymphocyte acute cell leukemia cell line, Raji: Burkitt's B-cell lymphoma were grown under standard culture conditions.

2.26 Proteomics of rhSP-D treated AML14.3D10 cells for identification of differentially expressed proteins

To understand the molecular mechanisms involved in the induction of rhSP-D mediated apoptosis, we carried out proteomic analysis of AML14.3D10 cell line (3×10^5 cells/ml),

cultured in 10 ml medium in 25cm2 tissue culture flasks, in the presence and absence of rhSP-D (10 µg/ml) (5% CO2, 37°C). The cell line was treated with rhSP-D for 48 h, a time period that would sufficiently prime the cells for a distinct pathway, before these cells extensively expressed proteins related to the apoptotic pathways. After harvesting, the cells were washed twice with cold salt free solution of Tris buffer sucrose (250 mM sucrose, 10 mM Tris buffer, pH 7.2) at 4°C. Total cell protein was extracted by sonication and interfering substances were removed by TCA/acetone preparation. The lyophilized sample was solubilized in the rehydration buffer (8 M Urea, 2% CHAPS, DTT) with the protease inhibitor cocktail (Roche) and PMSF (Sigma) and estimated by the Bradford assay. Proteins (400µg) untreated or rhSP-D treated cells were separated in first dimension by the iso-electricfocusing on IPG strips, 17 cm, pI range 5-8 (Biorad) on Protean isoelectric focusing (IEF) cell system (Biorad, Hercules, CA) and then in second dimension by SDS-PAGE on 12% Laemmli gels, following the manufacturers' protocol. The gels were stained with reversible silver stain or colloidal coomassie blue G250. 2-DE gel images were acquired using Fluor-S MultiImager (Bio-Rad) and image analysis was carried out using PDQuest Image Analysis Software version 7.2 to identify protein spots showing differential expression in the treated AML14.3D10 cells. The images were taken under uniform settings for each experiment. For this, 3-4 major spots in different areas of the gel were used for fixing the coordinates. 2DE gels for rhSP-D treated and untreated cells were normalized for small variations in staining or protein loads using total optical density of the protein spots. Only those spots with 3-fold or more changes in expression intensity were selected for further MALDI-TOF-MS. The protein spots were picked from colloidal coomassie blue protein gels for the protein identifications by MALDI-TOF-MS. The colloidal coomassie blue protein staining showed a similar protein profile to that of silver stained gels and had sensitivity comparable to silver staining. These selected proteins spots were excised and subjected to trypsin digestion. The tryptic digest was reconstituted in 0.6µl buffer (50% ACN/ 0.1% TFA) and analyzed using 4800 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems) in positive ion reflector mode. The instrument was calibrated to <10ppm accuracy using calibration mixture of standard peptides in the mass range 800-4000m/z. MS data of the tryptic digests was acquired in an automatic mode. PMF (peptide mass fingerprinting) data was interrogated for protein identification with NCBI (National Center for Biotechnology Information) database (*Homo sapiens*) using the Mascot search program (Matrix Sciences). The analysis was done on global proteomic

solutions (GPS) software (Applied Biosystems). The search parameters were partial methionine oxidation, no fixed modification, one missed cleavage and a mass tolerance of 100ppm, Mascot score ≥55, molecular mass unrestricted; sequence coverage > 10%. The MALDI-TOF analysis was repeated twice (from two gels) for all the samples followed by the search.

For confirmation of protein identification by PMF, the peptides were further subjected to fragmentation by tandem MS (MS/MS). The MS/MS ion spectra of the peptides were interrogated with NCBI database (*Homo sapiens*) for confirmation of the protein IDs generated with PMF. A peptide showing individual ion score of ≥30 was considered as significant identification. The protein IDs that showed significant score after MS and/or MS/MS analysis were considered. The functions and functional category of proteins were assigned from the ExPASy database for biochemical pathways (http://www.expasy.ch/tools/pathways/).

Chapter 3

rhSP-D with eosinophilic cell line

ABSTRACT

SP-D has been shown to reduce peripheral and pulmonary eosinophilia in allergic murine models. SP-D knock out mice show hypereosinophilia, which can be treated by exogenous delivery of rhSP-D, thus, it has been shown that rhSP-D directly binds to eosinophils but induces apoptosis only in sensitized or IL-5 primed eosinophils. This increase in apoptosis also overlaps with increase in oxidative burst and arrest of cells in G2 phase of cell cycle. Here, a proteomic and qPCR analysis is being reported investigating the pathway involved in rhSP-D induced apoptosis using an eosinophillic cell line AML14.3D10. These studies were also extended to other leukemic cell lines, which are commonly available, Jurkat T-cells and Raji B cells. The results appear to suggest involvement of p53 pathway and modulation of a number of signature proteins which fall in the categories of oxidoreductases, chaperones, ubiquitin-proteasome pathways, translation and transcription, RNA binding and metabolism; inflammation and survival; cytoskeleton; vesicle fusion, synthesis and trafficking; metabolic enzymes; energy metabolism and others. It is also evident that not all signature protein categories are similar in the three cell lines; therefore, further validation experiments are required with respect to rhSP-D induced apoptosis induction. Here we report using ELISA and microscopy techniques that AML14.3D10, Raji and Jurkat cells bind rhSP-D in a dose dependent manner and induce apoptosis in a time dependent manner, which is maximal at 48 hrs. In addition to validating proteomic profiling, qRT-PCR was used to validate a number of target genes representing different proteomic clusters. Although the overall transcriptional profile related to cell apoptosis by rhSP-D was overlapping and similar, they were clear differences between the three cell lines used. These results suggest that rhSP-D can target an overlapping set of genes in susceptible cell lines in a differential way.

3.1 INTRODUCTION

3.1.1 Role of eosinophils in pulmonary inflammation

Immune system plays an important role in the balance between the protection from the foreign bodies and protection from the self-damage and link in innate and adaptive immunity. In case of allergic disorders the balance is disturbed leading to the adverse effects on the host. The initial signals significant, at the onset of allergy are associated with the effector functions of the T cells, mast cells and eosinophils (Fulkerson, Rothenberg 2013, Holgate 1999). Eosinophils are pathologically linked to allergy and in allergic asthma, which is T-cell mediated disorder, mast cells cause the chronic inflammation and activated eosinophils disordered airway function which becomes the characteristic feature of the disease (Holgate 1999). Disordered airway functioning is caused due to the inflammation of the airways and thus leading to the increase in activated and degranulated eosinophils, which becomes the characteristic feature of asthma(Fulkerson, Rothenberg 2013, Wardlaw, Brightling et al. 2000, Uhm, Kim et al. 2012). Eosinophils whose function is immunoregulation can produce a wide range of cytokines like IL-4, IL-5, IL-6, IL-13 (Th2-type), IFN-γ, IL-12(Th1-type) and IL-1, IL-2, IL-3, IL-16, TNF-α, GM-CSF (nonpolarizing) IL-10, IL-8 (immunoregulatory) and IL-8, RANTES, Eotaxin, MIP-1α (chemokines) which induce the proinflammatory responses (Giembycz, Lindsay 1999, Spencer, Szela et al. 2009). TGF-β, heparin-binding EGF, PDGF-β, VEGF and IL-4 produced by eosinophils cause the tissue remodeling (Halwani, Vazquez-Tello et al. 2013, Venge 2010). NADPH oxidase, which is cytotoxic to mammalian cell lines, is higher in eosinophils when compared to other phagocytes and was observed to be affected in Crohn's disease, atopic dermatitis and allergic asthma (Yagisawa, Yuo et al. 1996, Giembycz, Lindsay 1999).

Lipid mediators from the tissue-recruited eosinophils are the cause for the tissue inflammation, bronchoconstriction, airway remodeling and bronchial hyper-reactivity (Luna-Gomes, Bozza et al. 2013). Eosinophils when activated on the recruitment to lung tissues release toxic granule proteins that harm epithelial cells, inducing airway smooth muscle contraction, increasing vascular permeability and also activate the mast cells, neutrophils, platelets and goblet cells (Wardlaw, Brightling et al. 2000, Kita 2013, Rosenberg, Dyer et al. 2013, Uhm, Kim et al. 2012, Bousquet, Jeffery et al. 2000,

Giembycz, Lindsay 1999, Walsh 2001). Eosinophils in the blood and tissues of atopic patients get delayed in elimination by apoptosis due to the increased stability of the GM-CSF mRNA after they are primed by cytokines (GM-CSF, IL-3 and IL-5). Accumulated eosinophils cause more damage to the allergic tissue, asthmatic lung by severe allergic inflammation and asthmatic symptoms (Duncan, Lawrie et al. 2003, Wong, Hu et al. 2010)

3.1.2 Studies involving an eosinophillic cell line

Acute myeloid leukemia 14.3D10 (AML14.3D10) cell line is from the patient with FAB M2 acute myeloid leukemia, which exhibits advanced eosinophillic differentiation. Depending on the patient's age and other lab tests, the AML are classified into two subtypes (as it generally doesn't form tumours); the French-American-British (FAB) and the World Health Organization. And the AML FAB M2 is acute myeloblastic leukemia with maturation. Baumann et al. picked a sub-clone (AML14.3D10) from the parental AML cell line, which showed an advanced eosinophillic phenotype and proliferated vigorously with a multiplying time of 48hrs even in the absence of cytokine supplementation (Baumann, Paul 1998). The cell line produces GM-CSF endogenously in turn helping its proliferation and also is seen the autocrine activation of intracellular cytokine (IL-3/GM-CSF/IL-5) signaling pathways and rapidly proliferate eosinophil like cells (Paul, Mahrer et al. 1997).

Sublines of Human promyelocytic leukemia cell line (HL60) can differentiate into eosinophils with the cytokine induction or chemical stimuli (Tomonaga, Gasson et al. 1986), but the eosinophilic character is terminally exhibited and incomplete differentiation is observed when compared to the AML14.3D10 cell lines.

AML14.3D10 shows the similar characteristic features to that of allergic donors like that of in vivo priming of eosinophils by cytokines (GM-CSF, IL-3 and IL-5) and more stable GM-CSF mRNA (Koenderman, van der Bruggen et al. 1996, Shen, Esnault et al. 2005). Eosinophils transform from quiescent eosinophils to an activated state through an exposure to an agent indicated as priming, which becomes more susceptible to degranulation, cytokine production and chemotaxis. These cells not only produce GM-CSF endogenously helping in proliferation, but also inducing intracellular signaling pathways of IL-5, IL-3 and GM-CSF, thus making them good to study instead of allergic

patient samples directly (Paul, Mahrer et al. 1997). Post-translational processing of major basic protein study, crystallographic structure of Charcot-Leyden crystals study (Leonidas, Elbert et al. 1995) has been done on these cell lines. Calcium ionophore stabilizes the GM-CSF mRNA in the AML14.3D10 cells (Ruth, Esnault et al. 1999). IL-8 is increased due to the autocrine production of GM-CSF by the human-recombinant histamine-releasing factor in these cells and IL-4 is increased when stimulated with reactive unreactive lectins (Hoffmann, Dahl et al. 2003).

3.1.3 Interaction of SP-D with Eosinophils

Treatment of AML14.3D10 cells with rhSP-D induced an increase in the oxidative burst and time/concentration dependent apoptosis showing the behavioral similarities to that of the allergic patients' eosinophils (Mahajan, Pandit et al. 2013). RhSP-D induced G2/M phase cell cycle arrest via activation of G2/M checkpoints (Increased p21 levels and Tyr 15 phosphorylation of cdc2). Activated p53, cleaved caspase-9 and poly (ADP-ribose) polymerase (PARP) increased showing a potential role of SP-D in the alleviation of eosinophilia and allergic diseases or eosinophil-associated diseases.

Eosinophils from healthy donors and allergic patients have shown calcium- and carbohydrate-dependent binding through the CRD region (rhSP-D) of the Surfactant protein D (Mahajan et al., 2008). Eotaxin-triggered chemotaxis and ECP-degranulation of IgG and serum of allergic patients is regulated by SP-D and was shown to bind to eosinophils in a calcium and carbohydrate-dependent manner involving the CRD region and reduced stimuli-mediated degranulation and chemotaxis in eosinophils from healthy individuals indicating an anti-inflammatory role of SP-D (von Bredow, Hartl et al. 2006). Gal-9 was shown to be responsible for the induction of the oxidative burst and CD69 in the eosinophils from healthy individuals, but suppressed phorbol mysistate acetate-induced oxygen radical production (Hirashima, Kashio et al. 2004). Increased CD69 expression with increased apoptosis was observed in A.E7 T cells on binding TCR with galectin-1 showing that CD69 expression is an activation associated marker inducing apoptosis (Stimuli results in the cell apoptosis or proliferation) (Chung, Patel et al. 2000, Maino, Suni et al. 1995). Eosinophil degranulation and other functional activities increase is not linked to oxidative burst and CD69 expression (Winqvist, Olofsson et al. 1984). There was no induction of apoptosis in eosinophils derived from healthy individuals, which indicated to have different pathways for degranulation and

superoxide production and hence when the healthy individual eosinophils were primed with cytokines (IL-5) or the AML14.3D10 cell lines were used, they showed the similar CD69 expression, oxidative burst and apoptosis(Mahajan, Madan et al. 2008).

SP-D knockout murine models of allergy rescued hyper eosinophilia partially and down-regulated pulmonary and peripheral eosinophilia when rhSP-D was administered exogenously (Madan, Kishore et al. 2001, Singh, Madan et al. 2003, Vandivier, Ogden et al. 2002). CRD region of SP-D has been shown to be crucial in the binding by the mutation model in an ovalbumin-induced model of eosinophilic pulmonary inflammation (Takeda, Miyahara et al. 2003). RhSP-D binding to eosinophils derived from healthy donors was 1.5 to 3-fold higher when compared to allergic patients, while rhSP-D-induced oxidative burst, CD69 expression and apoptosis was seen for allergic patients and no significant change was observed in healthy donors eosinophils. The interactive differences between the allergic and healthy donors suggest that there is a possibility of different sites on the CRD as the binding is inhibited with various concentrations of maltose and EDTA.

3.1.4 Induction of apoptosis by SP-D in activated or sensitized eosinophils

SP-D increased significantly in airway inflammation models mediating regulation of immune cells and pulmonary homeostasis indicating its crucial role as shown in BALB/c mice sensitized and challenged with ovalbumin and in rat BAL fluid proteins associated with oil mist exposure (Lee, Chen et al. 2006, Zhao, Zhu et al. 2005). RhSP-D-mediated induction of apoptosis in cancer cell lines and the 2-DE proteomics of SP-D treated AML14.3D10 cells revealed various involvements of p53 pathway proteins and their binding to rhSP-D, indicating its involvement in apoptosis. RhSP-D induced changes in tyrosine phosphoprotein expression profile of eosinophillic cells. Oxidative burst, p53 activation and downregulation of high-mobility group A1 (HMGA1) were observed, indicating a potent antiallergic mechanism of SP-D(Mahajan, Pandit et al. 2013). Proteomic profile of rhSP-D-treated (10µg/ml) AML14.3D10 cells for 48hrs showed 134 proteins that were differentially expressed and showed a three-fold change in comparison to untreated cell lines in the silver-stained 2-DE gels. Peptide mass fingerprinting (PMF) analysis identified 75 proteins, out of which 52 were unregulated and 23 proteins were down regulated (Refer Table 3.1) (Mahajan, Pandit et al. 2013). Major functional categories of these proteins were oxidoreductases, chaperones,

ubiquitin-proteasome pathways, translation and transcription, RNA binding and metabolism; inflammation and survival; cytoskeleton; vesicle fusion, synthesis and trafficking; metabolic enzymes; energy metabolism and others.

3.2 RESULTS

3.2.1 Purification, Expression and characterization of rhSP-D

The construct pUK1D containing 8gly-x-y collagen region, neck region and CRD region was transformed into BL21λ DE3 pLysS and checked for expression, following induction with IPTG. RhSP-D was expressed relatively higher to total *E.coli* extracts (Figure 3.1). The recombinant protein accumulated intracellularly as inclusion bodies, which can be recovered in soluble fractions after denaturation and renaturation cycle using urea. Finally, refolded material bound to maltose-agarose column that could be eluted using EDTA (Figure 3.1). The finally purified rhSP-D was recognized by polyclonal antibodies raised against native human SP-D in western blot (Figure 3.2). That the recombinant protein formed a trimer in solution was confirmed by BS3 crosslinking experiment, where the protein formed trimmers in a time and dose dependent manner (Figure 3.3). For further assays, endotoxins were reduced before being challenged to eosinophils, jurkat and B cells. Accepted endotoxin levels are below 1 EU/mg (<0.1 ng/mg) and considered as safe limit

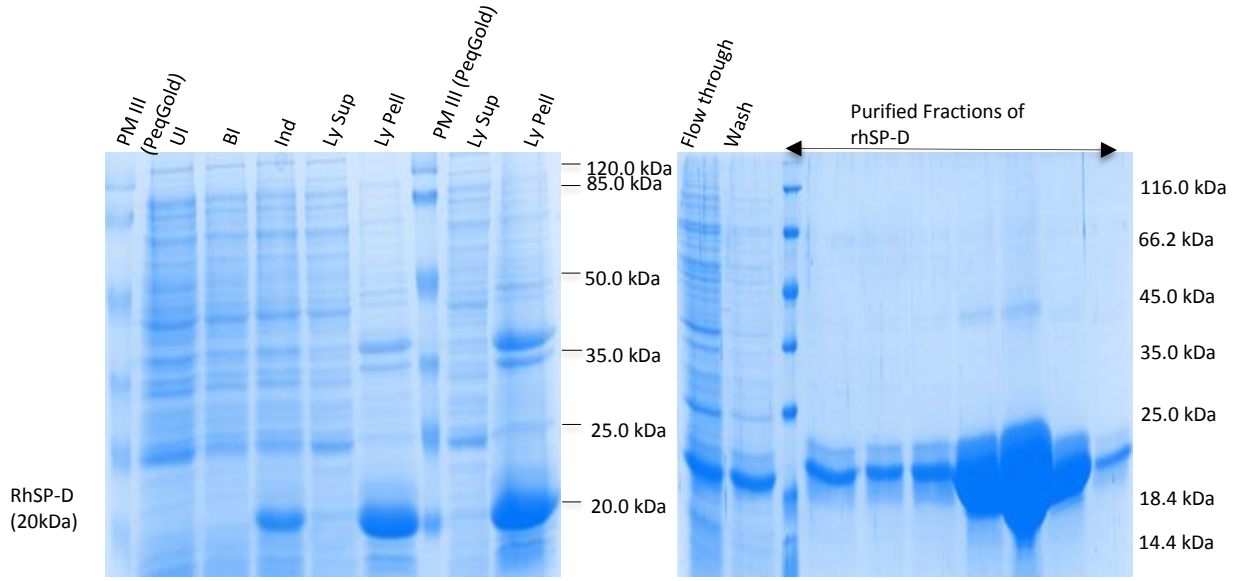


Figure 3.1 SDS–PAGE (12% w/v) analyses of rhSP-D at various stages of expression and purification. A recombinant fragment of human SP-D containing homotrimeric neck and CRD regions (rhSP-D) expressed as inclusion bodies using Escherichia coli BL21 (λDE3) pLysS as hosts under T7 promoter. Three hours after induction with 0.4 mM IPTG, the rhSP-D accumulated as an over-expressed protein of 20 kDa (Ind) compared with uninduced cells (UI). Bacterial cells were sonicated and the inclusion bodies in Lysis pellet (Ly Pell) containing insoluble rhSP-D. Purification of Insoluble rh SP-D were refolded via a denaturation and renaturation procedure and the soluble fraction was further purified using Q-Sepharose ion-exchange chromatography. The peak fractions containing rhSP-D were subsequently affinity purified on a maltose–agarose column (Purified fractions of rhSP-D).

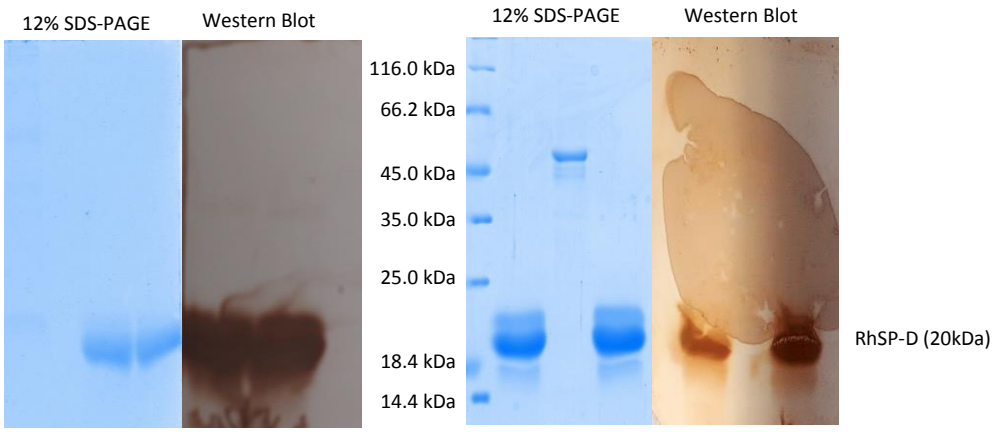


Figure 3.2 SDS–PAGE (12% w/v) analyses of rhSP-D after purification by western blot. Proteins were transferred on to the nitrocellulose membrane at 320mA in transfer discarded and wells were blocked with 5% BSA in PBS for 2 hours at room temperature. The anti-SP-D (1:1000) was added to the membrane added in Calcium buffer (5mM in $CaCl_2$) and incubated overnight at room temperature and following morning membrane was washed 3 times with PBS + 0.02% Tween. Bound proteins were probed with strep HRP (1:1000) and detected using DAB (3,3'-diaminobenzidine tetra hydrochloride; MW = 214.1) as a substrate. MBP (43kDa) is used as control in the second gel.

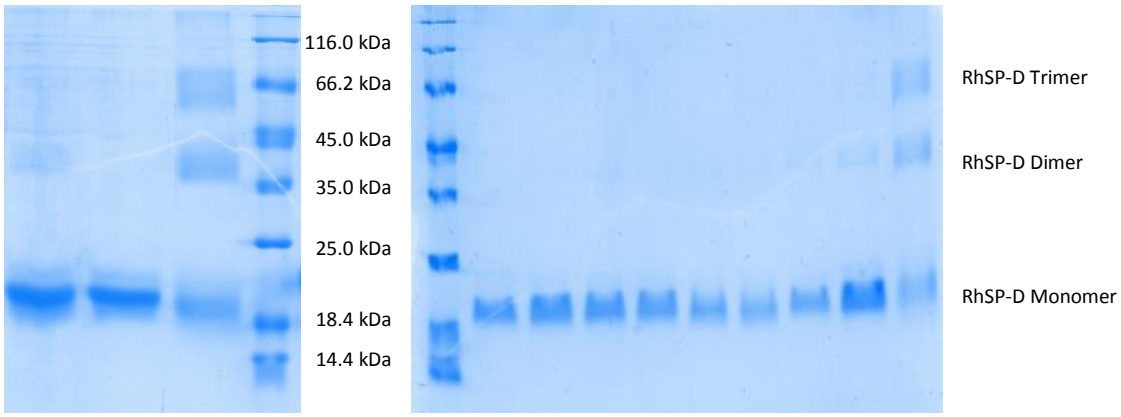


Figure 3.3 SDS–PAGE (12% w/v) analyses of rhSP-D after purification. Cross-linking of protein interactions among themselves to know if they form dimers and trimers to confirm the interactions using the non-cleavable NHS-ester cross-linker, bissulfosuccinimidyl suberate (BS3) in dose-dependent manner. Treatment of rhSP-D with three 0.01mM BS3 at different time intervals of 0, 1, 2, 4, 6, 8, 16, 32 minutes respectively (in the second gel) and the lane 10 showing 1mM BS.

3.2.2 Interaction of rhSP-D with AML14.3D10, Raji and Jurkat cells.

500 cells were seeded per well in carbonate bicarbonate buffer and left overnight at 4°C and came in rhSP-D. RhSP-D showed a dose-dependent binding to the eosinophils showing the interactions with protein (Figure 3.4). AML14.3D10 cells were incubated with rhSP-D for 24hrs and 48 hrs. (Figure 3.5 and 3.6) and then the percentage of apoptosis was calculated by counting the number of cells, which were green (apoptosis due to the Annexin V labelled with FITC), to the number of cells (DAPI labelled) and necrotic cells or later apoptotic cells by the Cy3 (7-AAD) under the fluorescence microscopy (figure 3.9). Apoptosis in the cells incubated with rhSP-D was found to be 60 to 80 % after incubating with rhSP-D. There was increase in Cy3 binding after 48 hrs indicating later apoptosis. AML14.3D10 also showed dose dependency with the concentrations of rhSP-D (10, 5, 2.5 and 1.25 µg/ml) in apoptosis ranging from 60 to 10% respectively after 24 and 48 hrs (Figure 3.7 and 3.8).

Raji cells were incubated with rh SP-D for 24hrs and 48 hrs (Figure 3.10 and 3.11) and then the percentage of apoptosis was calculated by counting the number of cells under the fluorescence microscopy (Figure 3.12), which were green (apoptosis due to the Annexin V labelled with FITC), to the number of cells (DAPI labelled) and necrotic cells or later apoptotic cells by the Cy3 (7-AAD). Apoptosis in the cells incubated with rhSP-D was found to be 40 to 50 % after incubating with rhSP-D. There was increase in Cy3 binding after 48 hrs indicating later apoptosis.

Jurkat cells were incubated with rhSP-D for 24hrs and 48 hrs (Figure 3.13 and 3.14) and then the percentage of apoptosis was calculated by counting the number of cells under the fluorescence microscopy (Figure 3.15), which were green (apoptosis due to the Annexin V labelled with FITC), to the number of cells (DAPI labelled) and necrotic cells or later apoptotic cells by the Cy3 (7-AAD). Apoptosis in the cells incubated with rhSP-D was found to be 60 to 70 % after incubating with rhSP-D. There was increase in Cy3 binding after 48 hrs indicating later apoptosis.

3.3.4.a ELISA

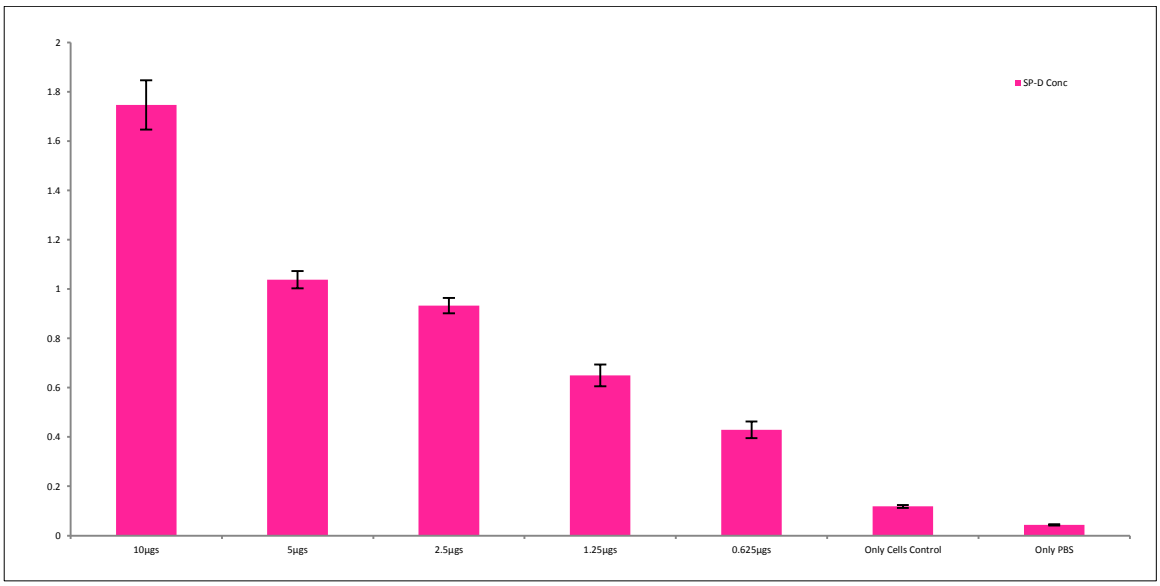


Figure 3.4 **ELISA showing the direct binding of eosinophils (AML14.3D10 cells) to rhSP-D.** Eosinophils were coated (500cells Per well) and left overnight at 4°C. Following morning, ~~contents~~ culture supernatant were discarded and wells were blocked with 2% BSA in PBS for 2hrs at 37 degrees. The wells were washed with PBS+0.05% tween for three times and different concentrations (10, 5, 2.5, 1.25 and 0.625µgs/well) of rhSP-D were added in **Calcium buffer** (**5mM $CaCl_2$**) and incubated for 1.5 hrs at 37 degrees and 1.5 hrs at 4°C, followed by the three washes of PBS+0.05% tween and the bound SP-D was detected by using anti-SP-D (1:1000) and protein HRP conjugate (1:1000). Colour was developed with OPD.

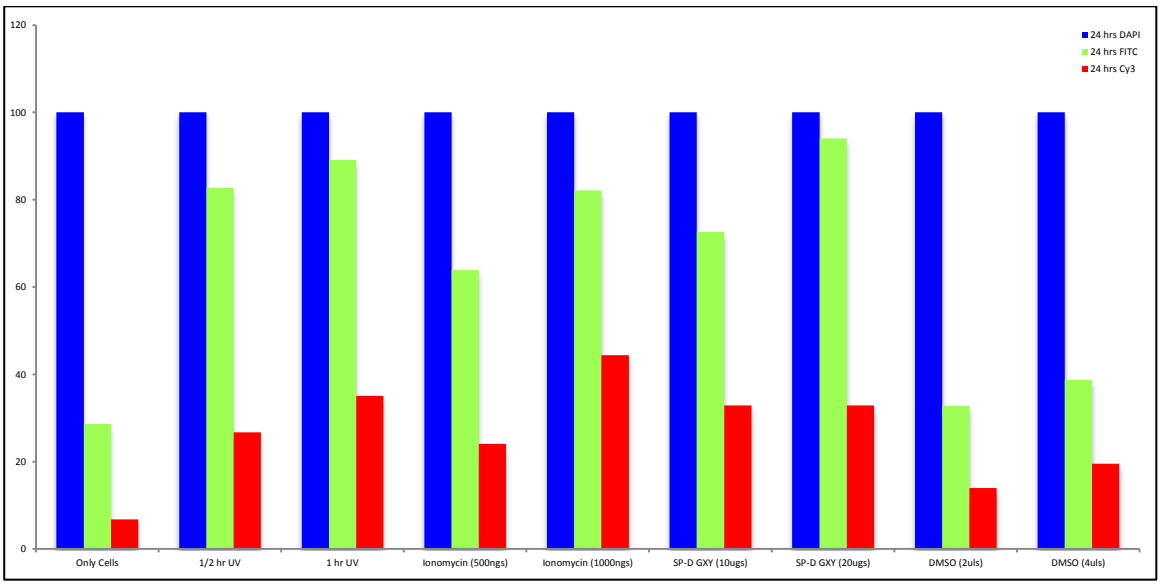


Figure 3.5 **Apoptosis induced in percentage is shown against various methods (UV irradiation, Ionomycin).** Induction of apoptosis in the **AML14.3D10 cells** was seen using the FITC Annexin V apoptosis detection kit with 7-AAD from Biolegend under the fluorescence microscopy. 1 million AML14.3D10 cells were treated with UV for half an hour and one hour separately using the UV strata linker 1800, 500ngs and 1000ngs of Ionomycin in DMSO were added to the cells, two different concentrations of rhSP-D was added to the cells with negative controls being only cells and cells treated with DMSO and left the cells at 37°C incubator (with 5% CO_2) for **24 hrs**.

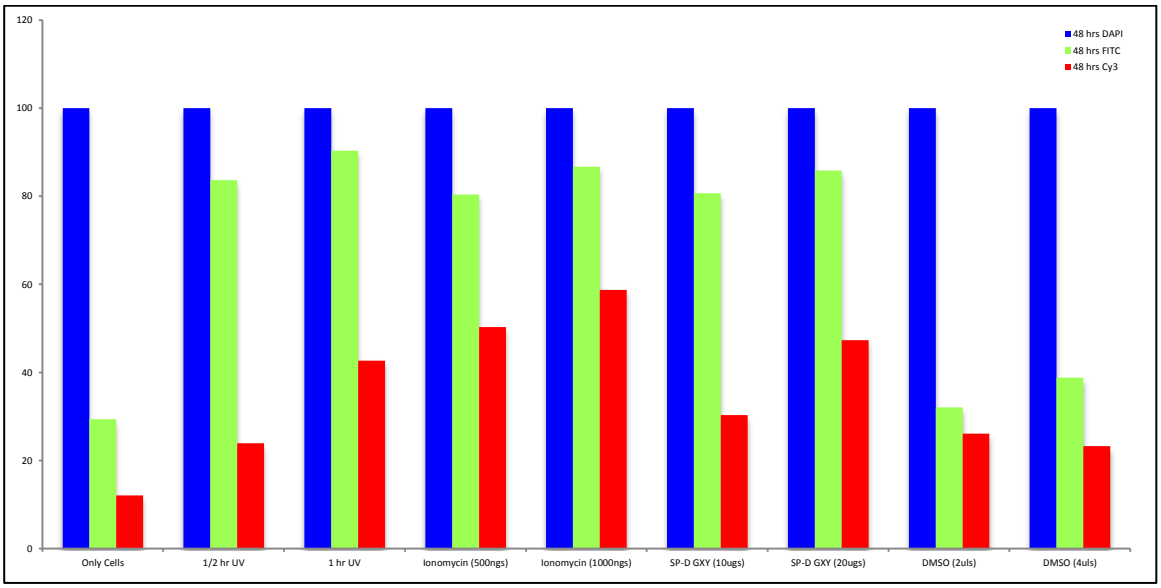


Figure 3.6 **Apoptosis induced in percentage is shown against various methods (UV irradiation, Ionomycin).** Induction of apoptosis in the **AML14.3D10 cells** was seen using the FITC Annexin V apoptosis detection kit with 7-AAD from Biolegend under the fluorescence microscopy. 1 million AML14.3D10 cells were treated with UV for half an hour and one hour separately using the UV strata linker 1800, 500ngs and 1000ngs of Ionomycin in DMSO were added to the cells, two different concentrations of rhSP-D was added to the cells with negative controls being only cells and cells treated with DMSO and left the cells at 37°C incubator (with 5% CO_2) for **48 hrs**.

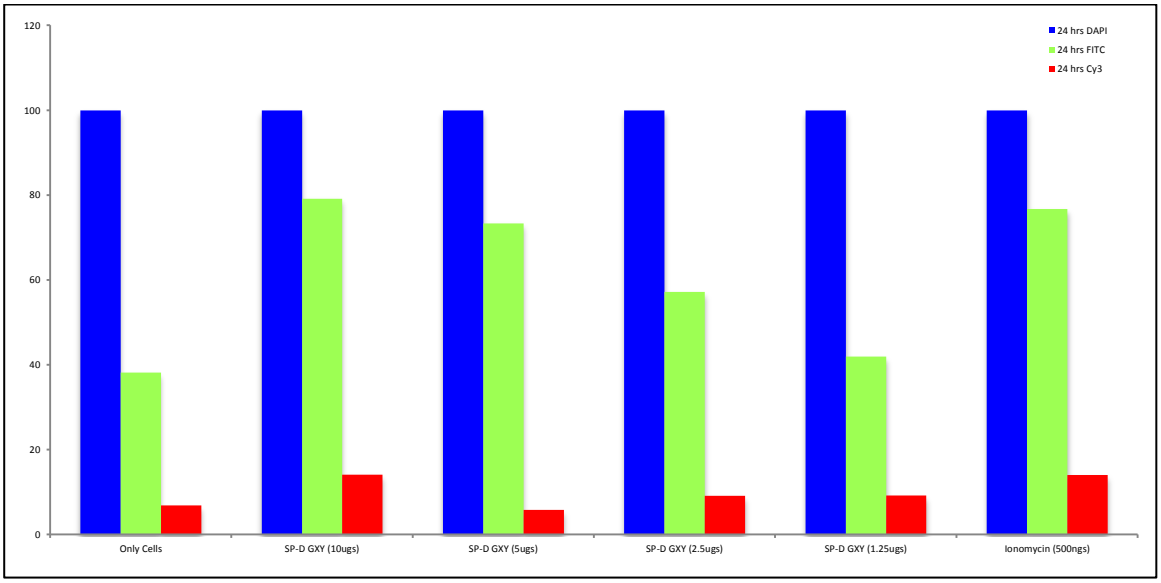


Figure 3.7 **Apoptosis induced in percentage is shown against different concentration of rhSP-D (10, 5, 2.5 and 1.25 µgs per ml.** Induction of apoptosis in the **AML14.3D10 cells** was seen using the FITC Annexin V apoptosis detection kit with 7-AAD from Biolegend under the fluorescence microscopy. 1 million AML14.3D10 cells were treated with 500ngs of Ionomycin in DMSO which has been a positive control, different concentrations of rh SP-D was added to the cells with negative controls being only cells and left the cells at 37°C incubator (with 5% CO_2) for **24 hrs**.

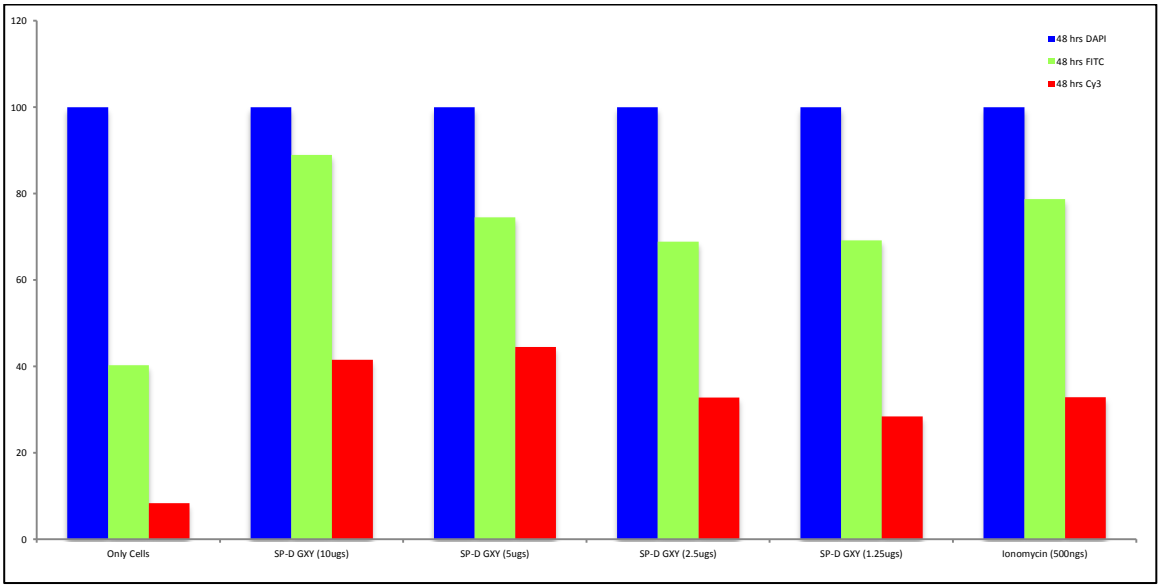


Figure 3.8 **Apoptosis induced in percentage is shown against different concentration of rhSP-D (10, 5, 2.5 and 1.25 µgs per ml.** Induction of apoptosis in the **AML14.3D10 cells** was seen using the FITC Annexin V apoptosis detection kit with 7-AAD from Biolegend under the fluorescence microscopy. 1 million AML14.3D10 cells were treated with 500ngs of Ionomycin in DMSO which has been a positive control, different concentrations of rh SP-D was added to the cells with negative controls being only cells and left the cells at 37°C incubator (with 5% CO_2) for **48 hrs**.

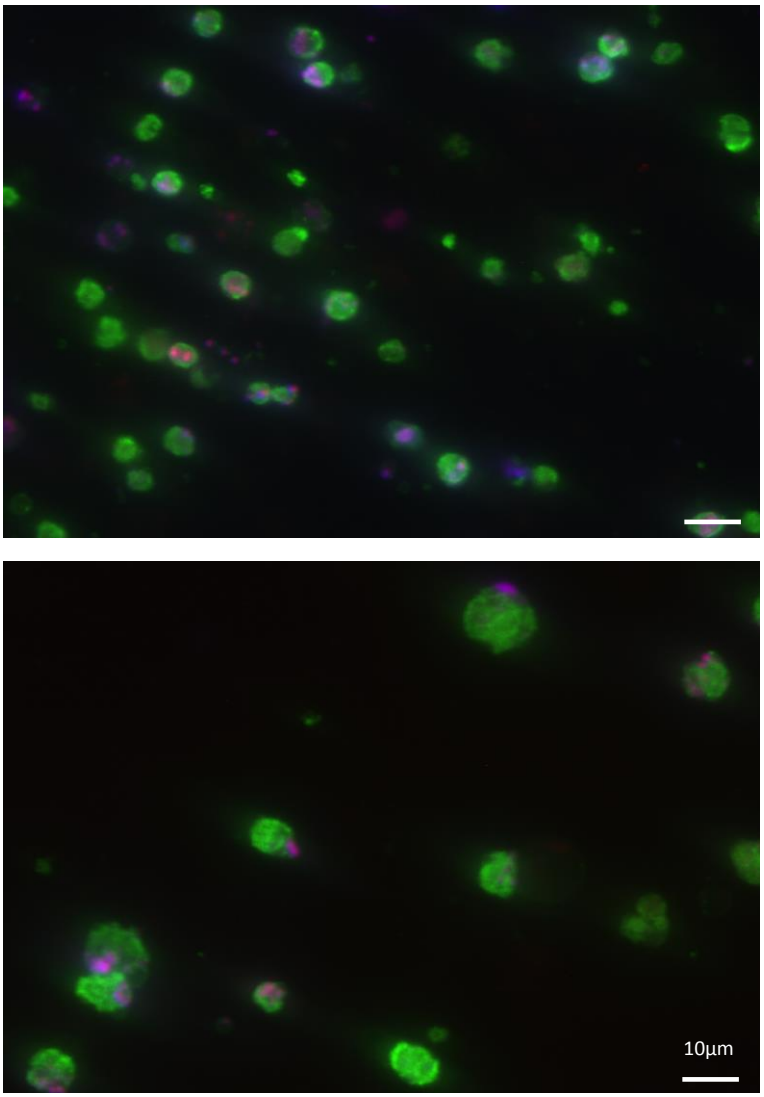


Figure 3.9 **AML14.3D10 cells seen under fluorescence microscopy after the treatment with 10µgs rhSP-D.** FITC Annexin V apoptosis detection kit with 7-AAD from Biolegend was used to detect apoptosis and can be seen under the fluorescence microscopy. 1 million AML14.3D10 cells were treated with rhSP-D and left the cells at 37°C incubator (with 5% CO_2) for **24 hrs and 48hrs**.

3.3.4.b Rh SP-D AND RAJI cells

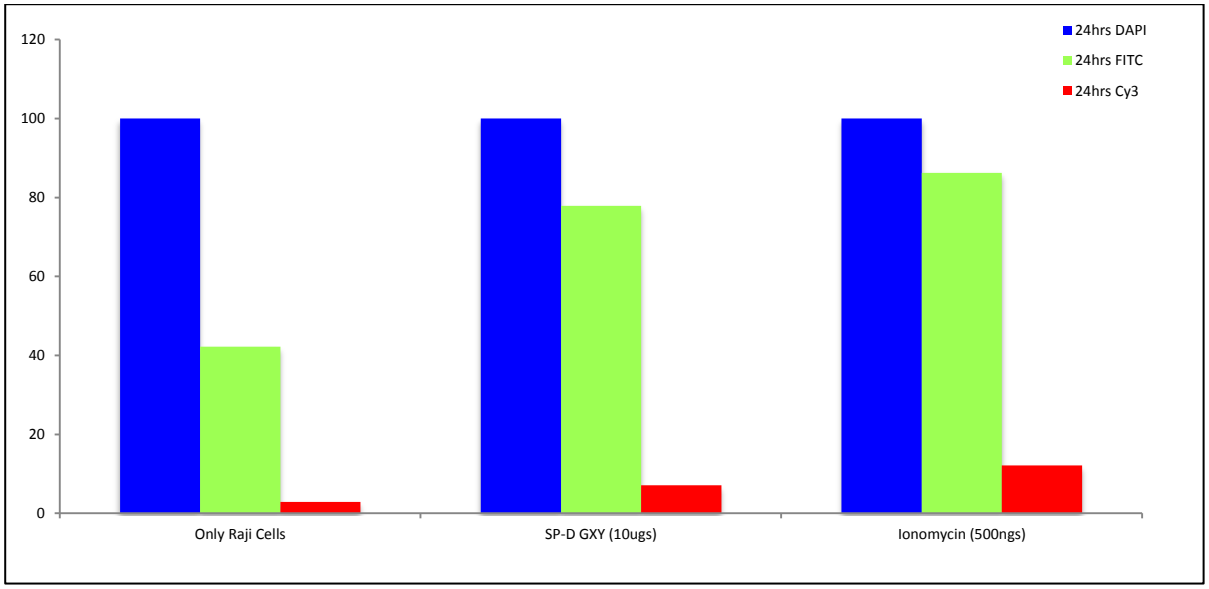


Figure 3.10 **Apoptosis induced in percentage is shown for rhSP-D (10µgs per ml), Ionomycin (500ngs per ml).** Induction of apoptosis in the **Raji cells** was seen using the FITC Annexin V apoptosis detection kit with 7-AAD from Biolegend under the fluorescence microscopy. 1 million Raji cells were treated with 500ngs of Ionomycin in DMSO used as a positive control against negative controls being only cells and 10ugs per ml of rhSP-D was added to the cells with and left at 37°C incubator (with 5% CO_2) for **24 hrs**.

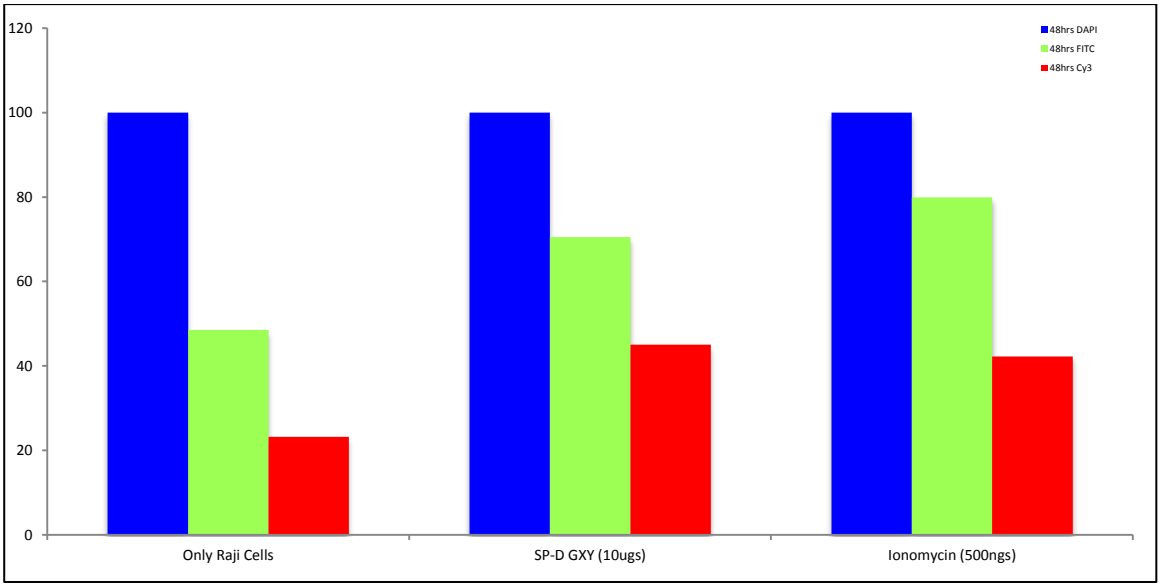


Figure 3.11 **Apoptosis induced in percentage is shown for rhSP-D (10µgs per ml), Ionomycin (500ngs per ml).** Induction of apoptosis in the **Raji cells** was seen using the FITC Annexin V apoptosis detection kit with 7-AAD from Biolegend under the fluorescence microscopy. 1 million Raji cells were treated with 500ngs of Ionomycin in DMSO used as a positive control against negative controls being only cells and 10ugs per ml of rhSP-D was added to the cells with and left at 37°C incubator (with 5% CO_2) for **48 hrs**

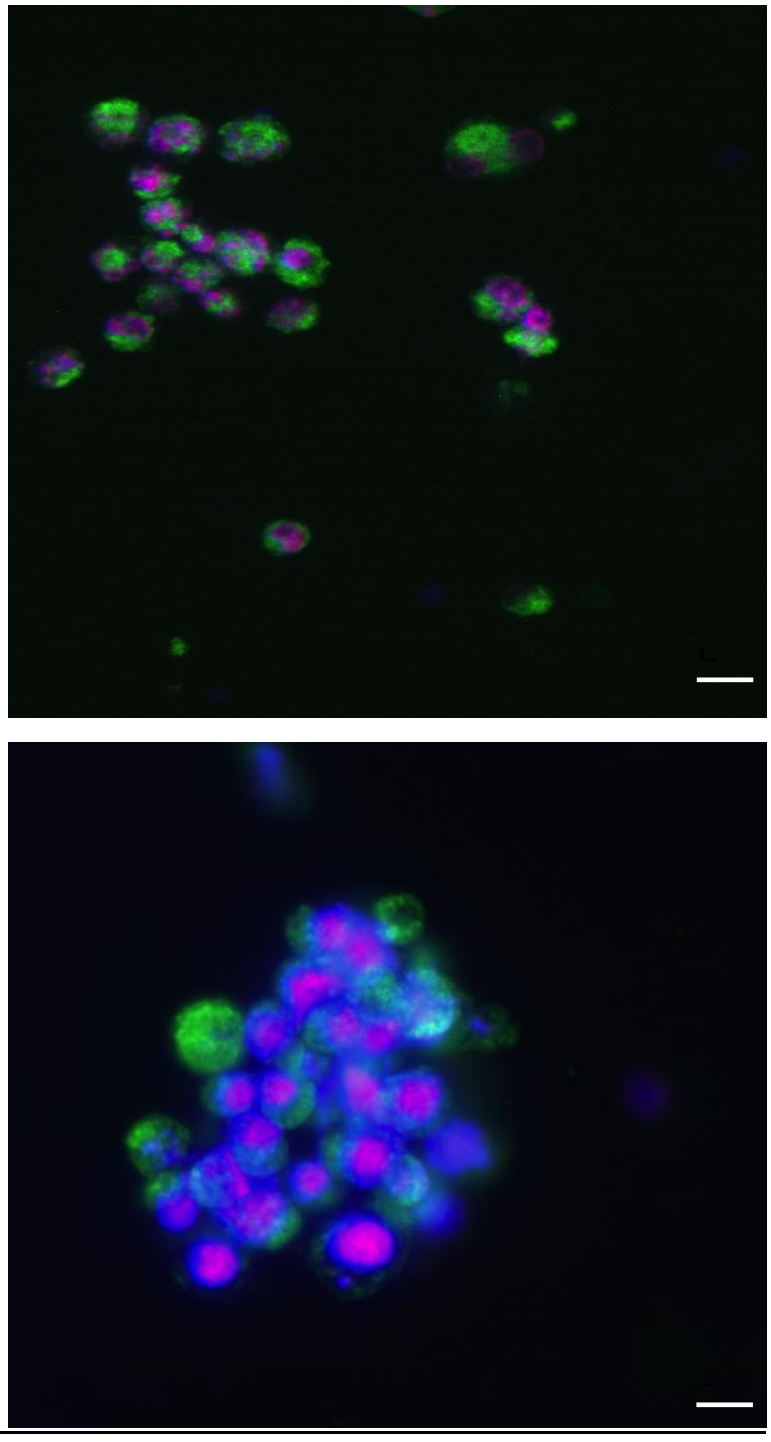


Figure 3.12 Raji cells seen under fluorescence microscopy after the treatment with 10µgs rhSP-D. FITC Annexin V apoptosis detection kit with 7-AAD from Biolegend was used to detect apoptosis and can be seen under the fluorescence microscopy. 1 million Raji cells were treated with rhSP-D and left the cells at 37°C incubator (with 5% CO_2) for **24 hrs and 48hrs**.

3.3.4.c Rh SP-D AND Jurkat cells

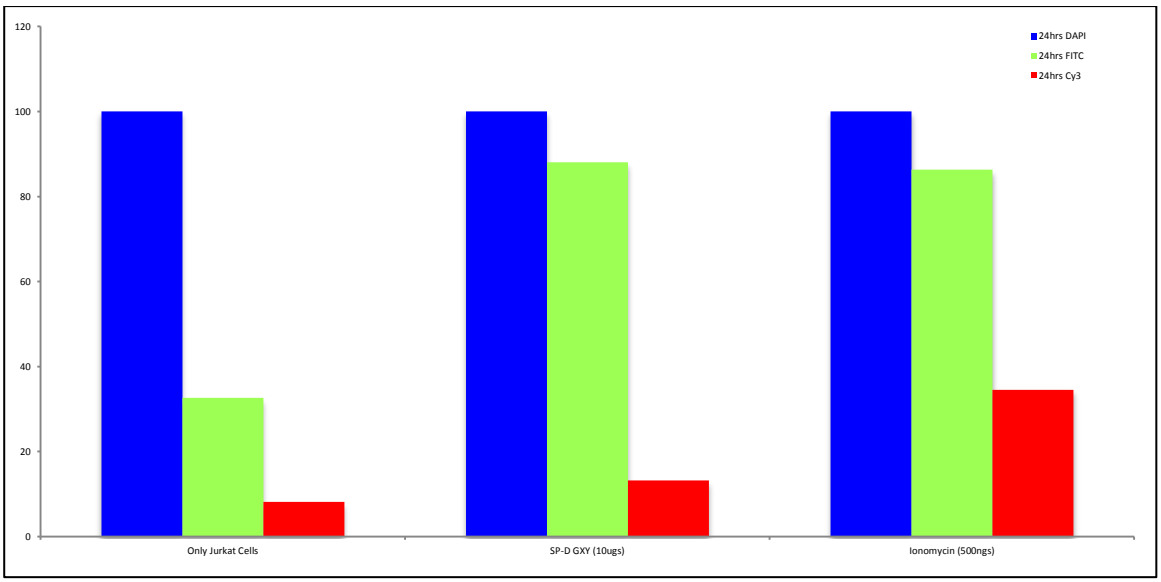


Figure 3.13 **Apoptosis induced in percentage is shown for rhSP-D (10µgs per ml), Ionomycin (500ngs per ml).** Induction of apoptosis in the Jurkat cells was seen using the FITC Annexin V apoptosis detection kit with 7-AAD from Biolegend under the fluorescence microscopy. 1 million **Jurkat** cells were treated with 500ngs of Ionomycin in DMSO used as a positive control against negative controls being only cells and 10ugs per ml of rhSP-D was added to the cells with and left at 37°C incubator (with 5% CO_2) for **24 hrs**.

Table 3.1 Function and functional categories of differentially expressed proteins of AML14.3D10 cells on treatment with rhSP-D

Protein Name	Functions	Up or downregulation of protein
	Chaperones and Heat shock proteins	
GRP78 precursor	Chaperone activity, protein metabolism	Increased
Chaperonin	Chaperone activity, protein metabolism	Increased
Calreticulin precursor variant	Chaperone activity, protein metabolism	Increased
Heat shock 70kDa protein 9B precursor	Heat-shock cognate protein	Increased
PREDICTED: similar to Copper chaperone for superoxide dismutase	The copper chaperone for superoxide dismutase (CCS) is an intracellular metallochaperone required for incorporation of copper into the essential antioxidant enzyme copper/zinc superoxide dismutase (SOD1).	Increased
Tubulin-specific chaperone a	Tubulin cofactor A gene silencing in mammalian cells induces changes in microtubule cytoskeleton, cell cycle arrest and cell death and G1 cell cycle arrest.	Decreased
	Oxidoreductases	
Glutathione synthetase	Ligase activity, metabolism activity	Increased
Chain A, Crystal Structure Of The Alpha Subunit Of Human S-Adenosylmethionine Synthetase 2	The protein is required for the (S-Adenosylmethionine (SAM, AdoMet). SAM is the important methyl donor used for synthesis of nucleic acids, phospholipids, creatine, and polyamines and for methylation of many bioactive molecules and in oxidative stress of hyperoxia, a part of SAM generated is directed toward cysteine/GSH in the transsulfuration pathway.	Increased
Methionine adenosyltransferase II, alpha (MAT)	In oxidative stress of hyperoxia, MAT increases progressively and part of SAM generated is directed toward cysteine/GSH in the transsulfuration pathway.	Increased
Thioredoxin-like 1 variant	Oxidoreductases	Increased
Mitochondrial ATP synthase, H+ transporting F1 complex beta subunit	Oxidoreductase activity: Protein metabolism	Increased
Thioredoxin domain containing 5 isoform 2	Protein-disulfide isomerase. Its expression is induced by hypoxia and its role may be to protect hypoxic cells from apoptosis.	Increased
Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 complex III of mitochondria	Reductase, Metabolism ; Catalytic activity; Energy pathways	Almost absent
	Energy Metabolism pathways	
Pyruvate dehydrogenase E1-beta subunit precursor	Energy Metabolism; Catalytic activity; Energy pathway	Increased
Chain A, X-Ray Crystal Structure Of A Chemically Synthesized Ubiquitin	Involved in ubiquitin-specific protease activity.	Decreased

Chain B, Crystal Structure Of The Chip-Ubc13-Uev1a Complex	The protein is involved in chaperoned ubiquitylation: CHIP U box E3 ubiquitin ligase.	Decreased
Proteasome 26S ATPase subunit 4 isoform 1	The proteasome cleaves peptides in an ATP and ubiquitin-dependent process in a non-lysosomal pathway.	Increased
PREDICTED: proteasome 26S ATPase subunit 3 isoform 2	Protein metabolism: Ubiquitin specific protease activity	Increased
	Metabolism enzymes	
Membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1 (MTCBP-1)	MTCBP-1 acts as an eukaryotic aci-reductone dioxygenase (ARD) in the methionine salvage pathway.	Decreased
Spermidine synthase	Spermidine synthase have been shown to be inducible during lectin induced lymphocyte activation, cell proliferation, such as liver regeneration and liver compensatory growth, hormone-induced growth of tissues.	Increased
	Ubiquitin proteasome pathways	
Chain A, Crystal Structure Of The Alpha Subunit Of Human S-Adenosylmethionine Synthetase 2	S-Adenosylmethionine (SAM, AdoMet) is the most important methyl donor used for synthesis of nucleic acids, phospholipids, creatine, and polyamines and for methylation of many bioactive molecules.	Increased
Glutathione synthetase	Ligase activity, metabolism activity	Increased
Aminoacylase 1-like 2	Glutamate carboxypeptidases	Increased
Chain A, Triosephosphate Isomerase (Tim) complexed with 2-Phosphoglycolic Acid	Intramolecular Oxidoreductase/Catalytic activity	Decreased
TALDO1 protein	The enzyme catalyses the reversible transfer of a dyhydroxyacetone moiety, derived from fructose-6 phosphate to erythrose-4-phosphate yielding sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate.	Increased
Guanine monophosphate synthetase (GMP synthetase)	In the guanine nucleotide pathway, GMP synthetase catalyzes the amination of XMP to GMP.	Decreased
Pyrophosphatase 1	PPases catalyze the hydrolysis of pyrophosphate to inorganic phosphate, which is important for the phosphate metabolism of cells.	Increased
Fatty acid binding protein 5 (psoriasis-associated)	These proteins are a family of small, highly conserved, cytoplasmic proteins that bind long-chain fatty acids and other hydrophobic ligands. It is thought that FABPs roles include fatty acid uptake, transport, and metabolism.	Decreased
OXCT Succinyl-CoA:3-Oxoacid CoA Transferase	Acyl CoA:acetate/3-ketoacid CoA transferase, alpha subunit (Lipid metabolism)	Increased
	Cytoskeleton	
Actin-like 6A isoform 2	DNA binding protein, The actin-related proteins are involved in diverse cellular processes, including vesicular transport, spindle orientation, nuclear migration and chromatin remodeling.	Increased
F-actin capping protein alpha-1 subunit	The protein regulates growth of the actin filament by capping the barbed end of growing actin filaments.	Increased

Tropomodulin 3 (ubiquitous)	**Tropomodulin is a novel tropomyosin regulatory protein that binds to the end of erythrocyte tropomyosin and blocks head-to-tail association of tropomyosin along actin filaments.**	Increased
Tubulin, beta 5	**Overexpression of a mouse class V beta-tubulin cDNA in mammalian cells produces a strong, dose-dependent disruption of microtubule organization, increased microtubule fragmentation, and a concomitant reduction in cellular microtubule polymer levels. These changes also disrupt mitotic spindle assembly and block cell proliferation.**	Increased
Tubulin, beta	**Microtubule regulation in mitosis: tubulin phosphorylation by the cyclin-dependent kinase Cdk1. As above.**	Increased
ACTB protein	**An ubiquitous protein involved in the formation of filaments that are a major component of the cytoskeleton. Interaction with myosin provides the basis of muscular contraction and many aspects of cell motility.**	Increased
Cofilin 1 (non-muscle)	**Cytoskeleton associated protein: cytoskeleton organization and biogenesis**	Decreased
	RNA binding and metabolism associated proteins	
Heterogeneous nuclear ribonucleoprotein H1	**RNA binding protein; Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism**	Increased
Heterogeneous nuclear ribonucleoprotein K isoform a variant	**RNA binding protein; Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism**	Increased
Chain A, Solution Structure Of Rrm Domain in HNRPC	**Single HNRPC tetramers bind 230-240 nucleotides. May play a role in the early steps of spliceosome assembly and pre-mRNA splicing.**	Increased
SYNCRIP protein (hnRNP-Q, synaptogamin binding)	**RNA binding protein; Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism NS1 associated protein**	Increased
NOP17	**Involved in pre-rRNA processing.**	Increased
Splicing factor, arginine/serine-rich 7	**RNA binding protein; Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism**	Increased
Ribonuclease HI, large subunit	**Rnase HI is the major enzyme and shows increased activity during DNA replication**	Increased
KH-type splicing regulatory protein	**K homology RNA-binding domain, type I. KH binds single-stranded RNA or DNA. It is found in a wide variety of proteins including ribosomal proteins, transcription factors and post-transcriptional modifiers of mRNA. mRNA export from nucleus; nuclear mRNA splicing, via spliceosome; regulation of transcription, DNA-dependent; RNA splicing.**	Decreased
Far upstream element-binding protein	**This is a ssDNA binding protein that activates the far upstream element (FUSE) of c-myc and stimulates expression of c-myc in undifferentiated cells. This protein has been shown to function as an ATP-dependent DNA helicase.**	Decreased
Basic transcription factor 3 isoform B	**This protein forms a stable complex with RNA polymerase IIB and is required for transcriptional initiation.**	Decreased
Core-binding factor, beta subunit isoform 1	**The protein is the beta subunit of a heterodimeric core-binding transcription factor belonging to the PEBP2/CBF transcription factor family which master-regulates a host of genes specific to hematopoiesis (e.g., RUNX1) and osteogenesis (e.g., RUNX2).**	Absent
Homeobox prox 1	**Transcription regulatory protein :Transcription regulatory activity**	Absent

Ribosomal protein S12	**40S ribosomal protein S12: This is a ribosomal protein and component of the 40S subunit. The protein belongs to the S12E family of ribosomal proteins. Increased expression of this gene in colorectal cancers compared to matched normal colonic mucosa has been observed.**	Decreased
Ribosomal protein P0	**A ribosomal protein that is a component of the 60S subunit. It is a neutral phosphoprotein with a C-terminal end that is nearly identical to the C-terminal ends of the acidic ribosomal phosphoproteins P1 and P2. The P0 protein can interact with P1 and P2 to form a pentameric complex consisting of P1 and P2 dimers, and a P0 monomer.**	Decreased
This CDS feature is included to show the translation of the corresponding V_region		Decreased
High-mobility group 1. Nuclear phosphoprotein HMGA1a	**High-mobility group A1 (HMGA1) overexpression and gene rearrangement are frequent events in human cancer. HMGA1 overexpression promoted HIPK2 relocalization in the cytoplasm and inhibition of p53 apoptotic function.**	Decreased
Chain A, Pkci-1-Apo+zinc	**Protein Kinase C Interacting (inhibitory). Protein PKCI and related proteins belong to the ubiquitous HIT family of hydrolases that act on alpha-phosphates of ribonucleotides.**	Decreased
Platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit 29kDa	**PAF acetylhydrolase (PAF-AH). PAF and PAF-AH are key players in inflammation and in atherosclerosis.**	Decreased
	Vesicle fusion, synthesis and trafficking	
Epsilon subunit of coatomer protein complex isoform c	**This is required for budding from Golgi membranes, and is essential for the retrograde Golgi-to-ER transport of dilysine-tagged proteins.**	Increased
p47 protein isoform a	**p47 is an adaptor molecule of the cytosolic ATPase p97. The principal role of the p97-p47 complex is to regulate membrane fusion events.**	Increased
Cytoskeleton associated protein	**Cytoskeleton-associated proteins (CAPs) are involved in the organisation of microtubules and transportation of vesicles and organelles along the cytoskeletal network.**	Increased
Aryl hydrocarbon receptor interacting protein		Increased
Hypothetical protein		Increased
PREDICTED: similar to Putative S100 calcium-binding protein A11 pseudogene	**The S-100 domain is a subfamily of the EF-hand CaBPs, expressed exclusively in vertebrates, and implicated in intracellular and extracellular regulatory activities expressed in leukocytes.**	Absent

Table 3.2 Identification of proteins of AML14.3D10 cell line binding to rhSP-D.

Spot No.	Locus tag	Protein name	Theo. Mass (Da)	Theo. pI	MS data			MS/MS data				
					No of peptides matched	% Seq Cov	Mascot score	No of peptides matched	Total Score	3 Peptides matched	Peptide	Peptide Score
1	gi|54648253	KHSRP protein	73307	8	31/50	44	309	6	211	IINDLLQSLR AINQQTGAFVEISR	385-394 449-462	62 75
2	gi|54648253	KHSRP protein	73307	8	18/44	26	132					
3	gi|4826998	SFPQ protein (splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated))	55549	9.89	14/40	34	137					
4	gi|38014635	SFPQ protein	55549	9.89	14/40	36	136	3	60	LFVGNLPADITEDEFKR	226-242	36
5	gi|11527777	PREDICTED: similar to heterogeneous nuclear ribonucleoprotein L isoform a isoform 3	60822	6.65	33/50	45	302	3	130	NPNGPYPYTLK VFNVFCLYGNVEK	569-579 399-411	35 72
6	gi|4557014	Catalase	59947	6.9	21/36	45	245	6	172	LFAYPDTHR AFYVNVLNEEQR NAIHTFVQSGSHLAAR LGPNYLHIPVNCPYR	355-363 445-456 507-522 366-380	35 40 39 36

7	Gi|109124618	PREDICTED: similar to heterogeneous nuclear ribonucleoprotein L isoform a isoform 3	60822	6.65	27/50	31	195					
8	Gi|109124618	PREDICTED: similar to heterogeneous nuclear ribonucleoprotein Lisoform a isoform 3	60822	6.65	27/50	31	178					
9	gi|14141159	heterogeneous nuclear ribonucleoprotein H3 isoform b	35273	6.36	11/26	34	124					
10	gi|2098448	Chain , Carbonic Anhydrase	28776	6.63	12/50	60	138	5	135	YAAELHLVHWNTKAVQQPDGLAVLGIFLK	112-124 131-146	31 37
11	gi|5031753	heterogeneous nuclear ribonucleoprotein H1	49484	5.89	26/50	63	274	4	131	HTGPNSPDTANDGFVR STGEAFVQFASQEIAEK ATENDIYNFFSPLNPVR	99-114 151-167 300-316	32 36 33
12	gi|5031753	heterogeneous nuclear ribonucleoprotein H1	49484	5.89	22/50	55	229	6	290	GLPWSCSADEVQR HTGPNSPDTANDGFVR STGEAFVQFASQEIAEK ATENDIYNFFSPLNPVR YVELFLNSTAGASGGAYEHR	17-29 99-114 151-167 300-316 356-375	51 37 79 84 35

13	gi|16876910	heterogeneous nuclear ribonucleoprotein F	45985	5.38	17/49	49	161	4	110	YIEVFKSSQEEVRATENDIYNFFSPLNPVR	180-192 300-316	32 43
14	gi|16876910	heterogeneous nuclear ribonucleoprotein F	45985	5.38	12/40	34	111	2	69	YIEVFKSSQEEVRATENDIYNFFSPLNPVR	180-192 300-316	32 37

3.3.4.d Real Time qPCR RESULTS

Based on proteomics data, a select group of target genes (as listed below) were identified for qPCR analysis from experiments where rhSP-D was used to induce apoptosis in AML14.3D10, Raji and Jurkat.

1. **MTCBP-1** Membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1
2. **PRDX3** Peroxiredoxin 3 isoform b
3. **SOD2** Chain A, X-Ray Crystal Structure For Human Manganese Superoxide Dismutase, Q143a
4. **UQCRFS1** Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
5. **HMGA1** High-mobility group I (HMGA1a)
6. **MDM2** The murine double minute 2 (mdm2) gene encodes a negative regulator of the p53 tumor suppressor
7. **PKCI-1** Chain A, Pkci-1-Apo+zinc
8. **AIP** Aryl hydrocarbon receptor interacting protein
9. **S100A11P** Putative S100 calcium-binding protein A11 pseudogen
10. **CBFB** Core-binding factor, beta subunit isoform 1
11. **P53** Inhibits the development and growth of tumors (in addition to other functions)
12. **Fas** The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor contains a death domain
13. **Bax** Accelerates programmed cell death by binding to, and antagonizing the apoptosis repressor BCL2 or itsadenovirus homolog E1B 19k protein

Bax Accelerates programmed cell death by binding to, and antagonizing the apoptosis repressor BCL2 or itsadenovirus homolog E1B 19k proteinThe effect on gene expression by AML14.3D10 is different from Raji and Jurkat (Figure 3.16, 3.17 and 3.18) in some of the genes. In AML14.3D10, HMGA-1 is down regulated and correlates to the proteomics data, where as in Raji and Jurkat it has seen to be upregulated after 48 hours (Figure 3.20). Similarly, for the genes MTCB-1 (have been persistently increasing in Raji), PRDX-3, SOD-2 (Figure 3.19), UQCRSFS-1 (Figure 3.20), S100A11P, PKCL-1, MDM-2 (Figure 3.20 and 3.21) (Again persistent increase in Raji) were seen. AIP increase was not observed in AML14.3D10 cells, but seen increase in

Raji and Jurkat cells with time till 48 hours. Gene CBFB was not expressed in AML14.3D10 cells, while this gene expression was seem to be increasing in Raji and Jurkat cells. P53, Fas and Bax gene (Figure 3.22) expression increased with time till 45 mins for AML cells, and consistently the increased in Raji and Jurkat till 48 hours

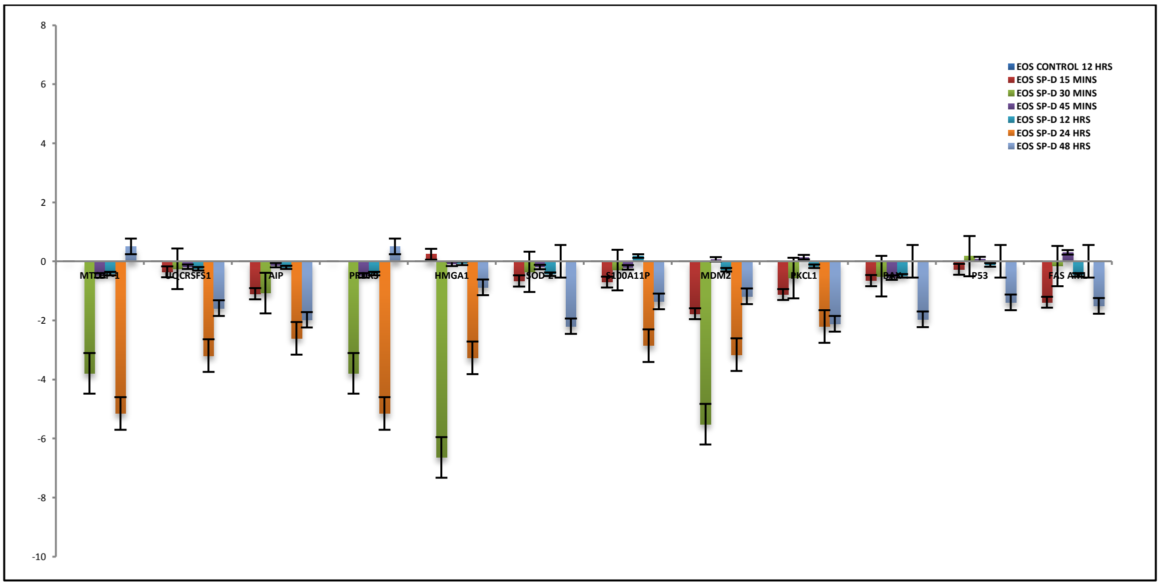


Figure 3.14 **Expression of Genes by AML14.3D10 cells in vitro during incubation with rhSP-D.** Cells were incubated with rhSP-D for 12 hrs, 30min, 45min, 60 min, 2hrs, 6hrs, 12hrs, 24hrs and 48 hours. The expression of the genes was measured using the real time qPCR and the data was normalized to 18sRNA gene expression as control. Relative expression (RQ) was calculated using the comparative Ct method with cells incubated with PBS for 12 hrs as the calibrator. The RQ value was calculated using the formula; RQ=$2^{\Delta\Delta Ct}$ and the experiment was done in triplicates. Error bars represent the ±standard error of the mean.

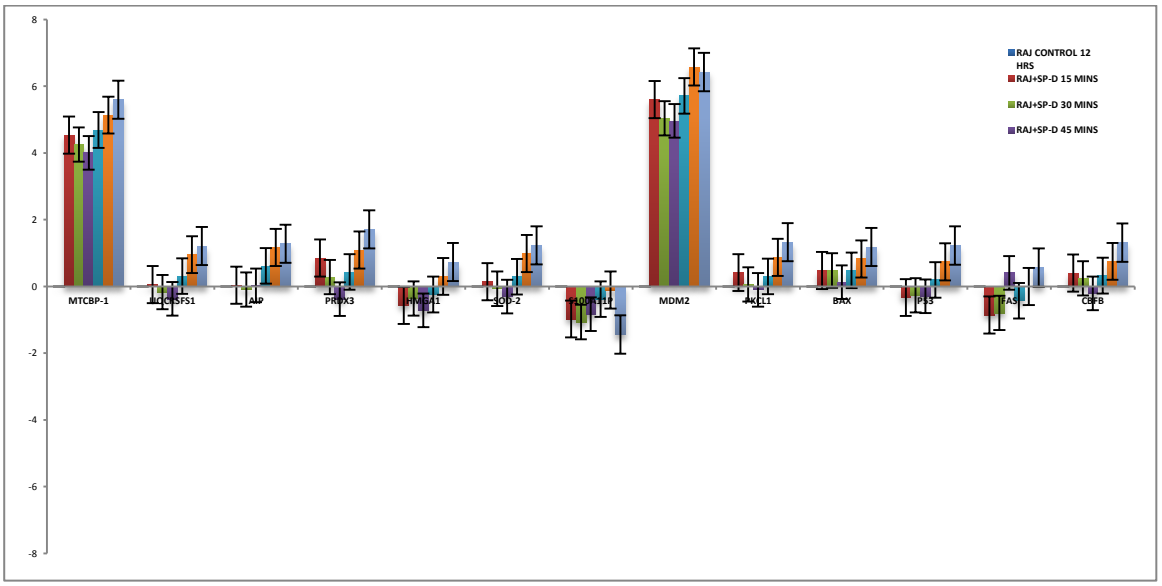


Figure 3.15 **Expression of Genes by Raji cells in vitro during incubation with rhSP-D.** Cells were incubated with rhSP-D for 15 min, 30min, 45min, 12hrs, 24hrs and 48 hours. The expression of the genes was measured using the real time qPCR and the data was normalized to 18sRNA gene expression as control. Relative expression (RQ) was calculated using the comparative Ct method with cells incubated with PBS for 12hrs as the calibrator. The RQ value was calculated using the formula; $RQ=2^{\Delta\Delta Ct}$ and the experiment was done in triplicates. Error bars represent the ±standard error of the mean.

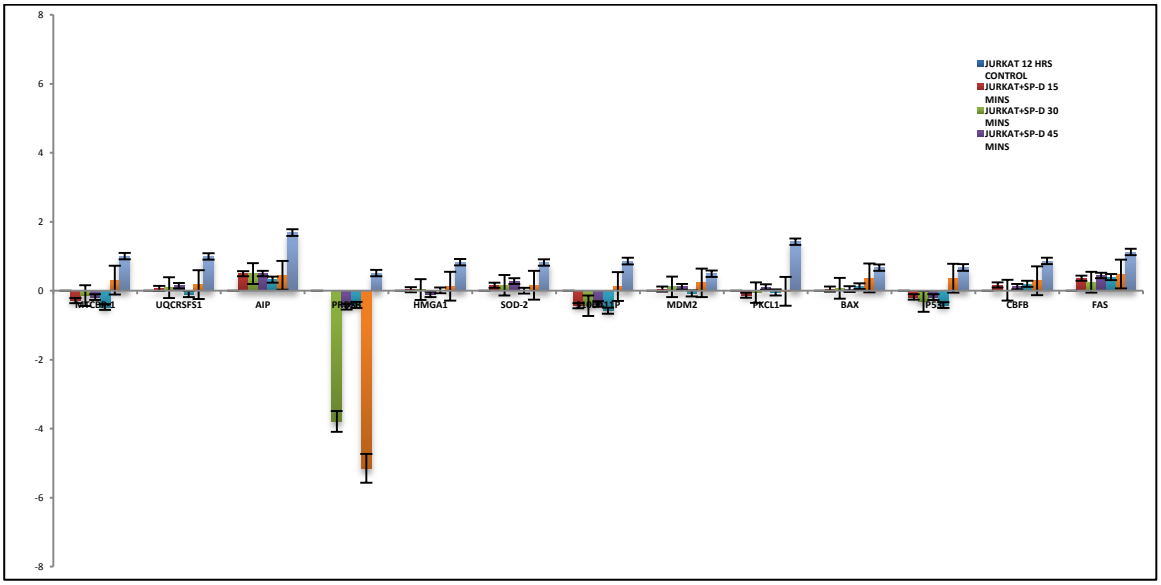


Figure 3.16 **Expression of Genes by Jurkat cells in vitro during incubation with rhSP-D.** Cells were incubated with rhSP-D for 15 min, 30min, 45min, 12hrs, 24hrs and 48 hours. The expression of the genes was measured using the real time qPCR and the data was normalized to 18sRNA gene expression as control. Relative expression (RQ) was calculated using the comparative Ct method with cells incubated with PBS for 15 mins as the calibrator. The RQ value was calculated using the formula; $RQ=2^{\Delta\Delta Ct}$ and the experiment was done in triplicates. Error bars represent the ±standard error of the mean.

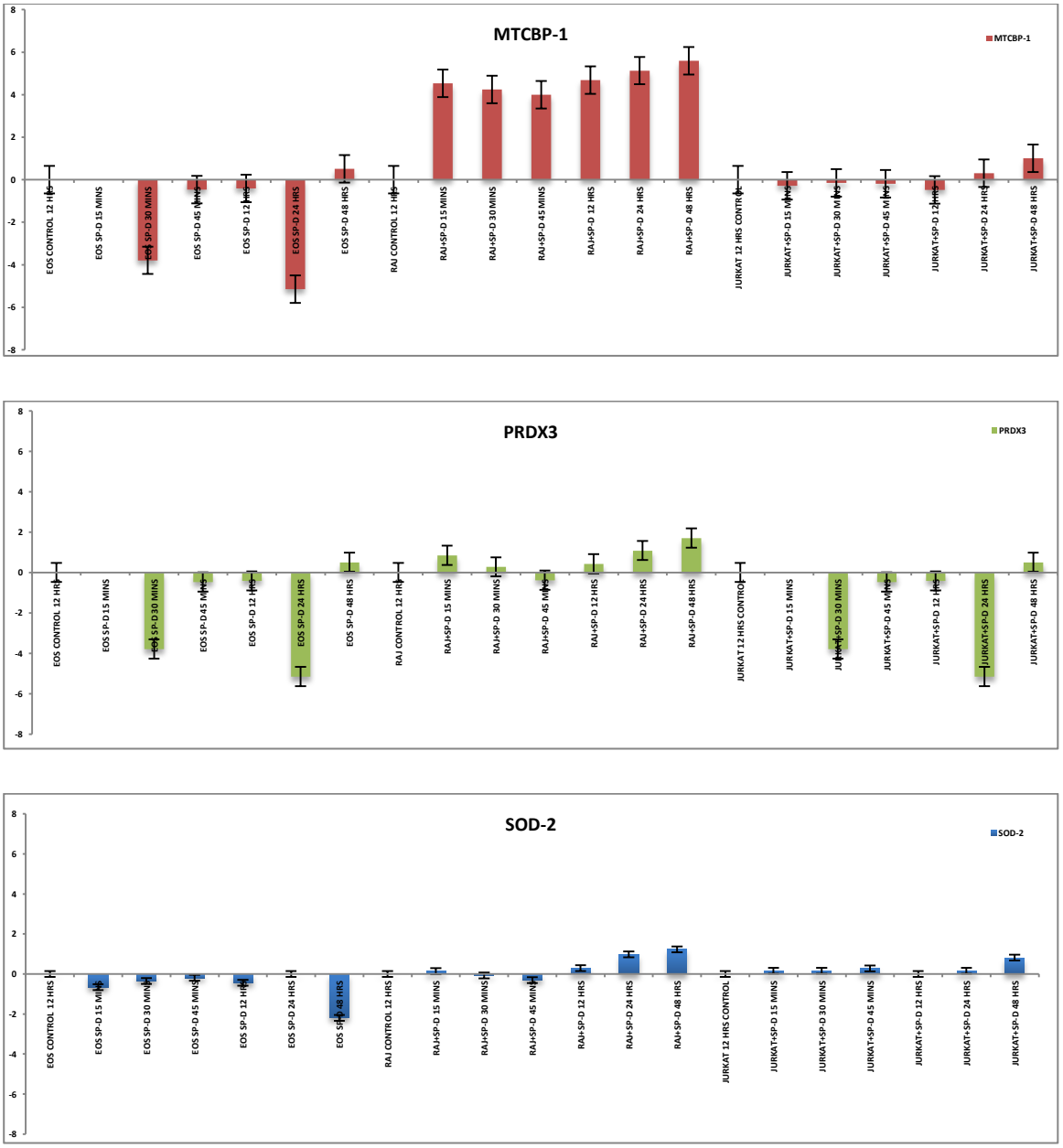
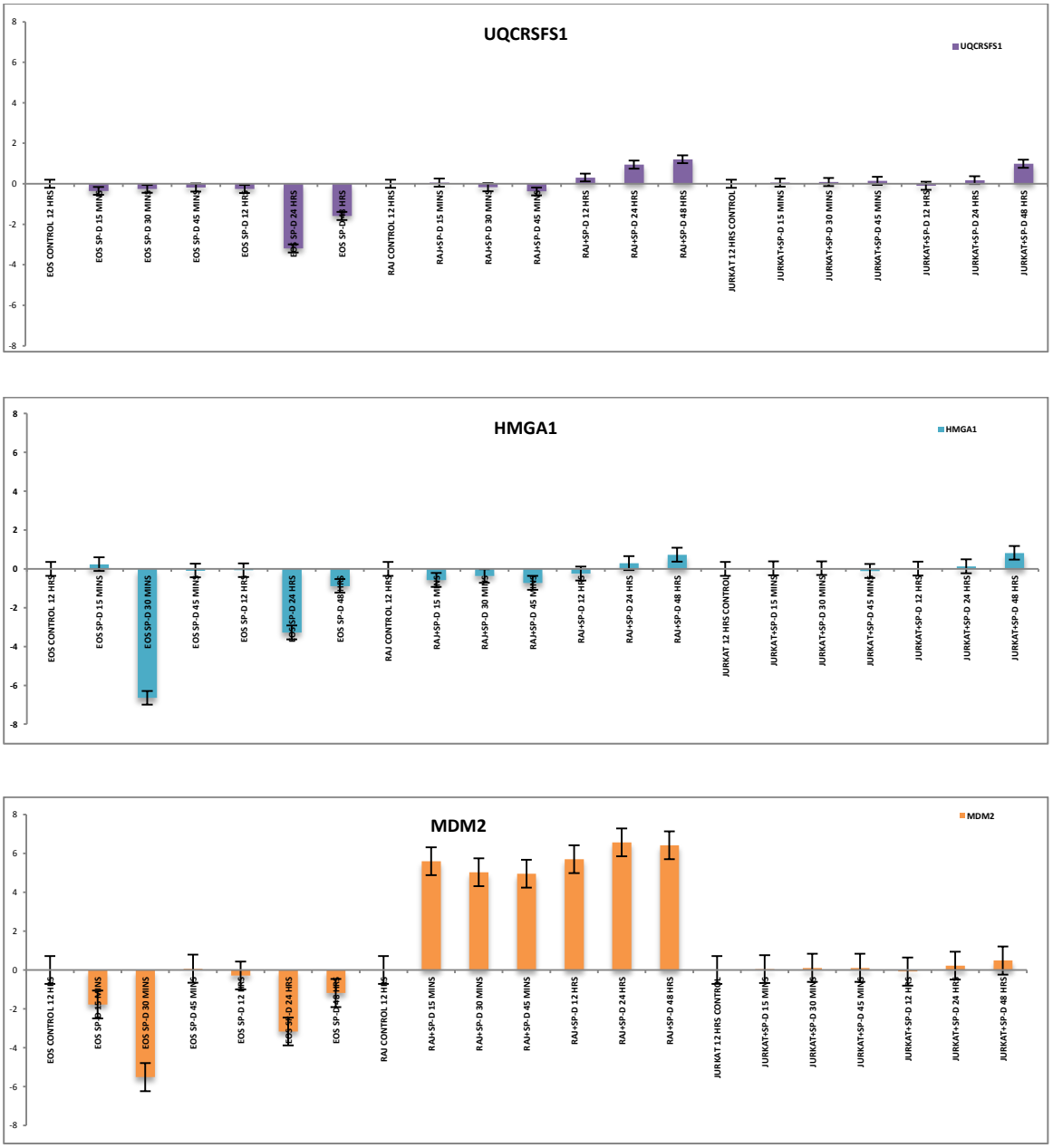


Figure 3.17 **Comparative expression of Genes (MTCBP-1, PRDX-3 and SOD-2) by AML14.3D10, Raji and Jurkat cells in vitro during incubation with rhSP-D.** Cells were incubated with rhSP-D for 15 min, 30min, 45min, 12hrs, 24hrs and 48 hours. The expression of genes was measured using the real time qPCR and the data was normalized to 18sRNA gene expression as control. Relative expression (RQ) was calculated using the comparative Ct method with cells incubated with PBS for 12 hrs as the calibrator. The RQ value was calculated using the formula; $RQ=2^{\Delta\Delta Ct}$ and the experiment was done in triplicates. Error bars represent the ±standard error of the mean.



§

Figure 3.18 **Comparative expression of Genes (UQCRSFS-1, HMGA-1 and MDM-2) by AML14.3D10, Raji and Jurkat cells in vitro during incubation with rhSP-D.** Cells were incubated with rhSP-D for 15 min, 30min, 45min, 12hrs, 24hrs and 48 hours. The expression of genes was measured using the real time qPCR and the data was normalized to 18sRNA gene expression as control. Relative expression (RQ) was calculated using the comparative Ct method with cells incubated with PBS for 12 hrs as the calibrator. The RQ value was calculated using the formula; $RQ=2^{\Delta\Delta Ct}$ and the experiment was done in triplicates. Error bars represent the ±standard error of the mean.

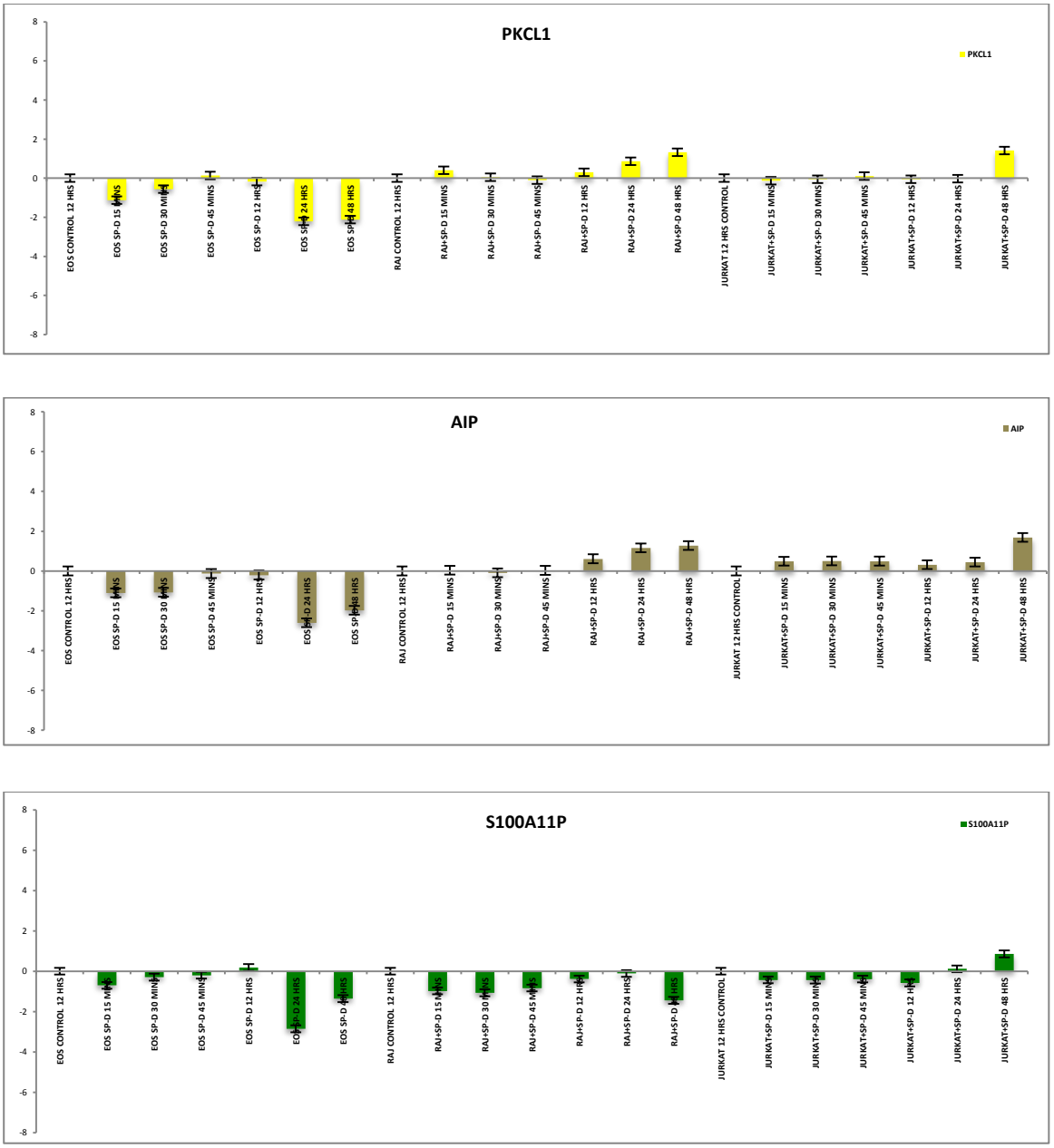


Figure 3.19 **Comparative expression of Genes (PKCL-1, AIP and S100A11P) by AML14.3D10, Raji and Jurkat cells in vitro during incubation with rhSP-D.** Cells were incubated with rhSP-D for 15 min, 30min, 45min, 12hrs, 24hrs and 48 hours. The expression of genes was measured using the real time qPCR and the data was normalized to 18sRNA gene expression as control. Relative expression (RQ) was calculated using the comparative Ct method with cells incubated with PBS for 12hrs as the calibrator. The RQ value was calculated using the formula; $RQ=2^{\Delta\Delta Ct}$ and the experiment was done in triplicates. Error bars represent the ±standard error of the mean.

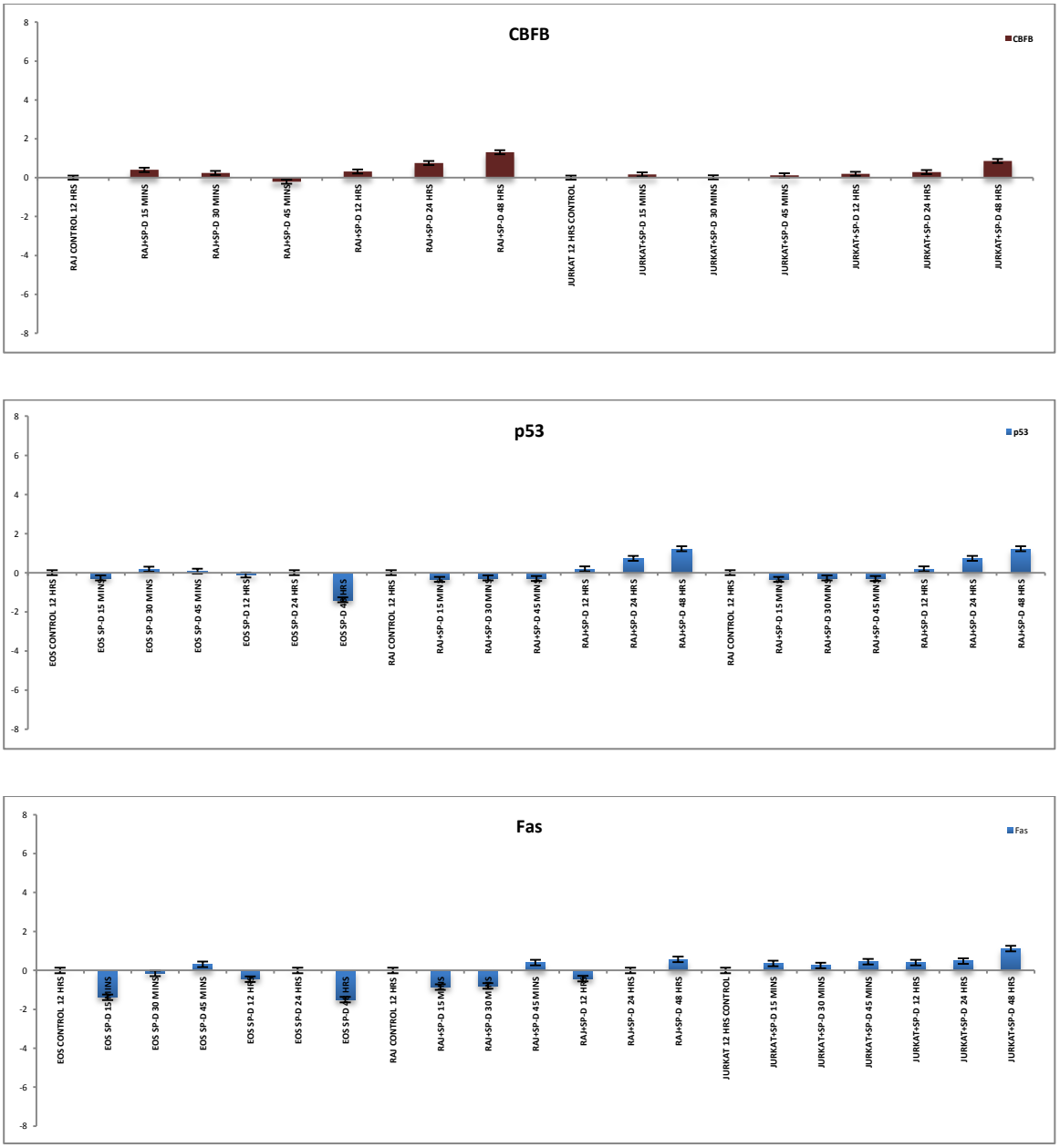


Figure 3.20 Comparative expression of Genes (CBFB, p53 and Fas) by AML14.3D10, Raji and Jurkat cells in vitro during incubation with rhSP-D. Cells were incubated with rhSP-D for 15 min, 30min, 45min, 12hrs, 24hrs and 48 hours. The expression of genes was measured using the real time qPCR and the data was normalized to 18sRNA gene expression as control. Relative expression (RQ) was calculated using the comparative Ct method with cells incubated with PBS for 12hrs as the calibrator. The RQ value was calculated using the formula; $RQ=2^{\Delta\Delta Ct}$ and the experiment was done in triplicates. Error bars represent the ±standard error of the mean.

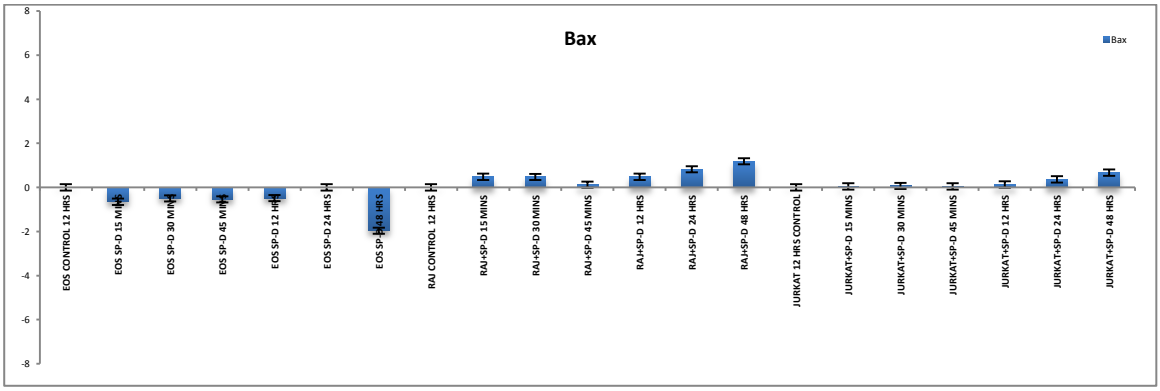


Figure 3.21 **Comparative expression of Gene Bax by AML14.3D10, Raji and Jurkat cells in vitro during incubation with rhSP-D.** Cells were incubated with rhSP-D for 15 min, 30min, 45min, 60 min, 2hrs, 6hrs, 12hrs, 24hrs and 48 hours. The expression of genes was measured using the real time qPCR and the data was normalized to 18sRNA gene expression as control. Relative expression (RQ) was calculated using the comparative Ct method with cells incubated with PBS for 15 mins as the calibrator. The RQ value was calculated using the formula; $RQ=2^{\Delta\Delta Ct}$ and the experiment was done in triplicates. Error bars represent the ±standard error of the mean.

DISCUSSION

Proteomic profile of rhSP-D-treated AML14.3D10 cells & identification of differentially expressed proteins comparative analysis of silver-stained 2-DE gels of AML14.3D10 cells treated with rhSP-D (10 mg/ml) for 48 h led to the identification of a total of 134 proteins that were differentially expressed and showed a threefold change in comparison to the untreated cell line (Mahajan, Pandit et al. 2013). The cell line was treated with rhSP-D for 48 h, based on the earlier results, defining it as a time period that would sufficiently prime the cells for a distinct pathway, before these entered and extensively expressed proteins related to the apoptotic pathways. Peptide mass fingerprinting (PMF) analysis after matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry and/or matrix-assisted laser desorption/ionization–time-of-flight/time-of-flight–tandem mass spectrometry analysis resulted in the identification of 75 proteins out of 134. Of these 75 proteins, 52 proteins that were identified were upregulated, while 23 protein spots were down regulated following rhSP-D treatment of the cell line (Mahajan, Pandit et al. 2013)

3.3.1 PROTEOMICS DATA

Proteomic profiling of AML14.3D10 treated with rhSP-D

SP-D increased significantly in airway inflammation models mediating regulation of immune cells and pulmonary homeostasis indicating its crucial role as shown in BALB/c mice sensitized and challenged with ovalbumin and in rat BAL fluid proteins associated with oil mist exposure (Lee, Chen et al. 2006, Zhao, Zhu et al. 2005). RhSP-D-mediated induction of apoptosis in cancer cell lines and the 2-DE proteomics of SP-D treated AML14.3D10 cells revealed various involvement of p53 pathway proteins and their binding to rhSP-D, indicating its involvement in apoptosis. Rh SP-D induced changes in tyrosine phosphoprotein expression profile of eosinophillic cells. Oxidative burst, p53 activation and downregulation of high-mobility group A1 (HMGA1) were observed, indicating a potent antiallergic mechanism of SP-D (Mahajan, Pandit et al. 2013). Proteomic profile of rhSP-D-treated (10µg/ml) AML14.3D10 cells for 48hrs showed 134 proteins that were differentially expressed and showed a three-fold change in comparison to untreated cell lines in the silver-stained 2-DE gels. Peptide mass fingerprinting (PMF) analysis identified 75 proteins, out of which 52 were upregulated and 23 proteins were down regulated (figure 3.16) (Mahajan, Pandit et al. 2013). Major

functional categories of these proteins were oxidoreductases, chaperones, ubiquitin-proteasome pathways, translation and transcription, RNA binding and metabolism; inflammation and survival; cytoskeleton; vesicle fusion, synthesis and trafficking; metabolic enzymes; energy metabolism and others.

Following, two dimensional gel electrophoresis, a far western blot was performed to examine proteins in the apoptosed or rhSP-D apoptosed eosinophillic cell lines, 14 proteins were identified as rhSP-D binding proteins (Figure 3.17 and Table 3.2). Tyrosine phosphorylation protein analysis of rhSP-D apoptosed eosinophils revealed reduction in the tyrosine phosphorylation of at least 7 proteins (figure 3.18). The eosinophillic cell line AML14.3D10 appears to produce number of tyrosine phosphorylated proteins constitutively.

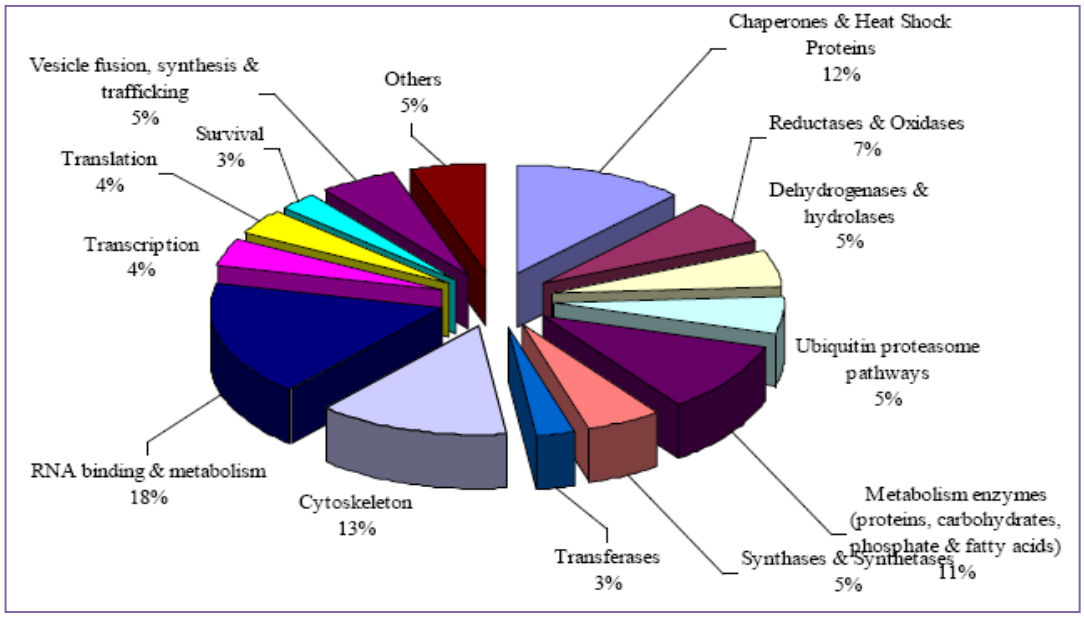


Figure 3.22 Pie chart grouping of differentially expressed proteins of AML14.3D10 eosinophilic leukemia cell line on treatment of cell line with pulmonary innate immune molecule rhSP-D (3.0-fold change) (n = 75) in functional classes annotated using expasy (Expert Protein Analysis System) database (http://expasy.org/uniprot) (Mahajan, Gautam et al. 2014).

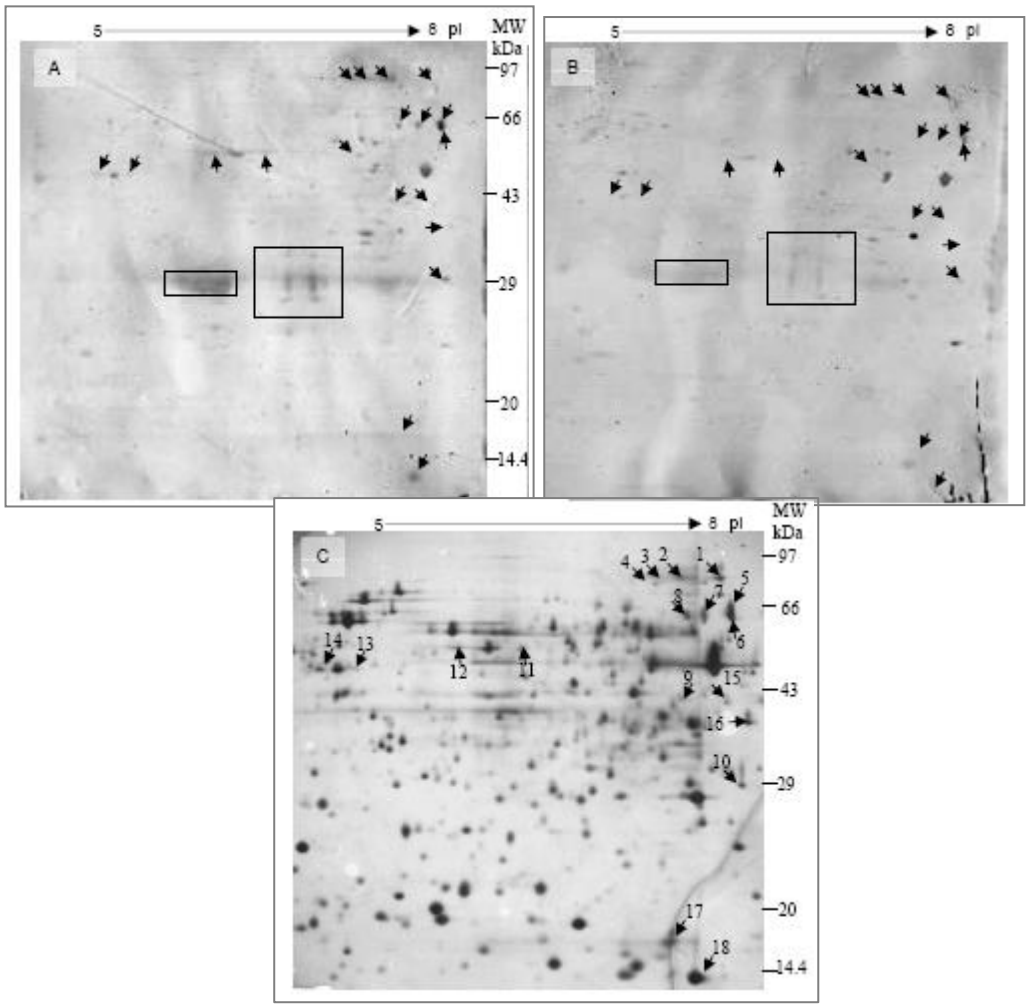


Figure 3.23 Two DE-western blot for detection and identification of AML14.3D10 cell line proteins binding to rhSP-D. The arrows on blot image (A) mark immunoreactive proteins of the cell line showing binding to rhSP-D; (B) show the absence of these spots on the control blot (western blot in the absence of rhSP-D). (C) 2DE-gel (colloidal coomassie stained) showing the corresponding protein spots (18 protein spots) used for identification by MALDI-TOF-MS and MALDI-TOF-MS-MS. A total of 14 proteins were identified and are listed in Table 2. For this, the proteins (350 µg) were separated by isoelectric focusing on IPG strips, 11 cm, pI 5-8 and then SDS-PAGE 12.0%; MW, molecular weight marker (kDa). Two clusters of rhSP-D interacting proteins were observed on the blot could not be traced back to appropriate spots on the gel and hence, were not subjected to PMF analysis.

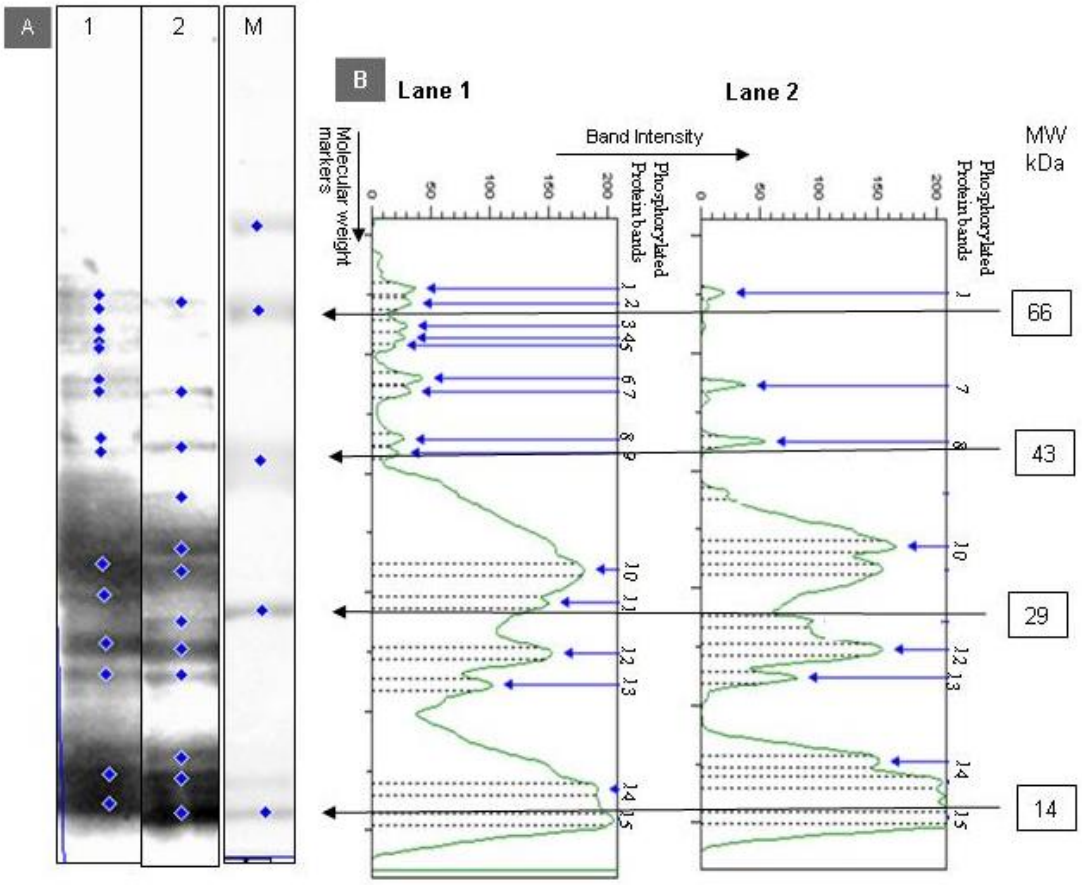


Figure 3.24 Tyrosine phosphorylation protein profile of rhSP-D treated AML14.3D10 cells. (A) AML14.3D10 cells constitutively contained a number of detectable tyrosine phosphorylated species (lane 1). The cell line treatment with rhSP-D resulted in marked decrease in tyrosine phosphorylation of seven protein bands (lane 2). (B) The phosphorylated protein bands in lane 1 and lane 2 were compared by band intensity diagram using the ImageQuant TL v2005. The blue arrows correspond to approximate molecular weight of the protein bands showing protein phosphorylation in lane 1 and lane 2 (X-axis marked by standard molecular weight, lane M, with black arrows). Lane M, Molecular weight marker, MW (kDa).

Eight of 14 spots that were identified by PMF belonged to the family of hnRNPs that included hnRNP L, hnRNP H1, hnRNP H3 and hnRNP F. Four other identified proteins belonged to the family of RNA-splicing proteins that included two isoforms, each of KHSRPs and proteins belonging to SR family of splicing factors. The hnRNP H and hnRNP F are hnRNP H group protein members that exhibit extensive sequence homology and are involved in mRNA processing. hnRNP H is found to be associated with nuclear matrix proteins and is involved in splicing regulation as part of the intronic splicing enhancer complex in the c-Src neuronal-specific N1 exon, as discussed earlier (Holzmann, Korosec et al. 1997, Chou, Rooke et al. 1999). The hnRNP F is known to interact with the nuclear cap-binding protein complex and is also involved in regulation of intronic splicing (Gamberi, Izaurralde et al. 1997). It has also been recently proposed to have a role in transcription via its interaction with the TATA-binding protein (Yoshida, Makino et al. 1999). HnRNP L, on the other hand, has been shown to increase RNA stability. The other four proteins that showed binding to rhSP-D belonged to the family of RNA-splicing proteins that included two isoforms each of KHSRPs required for decay of AU-rich elements-containing mRNAs and proteins belonging to SR family of splicing factors.

The most striking common feature between rhSP-D-modified protein profile and rhSP-D-binding proteins in 2-D immunoblotting experiments was the presence of a range of RNA binding and RNA splicing-related proteins. The importance of interaction of these RNA-binding proteins to rhSP-D, however, remains to be examined. We speculate that SP-D binding to RNA-binding proteins may be involved in interactions that may affect specific changes in mRNA processing and, most probably, the rates of translation and/or protein degradation. Though, there is no direct evidence of SP-D entering the cells or interacting with RNA-binding proteins, the data suggest further investigation to substantiate this observation.

The role of tyrosine protein phosphorylation in lymphocyte activation is well documented (Sefton, Campbell 1991).

The eosinophilotropic cytokines-IL-5, IL-3, and GM-CSF have also been shown to cause the induction of tyrosine phosphorylation of multiple cellular substrates (van der Bruggen, Kanters et al. 1998). The results also showed that programmed eosinophil and neutrophil death are regulated by early events of signal transduction pathways

such as tyrosine phosphorylation (Yousefi, Green et al. 1994). Since tyrosine phosphorylation plays a major role in the trans- membrane signal transduction through most cell surface receptors, we analyzed the effect of rhSP-D on the total tyrosine phosphoprotein expression pattern of AML14.3D10 cells.

Tyrosine phosphoprotein expression profile of rhSP-D- treated AML14.3D10 cells constitutively contained a number of detectable tyrosine-phosphorylated species. Remarkably, following incubation with rhSP-D, there was a marked decrease in tyrosine phosphorylation of several protein species (Figure 3.18). Of the 15 tyrosine phosphorylation positive bands, 7 protein bands of 69.7, 63.5, 60.7, 58.9, 53.3, 44 and 31 kDA showed a decrease in tyrosine phosphorylation profile. The study suggested a significant effect of rhSP-D treatment on intracellular signaling of AML14.3D10 cell line. Tyrosine phosphorylation of rhSP-D-treated AML14.3D10 cells showed significant changes in the protein phosphorylation profile, suggesting that rhSP-D interaction with its receptors on eosinophilic cells results in the initiation of signaling. In view of the evidences of altered tyrosine phosphoprotein expression pattern obtained from this pilot study, we are now planning to use peptide chip array to study the differential kinome activity profiling of AML14.3D10 cells on treatment with rhSP-D. The study may reveal tyrosine and serine/ threonine kinases for their role in rhSP-D-mediated apoptosis in these cells.

From the proteomics data, it has been observed that many survival and inflammation related proteins like high mobility group A (HMGA1) and chain A of PKC interacting (Inhibitory)-1-Apo+Zinc protein were downregulated in AML14.3D10 cell line treated with rhSP-D. The HMGA1 protein facilitates chromatin functions by modulating the binding of transcription factors to target DNA, facilitate transcription and stimulates the formation of homeodomain-interacting protein kinase 2 (HIPK2), a p53 proapoptotic activator (Pierantoni, Rinaldo et al. 2007)and thus inhibits p53-mediated apoptosis. Hence decreased apoptosis is expected to increase the p53-mediated apoptosis and the proteomics data confirmed the decrease in HMGA1 protein expression by the AMl14.3D10 cells when treated with rhSP-D indicating as one of the mechanisms to induce mechanisms. The qPCR analysis of HMGA1 gene exactly correlates with the proteomics data when the AML14.3D10 cells were treated with rhSP-D. There was decrease in gene expression was observed after 15 mins of treatment and continued till

48 hours of treatment. Decrease in the ubiquitin level and CHIP U box E3 ligase protein, upregulation of hnRNPK and downregulation of HMGAI leads to p53 mediated apoptosis in the eosinophillic cells (Tan, Qu et al. 2000, Chou, Rooke et al. 1999, Pierantoni, Rinaldo et al. 2007, Mahajan, Gautam et al. 2014). Membrane-type I matrix metalloproteinase (MT1-MMP) cytoplasmic tail binding protein (MTCBP-1), an metabolism enzyme, which acts as an eukaryotic acireductone dioxygenase (ARD) in the methionine salvage pathway (Hirano, Gotoh et al. 2005) decreased there is downregualtion of the gene was also observed in the qPCR analysis till 48 hours. Aryl hydrocarbon receptor interacting protein (AIP) was seen to upregualted in the protein expression, whereas gene expression showed downregualtion before 45 mins and 12 hours treatment of cells with rh SP-D, whereas in Raji and Jurkat cells, the AIP showed upregulation of the gene with time. AIP has conserved regions of three tetratricopeptide repeat (TPR) domain and a FK506 binding protein-type peptidyl-prolyl cis-trans isomerase (FKBP-PPI) domain, analogous to a related domain of immunophilin proteins, which act as receptors for the immunosuppressive drugs like cyclosporin A and FK506 (Carver, Bradfield 1997). Decrease in AIP expression shows the susceptibility of treated eosinophillic cells to immunosuppressive drugs. Ubiquinol-cytochrome c reductase was absent in the proteomic profile and the gene expression got downregulated along with the peroxiredoxin 3. Increased mitochondrial generation of reactive oxygen species, irreversible DNA damage of mitochondria, membrane lipids and proteins to mitochondrial antioxidant defense mechanisms can lead to mitochondrial dysfunction, which can eventually trigger intrinsic pathway of apoptosis and cell death (Turrens 2003).

Chapter 4

SP-D in pancreatic cancer

Abstract

The effect of rhSP-D on eosinophils and tumour cell line prompted the question of SP-D can induce apoptosis of cancer cells. Thus, we examined the effect of overexpressing full length SP-D in a range of pancreatic cancer cells. When human and mouse pancreatic cells were examined by qPCR for SP-D mRNA expression, one of the most aggressive pancreatic cell line patu8988t appears to express SP-D dramatically compared to PanC1 and L3.6. Compared to Human pancreatic cancer cell lines, cell lines derived from transgenic mice overexpressing wild or mutant p53 produced very little amount SP-D. These results were consistent with the western blots, where the total cell extracts were probed with anti-human SP-D antibodies. Subsequently, full length SP-D was overexpressed using HEK construct in Patu8988t which appeared to suppress migration or rather metastatic migration of pancreatic cancer cells in a wound closure assay. Suppression of metastatic potential by SP-D was concomitant with downregulation of Snai1, Zeb1a and Vementin 1, which are EMT related genes involved in cancer mestastasis. It is clear that SP-D may have an immunosurveillance role to play in not only inducing apoptosis in p53 sufficient cancer cells in also restricting cancer metastasis.

4.1 Introduction

p53 is the tumour suppressor gene which regulates numerous cell life and death (Vogelstein, Lane and Levine, 2000) . It monitors several cell cycle checkpoints, cell cycle arrest like apoptosis and senescence with network of upstream sensors and downstream effectors (Sperka, Wang and Rudolph, 2012; Reinhardt and Schumacher, 2012) , mitogenic oncogenes (c-myc, K-Ras or NFAT) signals lead to apoptosis or senescence (Sperka, Wang and Rudolph, 2012; Reinhardt and Schumacher, 2012; Dimauro and David, 2010; Sherr, 2001) . $P19^{ARF}$ is a crucial sensor for the oncogenic signals, which is encoded, in an alternate reading frame (ARF) with $p16^{INK4a}$ by the tumours suppressor locus CDKN2A (Sherr, 2001). $p16^{INK4a}$ protects p53 from MDM2 degradation, stabilizing p53 which is seen to bind to promoters of more than 300 target genes relating to cell growth control (Banin *et al.*, 1998) . p21 (WAF1/CIP1), which is an important downstream gene target of p53 is encoded by the gene CDKN1A gene binds to cyclin-CDK2 and cyclin-CDK1 complexes inhibiting their activity, regulating the cell cycle progression at the G1 phase (Sperka, Wang and Rudolph, 2012; Stiewe, 2007; Coqueret, 2003; Sherr, 2001; Banin *et al.*, 1998; Ginsberg *et al.*, 1991) . p53 reacts with transcriptional activators and inhibitors with histone deacetylase complexes and intrinsic histone-modifying activities which interfere with the regulation of cell death (Jafarnejad and Li, 2012; Vernier *et al.*, 2011) . PML and PTEN, which are tumour suppressors, stabilize p53 transcriptional activity altering phosphorylation and acetylation (formation and maintenance of senescent phenotype) in response to Ras signalling activation (Nardella *et al.*, 2011; Chen *et al.*, 2005; Pearson *et al.*, 2000) . Mutational p53 inactivation leads to disruption of senescence pathways and causes carcinogenesis in many tumours and model systems indicating the importance of p53 in senescence and tumour suppression (Nardella *et al.*, 2011; Stiewe, 2007; Chen *et al.*, 2005; Hingorani *et al.*, 2005; Pearson *et al.*, 2000) . P53 inactivation on chromosome 17p was known in many (50-75%) pancreatic cancers on the second allele (Scarpa *et al.*, 1993) . Genetic loss and frequently modified mutation (R175P) of p53 makes K-Ras to overcome senescence and promote the growth of precursor lesions to bigger tumours in the murine models (Morton *et al.*, 2010; Hingorani *et al.*, 2005) .

Pancreas is part of the digestive system, which is a glandular organ made up of Endocrine and Exocrine. Endocrine secretes insulin, glucagon and stomatostatin, whereas Exocrine secretes various other digestive enzymes and releases 1500 to

2000mls of alkaline fluid that is iso-osmotic into the small intestine per day. Exocrine is made up of acinar and ductal cells, which store and secrete active enzymes (amylase, lipase) and inactive enzymes (Zymogens, trypisinogen) (Ogami and Otsuki, 1998) . Secretin is the hormone released from the duodenal S-cells, which stimulates bicarbonate the pancreatic (ductal cells) secretions when there is gastric acid influx into the small intestine thus neural, hormonal, and mucosal factors play an important role (Bayliss and Starling, 1902) . Cholecystokinin (CCK) is released from the duodenal endocrine I-cells in response to proteins and fats in the small intestine, which stimulates acinar (Murphy *et al.*, 2008) cells and stimulates the vagal nerve responses which in turn activate the muscarinic acetylcholine receptors on the acinar cell resulting in the release of pancreatic enzymes.

Pancreatic Cancer in known to be extremely aggressive, chemo-resistant and lacks in prognosis. Reasons for this cancer have been numerous, pancreatic injury, inflammation are known to be important factors in the pancreatic cancer (Kolodecik *et al.*, 2014; Yadav and Lowenfels, 2013) . Intracelluar activation of transcription factor NF-κB along with the pancreatic enzymes leads to acute pancreatitis (AP). Chronic Pancreatitis (CP) is a fibro-inflammatory disease, which involves the pancreatic parenchyma being replaced by the fibrotic tissue. Acinar cell damage, mononuclear cell infilteration, and fibroses could be seen histologically (Shrikhande *et al.*, 2003) . CP was a risk factor for pancreatic cancer and meta-analysis show a risk of 13.3 for developing cancer (Raimondi *et al.*, 2010) , and occurs in three types of precancerous lesions: Pancreatic intraepithelial neoplasia (PanINs), intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms (MCN). These precursor lesions later evolve into pancreatic ductal adenocarcinoma (PDAC) (Yonezawa *et al.*, 2008) . Less than 5% of patients with CP enter into the disease inspite of the link (Raimondi *et al.*, 2010) . Frequent Pancreatic injury, genetic susceptibity, environmental factors (smoking, alcohol consumption, obesity which lead to oxidative stress), impaired autophagy, etc., which triggers the activation of inflammatory pathways, can lead to stimulating the stellate cells increasing fibrosis to chronic disease (Peery *et al.*, 2012; Spanier *et al.*, 2008) .

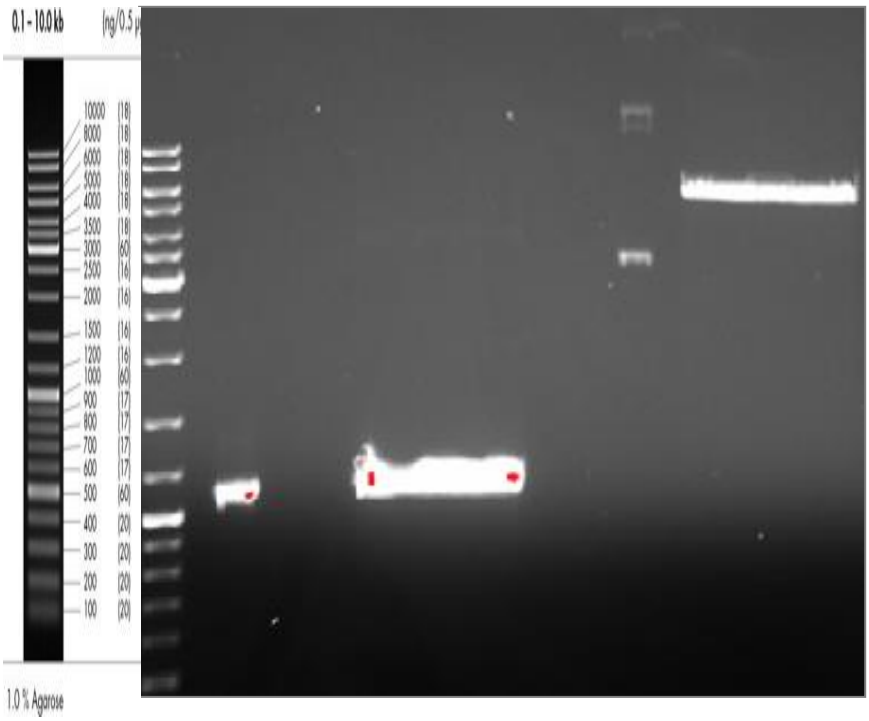
Activation of oncogenic Kras mutations through inflammation and changed tumour suppressor proteins like p53 and p16 ending up in the pancreatic cancer (Kolodecik *et al.*, 2014).

Notch signaling has crucial role in the embryonic development, adult tissue homeostasis and under abberant conditions, it can lead to many cancers like lung, colorectal, breast cancer and leukaemias (Rizzo *et al.*, 2008; Koch and Radtke, 2007) . It controls the central cellular processes; stemmness, differentiation, proliferation and survival (Hurlbut *et al.*, 2007; Roy, Pear and Aster, 2007) Notch ligands like Jag1, Jag2, DLL1, DLL3, DLL4 bind to notch receptors, which are cleaved later extracellularly by ADAM-type proteases and intracellularly by g-secretase are seen in the notch pathways, resulting in notch intracellular domain (NICD) translocating to the nucleus building up transcription activator complex with transcription factor CSL and coactivators, mastermind-like factors (Maml 1, 2,3). Hes and Hey1 which are notch target genes get activated due to the complex undergoing transcription (Koch and Radtke, 2007) . Mouse models for pancreatic cancer where inhibition of notch signaling by a gamma-secretase inhibitor (GSI) blocked the tumour formation completely (Plentz *et al.*, 2009) .

4.2 RESULTS

4.2.1 CLONING OF FULL LENGTH SP-D

Cloning of full length SP-D was done in an eukaryotic expression vector pSecTagB (Invitrogen) (Figure 4.4) with Igk-chain leader sequence. DNA fragment encoding for the full length sequence of SP-D was amplified using RT-PCR from cDNA using the following primers: 5'-**GGGAAGCTT**GCAGAAATGAAGACCTACTCCCAC-3' as sense and 5'-**GGGGGATCC**TCAGAACTCGCAGACCACAAGAC-3' as antisence (the blue nucleotides indicate the restriction endonuclease recognition sites). DNA fragment encoding the full length SP-D was amplied from pcDNA SP-D containing full length SP-D (provided by Dr. Kishore). The amplified PCR product (Figure 4.2) was double digested (BamHI/Hind III), agarose gel eluted and cloned into pSecTagB that was also double digested (BamHI/Hind III) and agarose gel eluted by ligation. The ligation was checked after the clone was transformed into the E.coli (BL21 λ DE3), plasmid isolated and restriction enzyme digested (BamHI/HindIII). The presence of the DNA fragment at appropriate molecular weight (1040bps) was checked by running 1% agarose gel with the ladder (Figure 4.3). The plasmids with the full length SP-D fragment were further confirmed by sending them for sequencing (Sequence data is enclosed in appendix section).



1% Agarose gel electrophoresis showing full length SP-D Uncut FL SP-D in the well 2 is followed by an empty well and the double digested (Hind III/Bam H I) fragment of FL SP-D is the well 4. Uncut pSecTagB and cut (Hind III/Bam H I) pSecTagB are seen in wells 6 and 7 respectively.

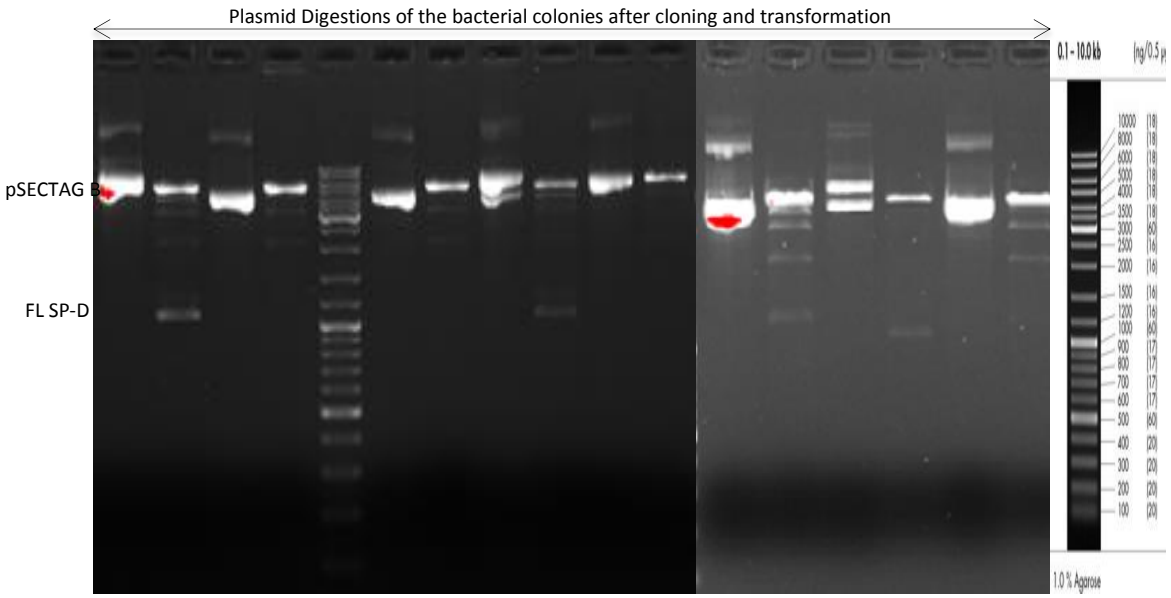


Figure 2.2 1% Agarose gel electrophoresis showing full length SP-D restriction digestion. Eco R 1/Bam H III restriction digestions for the plasmids isolated after cloning and transformations. Lower bands at approximately 1000bps show the FL SP-D plasmids and the higher bands at approximately at 5000bps are the pSecTag B plasmids. Plasmids picked from the (a.) 100_6 colony (Wells 6& 7) and 200_3 colony (wells 13 & 14) (b.) 200_9 colony (wells 4 & 5) were sent for sequencing.

4.2.2 TRANSFECTION OF FULL LENGTH SP-D IN HEK 293

HEK 293 cells were cultured in DMEM medium containing 10% fetal calf serum and antibiotics. The purified plasmids pSecTagB-FL-SP-D and pSecTagB were transfected with Lipofectamine LTX® plus (Life technologies) according to the manufacturer's instructions respectively. 24 to 48 hrs after transfection, the supernantants were checked for the full length SP-D protein expression by dot blot (Figure 4.5) and western blot (Figure 4.6) using anti SP-D.

Further upscaling was done by adding Zeocin (Sigma) to the culture, being a selective antibiotic for pSecTagB, only clones in the HEK293 cells grow and survive. The cells were further grown and the supernatants collected, maltose agarose purified and the fractions collected to get an yield of 100ugs per 250mls of Full length SP-D (figure 4.7).

a 

b 

Figure 2.3 Dot Blot showing the positive results for the supernatants for the full length SP-D transfection (a.) Dot 1. FL SP-D Flow through, Dot 2. Wash, Dot 3 is Fraction 1 and Dot 4 is Fraction 2 after the maltose agarose purification. (b.) Dot 1 is Fraction 3, dot 3 fraction 4 and dot 5 is Fraction 5 and Dots 2, 4 and 6 are flow through, fraction1 and fraction 2 of pSecTagB after maltose agarose purification.

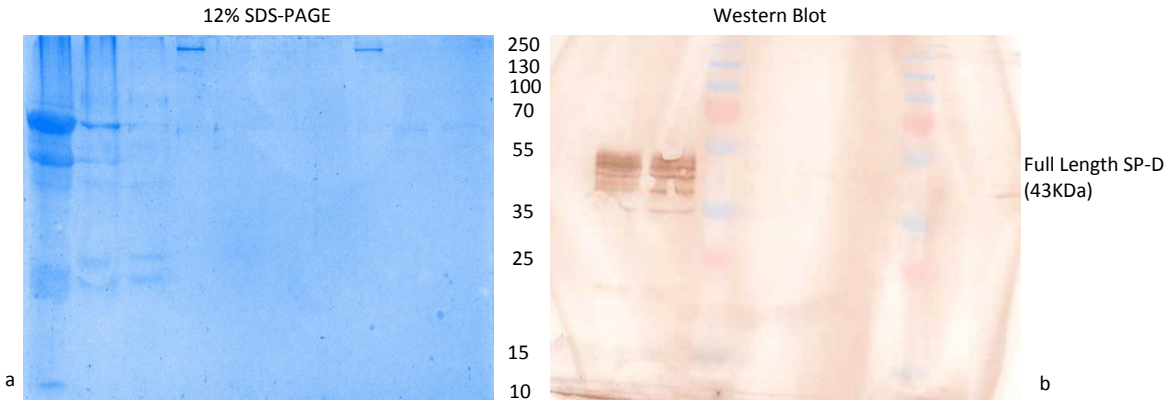


Figure 2.4 (a). 12% SDS-gel showing the corresponding wells after the transfer onto the nitrocellulose membrane. (b).Western Blot results confirming the small scale transfection results, wells 2 and 3 are the fractions 1 and 2 collected after the purification of the 25ml supernatants collected from the FL SP-D transfected HEK cells after passing through the agarose gel.

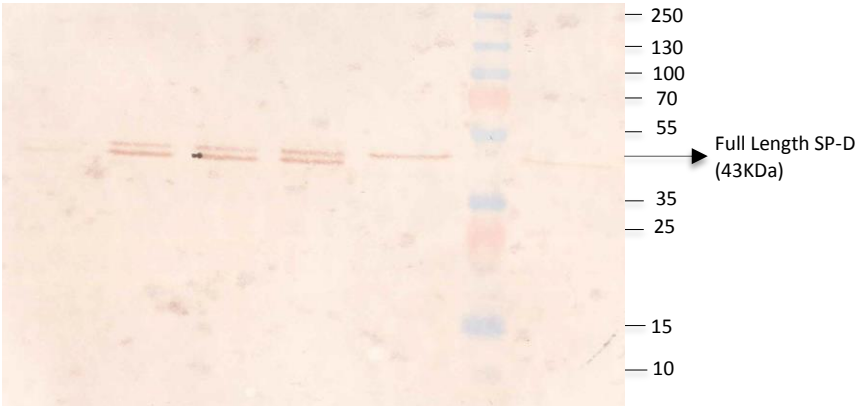
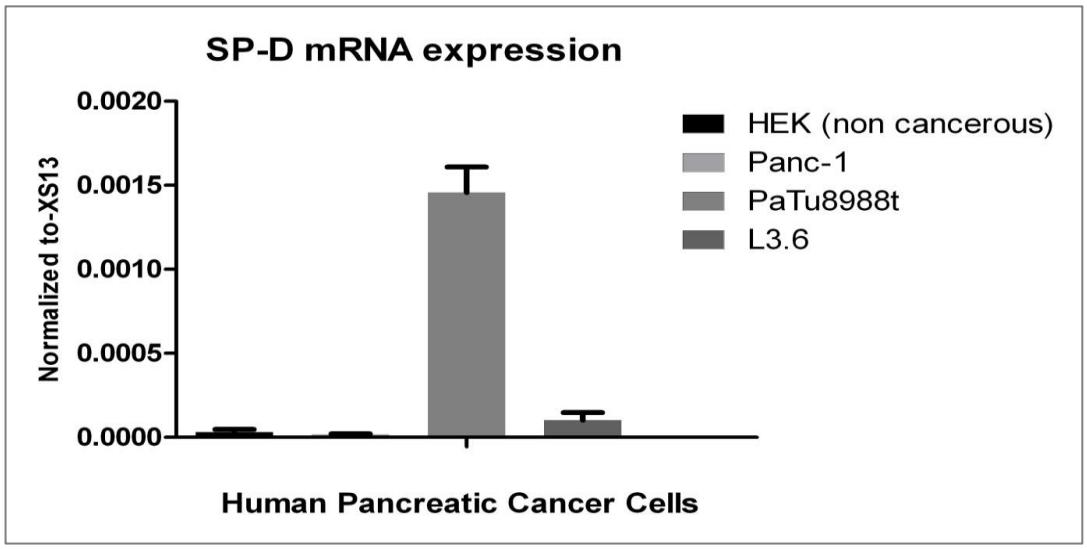


Figure 2.5 Western Blot confirming the expression of FL SP-D after transfection using Lipofectamine LTX reagent. Laness 10, 9 and 8 are the supernatant, flow through and wash respectively lane followed by rainbow marker (Lane 7) and fractions 1 to 6 in lanes 6 to 1.

4.2.3 SP-D IN PANCREATIC CANCER

SP-D mRNA was endogenously expressed in the pancreatic cell lines (Panc-1, PaTu8988t and L3.6), and the PaTu 8988t pancreatic cell line expressed the most (Figure 4.8). Genetically engineered mice models (p53 mutant and wild types) expressed very low when compared to the human cancer cells (Figure 4.8). Further, the expression was confirmed at the protein level by western blot for the cancer cell lines using the SP-D polyclonal antibodies and cyclin D(Figure 4.9).
Further, to the PaTu8988t SP-D overexpression, this cell lines were used for the would healing assay and have been seen to inhibit sna1, ZEB1 and Vementin which are the genes involved in the EMT pathways and help in metastasis (Figure 4.10 and Figure 4.11). This was also further confirmed with the western blot (Figure 4.12)



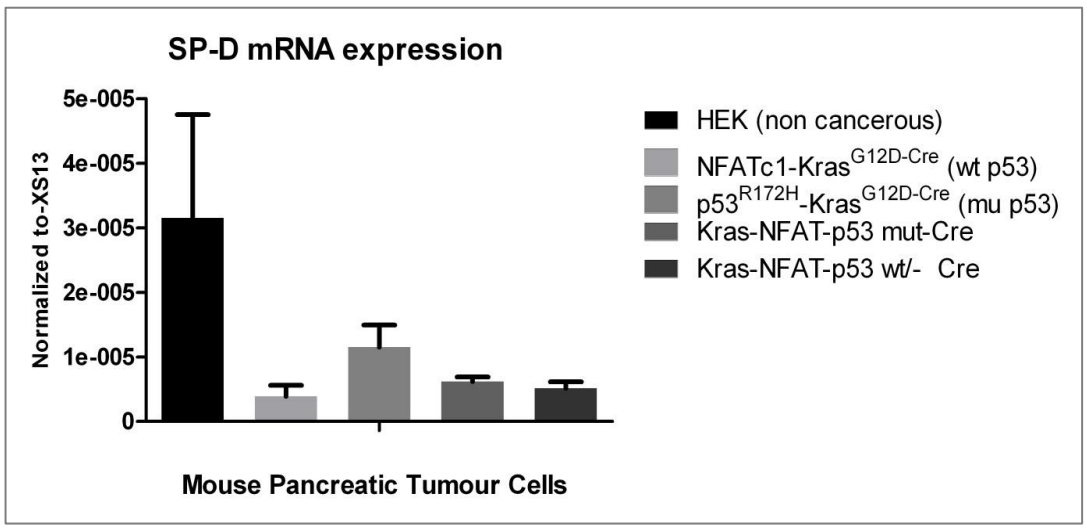


Figure 2.6 qRT-PCR analysis of SP-D expression levels in human pancreatic cancer cells, the mRNA expression levels were compared to normal human embryonic kidney cells (HEK). (PANC-1, Human pancreatic carcinoma, epithelial-like cell line cells non-endocrine pancreatic cancer for tumorigenicity, Human pancreas adenocarcinoma is PaTu8988t [from the liver metastasis of a primary pancreatic adenocarcinoma] and L3.6 cell line is metastatic cell line from pancreas to liver). Genetically engineered mouse models of metastatic cancer in which the resulting tumors recapitulate the genetic alterations and histological progression of the human disease. In these models, tumors develop within their appropriate microenvironment and undergo changes in their gene expression programs that endow them with the ability to invade blood and lymphatic vessels, survive in circulation, enter various distant organs, and ultimately grow into new tumor lesions.

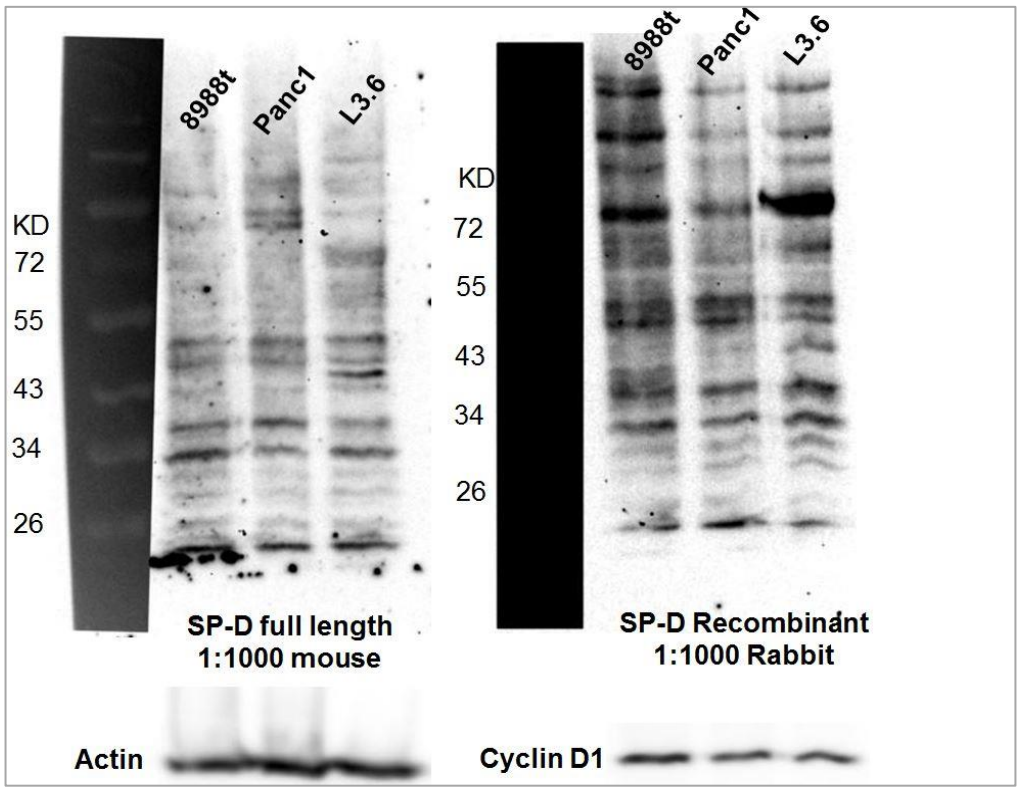


Figure 2.7 SP-D and Cyclin-D1 protein expression of pancreatic lysates from PaTu8988t, Panc1 and L3.6 cells, analyzed by western blot.

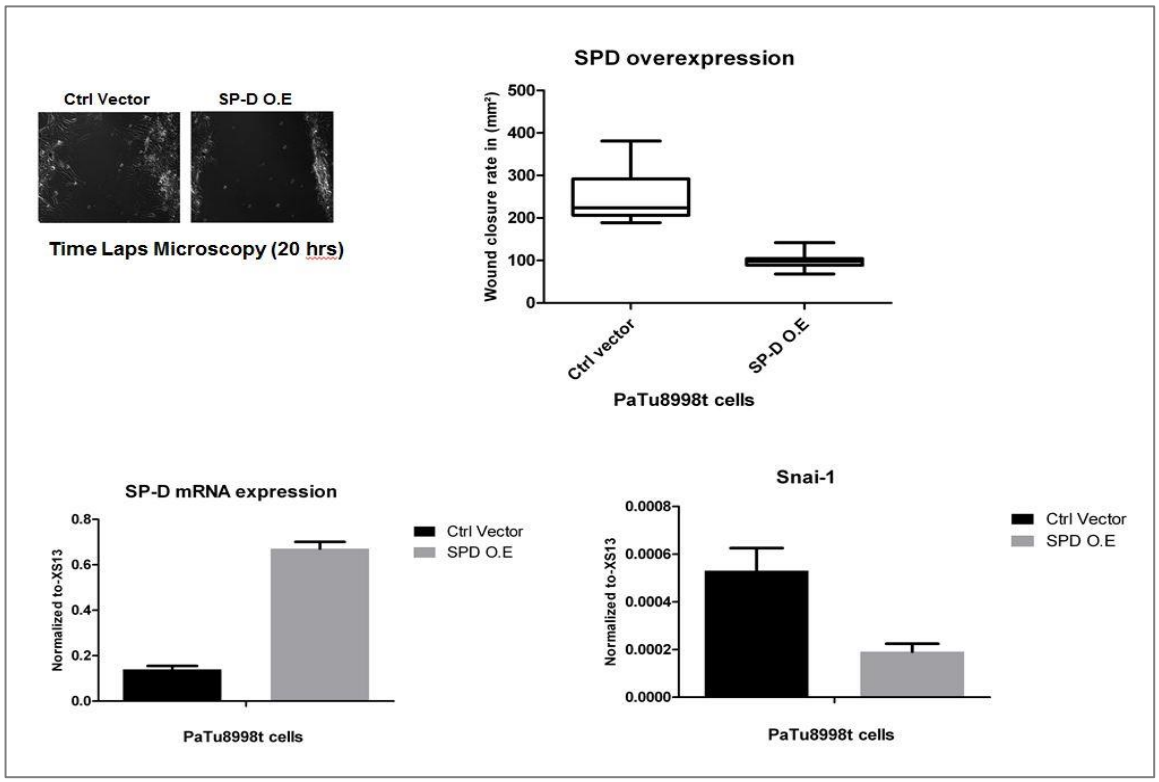


Figure 2.8 Wound healing assay after SP-D overexpression in PaTu8988t pancreatic cancer cells (left panel). Time laps analyzer software was used to quantify the wound closure rate.
SP-D and EMT-related gene expressions after SP-D overexpression in PaTu8988t pancreatic cancer cells, analyzed by real time PCR.

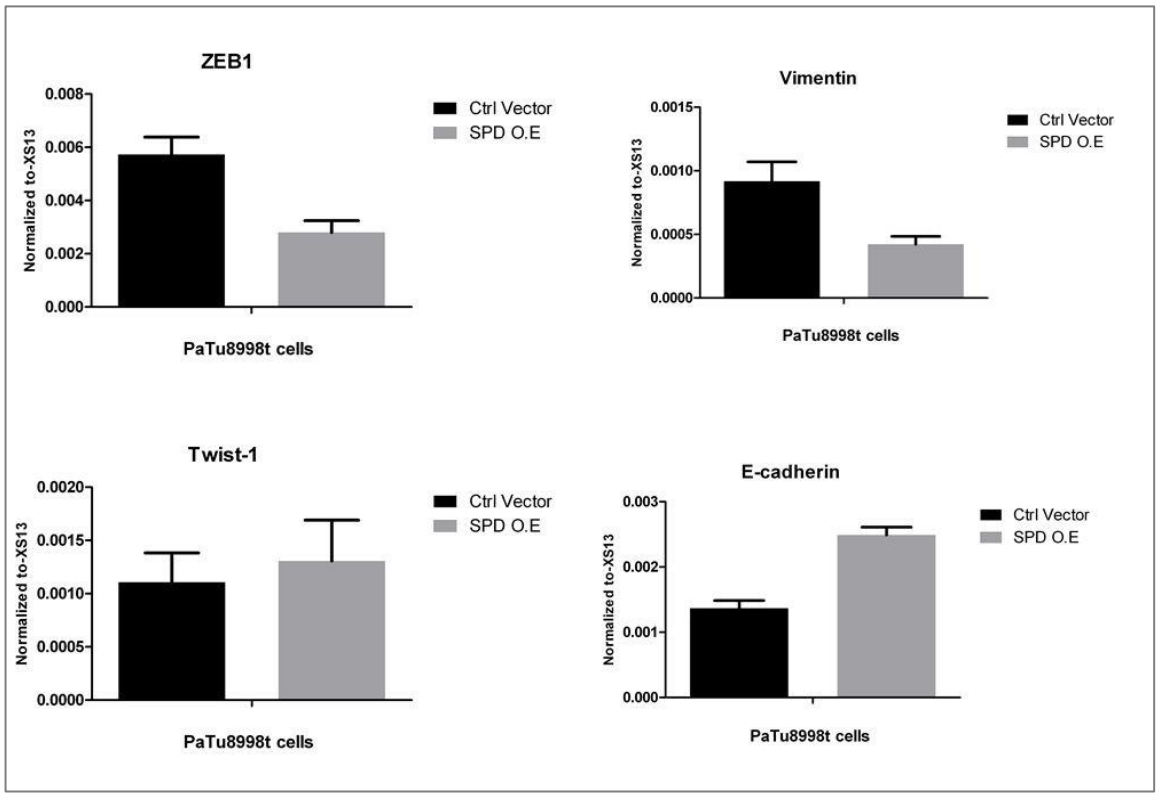


Figure 2.9 Wound healing assay after SP-D overexpression in PaTu8988t pancreatic cancer cells (left panel). Time laps analyzer software was used to quantify the wound closure rate.
SP-D and EMT-related gene expressions after SP-D overexpression in PaTu8988t pancreatic cancer cells, analyzed by real time PCR.

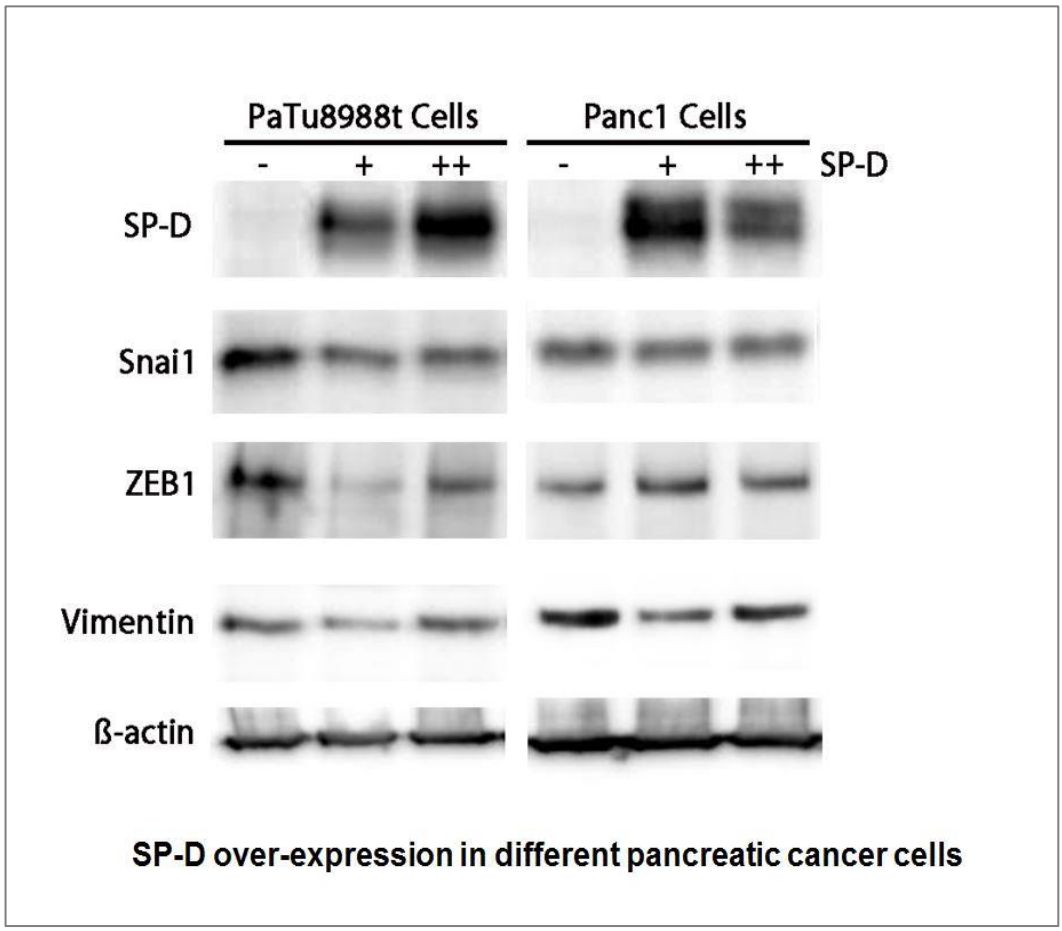


Figure 2.10 Representative protein expression levels upon SP-D overexpression in PaTu8988t and Panc1 pancreatic cancer cells

5.4 DISCUSSION

EMT is activated by signaling pathways like TGFb and FGF pathway helping in the stimulation of EMT activators, transcription factors repressing epithelial gene expression. Members of snail family, bHLH family and ZFH family (ZEB1 and ZEB2) are included (Thiery, 2009) . EMT activators activate cellular motility, maintain stem cell properties and also help in the cell survival (Mani *et al.*, 2008; Morel *et al.*, 2008) . miR-200 family of microRNAs has also been shown to play an important role (Shimono *et al.*, 2009; Wellner *et al.*, 2009) and that ZEB1 suppresses the expression of miR-200 family members (miR-141, -200a, b, c and -429), which in turn suppresses the expression of ZEB1 mRNA, leading to the double-negative ZEB/miR200 feedback loop (Brabletz and Brabletz, 2010) . Stem cell factor Bmi 1 being an additional factor of miR-200 family, overexpression of ZEB1 in tumours can indirectly, through the inhibition of miR-200 expression leads to stem cell properties as shown for breast and pancreatic cancer (Wellner *et al.*, 2009; Shimono *et al.*, 2009) ZEB1 can indirectly activate notch signaling by inhibiting miR-200 expression, stabilizing notch pathway, thus progressing tumour function showing that aberrant EMT activation can cause tumour metastasis (Brabletz and Brabletz, 2010) . Vimentin is ubiquitously expressed in normal mesenchymal cells and maintains cellular integrity and provides resistance against stress. It is recognized as a marker for epithelial-mesenchymal transition. It has been seen to overexpress in various epithelial cancers like prostrate, gastrointestinal, central nervous sytem tumours, breat cancer, malignant melanoma and lung cancer. Also seen to increase with the increase in the tumour or cancer and metastasis, and has been one of the target for the cure of cancer (Satelli and Li, 2011) Twist1 and Twist2 (Twist1–2), the two transcription factors belonging to the members of the basic helix-loop-helix family that are known as master transcriptional regulators of embryogenesis and developmental programs of mesenchymal cell lineages. Their role in oncogenesis in epithelium-derived cancer and in epithelial-to-mesenchymal transition has also been thoroughly characterized. Twist1–2 has been shown to play a crucial role of in the function and development of hematopoietic cells, as well as in survival and development of numerous hematological malignancies (Merindol *et al.*, 2014) . E-cadherin is a calcium-dependent cell-cell adhesion molecule, expressed in all mammalian epithelia with pivotal roles in epithelial cell behavior, tissue formation, and suppression of cancer.

It is a key molecule of the cadherin-catenin-cytoskeleton complex and establishes and maintains apiobasal polarity preserving epithelial cell survival and controlling proliferation. The role of E-cadherin (cadherin switching) in embryonic development and morphogenesis and has been shown that multiple mechanisms that disrupt E-cadherin function leads to cancer: inactivating somatic and germline mutations, epigenetic silencing by DNA methylation and epithelial to mesenchymal transition-inducing transcription factors, and dysregulated protein processing (van Roy and Berx, 2008) .

After full length SP-D was cloned and expressed, it was further analyzed in the pancreatic cancer cell lines and seen to be interfering with the metastasis genes. SP-D seems to be interacting with the EMT pathway genes like ZEB1, Vimentin and Sna1, making it a potent inhibitor in metastasis.

SP-D, which is expressed intracellularly, is likely bind to number RNA binding proteins and transcription factors. The likelihood of cancer becoming more metastatic and less metastatic is likely to be dictated by the levels of expression of endogenous expression of SP-D by cancer cells. Thus, the effect of SP-D on cancer cells can involve two routes. Exogenous SP-D is meant to induce apoptosis in cancer cells in a p53 depend manner while endogenous SP-D can negatively modulate metastasis, thus restricting the spread of tumour.

Chapter 5

SP-D and DC-SIGN interaction

Abstract

DC-SIGN, which is a receptor for a diverse range of pathogen target ligands, has recently been shown to bind to house dust mite (HDM) allergens leading to the promotion of Th2 response. Since SP-D is known to bind to allergens and modulate immune reactions to allergenic challenge, the over arching hypothesis here was to examine if SP-D by scavenging allergens can interfere with allergen-DC-SIGN interaction. Here we show that rhSP-D directly binds to tetrameric forms of DC-SIGN and DC-SIGNR. It was also found that both rhSP-D and recombinant DC-SIGN bind to timothy grass pollen (Phl p1) independently in a calcium and dose-dependent manner. The binding sites on Phlp1 appear to be overlapping since rhSP-D competed or inhibited binding of DC-SIGN to Phlp1. This raises the possibility that SP-D can modulate DC-SIGN driven Th2 response independent of its direct action on T-cells.

Introduction

DC-SIGN, a C-type lectin helps in binding to ICAM-3 on T-cell without the help of intergrin (in the presence of calcium), hence the name. Dendritic cells (DCs) play a pivotal role in the immune system in activating the primary immune response, also down regulating the immune response (Banchereau *et al.*, 2000) , in immune regulation, maintaining immune homeostasis and are known to be most powerful in presenting of antigens from pathogens, apoptotic and tumour cells. They play a key role in dendritic cell-T cell synapse and initial factor in auto-immune diseases, furthermore in immune escape of pathogens and tumours. Immunoregulatory factors of DCs are related to pattern recognition and immune regulation of the receptors on its surface. C-type lectin receptors (CLR) and Toll-like receptors (TLR) are a few examples of the many receptors on DCs which are studied (Zhou *et al.*, 2006) .

In the resting state, DC-SIGN tail (intracellular) is linked to a signalosome made up of Kinase suppressor of ras (KSR), a connector enhancer of kinase suppressor of Ras 1(KSR) and proto-oncogene serine/ threonine-protein kinase (Raf-1) by the adaptor protein lymphocyte-specific protein (LSP). DC-SIGN interaction with mannose containing ligands results in the recruitment of the upstream effectors Rho guanine nucleotide exchange factor 12 (LARG) and ras homolog family member A Rho A) to signalosome, and phosphorylating the Raf-1, which in turn the phosphorylation and acetylation of nuclear factor (NF)-κB subunit p65 and increasing the transcription factor of the IL12A, IL12B and IL6 genes. This whole reaction is reversed when fucose-bound DC-SIGN were added, as the signalosome might actively dissociate and the proinflammatory cytokines get suppressed. DC-SIGN though alone doesn't initiate changes in DC activation or cytokine secretion, modulates the signalling pathways with the help of other pattern-recognition receptors and acting like a signalling receptor (Gringhuis *et al.*, 2009) .

Evolutionarliy, high-mannose glycans are conserved and terminally modified glycans with fucosylation and sialylation are not, which is used by DC-SIGN to differentiate among its wide range of ligands to modulate TLRs into pro-(mannose) or anti-inflammatory (fucose) which is unique to C-type lectin family. High-mannose or mature glycoproteins can be detected by DC-SIGN in the higher mammals during cell damage

(otherwise stored Endoplasmic Reticulum during maturation of glycoproteins) or during pathogen invasion leading to trigger in pro-inflammation. DC-SIGN also triggers anti-inflammatory responses to Lewis-type glycan which are expressed in cells and developmentally regulated showing the dual functionality in immunological homeostasis which is fucose mediated and initiation of immune response against pathogens which is mannose-mediated.

In mammalian system, during the course of evolution the mechanism to recognize bacterial proteins, PAMPs were formed, which induce Th1 response. Complex carbohydrates drive the Th2 response to the parasites and allergens. Cell walls of fungi, helminths, pollens and bacteria contain polymers of glucose, which are absent in mammals and these carbohydrate moieties interact with the C-type lectin receptors (CLRs), which in turn promotes the Th2 biased immune responses, with the help of TLR4 in case of multicellular organisms. In case of allergens like Ara h 1, activates the human monocyte-derived dendritic cells and induces Th2-cytokine secreting cells through DC-SIGN. House dust mite (HDM) extract derive the epithelial chemokine production and DC leukotriene production, Der p 2 and Bermuda grass pollen (Cyn-dBG-60) bind to and signal through DC-SIGN and other CLRs. DC-SIGN binds to gp 120 and triggers CD4+ T cell Lung surfactant proteins SP-A and SP-D, secreted from the alveolar type II cells, which belong to collectin family play a major role in innate immunity during the respiratory pathogen infections and allergens. Lung being the most exposed organ in the body, these proteins play a crucial role, apart from surfactant homeostasis. These proteins have been shown to interact with varied number of pollen allergens, HDM extract, Der p 1, A. fumigatus, which mediate macrophages engulf them. The interaction of pathogens and allergens is through the recognition of carbohydrates and calcium dependent, inhibiting IgE specific binding to the carbohydrates of the allergens.

5.4 RESULTS

Expression and purification of recombinant DC-SIGN proteins

The constructs (pT5T) containing the DC-SIGN proteins were transformed into BL21 DE3 and checked for expression, following induction with IPTG, DC-SIGN tetramer (Figure 5.1), DC-SIGN R Tetramer (Figure 5.2), DC-SIGN monomer (Figure 5.3) and

DC-SIGN R monomer (Figure 5.4) were expressed. DC-SIGN protein accumulated intracellularly as inclusion bodies which were recovered in soluble fractions after denaturation and renaturation cycle using 1M NaCl salt buffer. Finally, refolded protein bound to mannon-agarose column was eluted using EDTA.

Binding of DC-SIGN proteins to rhSP-D and vice versa shown through western blot

Purified DC-SIGN proteins bound to rhSP-D, the binding was seen to more prominent for the tetramers (Figure 5.5), even though there is binding to monomers (Figure 5.7). rh SP-D was also shown to bind to all the four four DC-SIGN proteins (Figure 5.6).

Interaction of DC-SIGN proteins and rh SP-D (ELISA results)

DC-SIGN tetramer, DC-SIGN monomer, DC-SIGN R tetramer and DC-SIGN R monomer bind to SP-D in a dose dependent manner in the presence of Calcium (Figure 5.8), DC-SIGN tetramer, DC-SIGN monomer, DC-SIGN R tetramer and DC-SIGN R monomer bind to SP-D in a lesser dose dependent manner in the presence of EDTA (Figure 5.9).

Interaction of DC-SIGN proteins and rhSP-D with Phl p allergen

DC-SIGN tetramer, DC-SIGN R tetramer and SP-D GXY bind to Phl P allergen in a dose dependent manner in the presence of calcium, out of which SP-D binds the maximum. DC-SIGN monomer and DC-SIGN R monomer show very less binding (Figure 5.10). DC-SIGN tetramer, DC-SIGN R tetramer and SP-D GXY bind to Phl P allergen in a dose dependent manner in the presence of calcium, out of which DC-SIGN Tetramer binds the maximum. DC-SIGN monomer and DC-SIGN R monomer show very less binding (Figure 5.11). DC-SIGN tetramer, DC-SIGN R tetramer and DC-SIGN R monomer do not bind to Phl P allergen in the presence of EDTA, whereas, DC-SIGN monomer and SP-D GXY are showing no-specific binding (Figure 5.12).

Competitive ELISA

DC-SIGN tetramer/ rhSP-D and DC-SIGN R tetramer / rhSP-D bind Phl P allergen in a dose dependent manner in the presence of calcium against anti-DC-SIGN and not anti-SP-D. Constant concentration of DC-SIGN tetramer and DC-SIGN R Tetramer were added to the (Figure 5.13) DC-SIGN monomer/ rhSP-D and DC-SIGN R monomer / rhSP-D doesn't bind as well to Phl P allergen in the presence of calcium, when compared to tetramers (Figure 5.14) DC-SIGN tetramer and DC-SIGN R tetramer bind to Phlp allergen and SP-D in a dose dependent manner more when compared to DC-SIGN monomer and DC-SIGN R monomer binding to Phl P allergen and SP-D in the presence of Calcium (Figure 5.15). DC-SIGN tetramer/ rhSP-D and DC-SIGN R tetramer / rhSP-D binds to Phl P allergen in the presence of calcium, against anti DC-SIGN and not to anti-SP-D (Figure 5.16)

5.3.1 **DC-SIGN PROTEINS**

DC-SIGN proteins were purified after Expression and Purification of DC-SIGN Tetramer

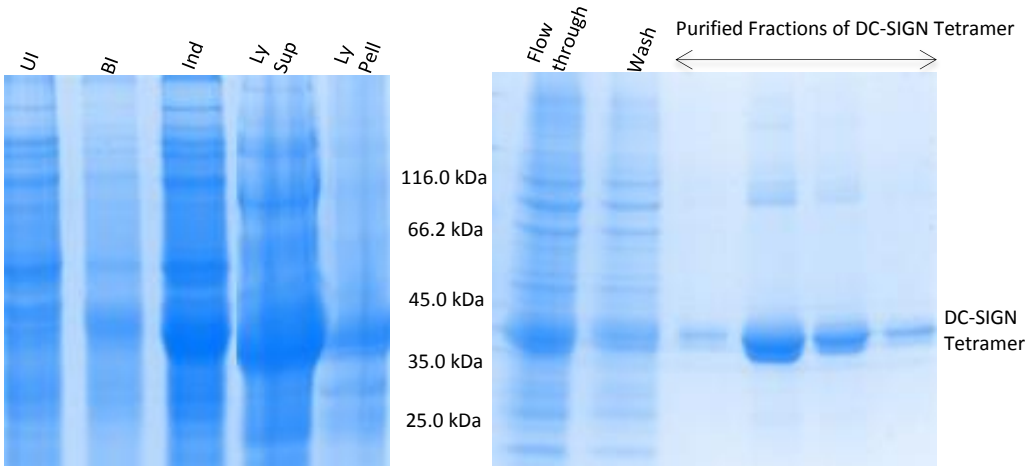


Figure 5.1 A recombinant **DC-SIGN tetramer** using Escherichia coli BL21 (λDE3) as hosts under T7 promoter. Three hours after induction with 0.4 mM IPTG, the accumulated as an over-expressed protein of 37 kDa (lane 3) compared with uninduced cells (lane 1). Bacterial cells were sonicated and the inclusion bodies (lane 4) containing insoluble DC-SIGN tetramer. Insoluble DC-SIGN tetramer was refolded via a denaturation and renaturation procedure and the soluble fraction was further purified using Q-Sepharose ion-exchange chromatography. The peak fractions showing were subsequently affinity purified on a mannan–agarose column (lane 9 to 12).

5.3.1.a Expression and Purification of DC-SIGN R Tetramer

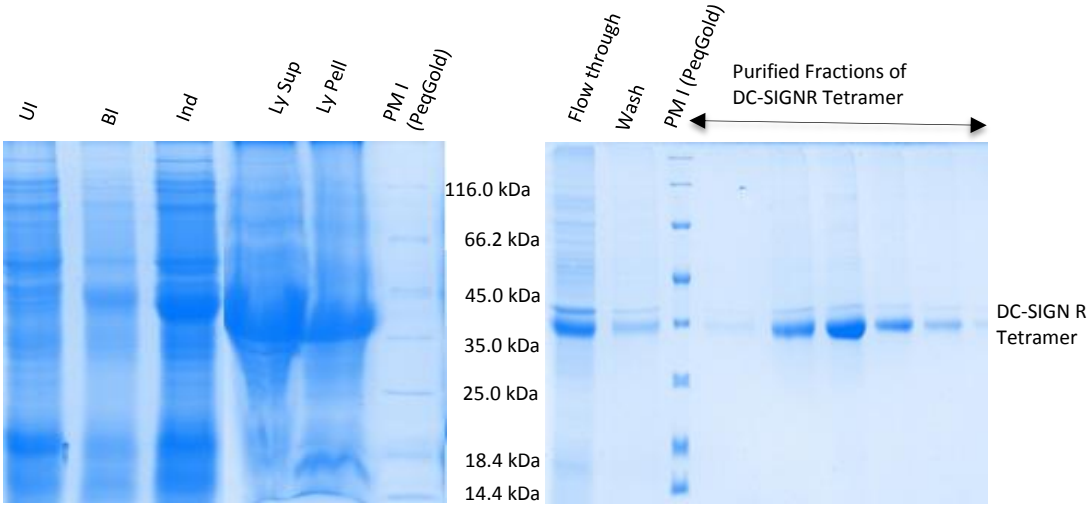


Figure 5.2 A recombinant **DC-SIGN R tetramer** using Escherichia coli BL21 (λDE3) as hosts under T7 promoter. Three hours after induction with 0.4 mM IPTG, the accumulated as an over-expressed protein of 37 kDa (lane 3) compared with uninduced cells (lane 1). Bacterial cells were sonicated and the inclusion bodies (lane 4) containing insoluble DC-SIGN R tetramer and the soluble fraction was further affinity purified on a mannan–agarose column (lane 11 to 14).

5.3.1.b Expression and Purification of DC-SIGN monomer

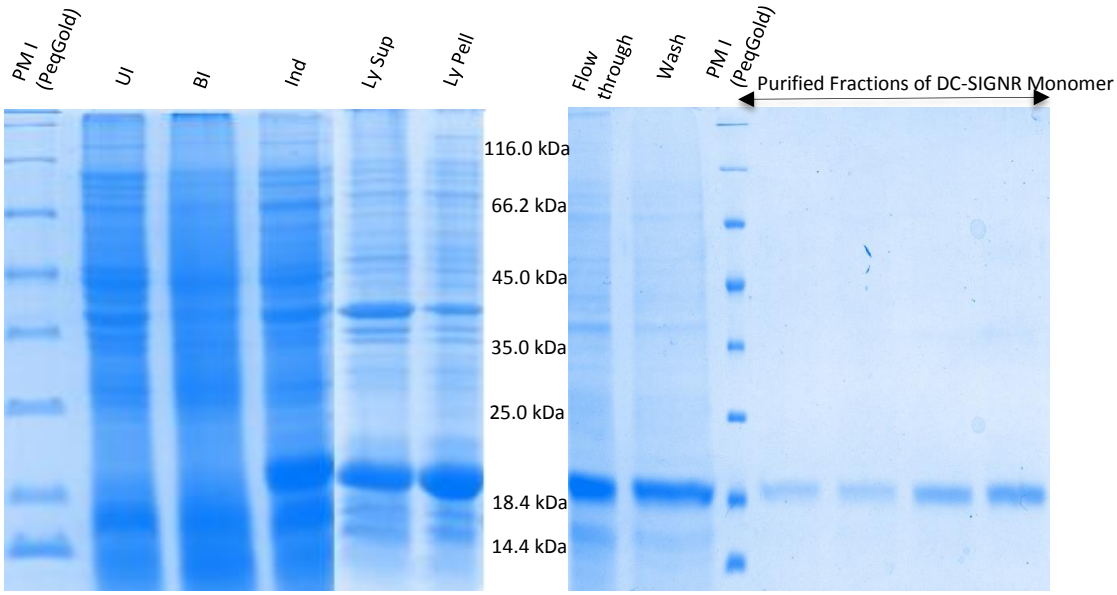


Figure 5.3 A recombinant **DC-SIGN monomer** using Escherichia coli BL21 (λDE3) as hosts under T7 promoter. Three hours after induction with 0.4 mM IPTG, the accumulated as an over-expressed protein of 18 kDa (lane 3) compared with uninduced cells (lane 2). Bacterial cells were sonicated and the inclusion bodies (lane 5) containing insoluble DC-SIGN monomer. Insoluble DC-SIGN monomer was refolded via a denaturation and renaturation procedure and the soluble fraction was affinity purified on a mannan–agarose column (lane 4 to 8).

5.3.1.c Expression and Purification of DC-SIGN R monomer

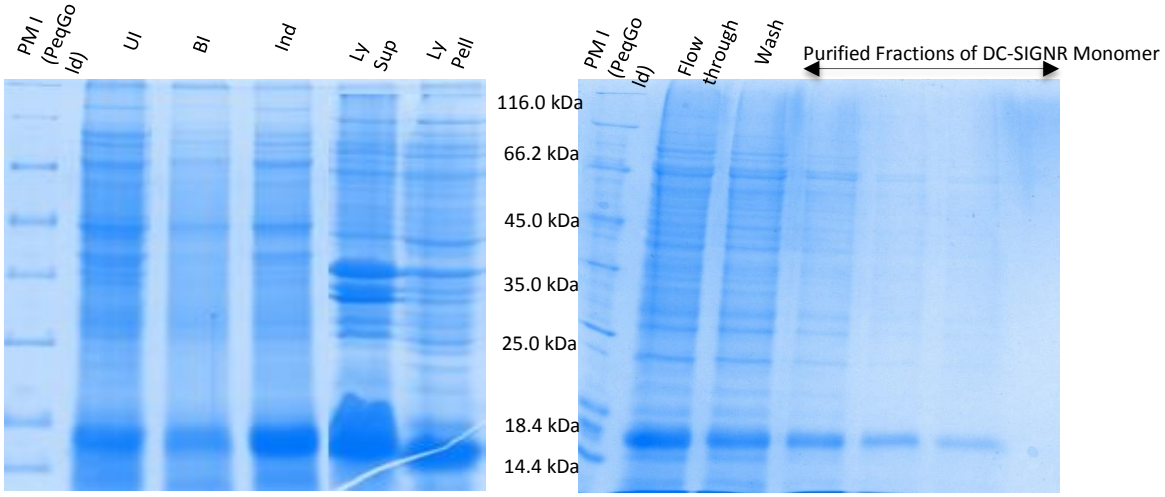
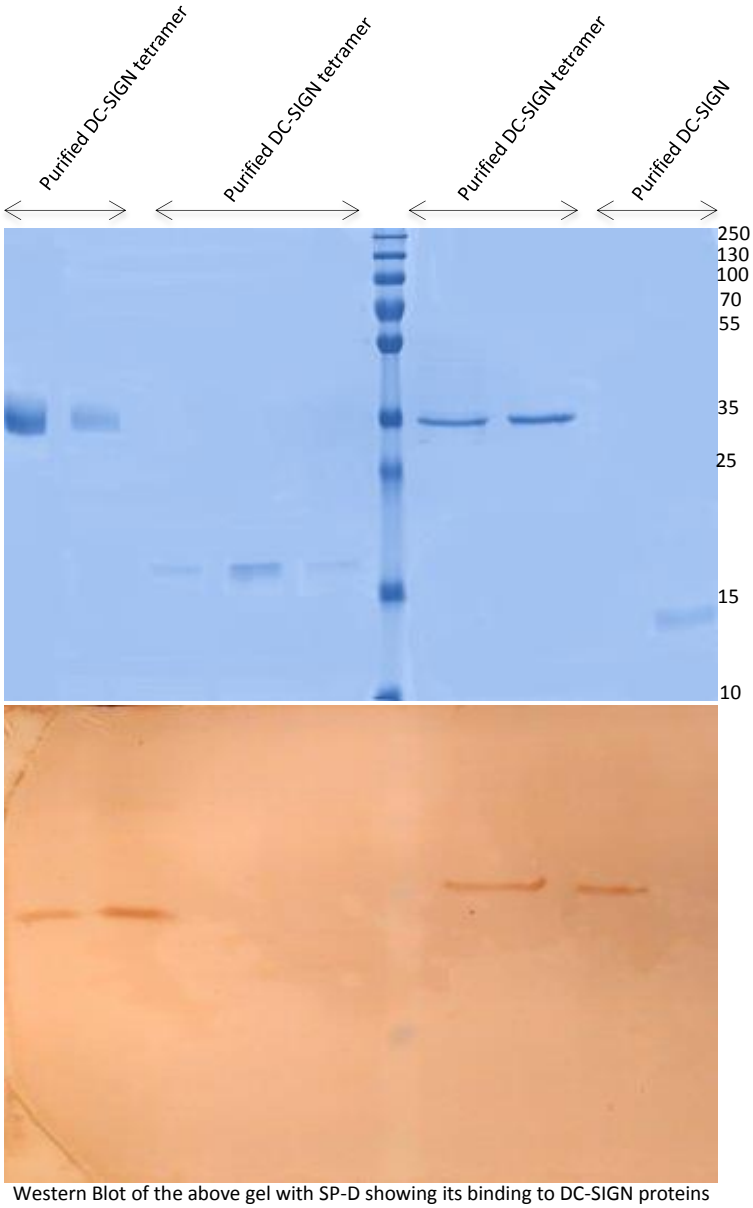


Figure 5.4 A recombinant **DC-SIGN R monomer** using Escherichia coli BL21 (λDE3) as hosts under T7 promoter. Three hours after induction with 0.4 mM IPTG, the accumulated as an over-expressed protein of 18 kDa (lane 4) compared with uninduced cells (lane 2). Bacterial cells were sonicated and the inclusion bodies (lane 5) containing insoluble DC-SIGN monomer. Insoluble DC-SIGN monomer was refolded via a denaturation and renaturation procedure and the soluble fraction was further purified using mannan–agarose column (lane 10 to 12).

5.3.1 Western Blot Results showing the binding between SP-D and DC-SIGN proteins

5.3.1 Purified DC-SIGN Proteins binding to rh SP-D



Western Blot of the above gel with SP-D showing its binding to DC-SIGN proteins

Figure 5.5 Binding of biotinylated rhSP-D to: DC-SIGN tetramer (lanes 1&2), DC-SIGN monomer (3 & 4), DC-SIGN R tetramer (6 & 7) and DC-SIGN R monomer (8 & 9)

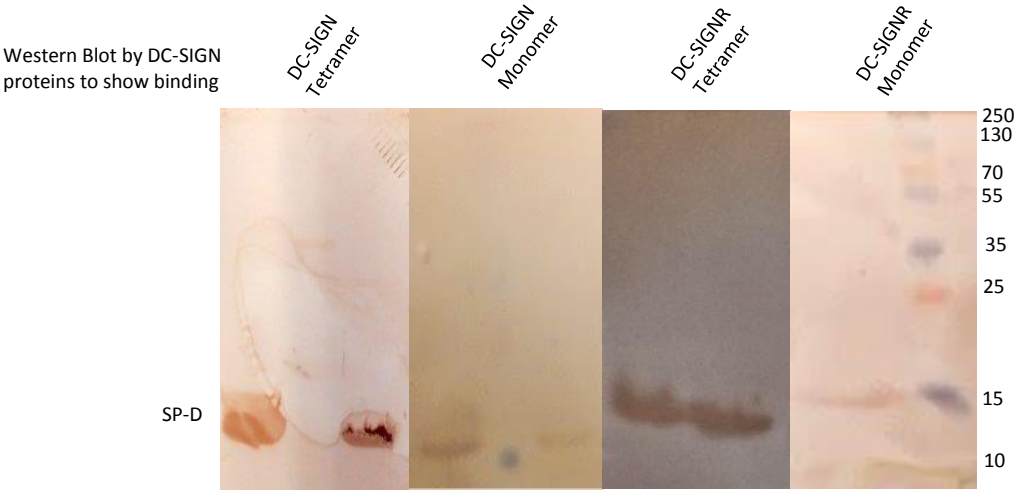


Figure 5.6 Binding of biotinylated DC-SIGN tetramer (lanes 1&3), DC-SIGN monomer (4 & 6), DC-SIGN R tetramer (7 & 8) and DC-SIGN R monomer (10) to rhSP-D.

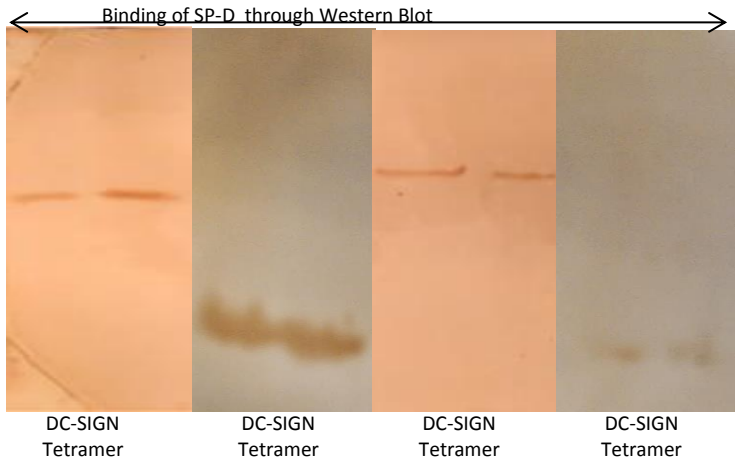


Figure 5.7 Western Blots of the DC-SIGN proteins (DC-SIGN tetramer/SP-D, DC-SIGN monomer/SP-D, DC-SIGN R Tetramer/SP-D and DC-SIGN R monomer/SP-D).

5.3.2 ELISA RESULTS:

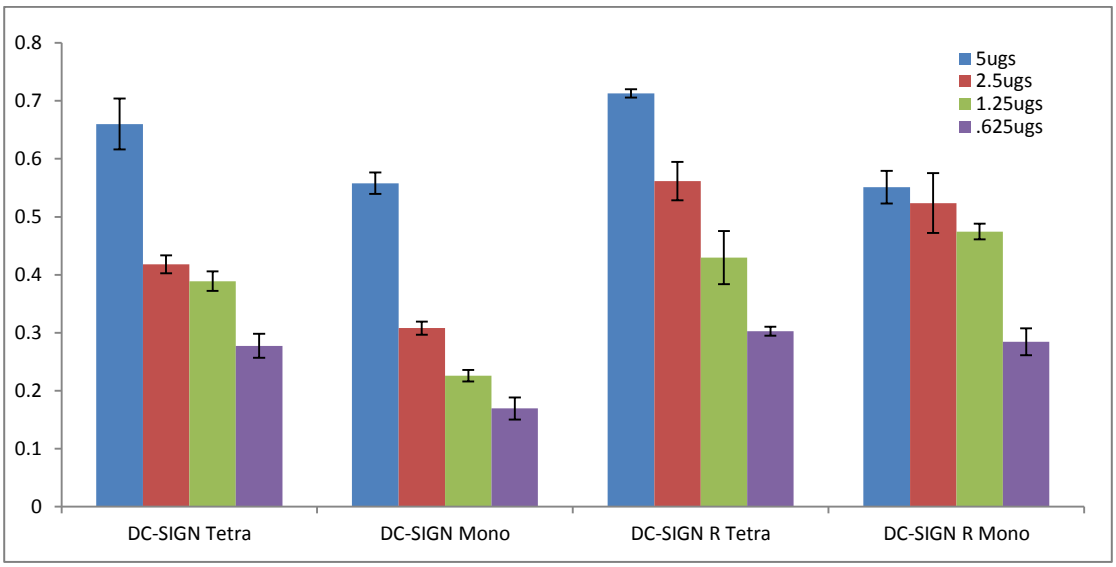


Figure 5.8 DC-SIGN tetramer, DC-SIGN monomer, DC-SIGN R tetramer and DC-SIGN-R monomer were coated at decreasing double dilutions from 5µg/well to 0.625µg/well in duplicates and left overnight at +4. Following Morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. The wells were then washed 3 times With PBS + 0.05% Tween and came in with **2.5ugs** of SP-D in **Calcium buffer**, left at 37 degrees for 1 and ½ hours and at 4 degrees for 1 and ½ hours. Following washes, bound SP-D was detected using anti-SP-D (1:1000) and Protein HRP conjugate (1:1000). Colour was developed using OPD.

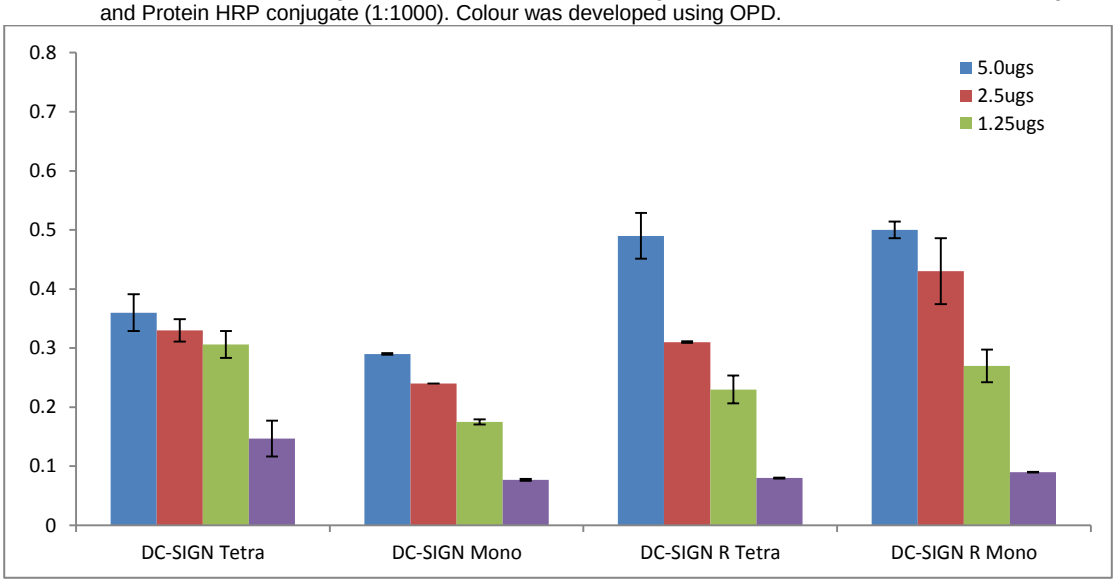


Figure 5.9 DC-SIGN tetramer, DC-SIGN monomer, DC-SIGN R tetramer and DC-SIGN-R monomer were coated at decreasing double dilutions from 5µg/well to 0.625µg/well in duplicates and left overnight at +4. Following Morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. The wells were then washed 3 times With PBS + 0.05% Tween and came in with **2.5ugs of SP-D** in **EDTA buffer**, left at 37 degrees for 1 and ½ hours and at 4 degrees for 1 and ½ hours. Following washes, bound SP-D was detected using anti-SP-D (1:1000) and Protein HRP conjugate (1:1000). Colour was developed using OPD.

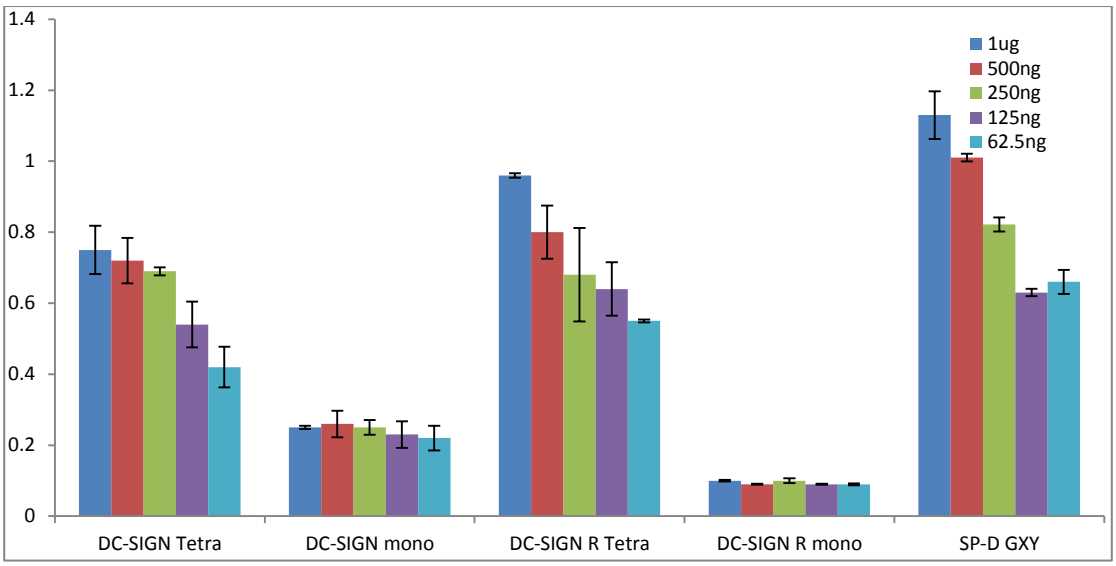


Figure 5.10 Phl p was coated at different concentrations (1, 0.5, 0.25, 0.125, and 0.0625 µgs/well) and left overnight at +4. Following Morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. The wells were then washed 3 times With PBS + 0.05% Tween and 5µg/well of Biotinylated DC-SIGN tetramer, DC-SIGN monomer, DC-SIGN R tetramer and DC-SIGN R monomer of **5µgs each** were added in **Calcium buffer** (**5mM in $CaCl_2$**) and incubated at 1.5 hours at 37 degrees and 1.5 hours At +4. Bound proteins were probed with strep HRP (1:1000) and detected using OPD.

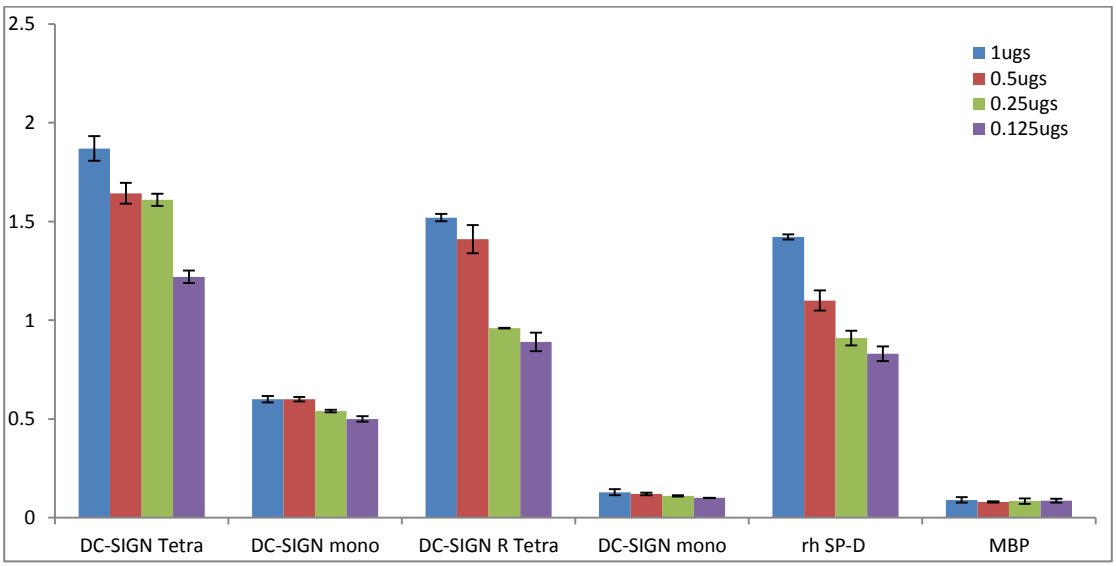


Figure 5.11 Phl p was coated at different concentrations (1, 0.5, 0.25, 0.125, and 0.0625 µgs/well) and left overnight at +4. Following Morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. The wells were then washed 3 times With PBS + 0.05% Tween and 5µg/well of Biotinylated DC-SIGN tetramer, DC-SIGN monomer, DC-SIGN R tetramer and DC-SIGN R monomer of **2.5µgs each** were added in **Calcium buffer** (**5mM $CaCl_2$**) and incubated at 1.5 hours at 37 degrees and 1.5 hours At +4. Bound proteins were probed with strep HRP (1:1000) and detected using OPD.

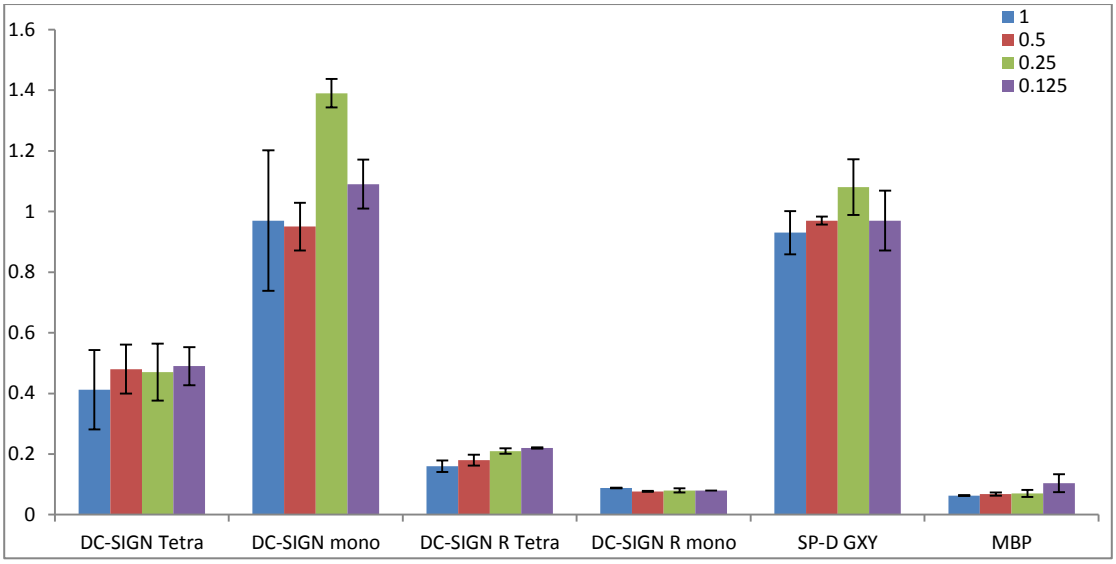


Figure 5.12 Phl p was coated at different concentrations (1, 0.5, 0.25, 0.125, and 0.0625 µgs/well) and left overnight at +4. Following Morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. The wells were then washed 3 times With PBS + 0.05% Tween and 5µg/well of Biotinylated DC-SIGN tetramer, DC-SIGN monomer, DC-SIGN R tetramer and DC-SIGN R monomer of **2.5µgs each** were added in **EDTA buffer** (**5mM EDTA**) and incubated at 1.5 hours at 37 degrees and 1.5 hours At +4. Bound proteins were probed with strep HRP (1:1000) and detected using OPD.

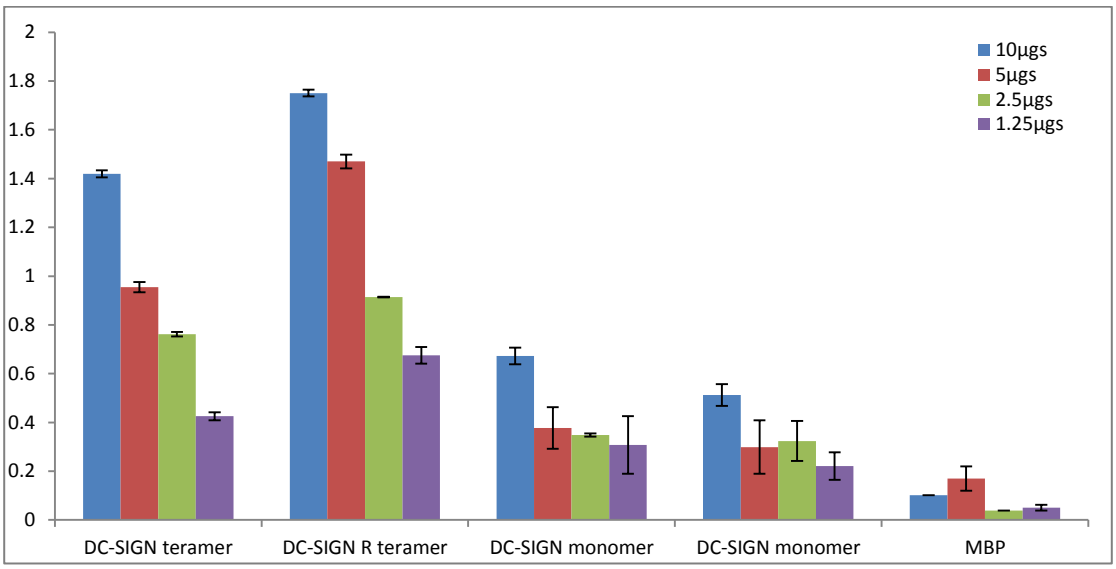


Figure 5.13 Phl p was coated (250ngs/well) and left overnight at +4. Following Morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. The wells were then washed 3 times With PBS + 0.05% Tween. DC-SIGN tetramer, DC-SIGN monomer, DC-SIGN R tetramer and DC-SIGN R monomer of different concentrations **(10 µgs, 5 µgs, 2.5µgs and 1.25 µgs)** were added in **Calcium buffer (5mM $CaCl_2$)** and incubated at 1.5 hours at 37 degrees and 1.5 hours At +4. Bound proteins were allowed to interact with constant concentrations of Biotinylated SP-D of **2.5µgs** which was then probed with strep HRP (1:1000) after the PBS-tween washes and detected using OPD.

5.3.3 COMPETITIVE ELISA RESULTS

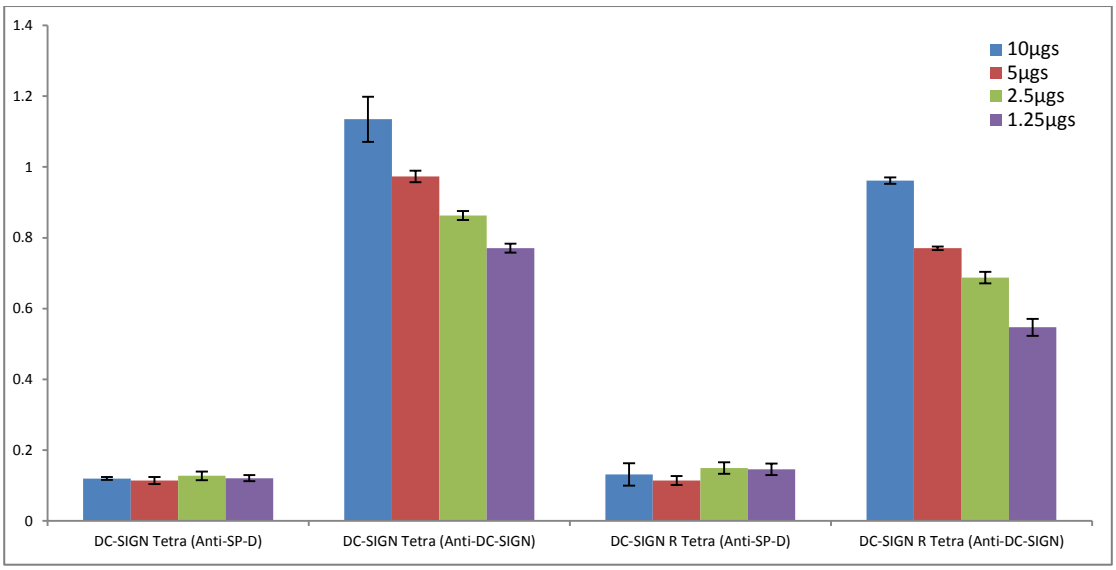


Figure 5.14 Phl p was coated (250ngs/well) and left overnight at +4. Following Morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. The wells were then washed 3 times With PBS + 0.05% Tween DC-SIGN tetramer, DC-SIGN R tetramer and SP-D of constant concentrations **(2.5µgs)** were added in **Calcium buffer (5mM $CaCl_2$)** and incubated at 1.5 hours at 37 degrees and 1.5 hours At +4. And Anti SP-D and Anti-DC-SIGN was then probed with Protein A HRP (1:1000) after the PBS-tween washes and detected using OPD .

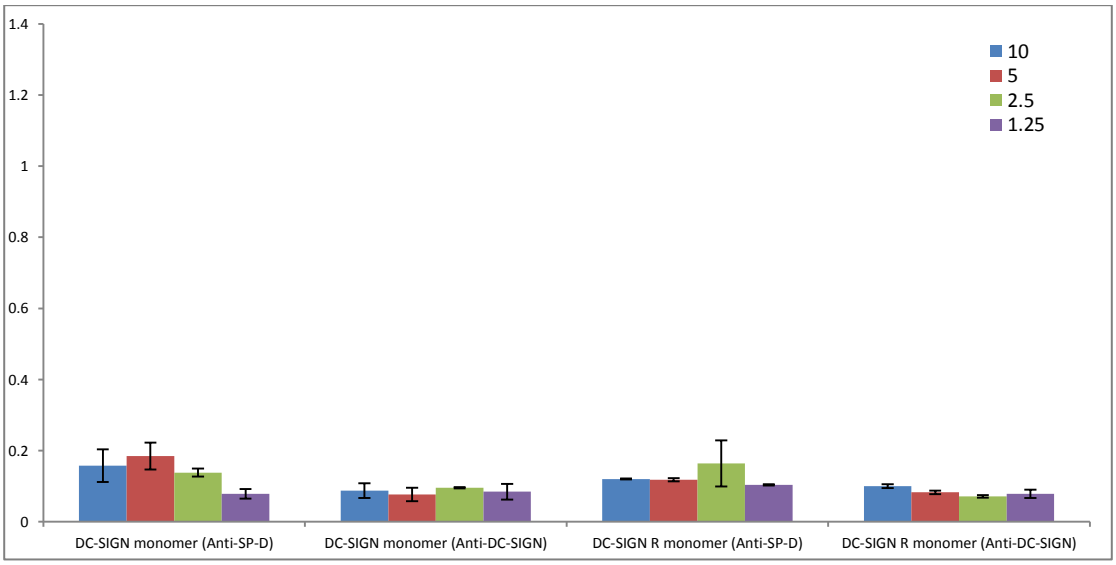


Figure 5.15 Phl p was coated (250ngs/well) and left overnight at +4. Following Morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. The wells were then washed 3 times With PBS + 0.05% Tween and DC-SIGN R tetramer and DC-SIGN R monomer **(2.5µgs)** were added in **Calcium buffer (5mM $CaCl_2$)** and incubated at 1.5 hours at 37 degrees and 1.5 hours At +4. Bound proteins were allowed to interact with different concentrations of SP-D **(10 µgs, 5 µgs, 2.5µgs and 1.25 µgs)** which and Anti SP-D and Anti-DC-SIGN was then probed with Protein A HRP (1:1000) after the PBS-tween washes and detected using OPD.

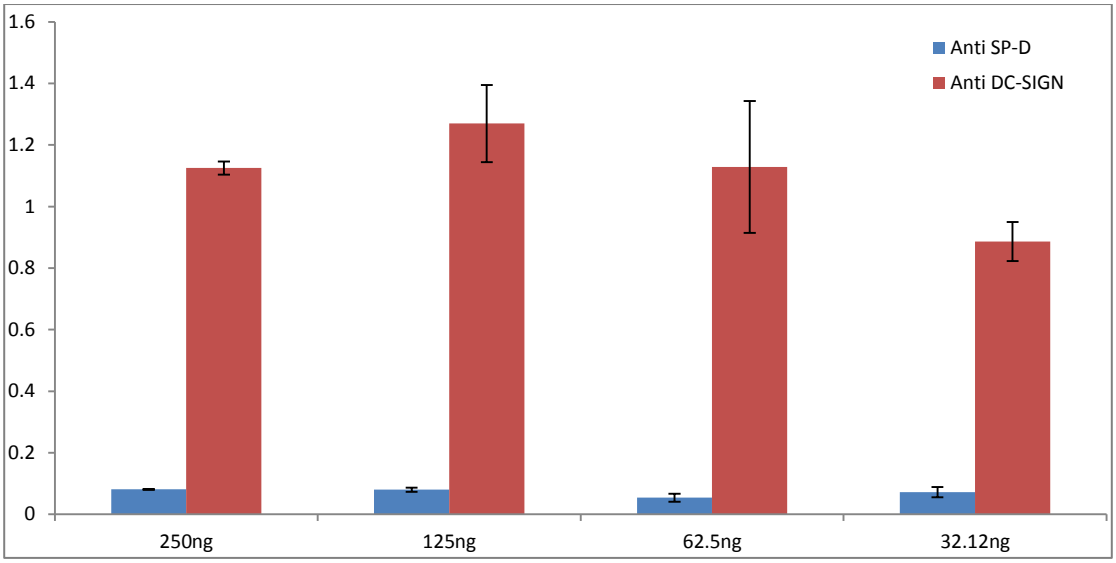


Figure 5.16 Phl p was coated (250ngs/well, 125ng/well, 62.5ng/well and 32.12ng/well) and left overnight at +4. Following Morning contents were discarded and wells were blocked with 2% BSA in PBS for 2 hours at 37 degrees. The wells were then washed 3 times With PBS + 0.05% Tween DC-SIGN tetramer, DC-SIGN monomer of constant concentrations **(5µgs)** were added in **Calcium buffer (5mM $CaCl_2$)** and incubated at 1.5 hours at 37 degrees and 1.5 hours At +4. Bound proteins were allowed to interact with different concentrations of SP-D **(10 µgs, 5 µgs, 2.5µgs and 1.25 µgs)** and after washing Anti SP-D and Anti-DC-SIGN was then probed with Protein A HRP (1:1000) after the PBS-tween washes and detected using OPD.

5.4 DISCUSSION

Dendritic cells are antigen-presenting cells that regulate innate and adaptive immune responses playing an important role in pathogenesis of asthma and allergy. (Banchereau *et al.*, 2000) . It is known that allergens activate DCs and induce pathogenesis of T helper cell type 2 (Th2) mediated allergy diseases, but the exact mechanisms are not clear yet. Molecules derived from pollen grains like phytoprostanes trigger DCs into Th2 antigen-presenting cells (Allakhverdi *et al.*, 2005; Traidl-Hoffmann *et al.*, 2005) . Dermatophagoides pteronyssinus (House dust mite) allergens (Der p) induced differential changes in DCs from the HDM allergy patients (Hammad *et al.*, 2003; Hammad *et al.*, 2001) . Dermatophagoides farinae allergens (extracts) have shown to induce Th2 cytokine response irrespective of previous in vitro or in vivo sensitization and can directly activate alveolar macrophages (innate immune cells) (Huang *et al.*, 2011; Chen *et al.*, 2003; Yu and Chen, 2003) . HDMs activated TLR4 on the pulmonary epithelium, eliciting allergen induced Th2 response releasing IL-25, IL-33 and thymic stromal lymphopoietin (Hammad *et al.*, 2009) . HDM-induced nitric oxide and TNF-α production of activated alveolar macrophages occurred through the stimulation of CD14/TLR4 surface receptors (Liu *et al.*, 2005) . Mite allergens bind to the CLR Dectin-2 on DCs, activating arachidonic acid metabolism, which triggers the innate immune cells eliciting the allergic inflammation (Barrett *et al.*, 2009) . The research done till date indicate that allergen-induced DC activation and inflammation can be mediated by the TLRs or CLRs on the Antigen presenting cells (Hammad *et al.*, 2009; Barrett *et al.*, 2009; Furmonaviciene *et al.*, 2007; Liu *et al.*, 2005) .

Binding of endogenous ligands containing oligosaccharides activates the host immunity at various levels like DC maturation and migration, T cell priming and immunomodulation (Th1 to Th2). DC-SIGN and L-SIGN have been shown to behave like PRRs to detect and bind to exogenous ligands of various invading pathogens, part from immune activation, pathogen uptake and dissemination, modulation of host immunity (Svajger *et al.*, 2010; Geijtenbeek, den Dunnen and Gringhuis, 2009; Geijtenbeek and Gringhuis, 2009) Respiratory syncytial virus glycoprotein G was shown to interact with DC-SIGN and L-SIGN and stimulate ERK1 and ERK2 phosphorylation and when neutralized, it reduced ERK1/2 phosphorylation projecting immunomodulation (Johnson, McLellan and Graham, 2012)

Cultured monocyte-derived dendritic cells (MDDCs) from peripheral blood mononuclear cells of Der p sensitive allergic asthmatics and nonallergic humans, immature MDDCs internalized Der p allergen through DC-SIGN and upon maturation promoted Th2 polarization on naïve CD4+T cells, indicating an important role of DC-SIGN in innate immune response of DCs. Allergens trigger the DC cytokine release, polarize immune response of naïve CD4+T cells indicating the allergen-induced inflammatory response in allergic diseases. With the addition of anti-DC-SIGN, the MDDCs did not release the IL-6 and IL-12 cytokines, showing that DC-SIGNs role in the allergen-induced Th2 response, which was absent in LPS induced response. Ara h 1, a major allergen from Arachis hypogaea (Peanut), soluble egg antigen of Schistosoma mansoni were able to polarize Th2 response by their interactions with DC-SIGNs on MDDCs (van Liempt *et al.*, 2007; Shreffler *et al.*, 2006) .

When SP-D binds to pathogens or allergen, AMs get activated releasing NO and TNF-α production and when the MH-S cells were treated SP-D and then Der p was added it showed no NO and TNF- α production concluding the role of SP-D in allergy and immune tolerance. SP-D was shown to bind to CD14 molecule inhibiting its binding to Der p and thus stopping the Der p-induced NF-κB activation via the CD14/TLR4 receptors. Allergen-induced AMs and DCs activation

SP-D modulates DC-SIGN expression, pretreatment of both allergic patients with SP-D pretreatment of allergic and control MDDCs prevented the Der p-induced reduction of DC-SIGN (Liu *et al.*, 2005) .

Phlp allergen from Timothy grass (Phleum pretense) pollen extract is binding to SP-D and DC-SIGN proteins, showing the allergen interactions with SP-D and its role in immunomodulation. DC-SIGN binding to Phlp allergen can trigger the Th2 response as seen earlier. It has been shown for the first time that DC-SIGN and its related interact with SP-D, which leads to hypothesize that SP-D plays a major in the Allergen-mediated immune response. Interaction of Phlp allergen and DC-SIGN triggers the Th2 response, but in the response SP-D this could be avoided. In the presence of SP-D, Phlp allergen could bind to SP-D and the allergic responses could be decreased.

Currently, co-culture experiments are being planned in which HEK cells transfected with DC-SIGN are being mixed with PBMCs derived from Phlp1 sensitized patients to examine proliferative and cytokine response in the presence of SP-D with and without

Phlp1 allergenic challenge. Similar experiments will be performed using macrophage cell lines overexpressing DC-SIGN on the cell surface. In order to confirm the modulation of Th2 response by SP-D, DC-SIGN over expressing cells will be subjected to interference RNA and co-culture experiments as explained above.

Perspectives

This thesis has examined an ever evolving idea that SP-D is a general immunosurveillance molecule of innate immunity at the outset full length human SP-D was produced in HEK cells while a recombinant fragment of human SP-D (rh SP-D) was expressed in *E.coli* consistent with recently reported work by Mahajan et al, 2008, rh SP-D was able to induce apoptosis in three leukemic cell lines. Proteomic analysis of rhSP-D treated AML14.3D10 cell line yielded a number of signature changes, which were validated by quantitative transcriptional analysis. Although, induction of apoptosis by rhSP-D was a consistent phenomenon in eosinophillic, Jurkat and Raji cell lines, the role of p53 and HMGA1 do not appear to be overlapping, it is likely that there are other apoptotic pathways involved in this process that needs further investigation. Thus, our group has initiated examining the susceptibility of a range of cancer cell lines for proteomic, transcriptosomic and array analysis. The most novel data in the thesis comes from the experiments using pancreatic cancer cell line where endogenous overexpression of full length SP-D appears to inhibit metastatic properties of the cancer cells. This is consistent with downregulation of cellular factors which are known to be involved in tumour invasion and matastasis. Experiments where the same cells are challenged using exogenous SP-D are in progress. This work opens up the possibility of using gene therapy to target a number of solid tumours.

Experiments with DC-SIGN appear to suggest that SP-D is most likely to modulate DC-SIGN mediated allergenic response. Since, SP-D has also been shown to modulate HIV infection in antigen presenting cells where DC-SIGN is a cognant receptor for the pathogen, this novel interaction is likely to alter infectivity of HIV-1. Currently, using HEK expressing surface DC-SIGN, we are trying to ascertain if HIV-1 infection can be modulated by rhSP-D in a coculture system. Using indicator Assay, HIV-1 infection is quantified using TZM-bl cells. These cells express luciferase as well as β-galactosidase genes under the control of the HIV-1 LTR promoter. HIV-1 strains being used: HIV-1LAI, HIV-1 IN93/905, HIV-1 98/IN/017 and HIV-1 96USNG31. HIV-1 is pretreated with SP-D and rhSP-D. After 48 hours, fixed cells in 1% glutaraldehyde stained with X-gal staining solution for 24 h at 37°C. The blue stained (β-gal expressing) foci counted under the microscope.

For allergen interaction DC-SIGN overexpressing macrophage cell lines are used in the presence of Th1 or Th2 cells and challenged with rhSP-D and allergens. The readouts are being used for T-cell proliferative and production of these cytokines.

This notion of SP-D being a general policing molecule for activated or transformed cell is reminiscent of its relationship with the classical pathways first subcomponent C1q. C1q has been proposed as not only a PAMP recognizing PRR but also DAMP (Danger associated molecular patterns). In this way, C1q cannot only recognize non-self but also altered self, which is threat to homeostasis. It is evident that SP-D has evolved not only to recognize PAMP and subsequent clearance of pathogens, but also to downregulate inflammatory processes in order to resolve inflammation which otherwise will lead to tissue injury. However, its ability to induce apoptosis via CRD domain in transformed cells seems novel observation that needs extensive investigation. Thus, SP-D present in serum and being locally made in the tissue constitutively has a far broader implication in immune regulation and homeostasis that hitherto appreciated.

References;

Akihiro, F., Makoto, Y., Masahiro, O., Hiroto, N., Takanori, K., Koji, H., Atsushi, M., Satoru, T., Tohru, I., Ko, S., Takeshi, A. and Masaru, K. (2004) "Gastric Dysmotility Associated with Accumulation of Mitochondrial A3243G Mutation in the Stomach", *Internal Medicine,* vol. 43, no. 12, pp. 1126--1130.

Akuthota, P., Xenakis, J.J. and Weller, P.F. (2011) "Eosinophils: offenders or general bystanders in allergic airway disease and pulmonary immunity?", *Journal of innate immunity,* vol. 3, no. 2, pp. 113-119.

Alcorn, J.F. and Wright, J.R. (2004) "Surfactant protein A inhibits alveolar macrophage cytokine production by CD14-independent pathway", *American journal of physiology.Lung cellular and molecular physiology,* vol. 286, no. 1, pp. L129-36.

Allen, M.J., Voelker, D.R. and Mason, R.J. (2001) "Interactions of surfactant proteins A and D with Saccharomyces cerevisiae and Aspergillus fumigatus", *Infection and immunity,* vol. 69, no. 4, pp. 2037-2044.

Asano, Y., Ihn, H., Yamane, K., Yazawa, N., Kubo, M., Fujimoto, M. and Tamaki, K. (2001) "Clinical significance of surfactant protein D as a serum marker for evaluating pulmonary fibrosis in patients with systemic sclerosis", *Arthritis and Rheumatism,* vol. 44, no. 6, pp. 1363-1369.

Awasthi, S., Magee, D.M. and Coalson, J.J. (2004) "Coccidioides posadasii infection alters the expression of pulmonary surfactant proteins (SP)-A and SP-D", *Respiratory research,* vol. 5, pp. 28.

Ballard, P. (2000) "Scientific rationale for the use of antenatal glucocorticoids to promote fetal development.", *Pediatr.Rev.,* vol. 1, pp. E83.

Barlo, N.P., van Moorsel, C.H., Ruven, H.J., Zanen, P., van den Bosch, J.M. and Grutters, J.C. (2009) "Surfactant protein-D predicts survival in patients with idiopathic pulmonary fibrosis", *Sarcoidosis, vasculitis, and diffuse lung diseases : official journal of WASOG / World Association of Sarcoidosis and Other Granulomatous Disorders,* vol. 26, no. 2, pp. 155-161.

Barr, F.E., Pedigo, H., Johnson, T.R. and Shepherd, V.L. (2000) "Surfactant protein-A enhances uptake of respiratory syncytial virus by monocytes and U937 macrophages", *American journal of respiratory cell and molecular biology,* vol. 23, no. 5, pp. 586-592.

Benne, C.A., Benaissa-Trouw, B., van Strijp, J.A., Kraaijeveld, C.A. and van Iwaarden, J.F. (1997) "Surfactant protein A, but not surfactant protein D, is an opsonin for influenza A virus phagocytosis by rat alveolar macrophages", *European journal of immunology,* vol. 27, no. 4, pp. 886-890.

Benne, C.A., Kraaijeveld, C.A., van Strijp, J.A., Brouwer, E., Harmsen, M., Verhoef, J., van Golde, L.M. and van Iwaarden, J.F. (1995) "Interactions of surfactant protein A with influenza A viruses: binding and neutralization", *The Journal of infectious diseases,* vol. 171, no. 2, pp. 335-341.

Bordet, J. (1906) "Les phénomènes d'absorption de la conglutinin du serum de boeuf.", *Ann.Inst.Pasteur,* vol. 49, pp. 260.

Borron, P. (2000) "Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo.", *Am.J.Physiol.Lung Cell Mol.Physiol.,* vol. 278, pp. L840.

Borron, P., McIntosh, J.C., Korfhagen, T.R., Whitsett, J.A., Taylor, J. and Wright, J.R. (2000) "Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo", *American journal of physiology.Lung cellular and molecular physiology,* vol. 278, no. 4, pp. L840-7.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y. and Zychlinsky, A. (2004) "Neutrophil extracellular traps kill bacteria", *Science (New York, N.Y.),* vol. 303, no. 5663, pp. 1532-1535.

Brinkmann, V. and Zychlinsky, A. (2012) "Neutrophil extracellular traps: is immunity the second function of chromatin?", *The Journal of cell biology,* vol. 198, no. 5, pp. 773-783.

Brinkmann, V. and Zychlinsky, A. (2007) "Beneficial suicide: why neutrophils die to make NETs", *Nature reviews.Microbiology,* vol. 5, no. 8, pp. 577-582.

Chen, C.-. (2001) "Stoichiometry of Complexes between Mannose-binding Protein and Its Associated Serine Proteases. DEFINING FUNCTIONAL UNITS FOR COMPLEMENT ACTIVATION", *The Journal of biological chemistry,* vol. 276, no. 28, pp. 25894; 25894-25902; 25902.

Chiba, H. (2002) "Human Surfactant Protein D (SP-D) Binds Mycoplasma pneumoniae by High Affinity Interactions with Lipids", *The Journal of biological chemistry,* vol. 277, no. 23, pp. 20379; 20379-20385; 20385.

Chow, O.A., von Kockritz-Blickwede, M., Bright, A.T., Hensler, M.E., Zinkernagel, A.S., Cogen, A.L., Gallo, R.L., Monestier, M., Wang, Y., Glass, C.K. and Nizet, V. (2010) "Statins enhance formation of phagocyte extracellular traps", *Cell host & microbe,* vol. 8, no. 5, pp. 445-454.

Chuammitri, P., Ostojic, J., Andreasen, C.B., Redmond, S.B., Lamont, S.J. and Palic, D. (2009) "Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils", *Veterinary immunology and immunopathology,* vol. 129, no. 1-2, pp. 126-131.

Cockshutt, A.M., Weitz, J. and Possmayer, F. (1990) "Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro", *Biochemistry,* vol. 29, no. 36, pp. 8424-8429.

Crouch, E., Hartshorn, K. and Ofek, I. (2000) "Collectins and pulmonary innate immunity", *Immunological reviews,* vol. 173, pp. 52-65.

Crouch, E., Parghi, D., Kuan, S.F. and Persson, A. (1992) "Surfactant protein D: subcellular localization in nonciliated bronchiolar epithelial cells", *The American Journal of Physiology,* vol. 263, no. 1 Pt 1, pp. L60-6.

Crouch, E., Persson, A., Chang, D. and Heuser, J. (1994) "Molecular structure of pulmonary surfactant protein D (SP-D)", *The Journal of biological chemistry,* vol. 269, no. 25, pp. 17311-17319.

Crouch, E., Rust, K., Marienchek, W., Parghi, D., Chang, D. and Persson, A. (1991) "Developmental expression of pulmonary surfactant protein D (SP-D)", *American journal of respiratory cell and molecular biology,* vol. 5, no. 1, pp. 13-18.

Crouch, E. and Wright, J.R. (2001) "Surfactant proteins a and d and pulmonary host defense", *Annual Review of Physiology,* vol. 63, pp. 521-554.

Dahl, M.R., Thiel, S., Matsushita, M., Fujita, T., Willis, A.C., Christensen, T., Vorup-Jensen, T. and Jensenius, J.C. (2001) "MASP-3 and Its Association with Distinct Complexes of the Mannan-Binding Lectin Complement Activation Pathway", *Immunity,* vol. 15, no. 1, pp. 127-135.

Downing, J.F., Pasula, R., Wright, J.R., Twigg, H.L.,3rd and Martin, W.J.,2nd (1995) "Surfactant protein a promotes attachment of Mycobacterium tuberculosis to alveolar macrophages during infection with human immunodeficiency virus", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 92, no. 11, pp. 4848-4852.

Eggleton, P. and Reid, K.B. (1999) "Lung surfactant proteins involved in innate immunity", *Current opinion in immunology,* vol. 11, no. 1, pp. 28-33.

Eisner, M.D., Parsons, P., Matthay, M.A., Ware, L., Greene, K. and Acute Respiratory Distress Syndrome Network (2003) "Plasma surfactant protein levels and clinical outcomes in patients with acute lung injury", *Thorax,* vol. 58, no. 11, pp. 983-988.

Ermert, D., Urban, C.F., Laube, B., Goosmann, C., Zychlinsky, A. and Brinkmann, V. (2009) "Mouse neutrophil extracellular traps in microbial infections", *Journal of innate immunity,* vol. 1, no. 3, pp. 181-193.

Ferguson, J. (2002) "Surfactant protein D inhibition of human macrophage uptake of Mycobacterium tuberculosis is independent of bacterial agglutination.", *J.Immunol.,* vol. 168, pp. 1309.

Ferguson, J.S., Voelker, D.R., McCormack, F.X. and Schlesinger, L.S. (1999) "Surfactant protein D binds to Mycobacterium tuberculosis bacilli and lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages", *Journal of immunology (Baltimore, Md.: 1950),* vol. 163, no. 1, pp. 312-321.

Fisher, A.B., Dodia, C. and Chander, A. (1994) "Inhibition of lung calcium-independent phospholipase A2 by surfactant protein A", *The American Journal of Physiology,* vol. 267, no. 3 Pt 1, pp. L335-41.

Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V. and Zychlinsky, A. (2007) "Novel cell death program leads to neutrophil extracellular traps", *The Journal of cell biology,* vol. 176, no. 2, pp. 231-241.

Gaiha, G.D., Dong, T., Palaniyar, N., Mitchell, D.A., Reid, K.B. and Clark, H.W. (2008) "Surfactant protein A binds to HIV and inhibits direct infection of CD4+ cells, but enhances dendritic cell-mediated viral transfer", *Journal of immunology (Baltimore, Md.: 1950),* vol. 181, no. 1, pp. 601-609.

Gaynor, C. (1995) "Pulmonary surfactant protein A mediates enhanced phagocytosis of Mycobacterium tuberculosis by a direct interaction with human macrophages.", *J.Immunol.,* vol. 155, pp. 5343.

Geertsma, M.F., Nibbering, P.H., Haagsman, H.P., Daha, M.R. and van Furth, R. (1994) "Binding of surfactant protein A to C1q receptors mediates phagocytosis of Staphylococcus aureus by monocytes", *The American Journal of Physiology,* vol. 267, no. 5 Pt 1, pp. L578-84.

Ghildyal, R., Hartley, C., Varrasso, A., Meanger, J., Voelker, D.R., Anders, E.M. and Mills, J. (1999) "Surfactant protein A binds to the fusion glycoprotein of respiratory syncytial virus and neutralizes virion infectivity", *The Journal of infectious diseases,* vol. 180, no. 6, pp. 2009-2013.

Giles, S.S., Zaas, A.K., Reidy, M.F., Perfect, J.R. and Wright, J.R. (2007) "Cryptococcus neoformans is resistant to surfactant protein A mediated host defense mechanisms", *PloS one,* vol. 2, no. 12, pp. e1370.

Green, P.J., Feizi, T., Stoll, M.S., Thiel, S., Prescott, A. and McConville, M.J. (1994) "Recognition of the major cell surface glycoconjugates of Leishmania parasites by the human serum mannan-binding protein", *Molecular and biochemical parasitology,* vol. 66, no. 2, pp. 319-328.

Grinberg, N., Elazar, S., Rosenshine, I. and Shpigel, N.Y. (2008) "Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic Escherichia coli", *Infection and immunity,* vol. 76, no. 6, pp. 2802-2807.

Groves, A.M., Gow, A.J., Massa, C.B., Laskin, J.D. and Laskin, D.L. (2012) "Prolonged injury and altered lung function after ozone inhalation in mice with chronic lung inflammation", *American journal of respiratory cell and molecular biology,* vol. 47, no. 6, pp. 776-783.

Haagsman, H.P. (1998) "Interactions of surfactant protein A with pathogens", *Biochimica et biophysica acta,* vol. 1408, no. 2-3, pp. 264-277.

Haagsman, H.P. and van Golde, L.M. (1991) "Synthesis and assembly of lung surfactant", *Annual Review of Physiology,* vol. 53, pp. 441-464.

Hallman, M., Ramet, M. and Ezekowitz, R.A. (2001) "Toll-like receptors as sensors of pathogens", *Pediatric research,* vol. 50, no. 3, pp. 315-321.

Hansen, S. (2003) "Functional aspects of SP-D and the derived bovine collectins: conglutinin, CL-43 and CL-46.", *Immunobiology,* vol. 205.

Hansen, S. (2000) "Purification and characterization of two mannan-binding lectins from mouse serum.", *J.Immunol.,* vol. 164, pp. 2610.

Harrod, K.S., Trapnell, B.C., Otake, K., Korfhagen, T.R. and Whitsett, J.A. (1999) "SP-A enhances viral clearance and inhibits inflammation after pulmonary adenoviral infection", *The American Journal of Physiology,* vol. 277, no. 3 Pt 1, pp. L580-8.

Hartley, C.A., Reading, P.C., Ward, A.C. and Anders, E.M. (1997) "Changes in the hemagglutinin molecule of influenza type A (H3N2) virus associated with increased virulence for mice", *Archives of Virology,* vol. 142, no. 1, pp. 75-88.

Hartshorn, K., Chang, D., Rust, K., White, M., Heuser, J. and Crouch, E. (1996) "Interactions of recombinant human pulmonary surfactant protein D and SP-D multimers with influenza A", *The American Journal of Physiology,* vol. 271, no. 5 Pt 1, pp. L753-62.

Hartshorn, K.L., Crouch, E., White, M.R., Colamussi, M.L., Kakkanatt, A., Tauber, B., Shepherd, V. and Sastry, K.N. (1998) "Pulmonary surfactant proteins A and D enhance neutrophil uptake of bacteria", *The American Journal of Physiology,* vol. 274, no. 6 Pt 1, pp. L958-69.

Hartshorn, K.L., Crouch, E.C., White, M.R., Eggleton, P., Tauber, A.I., Chang, D. and Sastry, K. (1994) "Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses", *The Journal of clinical investigation,* vol. 94, no. 1, pp. 311-319.

Hartshorn, K.L., Sastry, K., White, M.R., Anders, E.M., Super, M., Ezekowitz, R.A. and Tauber, A.I. (1993) "Human mannose-binding protein functions as an opsonin for influenza A viruses", *The Journal of clinical investigation,* vol. 91, no. 4, pp. 1414-1420.

Hartshorn, K.L., White, M.R., Shepherd, V., Reid, K., Jensenius, J.C. and Crouch, E.C. (1997) "Mechanisms of anti-influenza activity of surfactant proteins A and D: comparison with serum collectins", *The American Journal of Physiology,* vol. 273, no. 6 Pt 1, pp. L1156-66.

Hartshorn, K.L., White, M.R., Voelker, D.R., Coburn, J., Zaner, K. and Crouch, E.C. (2000) "Mechanism of binding of surfactant protein D to influenza A viruses: importance of binding to haemagglutinin to antiviral activity", *The Biochemical journal,* vol. 351 Pt 2, pp. 449-458.

Haurum, J.S., Thiel, S., Jones, I.M., Fischer, P.B., Laursen, S.B. and Jensenius, J.C. (1993) "Complement activation upon binding of mannan-binding protein to HIV envelope glycoproteins", *AIDS (London, England),* vol. 7, no. 10, pp. 1307-1313.

Hickling, T.P., Bright, H., Wing, K., Gower, D., Martin, S.L., Sim, R.B. and Malhotra, R. (1999) "A recombinant trimeric surfactant protein D carbohydrate recognition domain inhibits respiratory syncytial virus infection in vitro and in vivo", *European journal of immunology,* vol. 29, no. 11, pp. 3478-3484.

Hohwy, T., Otkjaer, K., Madsen, J., Soerensen, G., Nielsen, O., Vestergaard, C., Steiniche, T., Holmskov, U. and Lomholt, H. (2006) "Surfactant protein D in atopic dermatitis and psoriasis", *Experimental dermatology,* vol. 15, no. 3, pp. 168-174.

Holmskov, U. (2000) "Collectins and collectin receptors in innate immunity.", *APMIS,* vol. 108(Suppl. 100), pp. 7.

Holmskov, U., Malhotra, R., Sim, R.B. and Jensenius, J.C. (1994) "Collectins: collagenous C-type lectins of the innate immune defense system", *Immunology today,* vol. 15, no. 2, pp. 67-74.

Holmskov, U., Thiel, S. and Jensenius, J.C. (2003) "Collections and ficolins: humoral lectins of the innate immune defense", *Annual Review of Immunology,* vol. 21, pp. 547-578.

Ichijo, H. (1993) "Molecular cloning and characterization of ficolin, a multimeric protein with fibrinogen- and collagen-like domains.", *J.Biol.Chem.,* vol. 268, pp. 14505.

Ikeda, K. (1987) "Serum lectin with known structure activates complement through the classical pathway.", *J.Biol.Chem.,* vol. 262, pp. 7451.

Ishikawa, N., Hattori, N., Tanaka, S., Horimasu, Y., Haruta, Y., Yokoyama, A., Kohno, N. and Kinnula, V.L. (2011) "Levels of surfactant proteins A and D and KL-6 are elevated in the induced sputum of chronic obstructive pulmonary disease patients: a sequential sputum analysis", *Respiration; international review of thoracic diseases,* vol. 82, no. 1, pp. 10-18.

Jack, D.L., Klein, N.J. and Turner, M.W. (2001) "Mannose-binding lectin: targeting the microbial world for complement attack and opsonophagocytosis", *Immunological reviews,* vol. 180, no. 1, pp. 86-99.

Job, E.R., Deng, Y.M., Tate, M.D., Bottazzi, B., Crouch, E.C., Dean, M.M., Mantovani, A., Brooks, A.G. and Reading, P.C. (2010) "Pandemic H1N1 influenza A viruses are resistant to the antiviral activities of innate immune proteins of the collectin and pentraxin superfamilies", *Journal of immunology (Baltimore, Md.: 1950),* vol. 185, no. 7, pp. 4284-4291.

Johansson, M.W. (2014) "Activation states of blood eosinophils in asthma", *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* vol. 44, no. 4, pp. 482-498.

Jounblat, R., Kadioglu, A., Iannelli, F., Pozzi, G., Eggleton, P. and Andrew, P.W. (2004) "Binding and agglutination of Streptococcus pneumoniae by human surfactant protein D (SP-D) vary between strains, but SP-D fails to enhance killing by neutrophils", *Infection and immunity,* vol. 72, no. 2, pp. 709-716.

Kabha, K., Schmegner, J., Keisari, Y., Parolis, H., Schlepper-Schaeffer, J. and Ofek, I. (1997) "SP-A enhances phagocytosis of Klebsiella by interaction with capsular polysaccharides and alveolar macrophages", *The American Journal of Physiology,* vol. 272, no. 2 Pt 1, pp. L344-52.

Kahn, S.J., Wleklinski, M., Ezekowitz, R.A., Coder, D., Aruffo, A. and Farr, A. (1996) "The major surface glycoprotein of Trypanosoma cruzi amastigotes are ligands of the human serum mannose-binding protein", *Infection and immunity,* vol. 64, no. 7, pp. 2649-2656.

Kawasaki, N. (1989) "A serum lectin (mannan-binding protein) has complement-dependent bactericidal activity.", *J.Biochem.,* vol. 106, pp. 483.

Kawasaki, N. (1983) "Isolation and characterization of a mannan-binding protein from human serum.", *J.Biochem.,* vol. 94, pp. 937.

Kawasaki, T., Etoh, R. and Yamashina, I. (1978) "Isolation and characterization of a mannan-binding protein from rabbit liver", *Biochemical and biophysical research communications,* vol. 81, no. 3, pp. 1018-1024.

Kinder, B.W., Brown, K.K., McCormack, F.X., Ix, J.H., Kervitsky, A., Schwarz, M.I. and King, T.E.,Jr (2009) "Serum surfactant protein-A is a strong predictor of early mortality in idiopathic pulmonary fibrosis", *Chest,* vol. 135, no. 6, pp. 1557-1563.

Kishore, U., Greenhough, T.J., Waters, P., Shrive, A.K., Ghai, R., Kamran, M.F., Bernal, A.L., Reid, K.B., Madan, T. and Chakraborty, T. (2006) "Surfactant proteins SP-A and SP-D: structure, function and receptors", *Molecular immunology,* vol. 43, no. 9, pp. 1293-1315.

Kishore, U. and Reid, K.B. (2001) "Structures and functions of mammalian collectins", *Results and problems in cell differentiation,* vol. 33, pp. 225-248.

Kitaichi, N., Kitamura, M., Namba, K., Ishida, S. and Ohno, S. (2010) "Elevation of surfactant protein D, a pulmonary disease biomarker, in the sera of uveitis patients with sarcoidosis", *Japanese journal of ophthalmology,* vol. 54, no. 1, pp. 81-84.

Knudsen, L., Ochs, K., Boxler, L., Tornoe, I., Lykke-Sorensen, G., Mackay, R.M., Clark, H.W., Holmskov, U., Ochs, M. and Madsen, J. (2013) "Surfactant protein D (SP-D) deficiency is attenuated in humanised mice expressing the Met(11)Thr short nucleotide polymorphism of SP-D: implications for surfactant metabolism in the lung", *Journal of anatomy,* vol. 223, no. 6, pp. 581-592.

Kondo, A., Oketani, N., Maruyama, M., Taguchi, Y., Yamaguchi, Y., Miyao, H., Mashima, I., Oono, M., Wada, K., Tsuchiya, T., Takahashi, H. and Abe, S. (1998) "Significance of serum surfactant protein-D (SP-D) level in patients with pulmonary tuberculosis", *Kekkaku : [Tuberculosis],* vol. 73, no. 10, pp. 585-590.

Korfhagen, T.R., LeVine, A.M. and Whitsett, J.A. (1998) "Surfactant protein A (SP-A) gene targeted mice", *Biochimica et biophysica acta,* vol. 1408, no. 2-3, pp. 296-302.

Koziel, H., Phelps, D.S., Fishman, J.A., Armstrong, M.Y., Richards, F.F. and Rose, R.M. (1998) "Surfactant protein-A reduces binding and phagocytosis of pneumocystis carinii by human alveolar macrophages in vitro", *American journal of respiratory cell and molecular biology,* vol. 18, no. 6, pp. 834-843.

Kuan, S.F., Rust, K. and Crouch, E. (1992) "Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an Escherichia coli-binding protein in bronchoalveolar lavage", *The Journal of clinical investigation,* vol. 90, no. 1, pp. 97-106.

Kuang, Z., Hao, Y., Hwang, S., Zhang, S., Kim, E., Akinbi, H.T., Schurr, M.J., Irvin, R.T., Hassett, D.J. and Lau, G.W. (2011) "The Pseudomonas aeruginosa flagellum confers resistance to pulmonary surfactant protein-A by impacting the production of exoproteases through quorum-sensing", *Molecular microbiology,* vol. 79, no. 5, pp. 1220-1235.

Kucejko, W., Chyczewska, E., Naumnik, W. and Ossolinska, M. (2009) "Concentration of surfactant protein D, Clara cell protein CC-16 and IL-10 in bronchoalveolar lavage (BAL) in patients with sarcoidosis, hypersensivity pneumonitis and idiopathic pulmonary fibrosis", *Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society,* vol. 47, no. 2, pp. 225-230.

Kudo, K., Sano, H., Takahashi, H., Kuronuma, K., Yokota, S., Fujii, N., Shimada, K., Yano, I., Kumazawa, Y., Voelker, D.R., Abe, S. and Kuroki, Y. (2004) "Pulmonary collectins enhance phagocytosis of Mycobacterium avium through increased activity of mannose receptor", *Journal of immunology (Baltimore, Md.: 1950),* vol. 172, no. 12, pp. 7592-7602.

Kuroki, Y., Tsutahara, S., Shijubo, N., Takahashi, H., Shiratori, M., Hattori, A., Honda, Y., Abe, S. and Akino, T. (1993) "Elevated levels of lung surfactant protein A in sera from patients with idiopathic pulmonary fibrosis and pulmonary alveolar proteinosis", *The American Review of Respiratory Disease,* vol. 147, no. 3, pp. 723-729.

Kuroki, Y. and Voelker, D.R. (1994) "Pulmonary surfactant proteins", *The Journal of biological chemistry,* vol. 269, no. 42, pp. 25943-25946.

Kuronuma, K., Sano, H., Kato, K., Kudo, K., Hyakushima, N., Yokota, S., Takahashi, H., Fujii, N., Suzuki, H., Kodama, T., Abe, S. and Kuroki, Y. (2004) "Pulmonary surfactant protein A augments the phagocytosis of Streptococcus pneumoniae by alveolar macrophages through a casein kinase 2-dependent increase of cell surface localization of scavenger receptor A", *The Journal of biological chemistry,* vol. 279, no. 20, pp. 21421-21430.

Lacaze-Masmonteil, T. (1995) "Pulmonary Surfactant Protein A (SP-A) Is Expressed by Epithelial Cells of Small and Large Intestine", *The Journal of biological chemistry,* vol. 270, no. 20, pp. 12162; 12162-12169; 12169.

Le, Y., Tan, S.M., Lee, S.H., Kon, O.L. and Lu, J. (1997) "Purification and binding properties of a human ficolin-like protein", *Journal of immunological methods,* vol. 204, no. 1, pp. 43-49.

Lee, J.J. and Lee, N.A. (2005) "Eosinophil degranulation: an evolutionary vestige or a universally destructive effector function?", *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* vol. 35, no. 8, pp. 986-994.

Lekkala, M., LeVine, A.M., Linke, M.J., Crouch, E.C., Linders, B., Brummer, E. and Stevens, D.A. (2006) "Effect of lung surfactant collectins on bronchoalveolar macrophage interaction with Blastomyces dermatitidis: inhibition of tumor necrosis factor alpha production by surfactant protein D", *Infection and immunity,* vol. 74, no. 8, pp. 4549-4556.

Leon, M.A. and Yokohari, R. (1964) "Conglutination: Specific Inhibition by Carbohydrates", *Science (New York, N.Y.),* vol. 143, no. 3612, pp. 1327-1328.

Leth-Larsen, R., Zhong, F., Chow, V.T., Holmskov, U. and Lu, J. (2007) "The SARS coronavirus spike glycoprotein is selectively recognized by lung surfactant protein D and activates macrophages", *Immunobiology,* vol. 212, no. 3, pp. 201-211.

LeVine, A.M., Bruno, M.D., Huelsman, K.M., Ross, G.F., Whitsett, J.A. and Korfhagen, T.R. (1997) "Surfactant protein A-deficient mice are susceptible to group B streptococcal infection", *Journal of immunology (Baltimore, Md.: 1950),* vol. 158, no. 9, pp. 4336-4340.

LeVine, A.M., Gwozdz, J., Stark, J., Bruno, M., Whitsett, J. and Korfhagen, T. (1999) "Surfactant protein-A enhances respiratory syncytial virus clearance in vivo", *The Journal of clinical investigation,* vol. 103, no. 7, pp. 1015-1021.

Lim, B.L., Wang, J.Y., Holmskov, U., Hoppe, H.J. and Reid, K.B. (1994) "Expression of the carbohydrate recognition domain of lung surfactant protein D and demonstration of its binding to lipopolysaccharides of gram-negative bacteria", *Biochemical and biophysical research communications,* vol. 202, no. 3, pp. 1674-1680.

Limper, A.H., Crouch, E.C., O'Riordan, D.M., Chang, D., Vuk-Pavlovic, Z., Standing, J.E., Kwon, K.Y. and Adlakha, A. (1995) "Surfactant protein-D modulates interaction of Pneumocystis carinii with alveolar macrophages", *The Journal of laboratory and clinical medicine,* vol. 126, no. 5, pp. 416-422.

Lin, A.M., Rubin, C.J., Khandpur, R., Wang, J.Y., Riblett, M., Yalavarthi, S., Villanueva, E.C., Shah, P., Kaplan, M.J. and Bruce, A.T. (2011) "Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis", *Journal of immunology (Baltimore, Md.: 1950),* vol. 187, no. 1, pp. 490-500.

Lin, F.C., Chen, Y.C. and Chang, S.C. (2008) "Clinical importance of bronchoalveolar lavage fluid and blood cytokines, surfactant protein D, and Kerbs von Lungren 6 antigen in idiopathic pulmonary alveolar proteinosis", *Mayo Clinic proceedings,* vol. 83, no. 12, pp. 1344-1349.

Lomas, D.A., Silverman, E.K., Edwards, L.D., Locantore, N.W., Miller, B.E., Horstman, D.H., Tal-Singer, R. and Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints study investigators (2009) "Serum surfactant protein D is steroid sensitive and associated with exacerbations of COPD", *The European respiratory journal,* vol. 34, no. 1, pp. 95-102.

Madan, T., Eggleton, P., Kishore, U., Strong, P., Aggrawal, S.S., Sarma, P.U. and Reid, K.B. (1997a) "Binding of pulmonary surfactant proteins A and D to Aspergillus fumigatus conidia enhances phagocytosis and killing by human neutrophils and alveolar macrophages", *Infection and immunity,* vol. 65, no. 8, pp. 3171-3179.

Madan, T., Kishore, U., Shah, A., Eggleton, P., Strong, P., Wang, J.Y., Aggrawal, S.S., Sarma, P.U. and Reid, K.B. (1997b) "Lung surfactant proteins A and D can inhibit specific IgE binding to the allergens of Aspergillus fumigatus and block allergen-induced histamine release from human basophils", *Clinical and experimental immunology,* vol. 110, no. 2, pp. 241-249.

Madan, T., Kishore, U., Singh, M., Strong, P., Clark, H., Hussain, E.M., Reid, K.B. and Sarma, P.U. (2001) "Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by Aspergillus fumigatus antigens and allergens", *The Journal of clinical investigation,* vol. 107, no. 4, pp. 467-475.

Madan, T., Reid, K.B., Clark, H., Singh, M., Nayak, A., Sarma, P.U., Hawgood, S. and Kishore, U. (2010) "Susceptibility of mice genetically deficient in SP-A or SP-D gene to invasive pulmonary aspergillosis", *Molecular immunology,* vol. 47, no. 10, pp. 1923-1930.

Madsen, J. (2000) "Localization of lung surfactant protein D (SP-D) on mucosal surfaces in human tissues.", *J.Immunol.,* vol. 164, pp. 5866.

Madsen, J., Mollenhauer, J. and Holmskov, U. (2010) "Review: Gp-340/DMBT1 in mucosal innate immunity", *Innate immunity,* vol. 16, no. 3, pp. 160-167.

Mahajan, L., Madan, T., Kamal, N., Singh, V.K., Sim, R.B., Telang, S.D., Ramchand, C.N., Waters, P., Kishore, U. and Sarma, P.U. (2008) "Recombinant surfactant protein-D selectively increases apoptosis in eosinophils of allergic asthmatics and enhances uptake of apoptotic eosinophils by macrophages", *International immunology,* vol. 20, no. 8, pp. 993-1007.

Malhotra, R., Haurum, J., Thiel, S., Jensenius, J.C. and Sim, R.B. (1993) "Pollen grains bind to lung alveolar type II cells (A549) via lung surfactant protein A (SP-A)", *Bioscience reports,* vol. 13, no. 2, pp. 79-90.

Malhotra, R., Haurum, J.S., Thiel, S. and Sim, R.B. (1994) "Binding of human collectins (SP-A and MBP) to influenza virus", *The Biochemical journal,* vol. 304 (Pt 2), no. Pt 2, pp. 455-461.

Manz-Keinke, H., Plattner, H. and Schlepper-Schafer, J. (1992) "Lung surfactant protein A (SP-A) enhances serum-independent phagocytosis of bacteria by alveolar macrophages", *European journal of cell biology,* vol. 57, no. 1, pp. 95-100.

Mason, R.J., Greene, K. and Voelker, D.R. (1998) "Surfactant protein A and surfactant protein D in health and disease", *The American Journal of Physiology,* vol. 275, no. 1 Pt 1, pp. L1-13.

Matsushita, M. and Fujita, T. (1992) "Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease.", *The Journal of experimental medicine,* vol. 176, no. 6, pp. 1497-1502.

Matsushita, M. and Fujita, T. (2001) "Ficolins and the lectin complement pathway", *Immunological reviews,* vol. 180, no. 1, pp. 78-85.

Mazur, W., Toljamo, T., Ohlmeier, S., Vuopala, K., Nieminen, P., Kobayashi, H. and Kinnula, V.L. (2011) "Elevation of surfactant protein A in plasma and sputum in cigarette smokers", *The European respiratory journal,* vol. 38, no. 2, pp. 277-284.

McCormack, F.X., Festa, A.L., Andrews, R.P., Linke, M. and Walzer, P.D. (1997) "The carbohydrate recognition domain of surfactant protein A mediates binding to the major surface glycoprotein of Pneumocystis carinii", *Biochemistry,* vol. 36, no. 26, pp. 8092-8099.

McIntosh, J.C., Swyers, A.H., Fisher, J.H. and Wright, J.R. (1996) "Surfactant proteins A and D increase in response to intratracheal lipopolysaccharide", *American journal of respiratory cell and molecular biology,* vol. 15, no. 4, pp. 509-519.

McNeely, T. (1994) "Aggregation and opsonization of type A but not type B Hemophilus influenzae by surfactant protein A.", *American journal of respiratory cell and molecular biology,* vol. 11, pp. 114.

McNeely, T.B. and Coonrod, J.D. (1993) "Comparison of the opsonic activity of human surfactant protein A for Staphylococcus aureus and Streptococcus pneumoniae with rabbit and human macrophages", *The Journal of infectious diseases,* vol. 167, no. 1, pp. 91-97.

Meschi, J., Crouch, E.C., Skolnik, P., Yahya, K., Holmskov, U., Leth-Larsen, R., Tornoe, I., Tecle, T., White, M.R. and Hartshorn, K.L. (2005) "Surfactant protein D binds to human immunodeficiency virus (HIV) envelope protein gp120 and inhibits HIV replication", *The Journal of general virology,* vol. 86, no. Pt 11, pp. 3097-3107.

Montalbano, A.P., Hawgood, S. and Mendelson, C.R. (2013) "Mice deficient in surfactant protein A (SP-A) and SP-D or in TLR2 manifest delayed parturition and decreased expression of inflammatory and contractile genes", *Endocrinology,* vol. 154, no. 1, pp. 483-498.

Moqbel, R. and Coughlin, J.J. (2006) "Differential secretion of cytokines", *Science's STKE : signal transduction knowledge environment,* vol. 2006, no. 338, pp. pe26.

Muniz, V.S., Baptista-Dos-Reis, R. and Neves, J.S. (2013) "Functional extracellular eosinophil granules: a bomb caught in a trap", *International archives of allergy and immunology,* vol. 162, no. 4, pp. 276-282.

Muniz, V.S., Weller, P.F. and Neves, J.S. (2012) "Eosinophil crystalloid granules: structure, function, and beyond", *Journal of leukocyte biology,* vol. 92, no. 2, pp. 281-288.

Nayak, A., Dodagatta-Marri, E., Tsolaki, A.G. and Kishore, U. (2012) "An Insight into the Diverse Roles of Surfactant Proteins, SP-A and SP-D in Innate and Adaptive Immunity", *Frontiers in immunology,* vol. 3, pp. 131.

Neth, O., Jack, D.L., Dodds, A.W., Holzel, H., Klein, N.J. and Turner, M.W. (2000) "Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition", *Infection and immunity,* vol. 68, no. 2, pp. 688-693.

Neves, J.S., Perez, S.A., Spencer, L.A., Melo, R.C., Reynolds, L., Ghiran, I., Mahmudi-Azer, S., Odemuyiwa, S.O., Dvorak, A.M., Moqbel, R. and Weller, P.F. (2008) "Eosinophil granules function extracellularly as receptor-mediated secretory organelles", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 105, no. 47, pp. 18478-18483.

Neves, J.S., Perez, S.A., Spencer, L.A., Melo, R.C. and Weller, P.F. (2009) "Subcellular fractionation of human eosinophils: isolation of functional specific granules on isoosmotic density gradients", *Journal of immunological methods,* vol. 344, no. 1, pp. 64-72.

Neves, J.S. and Weller, P.F. (2009) "Functional extracellular eosinophil granules: novel implications in eosinophil immunobiology", *Current opinion in immunology,* vol. 21, no. 6, pp. 694-699.

Neves, J.S., Radke, A.L. and Weller, P.F. (2010) "Cysteinyl leukotrienes acting via granule membrane-expressed receptors elicit secretion from within cell-free human eosinophil granules", *Journal of Allergy and Clinical Immunology,* vol. 125, no. 2, pp. 477-482.

Noah, T.L., Murphy, P.C., Alink, J.J., Leigh, M.W., Hull, W.M., Stahlman, M.T. and Whitsett, J.A. (2003) "Bronchoalveolar lavage fluid surfactant protein-A and surfactant protein-D are inversely related to inflammation in early cystic fibrosis", *American journal of respiratory and critical care medicine,* vol. 168, no. 6, pp. 685-691.

Nomori, H., Horio, H., Fuyuno, G., Kobayashi, R., Morinaga, S. and Suemasu, K. (1998) "Serum surfactant protein A levels in healthy individuals are increased in smokers", *Lung,* vol. 176, no. 6, pp. 355-361.

Ogasawara, Y., Kuroki, Y. and Akino, T. (1992) "Pulmonary surfactant protein D specifically binds to phosphatidylinositol", *The Journal of biological chemistry,* vol. 267, no. 29, pp. 21244-21249.

Ohlmeier, S., Vuolanto, M., Toljamo, T., Vuopala, K., Salmenkivi, K., Myllarniemi, M. and Kinnula, V.L. (2008) "Proteomics of human lung tissue identifies surfactant protein A as a marker of chronic obstructive pulmonary disease", *Journal of proteome research,* vol. 7, no. 12, pp. 5125-5132.

Ohmer-Schrock, D., Schlatterer, C., Plattner, H. and Schlepper-Schafer, J. (1995) "Lung surfactant protein A (SP-A) activates a phosphoinositide/calcium signaling pathway in alveolar macrophages", *Journal of cell science,* vol. 108 (Pt 12), no. Pt 12, pp. 3695-3702.

Ohtani, K. (2001) "The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells.", *J.Biol.Chem.,* vol. 276, pp. 44222.

Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Yamazaki, H., Shimada, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T. and Wakamiya, N. (1999) "Molecular cloning of a novel human collectin from liver (CL-L1)", *The Journal of biological chemistry,* vol. 274, no. 19, pp. 13681-13689.

Oliveira, S.H. and Lukacs, N.W. (2003) "The role of chemokines and chemokine receptors in eosinophil activation during inflammatory allergic reactions", *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ...[et al.],* vol. 36, no. 11, pp. 1455-1463.

O'Riordan, D.M., Standing, J.E., Kwon, K.Y., Chang, D., Crouch, E.C. and Limper, A.H. (1995) "Surfactant protein D interacts with Pneumocystis carinii and mediates organism adherence to alveolar macrophages", *The Journal of clinical investigation,* vol. 95, no. 6, pp. 2699-2710.

Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B, Bao JK. (2012 Dec) "**Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis.**", *Cell Prolif.,* vol. 45, no. 6, pp. 487-98.

Palić, D., Ostojić, J., Andreasen, C.B. and Roth, J.A. (2007) "Fish cast NETs: Neutrophil extracellular traps are released from fish neutrophils", *Developmental & Comparative Immunology,* vol. 31, no. 8, pp. 805-816.

Pasula, R., Downing, J.F., Wright, J.R., Kachel, D.L., Davis, T.E.,Jr and Martin, W.J.,2nd (1997) "Surfactant protein A (SP-A) mediates attachment of Mycobacterium tuberculosis to murine alveolar macrophages", *American journal of respiratory cell and molecular biology,* vol. 17, no. 2, pp. 209-217.

Persson, A., Rust, K., Chang, D., Moxley, M., Longmore, W. and Crouch, E. (1988) "CP4: a pneumocyte-derived collagenous surfactant-associated protein. Evidence for heterogeneity of collagenous surfactant proteins", *Biochemistry,* vol. 27, no. 23, pp. 8576-8584.

Persson, A.V., Gibbons, B.J., Shoemaker, J.D., Moxley, M.A. and Longmore, W.J. (1992) "The major glycolipid recognized by SP-D in surfactant is phosphatidylinositol", *Biochemistry,* vol. 31, no. 48, pp. 12183-12189.

Piboonpocanun, S., Chiba, H., Mitsuzawa, H., Martin, W., Murphy, R.C., Harbeck, R.J. and Voelker, D.R. (2005) "Surfactant protein A binds Mycoplasma pneumoniae with high affinity and attenuates its growth by recognition of disaturated phosphatidylglycerols", *The Journal of biological chemistry,* vol. 280, no. 1, pp. 9-17.

Pikaar, J.C., Voorhout, W.F., van Golde, L.M.G., Verhoef, J., Van Strijp, J.A.G. and van Iwaarden, J.F. (1995) "Opsonic Activities of Surfactant Proteins A and D in Phagocytosis of Gram-Negative Bacteria by Alveolar Macrophages", *Journal of Infectious Diseases,* vol. 172, no. 2, pp. 481-489.

Pilsczek, F.H., Salina, D., Poon, K.K., Fahey, C., Yipp, B.G., Sibley, C.D., Robbins, S.M., Green, F.H., Surette, M.G., Sugai, M., Bowden, M.G., Hussain, M., Zhang, K. and Kubes, P. (2010) "A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus", *Journal of immunology (Baltimore, Md.: 1950),* vol. 185, no. 12, pp. 7413-7425.

Polotsky, V. (1996) "Interactions of human mannose-binding protein with lipoteichoic acids.", *Infect.Immun.,* vol. 64, pp. 380.

Ragas, A., Roussel, L., Puzo, G. and Riviere, M. (2007) "The Mycobacterium tuberculosis cell-surface glycoprotein apa as a potential adhesin to colonize target cells via the innate immune system pulmonary C-type lectin surfactant protein A", *The Journal of biological chemistry,* vol. 282, no. 8, pp. 5133-5142.

Reading, P.C., Hartley, C.A., Ezekowitz, R.A. and Anders, E.M. (1995) "A serum mannose-binding lectin mediates complement-dependent lysis of influenza virus-infected cells", *Biochemical and biophysical research communications,* vol. 217, no. 3, pp. 1128-1136.

Reading, P.C., Holmskov, U. and Anders, E.M. (1998) "Antiviral activity of bovine collectins against rotaviruses", *The Journal of general virology,* vol. 79 (Pt 9), no. Pt 9, pp. 2255-2263.

Reid, K.B. (1998) "Functional roles of the lung surfactant proteins SP-A and SP-D in innate immunity", *Immunobiology,* vol. 199, no. 2, pp. 200-207.

Remijsen, Q., Vanden Berghe, T., Wirawan, E., Asselbergh, B., Parthoens, E., De Rycke, R., Noppen, S., Delforge, M., Willems, J. and Vandenabeele, P. (2011) "Neutrophil extracellular trap cell death requires both autophagy and superoxide generation", *Cell research,* vol. 21, no. 2, pp. 290-304.

Restrepo, C.I., Dong, Q., Savov, J., Mariencheck, W.I. and Wright, J.R. (1999) "Surfactant protein D stimulates phagocytosis of Pseudomonas aeruginosa by alveolar macrophages", *American journal of respiratory cell and molecular biology,* vol. 21, no. 5, pp. 576-585.

Rosseau, S., Guenther, A., Seeger, W. and Lohmeyer, J. (1997) "Phagocytosis of viable Candida albicans by alveolar macrophages: lack of opsonin function of surfactant protein A", *The Journal of infectious diseases,* vol. 175, no. 2, pp. 421-428.

Sano, H. (1999) "Pulmonary surfactant protein A modulates the cellular response to smooth and rough lipopolysaccharides by interaction with CD14.", *J.Immunol.,* vol. 163, pp. 387.

Sano, H., Kuronuma, K., Kudo, K., Mitsuzawa, H., Sato, M., Murakami, S. and Kuroki, Y. (2006) "Regulation of inflammation and bacterial clearance by lung collectins", *Respirology (Carlton, Vic.),* vol. 11 Suppl, pp. S46-50.

Sano, H., Sohma, H., Muta, T., Nomura, S., Voelker, D.R. and Kuroki, Y. (1999) "Pulmonary surfactant protein A modulates the cellular response to smooth and rough lipopolysaccharides by interaction with CD14", *Journal of immunology (Baltimore, Md.: 1950),* vol. 163, no. 1, pp. 387-395.

Sano, H., Nagai, K., Tsutsumi, H. and Kuroki, Y. (2003) "Lactoferrin and surfactant protein?A exhibit distinct binding specificity to F?protein and differently modulate respiratory syncytial virus infection", *European journal of immunology,* vol. 33, no. 10, pp. 2894-2902.

Sasaki, R., Soejima, T., Matsumoto, A., Maruta, T., Yamada, K., Ota, Y., Kawabe, T., Nishimura, H., Sakai, E., Ejima, Y. and Sugimura, K. (2001) "Clinical significance of serum pulmonary surfactant proteins a and d for the early detection of radiation pneumonitis", *International journal of radiation oncology, biology, physics,* vol. 50, no. 2, pp. 301-307.

Sawada, K., Ariki, S., Kojima, T., Saito, A., Yamazoe, M., Nishitani, C., Shimizu, T., Takahashi, M., Mitsuzawa, H., Yokota, S., Sawada, N., Fujii, N., Takahashi, H. and Kuroki, Y. (2010) "Pulmonary collectins protect macrophages against pore-forming activity of Legionella pneumophila and suppress its intracellular growth", *The Journal of biological chemistry,* vol. 285, no. 11, pp. 8434-8443.

Schelenz, S., Malhotra, R., Sim, R.B., Holmskov, U. and Bancroft, G.J. (1995) "Binding of host collectins to the pathogenic yeast Cryptococcus neoformans: human surfactant protein D acts as an agglutinin for acapsular yeast cells", *Infection and immunity,* vol. 63, no. 9, pp. 3360-3366.

Schuijs, M.J., Willart, M.A., Hammad, H. and Lambrecht, B.N. (2013) "Cytokine targets in airway inflammation", *Current opinion in pharmacology,* vol. 13, no. 3, pp. 351-361.

Schurch, S., Possmayer, F., Cheng, S. and Cockshutt, A.M. (1992) "Pulmonary SP-A enhances adsorption and appears to induce surface sorting of lipid extract surfactant", *The American Journal of Physiology,* vol. 263, no. 2 Pt 1, pp. L210-8.

Sever-Chroneos, Z., Krupa, A., Davis, J., Hasan, M., Yang, C.H., Szeliga, J., Herrmann, M., Hussain, M., Geisbrecht, B.V., Kobzik, L. and Chroneos, Z.C. (2011) "Surfactant protein A (SP-A)-mediated clearance of Staphylococcus aureus involves binding of SP-A to the staphylococcal adhesin eap and the macrophage receptors SP-A receptor 210 and scavenger receptor class A", *The Journal of biological chemistry,* vol. 286, no. 6, pp. 4854-4870.

Shakoori, T.A., Sin, D.D., Ghafoor, F., Bashir, S. and Bokhari, S.N. (2009) "Serum surfactant protein D during acute exacerbations of chronic obstructive pulmonary disease", *Disease markers,* vol. 27, no. 6, pp. 287-294.

Shepherd, V.L. (2002) "Pulmonary surfactant protein D: a novel link between innate and adaptive immunity", *American journal of physiology.Lung cellular and molecular physiology,* vol. 282, no. 3, pp. L516-7.

Simon, D., Simon, H.-. and Yousefi, S. (2013) "Extracellular DNA traps in allergic, infectious, and autoimmune diseases", *Allergy,* vol. 68, no. 4, pp. 409-416.

Stahlman, M.T., Gray, M.E., Hull, W.M. and Whitsett, J.A. (2002) "Immunolocalization of Surfactant Protein-D (SP-D) in Human Fetal, Newborn, and Adult Tissues", *Journal of Histochemistry & Cytochemistry,* vol. 50, no. 5, pp. 651-660.

Stover, C. (1999) "Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene.", *J.Immunol.,* vol. 162, pp. 3481.

Thiel, S. (2000) "Interaction of C1q and mannan-binding lectin (MBL) with C1r, C1s, MBL-associated serine proteases 1 and 2, and the MBL-associated protein MAp19.", *J.Immunol.,* vol. 165, pp. 878.

Thiel, S. (1995) "Ontogeny of human mannan-binding protein, a lectin of the innate immune system", *Pediatric allergy and immunology,* vol. 6, no. 1, pp. 20; 20-23; 23.

Thiel, S., Vorup-Jensen, T., Stover, C.M., Schwaeble, W., Laursen, S.B., Poulsen, K., Willis, A.C., Eggleton, P., Hansen, S., Holmskov, U., Reid, K.B. and Jensenius, J.C. (1997) "A second serine protease associated with mannan-binding lectin that activates complement", *Nature,* vol. 386, no. 6624, pp. 506-510.

Thielens, N. (2001) "Interaction properties of human mannan-binding lectin (MBL)-associated serine proteases-1 and -2, MBL-associated protein 19, and MBL.", *J.Immunol.,* vol. 166, pp. 5068.

Tino, M. (1996) "Surfactant protein A stimulates phagocytosis of specific pulmonary pathogens by alveolar macrophages.", *Am.J.Physiol.Lung Cell Mol.Physiol.,* vol. 270, pp. L677.

Townsend, R., Read, R.C., Turner, M.W., Klein, N.J. and Jack, D.L. (2001) "Differential recognition of obligate anaerobic bacteria by human mannose-binding lectin", *Clinical and experimental immunology,* vol. 124, no. 2, pp. 223-228.

Ueki, S., Melo, R.C., Ghiran, I., Spencer, L.A., Dvorak, A.M. and Weller, P.F. (2013) "Eosinophil extracellular DNA trap cell death mediates lytic release of free secretion-competent eosinophil granules in humans", *Blood,* vol. 121, no. 11, pp. 2074-2083.

Urban, C.F., Reichard, U., Brinkmann, V. and Zychlinsky, A. (2006) "Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms", *Cellular microbiology,* vol. 8, no. 4, pp. 668-676.

van de Graaf, E.A., Jansen, H.M., Lutter, R., Alberts, C., Kobesen, J., de Vries, I.J. and Out, T.A. (1992) "Surfactant protein A in bronchoalveolar lavage fluid", *The Journal of laboratory and clinical medicine,* vol. 120, no. 2, pp. 252-263.

van de Wetering, J.K., van Eijk, M., van Golde, L.M., Hartung, T., van Strijp, J.A. and Batenburg, J.J. (2001) "Characteristics of surfactant protein A and D binding to lipoteichoic acid and peptidoglycan, 2 major cell wall components of gram-positive bacteria", *The Journal of infectious diseases,* vol. 184, no. 9, pp. 1143-1151.

van de Wetering, J.K., van Remoortere, A., Vaandrager, A.B., Batenburg, J.J., van Golde, L.M., Hokke, C.H. and van Hellemond, J.J. (2004) "Surfactant protein D binding to terminal alpha1-3-linked fucose residues and to Schistosoma mansoni", *American journal of respiratory cell and molecular biology,* vol. 31, no. 5, pp. 565-572.

van Emmerik, L.C., Kuijper, E.J., Fijen, C.A., Dankert, J. and Thiel, S. (1994) "Binding of mannan-binding protein to various bacterial pathogens of meningitis", *Clinical and experimental immunology,* vol. 97, no. 3, pp. 411-416.

van Iwaarden, F., Welmers, B., Verhoef, J., Haagsman, H.P. and van Golde, L.M. (1990) "Pulmonary surfactant protein A enhances the host-defense mechanism of rat alveolar macrophages", *American journal of respiratory cell and molecular biology,* vol. 2, no. 1, pp. 91-98.

Van Iwaarden, J.F., Pikaar, J.C., Storm, J., Brouwer, E., Verhoef, J., Oosting, R.S., van Golde, L.M. and van Strijp, J.A. (1994) "Binding of surfactant protein A to the lipid A moiety of bacterial lipopolysaccharides", *The Biochemical journal,* vol. 303 (Pt 2), no. Pt 2, pp. 407-411.

van Iwaarden, J.F., van Strijp, J.A., Ebskamp, M.J., Welmers, A.C., Verhoef, J. and van Golde, L.M. (1991) "Surfactant protein A is opsonin in phagocytosis of herpes simplex virus type 1 by rat alveolar macrophages", *The American Journal of Physiology,* vol. 261, no. 2 Pt 1, pp. L204-9.

van Iwaarden, J.F., van Strijp, J.A., Visser, H., Haagsman, H.P., Verhoef, J. and van Golde, L.M. (1992) "Binding of surfactant protein A (SP-A) to herpes simplex virus type 1-infected cells is mediated by the carbohydrate moiety of SP-A", *The Journal of biological chemistry,* vol. 267, no. 35, pp. 25039-25043.

van Rozendaal, B.A., van Spriel, A.B., van De Winkel, J.G. and Haagsman, H.P. (2000) "Role of pulmonary surfactant protein D in innate defense against Candida albicans", *The Journal of infectious diseases,* vol. 182, no. 3, pp. 917-922.

Vasta, G.R. (2009) "Roles of galectins in infection", *Nature reviews.Microbiology,* vol. 7, no. 6, pp. 424-438.

Vasta, G.R., Ahmed, H., Nita-Lazar, M., Banerjee, A., Pasek, M., Shridhar, S., Guha, P. and Fernandez-Robledo, J.A. (2012) "Galectins as self/non-self recognition receptors in innate and adaptive immunity: an unresolved paradox", *Frontiers in immunology,* vol. 3, pp. 199.

von Kockritz-Blickwede, M., Goldmann, O., Thulin, P., Heinemann, K., Norrby-Teglund, A., Rohde, M. and Medina, E. (2008) "Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation", *Blood,* vol. 111, no. 6, pp. 3070-3080.

Voorhout, W.F., Veenendaal, T., Kuroki, Y., Ogasawara, Y., van Golde, L.M. and Geuze, H.J. (1992) "Immunocytochemical localization of surfactant protein D (SP-D) in type II cells, Clara cells, and alveolar macrophages of rat lung", *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,* vol. 40, no. 10, pp. 1589-1597.

Vuk-Pavlovic, Z., Standing, J.E., Crouch, E.C. and Limper, A.H. (2001) "Carbohydrate recognition domain of surfactant protein D mediates interactions with Pneumocystis carinii glycoprotein A", *American journal of respiratory cell and molecular biology,* vol. 24, no. 4, pp. 475-484.

Walenkamp, A.M., Verheul, A.F., Scharringa, J. and Hoepelman, I.M. (1999) "Pulmonary surfactant protein A binds to Cryptococcus neoformans without promoting phagocytosis", *European journal of clinical investigation,* vol. 29, no. 1, pp. 83-92.

Wang, J.Y., Kishore, U., Lim, B.L., Strong, P. and Reid, K.B. (1996) "Interaction of human lung surfactant proteins A and D with mite (Dermatophagoides pteronyssinus) allergens", *Clinical and experimental immunology,* vol. 106, no. 2, pp. 367-373.

Wardini, A.B., Guimaraes-Costa, A.B., Nascimento, M.T., Nadaes, N.R., Danelli, M.G., Mazur, C., Benjamim, C.F., Saraiva, E.M. and Pinto-da-Silva, L.H. (2010) "Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus", *The Journal of general virology,* vol. 91, no. Pt 1, pp. 259-264.

Weaver, T.E. and Whitsett, J.A. (1991) "Function and regulation of expression of pulmonary surfactant-associated proteins", *The Biochemical journal,* vol. 273(Pt 2), no. Pt 2, pp. 249-264.

Weikert, L.F., Edwards, K., Chroneos, Z.C., Hager, C., Hoffman, L. and Shepherd, V.L. (1997) "SP-A enhances uptake of bacillus Calmette-Guerin by macrophages through a specific SP-A receptor", *The American Journal of Physiology,* vol. 272, no. 5 Pt 1, pp. L989-95.

White, R.T., Damm, D., Miller, J., Spratt, K., Schilling, J., Hawgood, S., Benson, B. and Cordell, B. (1985) "Isolation and characterization of the human pulmonary surfactant apoprotein gene", *Nature,* vol. 317, no. 6035, pp. 361-363.

Williams, M.D., Wright, J.R., March, K.L. and Martin, W.J.,2nd (1996) "Human surfactant protein A enhances attachment of Pneumocystis carinii to rat alveolar macrophages", *American journal of respiratory cell and molecular biology,* vol. 14, no. 3, pp. 232-238.

Woodworth, B.A., Neal, J.G., Newton, D., Joseph, K., Kaplan, A.P., Baatz, J.E. and Schlosser, R.J. (2007) "Surfactant protein A and D in human sinus mucosa: a preliminary report", *ORL; journal for oto-rhino-laryngology and its related specialties,* vol. 69, no. 1, pp. 57-60.

Wright, J.R. (2005) "Immunoregulatory functions of surfactant proteins", *Nature reviews.Immunology,* vol. 5, no. 1, pp. 58-68.

Wright, J.R. (1997) "Immunomodulatory functions of surfactant", *Physiological Reviews,* vol. 77, no. 4, pp. 931-962.

Wright, J.R., Borchelt, J.D. and Hawgood, S. (1989) "Lung surfactant apoprotein SP-A (26-36 kDa) binds with high affinity to isolated alveolar type II cells", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 86, no. 14, pp. 5410-5414.

Wright, J.R. and Dobbs, L.G. (1991) "Regulation of pulmonary surfactant secretion and clearance", *Annual Review of Physiology,* vol. 53, pp. 395-414.

Wright, J.R., Wager, R.E., Hawgood, S., Dobbs, L. and Clements, J.A. (1987) "Surfactant apoprotein Mr = 26,000-36,000 enhances uptake of liposomes by type II cells", *The Journal of biological chemistry,* vol. 262, no. 6, pp. 2888-2894.

Wu, H., Kuzmenko, A., Wan, S., Schaffer, L., Weiss, A., Fisher, J.H., Kim, K.S. and McCormack, F.X. (2003) "Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability", *The Journal of clinical investigation,* vol. 111, no. 10, pp. 1589-1602.

Wu, Y.P., Liu, Z.H., Wei, R., Pan, S.D., Mao, N.Y., Chen, B., Han, J.J., Zhang, F.S., Holmskov, U., Xia, Z.L., de Groot, P.G., Reid, K.B., Xu, W.B. and Sorensen, G.L. (2009) "Elevated plasma surfactant protein D (SP-D) levels and a direct correlation with anti-severe acute respiratory syndrome coronavirus-specific IgG antibody in SARS patients", *Scandinavian Journal of Immunology,* vol. 69, no. 6, pp. 508-515.

Yanaba, K., Hasegawa, M., Takehara, K. and Sato, S. (2004) "Comparative study of serum surfactant protein-D and KL-6 concentrations in patients with systemic sclerosis as markers for monitoring the activity of pulmonary fibrosis", *The Journal of rheumatology,* vol. 31, no. 6, pp. 1112-1120.

Yong, S.J., Vuk-Pavlovic, Z., Standing, J.E., Crouch, E.C. and Limper, A.H. (2003) "Surfactant protein D-mediated aggregation of Pneumocystis carinii impairs phagocytosis by alveolar macrophages", *Infection and immunity,* vol. 71, no. 4, pp. 1662-1671.

Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I. and Simon, H.U. (2009) "Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps", *Cell death and differentiation,* vol. 16, no. 11, pp. 1438-1444.

Zasloff, M. (2002) "Antimicrobial peptides of multicellular organisms", *Nature,* vol. 415, no. 6870, pp. 389-395.

Zimmerman, P.E., Voelker, D.R., McCormack, F.X., Paulsrud, J.R. and Martin, W.J.,2nd (1992) "120-kD surface glycoprotein of Pneumocystis carinii is a ligand for surfactant protein A", *The Journal of clinical investigation,* vol. 89, no. 1, pp. 143-149.

Basu, S., Binder, R.J., Ramalingam, T. and Srivastava, P.K. (2001) 'CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin', *Immunity,* 14(3), pp. 303-313.

Baumann, M.A. and Paul, C.C. (1998) 'The AML14 and AML14.3D10 cell lines: a long-overdue model for the study of eosinophils and more', *Stem cells (Dayton, Ohio),* 16(1), pp. 16-24.

Botto, M., Dell'Agnola, C., Bygrave, A.E., Thompson, E.M., Cook, H.T., Petry, F., Loos, M., Pandolfi, P.P. and Walport, M.J. (1998) 'Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies', *Nature genetics,* 19(1), pp. 56-59.

Bousquet, J., Jeffery, P.K., Busse, W.W., Johnson, M. and Vignola, A.M. (2000) 'Asthma. From bronchoconstriction to airways inflammation and remodeling', *American journal of respiratory and critical care medicine,* 161(5), pp. 1720-1745.

Brinker, K.G., Martin, E., Borron, P., Mostaghel, E., Doyle, C., Harding, C.V. and Wright, J.R. (2001) 'Surfactant protein D enhances bacterial antigen presentation by bone marrow-derived dendritic cells', *American journal of physiology.Lung cellular and molecular physiology,* 281(6), pp. L1453-63.

Chung, C.D., Patel, V.P., Moran, M., Lewis, L.A. and Miceli, M.C. (2000) 'Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction', *Journal of immunology (Baltimore, Md.: 1950),* 165(7), pp. 3722-3729.

Clark, H., Palaniyar, N., Strong, P., Edmondson, J., Hawgood, S. and Reid, K.B. (2002) 'Surfactant protein D reduces alveolar macrophage apoptosis in vivo', *Journal of immunology (Baltimore, Md.: 1950),* 169(6), pp. 2892-2899.

Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L. and Gregory, C.D. (1998) 'Human CD14 mediates recognition and phagocytosis of apoptotic cells', *Nature,* 392(6675), pp. 505-509.

Duncan, C.J., Lawrie, A., Blaylock, M.G., Douglas, J.G. and Walsh, G.M. (2003) 'Reduced eosinophil apoptosis in induced sputum correlates with asthma severity', *The European respiratory journal,* 22(3), pp. 484-490.

Eggleton, P., Lieu, T.S., Zappi, E.G., Sastry, K., Coburn, J., Zaner, K.S., Sontheimer, R.D., Capra, J.D., Ghebrehiwet, B. and Tauber, A.I. (1994) 'Calreticulin is released from activated neutrophils and binds to C1q and mannan-binding protein', *Clinical immunology and immunopathology,* 72(3), pp. 405-409.

Eggleton, P., Tenner, A.J. and Reid, K.B. (2000) 'C1q receptors', *Clinical and experimental immunology,* 120(3), pp. 406-412.

Epstein, J., Eichbaum, Q., Sheriff, S. and Ezekowitz, R.A.B. (1996) 'The collectins in innate immunity', *Current opinion in immunology,* 8(1), pp. 29-35.

Fadok, V.A., Bratton, D.L., Rose, D.M., Pearson, A., Ezekewitz, R.A. and Henson, P.M. (2000) 'A receptor for phosphatidylserine-specific clearance of apoptotic cells', *Nature,* 405(6782), pp. 85-90.

Fadok, V.A., de Cathelineau, A., Daleke, D.L., Henson, P.M. and Bratton, D.L. (2001) 'Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts', *The Journal of biological chemistry,* 276(2), pp. 1071-1077.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L. and Henson, P.M. (1992) 'Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages', *Journal of immunology (Baltimore, Md.: 1950),* 148(7), pp. 2207-2216.

Fulkerson, P.C. and Rothenberg, M.E. (2013) 'Targeting eosinophils in allergy, inflammation and beyond', *Nature reviews.Drug discovery,* 12(2), pp. 117-129.

Giembycz, M.A. and Lindsay, M.A. (1999a) 'Pharmacology of the eosinophil', *Pharmacological reviews,* 51(2), pp. 213-340.

Giembycz, M.A. and Lindsay, M.A. (1999b) 'Pharmacology of the eosinophil', *Pharmacological reviews,* 51(2), pp. 213-340.

Haczku, A., Cao, Y., Vass, G., Kierstein, S., Nath, P., Atochina-Vasserman, E.N., Scanlon, S.T., Li, L., Griswold, D.E., Chung, K.F., Poulain, F.R., Hawgood, S., Beers, M.F. and Crouch, E.C. (2006) 'IL-4 and IL-13 form a negative feedback circuit with surfactant protein-D in the allergic airway response', *Journal of immunology (Baltimore, Md.: 1950),* 176(6), pp. 3557-3565.

Halwani, R., Vazquez-Tello, A., Sumi, Y., Pureza, M.A., Bahammam, A., Al-Jahdali, H., Soussi-Gounni, A., Mahboub, B., Al-Muhsen, S. and Hamid, Q. (2013) 'Eosinophils induce airway smooth muscle cell proliferation', *Journal of clinical immunology,* 33(3), pp. 595-604.

Haslett, C., Savill, J.S., Whyte, M.K.B., Stern, M., Dransfield, I. and Meagher, L.C. (1994) 'Granulocyte Apoptosis and the Control of Inflammation', *Philosophical Transactions of the Royal Society of London.Series B: Biological Sciences,* 345(1313), pp. 327-333.

Hirashima, M., Kashio, Y., Nishi, N., Yamauchi, A., Imaizumi, T.A., Kageshita, T., Saita, N. and Nakamura, T. (2004) 'Galectin-9 in physiological and pathological conditions', *Glycoconjugate journal,* 19(7-9), pp. 593-600.

Hoffmann, H.J., Dahl, C., Schiotz, P.O., Berglund, L. and Dahl, R. (2003) 'Lectins interact differentially with purified human eosinophils, cultured cord blood-derived mast cells and the myeloid leukaemic cell line AML14.3D10: induction of interleukin-4 secretion is conserved among granulocytes, but is not proportional to agglutination or lectin-glycoprotein interaction', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 33(7), pp. 930-935.

Holgate, S.T. (1999) 'The epidemic of allergy and asthma', *Nature,* 402(6760 Suppl), pp. B2-4.

Holmskov, U., Lawson, P., Teisner, B., Tornoe, I., Willis, A.C., Morgan, C., Koch, C. and Reid, K.B. (1997) 'Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein-D binding molecule', *The Journal of biological chemistry,* 272(21), pp. 13743-13749.

Holmskov, U., Malhotra, R., Sim, R.B. and Jensenius, J.C. (1994) 'Collectins: collagenous C-type lectins of the innate immune defense system', *Immunology today,* 15(2), pp. 67-74.

Kankaanranta, H., Lindsay, M.A., Giembycz, M.A., Zhang, X., Moilanen, E. and Barnes, P.J. (2000) 'Delayed eosinophil apoptosis in asthma', *The Journal of allergy and clinical immunology,* 106(1 Pt 1), pp. 77-83.

Kita, H. (2013) 'Eosinophils: multifunctional and distinctive properties', *International archives of allergy and immunology,* 161 Suppl 2, pp. 3-9.

Koenderman, L., van der Bruggen, T., Schweizer, R.C., Warringa, R.A., Coffer, P., Caldenhoven, E., Lammers, J.W. and Raaijmakers, J.A. (1996) 'Eosinophil priming by cytokines: from cellular signal to in vivo modulation', *The European respiratory journal.Supplement,* 22, pp. 119s-125s.

Koopmans, J.G., van der Zee, J.S., Krop, E.J., Lopuhaa, C.E., Jansen, H.M. and Batenburg, J.J. (2004) 'Serum surfactant protein D is elevated in allergic patients', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 34(12), pp. 1827-1833.

Korb, L.C. and Ahearn, J.M. (1997) 'C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited', *Journal of immunology (Baltimore, Md.: 1950),* 158(10), pp. 4525-4528.

Lee, Y.S., Chen, P.W., Tsai, P.J., Su, S.H. and Liao, P.C. (2006) 'Proteomics analysis revealed changes in rat bronchoalveolar lavage fluid proteins associated with oil mist exposure', *Proteomics,* 6(7), pp. 2236-2250.

Leonidas, D.D., Elbert, B.L., Zhou, Z., Leffler, H., Ackerman, S.J. and Acharya, K.R. (1995) 'Crystal structure of human Charcot-Leyden crystal protein, an eosinophil lysophospholipase, identifies it as a new member of the carbohydrate-binding family of galectins', *Structure (London, England : 1993),* 3(12), pp. 1379-1393.

Luna-Gomes, T., Bozza, P.T. and Bandeira-Melo, C. (2013) 'Eosinophil recruitment and activation: the role of lipid mediators', *Frontiers in pharmacology,* 4, pp. 27.

Madan, T., Kishore, U., Singh, M., Strong, P., Hussain, E.M., Reid, K.B. and Sarma, P.U. (2001) 'Protective role of lung surfactant protein D in a murine model of invasive pulmonary aspergillosis', *Infection and immunity,* 69(4), pp. 2728-2731.

Mahajan, L., Gautam, P., Dodagatta-Marri, E., Madan, T. and Kishore, U. (2014) 'Surfactant protein SP-D modulates activity of immune cells: proteomic profiling of its interaction with eosinophilic cells', *Expert review of proteomics,* .

Mahajan, L., Madan, T., Kamal, N., Singh, V.K., Sim, R.B., Telang, S.D., Ramchand, C.N., Waters, P., Kishore, U. and Sarma, P.U. (2008) 'Recombinant surfactant protein-D selectively increases apoptosis in eosinophils of allergic asthmatics and enhances uptake of apoptotic eosinophils by macrophages', *International immunology,* 20(8), pp. 993-1007.

Mahajan, L., Pandit, H., Madan, T., Gautam, P., Yadav, A.K., Warke, H., Sundaram, C.S., Sirdeshmukh, R., Sarma, P.U., Kishore, U. and Surolia, A. (2013a) 'Human surfactant protein D alters oxidative stress and HMGA1 expression to induce p53 apoptotic pathway in eosinophil leukemic cell line', *PloS one,* 8(12), pp. e85046.

Mahajan, L., Pandit, H., Madan, T., Gautam, P., Yadav, A.K., Warke, H., Sundaram, C.S., Sirdeshmukh, R., Sarma, P.U., Kishore, U. and Surolia, A. (2013b) 'Human surfactant protein D alters oxidative stress and HMGA1 expression to induce p53 apoptotic pathway in eosinophil leukemic cell line', *PloS one,* 8(12), pp. e85046.

Maino, V.C., Suni, M.A. and Ruitenberg, J.J. (1995) 'Rapid flow cytometric method for measuring lymphocyte subset activation', *Cytometry,* 20(2), pp. 127-133.

Malhotra, R., Thiel, S., Reid, K.B. and Sim, R.B. (1990) 'Human leukocyte C1q receptor binds other soluble proteins with collagen domains', *The Journal of experimental medicine,* 172(3), pp. 955-959.

Michalak, M., Corbett, E.F., Mesaeli, N., Nakamura, K. and Opas, M. (1999) 'Calreticulin: one protein, one gene, many functions', *The Biochemical journal,* 344 Pt 2, pp. 281-292.

Munitz, A., Bachelet, I., Eliashar, R., Moretta, A., Moretta, L. and Levi-Schaffer, F. (2006) 'The inhibitory receptor IRp60 (CD300a) suppresses the effects of IL-5, GM-CSF, and eotaxin on human peripheral blood eosinophils', *Blood,* 107(5), pp. 1996-2003.

Ogden, C.A., deCathelineau, A., Hoffmann, P.R., Bratton, D., Ghebrehiwet, B., Fadok, V.A. and Henson, P.M. (2001) 'C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells', *The Journal of experimental medicine,* 194(6), pp. 781-795.

Ohya, M., Nishitani, C., Sano, H., Yamada, C., Mitsuzawa, H., Shimizu, T., Saito, T., Smith, K., Crouch, E. and Kuroki, Y. (2006) 'Human pulmonary surfactant protein D binds the extracellular domains of Toll-like receptors 2 and 4 through the carbohydrate recognition domain by a mechanism different from its binding to phosphatidylinositol and lipopolysaccharide', *Biochemistry,* 45(28), pp. 8657-8664.

Palaniyar, N., Clark, H., Nadesalingam, J., Hawgood, S. and Reid, K.B. (2003) 'Surfactant protein D binds genomic DNA and apoptotic cells, and enhances their clearance, in vivo', *Annals of the New York Academy of Sciences,* 1010, pp. 471-475.

Paul, C.C., Mahrer, S., McMannama, K. and Baumann, M.A. (1997) 'Autocrine activation of the IL-3/GM-CSF/IL-5 signaling pathway in leukemic cells', *American Journal of Hematology,* 56(2), pp. 79-85.

Rosenberg, H.F., Dyer, K.D. and Foster, P.S. (2013) 'Eosinophils: changing perspectives in health and disease', *Nature reviews.Immunology,* 13(1), pp. 9-22.

Ruth, J.H., Esnault, S., Jarzembowski, J.A. and Malter, J.S. (1999) 'Calcium ionophore upregulation of AUUUA-specific binding protein activity is contemporaneous with granulocyte macrophage colony-stimulating factor messenger RNA stabilization in AML14.3D10 cells', *American journal of respiratory cell and molecular biology,* 21(5), pp. 621-628.

Sano, H., Chiba, H., Iwaki, D., Sohma, H., Voelker, D.R. and Kuroki, Y. (2000) 'Surfactant proteins A and D bind CD14 by different mechanisms', *The Journal of biological chemistry,* 275(29), pp. 22442-22451.

Savill, J. and Fadok, V. (2000) 'Corpse clearance defines the meaning of cell death', *Nature,* 407(6805), pp. 784-788.

Schagat, T.L., Wofford, J.A., Greene, K.E. and Wright, J.R. (2003) 'Surfactant protein A differentially regulates peripheral and inflammatory neutrophil chemotaxis', *American journal of physiology.Lung cellular and molecular physiology,* 284(1), pp. L140-7.

Schagat, T.L., Wofford, J.A. and Wright, J.R. (2001) 'Surfactant protein A enhances alveolar macrophage phagocytosis of apoptotic neutrophils', *Journal of immunology (Baltimore, Md.: 1950),* 166(4), pp. 2727-2733.

Sedgwick, J.B., Geiger, K.M. and Busse, W.W. (1990) 'Superoxide generation by hypodense eosinophils from patients with asthma', *The American Review of Respiratory Disease,* 142(1), pp. 120-125.

Shen, Z.J., Esnault, S. and Malter, J.S. (2005) 'The peptidyl-prolyl isomerase Pin1 regulates the stability of granulocyte-macrophage colony-stimulating factor mRNA in activated eosinophils', *Nature immunology,* 6(12), pp. 1280-1287.

Shult, P.A., Lega, M., Jadidi, S., Vrtis, R., Warner, T., Graziano, F.M. and Busse, W.W. (1988) 'The presence of hypodense eosinophils and diminished chemiluminescence response in asthma', *The Journal of allergy and clinical immunology,* 81(2), pp. 429-437.

Singh, M., Madan, T., Waters, P., Parida, S.K., Sarma, P.U. and Kishore, U. (2003) 'Protective effects of a recombinant fragment of human surfactant protein D in a murine model of pulmonary hypersensitivity induced by dust mite allergens', *Immunology letters,* 86(3), pp. 299-307.

Somersan, S. and Bhardwaj, N. (2001) 'Tethering and tickling: a new role for the phosphatidylserine receptor', *The Journal of cell biology,* 155(4), pp. 501-504.

Spencer, L.A., Szela, C.T., Perez, S.A., Kirchhoffer, C.L., Neves, J.S., Radke, A.L. and Weller, P.F. (2009) 'Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially', *Journal of leukocyte biology,* 85(1), pp. 117-123.

Takeda, K., Miyahara, N., Rha, Y.H., Taube, C., Yang, E.S., Joetham, A., Kodama, T., Balhorn, A.M., Dakhama, A., Duez, C., Evans, A.J., Voelker, D.R. and Gelfand, E.W. (2003) 'Surfactant protein D regulates airway function and allergic inflammation through modulation of macrophage function', *American journal of respiratory and critical care medicine,* 168(7), pp. 783-789.

Taylor, P.R., Carugati, A., Fadok, V.A., Cook, H.T., Andrews, M., Carroll, M.C., Savill, J.S., Henson, P.M., Botto, M. and Walport, M.J. (2000) 'A Hierarchical Role for Classical Pathway Complement Proteins in the Clearance of Apoptotic Cells in Vivo', *The Journal of experimental medicine,* 192(3), pp. 359-366.

Teder, P., Vandivier, R.W., Jiang, D., Liang, J., Cohn, L., Pure, E., Henson, P.M. and Noble, P.W. (2002) 'Resolution of lung inflammation by CD44', *Science (New York, N.Y.),* 296(5565), pp. 155-158.

Tedla, N., Bandeira-Melo, C., Tassinari, P., Sloane, D.E., Samplaski, M., Cosman, D., Borges, L., Weller, P.F. and Arm, J.P. (2003) 'Activation of human eosinophils through leukocyte immunoglobulin-like receptor 7', *Proceedings of the National Academy of Sciences of the United States of America,* 100(3), pp. 1174-1179.

Tomonaga, M., Gasson, J.C., Quan, S.G. and Golde, D.W. (1986) 'Establishment of eosinophilic sublines from human promyelocytic leukemia (HL-60) cells: demonstration of multipotentiality and single-lineage commitment of HL-60 stem cells', *Blood,* 67(5), pp. 1433-1441.

Uhm, T.G., Kim, B.S. and Chung, I.Y. (2012) 'Eosinophil development, regulation of eosinophil-specific genes, and role of eosinophils in the pathogenesis of asthma', *Allergy, asthma & immunology research,* 4(2), pp. 68-79.

Vandivier, R.W., Ogden, C.A., Fadok, V.A., Hoffmann, P.R., Brown, K.K., Botto, M., Walport, M.J., Fisher, J.H., Henson, P.M. and Greene, K.E. (2002) 'Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex', *Journal of immunology (Baltimore, Md.: 1950),* 169(7), pp. 3978-3986.

Venge, P. (2010) 'The eosinophil and airway remodelling in asthma', *The clinical respiratory journal,* 4 Suppl 1, pp. 15-19.

von Bredow, C., Hartl, D., Schmid, K., Schabaz, F., Brack, E., Reinhardt, D. and Griese, M. (2006) 'Surfactant protein D regulates chemotaxis and degranulation of human eosinophils', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 36(12), pp. 1566-1574.

Von Bredow, C., Hartl, D., Schmid, K., Schabaz, F., Brack, E., Reinhardt, D. and Griese, M. (2006) 'Surfactant protein D regulates chemotaxis and degranulation of human eosinophils', *Clinical & Experimental Allergy,* 36(12), pp. 1566-1574.

Walsh, G.M. (2001) 'Eosinophil granule proteins and their role in disease', *Current opinion in hematology,* 8(1), pp. 28-33.

Wang, Q., Teder, P., Judd, N.P., Noble, P.W. and Doerschuk, C.M. (2002) 'CD44 deficiency leads to enhanced neutrophil migration and lung injury in Escherichia coli pneumonia in mice', *The American journal of pathology,* 161(6), pp. 2219-2228.

Wardlaw, A.J., Brightling, C., Green, R., Woltmann, G. and Pavord, I. (2000) 'Eosinophils in asthma and other allergic diseases', *British medical bulletin,* 56(4), pp. 985-1003.

Winqvist, I., Olofsson, T. and Olsson, I. (1984) 'Mechanisms for eosinophil degranulation; release of the eosinophil cationic protein', *Immunology,* 51(1), pp. 1-8.

Wong, C.K., Hu, S., Cheung, P.F. and Lam, C.W. (2010) 'Thymic stromal lymphopoietin induces chemotactic and prosurvival effects in eosinophils: implications in allergic inflammation', *American journal of respiratory cell and molecular biology,* 43(3), pp. 305-315.

Wyllie, A.H., Kerr, J.F.R. and Currie, A.R. (1980) 'Cell Death: The Significance of Apoptosis', in *International Review of Cytology.* Academic Press, pp. 251-306.

Yagisawa, M., Yuo, A., Yonemaru, M., Imajoh-Ohmi, S., Kanegasaki, S., Yazaki, Y. and Takaku, F. (1996) 'Superoxide release and NADPH oxidase components in mature human phagocytes: correlation between functional capacity and amount of functional proteins', *Biochemical and biophysical research communications,* 228(2), pp. 510-516.

Zhao, J., Zhu, H., Wong, C.H., Leung, K.Y. and Wong, W.S. (2005) 'Increased lungkine and chitinase levels in allergic airway inflammation: a proteomics approach', *Proteomics,* 5(11), pp. 2799-2807.

Full length SP-D sequence results

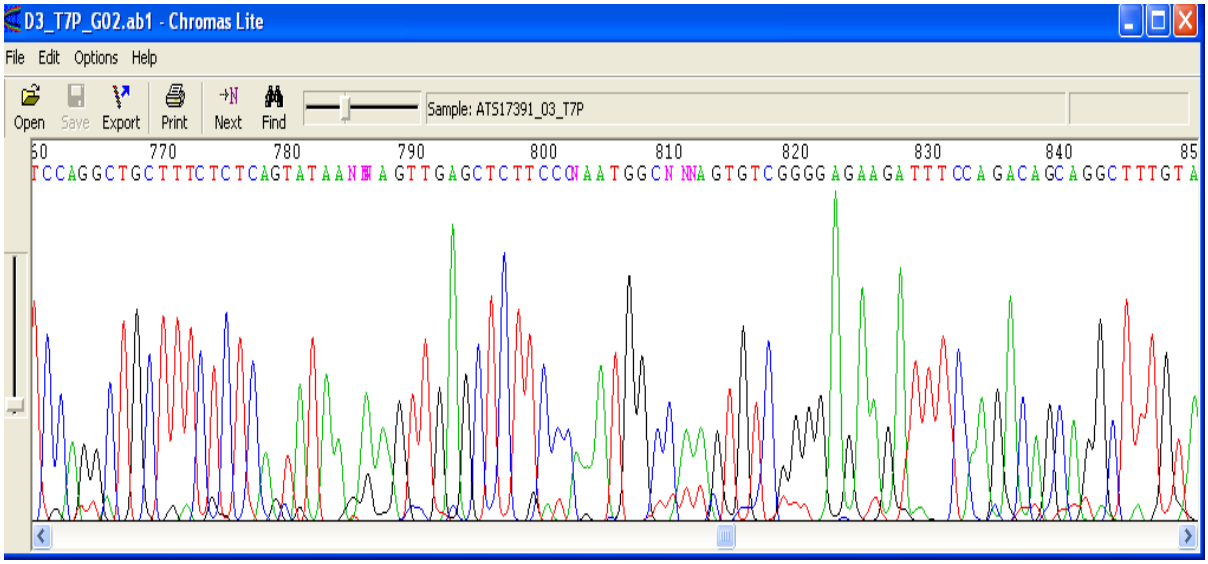
>gi|61699225:44-1171 Homo sapiens surfactant protein D (SFTPD), mRNA

Bases matched within the forward sequencing!

ATGCTGCTCTTCCTCCTCTCTGCACTGGTCCTGCTCACACAGCCCCTGGGCTACCTG
GAAGCAGAAATGAAGACCTACTCCCAGAGAACAATGCCCAGTGCTTGCACCCTGGTC
ATGTGTAGCTCAGTGGAGAGTGGCCTGCCTGGTCGCGATGGACGGGATGGGAGAGAG
GGCCCTCGGGGCGAGAAGGGGGACCCAGGTTTGCCAGGAGCTGCAGGGCAAGCAGGG
ATGCCTGGACAAGCTGGCCCAGTTGGGCCCAAAGGGGACAATGGCTCTGTTGGAGAA
CCTGGACCAAAGGGAGACACTGGGCCAAGTGGACCTCCAGGACCTCCCGGTGTGCCT
GGTCCAGCTGGAAGAGAAGGTCCCCTGGGGAAGCAGGGGAACATAGGACCTCAGGGC
AAGCCAGGCCCAAAAGGAGAAGCTGGGCCCAAAGGAGAAGTAGGTGCCCCAGGCATG
CAGGGCTCGGCAGGGGCAAGAGGCCTCGCAGGCCCTAAGGGAGAGCGAGGTGTCCCT
GGTGAGCGTGGAGTCCCTGGAAACACAGGGGCAGCAGGGTCTGCTGGAGCCATGGGT
CCCCAGGGAAGTCCAGGTGCCAGGGGACCCCCGGGATTGAAGGGGGACAAAGGCATT
CCTGGAGACAAAGGAGCAAAGGGAGAAAGTGGGCTTCCAGATGTTGCTTCTCTGAGG
CAGCAGGTTGAGGCCTTACAGGGACAAGTACAGCACCTCCAGGCTGCTTTCTCTCAG
TATAAGAAAGTTGAGCTCTTCCCAAATGGCCAAAGTGTCGGGGAGAAGATTTTCAAG
ACAGCAGGCTTTGTAAAACCATTTACGGAGGCACAGCTGCTGTGCACACAGGCTGGT
GGACAGTTGGCCTCTCCACGCTCTGCCGCTGAGAATGCCGCCTTGCAACAGCTGGTC
GTAGCTAAGAACGAGGCTGCTTTCCTGAGCATGACTGATTCCAAGACAGAGGGCAAG
TTCACCTACCCCACAGGAGAGTCCCTGGTCTATTCCAACTGGGCCCCAGGGGAGCCC
AACGATGATGGCGGGTCAGAGGACTGTGTGGAGATCTTCACCAATGGCAAGTGGAAT
GACAGGGCTTGTGGAGAAAAGCGTCTTGTGGTCTGCGAGTTCTGA

Bases matched within the forward sequencing!

ATGCTGCTCTTCCTCCTCTCTGCACTGGTCCTGCTCACACAGCCCCTGGGCTACCTG
GAAGCAGAAATGAAGACCTACTCCCAGAGAACAATGCCCAGTGCTTGCACCCTGGTC
ATGTGTAGCTCAGTGGAGAGTGGCCTGCCTGGTCGCGATGGACGGGATGGGAGAGAG
GGCCCTCGGGGCGAGAAGGGGGACCCAGGTTTGCCAGGAGCTGCAGGGCAAGCAGGG
ATGCCTGGACAAGCTGGCCCAGTTGGGCCCAAAGGGGACAATGGCTCTGTTGGAGAA
CCTGGACCAAAGGGAGACACTGGGCCAAGTGGACCTCCAGGACCTCCCGGTGTGCCT
GGTCCAGCTGGAAGAGAAGGTCCCCTGGGGAAGCAGGGGAACATAGGACCTCAGGGC
AAGCCAGGCCCAAAAGGAGAAGCTGGGCCCAAAGGAGAAGTAGGTGCCCCAGGCATG
CAGGGCTCGGCAGGGGCAAGAGGCCTCGCAGGCCCTAAGGGAGAGCGAGGTGTCCCT
GGTGAGCGTGGAGTCCCTGGAAACACAGGGGCAGCAGGGTCTGCTGGAGCCATGGGT
CCCCAGGGAAGTCCAGGTGCCAGGGGACCCCCGGGATTGAAGGGGGACAAAGGCATT
CCTGGAGACAAAGGAGCAAAGGGAGAAAGTGGGCTTCCAGATGTTGCTTCTCTGAGG
CAGCAGGTTGAGGCCTTACAGGGACAAGTACAGCACCTCCAGGCTGCTTTCTCTCAG
TATAAGAAAGTTGAGCTCTTCCCAAATGGCCAAAGTGTCGGGGAGAAGATTTTCAAG
ACAGCAGGCTTTGTAAAACCATTTACGGAGGCACAGCTGCTGTGCACACAGGCTGGT
GGACAGTTGGCCTCTCCACGCTCTGCCGCTGAGAATGCCGCCTTGCAACAGCTGGTC
GTAGCTAAGAACGAGGCTGCTTTCCTGAGCATGACTGATTCCAAGACAGAGGGCAAG
TTCACCTACCCCACAGGAGAGTCCCTGGTCTATTCCAACTGGGCCCCAGGGGAGCCC
AACGATGATGGCGGGTCAGAGGACTGTGTGGAGATCTTCACCAATGGCAAGTGGAAT
GACAGGGCTTGTGGAGAAAAGCGTCTTGTGGTCTGCGAGTTCTGA


D3_T7P_G02.ab1 - Chromas Lite
File Edit Options Help
Open Save Export Print Next Find
Sample: AT517391_03_T7P
760 770 780 790 800 810 820 830 840 85
TCCAGGCTGCTTTCTCTCAGTATAANNAGTTGAGCTCTTCCCNAATGGCNNNAGTGTCGGGGGAGAAGATTTCCAGACAGCAGGCTTTGTA