



Modulation of immune cell functions by human lung surfactant protein SP-D in allergic asthma

A thesis submitted for the degree of Doctorate of Philosophy

by

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Abstract

Lung surfactant protein D (SP-D) is a soluble pattern recognition and innate immune molecule, which has been shown to be protective against lung infection, allergy, asthma and inflammation. SP-D is composed of an N-terminal collagen region and a homotrimeric, C-terminal carbohydrate binding domain (CRD). A recombinant form of trimeric CRD region (rhSP-D) has been shown to offer protection against asthma and inflammation in murine models by bringing down IgE levels, eosinophilia, and causing T helper cell polarisation from a pathogenic Th2 to a protective Th1 phenotype. Thus, rhSP-D can provide a therapeutic effect by dampening asthmatic symptoms in mice. The therapeutic mechanisms include inhibition of allergen-IgE binding and histamine release by sensitized mast cells, downregulation of allergen/antigen-specific IgG and IgE antibodies, pulmonary and peripheral eosinophilia, a shift from Th2 to Th1 cytokine response, interference with airway remodelling processes, and apoptosis- induction in sensitised eosinophils from allergic patients.

The majority of the *ex vivo* and *in vivo* studies where a therapeutic effect of rhSP-D has been reported can not be explained by hitherto described candidate receptor involvement, especially CD91-calreticulin complex that requires collagen region for its cellular response. Thus, it is pertinent to examine at the cellular and molecular level how a trimeric lectin domain of human SP-D modulates immune cells. This was achieved by firstly expressing, purifying and characterising the recombinant rhSP-D and examining the interaction of rhSP-D with various immune cells such as macrophages, which are potent antigen presenting cells and play a crucial role in the maintenance of the inflammatory and humoral response to allergens. The highlight of this study is the demonstration that rhSP-D interferes with the co-operative binding of allergen-IgE complexes to B cells, and also downregulates expression of CD23, a low affinity IgE receptor (FcεRII), found on B cells. This suggests that inhibition of IgE-facilitated antigen presentation may represent a mechanism whereby SP-D suppresses Th2-driven allergic inflammation. In addition, this study is also the first to establish the calcium-dependent interactions between rhSP-D, CD23 and CD21. The possibility of formation of a trimolecular complex on the B cell surface may account for the suppression of IgE in therapeutic murine models since rhSP-D may interfere with CD21-CD23 mediated IgE production by primed B cells.

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Abbreviations

α -	Anti-
μ g	Microgram
Afu	<i>Aspergillus fumigatus</i>
AHR	Airway hyperreactivity
APC	Antigen presenting cell
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CCPM	Corrected counts per minute
cDNA	Complementary DNA
CNBr	Cyanogen bromide
COPD	Chronic obstructive pulmonary disease
CRD	Carbohydrate recognition domain
CRT	Calreticulin
CRPMI	Complete RPMI
DAB	3,3'-Diaminobenzidine
DAPI	Diamidino-phenylindole
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
Derp	<i>Dermatophagoides pteronyssinus</i>
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
DPPC	Dipalmitoylphosphatidylcholine
DVS	Divinyl sulfone
EBV	Epstein–Barr virus
ECP	Eosinophil cationic protein
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EPO	Eosinophil peroxidase
FAP	Facilitated antigen presentation
FAB	Facilitated allergen binding
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
HETE	Hydroxyeicosatetraenoic acids
HRP	Horseradish peroxidase
ICOSL	inducible costimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl β -D-1-thiogalactopyranoside

KO	Knock out
KDa	Kilo Dalton
LAL	Limulus amebocyte lysate
LB	Luria Broth
LT	Leukotriene
LPS	Lipopolysaccharide
Mac	Macrophage
MAP	multi-analyte panel
Mch	Methacholine
MBL	mannan-binding lectin
MBP	Major basic protein
MCP	Monocyte chemoattractant protein
Mdc	Myeloid dendritic cell
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
M.W	Molecular weight
mRNA	Messenger Ribonucleic acid
NFκB	Nuclear factor κ B
NKT cells	Natural killer T cells
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline (50 mM potassium phosphate; 150 mM NaCl; pH 7.2).
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll
PG	Prostaglandin
PHA	Phytohaemagglutinin
Phlp	<i>Phleum pratense</i>
PMSF	Phenylmethylsulfonyl fluoride
qPCR	quantitative polymerase chain reaction
RBC	Red blood cells
RNA	Ribonucleic acid
RNase	Ribonuclease
rhSP-D	Recombinant Surfactant Protein D
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RQ	Relative quantification
SCR	Short consensus repeat
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SEM	Standard error mean
SP-D	Surfactant Protein D
SIRP-α	Signal-regulatory protein alpha
SRS	Slow reacting substance
TCR	T cell receptor
TGF-β	Transforming growth factor-β

Th	T helper
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor alpha
5-HPETE	5-hydroperoxyeicosatetraenoic acid

Publications

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Chapter 1



Introduction

1.1 Collectins

Collectins are C-type lectins, which are structurally organised into an N-terminal triple-helical collagen-like domain and a carbohydrate recognition domain (CRD) that multimerise to yield very large structures (Thiel and Reid, 1989, Crouch, 1998, Jakel et al., 2013). The ability of collectins to bind to specific patterns of carbohydrates (neutral sugars), found on the surface of a range of microorganisms, is mediated by interactions of the CRD domain with the terminal monosaccharide residues that are distributed spatially in a pattern characteristic of microbial surfaces, and which, therefore, allow discrimination between self and non-self (Weis et al., 1998, Lu et al., 2002, Jakel et al., 2013). Binding to collectins can lead to direct agglutination or neutralization of microorganisms, opsonisation in order to present bound microbes directly to phagocytes (Lu, 1997), or complement activation via the lectin pathway (in the case of MBL only) (Wallis, 2002, Jakel et al., 2013). Consequently, collectins are considered important sugar pattern recognition molecules of the innate immune system that can interact directly with live pathogens, and therefore play important roles in the first line of defence against microbes. At present, collectin family is comprised of the following, mannan-binding lectin (MBL) (Kawasaki et al., 1983) two collectins only found in cattle; conglutinin and collectin-43 (CL-43) (Holmskov et al., 1993, Jensenius et al., 1994). Collectin liver 1 (CL-L1) (Kawai et al., 2002, Ohtani et al., 1999), CL-P1 (Selman et al., 2008, Ohtani et al., 2001), CL-46 (Hansen et al., 2002), as well as a recently identified collectin 11 (CL-11 or CL-K1) (Hansen et al., 2010, Selman and Hansen, 2012). The other two members, SP-A and SP-D, are both synthesized and secreted by airway type II and Clara cells, and therefore are also referred to as 'lung collectins'. In humans, SP-D has also been found to be expressed at various extra pulmonary sites; such as trachea, brain, testis, salivary gland, heart, prostate gland, kidney, pancreas, small intestine, placenta, mammary gland, stomach, as well as in parotid gland (Madsen et al., 2000).

1.2 Pulmonary Surfactant

Pulmonary surfactant covers the epithelial lining of alveoli. It serves to lower the surface tension between air and alveolar fluid and therefore prevents alveolar collapse at the end of the expiration (Oyarzun and Clements, 1977). Surfactant has also been shown to play an important role in pulmonary host defence (Crouch and Wright, 2001, Malhotra et al., 1994). Pulmonary surfactant consists of 90% lipids as well as four surfactant proteins (SP-A, SP-B, SP-C and SP-D, 5-10% by weight) (Kishore et al., 2005, Kishore et al., 2006, Jakel et al., 2013, Wright, 2004).

1.3 Surfactant Metabolism

Alveolar type II epithelial cells are mainly associated with the synthesis and secretion of surfactant. Following synthesis, surfactant phospholipids are stored as thoroughly packed bilayers in lamellar bodies, which function as the intracellular storage of surfactant. Lamellar bodies are secreted by type II cells by exocytosis into the alveoli. The lamellar body phospholipids rearrange in the alveoli into an expanded membrane lattice called tubular myelin (Williams, 1977), from which the phospholipid layer is formed at the air-fluid interface just above the alveolar epithelium. The hydrophobic surfactant proteins, SP-B and SP-C are also secreted in the lamellar bodies together with the phospholipids (Gobran and Rooney, 2001). The hydrophilic SP-A (Ikegami et al., 1992, Osanai et al., 1998) and SP-D (Crouch et al., 1991, Voorhout et al., 1992) are, however, secreted independently of lamellar bodies from the airway epithelial cells (Clara cells) (Phelps and Floros, 1991, Crouch et al., 1992), and associate with surfactant lipids in the alveolar lumen (Jakel et al., 2013).

1.4 Surfactant Lipids

The most abundant lipid group present in the pulmonary surfactant is phospholipids (~90% of total lipids), and the main phospholipid present is phosphatidylcholine (PC), with higher than approximately 50% of PC being disaturated. Saturated PC largely consists of dipalmitoyl phosphatidylcholine (DPPC) (Akino, 1978) and is the major surface active component of surfactant and essential for its biophysical function (Oyarzun and Clements, 1977). The second most abundant phospholipid is phosphatidylglycerol (PG) (5-10% of total phospholipids), while phosphatidylinositol (PI) accounts for a minor part of phospholipids (Kuroki et al., 1991, Jakel et al., 2013).

1.5 Structural organization of collectins

Collectins are characterised by polypeptide chains that are composed of four distinct regions (see figure 1.3): (i) a short N-terminal region that contains cysteine residues which are involved in the assembly, via disulphide bridges, of the monomers into higher order oligomers; (ii) a collagen-like region characterized by repetitive triplet Gly-Xaa-Yaa sequences (where X and Y can be any residue) which is capable of trimerizing into a triple helix (resulting in formation of a trimeric subunit); (iii) a short α -helical coiled-coil domain, termed the 'neck' region, which initiates trimerization of three monomers due to a heptad repeat of hydrophobic residues, resulting in strong hydrophobic interactions between the polypeptide chains in this domain; and (iv) a C-type CRD that can recognize glycan structures in a calcium-dependent manner (Lu et al., 2002, Holmskov et al., 2003). The polypeptides thus assemble into trimeric subunits, and these bind together, covalently and non-covalently via the N-terminal regions to form multimers.

SP-A is found mainly as octadecamers (hexamers of trimers). The hexamer has a "bunch-of-tulips" shape, found also in complement C1q, the ficolins and MBL. SP-D similarly is

assembled from trimeric subunits. A large cross-shaped tetramer of subunits is a common form (Kishore et al., 2006). The affinity of a single CRD interaction with carbohydrate structures is low but the trimeric arrangement of these CRDs and the polymerisation of the subunits allows simultaneous and multivalent interactions of higher avidity with multiple surface carbohydrate structures. This enables not only biologically relevant target recognition, but also requires matching arrangements of glycan structures present on the surface of a target before efficient binding can take place, thus contributing to distinguishing self from non-self. Although the collectins share a number of structural similarities, there are also many dissimilarities as listed below (Drickamer, 1995, Shrive et al., 2009).

The collectin trimeric subunits can associate into various forms of higher order oligomers, stabilized via interchain disulphide bonds between the N-terminal domains. SP-D is organized as a tetramer of these collagenous trimers, generating dodecameric cruciform structures (Lu et al., 1993). SP-D can also form higher order oligomeric 'fuzzy ball' complexes (Crouch et al., 1994). In addition to differing in oligomerization, the collectins also differ in the length of their collagen domains, number and distribution of cysteine residues located in the N-terminal domain and collagen domain, and distribution of N-linked oligosaccharides. Furthermore, differences occur in hydroxylation of proline residues, the degree of O-linked carbohydrate modification of the collagen domain, and carbohydrate binding selectivity of the CRDs. All these factors have an impact on the functional properties of the collectins and their interaction with targets and cells of the immune system (Jakel et al., 2013).

1.6 The lung collectins SP-A and SP-D

1.6.1 Surfactant protein SP-A

SP-A is a large glycoprotein made up of multiple copies of (in humans) two polypeptides, SP-A1 and SP-A2 (each of about 30 kDa). These are products of separate genes, but are 97% identical in amino acid sequence. In other mammals, only one SP-A polypeptide is expressed. These polypeptides assemble into subunits of ~90 kDa, which then form larger assemblies of up to ~540 kDa (Lawson and Reid, 2000). SP-A has functions in surfactant metabolism and in pulmonary host defense, which have been studied both *in vitro* and *in vivo*.

The role of SP-A in surfactant metabolism *in vitro* has been extensively investigated (Hawgood and Poulain, 2001). It takes part in surfactant pool size regulation by inhibiting surfactant secretion from type II cells (Rice et al., 1987, Dobbs et al., 1987). SP-A associates rapidly with the secreted lamellar bodies (Voorhout et al., 1991) and assists to form and maintain the tubular myelin structure (Suzuki et al., 1989, Williams et al., 1991, Klein et al., 2002). SP-A can bind to surfactant phospholipids (King and Macbeth, 1979, Ross et al., 1986), and there is evidence that it improves surface activity by facilitating the adsorption of surface-active material to the air-fluid interface (Schurch et al., 1992). SP-B is presumed to be the primary surfactant protein needed for phospholipid adsorption, and the role of SP-A in surfactant surface activity may be secondary, i.e. synergistic or regulatory (Hawgood and Poulain, 2001). SP-A also takes part in the recycling of surfactant and facilitates the uptake of phospholipids into type II cells (Wright et al., 1987, Wright and Youmans, 1995) and alveolar macrophages.

1.6.2 Surfactant protein SP-D

SP-D is a hydrophilic glycoprotein made up of twelve identical 43 kDa polypeptides with a total molecular weight of ~520 kDa (Batenburg, 1992, Kuroki and Voelker, 1994). SP-D

has a single type of polypeptide chain, with a much longer collagenous region than SP-A. The polypeptides form trimeric subunits (130kDa), which then form a tetramer of subunits, in a cross (cruciform) shape, which is very large (8-9nm diameter). The dodecamer of 520 kDa is the dominant form, but natural human and bovine SP-D can include a high proportion of trimers and dimers of the 130kDa subunit (Lu et al., 1993). SP-D dodecamers may also self-associate at their N-termini to form much more highly ordered stellate multimers (“fuzzy ball” structures) with peripheral arrays of trimeric CRDs (Crouch et al., 1994, Hartshorn et al., 1996). These multimers are not dissociated by EDTA or competing sugars, and are cross-linked by disulphide and non-disulphide bonds. They show higher apparent binding to a variety of ligands and are more efficient in mediating microbial aggregation (Hartshorn et al., 1996).

The main role of SP-D is in the pulmonary host defence. SP-D can bind to the surface of alveolar type II cells (Herbein et al., 2000) as well as alveolar macrophages (Kuan et al., 1994). SP-D does not substantially bind to surfactant aggregates. However, it binds to phosphatidylinositol (PI) (Ogasawara et al., 1992, Persson et al., 1992) and promotes the formation of tubular structures of phospholipids in the presence of PI, SP-B, and calcium (Poulain et al., 1999). Studies using SP-D gene deficient (-/-) mice also suggest a role for SP-D in surfactant homeostasis. The lungs of SP-D -/- mice contain enlarged alveoli, accumulation of surfactant phospholipids, increased numbers of large foamy alveolar macrophages, as well as abnormal type II cells and surfactant structure (Korfhagen et al., 1998, Botas et al., 1998). Although SP-A and SP-D are referred to as ‘lung collectins’, these proteins and/or their mRNAs are also expressed, albeit at lower level, in many other tissues including gastric and intestinal mucosae, mesothelial tissues, synovial cells, middle ear and in the peritoneal cavity (van Roozendaal et al., 2001). SP-A and SP-D are also detectable in human blood plasma (Hoegh et al., 2009).

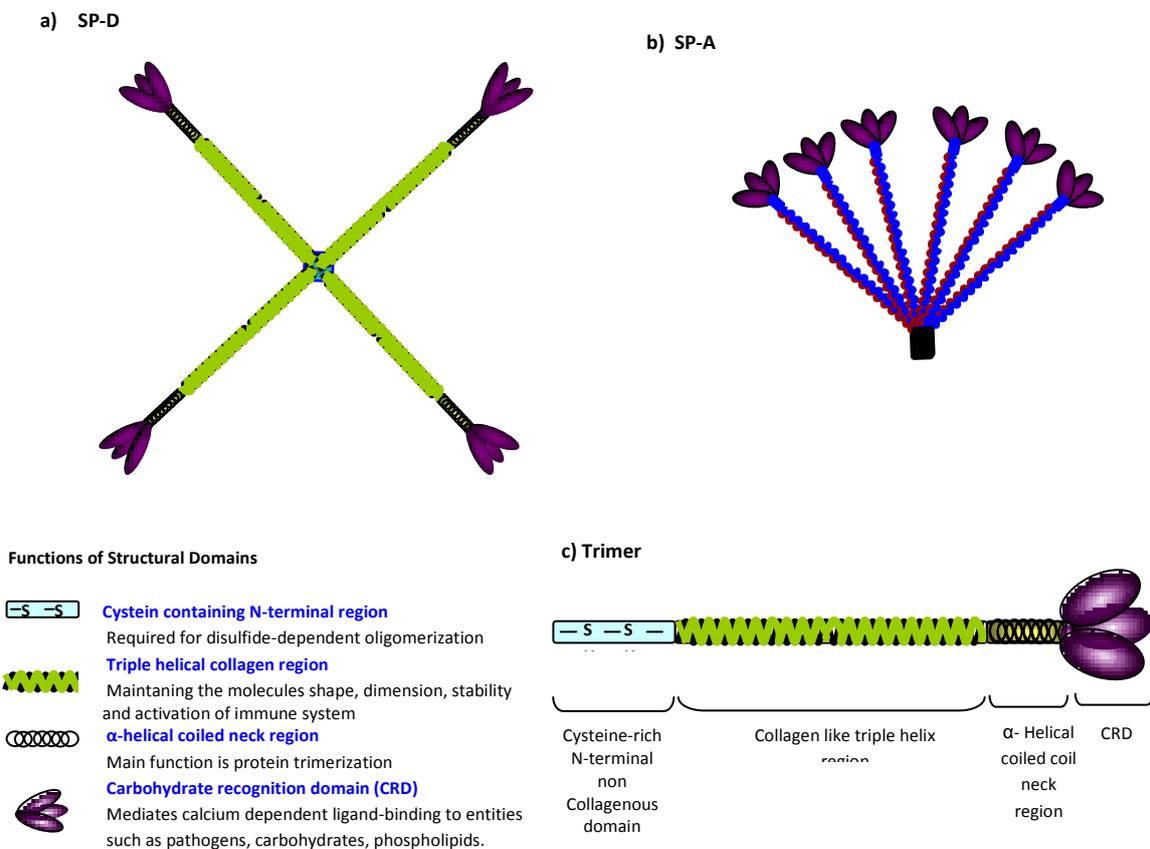


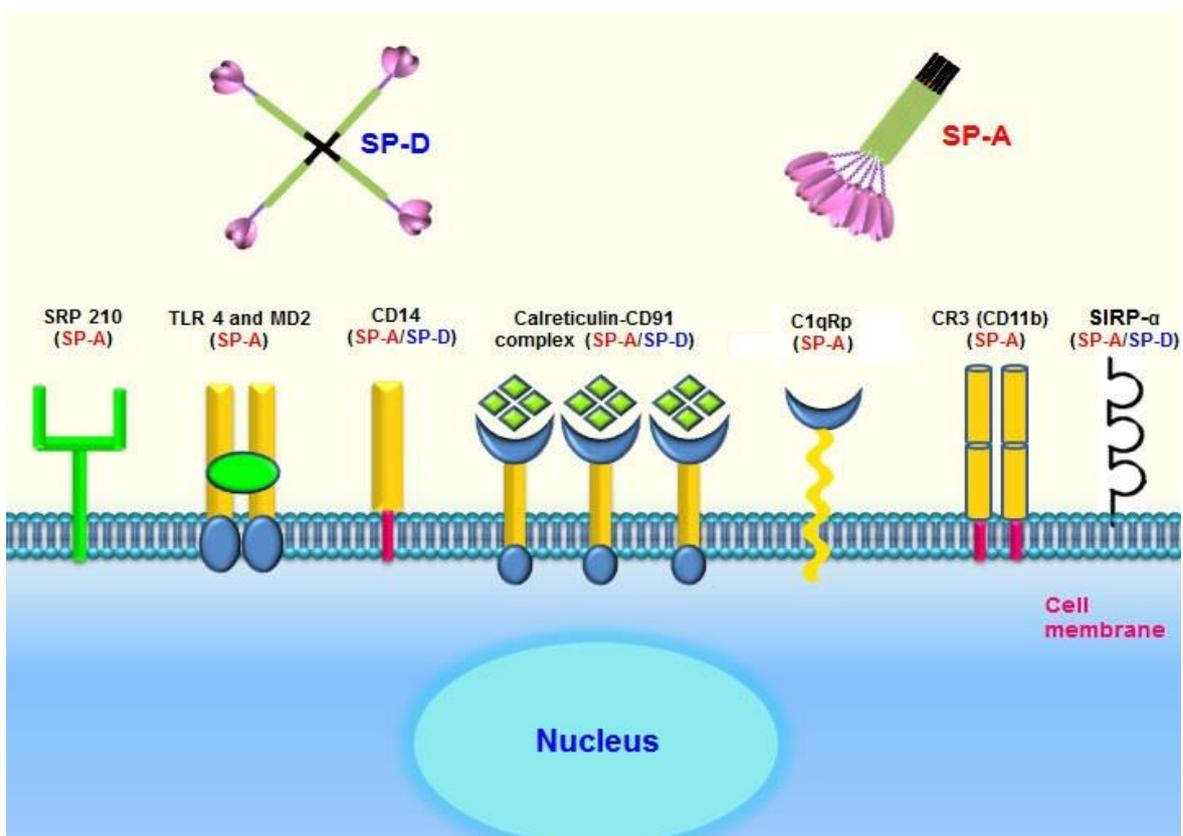
Figure 1.1 Structures of SP-A and SP-D

SP-A and SP-D are large hydrophilic proteins and belong to the collectin (collagen-lectin) subgroup of the C-type lectin superfamily. SP-A and SP-D are large oligomeric structures, each assembled from multiple copies of single polypeptide chains (except the human SP-A). SP-A has a hexameric structure with 6 subunits (each composed of three 35-kDa polypeptide chains) of 105 kDa each, which assemble to form a 630 kDa molecule. SP-D, is a 43 kDa glycoprotein, composed of 130-kDa oligomers (each composed of three identical polypeptide chains of 43-kDa). SP-D forms a crossbar-like structure composed of 12 subunits arranged in four trimeric subunits. The primary structure of SP-A and SP-D are organized into 4 regions, a non collagen region involved in the formation of inter-chain disulfide bonds (-SH-SH-), a collagen region composed of Gly-X-Y repeats where X and Y can be any amino acids (generally proline or hydroxyproline), a neck peptide, and C-type (calcium dependent) carbohydrate-recognition domains (CRDs). SP-D is a large oligomeric structure and dodecamer (12 mer), consisting of four trimeric subunits. (Qaseem et al., 2013)

1.7 Ligands and receptors

SP-A and SP-D are able to bind to a wide range of microorganisms and apoptotic cells, facilitating their clearance through various mechanisms. These collectins also participate in the clearance of other complex organic materials, such as pollens (Malhotra et al., 1993) and house dust mite allergens (Wang et al., 1996b). SP-A and SP-D also have the capacity to modulate leukocyte function, further enhancing the clearance of pathogens (Hickling et al., 2004, Lawson and Reid, 2000). An overview of the major ligands and receptors of SP-A and SP-D is presented below (Figure 1.2) (Jakel et al., 2013).

Figure 1.2: SP-A and SP-D receptors



1.8 Interactions with carbohydrates

SP-A and SP-D recognise bacteria, fungi and viruses by binding mainly to surface mannose, fucose and N-acetylglucosamine residues, and lipids. The majority of these interactions are mediated through the CRDs and are calcium-dependent. A notable exception is the Herpes Simplex Virus, which appears to bind to the N-linked oligosaccharides of SP-A (Van Iwaarden et al., 1992). SP-A and SP-D bind to a broad and overlapping range of microbial targets, but their precise modes of interaction, and the effects of the interaction can differ markedly.

The carbohydrate binding specificity of the C-type lectins is determined by a network of coordination and hydrogen bonds that stabilizes the ternary complex of protein, calcium ion and carbohydrate. SP-A and SP-D show differences in relative saccharide selectivity. Whereas the order of binding preference of SP-A for mono- or disaccharides follows: mannose, fucose > glucose, galactose >> N acetyl glucosamine (GlcNac) (Childs et al., 1992), human SP-D prefers interaction with maltose > glucose, mannose, fucose > galactose, lactose, glucosamine > N-acetylglucosamine (Persson et al., 1990). These differences in ligand specificity are likely due to the distribution of non-conserved residues that are located near the ligand-binding pocket of the CRD. Other structural factors are involved that have an impact on the carbohydrate-binding properties of the collectins. The clustering of three CRDs results in the generation of a trimeric high avidity ligand binding site (Kishore et al., 1996) and makes it possible for two or three CRDs to interact simultaneously with closely-spaced carbohydrate structures. This multivalent binding depends on a matching arrangement between the three CRDs of the collectin, and the pattern of sugars present on the surface of a target. In addition, asymmetric orientation of the CRDs and charge distributions of the trimeric CRD surface might also affect ligand affinity and specificity. The differences in the oligomeric organization of SP-A

(hexameric) and SP-D (dodecameric) result in variations of both number and spatial distribution of their trimeric CRDs, influencing the binding specificity and avidity for carbohydrate ligand patterns present on biologically relevant particles (Jakel et al., 2013).

1.9 Interactions with lipids

The lung collectins also display interactions with lipids which are mediated by the CRD but can also require the neck domain (Kishore et al., 1996, Ogasawara et al., 1994, Palaniyar et al., 1998, Kuroki and Akino, 1991). SP-A binds to DPPC (Kuroki and Akino, 1991), the lipid A moiety of Gram-negative lipopolysaccharides (LPS) (Van Iwaarden et al., 1992) and several glycolipids, including galactosyl-ceramide and lactosyl-ceramide, which are abundant in the plasma membranes of eukaryotic cells, although the functional consequences of these interactions are not known. These interactions appear to involve recognition of both the ceramide and saccharide moieties (Childs et al., 1992, Kuroki et al., 1992). SP-A also aggregates surfactant phospholipids in the presence of calcium ions (Haagsman et al., 1990, Haagsman et al., 1991). With regard to SP-A, it was demonstrated that calcium ions can induce structural changes of the octadecamer which may exist in an “open-bouquet” or a “closed-bouquet” conformation in the absence or presence, respectively, of calcium ions (Palaniyar et al., 1998). As a consequence, these differences in conformation may result in different interaction of the SP-A head groups (trimeric CRDs) with the lipid membrane surface (Jakel et al., 2013).

SP-A is also tightly associated with lipids *in vivo*, and is involved in the formation of ordered arrays of lipid known as tubular myelin (Suzuki et al., 1989). As expected, mice genetically deficient in SP-A (SP-A $-/-$ mice) have very little tubular myelin, but they do not show significant aberration of pulmonary surfactant functions (Korfhagen et al., 1996). It has also recently been shown that Phosphatidylserine (PS), which becomes exposed on the outside of apoptotic cells, is a relevant binding molecule for human SP-A, but not for

SP-D (Jakel et al., 2010b). The binding of SP-A to PS is Ca^{2+} -dependent and is not inhibited by mannose, suggesting that the sugar-binding site of the CRDs of SP-A is not involved. Flow cytometry studies on apoptotic Jurkat cells and apoptotic neutrophils showed inhibition of the binding of annexin V by increasing concentrations of SP-A. Supporting these data, confocal microscopy data showed a co-localisation of annexin V and SP-A in late apoptotic but not early apoptotic cells. Further studies are needed to exploit the relevance of the interaction of SP-A with PS in the context of phagocytic uptake of apoptotic cells (Jakel et al., 2013).

SP-D interacts *in vitro* with PI in a calcium ion-dependent manner (Ogasawara et al., 1992, Persson et al., 1992, Ogasawara et al., 1994). It does not interact strongly with other phospholipid components of pulmonary surfactant but does have a strong association with glycosyl-ceramide (Kuroki et al., 1992). The interactions of SP-D with PI and glycosyl-ceramide are calcium-dependent and inhibited by competing monosaccharides, indicating a CRD-mediated interaction. However, there is evidence to suggest that PI also interacts with the α -helical coiled-coil region or other regions in the CRD not involved in sugar-binding (Ogasawara and Voelker, 1995, Kishore et al., 1996). An overview of the different lipids interacting with SP-A or SP-D is shown below (Table 1.1).

Table 1.1 Summary of binding of lung collectins to lipids

Lipid	Collectin	Mechanism	Reference
DPPC¹	SP-A	Ca ²⁺ -dependent, sugar-independent	(Kuroki and Akino, 1991)
Lipid A (LPS)	SP-A	Ca ²⁺ -dependent, sugar-independent	(Van Iwaarden et al., 1992)
Glycolipids (galactosylceramide, lactosylceramide)	SP-A	Ca ²⁺ -dependent, CRD involved	(Kuroki et al., 1992, Childs et al., 1992)
DPPC¹ PG²	SP-A	Ca ²⁺ -dependent	(Haagsman et al., 1990)
Phosphatidylserine (PS)	SP-A	Ca ²⁺ dependent, sugar independent	(Jakel et al., 2010b)
Phosphatidylinositol (PI)	SP-D	Ca ²⁺ -dependent, CRD involved	(Ogasawara et al., 1992, Persson et al., 1992, Ogasawara et al., 1994),
Glycosylceramide (GlcCer³, GalCer⁴)	SP-D	Ca ²⁺ -dependent, CRD involved	(Kishore et al., 1996, Ogasawara and Voelker, 1995)

Abbr.: Dipalmitoylphosphatidylcholine ¹, Phosphatidylglycerol ², Glucosylceramide ³, Galactosylceramide ⁴

1.10 Interaction with Nucleic Acids

DNA is often found on the surface of apoptotic cells (Savill and Fadok, 2000). SP-A and SP-D bind DNA and RNA from various sources including mice and bacteria (Palaniyar et al., 2004) and enhance the uptake of DNA by human monocytic cells (Palaniyar et al., 2003a, Palaniyar et al., 2003b). Hence, binding of DNA may be one mechanism by which SP-A and SP-D mediate phagocytosis of apoptotic cells (Jakel et al., 2013).

1.11 Interaction with Protein Acceptors (Ligands)

SP-A and SP-D bind to cell-surface proteins which act as receptors (i.e. they transmit a signal to a cell on binding of the collectin to the receptor protein). SP-A and SP-D also bind to proteins which are not receptors and do not transmit any signal to a cell. These are referred to here as acceptors, to distinguish them from receptors (Jakel et al., 2013). An overview of proteins interacting with SP-A or SP-D is also shown in (Table 1.2).

1.11.1 Glycoprotein-340 (Gp-340)

Gp-340 was first identified as an exclusively SP-D binding molecule purified from bronchoalveolar lavage (BAL) of alveolar proteinosis patients (Holmskov et al., 1997). The binding of SP-D to gp-340 is inhibited by EDTA, but not affected by maltose, suggesting the involvement of a calcium ion-dependent protein-protein interaction through the CRD. SP-A also interacts with gp-340 in a calcium-dependent, but lectin-independent manner (Tino and Wright, 1999). cDNA cloning has revealed that gp-340 is a member of a scavenger receptor cysteine rich superfamily consisting of multiple scavenger receptor type B domains (Holmskov et al., 1999). Gp-340 is in fact identical to salivary agglutinin - an immune scavenger component of saliva, which has been shown to bind to *Streptococcus mutans* and *Helicobacter pylori* (Ligtenberg et al., 2001). Gp-340 exists in both a soluble form, and in a form associated with alveolar macrophage membranes

(Holmskov et al., 1999). It is clear now that gp-340 is mainly a soluble SP-A and SP-D binding protein rather than a surface receptor. The possibility that gp-340 acts as an adaptor for SP-A and SP-D binding to surfaces has not yet been investigated (i.e. gp340 binds to some recognition motif on a particle, allowing SP-A and SP-D to bind the particle via gp340) (Jakel et al., 2013).

1.11.2 Myeloperoxidase (MPO)

MPO is an intracellular defense molecule of neutrophils, which becomes exposed on the outside of the cell upon apoptosis. MPO was identified as a novel binding molecule for SP-A and SP-D (Jakel et al., 2010a). The interaction is independent of Ca^{2+} , suggesting involvement of a non-lectin, non-calcium binding site. Both proteins bind directly to purified, immobilised MPO, and show inhibition of the binding of an anti-MPO monoclonal antibody (mAb) to late apoptotic cells. Fluorescence microscopic studies confirm that Anti-MPO mAb and SP-A/SP-D co-localise on late apoptotic neutrophils in a Ca^{2+} -independent manner (Jakel et al., 2013).

1.11.3 C1q

SP-A was shown to interact with complement protein C1q (Oosting and Wright, 1994, Watford et al., 2002). The lectin domain is not required for this interaction. Uptake of C1q coated beads by alveolar macrophages was enhanced by preincubation with SP-A (Watford et al., 2002). Again C1q may act as an adaptor, binding to a particle surface then itself forming a binding site for SP-A (Jakel et al., 2013).

1.11.4 Immunoglobulins (Ig)

Nadesalingam et al, reported that SP-D binds various classes of immunoglobulins, including IgG, IgM, IgE and secretory but not serum IgA (Nadesalingam et al., 2005). The interaction appears to be through the globular domains of SP-D, both with the Fab and Fc

domains in a Ca²⁺ dependent manner. IgG coated beads were aggregated by SP-D and enhancement of phagocytosis by murine macrophages was shown. It was also shown that SP-A binds to the Fc, rather than the Fab, region of IgG (Lin and Wright, 2006). Binding is calcium dependent but not inhibited by saccharides known to bind to SP-A's carbohydrate recognition domain. The binding of SP-A does not inhibit the formation of immune complexes or the binding of IgG to C1q. Recently, a recombinant fragment of human SP-D, composed of trimeric neck and CRDs, have been shown to bind specific IgE that abrogates IgE interaction with specific allergens (AS Qaseem et al, unpublished data).

1.11.5 Defensins

Human neutrophil defensins (HNPs) inhibit infectivity of enveloped viruses, including influenza A viruses. HNPs were found to bind to the neck and/or CRD of SP-D. This binding was specific because no, or minimal, binding to other collectins was found (Hartshorn et al., 2006). HNPs precipitated SP-D from BAL fluid and reduced the anti-viral activity of BAL fluid. HNP-1 and -2 differed somewhat in their independent anti-viral activity and their binding to SP-D. Doss et al (Doss et al., 2009) used surface plasmon resonance to evaluate binding of defensins to SP-D. Human beta-defensins, HD6, and human neutrophil peptide (HNP)-4 bound minimally to SP-D. However HNP-1-3 bound SPD with high affinity and inhibited SP-D mediated anti-viral activity (Jakel et al., 2013).

1.11.6 Decorin

Nadesalingam et al, describe the co-purification of human decorin, a 130-150 kDa proteoglycan and novel SP-D-binding protein, from amniotic fluid (Nadesalingam et al., 2003). The CRD region of SP-D binds in a calcium dependent-manner to the sulfated N-acetyl galactosamine moiety of decorin. Complement subcomponent C1q, a complement

protein that is known to interact with decorin core protein via its collagen-like region, partially blocks the interaction between decorin and native SP-D. C1q, however, does not block the interaction between decorin and rhSP-D. Furthermore, the core protein, obtained by chondroitin ABC lyase treatment of decorin, binds SP-D, but not SP-D neck and CRD region. These findings suggest that decorin core protein binds the collagen-like region of the SP-D. Collectively, CRDs of SP-D interact with the dermatan sulfate moiety of decorin via lectin activity and that the core protein of decorin binds the collagen-like region of SP-D in vitro (Jakel et al., 2013).

1.12 Interaction with Protein Receptors

1.12.1 SPR-210

A 210 kDa SP-A-binding protein was first purified from the macrophage cell line U937 and became known as SPR-210 (surfactant protein receptor 210 kDa) (Chroneos et al., 1996). This receptor is also found on type II cells and alveolar macrophages. The interaction between SP-A and SPR-210 takes place through the collagen region of SP-A. Antibodies against SPR-210 inhibit binding of SP-A to alveolar type II cells and alveolar macrophages and inhibit the SP-A-mediated uptake of *Mycobacterium bovis*. SPR-210 may thus be a functional cell-surface receptor on both alveolar type II cells and macrophages. The molecular identity of SPR-210 has later been described as myosin 18A (Yang et al., 2005). This protein, which is expressed in multiple isoforms, may be a candidate for a genuine SP-A receptor, but this requires further investigation (Jakel et al., 2013).

1.12.2 CD14

(Sano et al., 1999) reported that SP-A and SP-D bind to native as well as recombinant soluble CD14, a known receptor for LPS, which is a component of the outer membrane of

Gram-negative bacteria that is responsible for sepsis and induction of inflammation. The neck domain of SP-A binds to the leucine-rich peptide portion of CD14 whereas the lectin domain of SP-D interacts with the carbohydrate moiety of naturally occurring human CD14. Both SP-A and SP-D appear to modulate LPS/CD14 interactions (Sano et al., 2000, Jakel et al., 2013).

1.12.3 Calreticulin-CD91 complex

A common receptor for C1q, MBL and SP-A was described in 1990 (Malhotra et al., 1990) and identified later as calreticulin (Sim et al., 1998). However, calreticulin had been characterized mainly as an intracellular protein; and it took some further research to demonstrate how calreticulin functioned as part of a cell-surface receptor. It was shown (Ogden et al., 2001, Vandivier et al., 2002) that calreticulin binds to the cell-surface receptor CD91 and acts as an adaptor or coreceptor to bind the collagenous region of C1q, MBL and other proteins with similar collagenous structures (the collectins SP-A and SP-D, and also the ficolins) to the cell surface via CD91. Phagocytic uptake of apoptotic cells, mediated by MBL or C1q binding to the Calreticulin-CD91 complex was demonstrated (Vandivier et al., 2002). On cells which do not express CD91, HLA class I heavy chain (Arosa et al., 1999), or possibly CD59 (Ghiran et al., 2003) may act as calreticulin-binding proteins, allowing particles coated with C1q or MBL to adhere to the cells. The region within calreticulin which binds to the collagenous proteins has been identified (Stuart et al., 1997).

1.12.4 C1qRp

A ~126kDa glycoprotein designated C1qRp and later shown to be CD93, was reported to play a role in C1q/MBL/SP-A-mediated removal of pathogens and immune complexes by phagocytosis, as determined by the ability of antibodies against this receptor partially to block enhancement of phagocytosis by these three proteins (Nepomuceno et al., 1997).

However, this molecule was later shown to be an adhesion receptor (McGreal et al., 2002) which does not interact directly or indirectly with any of the C1q-collectin-ficolin proteins. Thus C1qRp is not a receptor for C1q, SP-A, SP-D etc, although in many databases it is still described as such (Jakel et al., 2013).

1.12.5 Signal-inhibitory regulatory protein α (SIRP α)

Gardai and co-workers (Gardai et al., 2003) reported that SP-A and SP-D can modulate cellular functions through CD91-calreticulin complex as well as via signal-inhibitory regulatory protein- α (SIRP- α), which is found to be expressed on various cells such as DC's, macrophages and neutrophils (Saito et al., 2010). Their data show that SP-A differentially engages either CD91-calreticulin or SIRP- α , depending on whether the lectin domain of SP-A is bound to a target. For example, in the absence of a pathogen, SP-A binds through its lectin domain to SIRP- α . In the presence of a foreign organism or cell debris, to which the lectin domain of SP-A binds, the free collagen-like region activates immune cells through CD91-calreticulin. Importantly, engagement of the different receptors elicits different responses. When SP-D binds to SIRP- α via CRD region inflammatory mediator production is inhibited due to activation of SHP-1 kinases. This activation inhibits p38 MAPK phosphorylation resulting in the blockage of NF- κ B activation and thus inhibition of inflammatory functions. (Guo et al., 2008, Jakel et al., 2013)

1.12.6 Alveolar Type II Cell Receptors

Both SP-A and SP-D have been shown to interact with and be internalized by alveolar type II cells. The interactions of SP-A and SP-D with type II cells could be related to surfactant homeostasis (Hawgood and Poulain, 2001). Several putative type II cell receptors for SP-A have been described. Two different SP-A binding proteins were identified using an anti-

idiotypic antibody approach, including a 30 kDa protein (Strayer et al., 1993) and a 55 kDa protein known as BP55 that multimerizes to 170–200 kDa (Wissel et al., 1996). Another SP-A binding protein was described by Kresch and co-workers (Yamada et al., 2006). Unlike SPR-210, none of these receptors appears to be expressed on macrophages. None of these proteins has been identified at the molecular level. Important future studies including cloning and characterizing the mechanism by which these receptors mediate type II cell function are needed (Jakel et al., 2013).

1.12.7 Toll-like receptors (TLR4 and MD-2)

Yamada et al, showed that SP-A interacts directly with TLR4 and MD-2, which are critical signalling receptors for lipopolysaccharides (LPS) (Yamada et al., 2006). SP-A bound to the recombinant soluble form of extracellular TLR4 domain (sTLR4) and MD-2, in a Ca^{2+} -dependent manner, via the CRD region. In addition, SP-A attenuated cell surface binding of smooth LPS and subsequent NF- κ B activation in TLR4/MD-2 expressing cells. These studies indicate that in studies on ligand specificity of TLRs, it may often be important to consider whether a potential ligand has already encountered one of the soluble innate immune recognition systems (complement, collectins, ficolins) before it encounters the TLR (Jakel et al., 2013). Furthermore, the high affinity binding of SP-D to a complex of recombinant soluble forms of Toll-like receptor 4 (TLR4) and MD-2 and its ability to down-regulate TNF- α secretion and NF- κ B activation elicited by rough and smooth LPS, in alveolar macrophages and TLR4/MD-2-transfected HEK293 cells has also now been demonstrated (Yamazoe et al., 2008).

1.12.8 CR3 (CD11b, CD18)

SP-A has been shown to modulate cell surface expression of CR3 on alveolar macrophages and enhance CR3-mediated phagocytosis (Gil et al., 2009). Complement receptor CR3

(CD11b, CD18) was expressed at reduced levels on the surface of alveolar macrophages from SP-A $-/-$ mice compared with wild type mice. Administration of intratracheal SP-A to SP-A $-/-$ mice induced the translocation of CR3 from alveolar macrophage intracellular pools to the cell surface. Intratracheal challenge with *Haemophilus influenzae* enhanced CR3 expression on the surface of alveolar macrophages from SP-A $-/-$ and SP-A $+/+$ mice, but relative expression remained lower in the SP-A $-/-$ mice at all the time points post-inoculation. SP-A augmented CR3-mediated phagocytosis in a manner that was attenuated by N-glycanase or collagenase treatment of SP-A, implicating the N-linked sugar and collagen-like domains in that function. The direct binding of CR3 to SP-A was calcium dependent and mediated by the I-domain of CR3 and to a lesser extent by the SP-A lectin domain (Jakel et al., 2013).

An overview of proteins interacting with SP-A or SP-D is given below.

Table 1.2 Summary of proteins interacting with SP-A or SP-D

Acceptor	Protein	Collectin	Mechanism	Reference
	Gp 340	SP-A / SP-D	Ca ²⁺ -dependent, sugar-independent	(Holmskov et al., 1997), (Tino and Wright, 1999)
	MPO	SP-A / SP-D	Ca ²⁺ -independent	(Jakel et al., 2010a)
	C1q	SP-A / SP-D	Ca ²⁺ -dependent, sugar-independent	(Oosting and Wright, 1994, Watford et al., 2002)
	Igs	SP-A	Ca ²⁺ -dependent	(Nadesalingam et al., 2005)
	Defensins	SP-D	Ca ²⁺ -dependent, via CRD / neck	(Hartshorn et al., 2006, Doss et al., 2009)
	Decorin	SP-A	Ca ²⁺ -dependent, via CRD and collagen-like region	(Nadesalingam et al., 2003)
Receptor	SPR-210	SP-A	via collagenous region	(Chroneos et al., 1996)
	CD14	SP-A / SP-D	neck domain of SP-A; lectin domain of SP-D	(Sano et al., 1999)
	Calreticulin-CD91 complex	SP-A / SP-D	via collagenous region	(Malhotra et al., 1990, Sim et al., 1998, Ogden et al., 2001, Vandivier et al., 2002)
	C1qRp	SP-A (and others?)	Not clear	(Nepomuceno et al., 1997), (McGreal et al., 2002)
	SIRP α	SP-A / SP-D	via lectin or collagenous domain	(Gardai et al., 2003)
	Alveolar Type II Cell Receptors	SP-A / SP-D	Not clear	(Strayer et al., 1993, Wissel et al., 1996, Kresch et al., 1998)
	TLR4 and MD-2	SP-A	Ca ²⁺ -dependent, sugar-independent	(Yamada et al., 2006)
	CR3 (CD11b, CD18)	SP-A	Ca ²⁺ -dependent	(Gil et al., 2009)

1.13 Epidemiology of Asthma

Asthma is defined by World Health Organisation (WHO) as an inflammatory condition of the airways, where there is a reduction in air flow caused by swelling due to the irritation of nerve endings in the airways. Currently asthma is known to afflict up to 300 million people worldwide (van Schayck, 2013, Braman, 2006), with approximately 5 million people affected in U.K as per figures provided by Asthma UK. This thus places a huge burden on U.K economy, where it has been revealed that it can cost NHS to the tune of £1 billion per annum, for providing care and treatment to affected persons. Asthma is thought to be caused by many environmental and genetic factors, which are yet to be clearly understood. It is has now been shown that there are many different types of asthma triggered by various different factors, such as allergies due to an inappropriate immune response to innocuous protein-based antigens (allergens), such as those from house dust mite, pollen and insect venom. This form of asthma is called allergic asthma, which belongs to Type 1 hypersensitivity, and is discussed below.

1.14 Hypersensitivity

According to Gell and Coombs classification, there are four major types of hypersensitivity reactions and these are classified as type I-IV hypersensitivity reactions with each type having distinct mechanisms of pathogenesis, cells and mediator molecules. Type I or immediate hypersensitivity reactions occur when allergens react with tissue cells such as mast cells that have been passively sensitised with specific IgE, leading to the release of mediators of inflammation (Figure 1.3).

1.15 Effector cells in allergic asthma

Some of the major characteristics associated with allergic asthma are chronic airway inflammation due to increased numbers of eosinophils, macrophages, and lymphocytes,

which can lead to airway remodeling with features such as subepithelial fibrosis, mucus metaplasia, and myocyte hyperplasia (Elias et al., 1999). Thus these cells which are currently known to contribute to the pathophysiology of allergic asthma, are discussed below in further detail.

1.15.1 Mast cells

Mast cells are effector cells of an early allergic response that produce various inflammatory mediators associated with the allergic asthma (Williams and Galli, 2000). Degranulation of mast cells occurs through allergen-IgE crosslinking via high affinity IgE receptor (FcεRI) and can lead to release of various mediators by mast cell as further explained below in Figures 1.4 & 1.5. Degranulation of mast cells is a major event in the early response to allergens in asthmatic patients (Williams and Galli, 2000). Mast cells can also play a role in enhancing IgE synthesis by B cells due to expressing CD40L (Grewal and Flavell, 1998). Furthermore, as well as playing a role in the early phase of asthma pathogenesis, they have also been implicated in airway remodelling via activation of fibroblasts (Gruber et al., 1997), as well as by releasing TGF-β (Williams and Galli, 2000).

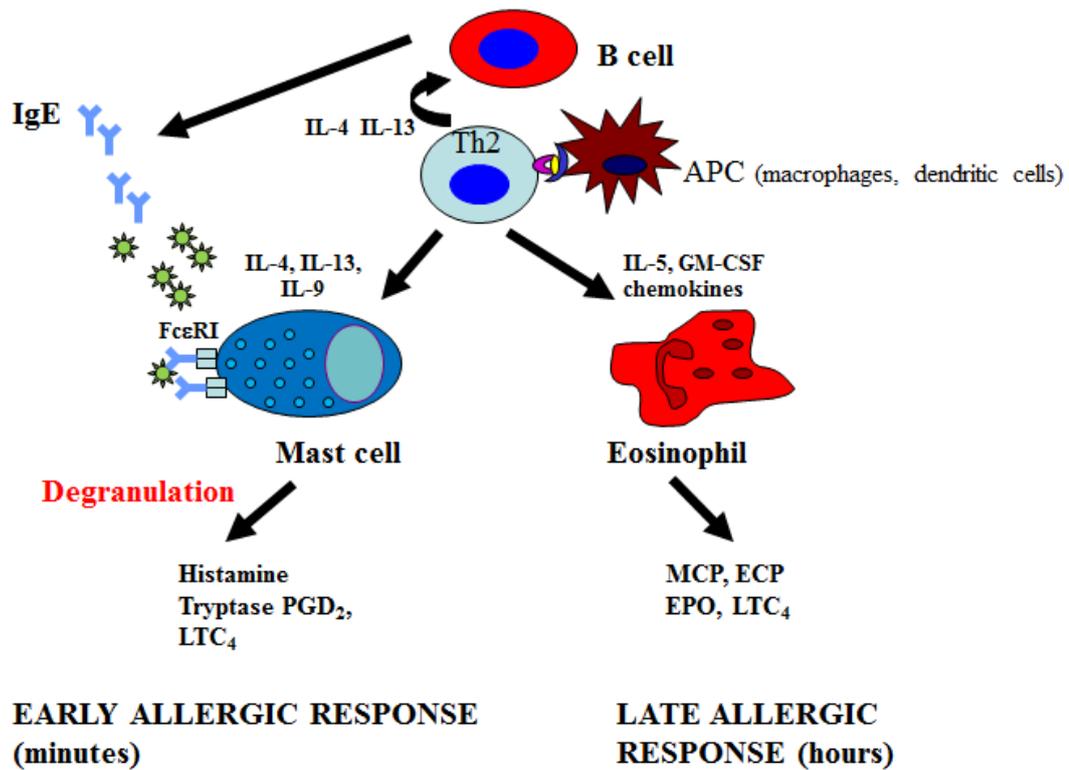


Figure 1.3: Early and late phase type I hypersensitivity

Acute phase allergic reactions occur within seconds to minutes of IgE receptor activation (mast cell mediator release) and resolving within an hour. Whereas in late phase allergic reaction, there is a delayed inflammatory response, peaking at 4-8 hours and persisting up to 24 hours following an intense acute phase reaction. (Adapted from (Hawrylowicz and O'Garra, 2005))

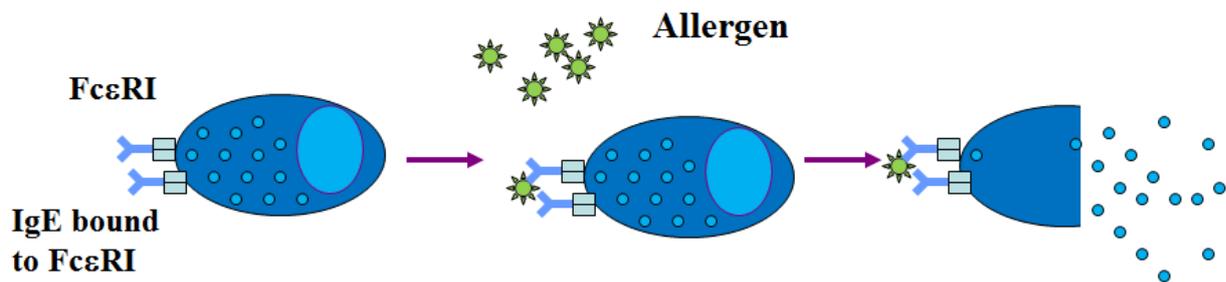


Figure 1.4: Pathophysiology of allergic asthma

Allergen binding to IgE via high affinity IgE receptor (FceRI) triggers release of preformed granules by mast cells containing i) vasoactive amines (Histamine, serotonin), which are short lived and lead to increased local blood flow & vascular permeability, ii) Leukotrienes which are inflammatory mediators leading to smooth muscle cell contraction (Bronchoconstriction), iii) Release of cytokines such as (IL-4 & TNF- α) which sustain inflammatory signal. Production of these mediators is thus initially responsible for early allergic response, which is associated with allergic inflammation which is a main pathophysiological response in allergic asthma.

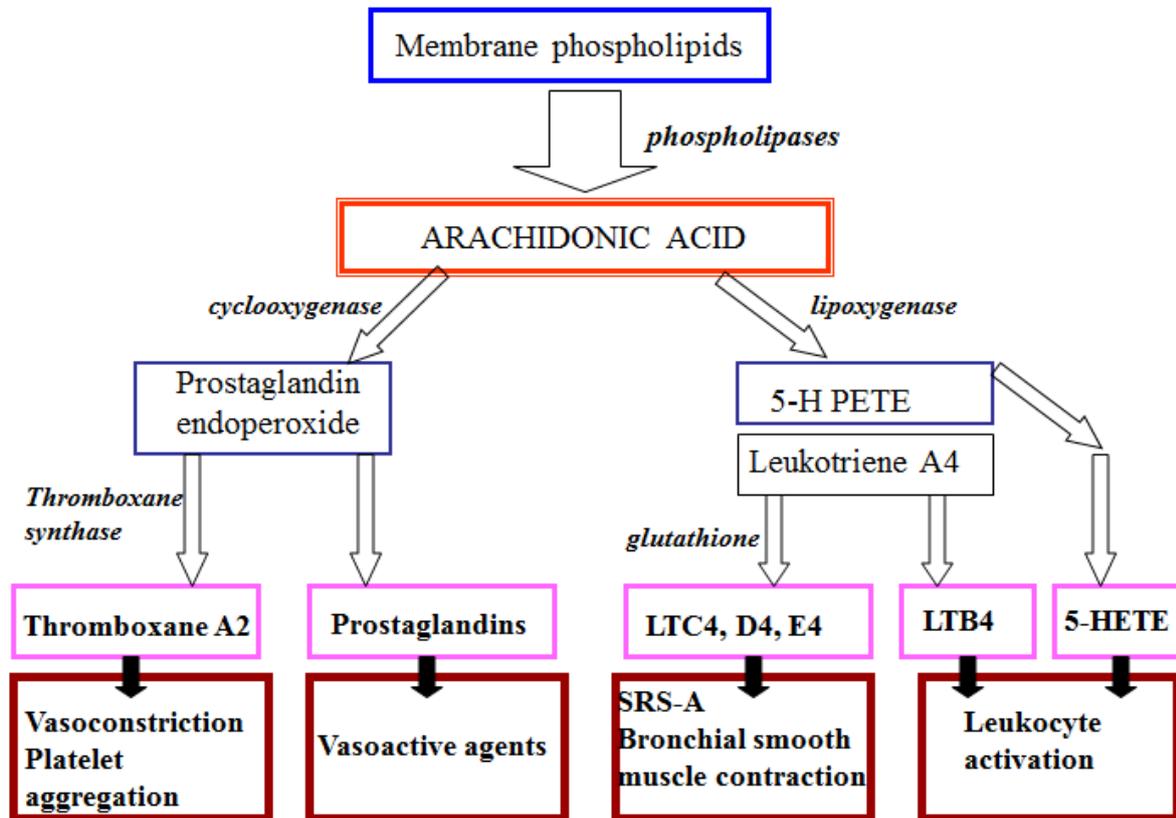


Figure 1.5: Role of arachidonic acid metabolism in the development of allergic asthma and inflammation.

Various mediators involved in the pathogenesis of asthma are produced through arachidonic acid metabolism. Two major products of this pathway are i) Prostaglandins, ii) Leukotrienes (LT), both of which are associated with various pathophysiological responses, which occur in allergic asthma. Prostaglandins (PG) are produced by undergoing further metabolism via cyclooxygenase pathway, whereas synthesis of leukotrienes occurs through further metabolism by lipoxygenase pathway, as depicted above.

1.15.2 B cells

B cells play a role in the pathogenesis of allergic asthma through their role in IgE production controlled by low affinity IgE receptor (FcεRII-CD23). The role of this low affinity IgE receptor (CD23) in IgE production is further discussed in Chapters 4 and 5. B cells require two signals provided by Th2 cells in order to produce IgE (Geha et al., 2003). These signals are in the form of IL-4 or IL-13 as well as a second co-stimulatory signal in the form of interactions between CD40 present on the B cells and CD40L found on Th2 cells. Production of IgE by B cells is associated with the pathogenesis of allergic asthma, which occurs upon crosslinking of allergen and IgE found on the surface of mast cells via high affinity IgE receptor (FcεR1), thus leading to degranulation of the mast cells (Robinson, 2004).

1.15.3 T cells

T cells have been shown to be important in controlling lung inflammation. T-cells develop in the thymus, and migrate to lymphoid tissue after being released in to the circulation, and return to the bloodstream if they encounter antigen (Petrie, 2003). Antigens are recognised by T cells via various T cell receptors (TCRs) found on their surface that can recognise specific antigens (Davis et al., 2003). However, they do not have the ability to directly interact with the allergen. The antigen thus has to be processed and presented via major histocompatibility complex class II (MHC class II) expressed on antigen presenting cells. There are two main types of T cells having different properties, which are defined by the expression of co-receptors that interact with MHC molecules (van der Merwe and Davis, 2003). CD4 effector T cells bind MHC class II molecules and provide defence against pathogens whereas CD8 effector T cells bind to MHC class I molecules, and are involved in the clearance of virally infected cells. However, it is CD4⁺ T cells which are implicated in allergic asthma and thus play an important effector role in asthma. Previous studies have

high lightened the role of CD4⁺ T cells in allergic asthma, where it has been shown that the levels of CD4⁺ T cells are elevated in the lungs of asthmatics, when compared with healthy subjects (Robinson et al., 1992b). CD4⁺ T cell sub population can be further subdivided in to Th1 and Th2, depending on their cytokine profiles, developmental pathways, chemokine receptor expression and functional properties. Th1 cells are characterised by the production of IFN- γ and are involved in pathogen defence, while Th2 are characterised by the production of IL-4, IL-5, IL-9, IL-13 and IL-25 and mediate immunity to helminth infection (Mosmann and Coffman, 1989). Th1 and Th2 cells also express different chemokine receptors, which play a role in the recruitment of specific cell types to the sites of inflammation (Gutierrez-Ramos et al., 2000).

1.15.4 The Th1/Th2 balance in allergic asthma

Allergic asthma is a complex chronic inflammatory disorder of the airway and T cells regulate this response in a major way (Murdoch and Lloyd, 2010). The cytokine secretion patterns of mouse CD4⁺ T cells define two major subsets of helper cells: Th1 cells and Th2 cells. Both cell types originate from a common naive precursor T cell, but have differences in cytokine profiles based on signals from the local environment. Allergic asthma is a Th2 associated disease (Barner et al., 1998) characterized by high levels of IL-4 and IL-13. Upon allergen exposure the antigen is taken up and processed by antigen presenting cells and subsequently presented to the T cells of the immune system. IL-13 is produced in the airway by T cells, eosinophils and mast cells and plays an important role in initiating and generating the physiological abnormalities that come to play in asthmatic diathesis (Robinson et al., 1992a, Cohn et al., 1997, Robinson et al., 1993). Th1 cells predominantly secrete IL-2, TNF- α , Interferon gamma (IFN γ) and lymphotoxin A (LTA), while Th2 cells produce mainly IL-4, IL-5, IL-6, IL-10 and IL-13 (Lucey et al., 1996, Abbas et al., 1996, Mosmann and Sad, 1996). Th1 responses have been implicated in cell-mediated immune responses against intracellular pathogens such as viruses and some bacteria, e.g. *Mycobacterium tuberculosis*, the causative organism of tuberculosis and sarcoidosis (Moller et al., 1996). Th2 responses play a key role in antibody-mediated immunity, which is required to control extracellular pathogens that are exposed to antibodies in blood and other body fluids. Th2 cells also help B cells make IgE antibodies by providing them with IL-4 and IL-13, both of which promote class switching. IgE mediated inflammation and responses are characterized by activated mast cells that release vasoactive substances and inflammatory mediators such as histamine and leukotrienes that recruit proinflammatory cells such as eosinophils to the tissue (Barner et al., 1998, Robinson et al., 1992a). Mast

cells, in turn, also release Th2 cytokines (IL-4 and IL-13) that can further induce IgE production.

1.15.5 Eosinophils

The eosinophil is an effector cell of the late phase allergic response, and has been shown to be a key player in allergic asthma associated inflammation (Kay et al., 1989). Eosinophils are granular leukocytes and contain toxic granule proteins such as major basic protein (MBP) and eosinophil cationic protein (ECP), which can kill microorganisms and parasites (Rothenberg and Hogan, 2006). They can also release mediators such as prostaglandins, leukotrienes and cytokines (Th2, Th1) and tissue remodelling growth factors, which can further amplify the inflammatory responses. An increase in eosinophil count has been noted in asthmatic subjects, where it has been shown to increase in asthmatics, thus further highlighting the role of eosinophils in propagating lung allergy (Bentley et al., 1992).

1.15.6 Antigen presenting cells (APCs)

Various cell types such as Dendritic cells, B cells, macrophages and eosinophils, have the ability to act as antigen presenting cells, however it is the dendritic cells which are the most potent APCs due to their ability to most effectively activate naïve T cells, and thus lead to activation of allergic response (Lambrecht and Hammad, 2003).

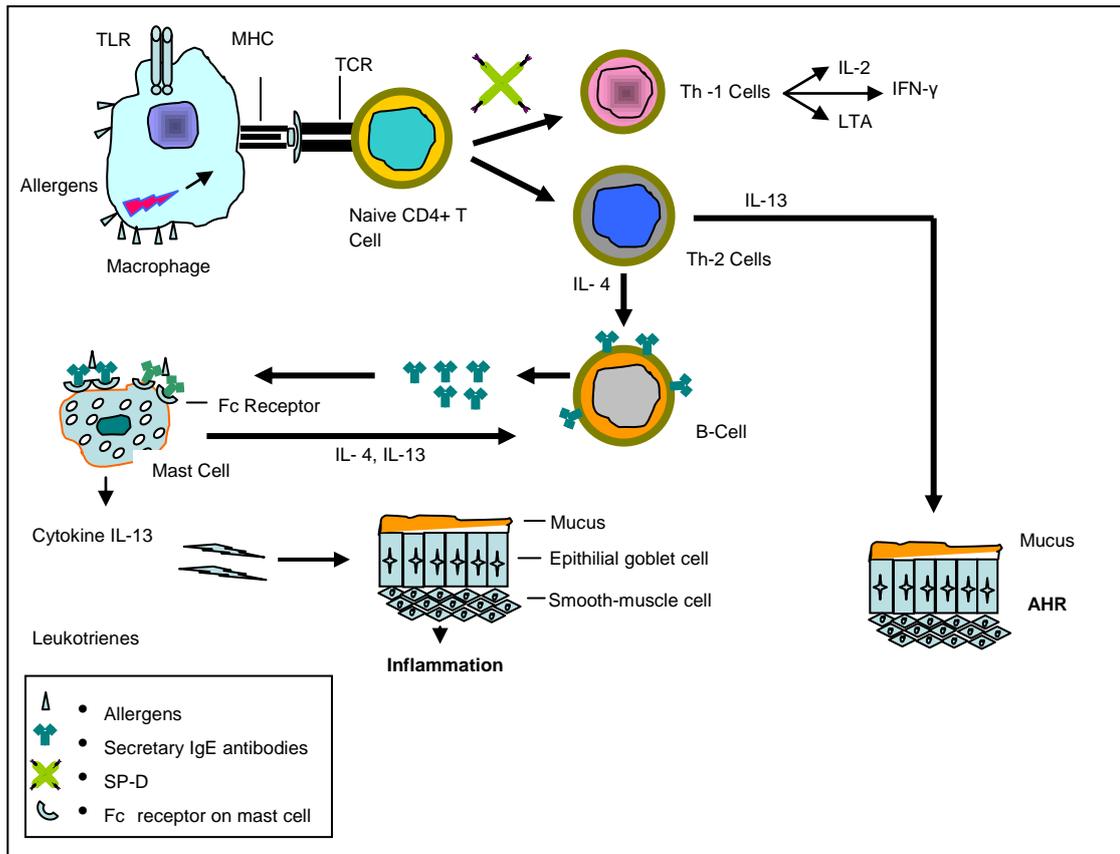


Figure 1.6: Sensitisation and re-exposure to an allergen leads to inflammatory responses typical in asthma.

The allergen is taken up and processed by antigen presenting cells (APCs) like macrophages and subsequently presented to the T cells. These changes trigger the production of cytokines like IL-4 and IL-13 by Th2 cells. IL-4 and IL-13 enhance class switching to IgE and regulate the clonal expansion of IgE committed secreting B-cells. The secreted IgE molecules bind to IgE specific Fc receptors on mast cells and a re-exposure to the allergen leads to cross linking of the bound IgE triggering the release of cytokines (IL-4 and IL-13), chemokines and pharmacological active mediators e.g. leukotrienes, histamine from mast cells causes smooth muscle contraction, increased vascular permeability vasodilation and drives the inflammatory response leading to infiltration of eosinophils, lymphocytes, macrophages and epithelial cells into the lung. The cytokine profile is predominantly of the Th2 type characterized by high levels of IL-4 and IL-13. SP-D, more known for its role in surfactant homeostasis and innate immunity has been shown to play a significantly protective role in asthma. Taken from (Qaseem et al., 2013).

1.16 Role of SP-D in modulating allergic asthma

SP-D has been shown to direct predominantly a Th1 response in vivo. Therapeutic treatment of sensitised mice with SP-D has shown to shift the ongoing Th2 cytokine profile typical in airway hyper-responsiveness and airway inflammation towards a protective Th1 response. Its relevance in directing a Th1 shift is corroborated in the SP-D^{-/-} mice which show an increase in Th2 cytokines over Th1 cytokines and a decreased IFN- γ /IL-4 ratio compared to wild type mice (Madan et al., 2005b). An overview of functions of SP-D in asthma is illustrated in Table 1.3.

This section discusses the relevance of SP-D in protecting against the characteristic pathophysiological phenotypes that constitute asthma, and its outcomes that may be crucial in the development and suppression of the complex immunological events in asthma and emphysema.

1.16.1 SP-D in Airway Hyper-responsiveness

Airway hyperresponsiveness (AHR) is caused by the inflammation of the asthmatic airway resulting in an increased bronchospastic response to a non-specific agonist such as MCh (Weinberger, 1993b, Weinberger, 1993a, White, 1993, Pueringer and Hunninghake, 1992). The role of SP-D in the development of AHR is not well defined but it has been implicated (Zhang et al., 2003). Elevations in SP-D levels have been shown in sensitised and allergen (OVA) challenged mice along with significant AHR (upon MCh inhalation) and increased eosinophils (Zhang et al., 2003). Upon rhSP-D administration to mice before challenge, AHR and eosinophil numbers in BAL reduced in a dose dependent manner, while the same therapy with a mutant SP-D with altered carbohydrate binding did not show any effect (Takeda et al., 2003b, Mahajan et al., 2008). Interestingly, C57BL/6J mice are relatively hypo-responsive and resistant to airway inflammation and AHR compared to other inbred

strains of mice (Zhang et al., 2003, Zhang et al., 1997). The C57BL/6J mice were shown to have higher basal SP-D levels than Balb/c mice after sensitisation and challenge. The elevated SP-D levels even during sensitisation could be consistent with the resistance of these mice to develop AHR.

1.16.2 SP-D association with goblet cell hyperplasia and mucus production

Allergic asthma is associated with a substantial increase in the mucus content of the airway epithelium (Rankin et al., 1996, Gavett et al., 1997, Kuperman et al., 1998, Zhu et al., 1999). Mucus hyperproduction in asthma results from a Th2-induced airway inflammation, significantly contributing to airway obstruction, wheezing and coughing (James and Carroll, 1995, Cohn et al., 1999). Although Th2 cells play an important role in mucus production, mucus could be induced in mice lacking IL-4 and IL-5 and corresponding inflammatory responses devoid of eosinophils or mast cells (Cohn et al., 1997, Cohn et al., 1999, Rankin et al., 1996). IL-13, along with its receptor the IL-4R, seems to be the critical cytokine involved in this pathway through which mucus production is induced (Grunig et al., 1998, Whittaker et al., 2002). Mucus is also an innate immune defence mechanism by which airway epithelium responds to pathogens and allergens. The Th2 inflammation in the airway stimulates epithelial cell hypertrophy and mucus metaplasia. The rhSP-D therapy in mice sensitised and challenged with OVA results in a significant decrease in goblet cell hyperplasia (Takeda et al., 2003b). SP-D expression has also been demonstrated in mucus cells of the rat gastric mucosa (Fisher and Mason, 1995) and on mucosal surfaces in numerous glands and organs (Madsen et al., 2000), where it may be involved in innate immunity. SP-D has been shown to be expressed in goblet cells of the airways, and the expression of SP-D strongly increases in sensitised and OVA-challenged mice in hyperplastic goblet cells (Kasper et al., 2002).

1.16.3 Role of SP-D in Inflammation and eosinophilia

Therapeutic administration of rhSP-D in a murine model of pulmonary allergy induced by allergens of *Aspergillus fumigatus* (Madan et al., 2001a) and dust mite (Singh et al., 2003) have been shown to lower pulmonary and blood eosinophilia, specific IgE levels, and pulmonary infiltration. On the other hand, SP-D $-/-$ mice exhibit a pronounced hypereosinophilia accompanied by an increase in intrinsic Th2 cytokines including IL-13 (Madan et al, 2005a). This hypereosinophilic phenotype can be reversed by administration of exogenous SP-D to SP-D $-/-$ mice. This clearly implies a role of SP-D in regulating eosinophilia and modulating lung immune response in the allergic condition (Madan et al., 2005b). In an *ex-vivo* study, SP-D and rhSP-D showed a direct, dose, carbohydrate and calcium dependent binding to human eosinophils. rhSP-D resulted in a differential effect on eosinophils from allergic patients with increase in CD69 expression, oxidative burst and apoptosis, while eosinophils from healthy controls were unaffected (Mahajan et al., 2008). Similarly, SP-D has been shown to reduce the *in vivo* eosinophilic inflammation in mice sensitised and challenged with ovalbumin (Takeda et al., 2003b). The eosinophil numbers in this study reduced by ~50% upon SP-D therapy. SP-D has been shown to induce production of IL-10, IL-12 and IFN- γ (Takeda et al., 2003a), which are all capable of influencing eosinophilic airway inflammation and AHR. Therefore, one of the protective anti-inflammatory effects of SP-D appears to be the induction of Th1 cytokines and shifting the predominant Th2 response that is typical of airway inflammation (Takeda et al., 2003b, Madan et al., 2001, Singh et al., 2003). SP-D could also resolve inflammation by clearing apoptotic cells by alveolar macrophages (Vandivier et al., 2002). Similar observations have been made by Mahajan et al, 2008 where SP-D showed increased binding to apoptotic eosinophils and resulted in their increased phagocytosis by macrophages. SP-D may thus be involved in resolution of eosinophilic inflammation following allergen provocation.

MMP-2, -9 and -12 have been shown to be crucial in the infiltration of inflammatory cells like eosinophils (Kumagai et al., 1999) and this finding has been supported by studies in murine models of antigen-induced airway inflammation (Kumagai et al., 1999, Corry et al., 2002, Cataldo et al., 2002). SP-D has also been shown to modify MMP production and thereby modulating inflammation (Wert et al., 2000a, Wert et al., 2000b, Cury et al., 1988). These effects of SP-D on MMP production are likely to be due to its interaction with alveolar macrophages, which have the capacity to secrete several members of the MMP family (Miyamura et al., 1994a, Cury et al., 1988, Campbell et al., 1991, Welgus et al., 1990). Therefore, common mediators like MMPs further suggest the role played by SP-D in inflammatory processes in the lung.

1.17 Role of SP-D in emphysema

Cigarette smoking is the most important risk factor for chronic obstructive pulmonary disease (COPD) and emphysema (Sandford et al., 2002). According to the Dutch hypothesis (Sluiter et al., 1991), the distinction between these two disorders is not absolute, with common factors contributing to both (Sluiter et al., 1991, Prescott et al., 1997). Tissue remodelling responses causing alveolar septal destruction and changes in compliance are characteristic of emphysema (Senior and Anthonisen, 1998). SP-D has been implicated in the development of emphysema. Furthermore, SP-D levels have been shown to be decreased in many clinical conditions like cystic fibrosis (Postle et al., 1999) and in smokers (Honda et al., 1996), possibly contributing to COPD and emphysema. Cigarette smoke induces apoptosis of alveolar macrophages, which could be one mechanism of lung damage leading to emphysema (Majo et al., 2001). SP-D *-/-* mice have been shown to develop progressive pulmonary emphysema and fibrosis in association with chronic inflammation (Wert et al., 2000b). These abnormalities are associated with increased activity of oxidant and MMP (MMP-2 and MMP-9) production by alveolar

macrophages contributing to inflammation and air space destruction leading to emphysema (Wert et al., 2000b). Conditional replacement of SP-D in the respiratory epithelium of SP-D $-/-$ mice does not reverse emphysema which is consistent with clinical observations (Zhang et al., 2002). Therefore, the value of SP-D therapy after the development of emphysema seems doubtful in reversing or decreasing this distress. SP-D is also a potent antioxidant due to its ability to act as a free radical chain terminator and inhibiting formation of lipid radicals (Bridges et al., 2000). In addition, it also contributes to regulation of MMP production (Trask et al., 2001, Wert et al., 2000c, Yoshida et al., 2001). The deficiency or reduction of SP-D in asthmatics and chronic smokers therefore could contribute to the development of emphysema.

1.18 Therapeutic potential of rhSP-D

Previous studies (Madan et al., 1997b, Strong et al., 2003) have implicated the role of trimeric CRD region in protecting animal models against asthma and inflammation by bringing down IgE levels, eosinophilia and causing T helper cell polarisation from pathogenic Th2 to a protective Th1 immune response. Thus, it appears that SP-D can target various components of therapeutic strategies that are currently being attempted in clinical trials. Furthermore, animal models of asthma have shown that rhSP-D (Figure 1.7) retains the immunomodulatory properties displayed by full-length SP-D, and has the ability to provide a long term therapeutic effect by dampening asthmatic symptoms in mice.

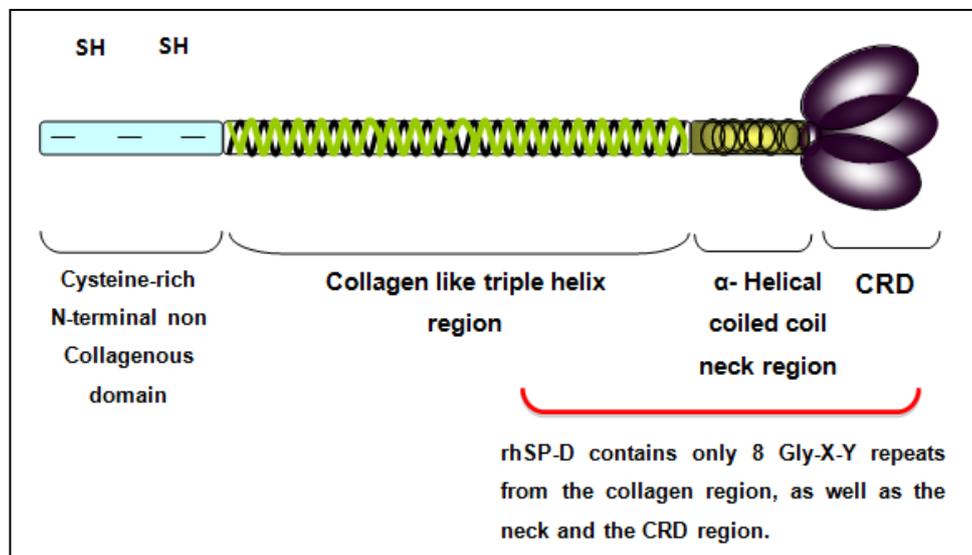


Figure 1.7: Structure of recombinant human SP-D (rhSP-D gly-x-Y)

A recombinant fragment of human SP-D (177 residues: Gly179 to Phe355) consisting of the globular CRD region (residues 236-355), and an α -helical coiled-coil neck region (residues 203-235) followed by short stretch of eight N terminal Gly-X-Y triplets with substitution of S for P in position 2 (residue 180) was expressed in Escherichia coli BL21(IDE3) pLysS.

Table 1.3: Functions of SP-D in asthma.

Functions of SP-D	References
Recognizes pathogen associated molecular patterns (PAMPs) and acts as an agglutinin, opsonin and immunomodulator thereby regulating proinflammatory responses induced by microbial components.	(Madan et al., 1997b, Wang et al., 1996b)
Contributes to immune homeostasis in the lung by recognizing and promoting removal of necrotic and apoptotic cells and enhances the uptake of these cells by alveolar macrophages,	(Vandivier et al., 2002, Schagat et al., 2001, Clark et al., 2002)
Decreases pollen induced IgE-dependent mast cell degranulation. Suppresses histamine release from basophils and mast cells together with antiproliferative effects on B and T lymphocytes	(Madan et al., 1997b, Wright, 2005, Wang et al., 1996b)
Inhibits allergen- induced proliferation of PBMCs in asthmatic children sensitive to mite allergens	(Wang et al., 1998)
Down-regulates allergen specific (Afu)-IgE, (Afu)-IgG, peripheral and pulmonary eosinophilia and competes with <i>Afu</i> -IgE to block subsequent histamine release from sensitised basophils from ABPA patients	(Madan et al., 1997b, Madan et al., 2001, Madan et al., 2005b)
Inhibits eosinophilic response in sensitised mice challenged with allergen therefore limiting the inflammatory changes during airway inflammation.	(Takeda et al., 2003b)
Exhibits immunomodulatory effects, inhibiting T cell proliferation and IL-2 production and also enhances chemotaxis of phagocytic cells.	(Wang et al., 1998, Borron et al., 1998)
Induces Th1 cytokines and shifting the predominant Th2 response.	(Takeda et al., 2003b)
Acts as a potent antioxidant and as a free radical chain terminator inhibiting formation of lipid radicals.	(Bridges et al., 2000)

1.19 General aims of this study

In this study, we seek to examine the underlying mechanisms by which SP-D confers protection against allergic asthma by using a truncated recombinant form of human SP-D (177 residues: Gly179 to Phe355), consisting of the globular CRD region (residues 236-355), an α -helical coiled-coil neck region (residues 203-235) preceded by short stretch of eight N terminal Gly-X-Y triplets with substitution of S for P in position 2 (residue 180), Figure 1.7.

Aim 1: To investigate the effect of rhSP-D on the immune response generated by a human monocytic cell line (THP-1) and monocytes when challenged with LPS. This will thus help to determine whether the production of pro-inflammatory versus anti-inflammatory cytokines is altered in these phagocytes in the presence of LPS, a known PAMP for TLRs.

Aim 2: To investigate the mechanism via which rhSP-D suppresses Th2 cell-driven allergic inflammation and its direct or indirect effect on the inhibition of the IgE-facilitated antigen presentation.

Aim 3: To investigate if rhSP-D can modulate IgE synthesis by primed B-cells by interfering with IgE interaction with CD23 and CD21.

Chapter 2



General Methods and Materials

2.1 Expression and purification of rhSP-D gly-x-y

The rhSP-D molecule was expressed in E.coli. Plasmid pUK-D1 containing cDNA for the neck and CRD of human SP-D under bacteriophage T7 promoter was used to produce rhSP-D (177 residues: Gly179 to Phe355) (Figure 1.7) in Escherichia coli BL21 (λ DE3) pLysS (Singh et al., 2003) (Dodagatta-Marri et al., 2013). The expression cassette included a short stretch of eight N terminal Gly-X-Y triplets with substitution of S for P in position 2 (residue 180), followed by the α -helical coiled-coil neck region (residues 203-235) and the globular CRD region (residues 236-355).

Vector Construction for expression of rhSP-D

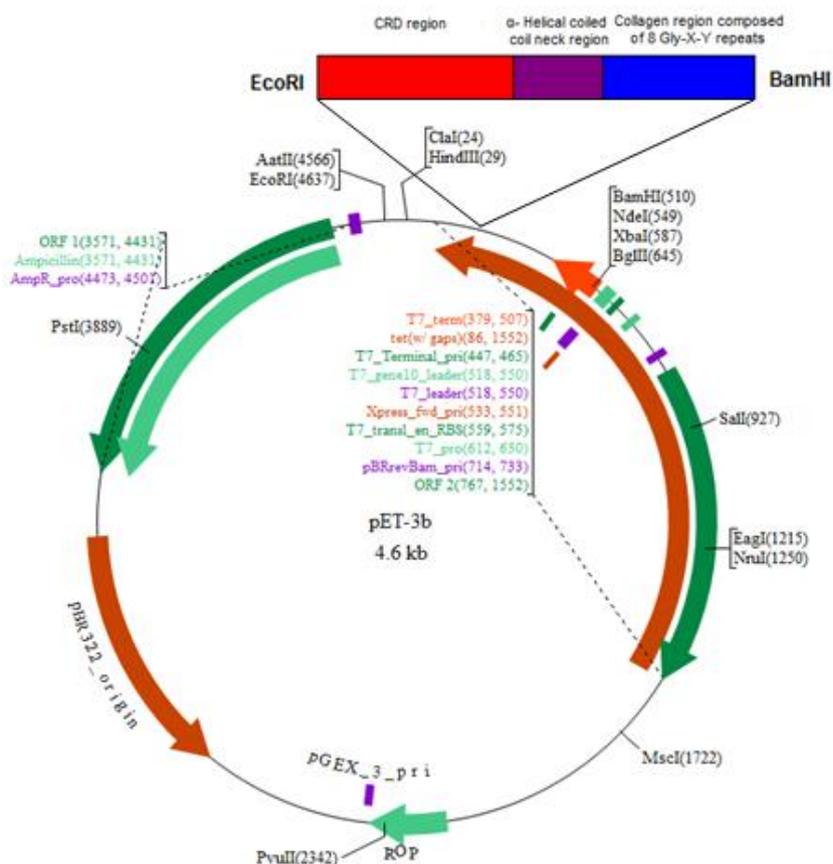


Figure 2.1: Schematic representation of the vector pET-3b

Vector pET-3b was used to express the truncated rhSP-D, which is composed of a CRD region, α -helical coiled neck region and collagen region composed of 8 Gly-X-Y repeats.

2.1.1 Preparation of Competent cells

A single colony of pLysS BL21 (λ DE3) was inoculated in 10ml of Luria Broth (LB) overnight at 37⁰C in a shaker. 500 μ l was removed from the overnight inoculum and placed inside a 50 ml falcon tube containing 25ml of LB medium along with 25 μ l of Chloramphenicol at a concentration of 50 μ g/ml. The resulting mixture was then placed inside the 37⁰C shaker, and optical density (O.D) at 600nm was measured after each hour until the O.D reached a value between 0.3-0.4 (Log Phase). After the log phase had been reached, the tube containing (LB + Chloramphenicol + pLysS) was centrifuged at 2000rpm for 5 minutes. Following centrifugation the supernatant was discarded, and 12.5ml of 0.1M CaCl₂ was added to the pellet. The cells were re-suspended and were placed in the temporary state of competence on ice for 1 hour. After one hour the cells were re-spun for 5 minutes at 2000 rpm. Finally, the supernatant was discarded, and the pellet was re-suspended in 2 ml of 0.1M CaCl₂ and stored on ice, ready to be transformed.

2.1.2 Transformation of cells

From the competent cells prepared as above, 200 μ l of cells were removed and placed inside a 10ml falcon tube containing 2 μ l (200ng) of (pUK-D1) Construct. The mixture was placed on ice for 1 hour, following which the mixture was subjected to Heat Shock at 42⁰C for 90 sec. Following heat shock the cells were placed on ice for a further 5 minutes, and then re-suspended in 800 μ l of LB. The mixture was placed inside a 37⁰C incubator for approximately 45 minutes. After incubation the cells were streaked out on anLB agar plate containing Ampicillin (100 μ g/ml), Chloramphenicol (50 μ g/ml) and left inside a 37⁰C incubator overnight to grow.

2.1.3 IPTG Induction

A single colony of transformed (pUK-D1) cells was grown in 25 ml LB along with 100 µg/ml ampicillin + 50 µg/ml chloramphenicol overnight in a shaker at 37°C. On the following morning, the overnight primary inoculum of 25 ml bacterial culture was inoculated into 1 litre LB along with 100 µg/ml ampicillin + 50µg/ml chloramphenicol and was grown to A600 of 0.6-0.8 inside a 37°C shaker, where the OD was measured at regular intervals. After the cells reached log phase (A600 @ 0.6-0.8) they were induced with 0.4 mM IPTG for 3 hours and harvested by centrifugation at 8500rpm for 15 minutes. Following centrifugation the supernatant was discarded and the pellet was stored at -20°C for further processing.

2.1.4 Cell Lysis

The cell pellet obtained after IPTG induction was re-suspended 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 spun for 1 hour in cold room at 4°C and then sonicated using a tip sonicator (10 cycles @ 30 s each).

2.1.5 Dialysis

The sonicate was harvested by centrifugation at 8500 rpm for 15 minutes and the rhSP-D recovered in the inclusion bodies was solubilized in 100ml buffer I (50 mM Tris-HCl pH 7.5, 100 mM NaCl) containing 10 mM 2-mercaptoethanol and 8 M Urea. The solubilized material was then dialyzed stepwise against buffer I containing 4 M urea, 2 M urea, 1 M urea and no urea, each for 2 hrs. The dialysate, clarified by centrifugation at 8500rpm for 15 mins, was then dialysed with calcium buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM CaCl₂, 0.05% Sodium Azide) for 3 hours to completely remove urea from the dialysate.

2.1.6 Protein purification by Affinity Chromatography

The rhSP-D was purified by affinity chromatography by passing through a Maltose-Sepharose column, which was prepared via the immobilization protocol shown below in section 2.1.7. The Maltose-Sepharose column (5ml) was rinsed with distilled water prior to use. After thorough rinsing of the column with d.H₂O, 50ml of affinity column buffer containing (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5mM CaCl₂, sodium azide-0.05%) was passed through. This was followed by the protein sample, which was passed through once. The bound protein was eluted by using elution buffer containing 5mM EDTA (25ml) (50mM Tris-HCL pH 7.5, 100mM NaCl, 5mM EDTA, Sodium Azide-0.05%). The eluted fractions were collected in 2ml eppendorf tubes and the U.V absorbance readings at A280 were obtained for each eluted fraction. Finally the presence of the purified protein was confirmed by SDS-PAGE.

2.1.7 Preparation of Maltose-Sepharose for Affinity Chromatography

In order to affinity purify rhSP-D, a Maltose-Sepharose column needed to be prepared as further described in (Dodagatta-Marri et al., 2014). Firstly, 100 ml of Sepharose 4B settled gel (Sigma) was activated with Divinyl Sulfone-DVS (Sigma). 100ml of Sepharose 4B settled gel was washed with 1 L water four times by centrifugation at 1000rpm for 5 minutes. (N.B- as Divinyl Sulfone (DVS) is highly toxic, all operations involved in activation and coupling were carried out in a fume hood).The washed gel was then resuspended in 100ml of 0.5M sodium carbonate pH 10 and stirred by magnetic stirring in fume hood. 10ml of DVS was slowly added drop wise over the duration of 15 minutes. Following DVS addition the gel suspension was stirred for a further one hour at room temperature. DVS-activated Sepharose 4B was then washed with 1 L water, and suction dried to a moist cake, and added to 1 L maltose solution containing 20% solution of maltose (Sigma) in 0.5 M sodium carbonate. The reaction mixture was stirred at room

temperature for 24 hr. The gel was then filtered and washed successively with 2 L each of water and 0.5 M sodium bicarbonate. The gel was then subsequently suspended in 100 ml, 0.5 M sodium bicarbonate containing 5 ml of 2-mercaptoethanol in a fume hood, and allowed to mix at room temperature for further 2 hours to block the excess vinyl reactive groups. Finally, the gel was washed with 2 L water. Maltose Sepharose gel was then stored in 0.02% sodium azide at 4°C.

2.2 SDS-PAGE

A 15% SDS-PAGE gel was used to determine the molecular weight of the protein samples, which were run along with a protein marker (Peq gold Protein marker 1-14.4 - 116 kDa, Peqlab Cat# 27-1010) having defined bands with specific molecular weights. The SDS-PAGE gel consists of a stacking gel on which the sample is loaded and a resolving gel on which the proteins are separated according to their electrophoretic mobility (M.W) by sieving effect. The resolving gel concentration depends upon the molecular weight range of the protein to be examined. 10ml of 15% resolving gel (2.3ml dH₂O, 5ml of 30% acrylamide, 2.5ml 1.5M Tris pH8.8, 0.1ml 10% SDS, 0.1ml 10% ammonium persulfate and 0.004ml of TEMED) was prepared and added to the SDS-PAGE assembly until it filled three quarter of the total volume available on the glass plates. Once the resolving gel had polymerised, 5% stacking gel (2.7ml H₂O, 0.67ml 30% acrylamide, 0.5ml 1.0M Tris pH6.8, 0.1ml 10% SDS, 0.4ml 10% ammonium persulfate and 0.005ml TEMED) was added on the top of the resolving gel and a comb inserted into the stacking gel for the formation of wells. After polymerisation of the stacking gel, the assembly was removed and placed into a tank containing 1X running buffer. The comb was carefully removed from the wells and the samples loaded on to the gel, which was run at 110 volts for 75 mins. The gels were stained for 3 hours in Coomassie blue stain (0.1% w/v Coomassie

R250, 10% acetic acid, 40% methanol 50% water) and destained using destaining solution (20% methanol, 10% acetic acid in water).

2.3 BCA Protein Assay

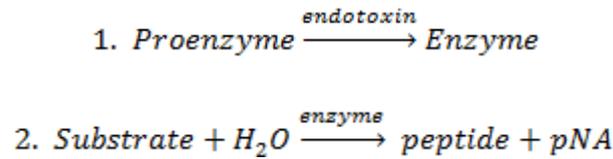
The Pierce BCA Protein Assay (Thermo Scientific Pierce, Product # 23225) was used for the colorimetric detection and quantitation of total protein.

2.4.1 Endotoxin removal from proteins

5ml of Polymyxin B agarose gel (Sigma, product # P1411) was packed in a 20ml column, and washed with 50ml of 1% sodium deoxycholate and then further rinsed with 50ml of autoclaved distilled H₂O to completely remove the sodium deoxycholate. After the rinsing of column, the protein solution was loaded into the column, and left to incubate at room temperature for half an hour after the protein solution had completely entered the gel. After the incubation period the protein solution was collected in 1.0-mL fractions. This LPS free rhSP-D protein was checked for purity by running it on 15% SDS PAGE. The protein was further quantified by measuring the absorbance at 280 nm.

2.4.2 Limulus Amebocyte Lysate (LAL) assay

Limulus Amebocyte Lysate (LAL) assay (QCL-1000-Lonza) was used according to the manufacturer's instructions provided with the kit, for detecting the total amount of endotoxin present after LPS removal from the affinity purified recombinant proteins. LAL assay utilises the initial part of the LAL endotoxin reaction to activate an enzyme which in turn releases p-nitroaniline (pNA) from a colorless synthetic substrate (Ac-Ile-Glu-Ala-Arg-pNA). The release of pNA leads to production of yellow color, which can be determined photometrically at 405-410nm after the reaction is stopped with the stop reagent.



The concentration of endotoxin in a sample was calculated from the absorbance values of solutions containing known amounts of endotoxin standard. The endotoxin concentration was found to be under 3 EU/ml.

2.5 BS³ Crosslinking Assay

A water soluble BS³ crosslinker (Thermo Scientific, UK) was used in order to show the trimerization of rhSP-D. BS³ cannot permeate cell membranes due it being charged; this thus confines BS³ crosslinking to the surface of intact cells. BS³ reacts with proteins via interaction between NHS ester group of crosslinker and primary amine group on protein. A schematic of the chemical reaction between BS3 and the protein is shown below in (figure 2.2).

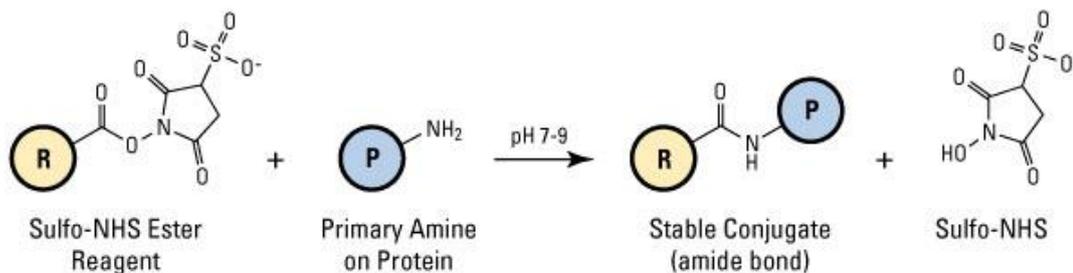


Figure 2.2: Chemical reaction between BS3 crosslinker and protein

Sulfo-NHS ester reaction scheme for chemical conjugation to a primary amine. (R) represents a labeling reagent or one end of a crosslinker having the sulfo-NHS ester reactive group; (P) represents a protein or other molecule that contains the target functional group (i.e., primary amine, -NH₂).

(Reference: <http://www.piercenet.com/method/amine-reactive-crosslinker-chemistry>)

This assay was carried out as per supplier's instructions. Briefly the procedure entailed preparation of 25mM stock BS³ cross-linking solution by dissolving 0.002g of BS₃ in

140µl of DMSO. From the stock 25mM BS³ solution the following concentrations were prepared for the cross linking experiment (0.01mM BS³, 0.1mM BS³, and 1mM BS³) in order to show the presence of monomer, dimer and trimer bands using various concentrations of BS³ cross linker by SDS-PAGE. This assay involved incubating 45µl of rhSP-D, which was dialysed against HEPES buffer consisting of 10mM HEPES pH 7.5, 100mM NaCl, 1mM EDTA) for 45 min at room temperature, with 5µl of various concentrations of BS³ cross-linker as prepared earlier. The cross-linking reaction was electrophoresed on a 15 % (w/v) acrylamide SDS-PAGE gel.

2.6 Biotinylation of proteins

Biotin is a vitamin that binds with high affinity to avidin and streptavidin proteins. Due to its relatively small size (244Da) biotin can be conjugated to many proteins without altering their biological activities. The labelled molecule then can be detected using streptavidin or avidin probes. N-Hydroxysuccinimide (NHS) ester-activated biotin is the most commonly used biotinylation reagent. NHS esters react with primary amino groups (-NH₂) in pH 7-9 buffers to form stable amide bonds (Figure 2.3).

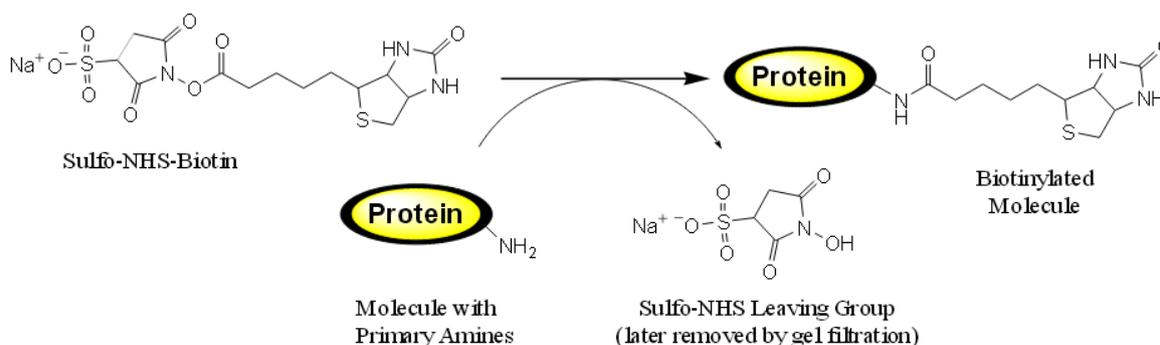


Figure 2.3: Reaction of Sulfo-NHS-Biotin with primary amine

(Reference: <http://www.piercenet.com/instructions/2161775.pdf>)

The extent of biotin labelling was determined by the using the following formulas supplied on the data sheet accompanying EZ-Link® Sulfo-NHS-Biotin (Thermo Scientific, product # 21338). The protein was biotinylated as per instructions provided by supplier. Briefly, the procedure entailed dialysing protein against PBS since it was eluted in a Tris buffer.

Immediately, prior to biotinylation a 10mM Sulfo-NHS-Biotin was prepared by dissolving 2.2mg in 500µl autoclaved distilled water, which was then added to the protein solution at an appropriate volume as calculated earlier. The mixture was incubated at room temperature for 30 min's at room temperature. The unreacted sulfo-NHS-biotin was removed by dialysis against affinity column buffer containing 50mM Tris-HCl pH 7.5, 100mM NaCl, 5mM CaCl₂.

The extent of biotin label incorporation can be further estimated by using a HABA assay.

2.7 Culture of THP-1 cells

THP-1 cells derived from a Human acute monocytic leukaemia (ATCC number TIB-202) were cultured from frozen cells stored in liquid nitrogen. One ampoule of THP-1 cells was thawed in a water bath at 37°C for 40 seconds, the contents were transferred into a 10ml falcon tube containing complete RPMI medium (cRPMI) consisting of 10% fetal bovine serum (Sigma-Aldrich, Cat no# F2442), 2 mM L-glutamine (Sigma-Aldrich, Cat no# G7513), 1% Pen/Strep (Sigma-Aldrich, Cat no# P0781) and 1mM Sodium Pyruvate (Sigma-Aldrich, Cat no# S8636). Since the cells were stored in DMSO, which is very toxic to cells at room temperature, there was thus the need to process the frozen cell quickly in order to retain good cell viability. Following the transfer of thawed cells into a falcon tube containing cRPMI, the cells were washed for 10mins at 1500rpm in an eppendorf 5810R centrifuge, following the first wash the supernatant was decanted and the cell pellet resuspended in fresh 10ml cRPMI. The cells were washed again as before and the supernatant was decanted. The cells were resuspended in 10ml cRPMI after the second wash and the cell viability was determined via Trypan Blue dye exclusion method, where equal volumes of cell suspension and Trypan Blue (0.4% w/v solution; Sigma) were mixed and cells counted using a haemocytometer with neubauer rulings. The cells were then re-suspended in cRPMI according to the cell count, and cultured in a T75 flask at 37°C

incubator with 5% CO₂. The cells were regularly checked in order to determine their health, and further passaged when required.

2.8 Freezing THP-1 cells

Surplus THP-1 cells were stored in order to ensure a constant supply of healthy THP-1 cells when required. THP-1 cells were counted using the method described above, and then spun for 10mins at 1500rpm. The supernatant was discarded and to the pellet an equal volume of FCS and of 20% DMSO (2ml DMSO + 8ml RPMI) were added. The cells were immediately placed at -80°C in a cryovial, since DMSO is very toxic to cells at room temperature. The THP-1 cells were stored at a density of 6×10^6 cells per cryovial in 0.5ml of FCS plus 0.5ml of 20% DMSO.

2.9 Indirect ELISA

ELISAs were conducted to detect the binding of biotinylated ligands to immobilised proteins. For indirect ELISAs, flat bottom 96-well polystyrene plates were coated with proteins or cells of interest in carbonate-bicarbonate buffer at specific concentrations and incubated overnight at +4°C. Following overnight incubation, in order to ensure the blocking of non-specific binding, 200µl of blocking buffer (1%BSA/PBS) was added to each well for two hours at room temperature. The plate was then washed four times with PBS + 0.05% Tween20 after blocking and the biotinylated protein was added in PBS at various concentrations (1-10µg/ml). Following the addition of biotinylated proteins to appropriate potential ligand wells, the plate was further incubated for 2 hours at 37°C, and then washed 4 x with PBS + 0.05% Tween20, after the incubation. 1/1000 dilution of HRP conjugated streptavidin (Biolegend, Cat# 405210) was added to each well and further incubated for 1 hour at 37°C. The 4 washing steps were repeated as before, and 200µL of

Sigma fast OPD (a chromogenic substrate, Cat# P9187) was added to develop the reaction, and the plate was analysed at 450nm on an ELISA plate reader.

2.10 Indirect Immunofluorescence of THP-1 cells

Indirect Immunofluorescence was carried out in order to visualize the binding of rhSP-D with THP-1 cells. Briefly, 8ml of cRPMI medium containing THP-1 cells was centrifuged at 2000rpm for 10min. The supernatant was removed following centrifugation and 6ml of PBS was added to the cells after re-suspension. The cells were then counted and distributed equally into two 2ml eppendorf tubes (approximately 20,000 cells/tube). The eppendorf tubes were then spun for 3mins at 3000rpm. Supernatant was removed following centrifugation and the cells were washed once in 1ml PBS containing 1% BSA at 3000rpm for 2mins. The supernatant was discarded and the cells re-suspended in 100µl of PBS/ 1% BSA Solution. Both eppendorf tubes were labeled accordingly “Control” containing (PBS + THP-1 cells only) and “Test” containing (THP-1 + 100µl of biotinylated 10µg/ml rhSP-D + 5mM CaCl₂). and were left to incubate at room temperature for 2hours at 37°C. Following this incubation period, both of the eppendorf tubes were washed four times in 1ml PBS containing 1% BSA solution at 3000rpm for 3 min. After washing, 2µl of FITC-Conjugated Streptavidin (stock concentration=0.5mg/ml) (BioLegend, Cat no# 405202) was added to both tubes and left to incubate for 1 hour at room temperature. Following this incubation period the cells were washed again three times with 1ml of PBS containing 1% BSA, and re-suspended in 200µl of PBS containing 1% BSA solution and then thoroughly mixed by vortexing. Cells were spun onto coverslips using a cytospin centrifuge (Cytospin 4, Thermo Scientific) for 5 min at 1300 rpm. The slides were viewed under fluorescence microscopes and the images of the cells from slides were captured under 100X objective of an Axioplan 2 Imaging fluorescence

microscope (Zeiss), equipped with a 50W mercury lamp. Image capture and enhancement was performed using Smart Capture 2 software (Digital Scientific, Cambridge, UK).

2.11 Lysing THP-1 cells

5 x 10⁶ THP-1 cells were harvested at 1500rpm for 10 min. The supernatant was discarded following centrifugation and the cells were re-suspended in 10ml of PBS. The cells were then transferred into a fresh 10ml falcon tube and spun again at 1500rpm for 10 mins. Following, centrifugation the supernatant was discarded, and cells resuspended by vortexing. 500µl of ice cold modified RIPA lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X 100, 1% Sodium Deoxycholate, 0.1% SDS and 1mmol/L PMSF was added to the cells, which were then incubated on ice for 25mins with constant mixing by vortexing. Following, the incubation period, THP-1 cell lysate was spun again for 10 minutes at 2000rpm, and the supernatant was stored for loading onto SDS PAGE.

2.12 Far-western blotting

Far western blotting was carried out to examine protein-protein interactions in order to detect proteins of interest. Here, two identical 15% SDS page gels were run, where one SDS gel underwent Coomassie blue staining and the second gel underwent protein transfer for the purpose of western blotting. The SDS PAGE gel undergoing protein transfer, was placed in a tray and flooded with 1X transfer buffer (20% Methanol; 25 mM Tris, 192 mM glycine, pH 8.3, 0.1% SDS) and allowed to equilibrate for 5 minutes. The gel was then sandwiched between nitrocellulose membrane and Whatman filter paper, which were further supported by fibre pads on either side. The entire sandwich was placed between the cassettes of the western blotting apparatus and the cassettes then mounted within the blotting tank. Entire blotting tank was filled with transfer buffer and the electro transfer was carried out at 100 V for at 2 hours. In order to avoid over-heating of the buffer, a

magnetic spin bar along with an ice pack were placed in the tank and the tank placed above a magnetic stirrer for the duration of the transfer. Following transfer, the nitrocellulose membrane was removed from the cassette, and placed on a petri dish. The membrane was washed with 1X TBS buffer (150 mM NaCl, 10 mM Tris pH8.0; 0.1% Tween 20) and blocked with 25ml of blocking solution (5 % w/v non-fat dried milk, 0.01% antifoam A (Sigma, Cat# A5633-25G), and 0.02% sodium azide) overnight on a shaker.

After incubation, the membrane was immersed into 10µg/ml of biotinylated protein for at least 2 hours at 37 degree C on a shaker. The membrane was then washed in 25ml washing buffer containing PBS + 0.02% Tween 20, three times for 10 minutes. After the washes, the membrane was again immersed in 1/1000 dilution of HRP conjugated Streptavidin in 10ml of blocking buffer; for at least 1 hour at 37⁰C degree C on a shaker. The membrane was washed as before in 25ml 1X TBS washing buffer for 10 minutes, after which it was transferred into a 10ml substrate solution containing DAB (SIGMA FAST™ 3,3'-Diaminobenzidine tablets, Cat # D4418) and developed until bands became visible. The reaction was stopped by pouring out the substrate solution, and rinsed in PBS and air dried for records.

2.13 Isolating peripheral blood mononuclear cells (PBMC) from human blood

Blood was extracted from volunteers, using butterfly needle and collected in a 60ml syringe containing 100µl of heparin solution (Heparin Sodium, preservative free, 1,000 I.U./ml, EAN:5012727-743011, Wockhardt UK Ltd). The heparinized blood was diluted with equal volume of RPMI 1640 medium (Sigma, Cat no# R8758) and mixed thoroughly. 10ml of blood/RPMI mix was then carefully overlaid on top of 4ml Ficoll-Paque™ Plus (GE Healthcare, Cat no# 17-1440-03) inside a 15ml falcon tube by using disposable plastic pasteur pipettes to ensure that the blood does not mix with Ficoll-Paque™. The tubes were

then spun at 400g for 35 mins at room temperature in order to separate the blood to its components by density-gradient centrifugation. Following centrifugation four layers were observed in the falcon tube:

1) Plasma, 2) PBMC (Interface), 3) Ficol, 4) RBC + Polymorphic nuclear leukocytes (RBC's, Eosinophil's, Neutrophils and Basophils). The upper layer containing plasma and platelets was drawn off using a sterile pipette, leaving the layer of mononuclear cells undisturbed at the interface. The interface was then carefully removed with pastette into a fresh 50ml falcon tube, and this process was repeated to ensure that all cells were obtained, and then topped up with RPMI to give a total volume of 50ml per tube. The falcon tubes were then spun at 2000rpm for 10min at room temperature in an eppendorf 5810R centrifuge, and the RPMI medium discarded following centrifugation. The cells were resuspended by gentle flicking of the tube and fresh 40ml of RPMI was added to the resuspended cells, which were again spun at 1500g for 10 min at room temperature. Following the second wash the RPMI was again discarded and the PBMC's were resuspended in RPMI media equivalent to original blood volume at the start. Following PBMC isolation the cell viability was determined by Trypan Blue dye exclusion method as described in section 2.7.

2.14 Primer Design

The following human primers sets were designed for real time q-PCR studies to examine the effect of rhSP-D in modulating the anti-inflammatory and pro-inflammatory genes expressed by both THP-1 cells and monocytes obtained from healthy human volunteers. Sequences taken from GenBank.

Primer	Strand	Primer Sequence
IL-1β F	FWD	GGACAAGCTGAGGAAGATGC
IL-1β R	REV	TCGTTATCCCATGTGTCGAA
IL-10 F	FWD	TTACCTGGAGGAGGTGATGC
IL-10 R	REV	GGCCTTGCTCTTGTTCAC
TGF-β F	FWD	GTACCTGAACCCGTGTTGCT
TGF-β R	REV	GTATCGCCAGGAATTGTTGC
TNF-α F	FWD	AGCCCATGTTGTAGCAAACC
TNF-α R	REV	TGAGGTACAGGCCCTCTGAT

2.15 LPS Stimulation of THP-1 cells

THP-1 cells were aliquoted, at a density of $\sim 2 \times 10^5$ cells per eppendorf tube, under various experimental conditions, and were stimulated with 100ng/ml of LPS from salmonella typhumurium strain SL1181 prepared in complete cell culture medium (Sigma-Aldrich, Cat no# L9516) for 5 hours. After this period the cell pellets were obtained by washing once with PBS and collected by centrifugation at 3,000 rpm for five minutes in an eppendorf 5810R centrifuge. The pellets obtained following centrifugation were stored at -20°C prior to extracting total RNA.

2.16 Real time Quantitative PCR studies

2.16.1 Extraction of total RNA

Total RNA was extracted using TRI Reagent by following supplier guidelines (Sigma-Aldrich, Cat no: T9424). The cell pellets were defrosted on ice and then loosened by flicking the tube gently and lysed in 1ml of TRI Reagent by repeated pipetting. Samples were then incubated for 10min at room temperature. Following the incubation period 0.2ml of chloroform per 1ml of TRI reagent was added, which was followed by vigorous shaking of samples for 15 seconds and incubated for further 15 minutes at room temperature. Samples were phase separated by centrifugation at 14,000rpm for 15mins at

4⁰C in an eppendorf 5810R centrifuge. The upper aqueous phase (~0.5ml) was transferred to a fresh eppendorf tube and RNA was precipitated by adding 0.5ml of 2-propanol per ml of TRI reagent. Samples were incubated for further 10mins at room temperature and centrifuged as above. The supernatant was carefully removed following centrifugation and the RNA pellet was washed once with 1ml of 70% ethanol (made with DEPC-water) and centrifuged again at 7,500rpm for 5mins at 4⁰C. The supernatant was removed carefully and the RNA was briefly dried for a period of 5-10mins and resuspended in 10µl of DEPC water.

2.16.2 DNase Treatment of Total RNA

The DNase treatment kit obtained from Sigma-Aldrich (Product Code: AMP-D1) was used to remove any contaminating DNA from RNA preparations. The following components were prepared in RNase-free PCR tubes: (RNA in 8µl water, 2µl of 10X Reaction Buffer, 2µl of Amplification Grade DNase I, 1 unit/µl). The contents inside each tube were gently mixed and then incubated for 15mins at room temperature. 2µl of stop solution was added to each tube following incubation period to bind calcium and magnesium ions and to inactivate DNase I. The tubes were then heated at 70⁰C for 10mins to denature both the DNase I and the RNA, and subsequently chilled on ice.

2.16.3 cDNA Synthesis

Complementary DNA (cDNA) was synthesized by using High Capacity RNA- to-cDNA Kit, supplied by Applied Biosystems (Product no: 4387406). On ice, 9µl of RNA samples were added to separate PCR tubes, each containing 10µl of 2x RT Buffer mix and 1µl of RT Enzyme Mix. The tubes were spun down briefly in centrifuge to eliminate any air bubbles and then placed inside a thermal cycler, where the reaction mixture was incubated

at 37⁰C for 60mins. The reaction was stopped by heating to 95⁰C for 5mins and then held at 4⁰C. The cDNA samples were stored at -20⁰C for subsequent real time q-PCR analysis.

2.16.4 Determination of RNA/cDNA quantity and purity

RNA/cDNA concentration and purity was determined via Nano Drop TM 2000c spectrophotometer (Nano Drop, Thermo Scientific). The absorption of ultra violet light was measured at 260nm and the purity was determined by using A_{260/280} ratio.

2.16.5 Real time q-PCR Analysis

Following cDNA synthesis, real time q-PCR analysis was performed in order to detect the amplification of the following cytokines (IL-1b, IL-10, TNF- α , TGF- β) by using the following instrument 7900 HT Fast Real-Time PCR System Applied Biosystems. Power SYBR[®] Green PCR Master Mix was prepared according to the supplier guidelines (Applied Biosystems[®], Catalogue No # 4368577). 9 μ l of the primer mix was plated accordingly in each well under various conditions. The synthesised cDNA obtained for each sample was diluted in the ratio of 1:4 (10 μ l of cDNA and 30 μ l DEPC water) and 1 μ l of various diluted cDNA samples were added to the appropriate wells, with a final volume of 10 μ l/well.

2.17 Biological materials

Timothy grass allergen (*Phelum pratense*) was obtained from Professor Stephen Durham's Allergy and Clinical Immunology laboratory at NHLI, Imperial College London. *Aspergillus fumigatus* culture filtrate was obtained from Dr Uday Kishore's lab. Allergic patients' sera as well as blood from well characterised atopic patients highly sensitised to timothy grass allergy, were obtained from Professor Stephen Durham's clinic at Royal Brompton Hospital London.

2.18 Maintenance of EBV-transformed B cell line

The EBV-transformed B cell line which had been specifically engineered to express high levels of CD23, as previously characterised and described by (van der Heijden et al., 1993), was maintained in vented-cap, canted-neck cell culture flasks from Falcon/VWR (Poole, UK) at 37 °C, 5% CO₂ and 95% relative humidity (RH). Growth medium consisted of RPMI 1640 supplemented with 1% (v/v) L-glutamine, 10% heat-inactivated FCS and 1% (v/v) penicillin/streptomycin mixture as described in detail before, (Shamji et al., 2006a).

2.19 Direct surface staining

Direct staining was carried out in order to show binding of rhSP-D to various cells within PBMCs obtained from atopic patients, by FACS using the following panel of antibodies: CD16 PE mouse anti-human (BD Pharmingen™, Cat# 555407, Isotype; Mouse IgG1 κ, Clone: 3G8); CD56 APC mouse anti-human (BD Pharmingen™, Cat# 555518, Isotype; IgG1, κ, Clone: B159); CD19 PerCP-Cy5.5 mouse anti-human (BD Pharmingen™, Cat# 561295, Isotype; IgG1, κ, Clone: HIB19); CD4 Pacific Blue mouse anti-human (BD Pharmingen™, Cat# 558116, isotype: IgG1, κ, Clone: RPA-T4); CD3 PE-Cy7 (BD Pharmingen™, Cat# 557851, isotype: IgG1, κ, Clone: SK7); CD8 AmCyan (BD Pharmingen™, Cat# 339188, isotype: IgG1, κ, Clone: SK1).

Firstly PBMC's were pre-treated with 10µg/ml of biotinylated rhSP-D along with 5mM CaCl₂, and were left to incubate at room temperature for 2 hours at 37°C. Following this incubation period, untreated and treated PBMC's were washed with PBS containing 1% BSA solution at 3000rpm for 3 mins. After washing, 2µl of FITC-Conjugated Streptavidin (Biolegend, Cat no# 405202) along with appropriate amount of staining mAb were added to appropriate samples and mixed well. Single stain and unstained compensation control

samples were also prepared. The samples were then incubated for 25mins at 4⁰C. Following the incubation period the samples were washed as before with 1ml PBS with 1% BSA at 1200rpm for 5mins, and the supernatant discarded. The cells were then fixed in 1% Paraformaldehyde (PFA) by resuspending the stained cells in 350µl of 1% PFA and analysed on a FACS Canto II flow cytometer using FACS DIVA software.

2.20 IgE-FAB assay

IgE-facilitated allergen binding (IgE-FAB) assay represents an in vitro model of facilitated allergen presentation. The IgE-FAB assay is reproducible, robust, sensitive and specific method suitable as a tool for monitoring inhibitory antibody function from patients receiving allergen immunotherapy (Shamji et al., 2006b). In this assay the binding of Phlp-IgE complexes to CD23 enriched B-cells pre-treated with rhSP-D, using sera obtained from 10 well characterised grass pollen allergic patients, was detected via flow cytometry. Briefly, CD23 enriched EBV transformed B cells were pre-treated with 5µg/ml rhSP-D in presence of 5mM CaCl₂ for an hour. In the meantime, stock indicator serum (20 µl) containing high concentration of grass pollen (*P. pratense*) specific IgE, was pre-incubated with 1 µg/ml allergen (5 µl) at 37 °C for 1 h to form allergen-IgE complexes. Following incubation period, 1×10⁵ EBV-transformed B cells (5 µl) were added to the allergen-IgE mixture and incubated for further 1 hour at 4 °C. Cells were then washed and bound complexes were detected using a polyclonal human anti-IgE PE-labelled antibody (using 20 µl of a 1/50 dilution for 45 minutes) by a FACS Canto II flow cytometer and analysed using FACS DIVA software. B cells were gated using forward scatter/side scatter parameters and a positive marker was set using cells incubated with indicator serum only. Five thousand cells were analysed and all samples were measured in triplicate.

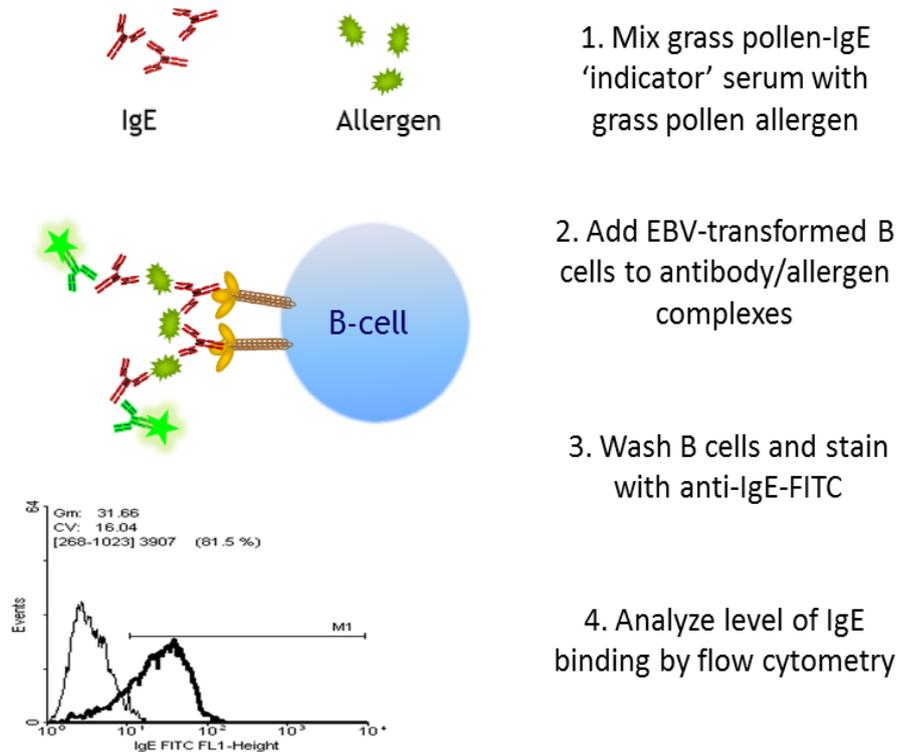


Figure 2.4: Schematic representation of IgE Fab assay

2.21 ³H-Thymidine incorporation assay

Effect of rhSP-D on antigen presentation & proliferation to T-cells was examined via a thymidine incorporation assay. The PBMCs obtained from 12 atopic patients highly sensitized to *phlp* (*Phelum pretense*) allergen, were incubated for 5 days under various conditions, i.e. in presence or absence of various concentrations of rhSP-D (5µg/ml, 10µg/ml), where 5µg/ml Phlp allergen was used to stimulate the proliferation of PBMCs. On day 5 the proliferation plates were pulsed by addition of 1µl of ³H thymidine (PerkinElmer, Cat# NET027X, specific activity: 20 Ci/mmol) to each well and cells harvested after 18 hours. A scintillation beta-counter was used to measure the radioactivity in order to determine the extent of cell proliferation which had taken place. In this assay, medium alone was used as a -ve control, and PHA was used as a +ve control.

2.22 Multiplex analysis

Multiplex assay was used to further characterise the cytokine and chemokine profiles of proliferating cells in order to determine if rhSP-D can inhibit allergen driven Th2 cytokine production. Thus the following 96 well plate based Millipore MILLIPLEX MAP (multi-analyte panel) kits were used according to instructions supplied. MILLIPLEX MAP Human Cytokine/Chemokine panel was used to detect the following analytes, IL-10, IL-8, RANTES, MDC, Eotakin, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 (Cat # HCYTOMAG-60K-09, Kit Lot# 2114660), and MILLIPLEX MAP Human TH17 Magnetic Bead Panel was used for detection of INF- γ , IL-17A, IL-12, IL-23, IL-27 and IL-33 (Cat# HTH17MAG-14K-08, Kit Lot # 2114641). The read out for this assay was obtained by processing the multiplex plates on a Luminix MAGPix analyser.

2.23 Statistical analysis

Statistical analysis was carried out using GraphPad prism 6. Because of the asymmetric and non-normal distribution, Wilcoxon matched-pairs signed rank test was used to analyse the results for IgE-Fab assay in order to see the difference in binding of allergen-IgE complexes to B cells, which were pre-treated with and without rhSP-D. The Mann Whitney test was used to evaluate the proliferative response of Phlp allergen stimulated PBMCs in presence of various concentrations of rhSP-D gly-X-Y, as well as used to analyse the cytokine response after multiplex analysis.

2.24 Expression and purification of C3dg and CD21 SCR 1-2

2.24.1 Preparation of BL21 (DE3) Competent cells

A single colony of BL21 (DE3) was inoculated in 10ml of Luria Broth (LB) overnight at 37⁰C in a shaker. 500 μ l was removed from the overnight inoculum and placed inside a 50 ml falcon tube containing 25ml of LB medium along with 25 μ l of 100 μ g/ml ampicillin.

The resulting mixture was then placed inside the 37⁰C shaker, and optical density (O.D) at 600nm was measured after each hour until the O.D value reached a value between 0.3-0.4 (Log Phase). After the log phase had been reached, the tube containing (LB + Ampicillin + BL21 (DE3)) was centrifuged at 2000rpm for 5 minutes. Following centrifugation the supernatant was discarded, and 12.5ml of 0.1M CaCl₂ was added to the pellet. The cells were re-suspended and were placed in the temporary state of competence in ice for 1 hour. After one hour the cells were re-spun for 5 minutes at 2000rpm. Finally, the supernatant was discarded, and the pellet was re-suspended in 2ml of 0.1M CaCl₂ and stored in ice, ready to be transformed.

2.24.2 Transformation of BL21 (DE3) cells with CD21 SCR 1-2 and C3dg plasmids

From the competent cells prepared earlier, 200µl of cells were removed and placed inside two separately labelled 10ml falcon tubes containing 1-2µl of pET15bCR2 (SCR1-2) and pET15bC3dg C1010A respectively, which were kindly donated by Professor David Isenman, University of Toronto . The mixtures were placed on Ice for 1 hour, following which the mixtures were subjected to heat shock at 42⁰C for 90 seconds. Following, heat shock the cells were placed on ice for a further 5 minutes, and then re-suspended in 800µl of L.B. The mixture was placed inside a 37⁰C incubator for approximately 45 minutes. After incubation the cells were streaked out on LB agar plate containing only Ampicillin (100µg/ml) and left inside a 37⁰C incubator overnight to grow.

2.24.3 C3dg Purification and coupling of purified C3dg to CNBr-activated Sepharose 4B.

C3dg is a ligand for complement receptor 2 or CD21, which is expressed on B-cell surface. Thus in order to purify CD21 SCR 1-2 domain for my study, firstly C3dg needed to be expressed and purified as described below using Plasmid pRT15bC3dg C1010A, kindly

donated by Professor David Isenman, in sufficient quantity and then coupled onto CNBr-activated Sepharose 4B, in order to prepare a C3dg column for affinity purifying CD21 SCR 1-2 domain.

The recombinant C3dg protein was expressed in the *Escherichia coli* host cell BL21(DE3) as described in (Isenman et al., 2010). One-liter cultures in LB-amp were grown at 37°C to A600 of ~0.6, and recombinant protein expression induced with 0.5 mM isopropyl b-D-thiogalactoside at 23°C for ~18 h. Cells were harvested in 10 mM sodium phosphate, 0.075 M NaCl, 2 mM EDTA, and 2 mM PMSF (pH 7.2) and sonicated to disrupt the cell membrane. The sonicated lysate, containing most of the recombinant C3dg protein, was dialyzed against loading buffer (20 mM sodium acetate, 20 mM NaCl, and 1 mM EDTA (pH 5.5)) and then loaded onto a 5ml column of CM-Sepharose Fast Flow (GE Health-Pharmacia, Piscataway, NJ). The protein was washed 10X in loading buffer, and eluted in the loading buffer to which were added 300mM NaCl and 0.1mM DTT. The eluted proteins were dialyzed against 10 mM sodium phosphate, 150mM NaCl, and 0.1 mM DTT (pH 7.2). The recombinant C3dg protein was ~80% pure at this stage. The purified C3dg protein was then coupled to CNBr-activated Sepharose 4B as per suppliers guidelines (GE Healthcare, Cat# 17-0430-01) to prepare a C3dg Sepharose column (~2mg C3dg per ml of sepharose).

2.24.4 CD21 SCR 1-2 expression and purification

Complement receptor 2-CR2 or CD21 found on the surface of B-cells and also a well-known ligand for low affinity IgE receptor CD23, is thought to play an important role in IgE regulation as shown previously by (Hibbert et al., 2005, Cooper et al., 2012). Thus in my study, the role of rhSP-D was examined in IgE synthesis and this therefore required the

production of a CD21 SCR 1-2 domain, which is one of the binding sites along with CD21 5-8 on CD21 molecule for CD23.

(SCR1-2) was bacterially-expressed and renatured from solubilized inclusion bodies as described by (van den Elsen and Isenman, 2011), using pET15bCR2(SCR1-2) expression plasmid. pET15bCR2(SCR1-2) in BL21(DE3) *E. coli* hosts were grown at 37°C in LB containing 100 µg/ml ampicillin until A600 was 0.6-0.7. Expression was induced with 1 mM IPTG and cells grown at 37°C for 3 hours. Then the cells were harvested, and were washed with PBS, the cell pellets obtained from 1L culture were re-suspended in 40 ml lysis buffer containing 10 mM sodium phosphate, 0.1 M NaCl, 2 mM EDTA, 1 mM PMSF, pH 7.2, DNase I (5U/ml, Clontech, Cat# 2270A) for 45 minutes at 37°C and then 1 hour at +4°C. The lysate was sonicated (18 cycles at 30 second each). Lysate supernatants were discarded and inclusion bodies were washed and solubilized in 20ml of solubilisation buffer at pH 7.2 containing 10 mM sodium phosphate, 0.1 M NaCl, 2 mM EDTA, 10 mM 2-mercaptoethanol and 6M Urea. The resolubilized material was further diluted in 80ml buffer consisting of 10 mM sodium phosphate, 0.1 M NaCl, 2 mM EDTA, 10 mM 2-mercaptoethanol each for 1 hour. CR2 (SCR1-2) was purified via affinity chromatography on a C3dg affinity column prepared earlier as described in the section above. The solubilised protein was dialysed against 2L of buffer containing 10 mM sodium phosphate, 25 mM NaCl, 0.02% NaN₃, pH 7.2, for loading onto the 5ml C3dg column equilibrated in the same buffer. The column was loaded and 2 ml fractions were collected. When the A280 decreased to near baseline, the column was washed with (~60 ml) of loading buffer made 75 mM in NaCl (10 mM sodium phosphate, 75 mM NaCl, pH 7.2). When the A280 was again at baseline, the NaCl concentration in the buffer was increased to 1.0 M (10 mM sodium phosphate, 1M NaCl, pH 7.2) to elute the bound CR2(SCR1-2).

Following purification the eluted fractions were run on SDS-PAGE to confirm the presence of purified protein.

2.25a IgE secretion assay

The role of rhSP-D in IgE synthesis was examined using PBMCs from four well characterised atopic patients sensitised to Phlp allergen, obtained via Ficoll-paque density gradient centrifugation as described in the section 2.13. The PBMCs were seeded at a density of 500,000 cells/well in triplicate under various conditions as shown in a table below (Table 2.1) in a total volume of 200 μ L/well, into a flat bottomed, 96-well plate. The PBMC's were incubated in the presence of IL-4 (20 ng/mL) and anti-CD40 (5 μ g/mL) (BD Pharmingen Purified mouse Anti-Human CD40, Cat # 555587) for 12 days at 37⁰C with 5% CO₂, in order to enable B-cells to undergo class switching to IgE synthesis. In addition to the switching factors, recombinant SP-D at 5 μ g/mL along with recombinant CD23 fragments (Figure 5a) (derCD23, 16 KDa) donated by Dr Andrew Beavil King's College London), (sCD23, 25 KDa) donated by Professor Vaughan Oosthuizen from Nelson Mandela Metropolitan University, South Africa at 25 ng/mL either in the presence or absence of recombinant CD21 SCR 1-2 at 1 μ g/mL were also added under various conditions shown below. Furthermore, it has been previously shown by (Bowles et al., 2011) that recombinant soluble CD23 at the concentration of 25ng/ml is the optimum concentration for stimulating production of IgE by B-cells. Following 12 day culture the total IgE was quantified in the collected supernatants, using ImmunoCAP Total IgE assay according to supplier's instructions (Phadia Total IgE, Cat# 10-9251-01) run on a fully automated sandwich ELISA based Phadia Laboratory ImmunoCap 100 system.

2.25b IgE secretion assay after optimisation

The immunomodulatory effects of SP-D on IgE synthesis was examined using PBMCs obtained from 10 well-characterized grass pollen allergic individuals. PBMC's were stimulated with *P. pratense* (5µg/mL), IL-4 (100ng/mL) (R&D systems, U.K). CD40L (100 ng/mL) (R&D systems, U.K), and IL-21 (100ng/ml) (Prospec-Tany, USA) in the presence of SP-D at 5 µg/mL and 5mM CaCl₂ for 14 days at 37⁰C in 5% CO₂. Total IgE was quantified in the cell culture supernatants using ImmunoCAP® Total IgE Fluoro-enzyme immunoassay.

Well no	Condition/well
1	25 ng/mL der CD23 only + IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
2	25 ng/mL sCD23 only + IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
3	5 µg/mL rhSP-D only+ IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
4	25 ng/mL der CD23 + 5 µg/mL rhSP-D+ IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
5	25 ng/mL sCD23 + 5 µg/mL rhSP-D+ IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
6	25 ng/mL der CD23+1 µg/mL CD21+ IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
7	25 ng/mL sCD23+1 µg/mL CD21+ IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
8	5 µg/mL rhSP-D+1 µg/mL CD21+ IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
9	25 ng/mL der CD23+5 µg/mL rhSP-D+1 µg/mL CD21+ IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
10	25 ng/mL sCD23+5 µg/mL rhSP-D+1 µg/mL CD21+ IL-4 (20 ng/mL) and anti-CD40 (5 µg/mL) + 5mM CaCl ₂
11	Negative control (no switch factors or SP-D, CD23 or CD21 added)
12	Positive control (switch factors added but no SP-D, CD23 or CD21)

Table 2.1: Conditions used in IgE secretion assay to examine the effect of rhSP-D on CD23-CD21 mediated IgE secretion

Chapter 3



Modulation of THP-1 and monocyte cell functions by rhSP-D

3.1 Introduction

SP-D is considered as one of the most important regulators of the alveolar macrophage activity (Kishore et al., 2005), and is also known to play a key role in the inflammatory processes as well as in the immune response in the lungs. SP-D has been shown to bind with great affinity to alveolar macrophages (Kuan et al., 1994, Miyamura et al., 1994a) and can enhance the phagocytosis of bacteria by further enhancing the activity of receptors on alveolar macrophages (Kudo et al., 2004). Lipopolysaccharide (LPS) a major component of the outer membrane of Gram-negative bacteria (Takeuchi et al., 1999), can activate macrophages, and induce an array of inflammatory mediators, (Raetz et al., 1991) by its ability to enter in to the lungs during infection by Gram negative bacteria or via inhaled airborne particles, leading to a cascade of events, which can lead to the development of acute lung injury.

SP-D has been shown to be able to modulate LPS induced inflammation due its ability to interact with various types of LPS (Kuan et al., 1992) through its CRD domain to the core regions of rough types of LPS (Kuan et al., 1992) as well as to the smooth form of LPS by inhibiting TNF- α secretion from alveolar macrophages (Yamazoe et al., 2008). SP-D does this by altering LPS receptor interaction by directly interacting with TLR4 and MD-2 through its CRD region and can inhibit the cell surface binding of smooth and rough LPS to TLR4/MD-2-expressing cells (Nie et al., 2008). Previously, it has also been reported that SP-D can bind to both the cellular and soluble form (sCD14) of CD14 (Kielian and Blecha, 1995, Wright, 1995). via the CRD domain (Sano et al., 2000), which is another receptor for LPS. Interaction of SP-D with LPS via CD14 and toll-like receptor 4 can induce the release of pro-inflammatory cytokines by alveolar macrophages (Dentener et al., 1993). This may therefore also interfere with the binding of LPS to CD14 (Sano et al.,

2000, Chaby et al., 2005), which in turn may lead to inhibition of inflammatory responses in the lung (Sano et al., 2000).

Thus, the objectives of this study were to firstly purify and characterise a truncated human recombinant fragment of SP-D (rhSP-D), composed of eight Gly-X-Y collagen repeat sequences, homotrimeric neck and lectin domains. After purification of rhSP-D, the next aim of this study was to examine the production of anti-inflammatory and pro-inflammatory cytokines upon interaction of rhSP-D with a monocytic cell line (THP-1) and monocytes obtained from healthy human subjects either in presence or absence of LPS. This line of research will further help to determine whether the protective effects of SP-D are localised to the CRD region or dependent on the collagen region.

3.2 Results

3.2.1 Purification and characterisation of recombinant human SP-D (gly-X-Y)

The recombinant human surfactant protein (rhSP-D) was expressed and purified in *E.coli* as described in the methods and materials (section 2.1). Following transformation, a pilot expression was carried out to examine the presence of rhSP-D. Samples were collected before and after induction with IPTG (section 2.1.3) for 3hours and run as described in (section 2.2) on a SDS-PAGE gel as shown below in (Fig 3.1) in order to visualise the presence of rhSP-D, which was confirmed by the presence of rhSP-D band at ~20kD as also previously demonstrated by (Mahajan et al, 2008).

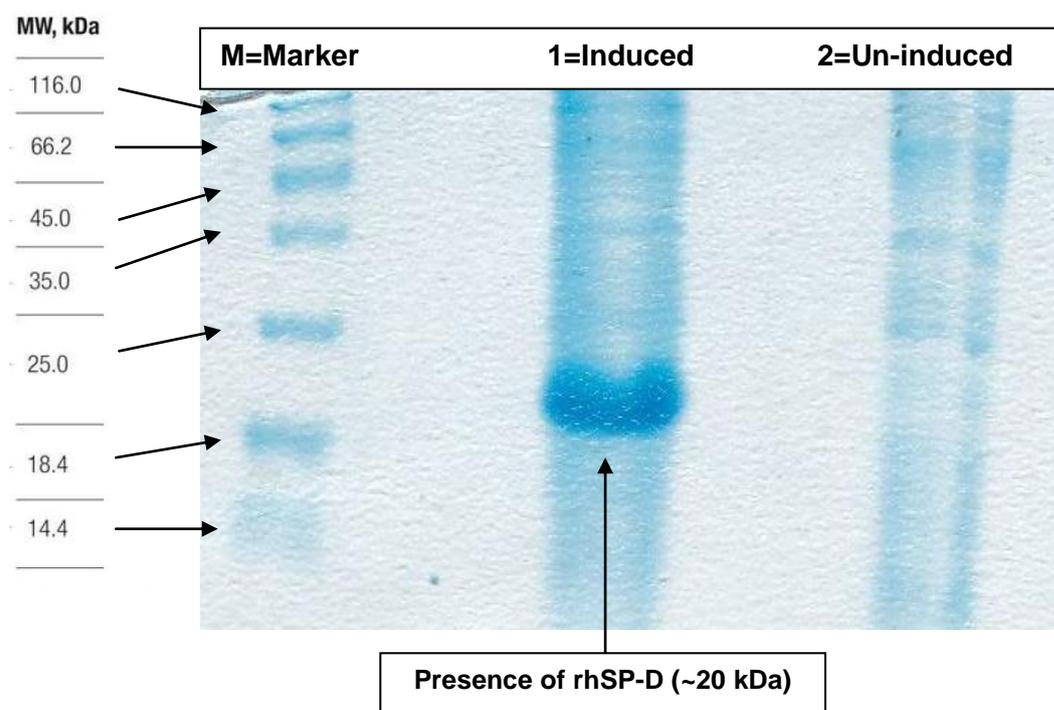


Figure 3.1: SDS-PAGE (15% w/v acrylamide) analyses of rhSP-D (8 Gly-X-Y) before and after 0.4mM IPTG induction:

A recombinant fragment of human SP-D containing homotrimeric neck and CRD regions (rhSP-D) was expressed as inclusion bodies using *Escherichia coli* BL21 (λ DE3) 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 (lane 1) compared with un-induced cells (lane 2). The molecular weight of the protein was confirmed by comparing with the protein marker (Lane M).

3.2.2 IPTG time course induction

After successfully expressing rhSP-D, the time course of IPTG needed to be ascertained in order to find out the optimum time point at which there is the greatest induction with IPTG. This was achieved by removing samples at various time points after induction, varying from 0 hrs to 5hrs, where the sample was collected after each hour. The samples obtained after each time point were loaded onto an SDS-PAGE gel as shown below in (Figure 3.2). From the gel the best protein expression can be observed three hours after induction with 0.4 mM IPTG, where the rhSP-D accumulated as an over-expressed protein of ~20 kDa (Lane 5).

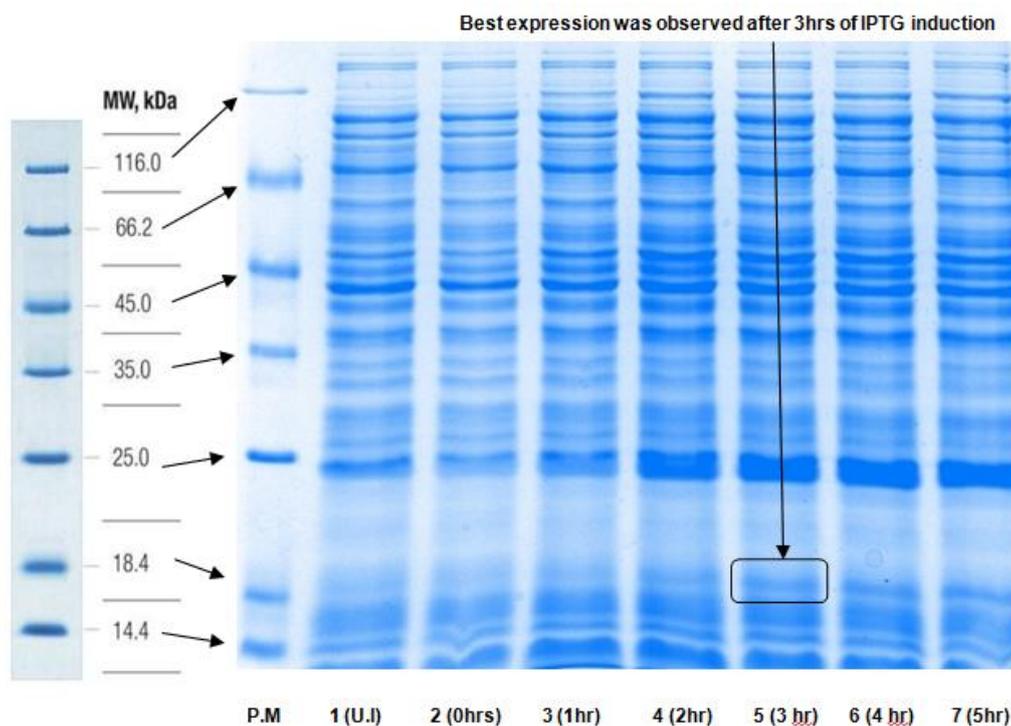


Figure 3.2: SDS-PAGE (15% w/v) analyses to show time course induction of rhSP-D with 0.4mM IPTG.

Best protein expression can be observed three hours after induction with 0.4 mM IPTG, where the rhSP-D accumulated as an over-expressed protein of ~20 kDa (lane 5) compared with un-induced cells (lane 1), 0hrs after IPTG induction (Lane 2), 1hr after IPTG Induction (Lane 3), 2hr after IPTG induction (Lane 4), 4hr after IPTG induction (Lane 6) and 5hr after IPTG induction (Lane 7). The molecular weight of the protein was confirmed by comparing with the protein marker (Lane P.M).

3.2.3 Affinity Chromatography

Upon confirmation of rhSP-D protein expression, the protein was subsequently purified as described in (section 2.1). The refolded protein obtained after step wise urea dialysis (section 2.1.5) was passed through affinity chromatography column (section 2.1.6) containing maltose sepharose which was also prepared as also described in (section 2.1.7). The eluted fractions were collected in 1.5ml eppendorf tubes and an SDS-PAGE gel was run as shown below in (Figure 3.3). To show the presence of purified rhSP-D protein as shown below. From SDS-PAGE gel it appears that the highest protein concentrations were eluted into first five fractions.

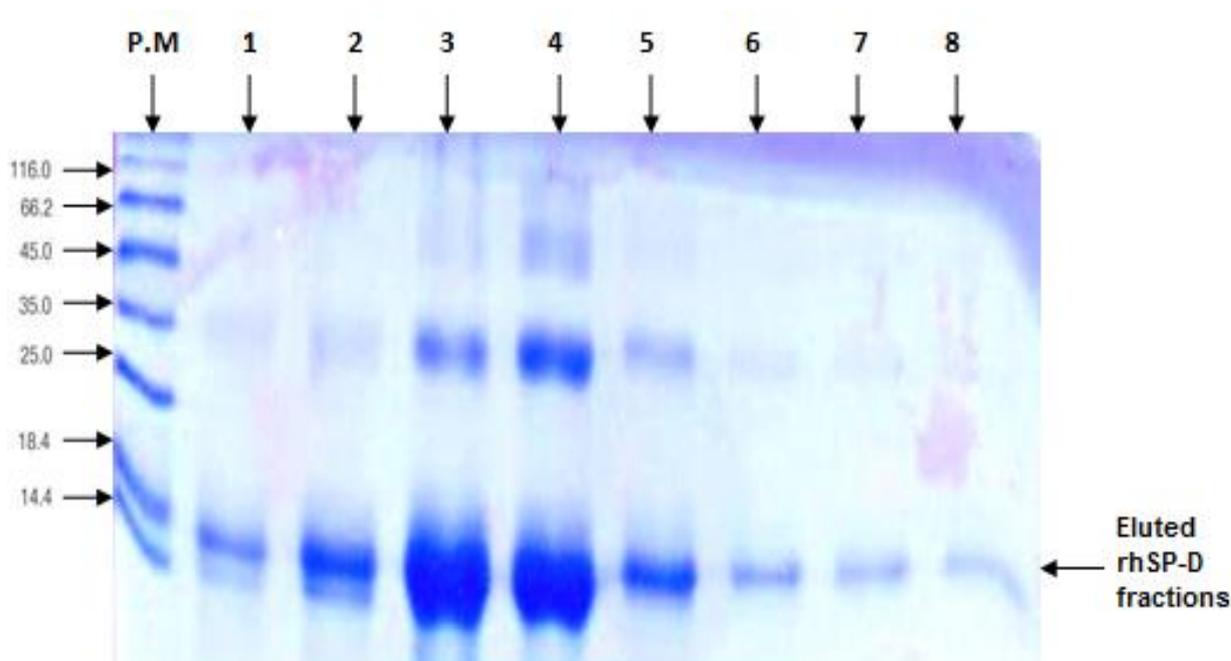


Figure 3.3: SDS-PAGE (15% w/v acrylamide), analyses shows the presence of purified rhSP-D after purification by affinity chromatography.

Purified rhSP-D fractions were run on a 15% SDS-PAGE gel to confirm the presence of protein. The rhSP-D protein bands were found in the region of approximately 18.5kDa when compared with protein marker (Lane P.M). From the gel it can be seen that the highest protein concentration was found in fractions 1-5 (Lanes 1-5)

3.2.4 Endotoxin removal from purified rhSP-D protein

Bacterial LPS was removed by following the method described in section 2.4.1, where the purified rhSP-D protein was passed through a column containing, Polymyxin B agarose gel. Following endotoxin removal the LPS free rhSP-D fractions were pooled together and SDS-PAGE (15% w/v) was run as shown below in (Figure 3.4) to further confirm the presence of purified rhSP-D after LPS removal . The LPS free protein was loaded in three wells, with each well containing different volumes of protein, 5 μ l (Lane 1), 10 μ l (Lane 2) and 15 μ l (Lane 2).

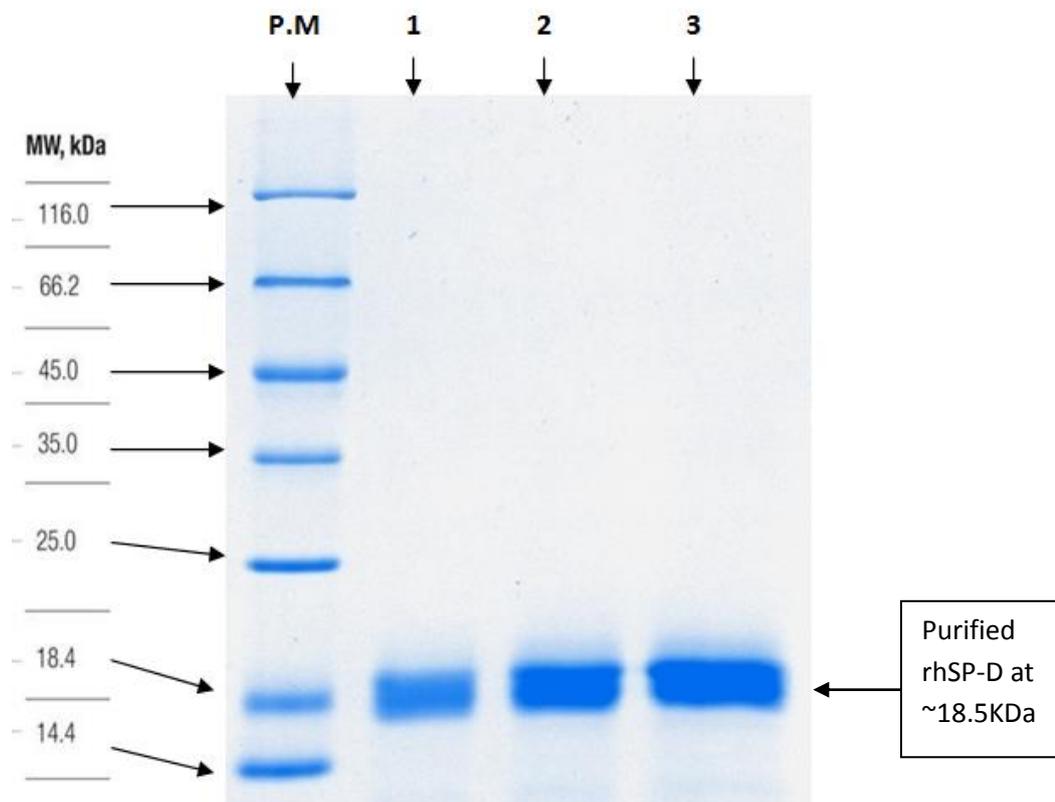


Figure 3.4: SDS-PAGE (15% w/v acrylamide) to show purity

The LPS free protein was loaded in three wells, with each well containing different volumes of protein, 5 μ l (Lane 1), 10 μ l (Lane 2) and 15 μ l (Lane 2) and compared with protein marker (Lane P.M). From the SDS-PAGE gel the presence of purified rhSP-D can be clearly observed following LPS removal.

3.2.5 BCA Protein Assay

The BCA Protein Assay was used as described in (section 2.3) for the colorimetric detection and quantitation of total protein following the purification of rhSP-D. The amount of protein was also further quantified by measuring the UV absorbance at 280nm. The highest fractions from each of the purified batches were pooled together separately and then prepared using BCA protein assay reagent, as detailed in (section 2.3) of materials and methods section. The O.D values were then determined at 562nm for both batch 1 and batch 2 pooled samples and were found to be at 1.73 and 2.18, respectively. The protein concentration of each sample was then determined by extrapolation using the plotted standard curve for BSA as shown below in (Figure 3.5). From the graph it was determined that the concentration for batch 1 was approximately 1.3mg/ml, whereas the concentration for batch 2 was found to be approximately 1.6mg/ml.

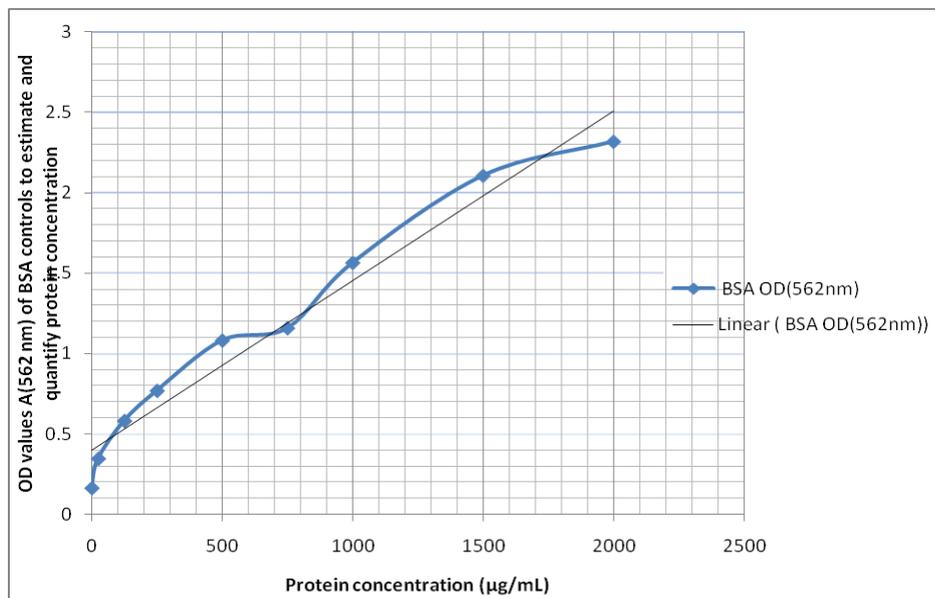


Figure 3.5: BSA standard curve for determining protein concentration

A standard curve was plotted for BSA standard vs. its concentration in µg/ml. A line of best fit was drawn and was used to extrapolate the protein concentrations for each sample of rhSP-D, which were prepared using BCA protein assay reagents and the O.D value determined at 562nm. The O.D values obtained for batch1 and batch 2 were found to be at 1.73 and 2.18, respectively. Thus by extrapolating the graph the protein concentrations of batch 1 and batch 2 were found to be approximately 1.3mg/ml and 1.6mg/ml respectively.

3.2.6 Limulus Amebocyte Lysate (LAL) assay

LAL test is a quantitative test for gram negative bacterial endotoxin. The LAL assay was carried out as described in (section 2.4.2) in order to detect the endotoxin concentration present in the affinity purified rhSP-D.

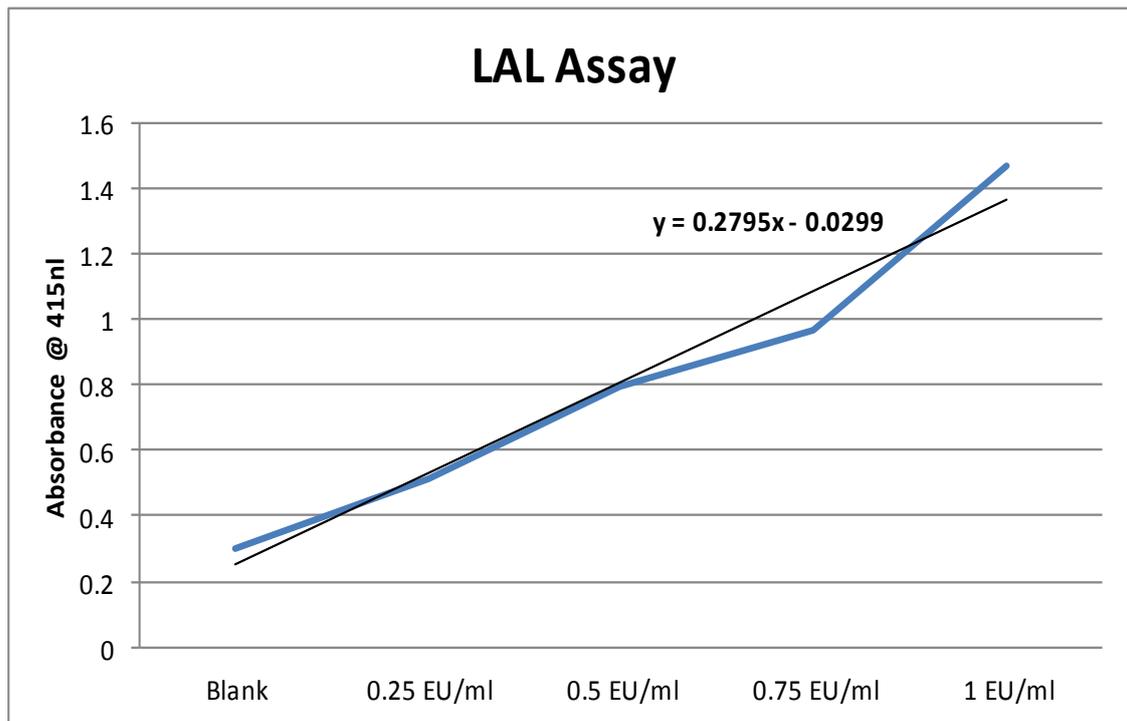


Figure 3.6: LAL assay to detect endotoxin concentration in rhSP-D preparation

The endotoxin level in the protein preparation was assessed by QCL-1000 Limulus amoebocyte lysate system (Lonza). The assay was linear over a range of 0.1–1.0 EU/ml (10 EU=1ng of endotoxin). From the standard graph obtained, a line of best fit was drawn and an equation was derived for this line of best fit. Thus from this equation, an approximate value for endotoxin content was estimated in the purified rhSP-D preparation, which was found to be in the region of 2.965EU/ml.

3.2.7 BS³ Crosslinking Assay

Since previous cross-linking studies have indicated that rhSP-D exists predominantly as a trimer in solution, a BS³ Cross-linking Assay as described under materials and methods (section 2.5), was carried out in order to observe the trimerization of rhSP-D. As it can be seen below in (Fig 3.7) the rhSP-D was incubated with various concentrations of BS³ cross linker in order to determine the best concentration of BS³ cross-linker which shows the trimerization of rhSP-D.

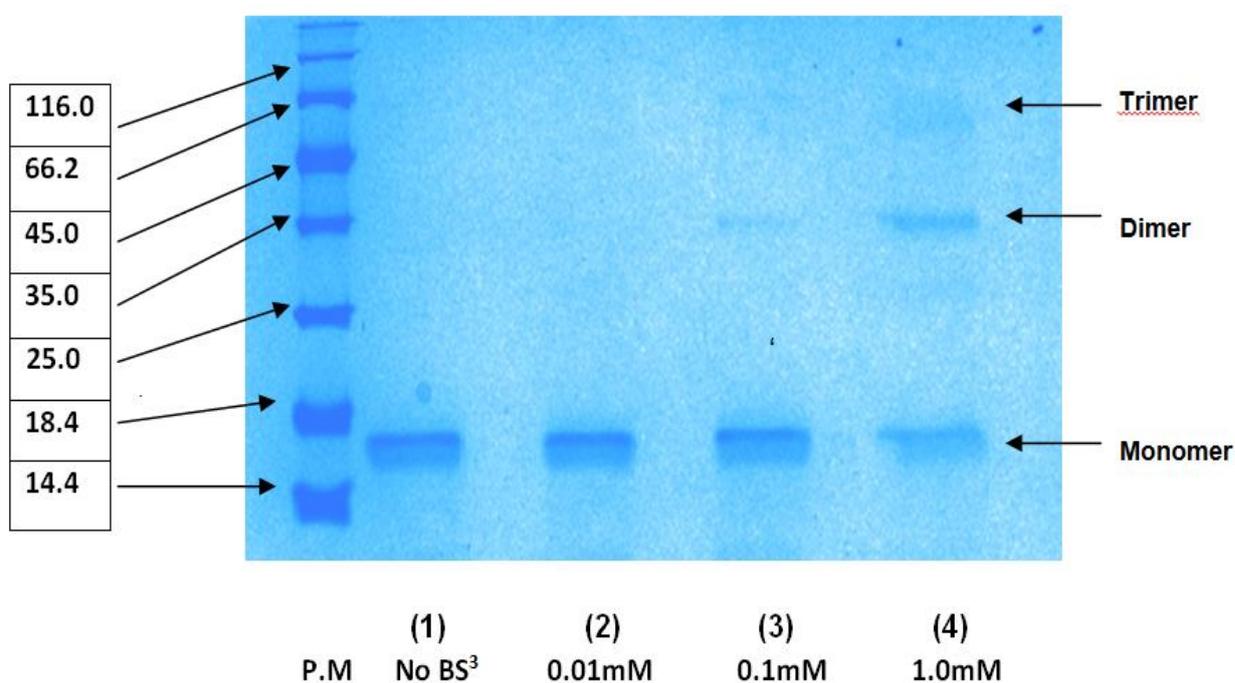


Figure 3.7: SDS-PAGE (15% w/v) to assess the trimerization of rh-SPD with various BS³ cross-linker concentrations

Three different concentrations of BS3 cross linker 0.01mM (Lane 1), 0.1mM (Lane 2) and 1.0mM (Lane 3) were incubated with rhSP-D and run on 15% w/v SDS-PAGE gel. These lanes were compared to control well containing no BS³ (Lane 1) and protein marker (Lane P.M)

3.2.8 Binding of rhSP-D to THP-1 cell line

THP-1 cells derived from human acute monocytic leukaemia (ATCC number TIB-202) were cultured as described in method and materials (section 2.7). THP-1 cell line was chosen as it is a suitable model for studying the interaction of rhSP-D with monocytes/macrophages. The THP-1 cells were checked on routine basis to examine the health of THP-1 cells prior to using them in experiments. Figure 3.8a and 3.8b below show the morphology of THP-1 cells as captured by inverted phase contrast microscope at objective 20 X and 40 X respectively.

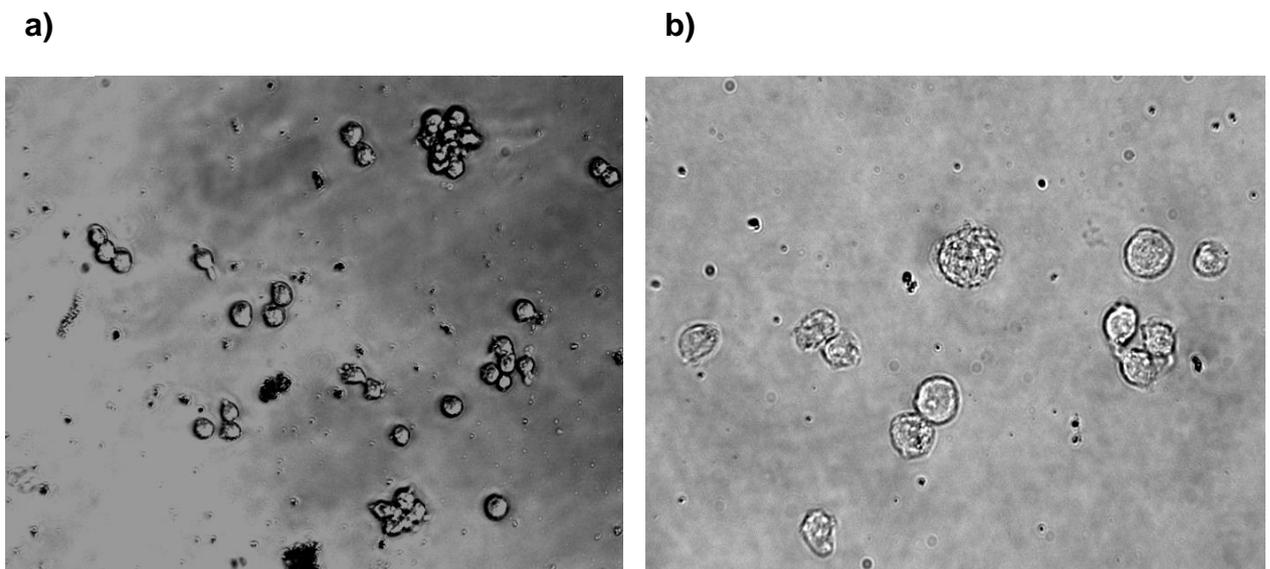


Figure 3.8: a) Photograph to show the morphology of THP-1 cells, captured by inverted phase contrast microscope at objective 20X; b) Photograph to show the morphology of THP-1 cells, captured by Inverted Phase contrast microscope at objective 40X.

3.2.9 Indirect Immunofluorescence to detect binding of rhSP-D to THP-1 cells

Indirect immunofluorescence microscopy was used in order to visualize the binding of rhSP-D at a concentration of 10 μ g/ml with THP-1 cells as described in methods and materials (section 2.10). From the photographs shown in fig 3.9 below, the untreated THP-1 cells (shown on left) are being compared with rhSP-D treated THP-1 cells (as shown on right). It can therefore be clearly seen that there is binding of rhSPD with THP-1 cells as depicted on photographs shown on right, where the THP-1 cells are giving higher fluorescence as compared to the cells on left due to the interaction of biotinylated rhSP-D with Streptavidin conjugated with FITC.

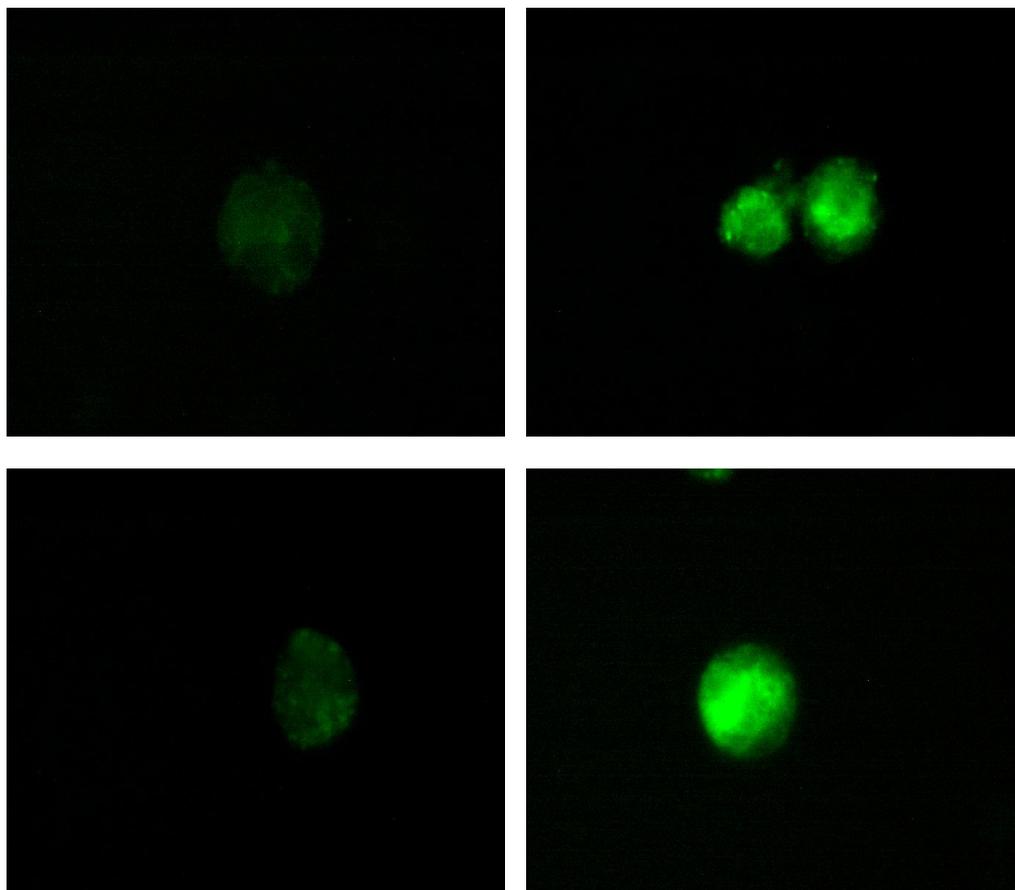


Figure 3.9: Indirect Immunofluorescence to visualise the binding of rhSP-D with THP-1 cells. Photographs on the left represent untreated THP-1 cells when treated with Streptavidin conjugated with FITC. These were used as control to compare the fluorescence emitted by the THP-1 cells when bound to biotinylated rhSP-D in presence of Streptavidin-FITC, as shown by photographs on the right.

3.2.10 Indirect ELISA to detect binding of rhSP-D with THP-1 cells

After having shown the binding of rhSP-D to THP-1 cells by Indirect Immunofluorescence microscopy, the next step was to determine the optimum concentration of rhSP-D which shows the best binding to THP-1 cells coated onto the ELISA plate. This was achieved by carrying out an Indirect ELISA as described in method and materials (section 2.9). From the results shown below in (Figure 3.10) the best binding of THP-1 cells to rhSP-D was observed with 5µg/ml rhSP-D and 1µg/ml rhSP-D respectively. However, when the concentration of rhSP-D was increased from 5µg/ml to 10µg/ml and 20µg/ml respectively, the binding of rhSP-D to THP-1 cells showed a downward trend as depicted in the graph below. This result further highlights the successful purification of trimeric rhSP-D, since at optimum concentration of 5µg/ml, all three trimeric heads are binding optimally to the cell, however at higher concentrations there is a competition between the trimeric heads and thus all three heads may not bind to the cell and hence they are more susceptible to washing due to weaker bonding.

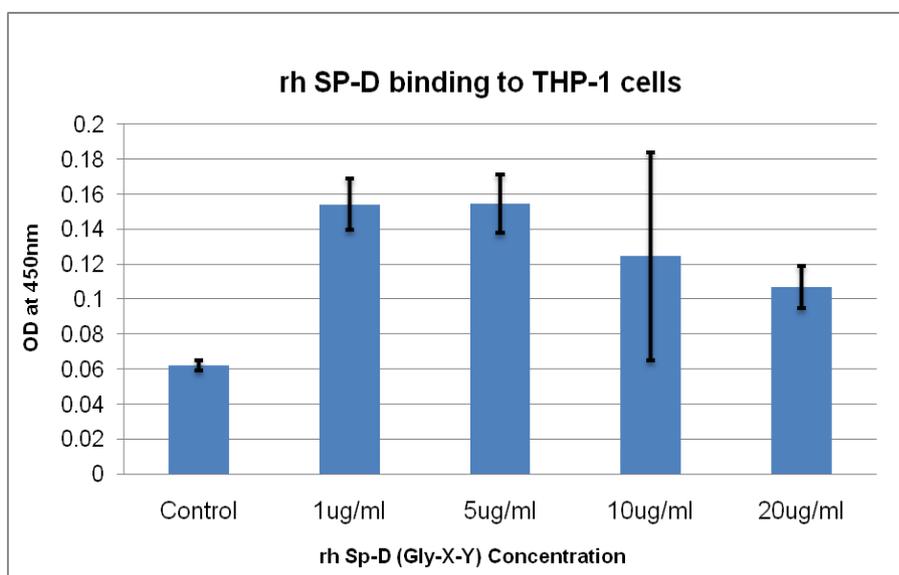


Figure 3.10: Determining the optimum binding concentration of recombinant SP-D to THP-1 cells

An Indirect ELISA, shows binding of rhSP-D to THP-1 cells. Where, it can be clearly seen that the optimum binding of rhSP-D to THP-1 cells takes place at the following concentrations of rhSP-D (5µg/ml and 1µg/ml respectively). Furthermore, as the concentration of rhSP-D is increased the binding of rhSP-D to THP-1 cells decreases.

3.2.11 Effect of rhSP-D on cytokine secretion by THP-1 cells

q-PCR study was carried out as described in method and materials section (section 2.16) to observe the effect of 5µg/ml rhSP-D on the secretion of the following pro-inflammatory cytokines: TNF-α and IL-1β and anti-inflammatory cytokines: TGF-β and IL-10 by THP-1 cells. It appears that the secretion by THP-1 cells of both pro-inflammatory cytokines IL-1β, and TNF-α, is amplified in presence of 5µg/ml rhSP-D.

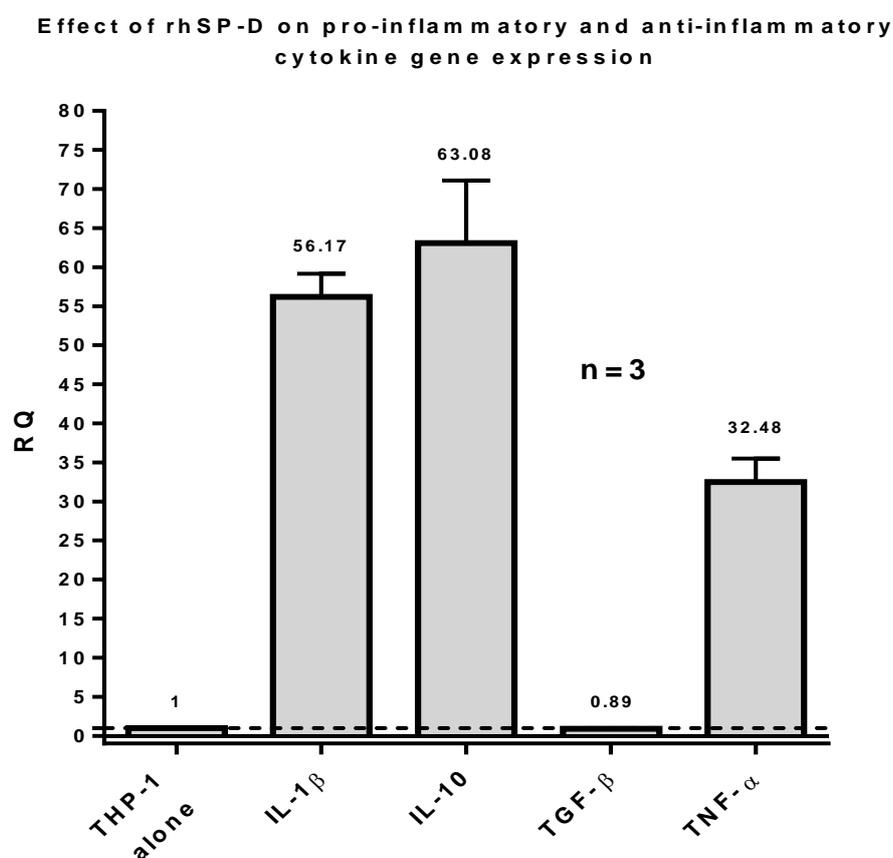


Figure 3.11: Expression of pro-inflammatory and anti-inflammatory genes by THP-1 cells in presence or absence of 5µg/ml rhSP-D.

RQ values obtained from q-PCR experiment were normalised to the control with RQ value of 1. These values were plotted in order to examine the expression levels of various genes by THP-1 cells in the presence of 5µg/ml rhSP-D. From the graph it can be seen that the level of IL-1β, IL10 and TNFα were enhanced, whereas there is a slight reduction in the level of TGF-β observed upon addition of 5µg/ml rhSP-D to THP-1 cells.

3.2.12 TNF- α expression following the stimulation of THP-1 cells with LPS E-coli.

After having examined the effect of rhSP-D on the expression of various anti-inflammatory and pro-inflammatory genes by THP-1 cells, we wished to examine the role of LPS in rhSP-D mediated inflammatory responses by monocytes obtained from three healthy donors ($n = 3$). However, in order to study this aspect the LPS conditions needed to be optimised, in order to determine the best LPS type, concentrations as well as time points, which elicit the optimum response. In these optimisation studies TNF- α was used as a target gene. Firstly, the time point for stimulation with LPS E-coli was determined by using 500ng/ml of LPS E. coli (Sigma Aldrich, UK; E. coli serotype O55:B5; source strain CDC 1644-70; Cat # L 2880).

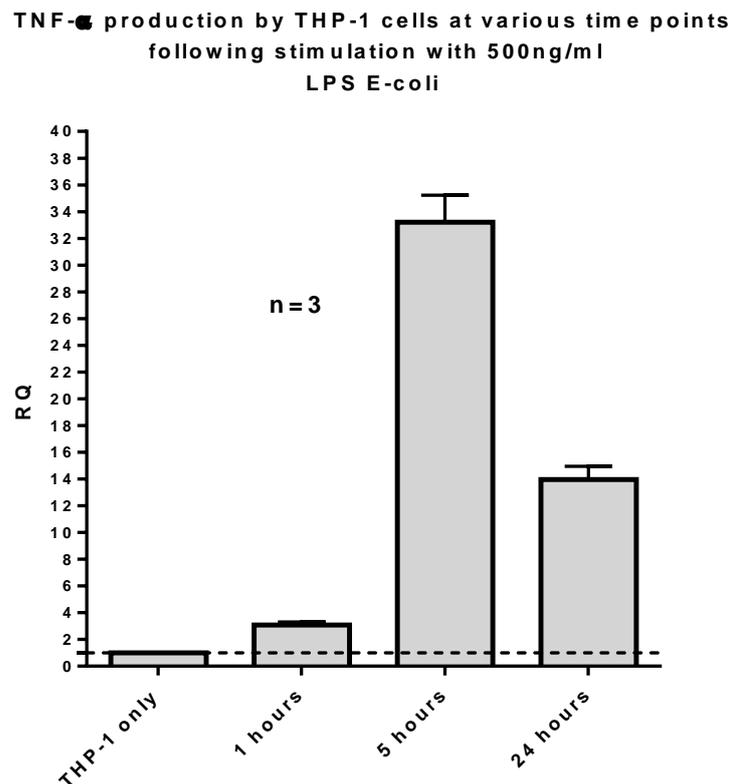


Figure 3.12: Expression of TNF- α by THP-1 cells at various time points following stimulation with 500ng/ml LPS E-coli.

TNF- α production was examined in order to determine the optimal time point of TNF- α expression following the stimulation of THP-1 cells with 500ng/ml of LPS E-coli. From the graph it can be seen that there is highest TNF- α expression after 5 hours of LPS E-coli stimulation.

3.2.13 The optimal time points for stimulation with LPS derived from *Salmonella typhimurium* were determined

The time point for stimulation with LPS *Salmonella typhimurium* was also determined by using 500ng/ml and 1µg/ml of LPS *Salmonella typhimurium*.

TNF- α production by THP-1 cells at various time points following stimulation with 500ng/ml and 1µg/ml of LPS *Salmonella typhimurium*

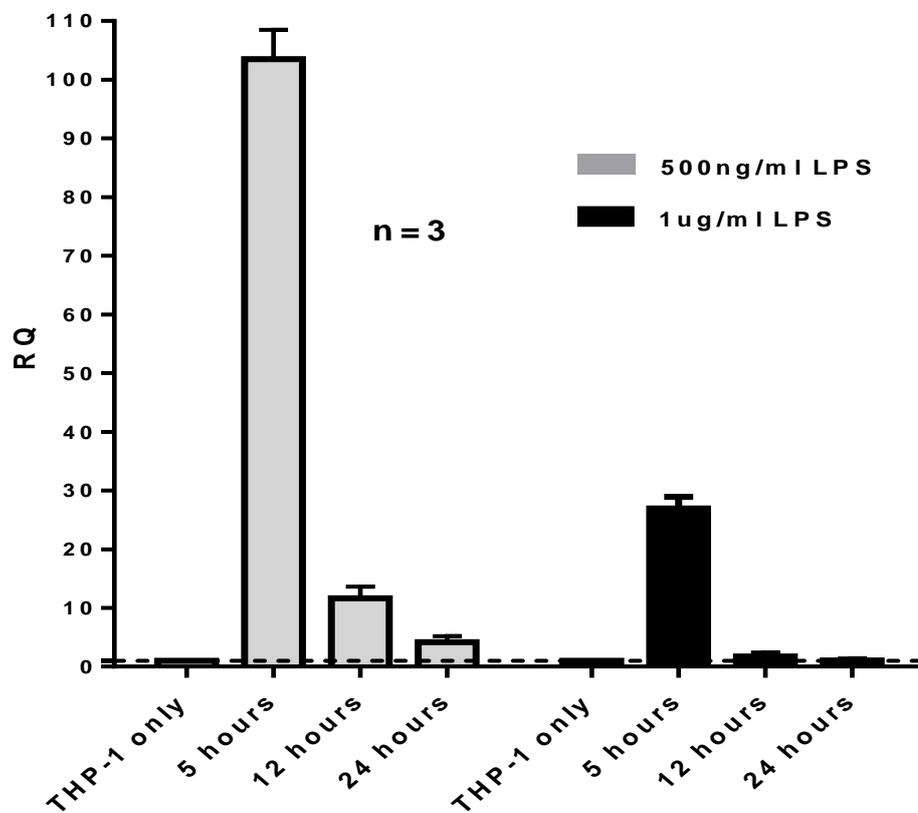


Figure 3.13: Expression of TNF- α by THP-1 cells at various time points following stimulation with 500ng/ml and 1µg/ml of LPS *Salmonella typhimurium*.

The optimal time points for stimulation with LPS-*Salmonella typhimurium* were determined, by examining for the TNF- α expression following the stimulation of THP-1 cells with 500ng/ml and 1µg/ml of LPS *Salmonella typhimurium* respectively. From the results obtained it can be clearly seen that following 5 hours of stimulation of THP-1 cells with 500ng/ml and 1µg/ml LPS *Salmonella typhimurium* gives the highest TNF- α expression.

3.2.14 Expression of TNF- α by THP-1 cells 5 hours after stimulation with various concentrations of LPS *Salmonella typhimurium*.

Having determined the optimum time point for stimulation with LPS *Salmonella typhimurium*, the concentration for LPS *Salmonella typhimurium* were also further optimised by using the following LPS concentrations; 50ng/ml, 100ng/ml and 500ng/ml, to examine the TNF- α expression by THP-1 cells after 5 hours stimulation.

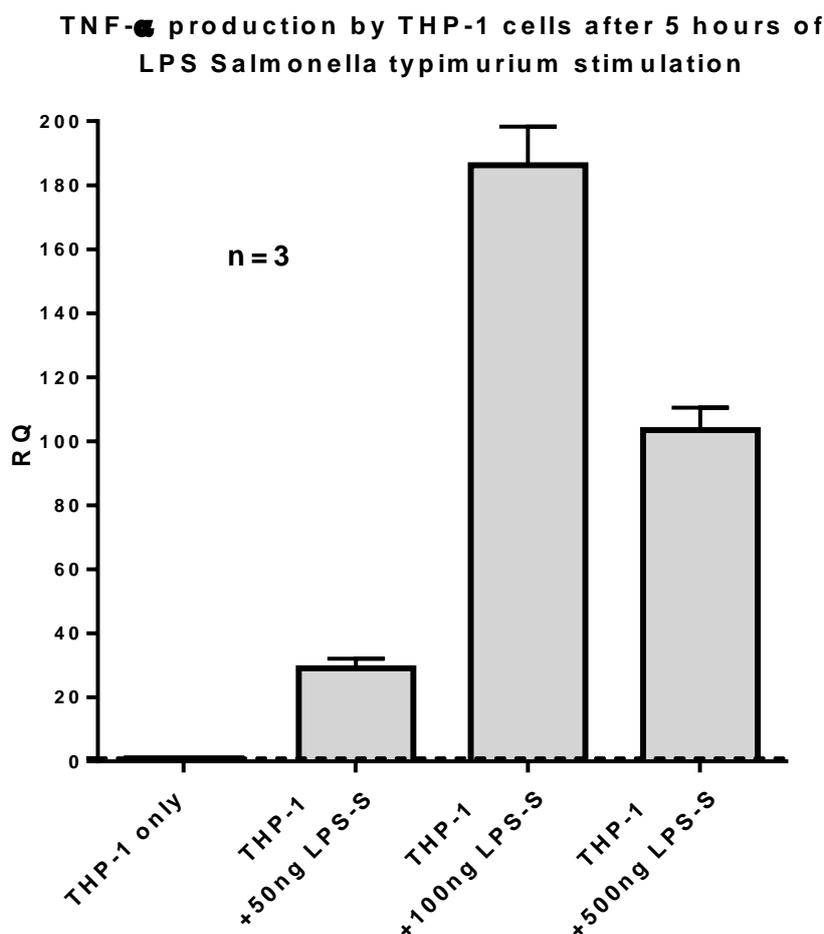


Figure 3.14: Expression of TNF- α by THP-1 cells 5 hours after stimulation with various concentrations of LPS *Salmonella typhimurium*.

Having determined the optimal time point for stimulation with LPS-*Salmonella typhimurium*, the optimal concentration of LPS-*Salmonella typhimurium* was also determined, by examining for the TNF- α expression 5 hours following the stimulation of THP-1 cells with various concentrations of LPS *Salmonella typhimurium* (50ng/ml, 100ng/ml and 500ng/ml). From the results obtained it can be clearly seen that stimulation of THP-1 cells with 100ng/ml LPS *Salmonella typhimurium* gives the highest TNF- α expression after 5 hours.

3.2.15 Expression of TNF- α by LPS stimulated THP-1 cells in presence or absence of 10 μ g/ml rhSP-D.

Having optimised the LPS concentrations and time points, LPS *Salmonella typhimurium* was selected for subsequent studies with monocytes. However, prior to studies with monocytes the effect of LPS stimulation on THP-1 cells treated with 10 μ g/ml rhSP-D was examined by looking at the TNF- α expression via q-PCR.

TNF- α production by THP-1 cells when stimulated with 100ng/ml LPS *Salmonella typhimurium* in presence of 10 μ g/ml rhSP-D

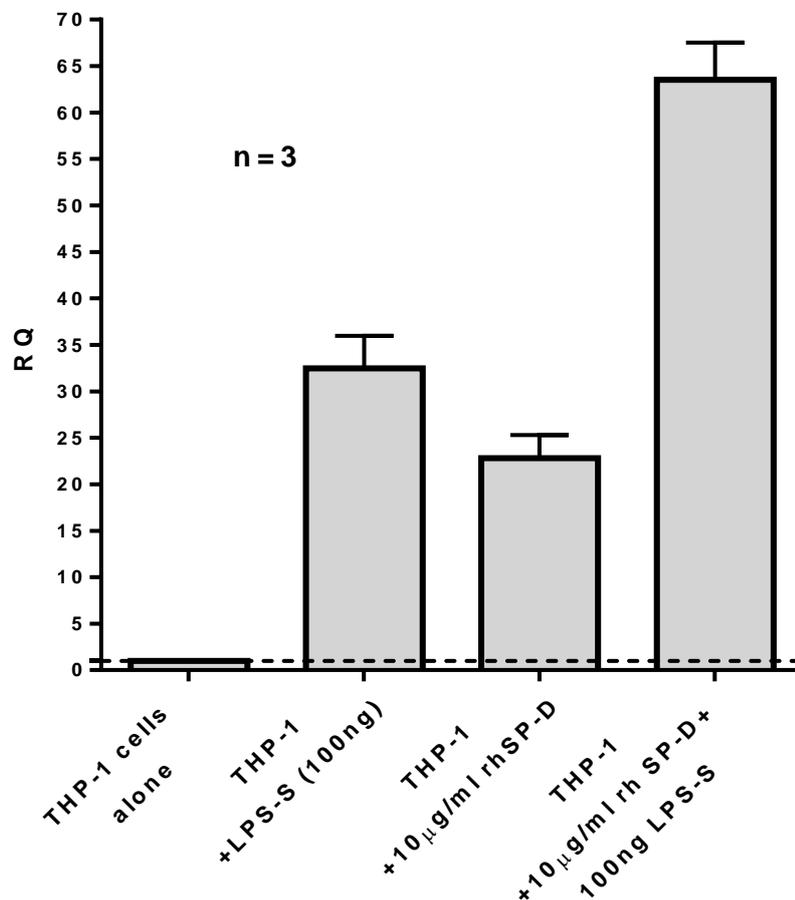


Figure 3.15: Expression of TNF- α by LPS stimulated THP-1 cells in presence or absence of 10 μ g/ml rhSP-D.

After having optimised the concentration and time point for LPS *Salmonella typhimurium*, the expression of TNF- α was again examined using the optimum conditions in presence of 10 μ g/ml rhSP-D. From the results obtained it can be seen that the expression of TNF- α is enhanced in the presence of rhSP-D, both with and without LPS stimulation.

3.2.16 Expression of IL-1 β by human monocytes in presence or absence of rhSP-D

Following initial optimisation experiments with THP-1 cell line, where the gene expression of various anti-inflammatory and pro-inflammatory genes was examined, these experiments were also repeated using monocytes obtained from 3 healthy individuals (Section 2.13). RQ values obtained from the q-PCR experiment were plotted in order to compare the level of IL-1 β expression by monocytes cells in presence or absence of 10 μ g/ml rhSP-D and with or without LPS stimulation.

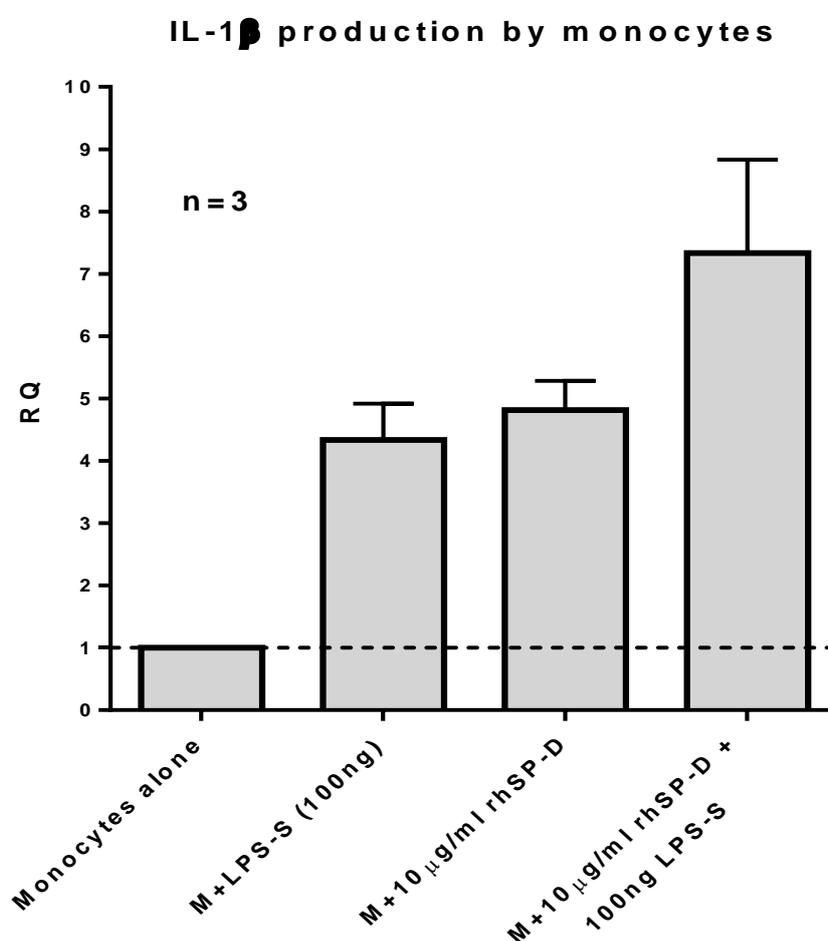


Figure 3.16: Expression of IL-1 β by monocytes in presence or absence of 10 μ g/ml rhSP-D.

From the graph it can be seen that the level of IL-1 β expression increases upon addition of rhSP-D to monocytes and this expression is further increased when monocytes are stimulated with 100ng/ml LPS-Salmonella typhimurium. Moreover, the amplification of IL-1 β by monocytes in presence of rhSP-D is also consistent with our earlier findings from the studies with THP-1 cell line (Figure 3.11).

3.2.17 Expression of IL-10 by human monocytes in presence or absence of 10µg/ml rhSP-D

RQ values obtained from the q-PCR experiment were plotted in order to compare the level of IL-10 expression by monocytes cells in presence or absence of 10µg/ml rhSP-D and with or without LPS stimulation.

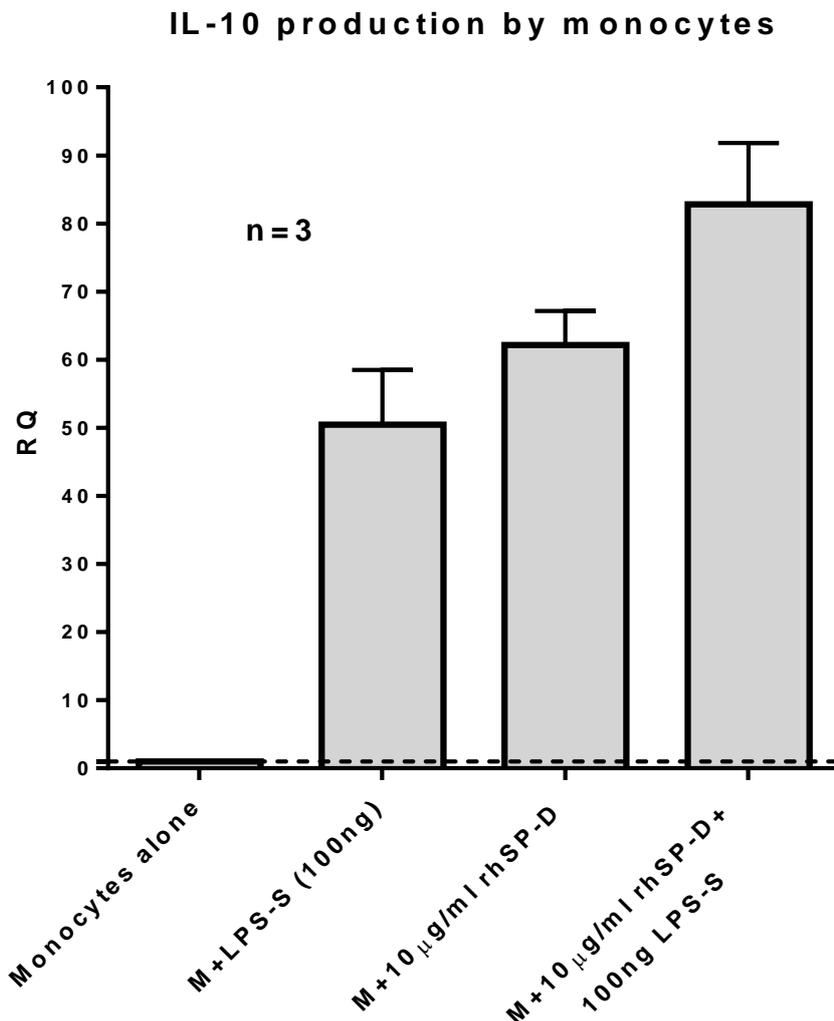


Figure 3.17: Expression of IL-10 by monocytes in presence or absence of 10µg/ml rhSP-D.

From the graph it can be seen that the level of IL-10 expression increases upon addition of rhSP-D to monocytes and this expression is further increased when monocytes are stimulated with 100ng/ml LPS-Salmonella typhimurium. Moreover, the amplification of IL-10 by monocytes in presence of rhSP-D is also consistent with our earlier findings from the studies with the THP-1 cell line (Figure 3.11).

3.2.18 Expression of TGF- β by human monocytes in presence or absence of 10 μ g/ml rhSP-D

RQ values obtained from the q-PCR experiment were plotted in order to compare the level of TGF- β expression by monocytes cells in presence or absence of 10 μ g/ml rhSP-D and with or without LPS stimulation.

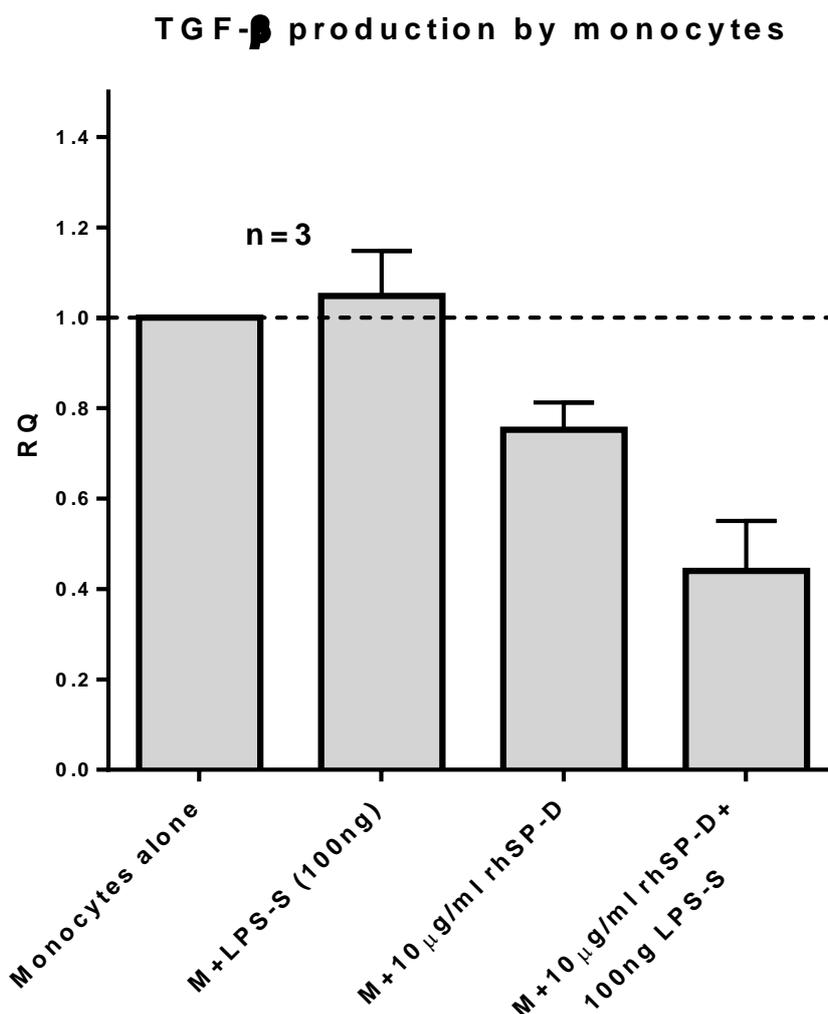


Figure3.18: Expression of TGF- β by monocytes in presence or absence of 10 μ g/ml rhSP-D.

From the graph it can be seen that the level of TGF- β expression actual decreases upon addition of rhSP-D to monocytes and this expression is further reduced when monocytes are stimulated with 100ng/ml LPS-Salmonella typhimurium. This decrease in the expression of TGF- β by monocytes in presence of rhSP-D is again consistent with earlier findings with THP-1 cell line (Figure 3.11).

3.2.19 Expression of TNF- α by human monocytes in presence or absence of 10 μ g/ml rhSP-D

RQ values obtained from the q-PCR experiment were plotted in order to compare the level of TNF- α expression by monocytes cells in presence or absence of 10 μ g/ml rhSP-D and with or without LPS stimulation.

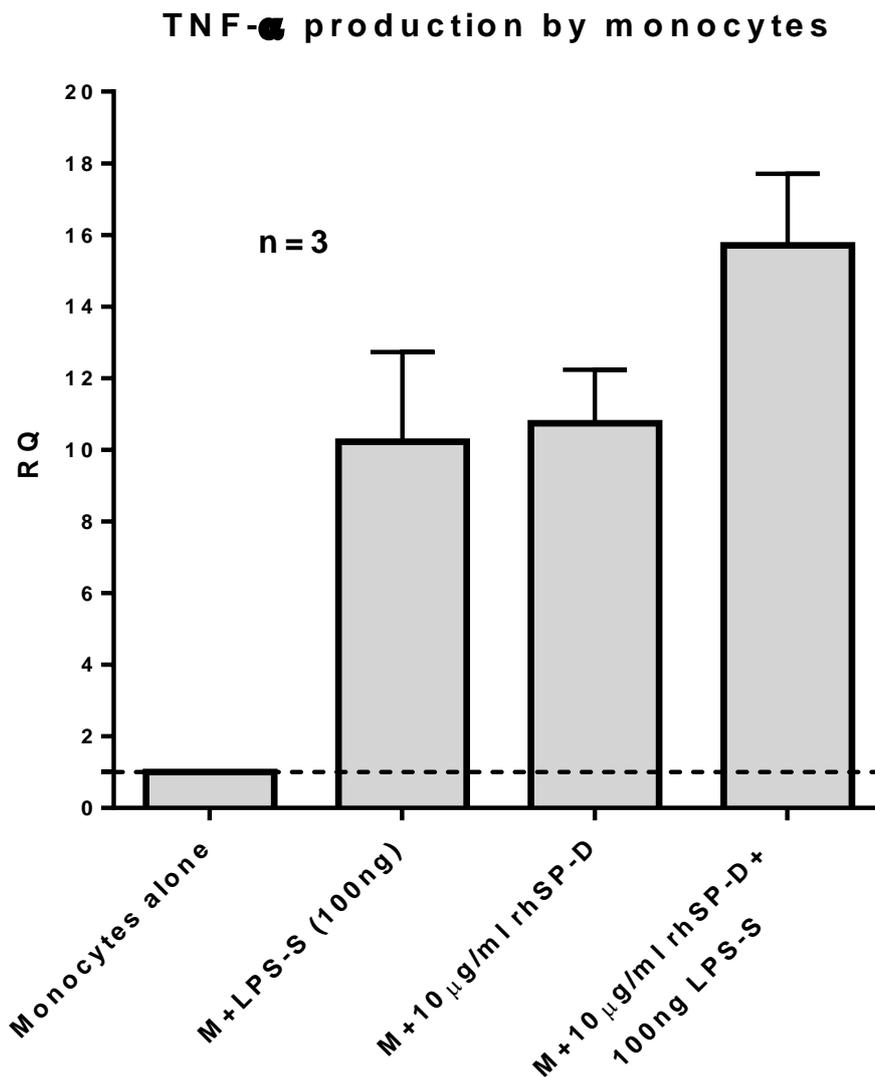


Figure 3.19: Expression of TNF- α by monocytes in presence or absence of 10 μ g/ml rhSP-D.

From the graph it can be seen that the level of TNF- α expression increases upon addition of rhSP-D to monocytes and this expression is further amplified when monocytes are stimulated with 100ng/ml LPS (*Salmonella typhimurium*). Finally, this increase in the expression of TNF- α by monocytes in presence of rhSP-D is again consistent with my earlier findings with THP-1 cell line (Figure 3.11)..

3.3 Discussion

This study examined the immunomodulatory properties of a truncated form of human recombinant SP-D (rhSP-D), which is composed of CRD region an α -helical neck region followed by eight Gly-X-Y collagen repeat sequences, in LPS mediated pulmonary inflammation. Following successful purification and characterisation of rhSP-D, the next aim of the study was to try and understand how the production of pro- and anti-inflammatory cytokine genes by THP-1 monocytic cell line derived from an acute monocytic leukemia patient, as well as monocytes derived from healthy individuals (n = 3) were modulated by rhSP-D in vitro.

The data obtained from this study thus indicates that rhSP-D can bind to THP-1 cells in presence of 5mM CaCl₂ as shown via indirect ELISA and by indirect immunofluorescence microscopy. Having confirmed the binding of rhSP-D with the THP-1 cells, the gene expression of pro-inflammatory cytokines (IL-1 β , TNF- α) as well as on anti-inflammatory cytokines (IL-10, TGF- β) was examined in the presence of 10 μ g/ml rhSP-D. From the results obtained it could be deduced that THP-1 cells when treated with rhSP-D have the ability to increase the gene expression of the above pro-inflammatory cytokines (IL-1 β , TNF- α) as well as also increase the expression of IL-10, which is an anti-inflammatory cytokine. However, the expression of TGF- β , an anti-inflammatory cytokine was found to be reduced upon addition of rhSP-D. Furthermore, the effect of rhSP-D on the secretion of the following pro-inflammatory cytokines (IL-1 β , TNF- α) and anti-inflammatory cytokines (IL-10, TGF- β) by LPS stimulation of monocytes obtained from healthy individuals was also examined. From the results obtained, it appears that the secretion by monocytes of two pro-inflammatory cytokines IL-1 β and TNF- α as well as IL-10 an anti-inflammatory cytokine is further amplified in the presence of rhSP-D by several fold when compared to the amplification level of un-stimulated monocytes. A decrease in TGF- β

expression was observed, with and without LPS stimulation when compared to the level of un-stimulated monocytes in absence of rhSP-D. These results obtained from human monocytes appear to be consistent with the results obtained using THP-1 monocytic cell line, where they both show the pro-inflammatory role of rhSP-D.

This pro-inflammatory effect observed with the rhSP-D, thus, appears to negate the earlier findings by (Yamazoe et al., 2008), where they showed that SP-D via its CRD region can inhibit LPS mediated pro-inflammatory response by inhibiting TNF- α secretion from alveolar macrophages in presence of LPS. This pro-inflammatory effect of rhSP-D could be somewhat explained though not completely, in light of earlier findings by (Gardai et al., 2003, Guo et al., 2008), where they proposed a dual role of SP-D by suggesting that SP-D may either enhance or suppress inflammatory responses depending on the binding orientation of SP-D. Moreover, the pro-inflammatory role of SP-D has been further highlighted in previous studies showing that when there is a deficiency of SP-D in relation to the numbers of pathogens present, i.e. in a diseased lung where a patient may be suffering from cystic fibrosis, the collagen region of SP-D may instead interact with the CD91/calreticulin complex on alveolar macrophages thus resulting in an increased expression of nuclear factor κ B (NF- κ B). (Voorhout et al., 1992, Hartl and Griese, 2006).

However, these studies used an intact SP-D molecule where orientation of collagen region dictated pro- or anti-inflammatory response, whereas in our study a truncated form of recombinant SP-D with a short collagen region was used, suggesting that this pro-inflammatory role of SP-D was mainly mediated by the CRD region rather than the collagen region. These results are interesting, since as earlier findings show that SP-D carries out its anti-inflammatory effects via its CRD region, which binds to the SIRP- α present on the surface of alveolar macrophages and therefore leads to suppression of NF-

κ B-mediated inflammation, through blocking the SH2 domain-containing tyrosine phosphatase (SHP-1), which results in an anti-inflammatory response (Kuan et al., 1994).

In summary, these results appear to suggest that rhSP-D may prevent the onset of LPS mediated pro-inflammatory response by the production of anti-inflammatory cytokines such as IL-10, which may lead to modulation of LPS mediated pulmonary inflammation. Further studies are required to ascertain if the interaction between TLR and LPS is modulated by rhSP-D.

Chapter 4



Regulation of B-cell mediated specific IgE synthesis by rhSP-D

4.1 Introduction

As discussed in Chapter 1, SP-D has been shown to play a protective role in pulmonary lung hypersensitivity via a range of mechanisms that include interference with allergen-IgE interaction, suppression of histamine release from sensitized basophils and mast cells and inhibition of IgE-allergen cross-linking (Madan, 2007, Madan et al., 1997c). The recombinant fragment of SP-D is composed of a carbohydrate recognition domain (CRD), an α -helical neck region and eight Gly-X-Y collagen triplets, has also been found to be as effective as full-length human SP-D in vitro and in vivo (Madan et al., 2005a, Wang et al., 1996a, Strong et al., 2002a, Madan et al., 2001, Singh et al., 2003).

The immunomodulatory role of SP-D has been further highlighted in various *in-vitro* and *in-vivo* studies, where it has been shown to be involved in pattern recognition of glycoprotein allergens (Miyamura et al., 1994b, Wang et al., 1996b, Madan et al., 1997a), as well as associated with dampening *Aspergillus fumigatus* (*Afu*) (Madan et al., 2001, Strong et al., 2002b) and house dust mite (*Dermatophagoides pteronyssinus*, *Der-p*) (Singh et al., 2003) induced allergic inflammation in murine models. In a study carried out by Madan et al, using BALB/c murine model of allergic bronchopulmonary aspergillosis (ABPA) caused by *Aspergillus fumigatus*, an opportunistic fungal pathogen, when challenged with SP-D, a marked reduction in specific IgE and IgG1 levels along with reduction in a peripheral blood eosinophils and pulmonary infiltration was observed (Madan et al., 2001). In addition SP-D was also found to alleviate allergic symptoms by shifting from the pathogenic Th2 response towards a protective Th1 response. These findings were further replicated in a study carried out by Strong et al, using C57BL/6 mouse models, which favour a Th1 response (Strong et al., 2002b, Hazlett et al., 2000). This study further showed the ability of SP-D to down regulate IgE and *Afu* specific IgG1 levels following challenge with rhSP-D. It is thus clear from the above evidences that SP-

D is able to modulate IgE driven allergic inflammation, however the exact mechanism/s by which SP-D confers these protective effects remain unclear and thus need to be further elucidated.

We thus hypothesised that a possible mechanism whereby SP-D may provide protection against allergy could be through interference with IgE-facilitated allergen presentation (FAP) (Figure 4a), which is dependent on the interaction of allergen-IgE complexes to low affinity IgE receptor (FcεRII or CD23) found on the surface of B-cells (Pirron et al., 1990). Here the allergen is captured by IgE leading to the formation of allergen-IgE complexes, which then bind to the surface of CD23 and are internalised. The allergen is then processed and presented to T cells via MHC class II molecules (Pirron et al., 1990). This interaction leads to increased production of Th2 cytokines which leads to further production of allergen specific IgE by B-cells, thus propagating the activation of T-cells due to increased levels of IgE in mucosal tissue, which in turn leads to further exacerbation of allergic inflammation.

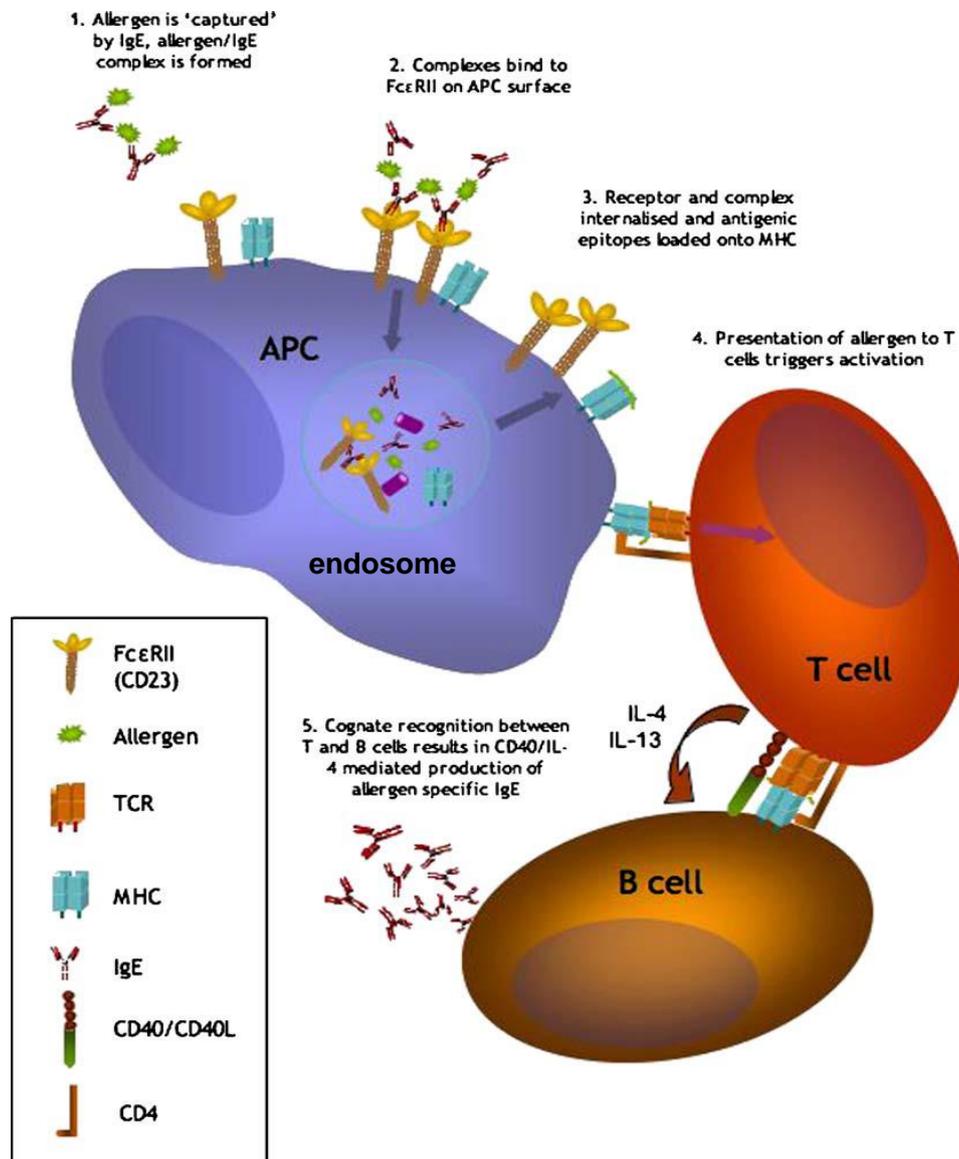


Figure 4a: IgE-facilitated allergen presentation (IgE-FAP)

The illustration above shows CD23 mediated IgE-FAP, where CD23 on the surface of a B cell leads to recognition by allergen-specific T cell. This interaction further promotes the production of allergen-specific IgE (Taken from (Wilcock et al., 2006)).

An IgE facilitated allergen binding assay (IgE FAB) was employed (Shamji et al., 2006a) in order to detect the binding of allergen-IgE complexes to rhSP-D pre-treated B cells, using B cells obtained from CD23 enriched EBV B-cell line, as well as using serum samples obtained from well characterised atopic patients, which were highly sensitised to Timothy grass (*Phleum pratense*-Phlp) allergen. The allergen-IgE complexes bound to the CD23 present on the surface of B-cells were detected via flow cytometry by anti-IgE antibodies (Shamji et al., 2006a). This assay was used, since as it is a robust in vitro assay for examining facilitated antigen presentation, as T-cell assays for readout of IgE-FAP are difficult and laborious to carry out, and require the generation and maintenance of long-term allergen-specific T cell clones (Shamji et al., 2006a).

Hence, this simplified flow cytometry based assay can be employed to examine the binding of allergen-IgE complexes to CD23 on the surface of B cells, which has been shown to correlate with T-cell activation and proliferation (van der Heijden et al., 1993). The effect of rhSP-D on antigen presentation and proliferation of CD4⁺ T cells was also examined via ³H-Thymidine incorporation assay, where proliferative responses of Phlp stimulated PBMCs obtained from well characterised patients highly sensitised to Phlp allergen were examined in the presence or the absence of rhSP-D. These proliferating cells were further characterised by using a multiplex assay, to examine the ability of rhSP-D to inhibit allergen driven Th2 cytokine production.

4.2 Results

4.2.1 rhSP-D binds *A. fumigatus* allergen

The interaction of rhSP-D with *Aspergillus fumigatus* (*Afu*) has been established in previous studies and was thus carried out in order to verify the biological activity of rhSP-D, which was expressed and purified for the current study, (section 2.1). In this direct binding ELISA the binding of rhSP-D with three week cultural filtrate of *A. fumigatus* was examined as described in method and materials (section 2.9). The results obtained as shown below in (Fig 4.2) show that 5 $\mu\text{g/ml}$ of rhSP-D was found to be the optimum concentration at which it binds best to 5 $\mu\text{g/ml}$ *Afu* coated wells. However, when the concentration of rhSP-D was increased from 5 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$, the binding ability of rhSP-D to *Afu* is slightly diminished (Fig 4.1).

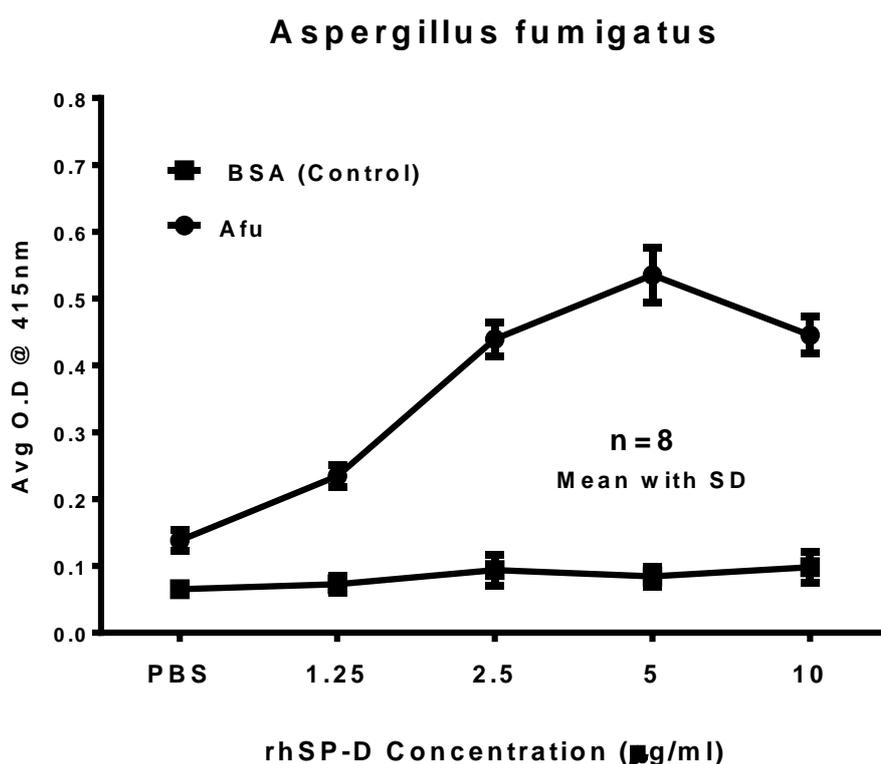


Figure 4.1: Binding of rhSP-D with *A. fumigatus*.

Binding of rhSP-D to *A.fumigatus* was confirmed by indirect ELISA. From the graph it can be seen that rhSP-D bound to 5 $\mu\text{g/ml}$ of 3 week *A.fumigatus* culture filtrate in a dose dependent manner, [0 $\mu\text{g/ml}$ (Optical density at 415nm=0.14), 1.25 $\mu\text{g/ml}$ (0.23), 2.5 $\mu\text{g/ml}$ (0.44), 5 $\mu\text{g/ml}$ (0.54), 10 $\mu\text{g/ml}$ (0.45)]. Data are presented as mean \pm SD (n=8).

4.2.2 rhSP-D binds *Phleum pratense* allergen

Following successful confirmation of the allergen-binding activity of rhSP-D, the interaction of rhSP-D with *Phlp* allergen was examined via indirect ELISA as described in (section 2.9), since firstly the interaction of rhSP-D with *phelum pratense* (*Phlp*) has not been previously established and also it was necessary to examine this interaction, since the patient samples used in this study were obtained from well characterised atopic patients highly sensitised to *Phlp* allergen. The results obtained as depicted below in (Fig 4.2) show that 5µg/ml of rhSP-D was found to be the optimum concentration at which it bound best to 5µg/ml *Phlp* allergen coated wells. However, as interaction of rhSP-D with *A.fu*, when the concentration of rhSP-D was increased from 5µg/ml to 20µg/ml, there was a reduction in the binding of rhSP-D to *Phlp* allergen.

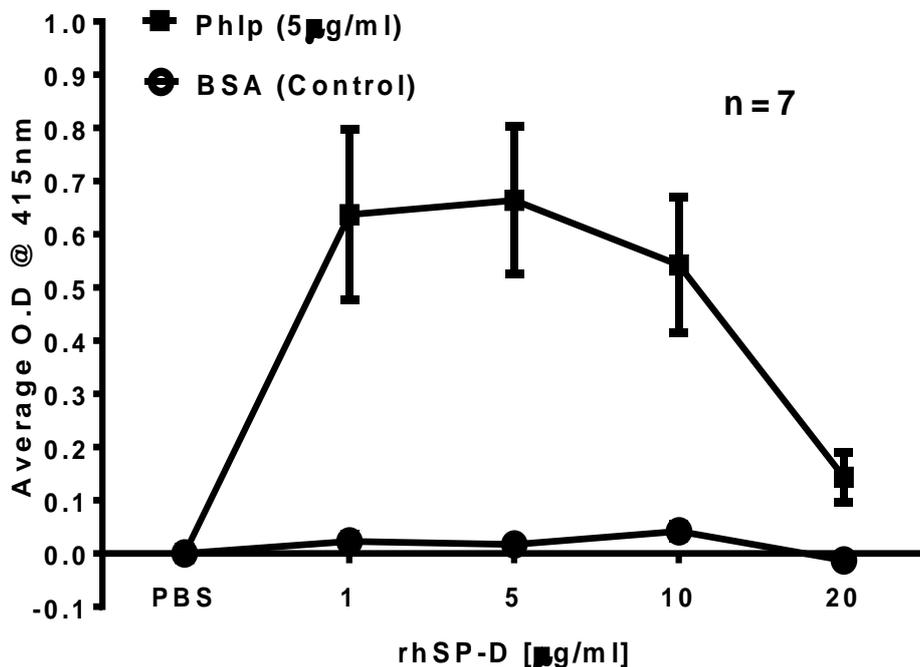


Figure 4.2: Binding of rhSP-D with *Phleum pratense* allergen

Binding of rhSP-D to *Phlp* allergen was shown by ELISA (n=7). From the graph it can be seen that rhSP-D at a concentration of 5µg/ml binds optimally to *Phlp* extract, Data are presented as mean ± SD of 7 independent experiments.

4.2.3 rhSP-D binds *Phleum pratense* allergen in a calcium dependent manner

The interaction of rhSP-D with *Phlp* allergen was further examined under various conditions via indirect ELISA as described in (section 2.9). Our results show that rhSP-D binds to *Phlp* allergen in dose dependent manner in presence of 5mM Calcium ions (Figure 4.3). However this interaction is partially inhibited by EDTA.

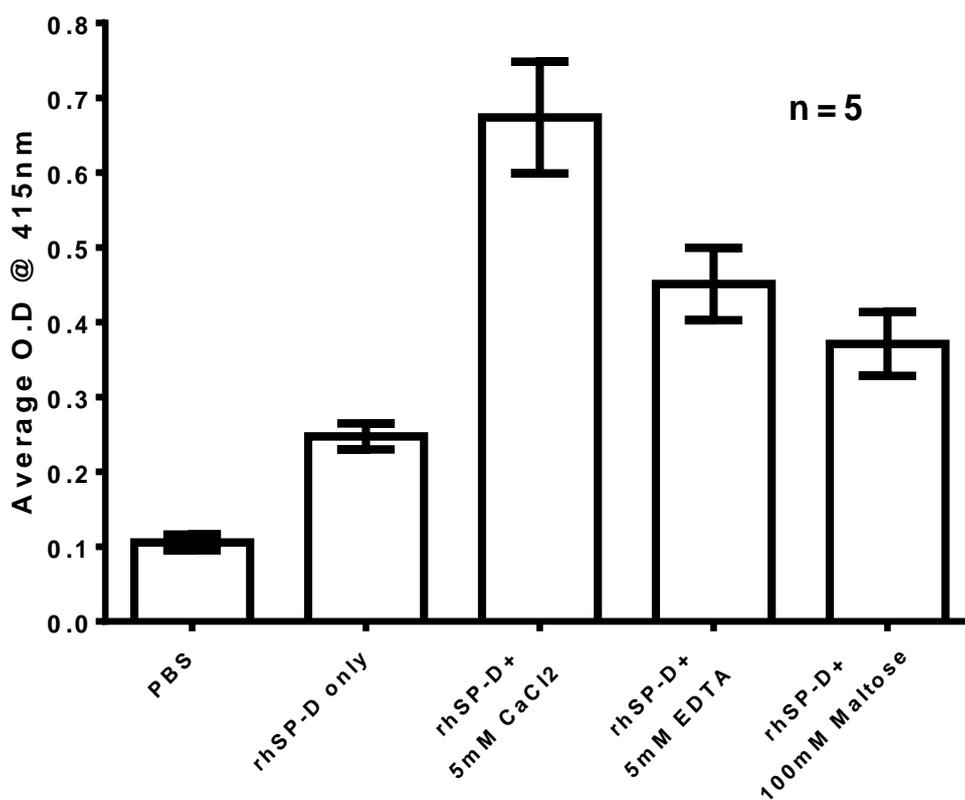


Figure 4.3: Binding of rhSP-D with *Phleum pratense* allergen under various conditions

This binding of rhSP-D with *Phlp* allergen was found to be calcium and carbohydrate dependent and was inhibited in the presence of 5mM EDTA ($P=0.0225$), as shown by ELISA ($n=5$). Data are presented as mean \pm SD.

4.2.4 rhSP-D binds to various proteins in *Phelum pratense* allergen extract

After having confirmed the binding of rhSP-D to *Phlp* allergen via Indirect ELISA, a far western blotting was also carried out as described in method and material (section 2.12) to detect the binding of rhSP-D with *Phlp* allergen (Figure 4.4), in order to identify which proteins in *Phlp* extract bind to rhSP-D.

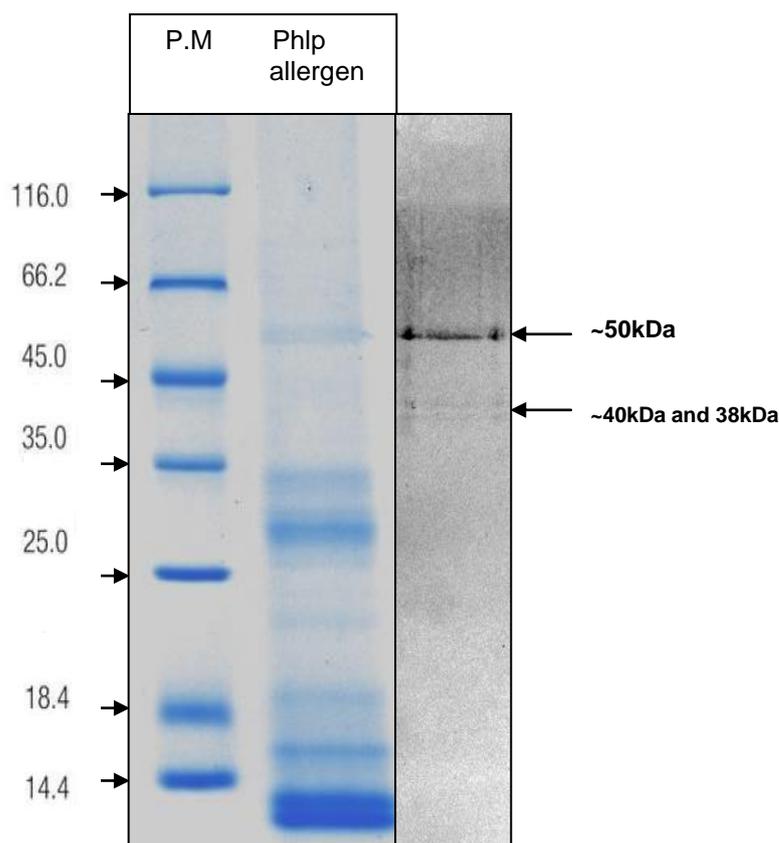


Figure 4.4: Western Blot to detect binding of rhSP-D to *Phlp* allergen

A western blot was carried out to detect the binding of rhSP-D to *Phlp* extract. From the blot shown above in figure 4.5, the binding of rhSP-D to three proteins around the region of 50kDa, 40kDa and 38kDa can be observed, when compared with a 15% w/v SDS-PAGE gel on which the *Phlp* allergen was run. Lane (PM) on the SDS-PAGE gel represents Protein marker, Lane 2 represents the *Phlp* allergen.

4.2.5 rhSP-D binds IgE

The interaction of rhSP-D with human IgE (Abcam, UK; Cat # ab65866) was also examined via indirect ELISA as described in (section 2.9). Our results show that rhSP-D binds to IgE in a dose dependent manner in presence of 5mM Calcium (Figure 4.5).

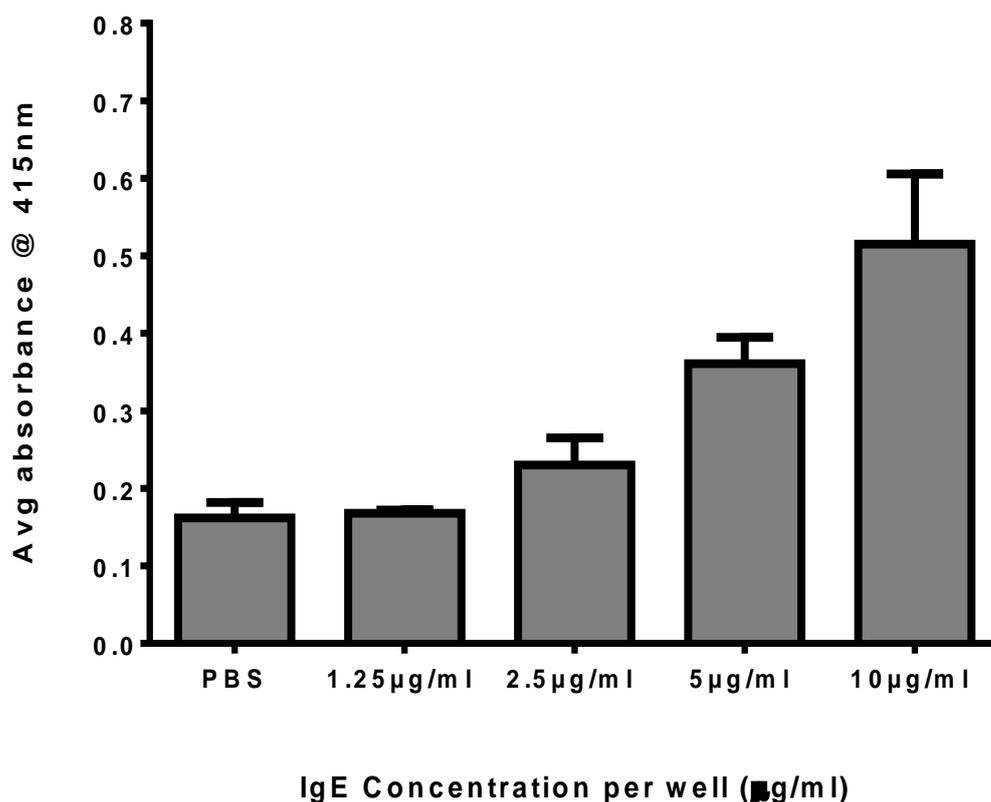


Figure 4.5: Binding of rhSP-D with IgE

Binding of rhSP-D to various concentrations of IgE was also confirmed via ELISA. From the graph below it appears that rhSP-D bound to IgE in a dose dependent manner. The concentration of rhSP-D used was 5µg/ml, which showed better binding with 10µg/ml IgE, the extent of this binding increased as the concentration of IgE was increased. Data are presented as mean ± SD.

4.2.6 Binding of SP-D to atopic PBMCs

SP-D is an innate immune molecule, which acts as a pattern recognition receptor and can thus bind to a range of immune cells. We thus sought to examine the binding of this recombinant fragment of SP-D to various cells present within PBMCs isolated from blood received from atopic donor as described in (section 2.14). Thus the binding of biotinylated rhSP-D (section 2.6) to PBMCs was shown by confocal microscopy (Figure 4.6a) as described in (section 2.10) as well as by direct staining via flowcytometry (section 2.19) by using PBMCs obtained from four well characterised atopic patients sensitised to *Phlp* allergen (Figure 4.6b). FITC conjugated rhSP-D bound to different cells with varying intensities; CD16 (PE)-63.4%, CD19 (PerCP-Cy5.5)-66.44%, CD3 (PE-Cy7)-79.1%, CD4 (Pacific Blue)-32.39%, CD8 (AmCyan)-55.61% and CD56 (APC)-67.1%.

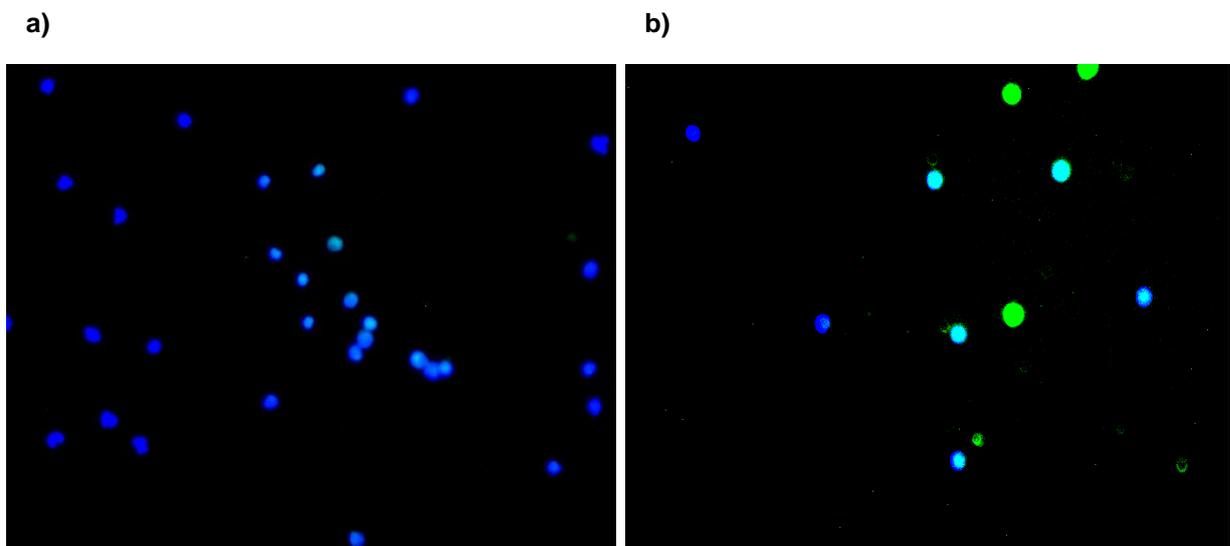


Figure 4.6a rhSP-D binding to PBMCs shown via confocal microscopy

The ability of rhSP-D to bind to various PBMC cell types was confirmed by confocal microscopy. a) represents DAPI stained PBMCs without rhSP-D, b) represents the binding of 5µg/ml biotinylated rhSP-D fluorescently labelled with FITC to DAPI stained PBMCs.

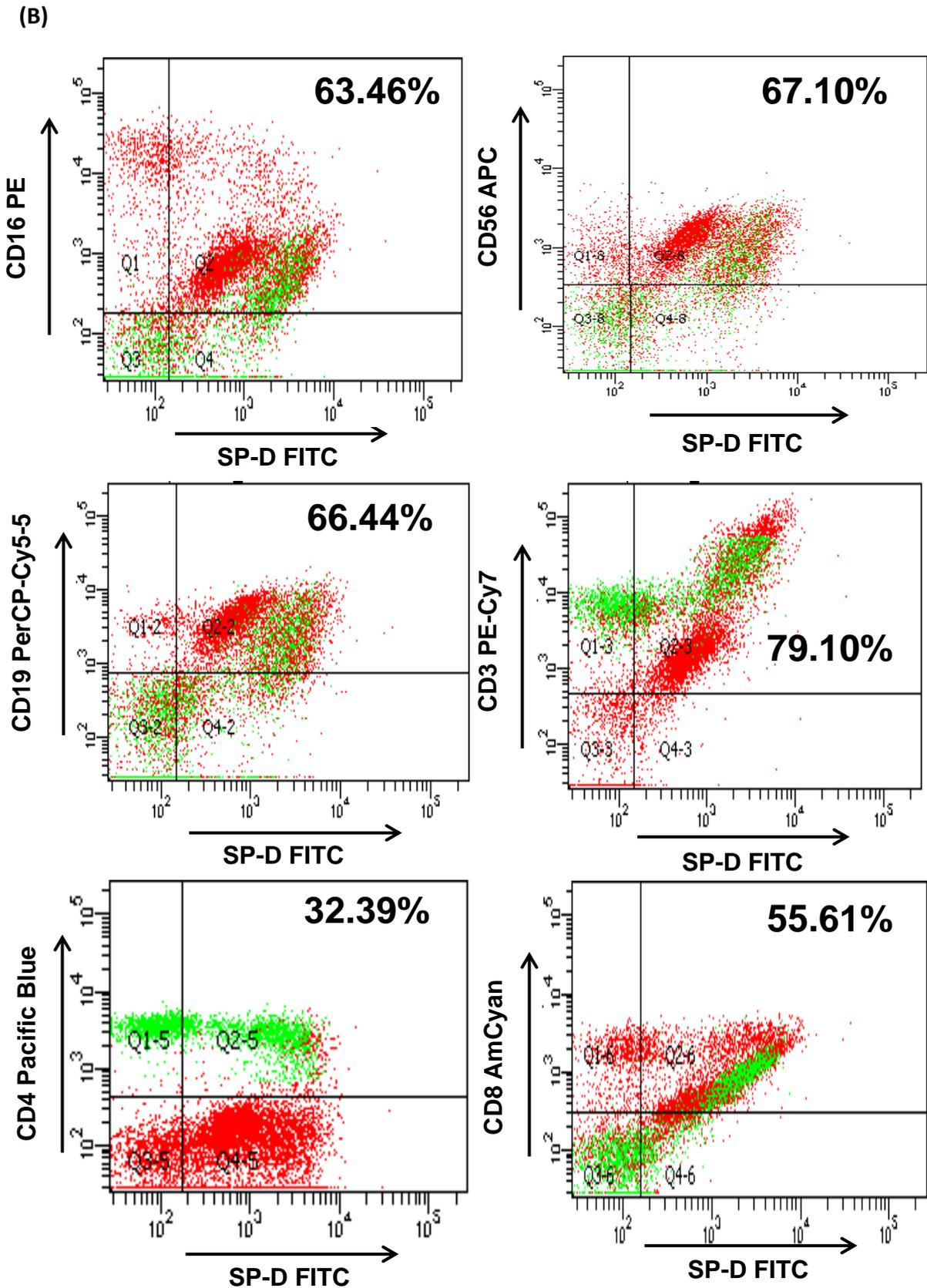


Figure 4.6b rhSP-D binding to various cells within PBMCs shown via flowcytometry

Having shown the binding of rhSP-D to PBMC by confocal microscopy, the binding intensity of rhSP-D to various specific cells (CD16, CD56, CD19, CD4, CD3, CD8) within the PBMCs were further examined via flowcytometry (n=4).

4.2.7 rhSP-D inhibits allergen-IgE complexes binding onto CD23 present on the surface of B-cells

We hypothesised that rhSP-D might suppress Th2 T-lymphocyte-driven allergic inflammation by inhibiting the IgE-facilitated antigen presentation. To test this hypothesis, we used an IgE FAB Assay as described in section 2.20, which is an in-vitro assay for facilitated antigen presentation. Here we examined the *Phlp*-IgE complexes binding on to CD23 enriched B-cells, which were pre-treated with rhSP-D by using serum obtained from ten well characterised grass pollen allergic patients (n=10). The results obtained show that the binding of allergen-IgE complexes to B cells was reduced by 40% (n=10, P=0.002; Wilcoxon matched-pairs signed rank test,) when CD23-enriched B cells were pre-treated with 5µg/ml rhSP-D (Figure 4.7).

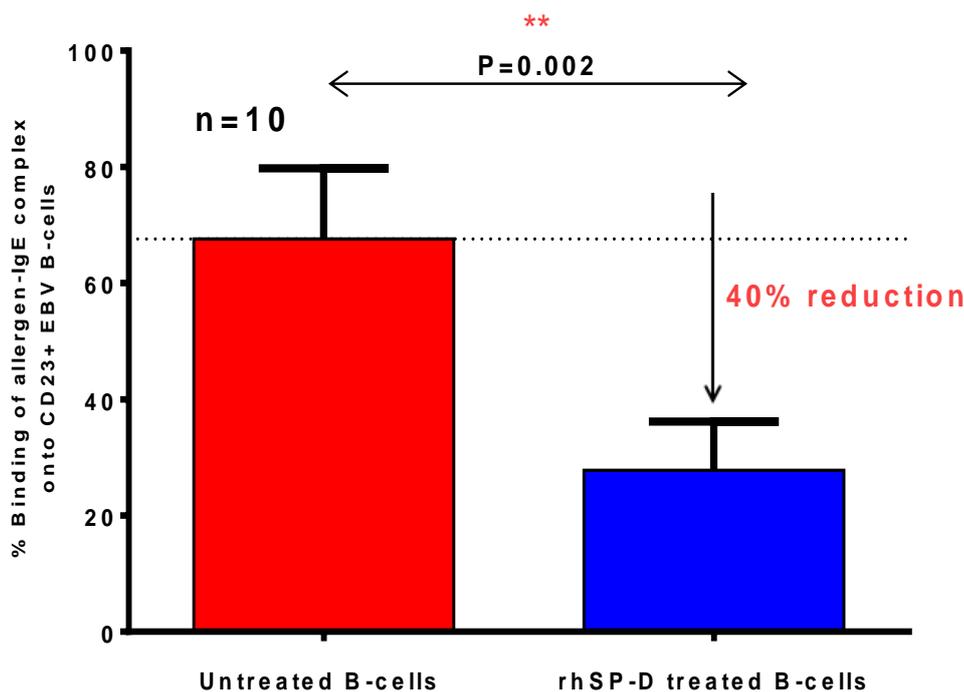


Figure 4.7 IgE Fab assay

Binding of allergen-IgE complexes to B cells was reduced by 40% (n=10, P=0.002) when CD23-enriched B cells were pre-treated with 5µg/ml rhSP-D for 1 hour at 37⁰c. Binding of Allergen-IgE complexes was detected using anti-IgE antibody. All data are shown as mean (±SD) percentage B cells bound to allergen-IgE complexes. *p* values were determined by Wilcoxon sign-rank test where ** represent *p*<0.01.

4.2.8 rhSP-D reduces CD23 expression

Furthermore, a reduction in CD23 expression on B-cells pre-treated with rhSP-D was also detected via flowcytometry (n=3, P<0.001), suggesting that interference of facilitated antigen presentation by rhSP-D is dependent on interaction between SP-D and CD23 (FcεRII), where reduction in CD23 expression may inhibit facilitated antigen presentation and thus allergen induced Th2 response.(Figure 4.8).

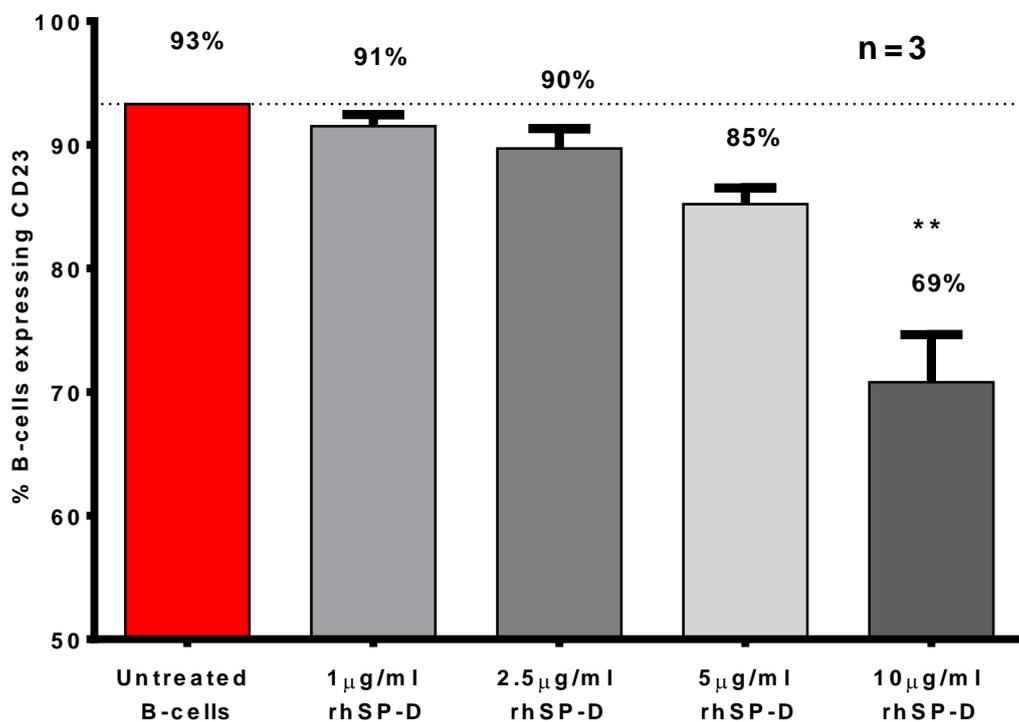


Figure 4.8 Reduction in CD23 expression of rhSP-D pre-treated B cells

A 24% reduction in CD23 expression of B-cells pre-treated with 10µg/ml of rhSP-D was observed via flowcytometry (n=3, P<0.001). These B-cells were obtained from EBV transformed B-cell line, that had been altered to give a higher expression of CD23. Results are shown as mean (±SD) percentage binding. *p* values were determined between treated and untreated by Wilcoxon sign-rank test where ** represent *p*<0.01.

4.2.9 rhSP-D inhibits allergen-induced T cell proliferation

Our next hypothesis involved examining whether rhSP-D can have an effect on antigen presentation and proliferation to CD4+ve T cells via ³H-Thymidine incorporation assay as described in (section 2.21) using PBMCs obtained from twelve well characterised atopic patients (n=12). The results obtained show that *Phlp* stimulated PBMCs that were pre-treated with both 5ug/ml and 10ug/ml of rhSP-D appeared to show a significant suppression of proliferation (Figure 4.9) to both allergen stimulated PBMCs as well as PHA stimulated PBMCs (Data not shown).

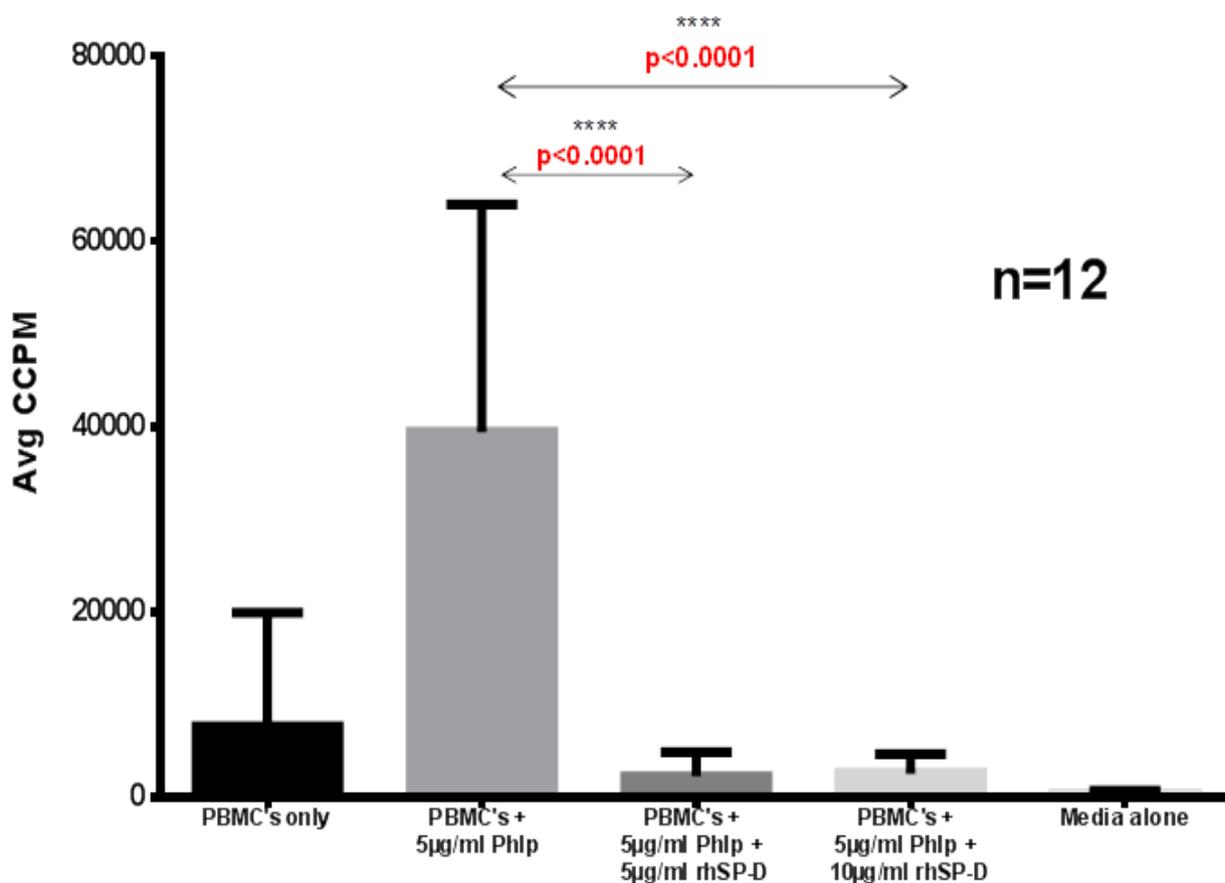


Figure 4.9 ³H-Thymidine incorporation assay

rhSP-D was found to significantly suppress the proliferation of phlp allergen stimulated PBMC's (n=12, $p < 0.0001$) at both 5µg/ml and 10µg/ml rhSP-D. Data are presented as mean \pm SD. p values were determined by wilcoxon sign-rank test where **** represents $p < 0.0001$.

4.2.10 rhSP-D inhibits allergen driven Th2 cytokine production

We then further characterised these proliferating cells by using a multiplex assay as described in (section 2.22), to see if rhSP-D can inhibit allergen driven Th2 cytokine production, and it appears that rhSP-D significantly inhibits IL-4, IL-5, IL-9, IL-10 and IL-13 (Figure 4.10).

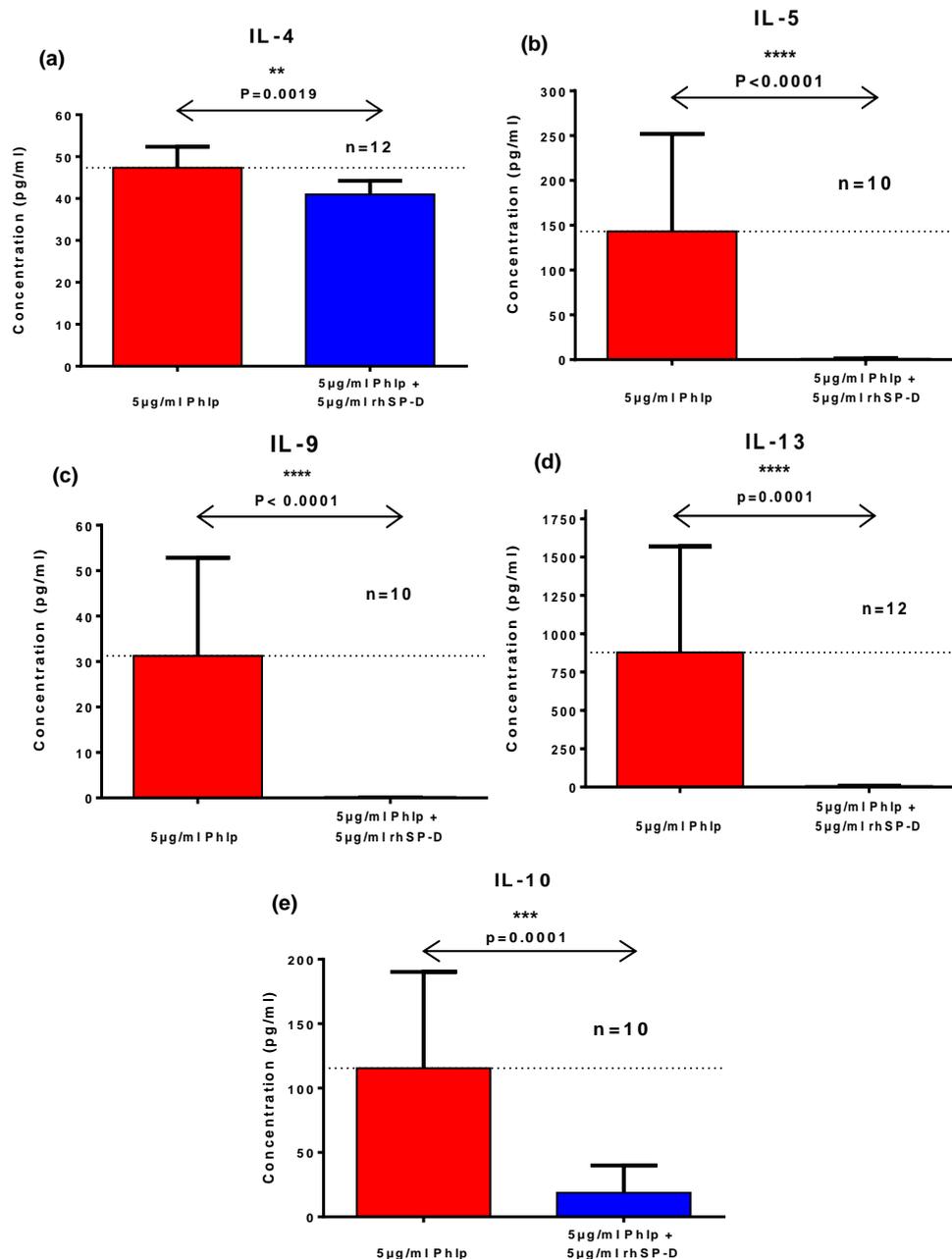


Figure 4.10 Allergy related Th2 cytokine suppression by rhSP-D

Data obtained from Multiplex analysis shows a clearly inhibition of Th2 cytokine responses (a) IL-4 (P=0.0019), (b) IL-5 (P<0.0001), (c) IL-9 (P<0.0001), (d) IL-13 (P<0.0001), (e) IL-10 (P<0.0001). Data are presented as mean \pm SD. *p* values were determined by wilcoxon sign-rank test where *, **, **** represent *p*<0.05, *p*<0.01 and *p*<0.0001 respectively.

4.2.11 rhSP-D inhibits allergen Th1 related cytokine production

The effect of rhSP-D on Th1 cytokine production was also looked at where we found that rhSP-D actually led to a slight reduction in the production of the following Th1 cytokines, INF- γ and IL12 (p70) (Figure 4.11), however this effect was not considered to be significant when compared to the inhibition of Th2 cytokine production by rhSP-D. This result is thus somewhat consistent with earlier findings by (Madan et al., 2005b), where they demonstrated that rhSP-D only suppresses pathogenic Th2 response but does not reduce Th1 expression.

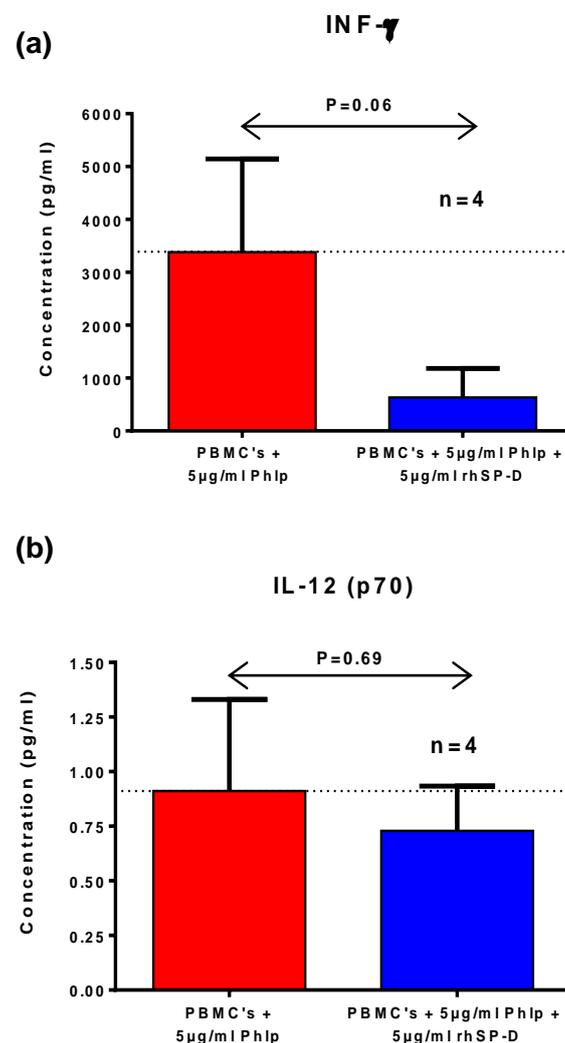


Figure 4.11 Th1 suppression by rhSP-D

Data obtained from Multiplex analysis also showed slight reduction in Th1 related cytokines, however these reductions in Th1 responses were not found to be statistically significant (a) INF- γ ($p=0.06$), (b) IL-12 ($p=0.69$). Data are presented as mean \pm SD. p values were determined by wilcoxon sign-rank test.

4.2.12 Effect of rhSP-D on allergen driven chemokine production

We also examined the effect of rhSP-D on allergen driven chemokine production and found that SP-D inhibits the production of Eotaxin and MDC, but no significant effect of SP-D was observed with IL-8 (CXCL8) and RANTES, (Figure 4.12).

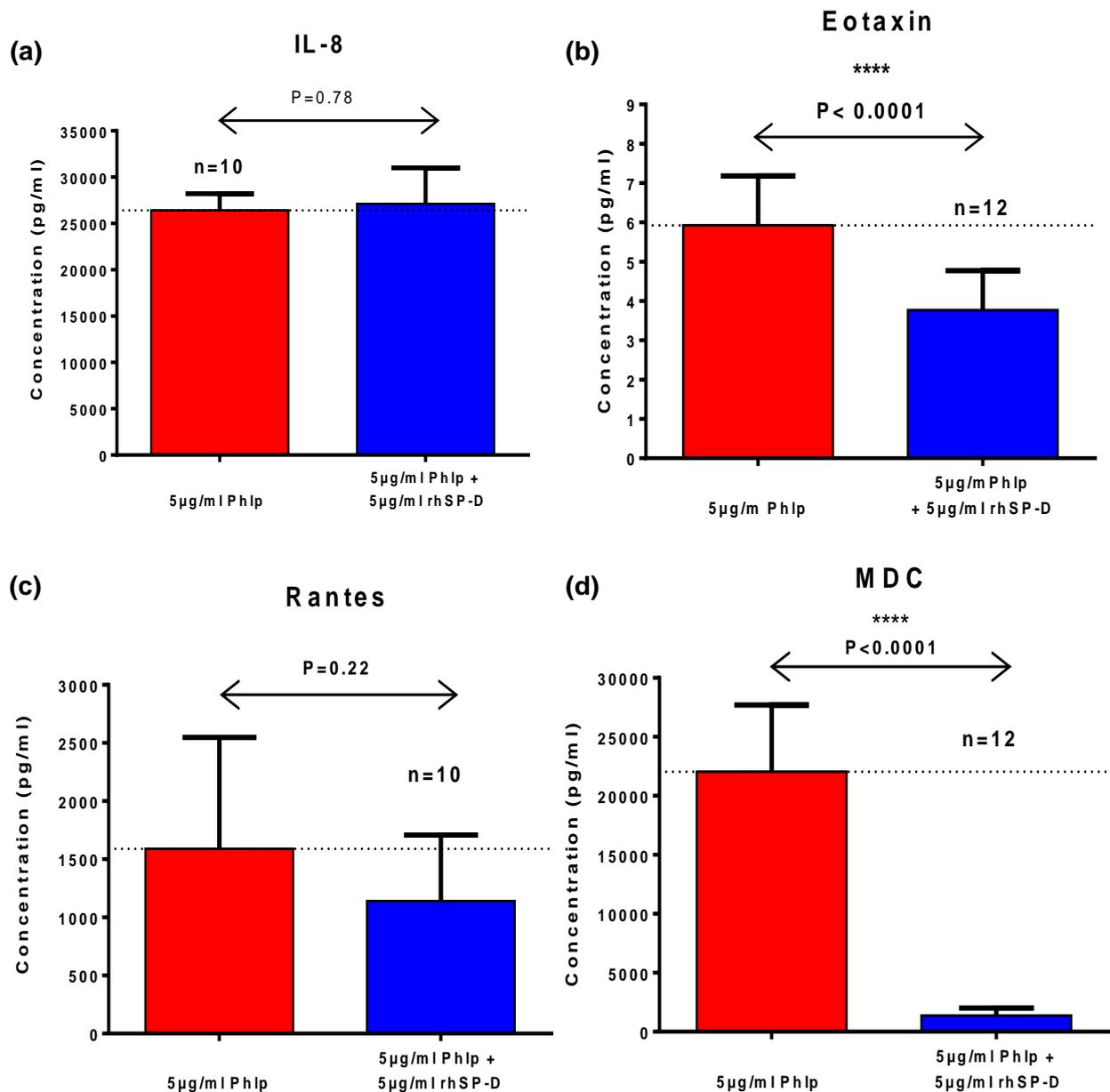


Figure 4.12 Effect of rhSP-D on chemokine production

Graphs obtained from Multiplex analysis showed the ability of SP-D to suppress Eotaxin ($p < 0.0001$) and MDC ($p < 0.0001$). Data are presented as mean \pm SD. p values were determined by wilcoxon sign-rank test where **** represents $p < 0.0001$.

4.2.13 rhSP-D inhibits pro-inflammatory and anti-inflammatory cytokine production

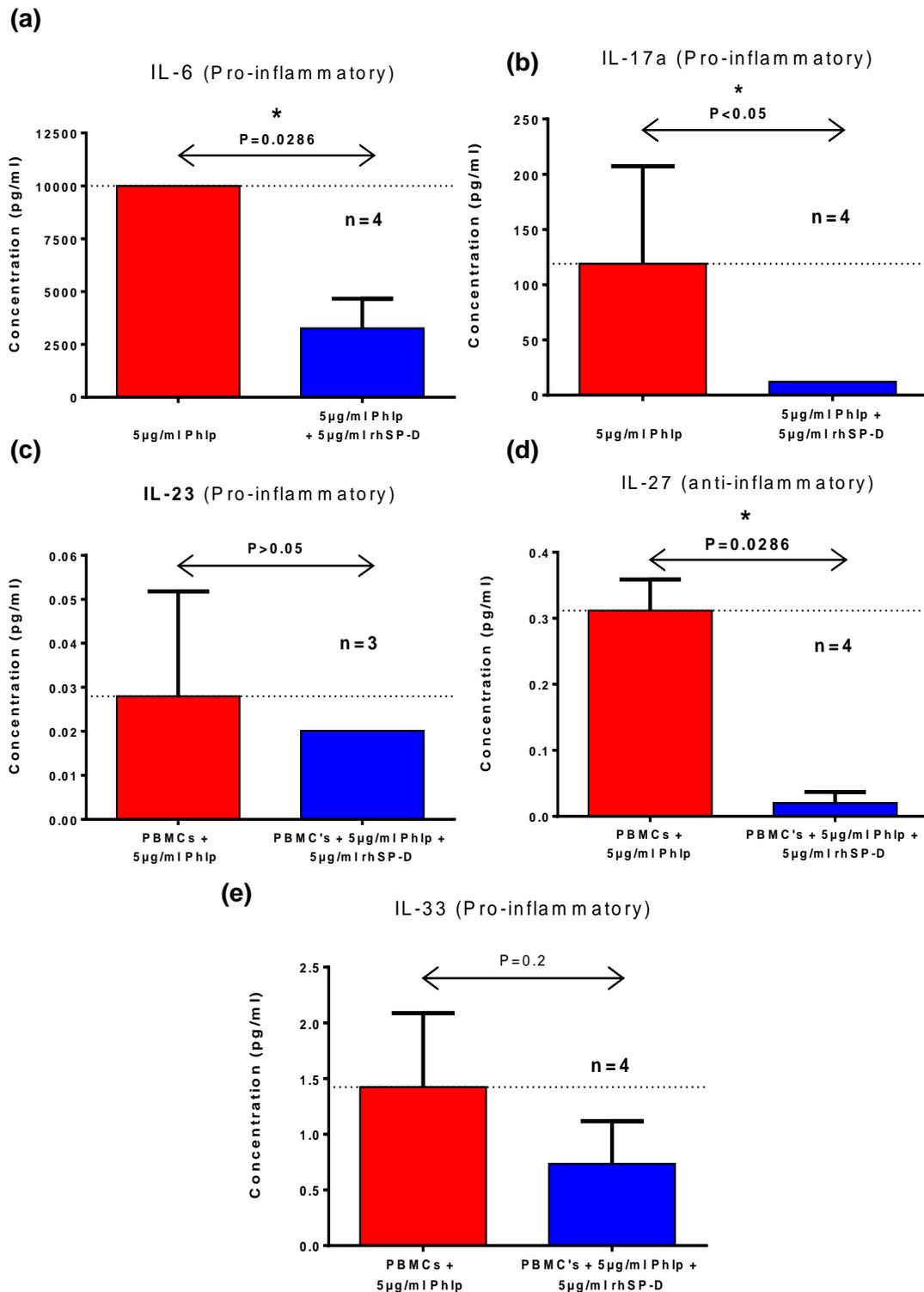


Figure 4.13 Suppression of pro-inflammatory and anti-inflammatory cytokine production by rhSP-D.

Data obtained from Multiplex analysis showed the ability to suppress various pro-inflammatory and anti-inflammatory responses. (a) IL-6 ($p=0.0286$), (b) IL-17a ($p<0.05$), (c) IL-23 ($p>0.05$), (d) IL-27 ($p<0.0286$), (e) IL-33 ($p>0.05$). Data are presented as mean \pm SD. p values were determined by wilcoxon sign-rank test where * represents $p<0.05$.

4.2.14 Reduced IgE-FAB vs ³H Proliferation assay vs Th2 cytokines (IL-4, IL-9)

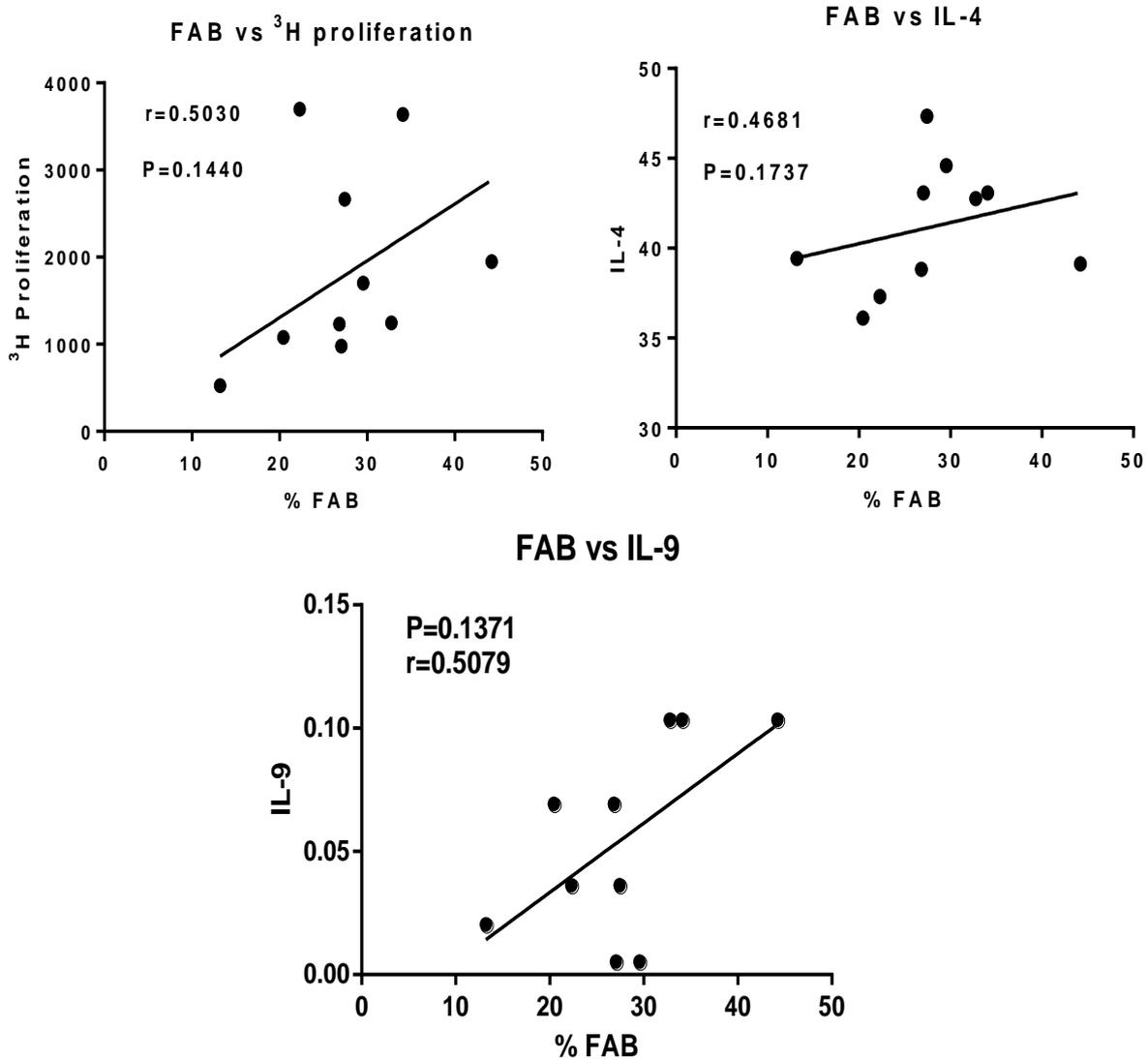


Figure 4.14: Correlation between reduced IgE-FAB vs ³H Proliferation assay vs Th2 cytokines (IL-4, IL-9)

There appeared to be a positive correlation though not significant between reduction in IgE-FAB vs reduction in proliferation as well as a positive correlation between reduced IgE-FAB and reduced TH2 cytokine response. *p* values were determined by non-parametric spearman rank correlation test .

4.3 Discussion

It has been reported that SP-D carries out a range of anti-allergic functions; however, the underlying mechanisms by how SP-D confers protection against allergy are still not yet well understood. We thus sought to further understand these underlying mechanisms involved in rhSP-D mediated protection against allergy by using timothy grass (*Phleum Pratense*) allergen as a model. We have established in this study that interference of rhSP-D with IgE-dependent mechanisms such as IgE facilitated allergen presentation responsible for allergic inflammation, can lead to suppression of Th2 driven allergic inflammation by inhibiting the IgE-facilitated antigen presentation, thus leading to reduction in allergic responses in-vivo.

Firstly, the binding of rhSP-D to *Phlp* extract needed to be established as the patient samples used in this study were obtained from atopic individuals who were highly sensitised to *Phlp* allergen. This aspect was examined via indirect ELISA, where it was found that rhSP-D binds to *Phlp* allergen in a dose dependent manner where 5µg/ml was observed to be the optimum concentration for binding with *Phlp* allergen. This binding was further confirmed by western blotting, which showed the ability of rhSP-D to bind to three proteins found in the *Phlp* extract around the region of 50kDa, 40kDa and 38kDa. Furthermore, this binding of rhSP-D with *Phlp* allergen was found to be calcium and carbohydrate dependent. This data thus corroborates with previous literature, showing the ability of SP-D to bind to certain allergens through its carbohydrate recognition domains, interacting with the carbohydrate residues on the allergen (Madan et al., 1997c). The binding of rhSP-D with IgE was also examined by ELISA, where it was found that rhSP-D binds to IgE in a dose dependent manner, where rhSP-D at a concentration of 10µg/ml showed optimum binding with 10µg/ml IgE coated wells. This thus further confirms that interaction of SP-D with IgE involves the neck/CRD region of SP-D as demonstrated previously with full length SP-D (Nadesalingam et al., 2005). Furthermore, the binding of SP-D via neck/CRD region to IgE

may take place through interaction with seven well identified glycosylation sites in the ϵ -chain at Asn¹⁴⁰, Asn¹⁶⁸, Asn²¹⁸, Asn²⁶⁵, Asn³⁷¹, Asn³⁸³ and Asn³⁹⁴, found in the constant region of IgE. (Arnold et al., 2004)

The ability of rhSP-D to bind to various cell types found in PBMCs was also confirmed by confocal microscopy. The intensity of rhSP-D binding to various PBMCs by flow cytometry was also examined; using PBMCs obtained from four grass allergic patients. FITC conjugated biotinylated rhSP-D bound to different cells with varying intensities due to different glycosylation properties of various cells, which express different amounts of carbohydrates on surface; CD16 (63.4%), CD19 (66.44%), CD3 (79.1%), CD4 (32.39%), CD8 (55.61%) and CD56 (67.1%). Moreover, this result further highlights the importance of CRD region in mediating the interaction with various cells due to interacting with carbohydrates present on the surface of these cells. After the initial optimisation studies, the next aim of this study was to examine the effect of rhSP-D in interfering with facilitated antigen presentation by preventing CD23-mediated IgE facilitated allergen binding to B cells, using an IgE-Facilitated Allergen Binding (IgE-FAB) assay previously validated by (Shamji et al., 2006a).

This assay was used to assess *Phlp*-IgE complexes binding to CD23 enriched B-cells that were pre-treated with 5 μ g/ml rhSP-D along with 5mM CaCl₂, using serum obtained from ten well characterised grass pollen allergic patients. This assay represents an *in-vitro* model of facilitated allergen presentation, where allergen-IgE complexes are incubated with an EBV-transformed B-cell line and complexes bound to CD23 on the surface of cells are detected by flow cytometry. This assay was used, as it has been previously shown that addition of serum from patients who have received allergen-specific immunotherapy can inhibit allergen-IgE complex binding to CD23 on B cells(Shamji et al., 2006a, Francis, 2008b, Wachholz et al.,

2003b). Furthermore, even though the read out obtained from this assay does not directly examine the antigen presenting capacity of B cells to T cells, this assay however has previously been shown to serve as a representative of this process (Wachholz et al., 2003a). IgE-FAB assay thus provides a simplified model as a substitute for facilitated allergen presentation without the need for carrying out T cell culture techniques, which can be difficult to quality control (Shamji et al., 2006a). The results obtained from this assay showed the ability of rhSP-D to significantly interfere with the co-operative binding of allergen-IgE complexes to B cells, by up to 40% (n=10, P=0.002) when CD23-enriched B cells were pre-treated with 5 µg/ml rhSP-D. This result thus proves our hypothesis and shows a possible mechanism by which rhSP-D can exert its therapeutic potential.

Furthermore, the effect of pre-treatment of CD23-enriched EBV-transformed B cells with rhSP-D was also determined by flow cytometry, where a 24% reduction in CD23 expression of B-cells pre-treated with 10µg/ml of rhSP-D was observed via flow cytometry (n=3, P<0.001). This is also a very interesting result, since as it has been previously shown by Boccafogli et al, that serum levels of sCD23 correlate with allergic seasonal symptoms (Boccafogli et al., 1997). Furthermore, additional literature also suggests the involvement of CD23 in IgE regulation (Gould and Sutton, 2008). This is therefore the first study to establish a link between SP-D and CD23, suggesting that interference of facilitated antigen presentation by rhSP-D is dependent on interaction between rhSP-D and CD23 (FcεRII), where reduction in CD23 expression will inhibit facilitated antigen presentation and thus allergen induced Th2 response. This interaction between SP-D and CD23 has been further evaluated in the next chapter, in order to better understand how SP-D can play a role in IgE regulation, since as previous literature indicates that it is important to further prevent the worsening of allergic symptoms occurring through CD23/IgE-mediated antigen presentation by B-cells (Mudde et al., 1995).

A link between increased allergen specific IgE found in the serum of atopic patients and increased allergen driven T cell proliferation has also been established *in vitro* (van der Heijden et al., 1993). Thus, the effect of rhSP-D on antigen presentation and proliferation to CD4⁺T cells was also examined via ³H-Thymidine incorporation assay since as it has been previously suggested that the results obtained via IgE Fab assay can correlate with reduction in T lymphocyte proliferation (Francis, 2008a). Here we compared the proliferation of untreated Phlp stimulated PBMCs, with PBMCs which were pre-treated with rhSP-D for 1 hour at 37⁰C in presence of 5mM CaCl₂, prior to stimulation with Phlp allergen. For this assay we used PBMCs obtained from 12 well characterised atopic patients highly sensitised to phlp allergen. We found that pre-treatment of PBMCs with rhSP-D shows significant suppression (P<0.0001) of allergen induced T-cell proliferation with both 5µg/ml and 10µg/ml of rhSP-D. The anti-proliferative effect of rhSP-D on *Phlp* stimulated PBMCs further conforms to an earlier study undertaken by Wang et al, where the inhibitory effect of SP-D was shown in *Der p* allergen-stimulated lymphocyte proliferation. (Wang et al., 1998)

We further characterised these proliferating cells by using a multiplex assay to examine the ability of rhSP-D to inhibit allergen driven Th2 cytokine production. We found that rhSP-D strongly inhibits allergen driven Th2 cytokine production by examining the following Th2 responses; IL-4 ($p=0.0019$), IL-5 ($p<0.0001$), IL-9 ($p<0.0001$), IL-13 ($p<0.0001$), IL-10 ($p<0.0001$). The reduction of Th2 responses by rhSP-D was as expected and these results corroborate with earlier findings. (Madan et al., 2001, Strong et al., 2003), however no significant effect of rhSP-D was observed with Th1 related cytokines; INF- γ ($p=0.06$), IL-12 P70 ($p=0.69$). Chemokines help inflammatory cells to infiltrate at the site of inflammation (Moser and Willimann, 2004). Thus, chemokine production by rhSP-D was also examined. rhSP-D was found to significantly suppress the production of Eotaxin ($p<0.0001$) and MDC ($p<0.0001$) but showed no significant effect on levels of IL-8 ($p>0.05$) and RANTES

($p=0.22$),. This thus suggests that SP-D induced inhibition of chemokine production would lead to lower infiltration and a decrease in inflammation. In case of allergic asthma, it will lead to reduced swelling of bronchial mucosa, which would ultimately lead to improvement of asthma symptoms. The production of various pro-inflammatory (IL-6, IL-17a, IL-2, IL-33) and anti-inflammatory (IL-27) cytokine responses were also examined in the presence of rhSP-D, where rhSP-D was found to significantly suppress IL-6 ($p=0.0286$), IL-17a ($p<0.05$) and IL-27 ($p=0.0286$), whereas no significant effect of rhSP-D was observed on the levels of IL-33 ($p=0.2$) and IL-23 ($p>0.05$).

Finally, the results obtained for the IgE FAB assay were correlated with the data obtained from T-lymphocyte proliferation assay, where it was found that there was a positive correlation between reduction in IgE-FAB vs reduction in proliferation. However this correlation did not appear to be statistically significant ($r=0.503$, $p=0.144$). Furthermore, the correlation between reduction in IgE-FAB and reduction in Th2 cytokine IL-4 ($r=0.4681$, $p=0.1737$), IL-9 ($r=0.5079$, $p=0.1371$) response was also examined. This again showed a positive correlation, however, this correlation did not reach statistical significance.

In summary, this study demonstrates the ability of SP-D to interfere with the binding of allergen-IgE complexes to B cells, as well as down regulating CD23 expression on B cells. Thus, inhibition of IgE-facilitated antigen presentation may represent a mechanism whereby SP-D suppresses Th2 T-lymphocyte-driven allergic inflammation.

Chapter 5



Interaction between rhSP-D, CD23 and CD21 and its effect on B cell functions

5.1 Introduction

CD23 is a low affinity IgE receptor (FC ϵ RII). It has been found to closely resemble the overall structure of the trimeric lectin domain of SP-D (Figure 5c). Like SP-D, CD23 is also a C-type lectin. CD23 is expressed on B-cells, macrophages, eosinophils and follicular dendritic cells (Beavil et al., 1995). Cell surface CD23 can be cleaved by various endogenous proteases, which can yield soluble fragments of different sizes (37, 33, 29, 25 & 16kDa). All of these soluble fragments exist in circulation in non-atopic patients. It has been previously shown that the fragments which possess the stalk region have the ability to form trimers, whereas the fragments which lack this stalk region cannot form trimers (McCloskey et al., 2007, Schulz et al., 1997). Furthermore, the length of this residual stalk, which depends on the cleavage site by the endogenous proteases, can determine its ability to trimerize (Schulz et al., 1997). These sCD23 fragments have been implicated in the pathophysiology of allergic inflammation by playing a role in IgE regulation. It has been reported that increased levels of these various soluble fragments of CD23 are observed in patients suffering from various allergy related disorders.

CD23 has been shown to bind to CD21, also known as complement receptor 2 (Aubry et al., 1992). CD21 is a 145-kDa type I transmembrane glycoprotein that binds the surface-fixed cleavage fragments of C3, iC3bC3dgC3d and serves as the receptor for Epstein-Barr virus EBV on B lymphocytes (Fingerhuth et al., 1984, Weis et al., 1984). CD21 was initially described as the receptor for the C3dg fragment of C3. CD21 is distinguished by the presence of short complement regulator (SCR) domains (also known as short consensus repeats). It is found on mature B-cells, FDC, epithelial cells, and some T-cells. CD21 is a receptor that plays an integral role in the immune system (Holers and Boackle, 2004). SCR 1-2 contains the binding site for C3d, and also contains binding sites for the low-affinity IgE receptor, CD23, and IFN- α . Importantly, a further binding site for CD23

occurs in the SCR 5-8 domains that is dependent upon the N-linked oligosaccharides within SCR 5-8 domains (Gilbert et al., 2006).

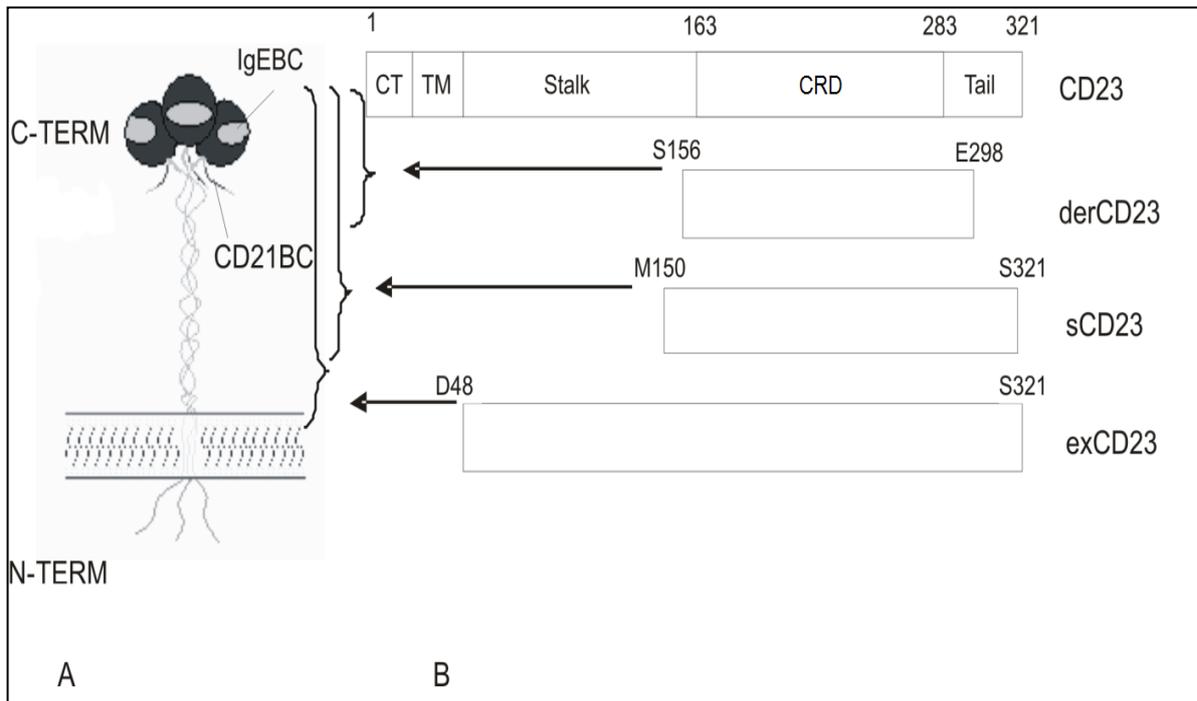


Figure 5a Structure of CD23

(A) CD23 structure shown with IgE binding site (IgEBC) and CD21 binding site (CD21BC) shown. (B) Furthermore, schematic structure of full length CD23 as well as the structure of truncated soluble constructs is also shown: derCD23 (Ser 156 – Glu 298), sCD23 (Met 150 – Ser 321) and exCD23 (Asp48- Ser321). (Bowles et al., 2011)

Triggering of CD21 by CD23 has been shown to increase IL-4-induced germline C ϵ transcription levels (Basaki et al., 2002) and have a synergistic effect on the expression of the ϵ transcript in B cells induced with the help of T-cells. Thus T-cell-related CD23 may interact with CD21 leading to an increase in IgE production in allergic patients. Adhesion pairing between CD23 and CD21 may either affect IgE production, or lead to further allergic exacerbation via numerous mechanisms. Cross-talk between the complement system and B cell receptor may take place upon the interaction of CD23 with CD21 and IgE thus leading to increase in IgE production (Gould and Sutton, 2008).

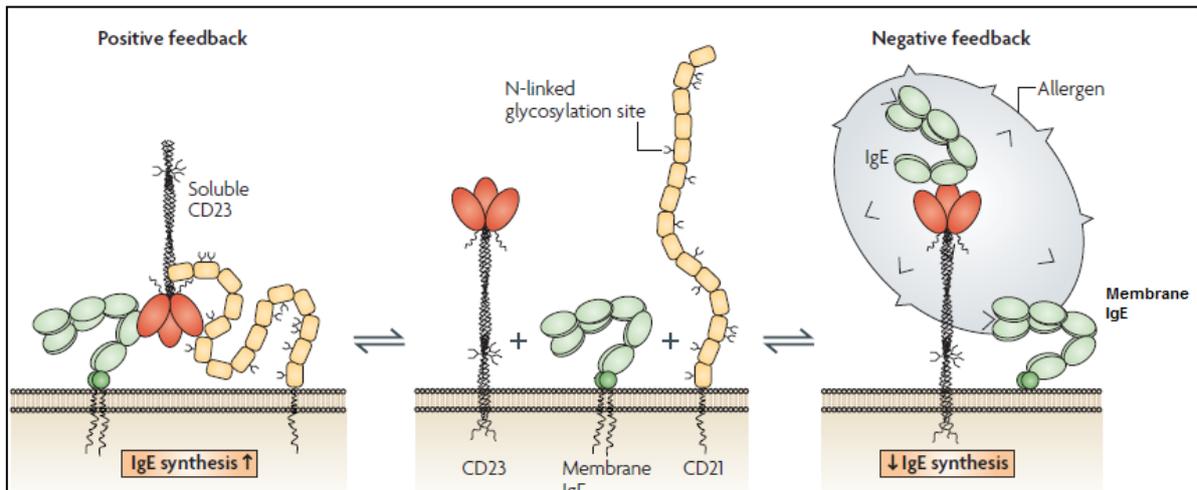


Figure 5b: Regulation of IgE synthesis by human CD23.

It has been shown in humans that a soluble trimeric CD23 can either up regulate or down regulate IgE synthesis and secretion upon interaction with CD21. Up regulation of IgE can occur through co-ligation of membrane bound IgE and CD21 present on the surface of B-cells, which are committed to IgE synthesis by soluble CD23 released upon the cleavage of membrane bound CD23. The signals generated upon this interaction can lead to enhanced production of IgE. Down regulation of IgE can however also occur upon co-ligation of IgE and CD23 by allergen-IgE complexes, where the competition between CD23 and CD21 for membrane IgE can lead to reduction in IgE synthesis (Gould and Sutton, 2008).

In the previous chapter, a link between CD23 and recombinant human SP-D (rhSP-D) was established, where it is being reported for the first time, that B-cells, pre-treated with rhSP-D, showed a reduced CD23 expression, when compared to untreated B cells. Here we sought to examine if there is a direct interaction between rhSP-D and CD23, and whether this interaction can positively or negatively regulate IgE synthesis (Gould and Sutton, 2008). We also examined the interaction of rhSP-D with CD21 SCR 1-2 fragment, which along with CD21 SCR 5-8 is the binding site for CD23 on CD21. The interaction of CD23 with CD21 SCR 1-2 is a protein-protein reaction, whereas the interaction between CD23 and CD21 SCR 5-8 is a lectin like interaction.

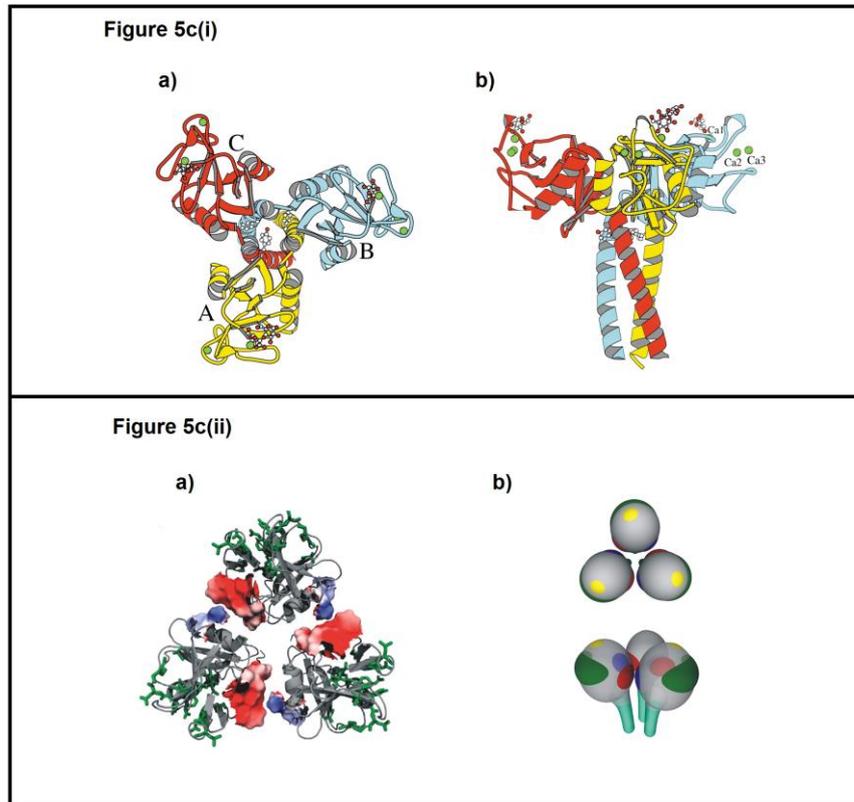


Figure 5c: Structural similarities between SP-D and CD23

CD23 is a low affinity IgE receptor (FC ϵ RII). It has been found to closely resemble the overall structure of the lectin domain of SP-D, since it too like SP-D belongs to a family of C-type lectins. Structure of recombinant human SP-D trimer is shown in Figure 5c(i), where (a) shows the molecular 3-fold axis of rhSP-D trimer whereas, (b) shows rhSP-D trimer when viewed perpendicular to the molecular 3-fold axis. Figure 5c(ii) shows the molecular structure (a) of CD23 trimer when viewed down, whereas (b) depicts both the down and the side views of CD23 trimer. Adapted from (Shrive et al., 2003, Hibbert et al., 2005)

We hypothesised that SP-D can regulate IgE synthesis by B cells via interfering with CD23-CD21 interaction. To establish this, we first examined the interactions between the rhSP-D and the two recombinant soluble CD23 fragments, yielded by the dust mite protease DerpI (derCD23-16kDa), and by ADAM10-mediated proteolysis (sCD23-25kDa), as well as with CD21 SCR 1-2 (14kDa). Here the binding of rhSP-D with soluble fragments of CD23 and CD21 SCR 1-2 was confirmed via solid phase binding assays. In addition, the effect of rhSP-D on IgE synthesis by co-incubating rhSP-D with soluble

CD23 fragments as well as CD21 SCR 1-2 as previously described in (Bowles et al., 2011) using PBMCs obtained from grass allergic patients was also examined.

5.2 Results

5.2.1 Detecting the presence of derCD23 and sCD23

Two recombinant fragments of sCD23 yielded by either the dust mite protease *Der pI* (derCD23-16kDa), a monomeric protein, which was expressed and purified in E-coli BL21 (DE3) as described before (Hibbert et al., 2005) was kindly donated by Dr Andrew Beavil King's College London, or ADAM10-mediated proteolysis (sCD23-25kDa), a trimeric protein which was expressed and purified in E-coli BL21 (DE3) as described in (Daniels et al., 2005) was donated by Professor Vaughan Oosthuizen, Nelson Mandela Metropolitan University, South Africa. Both of these fragments were used in this study. Moreover, both of these truncated forms of human CD23 have not been shown to possess any N-glycosylation sites (Hibbert et al., 2005). Thus, the first task of this study was to ascertain the presence of pure proteins by running SDS-PAGE (Figure 5.1) as described in (section 2.2) to confirm the purity of these protein fragments.

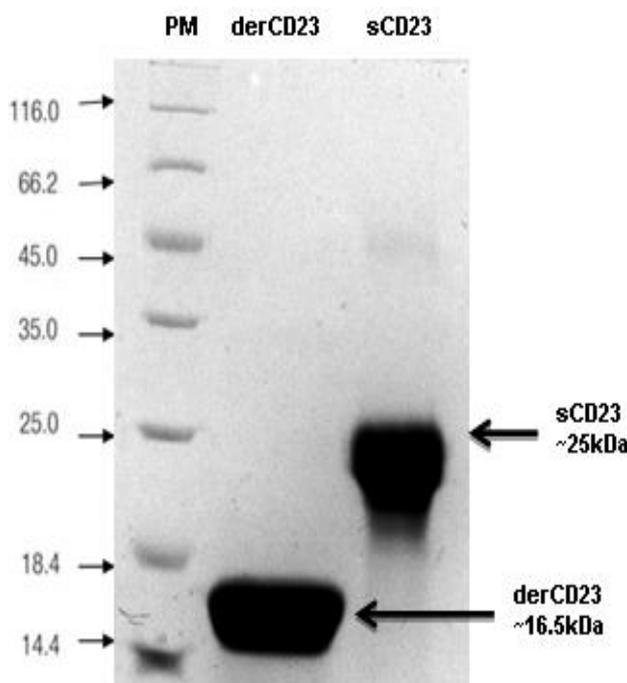


Figure 5.1: SDS-PAGE (15% w/v acrylamide) analyses to visualise the presence of sCD23 fragments.

Presence of derCD23 was indicated by a band in the region of 16.5kDa (lane 2), whereas molecular weight of sCD23 was also confirmed, where it was found to be in the region of 25kDa.

5.2.2 Expression of C3dg

C3dg, a ligand for CD21 needed to be expressed in sufficient quantity, in order to prepare a C3dg Sepharose column for purifying CD21 SCR 1-2. C3dg was expressed and purified in *E.coli* as described in the methods and materials (section 2.24.3). Following transformation as described in section 2.24.2, a pilot expression was carried out to examine the expression of C3dg. Samples were collected before and after induction with IPTG (section 2.1.3) for 3 hours and run as described in (section 2.2) on a 15% SDS-PAGE gel as shown below in (Fig 5.2) in order to visualise the presence of C3dg, which was confirmed by the presence of C3dg bands at ~38kDa as also previously shown in (Henson et al., 2001)

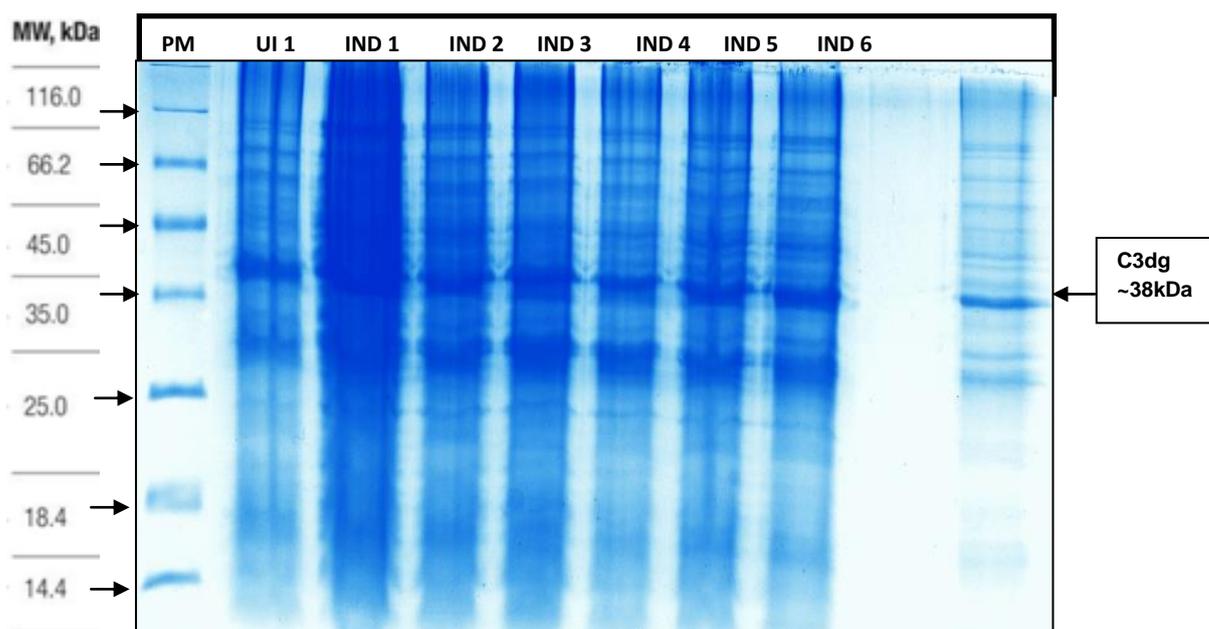


Figure 5.2: SDS-PAGE (15% w/v acrylamide) analyses of C3dg after induction

C3dg was expressed as inclusion bodies in *Escherichia coli* BL21 using pET15b expression plasmid. Three hours after induction with 0.4 mM IPTG, the rhSP-D accumulated as an over-expressed protein of ~38 kDa (lanes 3-8 from left) compared with un-induced cells (lane 2 and lane 10 from left). The molecular weight of the protein was confirmed by comparing with the protein marker (Lane 1 from left).

5.2.3 C3dg SDS-PAGE gel after lysis and sonication:

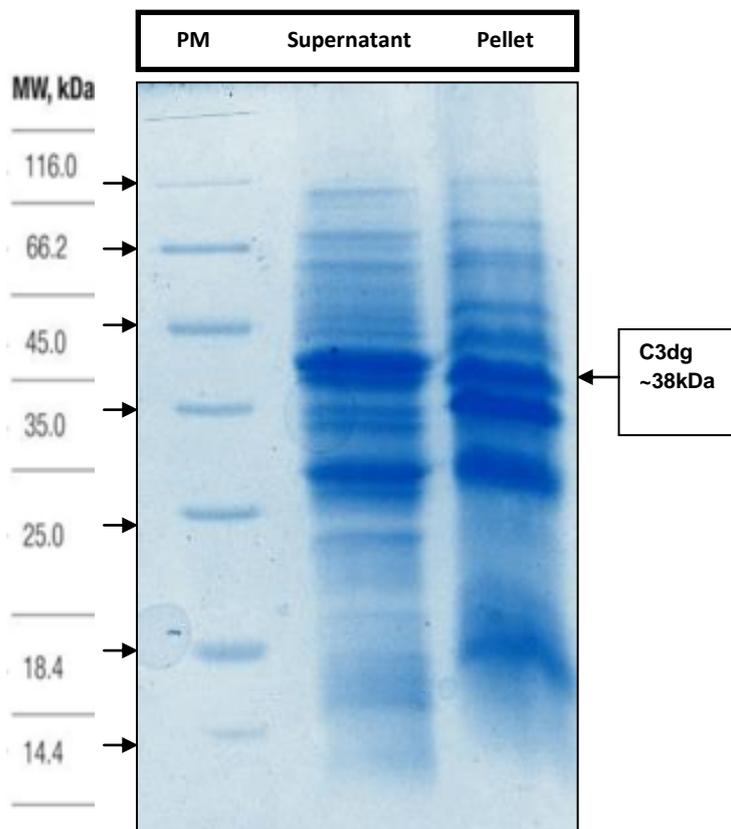


Figure 5.3: SDS-PAGE (15% w/v acrylamide) analyses of C3dg after cell lysis and sonication

Following successful verification of C3dg expression on a pilot scale, large scale cultures were grown as described in (Section 2.24.3) Cells were harvested in 10 mM sodium phosphate, 0.075 M NaCl, 2 mM EDTA, and 2 mM PMSF (pH 7.2) and sonicated to disrupt the cell membrane. From the SDS-PAGE gel a thicker band for C3dg can be observed in supernatant (Lane 2 from left).

5.2.4 C3dg after ion exchange

The sonicated lysate, containing most of the recombinant C3dg protein, was dialyzed as described in section 2.24.3 and then loaded onto a 5ml column of CM-Sepharose Fast Flow (GE Health-Pharmacia, Piscataway, NJ). The column was washed with 10 volumes of the loading buffer, and protein was eluted in the loading buffer composed of 300mM NaCl and 0.1mM DTT. The eluted proteins were further dialyzed against 10 mM sodium phosphate, 150mM NaCl, and 0.1 mM DTT (pH 7.2). The recombinant C3dg protein was approximately 80% pure after this stage (Figure 5.4).

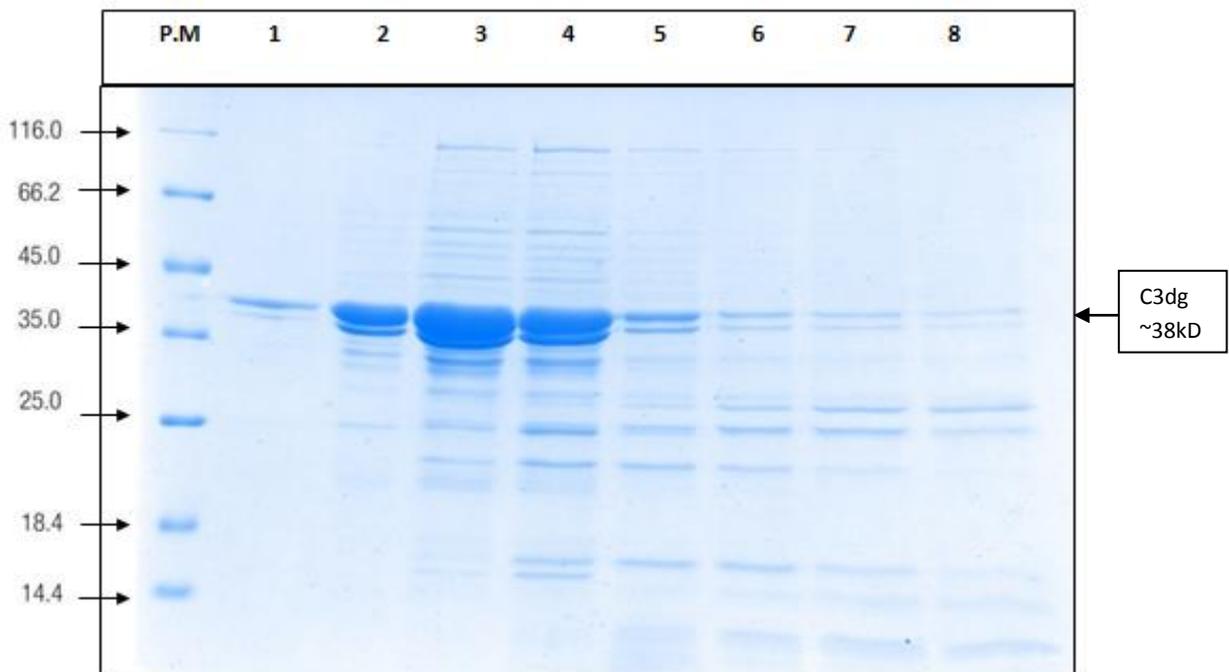


Fig 5.4 SDS-PAGE (15% w/v acrylamide) analyses of C3dg after ion exchange

Lanes 1-8 depict the C3dg fractions collected after ion exchange. From the SDS-PAGE gel it can be observed that most of the proteins were eluted in 2,3 and 4th fractions (Lane 2-4).

5.2.5 Purification of CD21 SCR 1-2

Following the preparation of C3dg affinity column, a truncated form of CD21 (complement receptor 2) ligand for CD23, was purified according to methods and materials (section 2.24.4). Firstly, transformation as described in section 2.24.2 was carried out followed by a pilot expression to examine the presence of CD21 SCR 1-2. Samples were collected before and after induction with IPTG (section 2.1.3) for 3 hours and run as described in section 2.2 on SDS-PAGE as shown below in Figure 5.5 in order to visualise the presence of CD21 SCR 1-2, which was confirmed by the presence of band at ~14.4kDa as also previously shown by Isenman et al. (van den Elsen and Isenman, 2011).

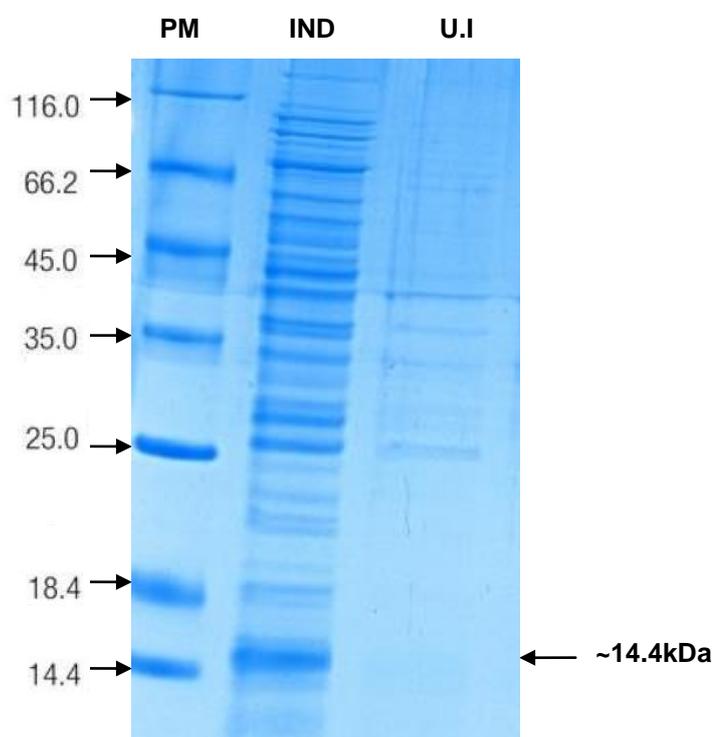


Figure 5.5: SDS-PAGE (15% w/v) analyses of CD21 SCR 1-2 after induction

CD21 SCR 1-2 was expressed as inclusion bodies in *Escherichia coli* BL21 using the pET15bCR2 (SCR1-2) expression plasmid. Three hours after induction with 1mM IPTG, CD21 SCR 1-2 was accumulated as an over-expressed protein at ~14.4 kDa (lane 2 from left) when compared with un-induced cells (lane 3 from left). The molecular weight of the protein was confirmed by comparing with the protein marker (Lane 1 from left).

5.2.6 Purification of CD21 SCR 1-2

CR2 (SCR1-2) was purified via affinity chromatography on a C3dg affinity column prepared as described in the section 2.24.4. The solubilised SCR 1-2 was dialysed against 2L of buffer (10 mM sodium phosphate, 25 mM NaCl, 0.02% NaN₃, pH 7.2), for loading onto the C3dg column equilibrated in the same buffer. The column was loaded and 2 ml fractions were collected. When the A280 decreased to near baseline, the column was washed with (~60 ml) with the loading buffer made with 75 mM in NaCl (10 mM sodium phosphate, 75 mM NaCl pH 7.2). When the A280 was again at baseline, the NaCl concentration in the buffer was increased to 1.0 M (10 mM sodium phosphate, 1M NaCl pH 7.2) to elute the bound CR2(SCR1-2). Following purification the eluted fractions were run on SDS-PAGE to confirm the presence of purified protein (Figure 5.6).

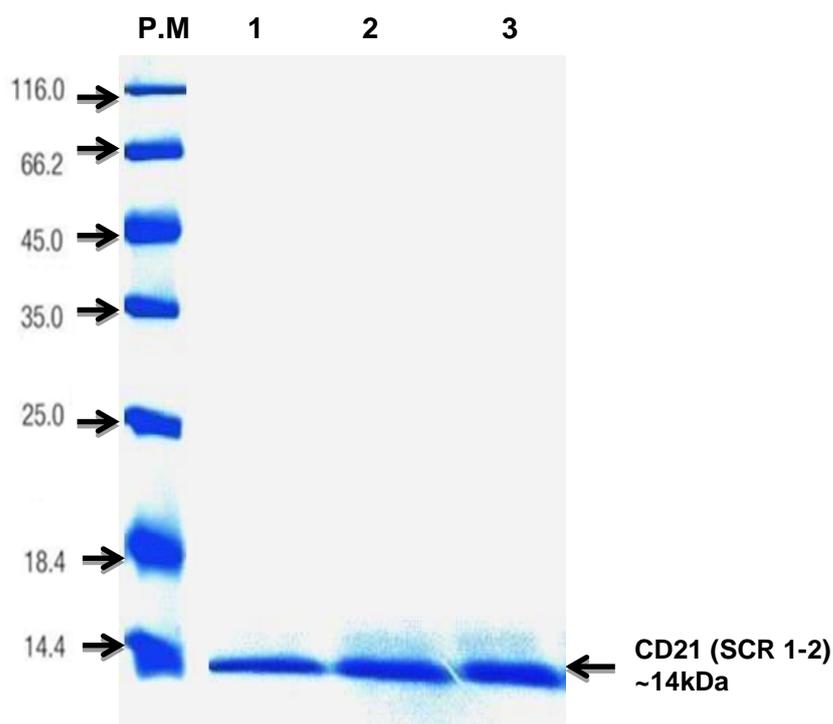


Figure 5.6: SDS-PAGE (15% w/v), analysis shows the presence of purified CD21 SCR 1-2 after purification by affinity chromatography.

Lanes 1-3 depict the CD21 SCR 1-2 fractions collected after elution from the C3dg affinity column. From the SDS-PAGE gel it was observed that the most of the proteins was eluted in the first three fractions.

5.2.7 Indirect ELISA to detect binding of rhSP-D with derCD23 and sCD23

The interaction of rhSP-D with to both derCD23 and sCD23 was examined via indirect ELISA as described in section 2.9, The results obtained as shown below in (Figure 5.7), show that 2.5µg/ml of rhSP-D was found to be the optimum concentration at which it bound best to 5µg/ml derCD23 and sCD23 coated wells.

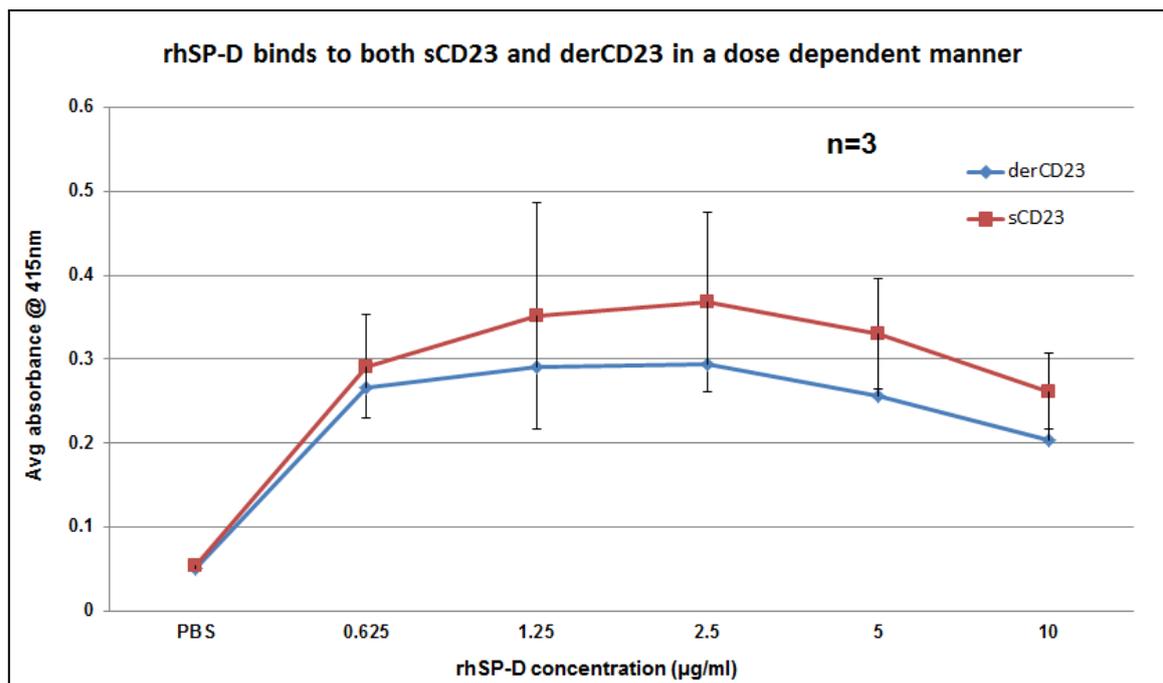


Figure 5.7: Binding of rhSP-D to derCD23 and sCD23

Binding of rhSP-D to derCD23 and sCD23 was detected by indirect ELISA. From the graph it can be seen that rhSP-D bound to both derCD23 and sCD23 in a dose dependent manner, (Negative control: PBS+2% BSA), Data are presented as mean ± SEM.

5.2.8 rhSP-D binds sCD23 in a calcium dependent manner

The interaction of rhSP-D with sCD23 was further examined under various conditions via indirect ELISA as described in section 2.9. Our results show that rhSP-D binds to sCD23 in calcium dependent manner in presence of 5mM Calcium. This interaction is inhibited by presence of EDTA (Figure 5.8).

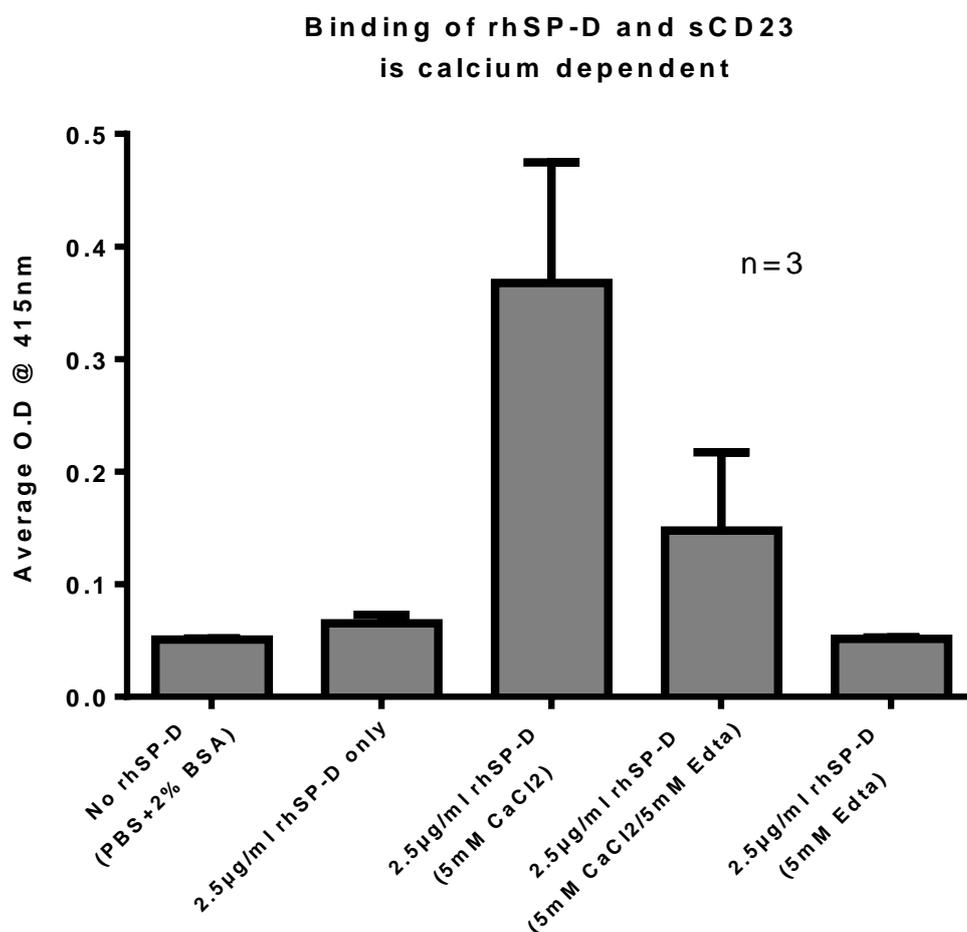


Figure 5.8: Binding of rhSP-D with sCD23 under various conditions

This binding of rhSP-D with sCD23 was found to be calcium dependent and was inhibited in the presence of 5mM EDTA, as shown by ELISA (n=3). Data are presented as mean \pm SEM.

5.2.9 rhSP-D binds derCD23 in a calcium dependent manner

The interaction of rhSP-D with derCD23 was further examined under various conditions via indirect ELISA as described in section 2.9. Our results show that rhSP-D binds to derCD23 in dose dependent manner in presence of 5mM Calcium. This interaction is inhibited by presence of EDTA (Figure 5.9).

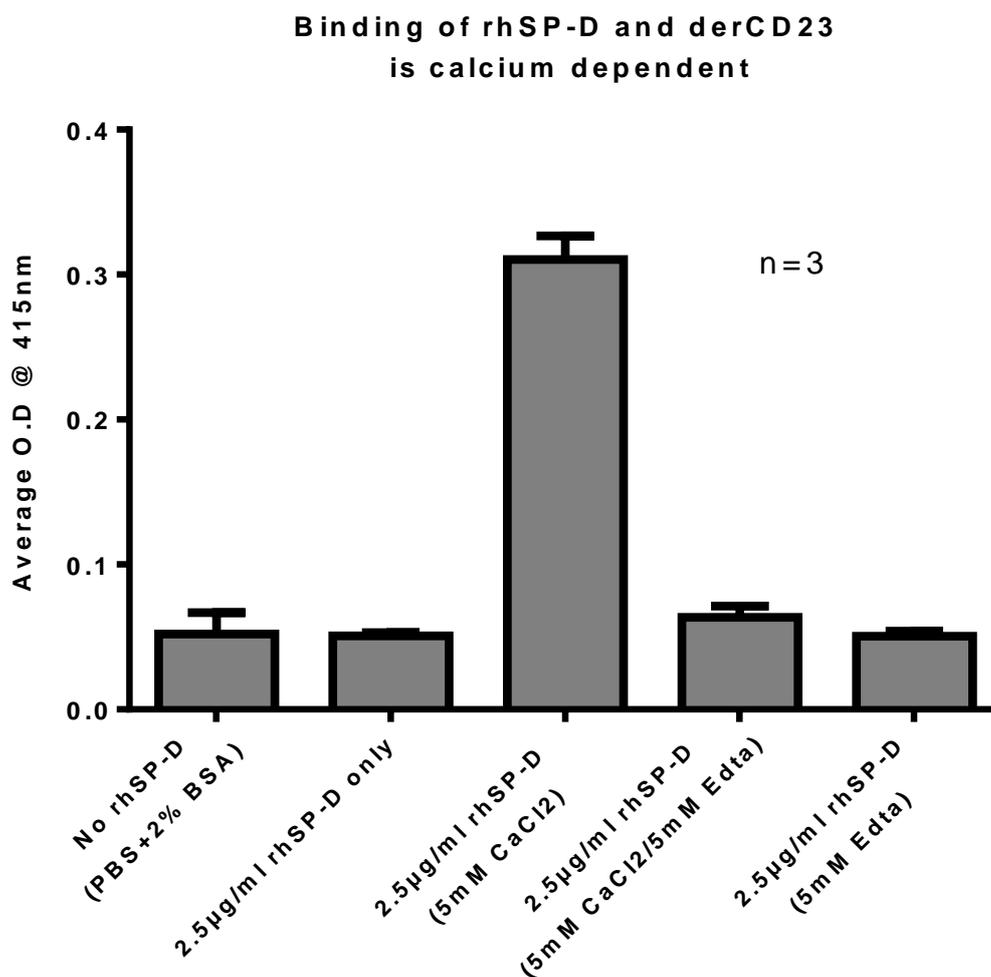


Figure 5.9: Binding of rhSP-D with derCD23 under various conditions

This binding of rhSP-D with derCD23 was found to be calcium dependent and was inhibited in the presence of 5mM EDTA, as shown by ELISA (n=3). Data are presented as mean \pm SEM.

5.2.10 Indirect ELISA to detect binding of rhSP-D with CD21 SCR 1-2

The interaction of rhSP-D both CD21 SCR 1-2 was examined via indirect ELISA as described in (section 2.9), The results obtained as shown below in (Figure 5.10) show that 2.5µg/ml of rhSP-D was found to be the optimum concentration at which it bound best to 5µg/ml CD21 SCR 1-2 coated wells.

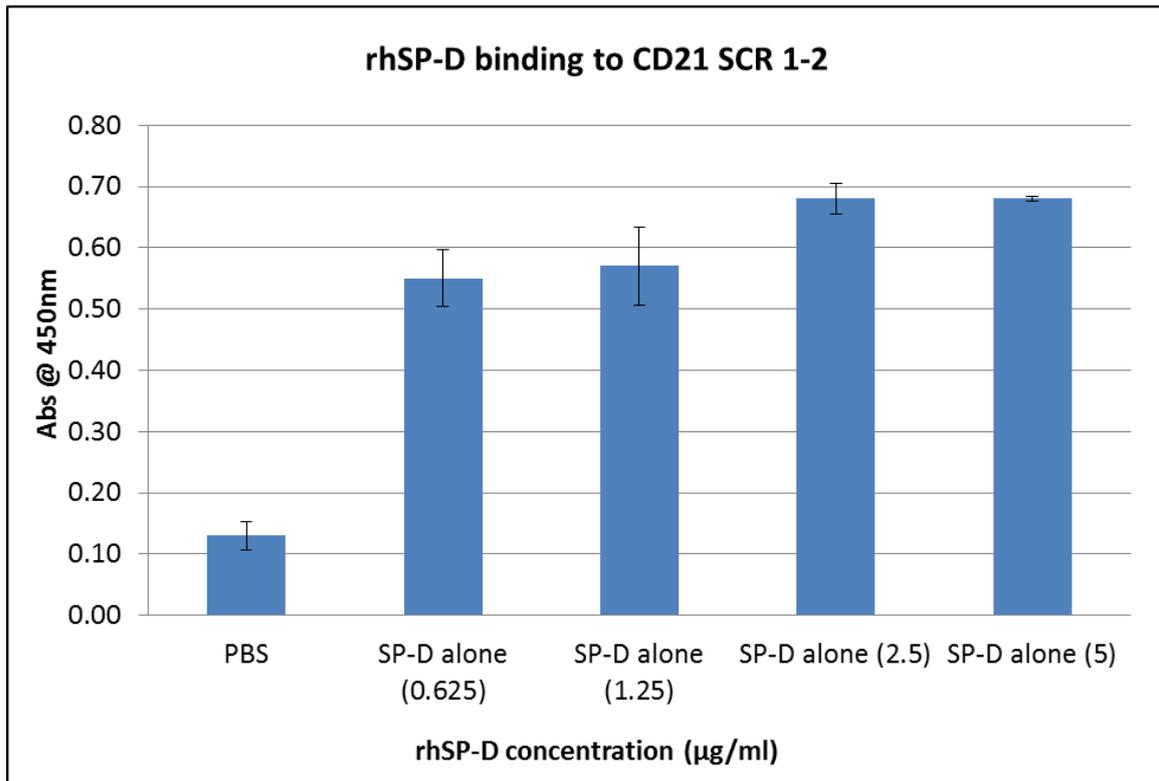


Figure 5.10: Binding of rhSP-D to CD21 SCR 1-2

Binding of rhSP-D to CD21 SCR 1-2 was detected by indirect ELISA (n=3). From the graph it can be seen that 2.5µg/ml rhSP-D bound to CD21 SCR 1-2 in a dose dependent manner. Data are presented as mean ± SEM.

5.2.11 rhSP-D binds CD21 SCR 1-2 in a calcium dependent manner

The interaction of rhSP-D with CD21 SCR 1-2 was further examined under various conditions via indirect ELISA as described in section 2.9. Our results show that rhSP-D binds to CD21 SCR 1-2 in a calcium dependent manner in presence of 5mM Calcium. This interaction is inhibited by presence of EDTA (Figure 5.11).

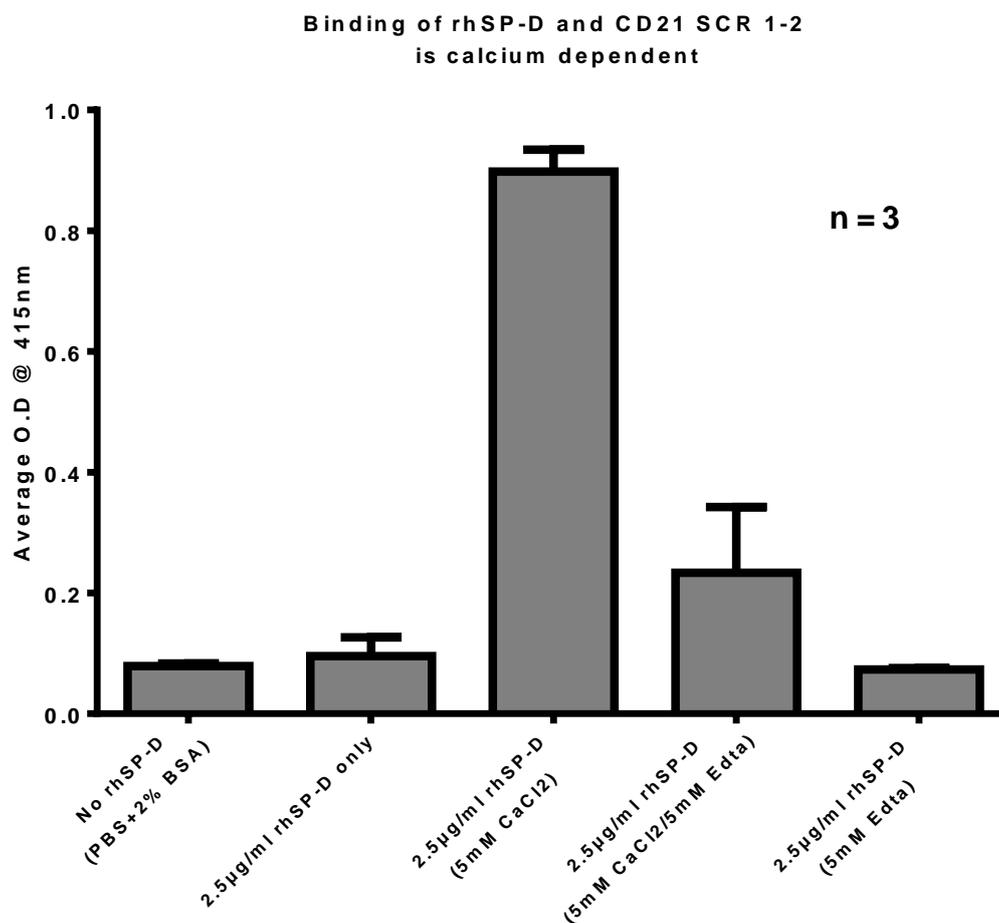


Figure 5.11: Binding of rhSP-D with CD21 SCR 1-2 under various conditions

This binding of rhSP-D with CD21 SCR 1-2 was found to be calcium dependent and was inhibited in the presence of 5mM EDTA, as shown by ELISA (n=3). Data are presented as mean ± SEM.

5.2.12 The effect of rhSP-D and CD23-CD21 interactions on IgE secretion

In this experiment recombinant human surfactant protein D (rhSP-D) was incubated with soluble CD23 fragments DerCD23 (Figure 5.12a) and sCD23 (Figure 5.12b) along with CD21 SCR 1-2 as described in section 2.26a according to a similar experiment undertaken as shown by (Bowles et al., 2011). PBMCs used in our experiment were obtained from four atopic patients, which were highly sensitised to Phlp allergen, in order to investigate the effect on IgE synthesis. The results obtained did not appear to show any conclusive effect of rhSP-D on IgE production in presence of both derCD23 and sCD23.

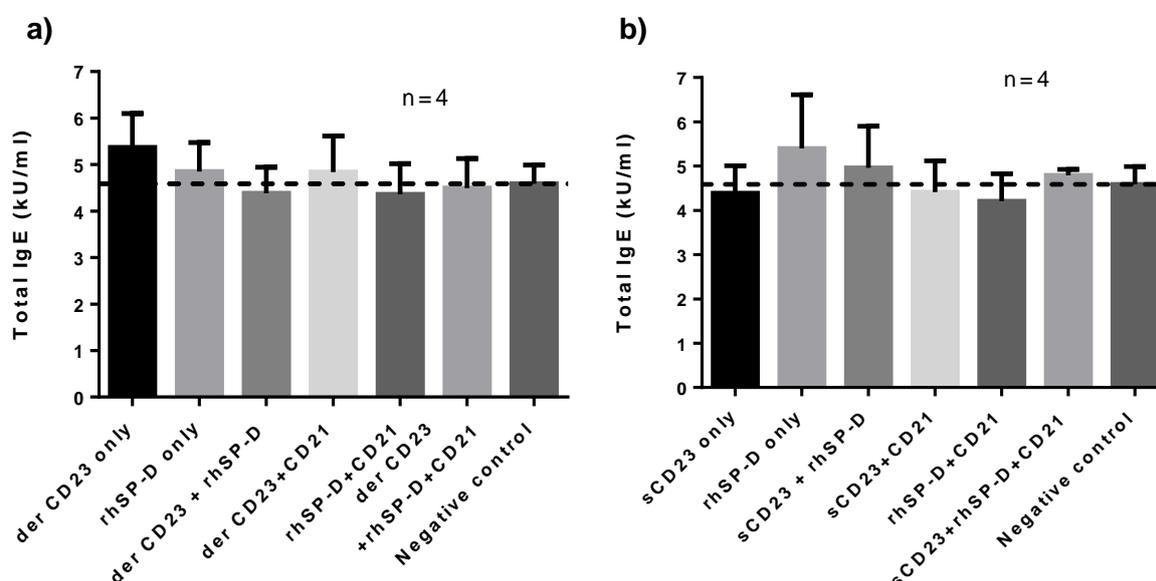


Figure 5.12: IgE production by rhSP-D in presence of derCD23 and sCD23

IgE secretion by PBMCs obtained from 4 atopic patients ($n = 4$) highly sensitised to Phlp allergen were treated with $5\mu\text{g/ml}$ rhSP-D in the presence or absence of derCD23 (Figure 5.12a), sCD23 (Figure 5.12b) and CD21 SCR 1-2. Data are presented as mean \pm SEM. PBMCs were cultured for 12 days with switch factors (IL-4 at 20 ng/mL and anti-CD40 at $5\text{ }\mu\text{g/mL}$) in the presence of either derCD23 or sCD23 (at 25 ng/mL) and also in both the presence and absence of CD21 SCR 1-2 (at $1\text{ }\mu\text{g/mL}$). Total IgE was measured in the cell supernatants ImmunoCAP® Total IgE Fluoroenzyme immunoassay. No significant effect on IgE production by rhSP-D in the presence of either derCD23 or sCD23 was observed. (N.B-Negative control contained no switch factors or proteins).

5.2.13 Effect of SP-D on IgE secretion

The immunomodulatory effects of SP-D on IgE synthesis in PBMCs obtained from allergic individuals when *in vitro* stimulated with grass pollen allergen in the presence of CD40L, IL-4 and IL-21 was determined as described in section 2.26b. CD40L and IL-4 induced IgE production and IL-21 further enhanced IgE synthesis from B cells in PBMCs cultures (60.97%; $p=0.3104$). SP-D inhibited CD40/IL-4 and IL-21 induced total IgE production (77.12%; $p=0.0195$). (Figure 5.13)

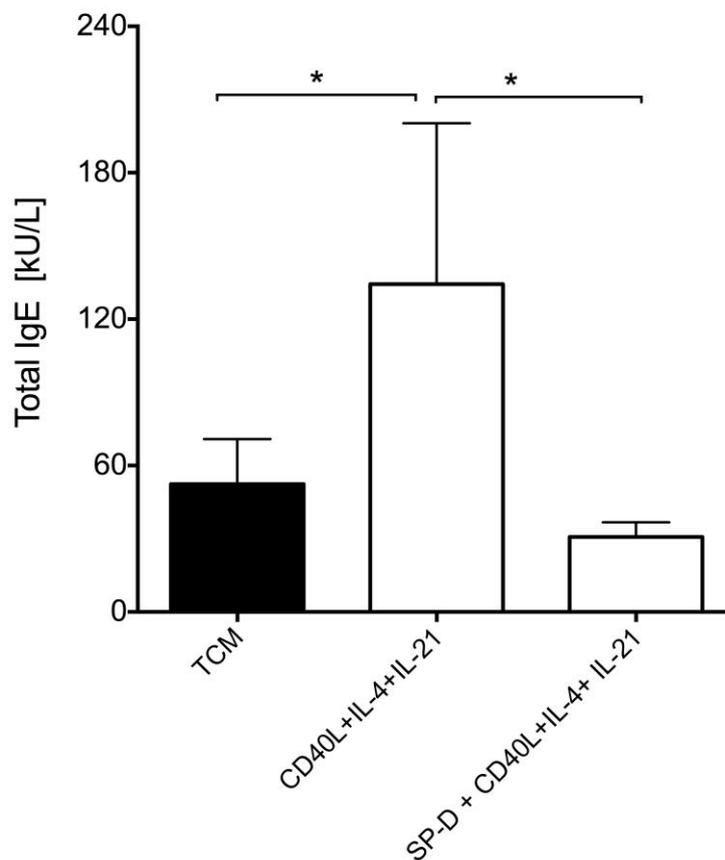


Figure 5.13. SP-D inhibits IgE production from B cells in PBMCs culture. PBMCs obtained from allergic individuals ($n = 10$) were stimulated with grass pollen allergen in the presence of IL-4, CD40L and IL-21 for 14 days. Total IgE production from B cells was measured in the cell culture supernatants by ImmunoCAP assay. All data are shown as mean (\pm SEM). p values were determined by Wilcoxon sign-rank test where * represent $p<0.05$.

5.3 Discussion

CD23 has been implicated in playing a regulatory role in IgE production and thus controlling the pathogenesis of allergic disease. Elevated CD23 expression of both membrane and soluble forms in allergic individuals has been established and appears to be dependent on IL-4 (McCloskey et al., 2007). Previous literature also indicates that CD23 can either positively or negatively regulate IgE production (Bonney et al., 1995, Delespesse et al., 1992, Gould et al., 2003), which is dependent on its oligomeric state (McCloskey et al., 2007) as well as the size of the CD23 fragment depending on the cleavage site by endogenous proteases (Aubry et al., 1992, Sarfati et al., 1992).

In the previous chapter, we established a link between rhSP-D and CD23 in modulating allergic response. Therefore, in this chapter we verified this relationship between SP-D and CD23 by firstly examining the interaction between them via indirect ELISA, where we found that rhSP-D binds to both derCD23 and sCD23 fragments in a dose and calcium dependent manner. The binding of rhSP-D to CD21 SCR 1-2 was also examined, as this along with CD21 SCR 5-8 is the site for its interaction with CD23. We found that rhSP-D as well as binding to CD23 also bound to CD21 SCR 1-2 in a calcium dependent manner. This is the first study to show the interaction of SP-D with both CD23 and CD21, which is dependent on interaction with CRD region. We thus propose a model as shown below in (Figure 5c) that SP-D may exert its anti-allergic effect by interfering with the CD21 and CD23 pairing by competing with CD23 for binding with membrane CD21 and membrane IgE, as this pairing with CD23 has been shown to be important in controlling IgE levels in vivo (Hibbert et al., 2005).

The data obtained from the initial IgE secretion assay, section 5.2.12, was analysed but no clear role of SP-D emerged in IgE production, this may be due to variations in the immune

responses between different individuals, it could also be due to using frozen PBMCs in this experiment, where there may be difference in the viability and functionality of these cells, which may vary over the two week culture period amongst different individuals, although, the cell viability was checked, at the time of setting up the cultures.

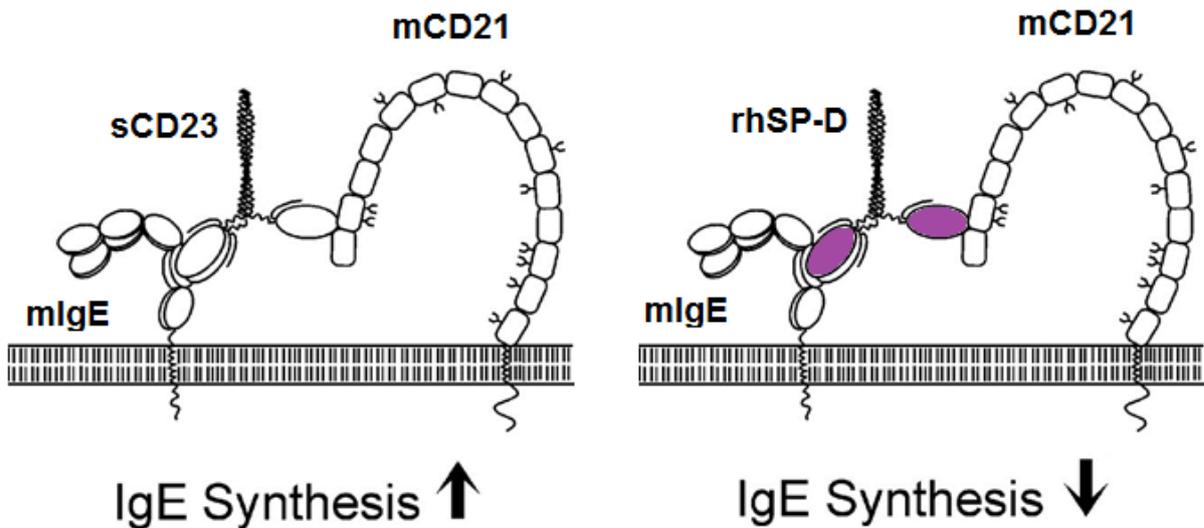


Figure 5c: Proposed model for SP-D mediated reduction in CD21-CD23 dependent IgE synthesis

It is previously known that cross-linking of membrane IgE and CD21 by soluble fragments of CD23 can lead to increase in IgE synthesis. We thus propose a novel mechanism, whereby SP-D may be able to play a role in IgE synthesis by competing with soluble CD23 for binding to both CD21 and membrane IgE on the B cell surface, since as SP-D shares a similar structural homology with CD23, due to both belonging to C-type lectin family and having the ability of SP-D to bind to both IgE and CD21. This interference of SP-D with CD21-CD23 interaction may lead to a reduction in IgE synthesis by B-cells.

A further reason for also not observing any significant effect by rhSP-D on IgE production could be due to using human PBMCs from atopic patients, which contain relatively low number of antigen specific memory B-cells, as compared to B-cells obtained from other lymphoid organs such as from tonsils, which are a rich source of memory B-cells and would thus have been more appropriate for our studies. This is further supported by a previous study by (McCloskey et al., 2007), where they showed the ability of soluble CD23 monomers to stimulate IgE production by B-cells isolated from human tonsils. However, due to the unavailability of B-cells from human tonsils, we examined IgE synthesis by rhSP-D in presence or absence of derCD23, sCD23 and CD21 SCR 1-2, using a simplified method described by (Bowles et al., 2011), where they cultured PBMCs obtained from atopic patients for 14 days in the presence of B-cell switch factors (IL-4 and CD40) along with 25 ng/mL soluble CD23, however we made a slight modification to this assay, where we reduced the culturing time period from 14 days to 12 days, due to time limitations.

The IgE secretion assay was further repeated under slightly modified conditions as described in section 2.25b, where we observed a clear suppressive effect of rhSP-D on IgE synthesis for the first time, section 5.2.13. This was shown by co-incubating the PBMCs isolated from well-characterised atopic individuals with rhSP-D for 14 days in the presence of B-cell switch factors IL-4, CD40L along with IL-21. IL-21 was used in this assay since it has been previously shown to enhance IL-4 mediated IgE production by isolated human B cells (Wood et al., 2004).

In summary, to our understanding this is the first study to establish interactions between rhSP-D, trimeric soluble CD23 fragments (derCD23, sCD21) as well as CD21 SCR 1-2. It was also found that these interactions of rhSP-D with CD23 and CD21 were calcium dependent. Furthermore, a novel function of rhSP-D in interfering with IgE secretion by PBMCs is being reported for the first time, where we observed a clear suppressive effect of SP-D on IgE synthesis. This data lends further support to our hypothesis that SP-D can modulate allergic inflammation by its ability to suppress IgE biosynthesis and thus provides a new direction for pursuing future line of investigations, in order to further evaluate the role played by rhSP-D in CD21-CD23 mediated IgE production.

Chapter 6



Conclusion and future directions

Pulmonary surfactant protein SP-D, in addition to performing surfactant functions, is also known to directly interact with pathogens and allergens, stimulate immune cells and manipulate cytokine and chemokine profiles during host's immune response. The pulmonary levels of SP-D have been shown to be altered in a range of pulmonary diseases including asthma, emphysema, pulmonary hypersensitivity, acute lung injury, chronic obstructive pulmonary disease, and respiratory distress syndromes. (Madan et al., 2001, Kishore et al., 2006).

Moreover, murine models of asthma have shown that when treated with recombinant form of human SP-D (rhSP-D), which is composed of eight Gly-X-Y collagen repeat sequences, homotrimeric neck and lectin domains but lacking the N-terminal region, retains the immunomodulatory properties displayed by full-length SP-D, and has the ability to provide a long term therapeutic effect by dampening asthmatic symptoms in mice, by suppressing IgE levels, eosinophilia, and pulmonary cellular infiltration and cause a T-cell polarisation from a pathogenic Th2 subset to a protective Th1 response (Madan et al., 2001, Strong et al., 2002b, Madan et al., 2005b). Hence, there was a need to further understand the underlying mechanism/s involved in providing these therapeutic effects by SP-D and how it is involved in manipulating the T-helper cell polarisation from pathogenic Th2 response to a protective Th1 response *in vivo*.

The first aim of this study was to purify and characterise recombinant human surfactant protein D (rhSP-D) in sufficient quantity, in order to undertake the purposed experiments. A truncated version of rhSP-D was used for this study since, as it has been previously demonstrated that rhSP-D retains the immunomodulatory properties displayed by the full-length SP-D. Furthermore, rhSP-D is also easier to purify in sufficient quantity compared to full length SP-D.

Following successful purification and characterisation of rhSP-D, The next aim of this study was to examine the immunomodulatory properties of this truncated fragment of SP-D (rhSP-D) on monocytic cell line (THP-1) and human monocytes in inflammation by examining the production of pro-inflammatory and anti-inflammatory cytokine genes in presence or absence of LPS. The results obtained from this part of the study appear to show that rhSP-D can enhance the production of pro-inflammatory cytokine genes (IL-1 β and TNF- α) as well as of anti-inflammatory cytokine gene (IL-10) in presence or absence of LPS, whereas it appeared to reduce the production of TGF- β , which is an anti-inflammatory cytokine. These results thus appear to be very interesting as they show the ability of rhSP-D to enhance the production of pro-inflammatory cytokine genes, where this pro-inflammatory effect of rhSP-D appears to be mainly localised to the CRD region. However, it may be possible that rhSP-D may prevent the onset of LPS mediated pro-inflammatory response, by production of anti-inflammatory cytokine genes such as IL-10 as we also observed.

Our next major aim was to explore a potential mechanism by which rhSP-D provides protection in allergic asthma. We thus hypothesised that a possible mechanism of SP-D mediated protection could be due to its ability to interfere with facilitated antigen presentation. We thus employed an IgE facilitated allergen binding assay (IgE FAB), which is a flow cytometric based assay that has previously validated by (Shamji et al., 2006a) to examine this aspect. This assay was used since as it represents an *in-vitro* model for facilitated antigen presentation, however it does not directly examine the antigen presenting capacity of B-cells to T-cells, but previous studies have shown this assay to be representative of this process (Wachholz et al., 2003a). This assay demonstrated for first time the ability of rhSP-D to interfere with facilitated antigen presentation by showing a

significant inhibition of allergen-IgE complexes binding to CD23 present on the B-cell surface.

Furthermore, the effect of rhSP-D on antigen presentation and proliferation to CD4⁺ T cells was also examined via ³H-Thymidine incorporation assay, as it has been previously reported that inhibition of allergen-IgE complexes to B cells can correlate with reduction in T-cell proliferative responses. Thus, the proliferative responses of Phlp stimulated PBMCs obtained from twelve well characterised patients highly sensitised to Phlp allergen, were examined in the presence or in the absence of rhSP-D, where we observed a significant reduction in T-cell proliferation in presence of rhSP-D. Finally we characterised these proliferating cells via multiplex assay and found the ability of rhSP-D to significantly inhibit allergen driven Th2 cytokine production.

This study is therefore the first to demonstrate the ability of SP-D to interfere with the co-operative binding of allergen-IgE complexes to CD23 on the surface of B cells, and thus also the first study to show down regulation of CD23 expression on B cells. Thus, inhibition of IgE-facilitated antigen presentation may represent a novel mechanism whereby SP-D suppresses Th2 T lymphocyte-driven allergic inflammation.

Having established a novel link between rhSP-D and CD23 expression on B-cells, we thus finally hypothesised that SP-D may be able to regulate IgE synthesis by interfering with CD23-CD21 interaction by competing with soluble CD23 fragments in binding to CD21, due to sharing structural homology with CD23, and thus preventing the interaction of soluble trimeric CD23 with CD21, which is necessary for up regulating IgE synthesis. In order to examine this aspect, the interactions between rhSP-D, CD21 SCR 1-2 (14kDa) as well as with the two recombinant soluble CD23 fragments,(yielded by the dust mite protease Der pI (derCD23-16kDa), and by ADAM10-mediated proteolysis (sCD23-

25kDa) were established via solid phase binding assays, in addition the effect of rhSP-D on IgE synthesis was also examined by co-incubating rhSP-D with soluble CD23 fragments as well as CD21 SCR 1-2, using PBMCs obtained from grass allergics. This study is the first to establish interactions between rhSP-D, trimeric soluble CD23 fragments (derCD23, sCD21) as well as CD21 SCR 1-2, where the binding of rhSP-D to these proteins was found to be calcium dependent. Moreover, we also examined the production of IgE secretion by PBMCs but did not observe any significant changes in IgE production by rhSP-D.

It is also worth noting that these studies were carried out by using a truncated fragment of human SP-D only, and thus in order to further validate, whether if the protective effects of SP-D are mainly localised to the CRD region, it will be worth repeating these experiments using full length SP-D in order to check for the effect of the multivalency of SP-D molecule. However it does appear through this study that the protective effects of SP-D are mainly localised to the CRD region.

In conclusion, the exciting data generated through this study provides further evidence of the therapeutic potential of rhSP-D to act as a novel immunomodulator in allergic asthma, and thus warrants a clinical trial to further test the efficacy of this truncated recombinant form of human SP-D.

Plans for future research:

1) Looking at the mechanisms by which rhSP-D modulates immunologic response to pulmonary allergens

Future avenues of research entail investigating the interaction between rhSP-D and DC, as well as looking at how it prevents the activation of the existing Th2 response but

facilitates the secretion of IL-12 and the induction of Th1 response in vitro and in vivo. These lines of investigation would help us understand a possible link between DC and rhSP-D-mediated polarization of Th response. Shifting of cellular responses from a predominantly Th2 to a Th1 cytokine profile, following treatment with SP-D and rhSP-D, appears central to the protective mechanism since IFN- γ , a Th1 cytokine, promotes cellular immunity and normally inhibits Th2 differentiation in response to IL-4. Furthermore, the role of SP-D in context of Th-17 cells has also not been looked at to date, which therefore makes it worthwhile to look at.

2) Examining the interaction of rhSP-D with Mast Cell Line

Since mast cells play a role in early allergic response, the interactions between rhSP-D and mast cells need to be further examined in order to assess whether rhSP-D can interfere with the degranulation of IgE coated mast cells after challenge with the appropriate antigen, since as degranulation of IgE coated mast cells can lead to exacerbation of asthma due to release of histamine which is responsible for bronchial constriction. The interactions between rhSP-D and LAD-2 mast cell line were thus briefly examined but not reported here. Other possibilities include examining whether rhSP-D can desensitise the mast cells, as well looking at the leukotriene pathways, C4, D4, and E4 to see whether binding of rhSP-D can inhibit these pathways, as these pathways are involved in early and late phase development of asthma. The role of rhSP-D in inhibiting COX-1 and COX-2 also needs to be looked at.

3) Examining the role of DC in priming specific Th1 and Th2 T cell responses in the presence of rhSP-D via Microarray analysis

The gene response in DC and T cells challenged with rhSP-D also needs to be investigated. This approach would entail identifying the genes, that are involved in

immunomodulation. Cultured human cells will be investigated using microarrays, to analyse the transcriptome of cells challenged with rhSP-D. A transcriptome profile for each of these interactions will be obtained, and by comparing gene expression profiles, the specific genes/pathways involved.

The aims are:

- (1) To determine the gene expression of cultured cells, when challenged with rhSP-D, in order to investigate which genes/pathways are involved in activation;
- (2) To determine how the cellular transcriptome response is altered in the presence of the full length and truncated SP-D.

Total RNA will be extracted from samples of SP-D treated and untreated cells and will be used for synthesis of cDNA by reverse transcription. Following the microarray analysis, Statistical analysis will also be carried out (Wilcoxon rank test) to compare expression intensities between the experimental groups.

Chapter 7



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