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Foreword

Bovine conglutinin was the first collectin protein to be identified in 1909 by Bordet and Gay, characterized by its property of agglutinating erythrocytes that had reacted with antibody and complement, which is now known to be due to the binding of the conglutinin to the carbohydrate site on iC3b, coated on the surface of the activator. However, it was not until the late 1980s, after conglutinin's molecular structure was fully established, that it became apparent that there is a family of proteins, which have C-type lectin-binding heads connected to triple helical collagen-like tails, which include mannose-binding lectin (MBL) and the lung surfactant proteins SP-A and SP-D, as well as conglutinin and another bovine collectin, CL-46.

The overall structural similarity between these collectins, and later the ficolins (which have fibrinogen-like globular heads connected to collagen-like tails), and the complement component C1q, did suggest that, like C1q, the collectins and ficolins, might be involved in the recognition and elimination of pathogenic microorganisms. This indeed turned out to be true, for MBL and the ficolins, which specifically bind, by their lectin-binding domains, to arrays of carbohydrate structures on the surfaces of pathogens, leading to the activation of the serum complement system, via their MBL-associated serine proteases (MASPs), in an analogous fashion to that seen by the interaction of C1q to immune aggregates, followed by the activation of its associated $C1r_2C1s_2$ protease complex, and consequently the complement cascade. However, it became very clear that the lung surfactant proteins, SP-A and SP-D, do not associate with proteases such as the MASPs, or the $C1r_2C1s_2$ complex, to activate the complement system, but mediate their innate immune properties by triggering receptors after binding their targets, and, as highlighted in this volume, appear to play important immune surveillance roles within quite a range of tissues besides the lungs.

The volume thus covers the recent advances with respect to the structures and functions of MBL, ficolins and MASPs, SP-A and SP-D, and neatly ends with a survey of bovine collectins, featuring conglutinin, the very first collectin to be identified, in which it is suggested that the protein may enhance the clearance of mycobacterial infection by *M. bovis* and *M. tuberculosis*.

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Preface

Collectins (collagen containing C-type lectins) are soluble molecular pattern recognition proteins endowed with the ability to discriminate between self and non-self. They are also capable of recognizing altered self including apoptotic and necrotic cells as well as tumor cells as a part of their immune surveillance activities. Since the discovery of mannan-binding lectin (MBL) as the recognition subcomponent of the complement lectin pathway, and that of surfactant protein A (SP-A) and D (SP-D) as molecules involved in surfactant homeostasis and pulmonary defense, the knowledge about the collectin family members has come a long way in the last two decades. This book is a tribute to all those brilliant researchers in the field who kept on discovering new knowledge on collectins with their imaginative ideas. Collectins are now recognized as potent mucosal immune players. They influence host–pathogen interaction in a complement-dependent and independent manner, regulate inflammation, dendritic cell maturation, T and B cell functions, in allergy, pregnancy, autoimmunity, and cancer. Thus, there could not have been a more pertinent time to publish a book exclusively on the collectins.

This book begins with a historical account of seminal discoveries on the lectin pathway (Mac Turner), followed by current state-of-the-art information on MBL structure-function and its importance in physiology and disease mechanisms (Doulami et al.). Next two chapters have focused on rather enigmatic MBL-associated serine proteases (MASPs) that continue to ignite debate and controversy about their association with MBL and independent roles in complement activation. Their involvement in processes other than the lectin pathway via selective targeting is vividly addressed by Gal and Dobo. The crystallographic structural details of MASPs and their parallel with C1r and C1s of the classical pathway are presented by Gaboriaud et al.

Collectins not only recognize both extracellular and intracellular pathogens, but they also activate the immune cells to effectively eliminate pathogens. Yasmin and Kishore provide a detailed account of various biological mechanisms underlying the broad spectrum anti-pathogenic activities of SP-A and SP-D. The next chapter describes the important role SP-D plays in protective immune response against allergen challenge (Singh et al.). SP-D (and a recombinant fragment of human SP-D

containing neck and trimeric lectin domains) can bind a range of allergens, inhibit IgE binding, dampen histamine release by activated basophils, and cause Th2 to Th1 polarization. Most crucially, a link between SP-D and B cell modulation has emerged that involves suppression of IgE synthesis by primed B cells ex vivo. An anti-cancer role of SP-D was discovered by serendipity, as a follow-up of the role of SP-D in pulmonary hypersensitivity. Activated or sensitized eosinophils seem convenient targets for SP-D-induced apoptosis. When these experiments were replicated using an eosinophilic-leukemic cell line, a link between SP-D and p53 pathway was unraveled. Subsequent studies have revealed that SP-D differentially binds to cancer cells, initiating signaling leading to their apoptotic death, as evident in leukemia, lung cancer, ovarian cancer, pancreatic cancer, and prostate cancer. Thakur et al. thus provide an insight into SP-D-mediated simultaneous targeting of multiple cellular signaling pathways including transcription factors, tumor cell survival factors, and protein kinases resulting in the efficient and selective killing of cancer cells.

Expression of collectins by mucosal epithelial cells at extra-pulmonary sites including male and female reproductive tracts has stimulated studies examining their role in pregnancy and testicular immune privilege. Testicular immune privilege is a recently discovered domain of collectins. Their expression is positively regulated by testosterone and LPS-induced inflammation. Salvaging of the LPS-impaired testicular barrier by SP-D highlights the contribution of collectins to male fertility (Rokade et al.). The chapter by Kale et al. on collectin-mediated regulation of feto-maternal cross talk discusses their role in the induction of parturition, implantation, and early pregnancy. Importantly, serum levels of collectins are dysregulated in pregnant women early on, weeks before they undergo spontaneous abortion and preeclampsia implicating them as predictive biomarkers of placental function.

Similar to their human counterparts, the bovine collectin family includes SP-A, SP-D, MBL-A, and MBL-C, conglutinin (CGN), collectin 43 (CL-43), and collectin 46 (CL-46): they have an integral role in bovine immunity and disease (Tsolaki et al.). This chapter also focuses on their genetic polymorphisms and predisposition of the bovine host to various infectious diseases.

We sincerely hope that this book provides an insight into the diverse and important functions collectins perform. We are grateful to all the contributing authors who have taken time out of their busy schedule to write such well-crafted chapters. We would also like to thank Alison Ball and Sofia Valsendur (Springer Nature) for their enormous patience while we were editing the book during the COVID-19 lockdown period.

Uxbridge, UK — Uday Kishore
Mumbai, India — Taruna Madan
Oxford, UK — Robert B. Sim

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About the Editors

Uday Kishore is a teacher and a scientist with special interest in innate immunity. He was the Founder Director of the Centre for Infection, Immunity and Disease Mechanisms, Brunel University London. He earned his BSc from S. P. Jain College, Sasaram, Bihar; MSc from Hindu College, Delhi; and PhD from the Department of Zoology, University of Delhi and CSIR Institute of Genomics and Integrative Biology, Delhi, India. After spending a year at the Salk Institute for Biological Studies, La Jolla, California, as a NASA Fellow, he moved to the University of Oxford for the majority part of his post-doctoral training, first at the MRC Immunochemistry Unit, Department of Biochemistry and then at the Weatherall Institute of Molecular Medicine, John Radcliffe Hospital. His previous positions include NASA fellow, Wellcome Trust International Fellow, and Alexander Humboldt fellow. He is also the recipient of MRC Investigator Prize, European Commission Young Scientist Prize, and Mother Teresa Excellence Award. Dr. Kishore has several adjunct and professorial positions internationally. He has authored nearly 200 research manuscripts, several book chapters, patents, and books. His research aims to understand how the innate immune components deal with self, non-self, and altered self. His team is examining the roles of complement proteins and C-type lectins (including collectins) in host–pathogen interaction, cancer, allergy, and pregnancy.

Taruna Madan has immensely contributed to the biomedical research on human collectins and deciphered their relevance in host defense, cancer surveillance, and reproductive health. She established the therapeutic potential of collectins, viz. SP-A, SP-D, and MBL against the fungal pathogen, *Aspergillus fumigatus*. Discovery of a direct anti-cancer role of SP-D by her team in leukemic and breast cancer cells via p53 and HMGA1 was subsequently validated by other groups using pancreatic, prostate, and ovarian tumor cells. With an aim to develop a vaginal microbicide for HIV-1, she established the potent and broad spectrum anti-HIV-1 activity of SP-D and elucidated SP-D-mediated reversal of HIV-1-induced pro-inflammatory gene signature. Taking cues from the significance of immunoregulation in pregnancy and male fertility, she demonstrated critical roles of collectins in the embryo implantation, placental development, preeclampsia, and spermatogenesis. Clinical studies by her group in HIV-1 sero-discordants, pre-eclamptic women, and infertile men have endorsed the translational potential of SP-D. She has authored over 100 research publications and 9 granted patents (Google scholar citations 3600, h-index 27, and i10 index 54). Dr. Madan has several national and international collaborations, including with Brunel University London, MRC Immunochemistry Unit, Oxford, Wythenshawe Hospital, Manchester, Brigham, and Women's Hospital, Boston, USA, and Pasteur Institut, Paris, France.

Robert B. Sim has now retired from posts at the Medical Research Council (UK) (MRC Immunochemistry Unit, Department of Biochemistry, Oxford University), Professor of immunology at Kingston University, UK, senior researcher at Department of Pharmacology, Oxford University, and Honorary Professor at the University of Leicester, Department of Infection, Immunity and Inflammation. He studied Biochemistry (BSc) at the University of Edinburgh and received his D. Phil (PhD) in Immunochemistry from the University of Oxford, MRC Immunochemistry Unit, Department of Biochemistry. He was an MRC/INSERM Research Fellow at Centre d'Etudes Nucleaires in Grenoble, and then returned to Oxford as a group leader in the MRC Immunochemistry Unit. His research interests are focused on the structure and functions of complement and other innate immune system proteins, and their interactions with microorganisms, altered host cells and macromolecules, and with synthetic materials. With more than 500 publications, Prof. Sim is considered a pioneer figure in the field of complement and collectin research.

Discovering the Role of Mannose-Binding Lectin (MBL) in Innate Immunity: The Early History

Malcolm Turner

Introduction

The so-called lectin pathway of complement activation was the third such molecular sequence to be described which results in enzymatic cleavage of the C3 component of complement. However its discovery was a gradual process arising from observations in different areas of the biological sciences. Three such strands of research predominated and these were pursued essentially independently of each other with little or no "cross talk" until 1989 when links began to be established. These areas of study were (1) Biochemical studies (especially in the field of liver biochemistry); (2) Identification of an immunodeficiency disorder and (3) Microbiology research.

The study of a relatively common immunodeficiency disorder will be used here to illustrate the clinical relevance of one of the initiating recognition molecules of the lectin pathway, namely mannose-binding lectin (MBL).

Early Studies on Defective Opsonisation

The most significant contributor to the early history of this particular narrative was John Soothill (1925–2004). His early research life was spent in John Squire's Department of Experimental Pathology in the University of Birmingham where one of his responsibilities was the co-ordination of the MRC trial of replacement IgG in patients with Bruton's agammaglobulinaemia. This background clearly facilitated his appointment in 1968 as the first Professor of Immunology at Great Ormond Street Hospital and the associated Institute of Child Health (Fig. 1).

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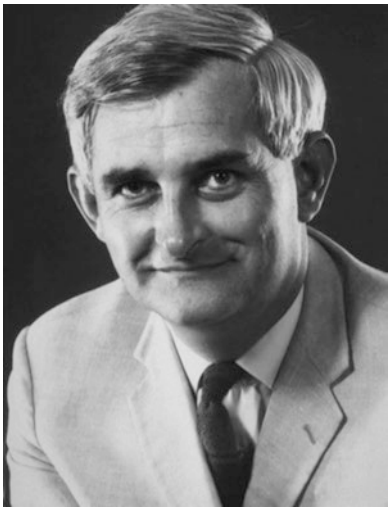

Fig. 1 John Soothill, Professor of Immunology at the Institute of Child Health, London from 1968 until 1985, pioneered studies on defective opsonization in childhood

After moving to London Soothill set about building a department with a major focus on immunodeficiency disorders involving both the cellular and humoral arms of the immune system. The author joined the team after a period as a postdoctoral research fellow in the Biochemistry Institute of the University of Uppsala in Sweden. In the decades after the 1930s the Biochemistry Institute had acquired a world leading reputation in the study of plasma proteins. One important result of this training experience which was to prove critical was the acquisition of a large −70 °C freezer for the storage of serum in the Institute of Child Health at a time when many hospital laboratories still relied heavily on −20 °C storage.

Always receptive to reports of potentially interesting patients Soothill was quick to follow up a paper in The Lancet describing a girl with frequent infections and a phagocytic defect (Miller et al. 1968). Accordingly, he began a systematic search for similar patients and over time collected some 40 serum samples, duly stored at −70 °C, from patients with frequent unexplained infections. It is also worth noting that because Great Ormond Street Hospital is a tertiary referral centre it inevitably concentrates subsets of patients with the most severe symptoms.

Soothill, ably assisted by his technician Betty Harvey, then tested these stored samples using the assay described by Miller and colleagues. This involved incubating heat killed baker's yeast with diluted serum and polymorphonuclear cells. The uptake of yeast particles was evaluated microscopically after appropriate staining and some 25% of the patients were found to have defective uptake (Soothill and Harvey 1976). Furthermore, it could be shown that this defect was due to poor opsonization by the serum rather than a defect of phagocytic function, precisely as demonstrated initially by Miller and colleagues.

As shown in Fig. 2, using sera with normal opsonic function resulted in the phagocytes ingesting three or more yeast particles whereas using sera with defective function was associated with little or no uptake of particles.

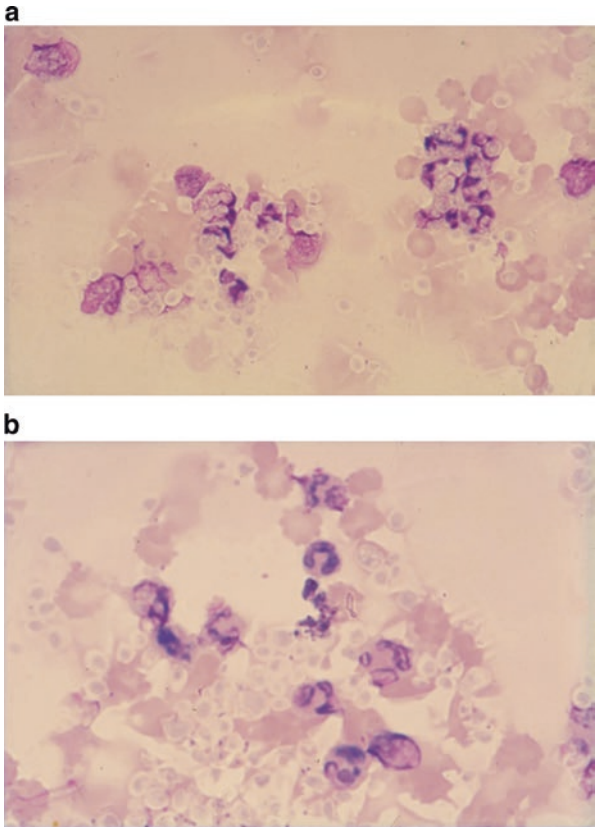

Fig. 2 Photomicrographs of stained polymorphonuclear cells incubated with heat killed baker's yeast in the presence of (**a**) serum proficient in opsonic activity and (**b**) serum deficient in opsonic activity

The microscopic assay was, however, time consuming and in 1978 my late colleague Roland Levinsky developed an automated version of the test based on Coulter Counter technology which was then used to study a cohort of apparently healthy school children (Levinsky et al. 1978). Surprisingly, this revealed that some 5–7% of such individuals had poor opsonic function, an observation which was consistently confirmed in a range of assays subsequently developed for routine use.

In the early1980s the author was encouraged to investigate the immunochemical basis of this defect since there was some scepticism among the wider immunological community regarding the significance of observations made using baker's yeast as targets in the assays. It was already well established at this time that two distinct families of opsonic molecules were important in antimicrobial immunity. These were (1) certain immunoglobulin molecules and (2) various cleaved fragments of complement component C3. Since the levels of immunoglobulin classes and subclasses had already been investigated and found to be normal in most of the patients with defective opsonization it was unlikely that these molecules were implicated in the disorder. Therefore C3 derived opsonins were assumed to be those playing a role in the development of the defect and an assay was devised to directly

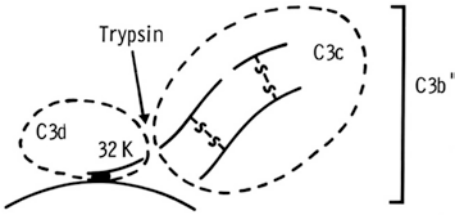

Fig. 3 Site of tryptic cleavage of surface bound C3b molecules. The released C3c fragments could be readily quantified by single radial diffusion

measure the deposition of C3 related molecules on various surfaces (Turner et al. 1981). In brief, serum diluted in VBS buffer containing calcium and magnesium was initially incubated with a suspension of the target organism or yeast zymosan before quenching the reaction with ice cold VBS-EDTA buffer. After centrifugation and washing steps, trypsin was incubated with the target preparations at 37 °C and the released C3c fragments were quantified in single radial diffusion plates containing anti-C3. The site of trypsin cleavage of a cell bound C3b molecule is illustrated in Fig. 3.

The development of this procedure, which was termed the C3c elution assay, provided an important investigative tool which clearly established that abnormal C3 deposition was implicated in the opsonic disorder. For example, dose response curves over a 30 min incubation period consistently indicated increasing deposition of C3b fragments from sera found to be normal in the Coulter Counter assay whereas there was always minimal deposition from sera found to be defective in the Coulter Counter assay. The assay was also used to show that adding small aliquots of normal sera could correct the defect but no serum with defective opsonization was ever shown to correct other sera with the defect. In later studies (Turner et al. 1986) the assay was employed to compare the deposition of C3b on the surfaces of *Candida albicans*, *Staphylococcus aureus* and *E. coli* using sera from five individuals with normal deposition on yeast particles with five sera having defective deposition on yeast. This study showed convincingly that the opsonic defect was a general phenomenon and not confined to yeast targets.

Despite anchoring the phenomenon of defective opsonization firmly in the complement system it was proving impossible to pinpoint the precise cause within the two known complement activation pathways. Total haemolytic complement values were normal in sera with the defect and immunochemical levels of C3, C4, Factor B, Properdin and Factor H were all normal as was functional Factor H (Turner et al. 1985). At this stage the data appeared to suggest that there was some previously unidentified co-factor required for full opsonic potential but this idea was met with some scepticism in some quarters when the data was presented at the sixth International Congress of Immunology held in Toronto in July 1986. It was argued that no additional factors or regulators were required for the expression of full complement potency. Despite this negativity we were fortunate to obtain further funding from Action Research in 1987 which permitted the investigation of various possibilities such as non functional variant alleles of critical complement components e.g., properdin. Later that year Mike Super was recruited as a graduate student to work on this project and our plan included the exploration of such possibilities in association with colleagues at the MRC Immunochemistry Unit in Oxford.

Biomedical research in the 1980s was underpinned by access to the world output of peer reviewed publications exactly as it is now but with one important difference. The retrieval of papers of relevance required visits to the library and the scouring of the weekly publication Current Contents. The choice of which journals were to be perused was made by the reader and the numbers of potentially relevant titles increased year on year. This was a particular problem with papers on aspects of the complement system which could be found in a wide variety of journals covering the medical and biochemical literature. However, a revolution in the retrieval process occurred in the late 1980s. Libraries began to install computer terminals and the output of individual journals collated by Current Contents on a floppy disk could be viewed on screen and, critically, it became possible to type in key words to ensure that papers of relevance were not missed whichever journal had accepted the submission. Accordingly, in 1988 such a literature search (see Fig. 4) identified a paper by Ikeda and colleagues working in Kyoto and published a few months previously in 1987. This described a lectin called mannan-binding protein (MBP) isolated from rat serum which had been found to activate complement through the classical pathway (Ikeda et al. 1987).

Already in 1988 it was known that C-reactive protein was an alternative initiator of complement activation by the so-called classical pathway but it did not seem likely to be involved in yeast opsonization since it does not bind to yeast mannan. However the rat lectin did appear to be worthy of further consideration as a potential candidate. As part of the follow up to this we would need to have access to both purified human MBP and preferably also antiserum to the protein. By happy

Fig. 4 Graduate student Michael Super undertaking an early computer based literature search in the library of the Institute of Child Health in 1988

coincidence we discovered shortly afterwards in discussions with Ken Reid and colleagues from the Oxford MRC Immunochemistry Unit that their group was also beginning to take an interest in the same area and that visiting scientists Steffen Thiel and Jinhua Lu were already purifying human MBP and intended to raise a rabbit antiserum in due course. It was agreed in principle that when these materials became available we would be able to have access to them on a collaborative basis.

With the availability of the rabbit anti-MBP antibody and an MBP standard it was possible to develop an ELISA procedure for the measurement of the protein in stored sera including samples from ten children known to have the opsonic defect, 59 healthy babies and 186 adult blood donors. Whereas both the adult blood donors and the healthy babies were found to have a wide range of MBP values, each of the ten children with the opsonic defect had extremely low levels of the serum lectin. Moreover it was possible to titrate in increasing levels of MBP into serum from an individual with the opsonic defect and demonstrate a dose dependent correction of the deficiency (Super et al. 1989).

A Question of Nomenclature

Once it was possible to ascribe a predominantly immunological role for the protein known up to then as mannan- (or mannose-) binding protein it created a potential confusion with the usage of MBP as an abbreviation. MBP was already used extensively in the immunological literature to denote three other proteins, namely myelin basic protein, major basic protein of eosinophils and maltose binding protein. Accordingly, at least in the immunological sphere, the abbreviation of MBL is now practically universal.

A Decade of Biochemical Studies

As the 1980s came to an end it was clear that there was strong evidence that the protein known as MBP had an important immunological function but for most of the decade MBP had been a protein in search of a function. The existence of serum lectins was first predicted by Robinson et al. (1975) and shortly afterwards Kawasaki et al. (1978) described the isolation and characterization of mannan-binding protein from rabbit liver. The same group subsequently isolated the protein from human serum (Kawasaki et al. 1983) from bovine serum (Kawasaki et al. 1985) and from rat serum (Oka et al. 1988). Progress was made on the basic structure of the protein (notably by Weis et al. 1991 and Weis and Drickamer 1994 and by Sheriff et al. 1994) and the structure of the gene encoding human serum mannose-binding protein was independently published by Sastry et al. (1989) and Taylor et al. (1989). Some of the key steps in this biochemical chronology are shown schematically in Fig. 5.

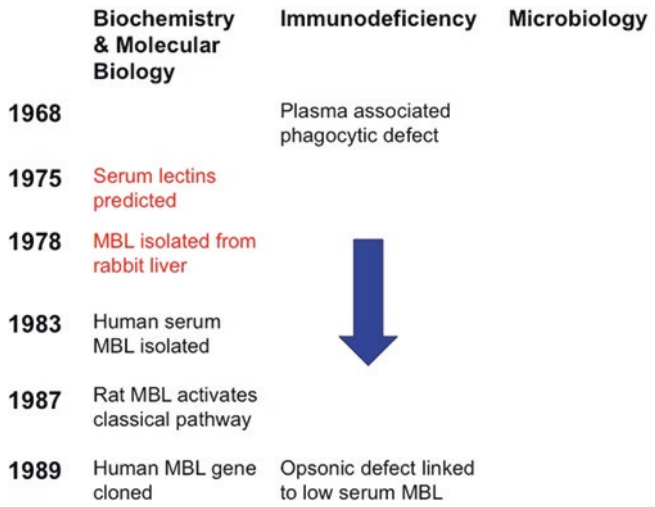

Fig. 5 Chronology of studies in three biomedical areas which were pursued essentially in isolation until 1989 when links began to be established between the various studies. Key findings in the biochemical area are highlighted in this scheme

In fact we now know that there are two human MBL genes but *MBL-1* is a pseudo-gene. The functional *MBL-2* gene located on chromosome 10 encodes the protein product found in serum and comprises four exons. Exon 1 encodes the signal peptide, a cysteine rich region and part of a glycine rich collagenous region. Exon 2 encodes the remainder of the collagenous region whilst exon 3 encodes the so-called neck region characterized by an alpha-helical coiled coil structure. Finally, exon 4 encodes the C terminal globular carbohydrate recognition domain (CRD). In the assembly of a functional MBL molecule three such polypeptide chains come together with the collagenous regions forming a triple helix stabilized by hydrophobic interactions and interchain disulphide bonds in the cysteine rich region. In serum the circulating MBL consists of a series of oligomers of this basic subunit ranging from dimers to hexamers but expression of functional binding to microbial surfaces appears to require higher order structures (tetramers to hexamers). The reason for this is that a single CRD binds only weakly to its target sugar group and high functional affinity (avidity) is only achieved through multiple CRD binding as is also the case with IgM antibody interactions with its target antigens. X-ray crystallographic studies reveal that the high order MBL oligomers adopt a sertiform or bouquet like structure facilitated by an interruption in the collagenous region which gives rise to a kink or hinge.

In 1990 it was possible to construct a model of the probable functional role of MBL in the activation of the complement system. In essence the hypothesis was that MBL acted as a mimic of C1q in the classical complement cascade. This model required MBL to interact with both C1r and C1s subcomponents of the C1 complex and the binding of the CRD domains of MBL to multiple sugar groups on a target

surface was believed to induce the autocatalytic activation of C1s leading in turn to cleavage of both C4 and C2 and the assembly of the C1-esterase enzyme. This model received powerful support from the study of Lu et al. (1990) but was destined to be challenged by the description in 1992 of an alternative model in which MBL was postulated to interact with a completely novel C1s-like serine protease (Matsushita and Fujita 1992). Although initially it appeared odd that the authors had sought to establish the involvement of another serine protease in the expression of MBL induced complement activation the explanation was provided in earlier studies that both Dr. Fujita and Dr. Matsushita had co-authored with Dr. Kawakami. These provide a link to the third strand of research which contributed significantly to our understanding of the role of MBL in immunity, namely microbiological research.

Microbiological Studies in the 1980s

In parallel with the previously described biochemical studies on serum MBL Masaya Kawakami and colleagues were publishing a series of papers on a family of bactericidal factors called RaRF present in the sera of a range of vertebrates. In particular, RaRF was found to react specifically with Ra chemotype strains of *Salmonella* and the R2 strains of *E. coli.* The authors proposed that these factors were necessary to resist infection by rough strains of enterobacteria.

Furthermore the authors were able to demonstrate that, after binding to bacteria, RaRF was able to activate complement thereby killing the organisms. In 1988 the authors presented evidence that RaRF had a polypeptide structure which resembled C1q and was partially collagenous (Ji et al. 1988). In the same paper the authors noted the close similarity between RaRF and the rat lectin described by Ikeda et al. (1987) and, significantly, they reported that whereas C4 and C2 components were required for complement activation by RaRF, C1 apparently was not required. Both Dr. Fujita and Dr. Matsushita were coauthors on this publication and these RaRF studies led them to question the precise mechanism of complement activation by MBL and describe the first of the three MBL associated serine proteases (MASPS) (Matsushita and Fujita 1992). In the same year Matsushita and Fujita also co-authored the paper which definitively demonstrated that RaRF was indeed identical to MBL (Matsushita et al. 1992).

In addition to the RaRF studies there was another area of microbiological research which should be acknowledged. In 1946 Sir Frank Macfarlane Burnet and John McCrea described three inhibitors in mammalian serum (called alpha, beta and gamma) which were able to inactivate influenza virus (Burnet and McCrea 1946). By 1969 the beta-inhibitors were known to be calcium dependent, non-sialylated, heat–labile glycoproteins present in mouse, guinea-pig, ferret and rabbit serum (Krizanova and Rathova 1969) but it was not until the 1990s that the beta-inhibitors in these species were finally shown to be mannose–binding lectins (Anders et al. 1990, 1994). The chronology of some of the key discoveries in the microbiology area are summarized schematically in Fig. 6.

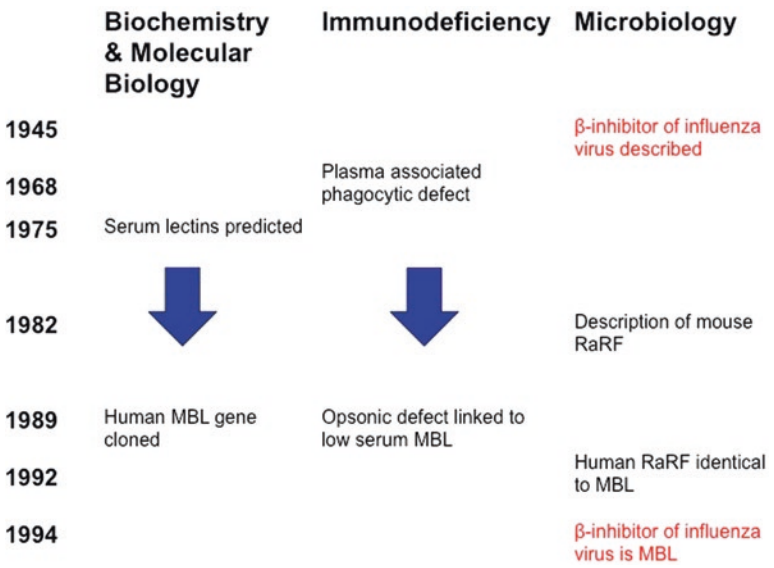

Fig. 6 Key dates of microbiological studies which were subsequently found to be relevant to our understanding of the role of MBL

Determination of the Underlying Cause of the Opsonic Deficiency

The association of low levels of MBL with the opsonic deficiency still begged the question of what was causing the low levels. A genetic explanation was clearly one possibility and the cloning of the human MBL gene in London by Taylor et al. (1989) provided ready local access to the technology required. As discussed previously the *MBL-2* gene comprises four exons arranged as follows. Exon 1 encodes a signal peptide, a cysteine rich region and part of the glycine-rich collagenous region, exon 2 encodes the remaining portion of the collagenous region and exon 3 encodes the so-called neck region characterized by an alpha-helical coiled coil structure. Finally, exon 4 encodes the carbohydrate recognition domain (CRD) which has a globular structure.

However, there were no observations to suggest where a putative mutation might be located and Mike Super commenced the nucleotide sequencing of individuals from three British families with the opsonic defect beginning at the C-terminal end of exon 4. This proved to be an unfortunate decision for him since, after working through exons 4, 3 and 2, but finding no evidence of any mutations, he had to leave London to take up a post doctoral position in the US and it was Michiko Sumiya's good fortune to take over the search and discover the first MBL mutation in exon 1 (Sumiya et al. 1991). This was a single point mutation at base 230 in codon 54 which resulted in a change of the translated amino acid in the peptide chain from a glycine to an aspartic acid. This has significant consequences because the integrity

of all collagenous proteins is based on assembling a triple helix in which every third residue is the smallest amino acid, glycine, situated exactly along the axis of the helix. Three aspartic acid residues, each having a bulky side chain, would not be readily accommodated in the axial position of the helix. Such disruption was already documented in the structural collagen molecules of patients with *osteogenesis imperfecta* (Sykes 1989) and is illustrated schematically in Fig. 7.

In essence the defect is not a primary genetic deficiency but rather a secondary functional deficiency in which individual 3-chain MBL subunits are unable to assemble into higher order oligomers with the capacity to make multipoint attachments to target sugar groups and hence overcome the intrinsic low affinity of such binding by an individual subunit of three CRDs. SDS-PAGE analysis of MBL from an individual homozygous for the codon 54 mutation shows most protein is indeed of low molecular weight compared to the MBL of an unaffected individual which is characterized by a spectrum of higher order oligomers (see Fig. 8).

Following Mike Super's departure the author recruited a new graduate student, Richard Lipscombe, to continue the early work on structural and functional aspects of MBL. In the first of a series of fruitful collaborative publications with former Institute of Child Health Research Fellow Yu Lung Lau, Richard initially confirmed the presence of the codon 54 mutation in a small group of Hong Kong Chinese

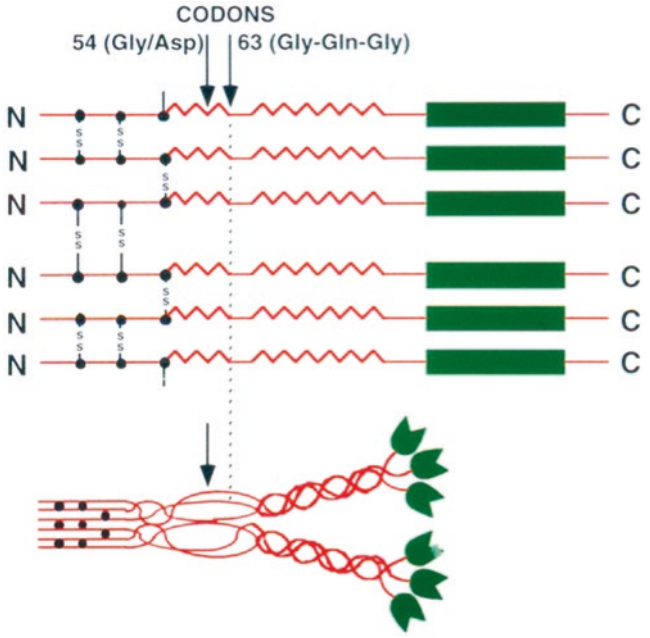

Fig. 7 Schematic showing the presumed effect of the codon 54 mutation in the *MBL-2* gene on the expressed protein product. A critical axial glycine residue is replaced with an aspartic acid having a bulky side chain and this disrupts the assembly of higher order oligomers essential for proper biological function

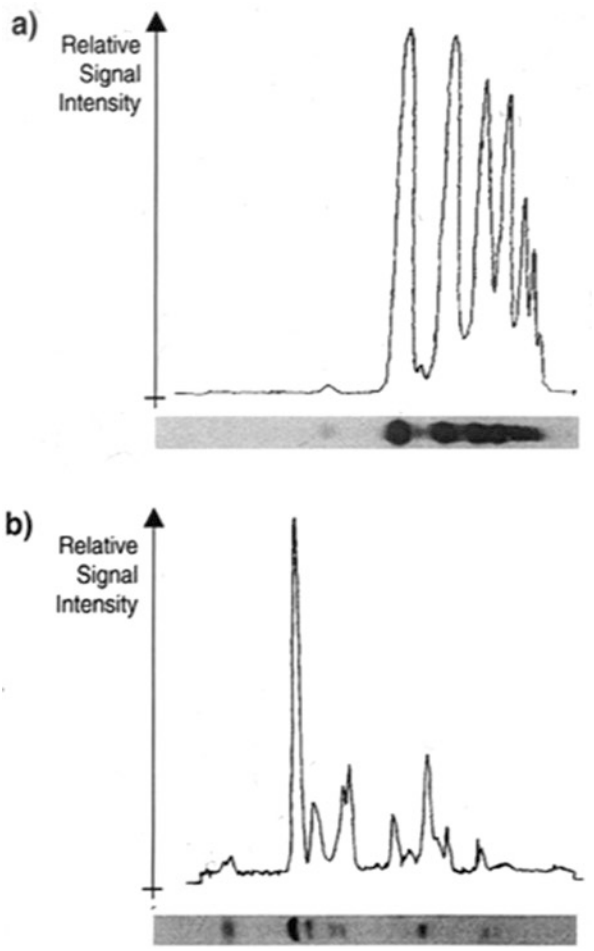

Fig. 8 SDS-PAGE analysis of human MBL from a subject with no codon 1 mutation and showing a spectrum of oligomeric forms (upper profile). In contrast, MBL from a subject homozygous for the codon 54 mutation is predominantly of low molecular weight (lower profile). See Lipscombe et al.(1995) for more details

(Lipscombe et al. 1992a). This suggested that the mutation had probably originated more than 40,000 years ago in a population ancestral to both modern day Asians and Europeans. Subsequently a series of population studies were undertaken, one of which included the identification of a second codon 1 mutation in a cohort from The Gambia in West Africa (Lipscombe et al. 1992b). The latter mutation in codon 57 results in a glutamic acid substitution for an axial glycine and, since it is associated with reduced levels of serum MBL, is presumed to result in disrupted assembly of high order oligomers of MBL protein as is the case with the codon 54 mutation.

Some Concluding Thoughts

The studies described in this chapter provided a reasonable model of the biological function of MBL as viewed in the mid 1990s. This is summarized schematically in Fig. 9.

Here the tetrameric form of MBL is shown with each of the four subunits composed of three identical chains terminating in a cluster of three carbohydrate recognition domains. Binding of these domains to repeating sugar groups on a microbial surface were assumed to bring about activation of the MBL-associated serine protease −2 molecules located near the "kink" in the collagenous regions. Nearby C4 and C2 molecules would be cleaved and biologically active C3 convertase generated. The formation of C3b fragments able to bind covalently to the microbial surface would then complete the first stage of the opsonic process. For an update on the latest findings regarding MBL-MASP activation mechanisms the reader should look elsewhere in this volume.

The initial association of frequent infections with the opsonic deficiency state suggested an important role for MBL in the innate immune system. However, the apparent maintenance of the codon 54 mutation over several 1000 years suggest some associated biological advantage and the presence of high frequencies of a similar independent mutation in Sub-Saharan Africa provides strong support for this view. Two hypotheses have been proposed and these may not be mutually exclusive. Firstly, the reduced levels of complement activating MBL could help to reduce the risk of host damage from the excessive release of inflammatory mediators particularly in tropical environments (Lipscombe et al. 1992b). Alternatively, it has been proposed that MBL deficiency may help to protect the host against intracellular parasitic infections which depend upon C3 opsonization and C3 receptor uptake in order to breach cellular defence mechanisms (Garred et al. 1992).

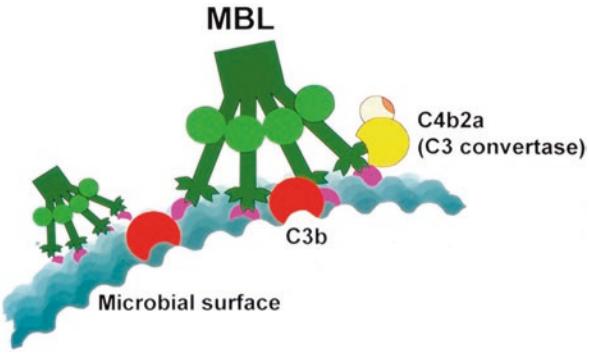

Fig. 9 Functional human MBL binds to sugar groups on microbial surfaces in association with MASP-2 moieties (shown in light green) to generate C4b2a complexes with C3 convertase activity. This leads to the deposition of C3b opsonins (shown in red) on the nearby surface

Many groups, including the author's, have undertaken various disease association studies with respect to serum MBL levels and these are the subject of another chapter within this volume.

In evaluating the importance of MBL deficiency in human disease the author has always advocated the need to seek evidence of effects beyond simple disease associations. For example, co-existing partial immunodeficiencies are by no means rare. Some 8% of the Caucasian population lack two of the four possible functional C4 genes and we know that 5% of the same ethnic group have very low MBL levels which means that one in 250 individuals would present with both partial deficiencies (Turner 1996). Similarly we reported that polymorphisms in exon-1 of the *MBL-2* gene, resulting in low plasma levels of MBL, were significantly overrepresented in patients with primary antibody deficiency and mycoplasma infections (Hamvas et al. 2005).

In support of the concept of MBL playing a role as a disease modifier we showed that individuals deficient in MBL were at risk of developing sepsis and systemic inflammatory response syndrome (SIRS) (Fidler et al. 2004).

The initial association of the opsonic deficiency with a clinical presentation of frequent infections in young children suggested that MBL must be particularly important in early life after maternally derived antibodies have decayed and the host is relatively antibody deficient, the so-called "window of vulnerability" (Super et al. 1989). However, it has also been suggested that MBL plays an important role in the early phase of many primary encounters with sugar rich pathogens, acting as an "ante-antibody" (Ezekowitz 1991). A unique opportunity to test this hypothesis arose when a novel human pathogen emerged in China in 2003. The coronavirus which caused severe acute respiratory syndrome (SARS) infected some 8098 individuals and resulted in 774 deaths. In a case controlled study of 569 patients Ip et al. (2005) found that serum levels of MBL were significantly lower in patients with SARS than in control subjects suggesting that MBL did indeed contribute to first-line host defence against SARS-CoV. We have shown that a wide range of different microorganisms are able to bind to MBL (Neth et al. 2000) and in a later study we also demonstrated that the MBL-MASP system can significantly enhance other complement activating pathways (Neth et al. 2002). It is not unreasonable to conclude that MBL is a major player in the initial immune response to many pathogens throughout life but is of particular importance in young infants.

Acknowledgments The author gratefully acknowledges the considerable input of many former colleagues involved in the development of this research area and Dr. Jonathan Turner for help with the preparation of this chapter.

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Mannose-Binding Lectin in Human Health and Disease

Christiana Doulami, Uday Kishore, Robert B. Sim, and Wilhelm Schwaeble

Introduction

Mannose-binding lectin (MBL), also known as mannose-binding protein or mannan-binding lectin, was first discovered by Kawasaki in 1978 (1978). Five years later, MBL was isolated from human and rat liver (Wild et al. 1983) and from a variety of species (Kozutsumi et al. 1980; Townsend and Stahl 1981; Kawasaki et al. 1983; Oka et al. 1988). MBL is mainly produced by the liver but low amounts have been found in the small intestine (Uemura et al. 2002).

The protein's first description occurred in 1968, a decade before its discovery, where the protein was referred to as having an opsonic activity in relation to immune deficiency. In this report, an infant with a serum dependent defect of phagocytosis was described (Miller et al. 1968). The girl who was suffering from severe dermatitis and recurrent bacterial infections, was found to lack a plasma component detected by the opsonization of baker's yeast. The phagocytic deficiency was improved by infusion of fresh plasma which resulted also in clinical improvement. Studies on serum from the patient's mother and her relatives, revealed that the condition was

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hereditary. Investigations by Super et al. (1989) found that 25% of hospitalized children with recurrent infections had an opsonic defect. Later, the missing plasma component in these children was linked to MBL and that the originally described phagocytic defect, was due to MBL deficiency (Turner 1996).

After the discovery of MBL, studies showed that the protein could activate the complement system upon binding to surfaces which displayed mannan (Kawasaki et al. 1983; Ikeda et al. 1987). Further studies revealed that MBL in serum was associated with a serine protease, and therefore called MBL-associated serine protease (MASP) (Matsushita and Fujita 1992). Subsequently, three MASPs, now named MASP 1, 2 and 3 were characterized. In serum, the MBL circulates in complex with MASPs and initiates the lectin pathway of the complement system when binding to its target and is also associated with coagulation events. In this review, we will give a comprehensive overview of the literature on MBL by discussing its structure, function, interaction with its serine proteases, genetics and its role in association with various pathologies.

MBL Structure

MBL is a collectin, which is a Group III C-type lectin. The C-type lectin superfamily is a large group of proteins that are characterized by the presence of one or more C-type lectin or carbohydrate recognition domains (CRDs) and is involved in innate defense systems (Van De Wetering et al. 2004). Collectins are characterized by a collagen-like region and a C-type carbohydrate domain (CRD) in their C-terminal end (Fig. 1a). The MBL CRD specifically recognizes a monosaccharide which exposes horizontal 3′- and 4′ OH groups (Kjaer et al. 2013). MBL is an oligomeric molecule of 25 kDa polypeptides that form a trimer, referred to as the subunit (Fig. 1b), that further assemble into oligomeric forms (Fig. 1c). Each subunit is made up of three identical polypeptide chains (Holmskov et al. 2003; Garred et al. 2006) (Fig. 1b). Each peptide chain consists of four regions: an N-terminal tail region, followed by a collagenous region, a short α-helical neck domain, and each chain terminates in the C-terminal calcium-dependent carbohydrate-recognition domain (CRD) (Garred et al. 2006) (Fig. 1a).

The N-terminal tail region consists of 21 amino acids (aa), three of which are cysteines (Fig. 1a). It has been shown that the N-terminal region provides stability to the subunit by intra-subunit disulfide bonds (Hansen and Holmskov 1998) and the cysteines seem to be responsible for the oligomerization of the subunit (Jensen et al. 2005). The collagenous region (59 aa) has 19 Gly-X-Y repeats (Gly-X-Y, where X and Y may be any amino acids) with a GlyGLn interruption at the eighth triplet (Fig. 1a). It is assumed that the break between the two collagen repeats induces a bend or flexibility, the so-called “kink” (Gal and Ambrus 2001). The collagenous region is followed by a short α-helical coiled-coil neck region (33 aa) which links the final domain, a globular CRD (115 aa) (Fig. 1a). The only crystal structures that have been determined by X-ray crystallography so far are these of the

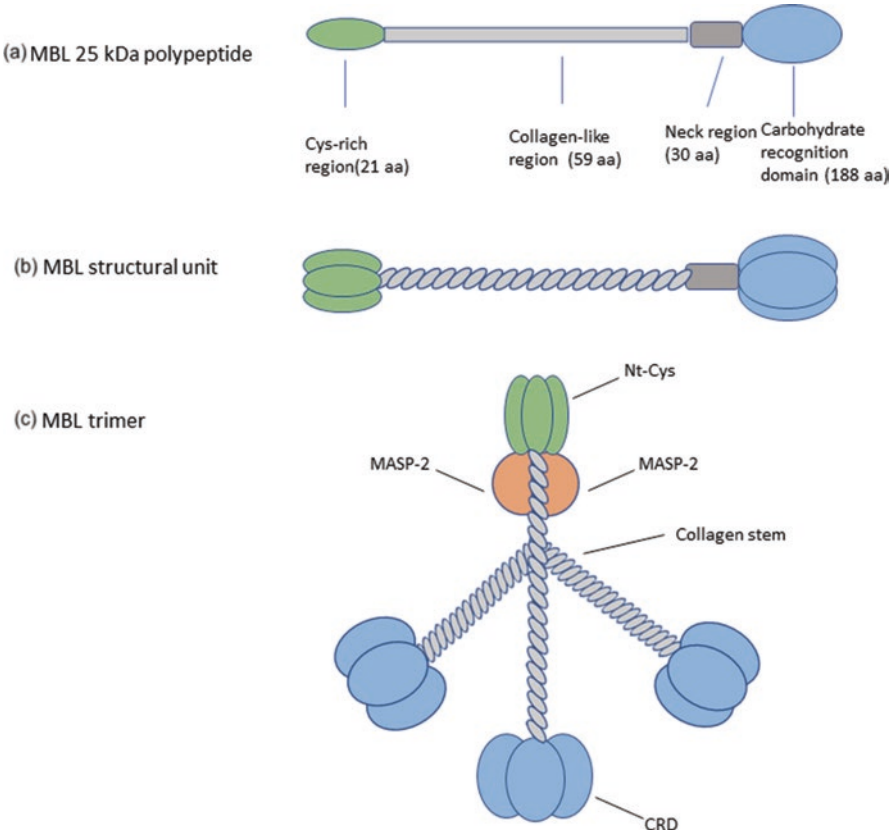

Fig. 1 Polypeptide, monomeric and oligomeric structures of mannose-binding lectin (MBL). An MBL monomer is a homotrimer of three polypeptides, each subunit consists of a cysteine-rich N-terminal region, a collagen-like domain with Gly-X-Y repeats and a C-terminal carbohydrate recognition domain (CRD) (**a**). The structural unit of MBL consists of three subunits (**b**) and the oligomer is composed of up to six structural units (corresponding to 18 subunits) linked by disulfide bonds (**c**)

CRDs with the α-helical coil neck region (PDB 1HUP, 1RTM) (Sheriff et al. 1994; Weis and Drickamer 1994) as well as the collagen-like peptide (PDB 3PON) (Gingras et al. 2011) and the primary structure of the peptide is solved (Ezekowitz et al. 1988).

Three polypeptide chains associate to form a homotrimeric subunit comprising the collagen-like triple helix, an α-helical coiled-coil, the so-called neck region, and three CRDs (Turner 1996) (Fig. 1c). The subunit is stabilized by disulfide bonds between cysteine residues and hydrophobic interactions in the N-terminal region (Lu et al. 1990) and these three cysteines form interchain disulfide bonds that mediate formation of higher oligomeric forms (Super et al. 1992).

A trimer of MBL is the basic building block that is organized in oligomers ranging from dimers and trimers and extend up to hexamers and octamers (Jensenius

et al. 2009) (Fig. 1c), each displaying 6–24 CRDs providing MBL high avidity towards targets with suitably displayed ligand (Dahl et al. 2001; Jensen et al. 2007). Moreover, the prominent forms of human MBL are trimers and tetramers, whereas pentamers and hexamers occur in much lower amounts (Lu et al. 1990; Teillet et al. 2005). It has been assumed that oligomeric MBLs form bouquet-like structures, called "bunch of tulips", which is similar to the structure of complement C1q (Lu et al. 1990). However, imaging studies have revealed that MBL adopts a near-planar, fan-shaped structure in solution (Jensenius et al. 2009; Miller et al. 2012; Nan et al. 2017).

MBL is secreted into the blood stream as a large multimeric complex and is primarily produced by the liver, although other sites of production, such as the intestine, have been proposed (Uemura et al. 2002). The protein circulates as a complex with MASPs which get activated upon binding to its ligands (Fig. 1c). The ligand specificity of MBL is determined by the CRD, which is able to bind a range of oligosaccharides including mannose, N-acetylglucosamine (GlcNAc) and L-fucose (Turner 1996).

Various Functions of MBL

Binding of MBL with Ligands and Pathogens

The key difference between innate and adaptive immunity exists in the flexibility, kinetics and specificity of the immune response (Beutler 2004). In innate immunity, a well-defined pool of germline-encoded pattern recognition receptors (PRRs) can bind to highly conserved structures termed as pathogen-associated molecular patterns (PAMPs), which can be found in large groups of invading microorganisms. The PRRs are either present on many effector cells of the immune system, e.g. macrophages or dendritic cells, or are secreted in the serum (Beutler 2004). Certainly, the arrangement of PAMPs, in which carbohydrates are involved, on the surfaces of bacteria, fungi, viruses and protozoans is distinct from the carbohydrates found in eukaryotes, thereby helping in distinguishing self (non-infectious) from non-self (infectious) (Janeway and Medzhitov 2002). Upon carbohydrate pattern recognition on a pathogen, the PRRs trigger effector cells to destroy the invading microorganism. Several classes of PRRs have now been identified, where the three general categories are: signaling, endocytic and secreted. MBL belongs to the last class (Nuytinck and Shapiro 2004). By having the role of an opsonin, the protein binds to microbial cell walls to tag them for recognition from the complement system and phagocytes.

It has been shown that the binding of a single MBL CRD to microbial carbohydrates is relatively weak, with a K_d of approximately 10^{-3} M (Turner 1996). Significant functional avidity can be achieved when multimeric interactions occur between the CRDs of the multimeric protein, altogether 18 in a hexameric MBL, and the cell surface carbohydrates (Lu et al. 1990). CRD's ability to recognize and

bind a range of oligosaccharides (Turner 1996), makes MBL a very important protein in first-line immune defense (Neth et al. 2000).

The bacterial targets that have been identified *in vitro* for MBL are numerous. Mannose and N-acetylglucosamine oligosaccharides, which MBL binds with high affinity, are present on the surface of various strains of Gram-positive and Gram-negative bacteria, fungi and yeast particles (Neth et al. 2000). It has been reported that MBL binds strongly to *Candida* species, *Aspergillus fumigatus, Staphylococcus aureus*, and β-hemolytic group A *streptococci*. An intermediate binding of MBL has been found for *Escherichia coli, Klebsiella* species, and *Haemophilus influenzae* type b. Contrary to β-hemolytic group B *streptococci, Streptococcus pneumoniae,* and *Staphylococcus epidermis* which MBL binds weakly (Neth et al. 2000). The latter observation suggests that MBL binding is significantly weakened by the presence of a capsule (Turner 1996), or by the addition of sialic acids on the bacterial surfaces (Eisen and Minchinton 2003). Consequently, various strains of one bacterial species may vary significantly with regards to binding of MBL (Thiel and Gadjeva 2009).

MBL's recognition and binding is not limited to microbial carbohydrates to initiate complement activation cascade, but the protein binds also to phospholipids (Kilpatrick 1998), nucleic acids (Palaniyar et al. 2004) and non-glycosylated proteins (Estabrook et al. 2004); characteristics that are probably related to the clearance of apoptotic cells and avoidance of autoimmunity (Nuytinck and Shapiro 2004). Engagement of MBL with apoptotic and necrotic cells has been reported where MBL facilitates the uptake of these cells by macrophages (Nauta et al. 2003).

Many publications suggest that MBL's involvement may be expanded above complement activation. It has been proven that MBL is able also to promote complement-independent opsonophagocytosis, modulate inflammation and probably promote apoptosis (Ogden et al. 2001; Nauta et al. 2003; Turner 2003; Saevarsdottir et al. 2004). Additionally, MBL and the MBL-associated serine proteases-1 and -2 have been associated with the coagulation system (Gulla et al. 2010; Dobó et al. 2016b) and recent studies demonstrated their involvement in the ongoing COVID-19 pandemic (Eriksson et al. 2020; Rambaldi et al. 2020). Therefore, MBL seems to play a central role in innate immunity, given its contribution in microbial recognition, clearance, inflammation and apoptosis. It is reasonable to expect that MBL deficiency may result in impaired host immune defenses, and consequently, have been associated with several diseases. Indeed, a huge number of studies has shown MBL's association with several diseases.

Physiological Role of MBL

As described briefly above, at least four distinct functions of MBL have been reported so far:

(a) activation of complement; (b) promotion of (complement-independent) opsonophagocytosis; (c) modulation of inflammation, and (d) promotion of apoptosis

(Turner 2003). MBL's association with the complement system has been studied extensively, whereas the other three MBL associated mechanisms are not so clear yet. In addition to these functions, recent studies describe the involvement of MBL beyond innate immunity. MBL and MASPs have been associated with the coagulation system where the proteins show coagulation factor-like activities (Gulla et al. 2010; Dobó et al. 2016b).

Activation of the complement system occurs via three pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). All pathways converge onto a common terminal pathway (TP) via generation of a C3-convertase, resulting in opsonization and lysis of invading pathogens and altered self-structures, and recruitment of inflammatory cells. The CP is activated through binding of C1q to immune complexes. The LP is activated when the complexes of MBL or ficolins or other collectins with MASPs bind to the carbohydrates on the surface of pathogens (Fujita et al. 2004). MASPs convert C2 and C4 into a C3-convertase, the C4b2a complex, which is the same complex generated by the CP. The alternative pathway is activated by C3b generated during the activation of the classical and lectin pathway and by other mechanisms; therefore, it is acting mainly as an amplification loop for those two complement pathways.

MBL's association with the complement system has mostly been reported to be involved in the lectin pathway, where the serine proteases MASP-1 and MASP-2 are the key enzymes of the pathway. While MASP-3 has been reported to be involved in the activation of the alternative pathway by activating the complement component pro-Factor D (Dobó et al. 2016a; Pihl et al. 2017).

Several reports have been shown that MBL is able to promote opsonophagocytosis in a complement-independent manner. The first report about this function of MBL was by Kuhlman et al. (1989) where the Gram-negative bacterium *Salmonella montevideo*, exposing a mannose-rich O-polysaccharide, was found to be ingested by monocytes in an MBL-dependent manner. The experiments were performed with both human MBL (20 μg/mL) and recombinant MBL (rMBL) (1 μg/mL) expressed in CHO cells (Kuhlman et al. 1989).

Super et al. (1992) performed further studies by studying the differences in functionality between the wild-type rMBL and the mutant (in codon 54) rMBL and showed that both forms of recombinant proteins had a similar potential of mediating the uptake of *Salmonella montevideo* by human neutrophils, suggesting that the mutant form didn't lose the ability to bind to the bacterium and opsonize it.

Direct opsonization was reported also by other studies. Hartshorn et al. (1993) reported that human MBL could bind to Influenza A virus which displays high mannose oligosaccharide and enhance the H_2O_2 production by neutrophils. Similar effect was observed also between wild type rMBL and mutant rMBL (mutation on codon 54).

Thus, MBL has a direct interaction with receptors on the surface of phagocytes. Several putative receptors or MBL- binding proteins have since been proposed. Amongst these receptors, cC1qR appears to be the main candidate, which was later found to be identical to the intracellular protein calreticulin (Malhotra et al. 1990). Other suggested receptors which are linked to promote opsonophagocytosis by

MBL, are the C1qRp (Tenner et al. 1995) and CR1 (Ghiran et al. 2000). C1qRp was subsequently found to be a mitochondrial protein and found not to bind MBL (McGreal et al. 2002; Norsworthy et al. 2004) but CR1, the complement C3b receptor, interacts with MBL (Ghiran et al. 2000; Jacquet et al. 2018) but this functional activity has to be further explored.

On the other hand, van Asbeck et al. (2008) showed that MBL is an opsonin only in the presence of complement. Since complement activation will amplify opsonisation, such findings depend on assay sensitivity. They performed opsonophagocytosis assays with complement-deficient sera and phagocytosis was not observed from the binding of MBL in the absence of downstream complement components, whereas phagocytosis was enhanced via C3b-dependent opsonization recognized by complement receptors on PMNs, suggesting that MBL doesn't act as a direct opsonin. Other studies have also reported similar results. Certainly, the phagocytic potential of MBL alone is less well understood and more studies must be done.

There is increasing evidence from studies which report that the protein has a major role in the modulation of inflammation, independent of complement activation (Jack et al. 2001) and MBL as a pleiotropic immunomodulator affects numerous cell types of innate and adaptive immunity. Studies have shown that MBL modifies cytokine response through a cooperation with Toll-like receptors 2 and 6 in the phagosome (Ip et al. 2008), or through LPS and Toll-like receptor 4 (Wang et al. 2011). Other studies reported that MBL is able to bind human monocytes and reduce inflammatory responses (Wang et al. 2013; Tang et al. 2015) and additionally interact with human T cells and suppress T cell activation (Zhao et al. 2017). Additionally, a recent publication showed that the protein can bind to human peripheral NK cells and regulate inflammatory cytokine production (Zhou et al. 2019). Nonetheless, the underlying mechanisms of MBL-mediated modulation of inflammation are not clear and further work is necessary to define these mechanisms, providing new therapeutic opportunities in inflammatory and autoimmune diseases.

It has been also reported that MBL takes part in a very important function of the immune system, in the recognition of altered or damaged self and subsequently in facilitating their clearance (Nauta et al. 2003; Stuart et al. 2005). MBL can bind apoptotic cells and initiate their uptake by macrophages (Ogden et al. 2001). It was suggested that calreticulin associates with CD91 through which MBL is bound on the macrophage surface and mediates uptake of apoptotic cells in addition to the uptake of microorganisms (Ogden et al. 2001; Duus et al. 2010).

Recent findings indicate a linkage of MBL to the coagulation system. The MBL-associated serine protease-1 (MASP-1) and -2 (MASP-2) have been shown to be responsible for the procoagulant activity of the lectin pathway by activating components of the coagulation system (Dobó et al. 2016b; Krarup et al. 2008; Gulla et al. 2010). MASP-1 acts as a thrombin-like enzyme by cleaving thrombin substrates such as fibrinogen and Factor XIII, proteins involved in the clotting process (Krarup et al. 2008) and thrombin-activatable fibrinolysis inhibitor (TAFI) which prevents fibrinolysis (Hess et al. 2012). Both MASP-1 and MASP-2 have been described to cleave and activate prothrombin to generate thrombin, leading to

the formation of a fibrin clot (Krarup et al. 2007; Dobó et al. 2016b). Additionally, studies revealed that the serine proteases MASP-1 and MASP-2 in complexes with their recognition molecules MBL or ficolins can generate insoluble fibrin clots (Gulla et al. 2010). Furthermore, products of coagulation events, such as fibrin or activated platelets, have been shown to activate MASP-1 and MASP-2 suggesting an ongoing crosstalk between the lectin pathway and the coagulation system in hypercoagulable states (Kozarcanin et al., 2016).Both complement and coagulation systems may play an important role in severe COVID-19, a disease caused by the severe acute respiratory coronavirus 2 (SARS-CoV-2), the etiological agent responsible for the ongoing pandemic. Recent reports describe coagulopathy and thrombotic events in patients with severe COVID-19 (Miesbach and Makris 2020). Thrombosis can be caused by endothelial injury, which is a central pathological component of COVID-19 pathophysiology (Ackermann et al. 2020; Rambaldi et al. 2020; Varga et al. 2020). Complement activation through the lectin pathway has been correlated with the complications of this disease. It was shown that MBL is strongly related to thrombosis and to plasma D-dimer levels, an indicator of coagulopathy (Eriksson et al. 2020). Furthermore, MASP-2 and products of lectin pathway activation can be found in affected microvasculature in biopsy specimens from patients with severe COVID-19 disease (Magro 2020) and MASP-2 has been reported to be associated with lung injury in coronavirus infection (Rambaldi et al. 2020). Therefore, inhibition of the complement system is investigated as a potential treatment for severe COVID-19. Recent studies have shown clinical improvement in COVID-19 patients following treatment with inhibitors for the complement components MASP-2 (Rambaldi et al. 2020), C3 (Mastaglio et al. 2020) or C5 (Diurno et al. 2020), suggesting that complement suppresion may provide an effective therapeutic approach to treat severe COVID-19 (Gao et al. 2020). A recent study showed clinical improvement and survival of all hospitalized patients with severe COVID-19 when treated with Narsoplimab (OMS721; Omeros Corporation, Seattle, WA), a MASP-2 inhibitor (Rambaldi et al. 2020). The observations from this study support further the involvement and importance of the lectin pathway in the pathophysiology of this disease, suggesting lectin pathway inhibition to be a promising treatment for lung injury and thrombosis in COVID-19 (Rambaldi et al. 2020).

MBL-Associated Serine Proteases

As a pattern recognition molecule, MBL binds to a range of sugars on viruses, bacteria, yeasts, fungi and protozoa and activates the complement system in an antibody and C1-independent manner (Turner 2003). The recognition domains of the MBL bind simultaneously to a pattern ligand to obtain complement activation. This activation is mediated by complexes of MBL with serine proteases, called MBL-associated serine proteases (MASPs). The name MASP is related to their association with MBL, but nowadays it is also known that MASPs can be found also in

complexes with L-ficolin (Matsushita et al. 2000), H-ficolin (Matsushita et al. 2002), M-ficolin and collectin 11.

These serine proteases are present as zymogens in the circulating complexes and become activated upon the pattern recognition molecules binding to their targets (Dobó et al. 2016b). Binding induces conformational changes in these complexes, which leads to either autoactivation of the serine proteases (Wallis 2007; Héja et al. 2012; Kjaer et al. 2013; Degn et al. 2014), or activation by neighboring complexes (Degn et al. 2014), resulting in the activation of the complement cascade. There are three MBL-associated serine proteases (MASPs) which form complexes with MBL oligomers, MASP-1 (Matsushita and Fujita 1992), MASP-2 (Thiel et al. 1997) and MASP- 3 (Dahl et al. 2001), and two non-enzymatic associated proteins, Map 44 (Degn et al. 2009) and Map 19 (Stover et al. 1999). MASP-1 and MASP-2 activate the lectin pathway of the complement system (Schwaeble et al. 2002; Héja et al. 2012), while MASP-3 is responsible for the activation of alternative pathway component, pro-Factor D (FD) (Iwaki et al. 2011; Dobó et al. 2016a; Pihl et al. 2017). MBL molecules circulate as a complex with MASPs and initiate the complement cascade only after binding to their targets, therefore the structural understanding of the MBL/MASP complex is important. Formation of the complex takes place at the collagenous region of MBL which interacts in a calcium-dependent manner with the CUB1-EGF-CUB2 domains of the MASPs (Thielens et al. 2001) (Fig. 2).

Several unsuccessful attempts have been made to determine a high-resolution structure of a lectin pathway complex and the reason might be the heterogeneity of components and their intrinsic flexibility (Gingras et al. 2011). Based on structural studies that were performed on smaller units, various models of MBL/MASPs complexes have been proposed, some of them are presented briefly here (Fig. 3).

Crystallography and structural studies of both MBL and MASPs have uncovered the binding site of the complex. The MASPs bind on the MBL's collagen-like domain on the C-terminal side of the hinge region (kink) formed by an interruption in the Gly-X-Y repeat pattern (Davies et al. 2003; Wallis et al. 2004). The binding site of MASPs lies within the three N-terminal domains (CUB1-EGF-CUB2) (Wallis 2007) (Fig. 2a). Studies have shown that all three N-terminal domains are necessary to reproduce the binding properties of full-length proteins. The CUB1-EGF domain alone can bind to MBL, but with lower affinity (Wallis 2007). It should be mentioned here that the binding sites for MASP-2, MASP-1 and -3 overlap, but are not identical (Wallis et al. 2004). A slight loss in affinity has been observed when the N-terminal portion of MBL's collagen-like domain is removed, which suggests that there may be differences in the interactions of MBL and the three MASPs (Wallis et al. 2004).

The interaction between the collagenous region of MBL and the N-terminal domains of MASPs is calcium dependent and the critical sites are located within the CUB1-EGF fragment of the MASPs (Wallis 2007; Kjaer et al. 2015) (Fig. 2a). From the available crystal structures (of the CUB1-EGF-CUB2 domain), two binding sites for Ca^{2+} have been identified (Feinberg et al. 2003; Teillet et al. 2008), but it is not clear whether the interaction between MBL and MASPs depends on a single

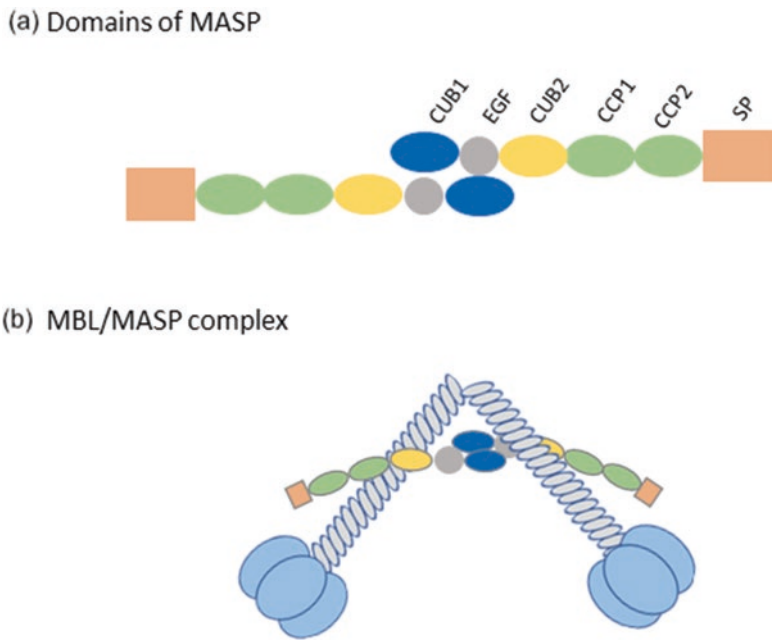

Fig. 2 Schematic model of MBL/MASP complex. (**a**) Domains of a MASP homodimer. (**b**) A schematic MBL oligomer is shown bound to a MASP dimer. The N-terminal domains CUB1-EGF-CUB2 of MASP bind to the collagen-like domain of the hinge region (kink) (not shown) of MBL (Davies et al. 2003; Wallis et al. 2004)

Ca^{2+} (Wallis 2007). The importance of Ca^{2+} has been shown in studies where experiments on MASP-2 deficiency (Stengaard-Pedersen et al. 2003; Sørensen et al. 2005) revealed that an amino acid substitution at position 105 (D105G) in the CUB1 domain of MASP-2 results in lower serum levels and failure of the protein to associate with MBL or ficolins. Asp105 provides one of the ligands for Ca^{2+} at site II in the CUB1 domain and the observed phenotype of the mutation could be explained with reduction in the MASPs ability to bind Ca^{2+} at site II, suggesting that Ca^{2+} is critical for MBL binding (Wallis 2007). The importance of Ca^{2+} has been shown also in studies of MBL deficiency on the collagen-like domain where mutations of Lys46 and Leu47 disrupt the interaction with MASPs, leading to elimination of detectable complement fixation (Wallis et al. 2004). Besides Ca^{2+}, it has been observed that glycosylated residues (glycosylgalactosyl-5-hydroxylysine) are flanked at the binding site of MASPs, suggesting prevention of non-specific interactions with other macromolecules (Wallis 2007).

Most of the reports agree that the complex consists of an MBL oligomer and MASPs dimers (Feinberg et al. 2003; Gingras et al. 2011; Kjaer et al. 2015; Nan et al. 2017) (Fig. 2b), but the studies are contradicting whether MBL is complexed with one or two different types of MASPs. Mayilyan et al. (2006) reported that each oligomer in the circulation binds only one type of MASP, therefore MBL-$(MASP\text{-}2)_2$

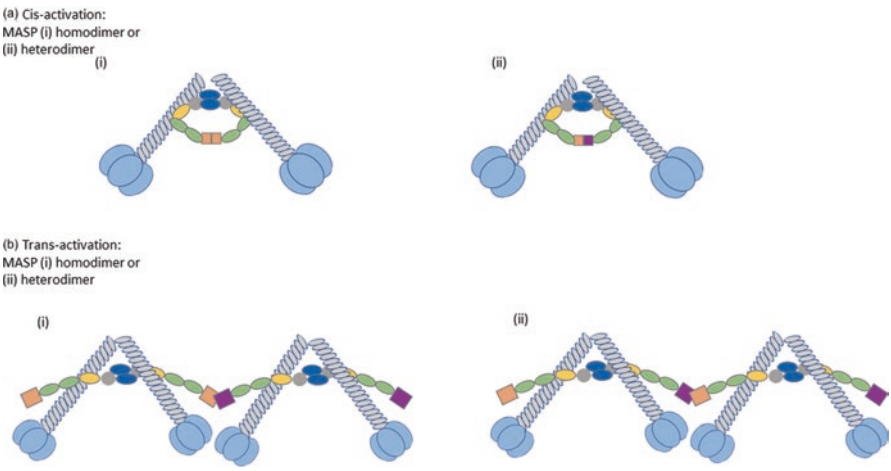

Fig. 3 Schematic models of suggested MBL/MASP complexes in the lectin pathway (LP). The suggested models include both MASP homodimers (orange/orange or violet/violet SP domains in one dimer) and heterodimers (orange/violet SP domains). (**a**) MASP dimer in MBL which forms a triangular structure. Flexibility between CUB2 and CCP1 domains of MASP will form a structure where the SP domains of the homo- or heterodimer are able to auto-activate themselves (cis-activation) upon MBL's binding to its target (Feinberg et al. 2003; Teillet et al. 2008). (**b**) Two MBL/MASP complexes where one MBL/MASP complex activates neighboring MASP molecules on an MBL/MASP complex. The inter-molecular activation is called trans-activation (Wallis et al. 2010; Degn et al. 2014)

or MBL-$(MASP\text{-}1)_2$ will occur rather than complexes with more than one type of MASPs (Fig. 3a(i)). This has been verified by other reports showing that dimers and the trimeric and tetrameric predominant oligomers of MBL bind to one homodimer of MASP (Wallis et al. 2004; Teillet et al. 2005). However, other studies have reported that in higher oligomers within a single MBL/MASP complex, different types of MASPs can be found (Degn et al. 2012, 2014) (Fig. 3a(ii)). In addition, it has been proposed that low-order MBL oligomers mainly associate with MASP-1 and Map 19, while higher oligomers preferentially form a complex with MASP-2 and MASP-3 (Dahl et al. 2001).

A variety of models have been proposed to explain the mechanisms of complement activation. However, a detailed description of the physical changes of the complex during complement activation has not been reported yet, mainly due to lack of structural information on the interactions between components. Feinberg et al. (2003) suggested that the MASP dimer fits in MBL by forming a roughly triangular structure where the CUB1-EGF-CUB2 dimer forms the base and the CCP1-CCP2-SP domains comprising the other two sides (Fig. 3a). Other structural studies have shown that MBL, in complex with dimeric MASP, obtains a quite well-defined cone-like structure (Kjaer et al. 2015), contrary to what has been reported for free MBL, which adopts a planar structure (Lu et al. 1990; Miller et al. 2012; Nan et al. 2017). The flexibility which is present at the collagen-CRD hinge in MBL and

possible further flexibility which is upstream at the putative collagen kink (Gal and Ambrus 2001), combined with the flexibility which probably exists in the EGF-CUB2 junction of MASP, would permit MBL to adapt spatially various ligand patterns (Gingras et al. 2011; Kjaer et al. 2015). In addition, flexibility between MASP domains would allow auto-activation of the SP domains (Fig. 3a). Some studies proposed flexibility between CUB2 and CCP1 of MASP which will form a "closed" MASP structure where the SP domains would be able to auto-activate themselves (Feinberg et al. 2003; Teillet et al. 2008). (Fig. 3a). In a recent report, Nan et al. (2017) showed that MASPs are much more flexible than previously thought, and suggested that the SP domains on MASPs could bend toward each other within the complex leading to intra-molecular MASP autoactivation upon MBL's binding to a mannose-coated surface. Intra-molecular autoactivation has also been shown in other reports, where it has been suggested that MASP in the complex can bend significantly and activate its partner, as occurs for the homologous to MASP, C1s and C1r within the C1q complex (Degn et al. 2012). Another possibility is the inter-molecular activation where an MBL/MASP complex activates neighboring MASP molecules on MBL/MASP complexes (Wallis et al. 2010; Degn et al. 2014). In this case, the MASP dimer protrudes roughly perpendicular from the primary axis of MBL and when the complex binds to its ligand the SP domain of the one complex will activate the other (Kjaer et al. 2015) (Fig. 3b). It should be highlighted that the proposed models of the above studies do not exclude one another; both intra- and inter-molecular interactions are possible for the activation and initiation of the complement lectin pathway (Fig. 3).

Despite the fact that numerous studies have been made on the MBL/MASP complexes, the exact composition of the complex and subsequently the mechanism of complement activation, remain unclear. Crucial steps to determine the structural aspects of the initiation complex are the understanding of how MASP CUB domains recognize the collagen stems in the MBL (Kjaer et al. 2013) and the understanding of the conformational changes of the serine proteases which lead to their activation and hence trigger the complement cascade.

Serum MBL Levels

MBL is produced primarily by the liver as an acute-phase reactant (Thiel et al. 1992), hence its blood level increases significantly in response to infection. The publication of the nucleotide sequence of MBL2 gene revealed several features in the 5′ region that were characteristic of acute phase proteins (Ezekowitz et al. 1988; Sastry et al. 1989; Taylor et al. 1989). These characteristics included a heat shock consensus element, three glucocorticoid responsive elements, and a sequence with a high degree of homology to amyloid A protein (Turner 1996). In 1992, Thiel et al. confirmed this finding by showing moderate increase in the concentration of MBL in plasma up to three-fold in patients after a major surgery, and in patients suffering a malaria episode (Thiel et al. 1992). Additional measurements from other studies

have also confirmed that MBL is indeed an acute phase protein. However, it should be noted that in comparison with the classical acute phase reactant C-reactive protein, the increase in MBL levels is modest, therefore, MBL can be only characterized as a weak acute phase reactant (Petersen et al. 2001).

MBL circulates in the serum with median levels of about 1.2 μg/mL (Garred et al. 1992); however, it has been observed that the concentration varies greatly between individuals (Turner 1996) from below 50 ng/mL to above 10 μg/mL. This variation between individuals originates primary from some identified polymorphisms in the coding sequence and promoter of the MBL gene (Turner 1996).

MBL Genetics and Deficiency

Deficiency of MBL is probably the most common human immunodeficiency and is associated with an increased risk of infections, especially in young children and in immunocompromised individuals (Summerfield et al. 1995; Petersen et al. 2001; Turner 2003). It has been reported that the deficiency may occur in about 15% of the population (Eisen et al. 2008), even though the MBL level cutoff for defining deficiency has not been agreed in the literature. Deficiency has been defined variably as <1000, <500, <200, or <100 ng/mL (Nuytinck and Shapiro 2004; Altorjay et al. 2010). The resolution of the molecular structure of the human MBL2 gene (Sastry et al. 1989; Taylor et al. 1989) revealed the molecular basis of MBL deficiency, which for approximately 20 years was measured functionally as defective yeast opsonization (Turner 1996).

Organization of the Human MBL2 Gene

Two human MBL genes have been found, MBL1 and MBL2, where MBL1 is a pseudogene (Guo et al. 1998) and only MBL2 encodes a protein product (Fig. 4); rodents have two distinct functional genes, known as mbl-a and mbl-c encoding two different forms of MBL. The mbl-a and mbl-c genes are located on different chromosomes, 14 and 19, respectively (White et al. 1994). In human the two genes are closely positioned on chromosome 10 (10q11.2-q21) (Sastry et al. 1989; Guo et al. 1998). MBL-2 comprises four exons interrupted by three introns (Fig. 4). Exon 1 encodes the signal peptide, a cysteine-rich domain and seven copies of a repeated Glycine-Xaa-Yaa motif typical for the triple helix formation of collagen structures. Exon 2 encodes the remainder of the collagenous region with 12 Glycine-Xaa-Yaa repeats. Exon 3 encodes a neck region and exon 4 a carbohydrate-binding domain (CRD) (Garred et al. 2006) (Fig. 4).

The promoter sequence of the MBL gene contains various elements which would be expected to enhance MBL transcription (Sastry et al. 1989; Taylor et al. 1989), indicating that MBL is an acute phase protein. The majority of MBL2 transcription

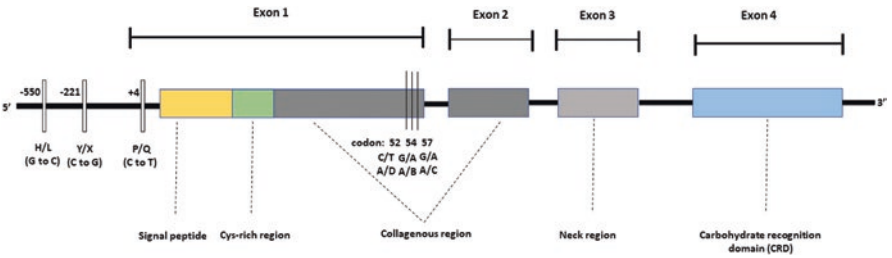

Fig. 4 Structure and organization of the MBL2 gene. Structural mutations and promoter polymorphisms are shown. MBL2 comprises four exons interrupted by three introns. Three base substitutions have been found in the collagen-like domain (at codons 52, 54 and 57) and three polymorphisms in promoter 1 at positions −550, −221, and in a 5′ untranslated region at position +4

is initiated by a promoter related to exon 1, although it has been found that the MBL2 gene contains an extra alternative promoter region approximately 1 kb upstream of exon 1, which may be responsible for 10–15% of MBL expression (Naito et al. 1999).

Deficiency

After resolving the molecular basis of the human MBL gene, mutations were found in the first of the four exons of the human gene (Super et al. 1989; Petersen et al. 2001). Six DNA polymorphisms in MBL2 gene are associated with variation in the expression of MBL2, and subsequently in the function of MBL. Three base substitutions have been found in the collagen-like domain and three polymorphisms in promoter 1 (Fig. 4).

The three mutations are at codon 52 (rs5030737; C>T; Arg>Cys), called D allele, codon 54 (rs1800450; G>A; Gly>Asp), called B allele and codon 57 (rs1800451; G>A; Gly>Glu), called C allele (Fig. 4). B, C and D alleles are referred to collectively as O, while A is the wild-type (Sumiya et al. 1991; Lipscombe et al. 1992; Madsen et al. 1994). The point mutation at codon 54 in exon 1 (B allele) causes a substitution of glycine with aspartic acid (GGC to GAC) (Sumiya et al. 1991). The structural substitution in codon 57 (C allele) causes a glycine to be replaced by glutamic acid (GGA to GAA) (Lipscombe et al. 1992); the third point mutation at codon 52 (D allele) causes a substitution of arginine with cysteine (CGT to TGT) (Madsen et al. 1994) (Fig. 4). Each of these mutations result in a similar phenotype: individuals who are heterozygous, A/B, A/C and A/D, for a mutant allele show a dominant decreasing effect on the functional levels of MBL serum. Specifically, studies have shown that heterozygotes for B and C alleles have an about ten times decrease in median protein concentration (Garred et al. 2003, 2016), while the D allele does not reduce the protein concentration to analogous degree (Garred et al. 2003).

All three variants contain a structural change in the collagen-encoded region of the molecule impairing the assembly and/or stability of the basic MBL structural unit; they do not effectively bind mannan and make probably the protein prone to degradation (Sumiya et al. 1991), resulting in low MBL serum concentration. Furthermore, the absence of a sufficient number of higher orders oligomers leads to inefficient complement-fixing activity of MBL (Yokota et al. 1995; Wallis and Drickamer 1999; Larsen et al. 2004). Specifically, it was suggested that the decreasing effect on the B and C alleles on the MBL serum concentration was due to incorrect assembly of the MBL triple helix, caused by disruption of the Gly-X-Y repeats of the collagenous region (Sumiya et al. 1991; Petersen et al. 2001), while the additional cysteine residue in D allele has been suggested to disrupt oligomer formation by generation of aberrant sulfide bonds (Wallis and Cheng 1999).

Additional to the structural mutation within the MBL2 gene, promoter polymorphisms have been identified. This identification showed that the level of MBL in plasma is also modulated at the transcriptional level (Madsen et al. 1995) and large inter-individual variations that have been observed may be explained by polymorphisms found in the promoter 1 region of MBL2 (Madsen et al. 1994, 1995). Particularly, the promoter polymorphisms that have been identified are: at position −550 (rs11003125; G>C; polymorphism H/L) and −221 (rs7096206; C>G; polymorphism X/Y), 7 and a P/Q variant in a 5'untranslated region at position +4, (C>T, rs7095891) (Madsen et al. 1998) (Fig. 4). Because of a linkage disequilibrium between the polymorphisms present in the promoter and the structural alleles in exon 1 of the MBL2 gene, only seven common haplotypes have been described (HYPA, LYPA, LXPA, LYQA, HYPD, LYPB and LYQC) (Madsen et al. 1998), and thus, there are 28 possible diplotypes, the frequency of which varies among populations (Garred et al. 2006). It has been shown that haplotypes with the wild-type A allele, HYPA and LYQA are related to high MBL levels, whereas LYPA is related to medium to low levels (Madsen et al. 1998; Garred et al. 2003).

Moreover, ethnic differences have also been studied and it has been observed that the occurrence of these haplotypes varies in different human populations (Nuytinck and Shapiro 2004), specifically low MBL level related genotypes exist in 10% of Caucasians and up to 40% of Africans (Madsen et al. 1995; Ezekowitz 2003; Garred 2008). It has been also reported that the B allele is frequent among Caucasians, South Americans and Asians; C allele is only frequent among Africans but is rare elsewhere, and D allele has low frequency among all populations (Garred et al. 2006).

The relation between MBL deficiency and several diseases has been reported in a significant amount of publications, where clinical significance of low serum levels of the protein has been described. It has been shown that MBL deficiency influences the susceptibility, and the course of diseases, including numerous types of infectious, autoimmune, cardiovascular diseases and even cancer (Nuytinck and Shapiro 2004).

MBL Associated with Diseases

An abundance of reports has been published which show correlation between MBL and disease. Many reports agree that MBL deficiencies are particularly associated with an increased risk for infections and autoimmune diseases; additionally, influence on the severity and course of several diseases has been shown (Kilpatrick 2002a, b; Eisen and Minchinton 2003; Summerfield 2003; Turner 2003). However, a lot of controversy is found in the literature about MBL's role in several diseases.

Certainly, MBL plays an important role during early childhood (6–18 months) when the adaptive immune system has not yet matured. In this age children are vulnerable to infection (Koch et al. 2001) and especially those who have been found homozygotic for MBL2 mutations. Immunosuppressed adult patients with autoimmune diseases or malignancies who carry MBL2 mutations have been also shown to have increased susceptibility to various-infectious related diseases (Neth et al. 2001; Schmiegelow et al. 2002; De Benedetti et al. 2007). Patients with co-existing low MBL levels and primary or secondary immune deficits are also prone to develop infectious diseases (Nuytinck and Shapiro 2004). On the other hand, MBL deficiency has been also associated with protection against diseases. MBL2 heterozygotic individuals may be protected against diseases such as tuberculosis (Hoal-Van Helden et al. 1999; Søborg et al. 2003).

The following brief overview will focus on selected examples of diseases that have been investigated for association of MBL deficiency (Table 1).

MBL and Infection

The main function of MBL is probably protection against pathogens which is achieved via opsonization and lysis (Super et al. 1989). MBL deficiency was first recognized as a functional opsonic defect in children with recurrent, unexplained infections (Super et al. 1989). Since then, many studies have been published about MBL's relation to infectious diseases.

Increased frequencies of MBL variant alleles have been found in patients with infections (Summerfield et al. 1995) and in patients with suspected immunodeficiency (Garred et al. 1995). Also, it has been shown that patients with insufficient MBL levels develop recurrent infections (Summerfield et al. 1995; Kakkanaiah et al. 1998; Garred et al. 2003; De Benedetti et al. 2007).

Acute respiratory tract infections seem to be associated with deficiency of the protein (Eisen 2010) (Table 1). It has been shown that MBL deficiency predisposes to invasive pneumococcal disease (Roy et al. 2002) and low serum levels caused by deficiency of the protein, are associated with increased death in patients with pneumococcal sepsis (Eisen et al. 2008). However, some reports have come to opposite conclusions about MBL's role in pneumococcal infection (Kronborg et al. 2002; Moens et al. 2006) and this association requires further investigation by

Table 1 Mannose-binding lectin and its association with various diseases

Disease field	Disease subtype/ subgroup	Outcome	Refs.
Respiratory Tract Infection	Pneumonococcal disease	O/O MBL2 genotype predispose to invasive pneumococcal disease	Roy et al. (2002)
Respiratory Tract Infection	Pneumonococcal disease	MBL deficiency doesn't seem to play a role in the outcome of invasive pneumococcal infection	Kronborg et al. (2002); Moens et al. (2006)
Respiratory Tract Infection	Pneumonococcal disease	Low MBL levels were associated with increased death due pneumococcal infection	Eisen et al. (2008)
Respiratory Tract Infection	Pneumonococcal disease	MBL2 genotypes are not involved in susceptibility to either P-CAP[a] or IPD[b]	García-Laorden et al. (2013)
Respiratory Tract Infection	Pneumonococcal disease	Role of MBL deficient genotypes in PM[c] (caused by *S. pneumoniae).* Children (<2 years) with MBL deficiency are in higher risk for PM	Bautista-Rodriguez et al. (2017)
Bacterial infection	Pneumonococcal infection	MBL does not drive lectin pathway activation on the surface of *S. pneumoniae*	Ali et al. (2012)
Respiratory Tract Infection	Pneumonococcal disease	Low MBL production could be associated with IPD in children <2 years	Muñoz-Almagro et al. (2014)
Viral infection	Influenza A viral infection	*In vivo* studies on mice about protection of MBL from IAV infection. Suggesting MBL deficiency as a risk factor for IAV infection	Chang et al. (2010)
Viral infection	Influenza virus-related critical illness	MBL deficiency is not a risk factor for severe influenza infection in children and young adults	Levy et al. (2019)
Viral infection	HIV	There is an association between undetectable serum MBL concentrations and susceptibility to HIV infection. But the course of HIV infection does not seem to be influenced by the level of MBL	Nielsen et al. (1995)
Viral infection	HIV	Homozygous carriers of MBL alleles are at increased risk of HIV infection	Garred et al. (1997)
Autoimmune diseases	Systemic lupus erythematosus	Increased frequency of MBL allele (codon 54) in patients with SLE, but it represents a minor risk factor for this disease. Studies in British SLE patients	Davies et al. (1995)
Autoimmune diseases	Systemic lupus erythematosus	MBL allele represents a risk factor for SLE in Spanish population and may affect susceptibility in an additive way with C4 null alleles	Davies et al. (1997)

(continued)

Table 1 (continued)

Disease field	Disease subtype/ subgroup	Outcome	Refs.
Autoimmune diseases	Systemic lupus erythematosus	MBL mutation on codon 54 may seems to be a minor risk factor for SLE. Study on SLE Chinese patients	Lau et al. (1996)
Autoimmune diseases	Systemic lupus erythematosus	Deficiencies of MBL predispose individuals to SLE. Study in SLE black patients	Sullivan et al. (1996)
Autoimmune diseases	Systemic lupus erythematosus	Not a significant association between MBL deficiency and SLE	García-Laorden et al. (2003)
Autoimmune diseases	Systemic lupus erythematosus	Patients with low MBL-producing genotype have a predisposition to develop SLE. Study in Indian females	Panda et al. (2013)
Autoimmune diseases	Systemic lupus erythematosus	A tendency of higher frequency of the B allele was observed in Spanish patients with SLE	Losada López et al. (2016)
Autoimmune diseases	CVD and SLE	MBL variant alleles are associated with an increased risk of arterial thrombosis. Study in Danish patients with SLE	Øhlenschlæger et al. (2004)
Autoimmune diseases	CVD and SLE	MBL deficiency is not determinant of CVD in SLE patients, independent of other risk factors	Kieninger-Gräfitsch et al. (2020)
Virus infection	Influenza	MBL doesn't increase susceptibility to severe influenza infection in pediatric patients	Levy et al. (2019)
Bacterial infection	Pulmonary tuberculosis	MBL-2 gene polymorphisms may be involved in the pathogenesis of PTB (pulmonary tuberculosis) and serum may be a biomarker for the diagnosis of PTB	Tong et al. (2019)
Autoimmune diseases	RA	MBL2 polymorphisms at codon 52, 54 and 57, as well as at promoter position −220, were not associated with increased risk to RA. (Meta-analysis)	Epp Boschmann et al. (2016)
	Neonatal sepsis	MBL is protective toward the development of neonatal sepsis and low MBL levels at birth are associated with an increased risk of hospital-acquired sepsis in infants	De Benedetti et al. (2007)
	Neonatal sepsis	Low MBL levels were not associated neither with gestational age or sepsis in infants	Hartz et al. (2018)
	Sepsis	MBL2 exon polymorphisms with low serum levels of MBL increase the risk of sepsis infection and septic shock in pediatric patients	Fidler et al. (2004)

(continued)

Table 1 (continued)

Disease field	Disease subtype/ subgroup	Outcome	Refs.
	Neonatal sepsis	MBL levels below 400 ng/mL increase the chances of developing sepsis	Dzwonek et al. (2008)
	Neonatal sepsis	No major impact on sepsis risk unless in infants between 32–36 gestational age	Hartz et al. (2017)
	Neonatal sepsis	The B allele of MBL2 exon 1 gene is an important risk factor for development of sepsis in premature infants	Özkan et al. (2012)

[a]*P-CAP* pneumococcal community-acquired pneumonia
[b]*IPD* invasive pneumococcal disease
[c]*PM* pneumococcal meningitis

performing clinical studies, particularly on MBL's contribution to the phagocytosis of *Streptococcus pneumoniae* and *S. aureus* (Eisen 2010).

It has been shown that MBL is able to protect from certain viral infections. MBL binds to microbial surface glycosylation residues and targets influenza A virus via direct neutralization in an complement independent-manner (Kase et al. 1999). Studies have shown MBL's protection also *in vivo* and it has been suggested that MBL deficiency may be a risk factor for influenza A virus infection (Chang et al. 2010). Nevertheless, in a recent report where 420 patients with confirmed influenza-critical illness were studied, MBL deficiency was not found to be a risk factor for very severe influenza infection in children and adolescents (Levy et al. 2019) (Table 1).

The role of MBL in HIV-1 infection has been studied extensively and many clinical studies have reported that MBL serum levels increase during infection. MBL recognizes the HIV-1 via the gp120 surface glycoprotein of the virus (Ying et al. 2004) and it has been reported that MBL's insufficiency predisposes to susceptibility to HIV-1 infection (Nielsen et al. 1995; Garred et al. 1997) (Table 1).

Neonatal Sepsis

Due to the immature immune system of newborns and lack of experience of their maturing adaptive immune system, the innate immune response represents a vital first-line defense mechanism against infections in newborns. Therefore, genetic and/or developmental variations in the innate immune system are probably of high importance in modulating the predisposition to invasive infections (De Benedetti et al. 2007).

Numerous studies have investigated the role of MBL in sepsis, the majority of them indicate an increased risk of sepsis in MBL deficient infants (Table 1). In particular, MBL2 gene mutation, which leads to reduced serum levels and functional impairment of the protein, has been shown to be associated with this condition (Gordon et al. 2006). However, it should be pointed out that not all studies confirm

this association (Hartz et al. 2017, 2018). (Table 1). An explanation of this discrepancy may be methodological differences, such as variation in definitions of infection, the sample size which was small in most of the studies, differences in definitions of MBL deficiency, and finally different average of gestational age (Keizer et al. 2014a, b). Studies have shown that serum MBL levels are strongly correlated with gestational age and show a postnatal increase, similar to ficolins and other complement proteins (Schlapbach et al. 2010; Sallenbach et al. 2011). However, this has not been verified by a recent study where it was shown that gestational age had no major influence on MBL and MASP-2 levels most probably due to different inclusion criteria (Hartz et al. 2018).

In summary, the majority of the available evidence indicates that reduced MBL levels are a risk factor for sepsis in neonatal age group. The importance of this condition is not debatable; therefore, further research is needed to identify patients at high risk achieving early diagnosis and treatment of sepsis.

Autoimmune Diseases

Several studies have indicated an association of MBL deficiency with susceptibility to autoimmune diseases (Monticielo et al. 2008). Particularly, numerous studies reported an increased frequency of mutant MBL alleles or low levels of the protein in patients with SLE in different ethnicities (Davies et al. 1995, 1997; Lau et al. 1996; Sullivan et al. 1996; Panda et al. 2013) (Table 1). Nevertheless, data remains controversial; the precise effects of MBL deficiency in relation to the development of SLE and disease progression, as well as the role of MBL as a biomarker in assessing SLE activity remain unclear (Losada López et al. 2016). A study about association of MBL deficiency in SLE Spanish patients revealed that there is only a tendency to a greater frequency of the B allele (Losada López et al. 2016), while another study shows that there is not a significant association between SLE and MBL deficiency (García-Laorden et al. 2003) (Table 1). The major cause of death in SLE is cardiovascular disease (CVD). This disease has also been reported to be associated with MBL deficiency (Øhlenschlæger et al. 2004; Font et al. 2007) (Table 1). On the other hand, several reports are showing that there is no such link (Larsen et al. 2018) (Table 1). Also, a recent publication that studied whether MBL deficiency, based on the blood concentrations (<1000 ng/mL), is associated with an increased incidence of CVD in SLE patients, showed that this is not the case (Kieninger-Gräfitsch et al. 2020) (Table 1). Large prospective studies with long follow-ups would be required to exclude definitely a role of MBL in SLE-associated CVD, as also for SLE.

MBL Replacement Therapy

For the past 30 years, MBL replacement therapies have been under development, using either plasma derived MBL (pdMBL) or recombinant MBL (rMBL). Some small case studies with pdMBL on MBL-deficient patients in the past years showed that opsonic activity was restored after receipt of MBL infusion (Valdimarsson et al. 1998; Garred et al. 2002). However, these case reports were performed with modest numbers of patients and a conclusion could not be drawn about the significant effect of pMBL. Nevertheless, phase I and early phase II studies have been performed with pdMBL which have established the plasma derived MBL infusions' safety and efficacy in MBL-deficient individuals (Valdimarsson 2003; Valdimarsson et al. 2004; Brouwer et al. 2009; Frakking et al. 2009).

Phase I studies have been also performed with rMBL (Petersen et al. 2006), which seems to preserve full restoration of functionality of the lectin pathway (Keizer et al. 2018), contrary to pdMBL (Keizer et al. 2014a, b), and therefore it has been proposed to consider new clinical substitution studies using rMBL instead (Keizer et al. 2018).

Conclusions and Perspectives

Unquestionably, MBL is a major component of the innate immunity, taking part in very important functions of the immune system. Therefore, it is reasonable that its deficiency has been associated with numerous different diseases. Association with increased susceptibility to certain infections and autoimmune diseases has been found in individuals who carry MBL2 allelic variants which predispose to low MBL serum levels; these individuals have either an unmatured immune system, carry a coexisting primary or secondary immune deficiency, or are immunocompromised. In those cases, low serum levels of MBL could be a potential biomarker and could be used to identify individuals at increased risk of developing disease. However, more research is needed to standardize MBL measurements (Kilpatrick 2003); this could be achieved by better understanding of the relation between genetic variants and MBL serum levels and determination when MBL serum concentration is associated with clinical relevance (Eisen et al. 2004).

Additionally, the role of MBL in the susceptibility to various diseases is often controversial. Potential reasons of inconsistencies among studies are the focus on the MBL2 gene polymorphisms, the relatively small study populations and the choice of controls (Keizer et al. 2014a, b). It has been observed that the frequencies of MBL polymorphisms show some variability within different populations or ethnicities, hence the selection of the control population is a crucial point. Generally, further cohort studies have to been performed with consistent parameters and greater number of study populations. Moreover, the number of clinical studies is still reduced and the majority of the past clinical studies that have been reported used a modest number of patients. Therefore, larger well-designed trials are necessary.

Finally, MBL replacement therapy may be an important application for the future to reduce the risk of certain diseases in children or immunosuppressed patients. For the better development of effective therapies, a better understanding of the structure of MBL, oligomerization and structural association with its serine proteases is important (Miller et al. 2012).

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Activities of MASPs, The Complement Proteases Associated with Collectins and Ficolins

Péter Gál and József Dobó

Brief History

Mannose-binding lectin (MBL)-associated serine proteases (MASPs) are the enzymatic components of the supramolecular complexes that are responsible for the activation of the lectin pathway of the complement system. The pattern recognition subunit of these complexes can be MBL, ficolins 1, 2, 3 (aka M-, L-, H-ficolins), collectin kidney 1 (CL-K1, aka CL-11) or collectin liver 1 (CL-L1, aka CL-10). Historically, the first clue that such complexes exist came from the discovery of the Ra-reacive factor (RaRF), a bactericidal factor that binds to Ra chemotype strains of Salmonella (Kawakami et al. 1982). Later, it turned out that RaRF is a complex consisting of MBL and a serine protease (P100, later named MASP-1) capable of cleaving both C4 and C2, consequently activating the complement cascade very similarly to the C1 complex of the classical pathway (Matsushita and Fujita 1992; Takayama et al. 1999). This discovery dismissed the earlier view that MBL is associated with C1r and C1s and activates the complement cascade through the classical pathway (Ikeda et al. 1987). When a second MASP (MASP-2) was discovered (Thiel et al. 1997) and it was shown that this minor component of the complex is actually responsible for C4 and C2 cleavage, it was tempting to believe that MASP-1 and MASP-2 act like C1r and C1s in the C1 complex. It was also shown in *in vitro* experiments, however, that MASP-2 can autoactivate and therefore it can initiate the complement cascade independently from any other proteases (Vorup-Jensen et al. 2000). The view that MASP-2 alone is sufficient for complement activation was the "central dogma" of the lectin pathway for about a decade and degraded the status of MASP-1 as being a supporting enzyme only. Finally, the picture became even more complicated when the third MASP enzyme (MASP-3) was discovered (Dahl et al. 2001). MASP-3 binds to collectins and ficolins but it

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does not cleave any downstream complement components (i.e. C4, C2, C3); it seemed that its proteolytic activity does not contribute to complement activation. Interestingly, no physiological inhibitor of MASP-3 has been identified.

Nevertheless, the domain structures of C1r, C1s, MASP-1, MASP-2 and MASP-3 are identical: the serine protease (SP) domain is preceded by five non-catalytic domains in the order CUB1-EGF-CUB2-CCP1-CCP2-SP (Fig. 1). This fact and other data indicate that these proteases are evolutionary closely related (Gál et al. 2007; Fujita et al. 2004). In which the MASPs differ from C1r and C1s is that truncated forms of MASPs have been identified in the circulation. MAp 19 (aka MAP-2, sMAP) is an alternative splice product of the *MASP2* gene containing the CUB1-EGF domain pair plus four unique C-terminal residues (Stover et al. 1999; Takahashi et al. 1999). Similarly, MAp 44 (aka MAP-1) is an alternative splice product of the *MASP1* gene consisting of the CUB1-EGF-CUB2-CCP1 domains and a 17 amino-acid-long peptide at the C-terminus (Degn et al. 2009; Skjoedt et al. 2010). MASP-3 is also an alternative splice product of the *MASP1* gene, the two proteases have identical non-catalytic domains (CUB1-EGF-CUB2-CCP1-CCP2) but they have individual SP domains. The *MASP1* gene is located on chromosome 3q27–28 in human. The level of the expression of the three distinct gene products is quite different depending on the site of the synthesis; MASP-1 is expressed mainly in the liver, MAp 44 is expressed almost exclusively in the heart, while the mRNA of MASP-3 can be detected in many tissues including liver, colon, heart, skeletal muscles, prostate, lung, and cervix. The human *MASP2* gene was mapped to chromosome 1p36.2–3 and the mRNAs of the *MASP2* gene products were detected almost exclusively in the liver. In the recent years, many functions of the different MASPs have been discovered. Most functions are connected to the activation of the complement system, but some functions reach far beyond the traditional complement cascade, and they have not yet been clarified entirely.

The Role of MASPs in Lectin Pathway Activation

The activation of the lectin pathway results in the generation of the classical/lectin pathway C3 convertase complex: C4b2a. The only enzyme, which is capable of cleaving C4 in the lectin pathway is MASP-2. The catalytic efficiency of C4 cleavage has been determined by independent research groups using enzymes from different sources (Ambrus et al. 2003; Rossi et al. 2001; Chen and Wallis 2004) (Table 1). These data show that MASP-2 is a very efficient enzyme at C4 cleavage (the k_{cat}/K_m values are in the 10^5–10^7 M^{-1} s^{-1} range), it is even more efficient than C1s. Kinetic and structural data prove that the CCP domains contribute to the efficient C4 cleavage through providing exosite for the substrate binding (Ambrus et al. 2003; Duncan et al. 2012; Kidmose et al. 2012). Experiments using chimeric molecules containing domains of C1s and MASP-2 proved that the CCP domains of MASP-2 are responsible for the superior C4 cleavage due to their high substrate recognition efficacy (Rossi et al. 2005). The high catalytic power of MASP-2

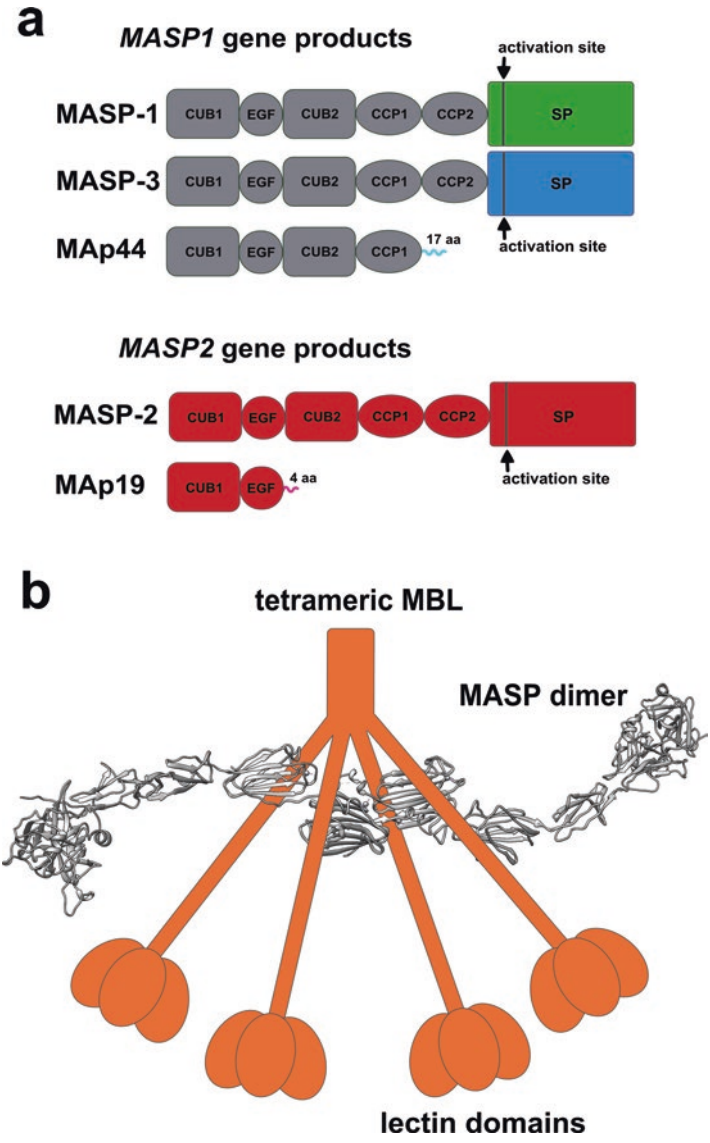

Fig. 1 Domain structure of MASPs and the structure of the MBL-MASP complex. (**a**) MASP-1, MASP-2 and MASP-3 share the same domain organization. The N-terminal CUB (**C**1r/C1s, sea urchin **U**egf and **b**one morphogenetic protein-1) domain is followed by an EGF (epidermal growth factor)-like domain and a second CUB domain. The catalytic region of these proteases consists of two CCP (complement control protein) domains and an SP (serine protease) domain. The one-chain zymogen form is converted to the active form by limited proteolysis at the activation site (arrow). (**b**) Schematic representation of an MBL-MASP complex consisting of a tetrameric MBL and a MASP dimer. Two MASP molecules form a dimer through the CUB1-EGF-CUB2 region and bind to the collagenous stalks of the MBL molecule. The catalytic regions (CCCP1-CCP2-SP) of the MASP protomers are protruding from the complex making possible the cleavage of the protein substrates (C2, C4)

Table 1 Specificity constants for proteolytic cleavage of C2, C4 and C3 by C1s and MASPs

		k_{cat}/K_m (M^{-1} s^{-1})			
Enzyme	Source	C2	C4	C3	References
C1s	Human plasma	4.3×10^5	2.45×10^6	ND[a]	Rossi et al. (2001)
MASP-2cf[b]	Insect cells	1.12×10^6	5.67×10^7	ND[a]	Rossi et al. (2001)
MASP-2	Insect cells	7.5×10^5	2.97×10^7	ND[a]	Rossi et al. (2001)
MASP-2cf[b]	*E. coli*	5.0×10^5	5.5×10^5	3.5×10^2	Ambrus et al. (2003)
MASP-2 CCP2-SP	*E. coli*	1.3×10^6	5.7×10^6	ND[a]	Ambrus et al. (2003)
rat MASP-2K[c]	CHO cells	1.3×10^6	2.8×10^7	ND[a]	Chen and Wallis (2004)
MASP-1cf[b]	*E. coli*	3.0×10^5	NS[d]	3.0×10^2	Ambrus et al. (2003)
rat MASP-1ent[e]	CHO cells	3.4×10^5	–	ND[a]	Chen and Wallis (2004)

[a]*ND* not determined
[b]*MASP-1/2cf* catalytic fragment containing the three C-terminal domains (CCP1-CCP2-SP)
[c]*MASP-2K* catalytically active MASP-2 mutant, in which the Arg^{424} was replaced with a Lys at the activation site
[d]*NS* not significant
[e]*MASP-1ent* catalytically active MASP-1 mutant, in which the ^{425}Lys-His-Ile-Ser-Arg sequence at the activation site was replaced with the recognition sequence of the enterokinase (Asp-Asp-Asp-Asp-Lys)

enables this enzyme to promote efficient lectin pathway activation in spite of its relatively low serum concentration (about 6 nM, 0.4 μg/mL) (Thiel et al. 2012). MASP-2 cleaves C2 with approximately the same rate as C1s. MASP-1 also cleaves C2, although with slightly lower efficiency than C1s and MASP-2. Taking into account the high serum concentration of MASP-1 (about 143 nM, 11 μg/mL) we can presume, that MASP-1 contributes significantly to the formation of the C3 convertase through C2 cleavage. Indeed, it was shown that MASP-1 generates about 60% of C2a present in the lectin pathway C3 convertases (Héja et al. 2012). Since MASP-1 does not cleave C4, it cannot generate C3 convertase complex alone. In *in vitro* experiments it was shown that complexes reconstituted from recombinant MASP-2 and MBL bind to mannan-coated surface and this binding induced the autoactivation of zymogen MASP-2 (Vorup-Jensen et al. 2000). The autoactivation ability of MASP-2 was verified by other laboratories, although the autoactivation capacity of MASP-2 is much less pronounced than that of MASP-1 (Rossi et al. 2001; Chen and Wallis 2004; Gál et al. 2005). Autoactivation of MASP-2 requires much higher concentration than the physiological one and a long incubation at 37 °C. MASP-1 zymogen, however, autoactivates about 3200-times more efficiently than MASP-2 zymogen indicating that MASP-1 is the first enzyme that autoactivates in the course of lectin pathway activation (Megyeri et al. 2013).

The first evidence indicating that MASP-1 plays a pivotal role in the activation of the lectin pathway came from the experiments using serum of MASP1/3$^{-/-}$ mouse (Takahashi et al. 2008). Although the authors claimed that there was a low level lectin pathway activation in this serum, their results showed practically no C4 deposition on mannan-coated surface. Since MASP-2 is the only enzyme in the lectin pathway which can cleave C4, it indicates that there is no active MASP-2 in

the absence of MASP-1. Addition of recombinant MASP-1 restored the lectin pathway activity in the serum of MASP1/3$^{-/-}$ mouse. Finally, using selective inhibitors against MASP-1 and MASP-2 unambiguously proved the central role of MASP-1 in the lectin pathway activation (Héja et al. 2012). SGMI-1, the highly specific MASP-1 inhibitor completely inhibited the lectin pathway in normal human serum. However, when the MASP-1 and MASP-2 proteases were pre-activated before the C4 deposition assay, only the MASP-2 inhibitor (SGMI-2) was efficient at attenuating the lectin pathway. These results clearly show that MASP-1 activates MASP-2 in the course of lectin pathway activation. Although MASP-2 can autoactivate *in vitro* under artificial conditions, this ability does not manifest under physiological conditions in normal human serum. It could be the consequence of its low serum concentration (the autoactivation of MASP-2 requires high enzyme concentration) and the fact that each MASP-2 is surrounded by multiple MASP-1 molecules on the activation surface (the molar ratio of MASP-1 to MASP-2 is approximately 24 to 1 in normal human serum). According to the current model of lectin pathway activation the first enzymatic step is the autoactivation of MASP-1 (Fig. 2). Activated MASP-1 then cleaves MASP-2 which in turn cleaves C4 into C4a and C4b. Deposited C4b binds C2 and the C4b-bound C2 is cleaved by both MASP-1 and MASP-2. This mechanism was corroborated by using serum of a 3MC syndrome patient whose blood lacks both MASP-1 and MASP-3 due to a mutation in the *MASP1* gene (Degn et al. 2012). The serum of this patient does not show lectin pathway activity (there is no C4 deposition), although it contains normal level of MASP-2. The lectin pathway activity could be restored by adding recombinant MASP-1, confirming that MASP-1 is indispensable to activate MASP-2 under physiological conditions. This mechanism was further confirmed by determining

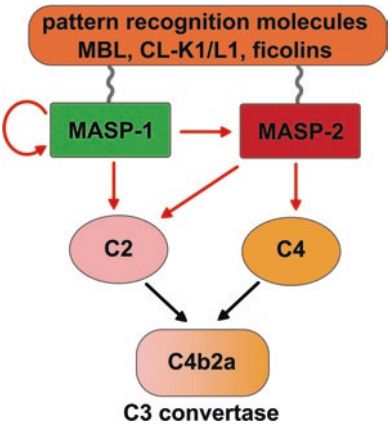

Fig. 2 Mechanism of lectin pathway activation. When the initiation complexes bind to the activator surface, MASP-1 autoactivates and then it activates neighboring MASP-2 molecules. MASP-2 cleaves C4, while C2 is cleaved by both MASP-1 and MASP-2. Red arrows represent proteolytic cleavage pointing from the enzyme to the substrate, while black arrows simply indicate conversion

the rate constants of the autoactivation and cross-activation reactions between MASP-1 and MASP-2 (Megyeri et al. 2013). The autoactivation capacity of zymogen MASP-1 ($k_{cat}/K_m = 4.5 \times 10^2\ M^{-1}\ s^{-1}$) is three-orders of magnitude higher than that of the zymogen MASP-2 ($0.14\ M^{-1}\ s^{-1}$). Activated MASP-1 cleaves zymogen MASP-2 very efficiently ($1.2 \times 10^4\ M^{-1}\ s^{-1}$); actually its rate constant is two orders of magnitude higher than that of the autocatalytic activation of MASP-2 ($6.0 \times 10^2\ M^{-1}\ s^{-1}$) where active MASP-2 cleaves zymogen MASP-2. The rate constants quantitatively highlight the central role of MASP-1 in the initiation of the lectin pathway.

The previously presumed analogy between the proteases of the classical and the lectin pathway has revived, although this analogy is far from perfect. C1r and C1s are present in equimolar concentration in the serum, while MASP-1 is much more abundant than MASP-2. C1r and C1s are present in the same complex with a well-defined stoichiometry as a tetramer ($C1qC1r_2C1s_2$), while MASP-1 and MASP-2 form dimers and mostly occupy separate complexes (e.g. distinct MBL-$(MASP\text{-}1)_2$ and MBL-$(MASP\text{-}2)_2$ complexes) (Dahl et al. 2001; Teillet et al. 2005; Mayilyan et al. 2006). C1r activates C1s inside the C1 complex, while MASP-1 activates MASP-2 probably on the activation surface where the distinct complexes bind next to each other (Degn et al. 2014). Another significant difference is that the function of C1r confines to the activation of C1s, whereas MASP-1 cleaves C2 beside MASP-2 during lectin pathway activation. The presence of the non-enzymatic components (MAp 19 and MAp 44) in the activation complexes of the lectin pathway may provide a regulation that does not exist in the classical pathway.

The above mechanism is valid for the human system. In other species, the enzymatic properties of MASPs can be different. In chicken, for example, MASP-1 is missing but there is a MASP-2-driven lectin pathway (Lynch et al. 2005). It seems very likely that the autoactivation capacity of chicken MASP-2 is much higher than that of human MASP-2, and the activation of chicken MASP-2 does not depend on the activation of other protease. In a mouse model of ischemic brain injury, it was suggested that a MASP-2-driven, MASP-1-independent C4-bypass activation route is responsible for the brain tissue damage (Orsini et al. 2016). The MASP-$2^{-/-}$ mice exhibited significantly reduced infarct volumes compared to the wild type mice, while the MASP-$1/3^{-/-}$ mice were not protected from the ischemia-reperfusion injury (IRI). It cannot be excluded that *in vivo*, during cerebral IRI MASP-2 activates on its own in the MASP-$1/3^{-/-}$ mice, however, *in vitro* experiments with MASP-1 deficient mouse sera do not support this. In the MASP-1 deficient sera no C4 deposition could be detected on mannan- or acetylated BSA-coated surface, although active MASP-2 is an extremely efficient protease at C4 cleavage (Machida et al. 2018). Recently, genome editing by CRISPR/Cas9 system was used to generate mice that mono-specifically lacked MASP-1 in the circulation while the expression of MASP-3 was intact (Hayashi et al. 2019). The sera of these mice failed to elicit C4 deposition on mannan-coated plates, corroborating that MASP-1 is essential for lectin pathway activation. Even if we suppose that the marginal autoactivation ability of MASP-2 may play a role in certain pathophysiological situations, in the sera of wild type mice, where the abundant MASP-1 molecules surround the

MASP-2 molecules on an activation surface, the inhibition of MASP-1 could suppress the possible autoactivation of MASP-2. The physiological role of MASP-1 can only be assessed in wild type mice by selectively inhibiting MASP-1.

Role of the MASPs in the Alternative Pathway Activation

The three activation pathways of the complement system (the classical, the lectin and the alternative pathways) have been considered as independent ones, since they are triggered by different activation surfaces and they use distinct serine protease components. They converge at the cleavage of C3; and from this point, the complement activation proceeds through the unified terminal pathway generating the membrane attack complex (C5b-9). Several interconnections between the different pathways have already been known for a long time. For example, the classical and the lectin pathway generate the same C3 convertase complex by cleaving the same components (C4 and C2), and the alternative pathway acts as an amplifying loop for the classical and the lectin pathways. The alternative pathway ensures an efficient complement activation regardless of the initiation pathway which generates the first C3b molecules (Harboe et al. 2009). It was also shown that the analog components of the classical/lectin and alternative pathway C3 convertases (e.g. C4b, C3b and C2a, Bb) can substitute each-other to a certain degree in *in vitro* experiments (Laich and Sim 2001). In the recent years, however, unexpected novel connections between the alternative and lectin pathways have been discovered, which fundamentally changed our view about the "independent" activation routes.

As we mentioned above, in the serum of the MASP-1/3 knockout mice there is no functional lectin pathway since MASP-2 remains in its zymogen form. It turned out, however, that the alternative pathway activity is also missing (or at least greatly reduced) in these sera: it cannot lyse rabbit erythrocytes and there is no C3 deposition on zymosan-coated plates in Mg^{2+}-EGTA buffer (Takahashi et al. 2010). The reason of this surprising phenomenon is that factor D (FD), the protease which is responsible for cleaving C3b-bound factor B (FB) is also in its zymogen form (pro-FD) in the serum of MASP1/3$^{-/-}$ mice. Previously the general consensus was that pro-FD is activated at the site of its synthesis (in the adipocytes) and it is secreted as a processed enzyme into the blood. Indeed, in the serum only cleaved FD was detected previously. The fact that in the sera of the MASP-1/3 knockout mice pro-FD is not processed suggests that MASP-1 and/or MASP-3 are involved in the processing of pro-FD. The first assumption was that MASP-1 is responsible for the cleavage, since *in vitro*, using purified components, MASP-1 can activate pro-FD. This assumption seemed to be reasonable, because several lines of structural and functional evidence indicated that MASP-1 has a more relaxed substrate specificity than the other complement proteases, which have typically one or two substrates (Dobó et al. 2009). Addition of recombinant MASP-1 to the serum of the knockout animals, however, failed to restore the alternative pathway activity. Another study suggested that MASP-3 is the protease that processes pro-FD and even zymogen

MASP-3 is effective (Iwaki et al. 2011). The picture became more controversial when it was shown that in the serum of a 3MC syndrome patient, where the lectin pathway was non-functional, significant alternative pathway activity could still be detected (Degn et al. 2012).

In order to clarify the role of the MASPs in the pro-FD activation we checked the catalytic efficiency of the three MASPs at the cleavage reaction using purified recombinant enzymes (Oroszlán et al. 2016). The activated form of all three MASPs cleaved pro-FD quite efficiently; the k_{cat}/K_M values were at the 10^3 M^{-1} s^{-1} range: MASP-1, 3.9×10^3; MASP-2, 7.2×10^3; MASP-3, 4.7×10^3. The zymogen form of MASPs, however, did not show significant activity. In the next experiment we added fluorescently labeled pro-FD to human serum and plasma and followed the conversion. Pro-FD was processed in plasma with a half life of ~5 h and in the serum with a half life of ~3 h. The fact that in EDTA plasma pro-FD is cleaved, unequivocally proves that there is a protease in the blood that is able to convert pro-FD into FD prior to the activation of the complement or the coagulation system. Addition of active MASP-1 and MASP-2 did not change the half life of pro-FD. It is very likely that the serpins, especially C1-inhibitor, immediately inactivated these proteases in the plasma. On the other hand, active MASP-3 (200 nM) was very effective: it reduced the half life of pro-FD from ~5 h to ~20 min. Obviously, MASP-3 preserved its activity which is in agreement with the fact, that there is no known inhibitor of this protease in the blood. The experiments using the selective inhibitors also confirmed the key role of MASP-3 in the pro-FD activation; SGMI-1 and SGMI-2 (MASP-1- and MASP-2-selective inhibitors) were inefficient while the MASP-3-selective inhibitor (TFMI-3) blocked the pro-FD conversion completely in plasma and partially in serum (Dobó et al. 2016).

We can conclude that MASP-3 is the exclusive activator of pro-FD in plasma or in "resting" blood, where none of the proteolytic cascade systems are triggered (Fig. 3). This mechanism ensures that nascent pro-FD is permanently activated in the blood even before the appearance of any danger signal. In MASP-3 deficient patients complement and coagulation proteases can serve as back up enzymes providing active FD locally, at the site of infection or injury. It was also shown, that in contrast to the previous assumptions, normal human plasma also contains some pro-FD besides the predominant FD (Wu et al. 2018). It is very unlikely though that MASP-3 can boost the alternative pathway during complement activation via cleaving the residual pro-FD at the activation surface, since only a small percentage of the normal FD level can support significant alternative pathway activity and there is no need to activate the entire pro-FD pool (Wu et al. 2018). The fact that only active MASP-3 can process pro-FD means that MASP-3 should be present in activated form in the circulation. Indeed, according to our measurements more than 70% of the MASP-3 molecules are activated in normal human plasma (Oroszlán et al. 2017). This means that zymogen MASP-3 is activated in the "resting" blood similarly to FD, although we do not know the mechanism yet.

Recently, a novel and unexpected link has been discovered between the lectin and the alternative pathway. We found that the proteolytic activity of MASP-1 is necessary for the efficient alternative pathway activation on LPS-covered surface

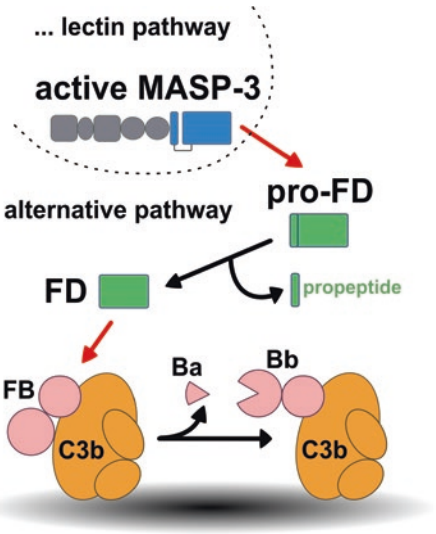

Fig. 3 The role of MASP-3 in the alternative pathway activation. MASP-3 is the exclusive activator of pro-FD in resting human blood. MASP-3 is present predominantly in its active form in the blood, and active MASP-3 cleaves and activates pro-FD. MASP-3 continually activates nascent pro-FD without the prior activation of the complement or the coagulation cascades. Red arrows represent proteolytic cleavage pointing from the enzyme to the substrate, while black arrows indicate conversion

while it has only little effect on zymosan-covered activation surface (Paréj et al. 2018). The MASP-1 specific inhibitor (SGMI-1) significantly reduced the C3 deposition on surfaces covered by LPS of different Gram-negative bacterial strains (e.g. E. coli, S. typhimurium, P. aeruginosa), while the effect was much less pronounced on yeast zymosan and it was negligible at lysing rabbit erythrocytes. A MASP-1 specific polyclonal antibody that was a strong inhibitor of the lectin pathway, also inhibited the LPS-driven alternative pathway, but barely affected the zymosan-driven alternative pathway activation. Depletion of MASP-1 from the normal human serum also confirmed the essential role of MASP-1 in the alternative pathway activation on activators representing the surfaces of Gram-negative bacteria. It seems obvious that MASP-1 directly (cleaving a complement component) or indirectly (activating a protease that cleaves a complement component, or inactivating an inhibitor of the alternative pathway) contributes to alternative pathway activation. We showed that MASP-1 does not cleave C3b-bound FB (does not activate the pro-convertase) and it is not the physiological activator of pro-FD. The MASP-1 specific inhibitor does not inhibit FD or the C3 convertase (C3bBb). On the other hand C3 is cleaved by both MASP-1 and MASP-2; although with low efficiency (Table 1). Interestingly, MASP-1 and MASP-2 cleaved C3i (C3 with a cleaved thioester bond) with 10–20 fold higher efficiency compared to the proteolysis of intact C3, but the physiological relevance of this reaction is unclear (Ambrus et al. 2003). It cannot be ruled out that the low C3 cleaving activity of

MASP-1 contributes to the initiation of the alternative pathway. We have shown, however, that the MASP-1 specific inhibitor attenuates the alternative pathway activation also on surfaces which are partially covered by C3b, suggesting that MASP-1 contributes to the amplification phase of the alternative pathway activation, as well. Taken together it is likely that the protein, which is cleaved by MASP-1 during the alternative pathway activation, is not among the core components of the alternative pathway.

The surface dependence of the MASP-1 action is also an intriguing question. Similar phenomenon was observed earlier using serum from properdin KO mice (Kimura et al. 2008). Properdin, which is a positive regulator of the alternative pathway, was essential for LPS-induced alternative pathway activation, while it was not necessary for zymosan-induced alternative pathway activation. It seems that zymosan, as a strong activator of the alternative pathway, does not need the contribution of properdin and MASP-1, while LPS as a weak activator does. It is very likely that the efficiency of the alternative pathway activation on a given surface depends on the ratio of the various activators and inhibitors present. In this respect it is worth mentioning that factor H (FH), the master regulator of the alternative pathway, binds to LPS more strongly than to zymosan. We can speculate that MASP-1 and properdin may counteract the action of FH on the LPS-covered surfaces.

Another study also supports the role of MASP-1 in the alternative pathway activation on bacterial surfaces. Serum fractions containing MBL-MASP-1 complexes were able to elicit C3b deposition on LPS-covered surface in the absence of C2, whereas complexes containing MASP-2 or MASP-3 failed to do so (Selander et al. 2006). Our experiments also demonstrated that inhibition of MASP-1 in C2-depleted serum attenuated C3b deposition on the surface of Gram-negative bacteria. Taken together, a large body of evidence shows that MASP-1 contributes to alternative pathway activation through a yet unknown mechanism. These findings further emphasize the role of MASP-1 in the antibody-independent defense against Gram-negative bacterial infection.

Cross-Talk Between the Lectin Pathway and Coagulation

The complement system is a proteolytic cascade system that is closely related to the other blood-borne cascade systems (i.e. contact, coagulation and fibrinolytic systems) (Krem and Di Cera 2002). In fact the serine proteases of the blood, all belonging to the chymotrypsin family (Family S1, MEROPS), form a network of proteases that can be divided into distinct cascade systems primarily for practical and didactical reasons only. We should keep in mind, however, that there are several interactions between these cascade systems, and the activation of one cascade system usually influences the activation of the others. (Markiewski et al. 2007; Conway 2015). For example, the complement and the coagulation systems are activated simultaneously at the site of the injury, where pathogen microorganisms

enter into the bloodstream and many interactions have been described between the two proteolytic cascade systems. Recently, a large body of evidences has accumulated indicating that the proteases of the lectin pathway, especially MASP-1, form important links between coagulation and complement (Dobó et al. 2014). It was shown that MBL-null mouse and MASP-1/-3 KO mouse both have prolonged bleeding times *in vivo* (Takahashi et al. 2011). Moreover, in disease models, it was demonstrated that these mice have significantly decreased $FeCl_3$-induced occlusive thrombogenesis (La Bonte et al. 2012; Pavlov et al. 2015).

MASP-1 is an atypical complement serine protease, since it has more substrates than other complement proteases (e.g. C1r, C1s, MASP-2). MASP-1 has been shown to influence the coagulation in several ways (Fig. 4). MASP-1 cleaves fibrinogen and factor XIII (plasma transglutaminase) promoting cross-linked fibrin formation (Hajela et al. 2002; Hess et al. 2012). MASP-1 cleaves the fibrinogen β-chain similarly to thrombin releasing the proinflammatory peptide fibrinopeptide B (Krarup et al. 2008). The fibrinogen α-chain, however, is cleaved differently by the two proteases. Factor XIII is also cleaved and activated by MASP-1, although the catalytic efficiency is lower compared to thrombin cleavage; the thrombin turnover rate of this substrate is about 650 times faster than that of MASP-1 under

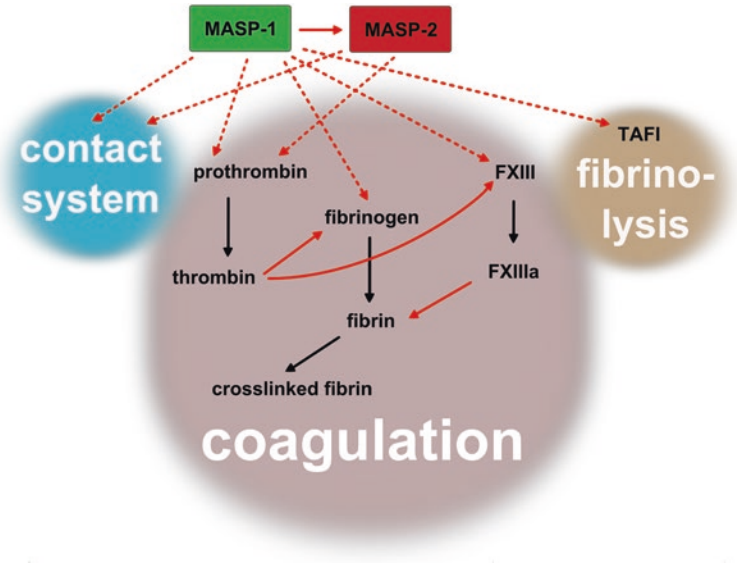

Fig. 4 Cross-talk between the lectin pathway and other blood-borne cascade systems. *In vitro* MASP-1 and MASP-2 can cleave certain components of the coagulation, contact and the fibrinolytic systems. The contribution of MASP-1 to the coagulation and thrombus formation was also corroborated *in vivo* by using mouse models. MASP-1 has thrombin-like activity and it was also shown to activate prothrombin. Red arrows indicate proteolytic cleavage pointing from the enzyme to the substrate, while black arrows indicate conversion. Dashed red arrows indicate cleavage reactions that were demonstrated *in vitro* but their *in vivo* relevance needs further confirmation

physiological circumstances. An interesting feature of the MASP-1 cleavage is that it prefers the Val34 (P4 residue) polymorphic variant of factor XIII to the Leu34 variant, while thrombin shows the opposite preference (Hess et al. 2012). It is speculated that this preference might be disadvantages since the activation of the Val34 variant may increase the probability of the development of unwanted thrombotic events. MASP-1 also cleaves thrombin-activatable fibrinolysis inhibitor (TAFI). Besides being an inhibitor of fibrinolysis, TAFI has direct impact on complement activation, since it is a carboxypeptidase (carboxypeptidase B), and it inactivates the anaphylatoxins (C3a, C5a) very efficiently (Nishimura et al. 2007). MASP-1 can also facilitate fibrin formation in an indirect way, via activating prothrombin. It was shown that addition of MASP-1 to whole blood and platelet-poor plasma samples accelerated the clotting process in a prothrombin-dependent manner (Jenny et al. 2015a). Using purified proteins, it was also demonstrated that MASP-1 cleaves and activates prothrombin in a unique fashion (different from the cleavage pattern of factor Xa and thrombin) generating several active alternative thrombin species (Jenny et al. 2015b). The coagulation promoting effect of MASP-1 was also demonstrated using a microvascular, whole blood flow model, which simulates blood flow through microchannels cultured with endothelial cells representing a situation close to human physiology (Jenny et al. 2018). Besides MASP-1, MASP-2 was also shown to activate prothrombin and to elicit fibrin deposition on the surface of the bacterium *S. aureus* (Krarup et al. 2007). Since MASP-1 is much more abundant than MASP-2, and MASP-2 is activated by MASP-1, it is very likely that MASP-1 plays the major role in the procoagulant activity of the lectin pathway. This procoagulant activity may represent an ancient form of innate immunity. The fibrin clot formation can prevent the spread of the microorganism in the body and facilitate its removal by phagocytosis.

A recently discovered intriguing aspect of the interaction between the complement and the coagulation cascades is that the platelet activation and fibrin formation resulted in the activation of MASP-1 and MASP-2 (Kozarcanin et al. 2016). Both activated platelets and fibrin clots bind lectin pathway components and activate the serine proteases, MASP-1 and-2, which can act as a positive feedback loop to boost the coagulation. This observation further supports the view that the lectin pathway is involved in thrombotic events. Although the exact mechanism has yet to be determined, it seems likely that platelets and fibrin clots activate the lectin pathway differently. The activated platelets generate mainly MASP/C1-inhibitor complexes while on the cross-linked fibrin predominantly MASP/antithrombin complexes can be detected. Taken together, we can conclude that the evolutionary related cascade systems act in a concerted way to eliminate dangerous particles and to restore homeostasis, however the concurrent overactivation of the complement and coagulation systems can lead to the development of disease conditions such as ischemia reperfusion injury, thromboinflammation and atherosclerosis (Ekdahl et al. 2016).

Endothelial Cell Activation by MASP-1

One of the important proinflammatory functions of the complement system is the activation of leukocytes and endothelial cells. The anaphylatoxins (C3a and C5a), soluble proteolytic products of the complement cascade activation, bind to their cognate receptors (C3aR, C5aR1, C5aR2). These receptors are G-protein coupled receptors (GPCRs) and elicit cell activation through increasing the intracellular calcium concentration and triggering signal transduction events mediated by protein kinases. Since MASP-1 and MASP-2 drive the activation of the lectin pathway, these proteases are indirectly responsible for cell activation. During coagulation, the split products of certain coagulation components (e.g. fibrinopeptides) trigger similar cell activation events. Thrombin, the executive protease of the coagulation, however, is able to activate endothelial cells and platelets directly, via the cleavage of protease activated receptors (PARs) on the cell surface (Coughlin 2000). The PARs are also GPCRs, which carry their own ligands (tethered ligand) on the N-terminal end of the polypeptide chain. These ligands become exposed after a protease cleaves in the N-terminal region and unmask a new N-terminus. Cleavage of PAR1 and PAR4 results in morphological changes in endothelial cells and trigger the release of cytokines and other vasoactive substances. Recently, it has been shown that MASP-1 is also able to cleave PARs and stimulate endothelial cells (Megyeri et al. 2009, 2014) (Fig. 5). Using peptide substrates representing the N-terminal regions of the PARs, the kinetic constants of the cleavage reactions have

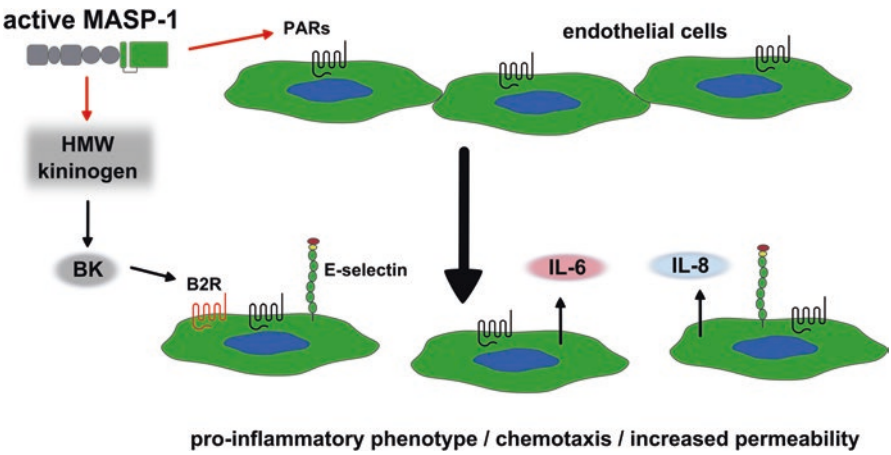

Fig. 5 The role of MASP-1 in endothelial cell activation. MASP-1 can activate endothelial cells by direct and indirect manner. MASP-1 cleaves PARs on the surface of the endothelial cells resulting in the release of chemotactic and pro-inflammatory cytokines, up-regulating E-selectin expression, and also changing endothelial permeability. MASP-1 also cleaves HK and liberates bradykinin. Bradykinin binds to the B2R receptor and induces pro-inflammatory changes in the endothelial cells. Red arrows indicate proteolytic cleavage pointing from the enzyme to the substrate, while black arrows indicate conversion or cellular processes

been determined. MASP-1 cleaved PAR1, PAR2 and PAR4 peptides quite efficiently (k_{cat}/K_M values are 1.1×10^4, 1.5×10^4 and 1.8×10^5 M^{-1} s^{-1}, respectively). Although thrombin is more efficient protease at PAR1 cleavage (1.6×10^6 M^{-1} s^{-1}) than MASP-1, the PAR4 substrate is cleaved by the two proteases with similar efficiency (k_{cat}/K_M value for thrombin: 5.3×10^5 M^{-1} s^{-1}). In cultured human umbilical vein endothelial cells (HUVECs), MASP-1 treatment elicited typical intracellular calcium response, in a similar manner and extent as thrombin. MASP-1 also induced NF-κB nuclear translocation and initiated p38 MAPK signaling. It was also demonstrated that MASP-1 cleaves PAR4 on the surface of HUVECs. These findings unequivocally proved that MASP-1 is able to cleave PARs and trigger pro-inflammatory reactions in the endothelial cells. Other studies corroborated the PAR cleavage-mediated pro-inflammatory role of MASP-1 in endothelial cells. Activation of various signal transduction pathways (e.g. p38 MAPK, pCREB, NFκB, JNK) resulted in cytokine production and altered adhesion molecule pattern in HUVECs (Jani et al. 2014, 2016). MASP-1 induced IL-6 and IL-8 production and it increased the expression of E-selectin and decreased that of ICAM-2. The supernatant of MASP-1-stimulated cells served as chemoattractant for neutrophil granulocytes and the altered adhesion molecule pattern of HUVECs resulted in increased adherence of differentiated PLB-985 cells (neutrophil granulocyte model cells). The neutrophil granulocytes are the predominant contributors to the early cellular response against invading pathogenic microorganisms. MASP-1 forms a link between the humoral and the cellular innate immune response in two ways: in a direct way through activating endothelial cells by PAR cleavage, and in an indirect way through recruiting neutrophil granulocytes by the secreted cytokines and increasing their adhesion to the endothelial cells. A very recent report has demonstrated that MASP-1 dose-dependently increased endothelial permeability (Debreczeni et al. 2019). This effect is mediated by PAR1 and Rho-kinase, and may foster the elimination of invading pathogens by facilitating the extravasation of soluble and cellular components of the immune system to the site of the infection.

A microarray-based transcriptome analysis of inflammation-related gene expression also confirmed the direct proinflammatory effect of MASP-1 on endothelial cells (Schwaner et al. 2017). Analysis of a set consisting of 884 inflammation-related genes showed that the proteolytic activity of MASP-1 changed the expression of 30 genes. The effect of MASP-1 on the gene expression was found to be a rapid process it occurred in the first 2 h of activation. The decisive role of the p38 MAPK and NFκB pathways in the MASP-1-induced pro-inflammatory process was also verified.

Another study demonstrated that MASP-1 can activate hepatic stellate cells (HSCs) and accelerate fibrosis progression in hepatitis C virus liver disease (Saeed et al. 2013). Although the mechanism of this activation has not been deciphered, it is likely that the cleavage of PARs plays a role, since the PARs are expressed at the protein level on quiescent stellate cells, and thrombin also exerts multiple actions on HSCs.

In order to identify other MASP-1 substrates among the serum proteins a proteomic approach was applied. 2D differential gel electrophoresis of MASP-1 treated

serum samples revealed that high-molecular-weight kininogen (HK) is a potential substrate of MASP-1 (Dobó et al. 2011). Using purified HK and MASP-1 we proved that MASP-1 cleaves HK and liberates the vasoactive peptide bradykinin (Fig. 5). Moreover, another study showed that MASP-1 treatment significantly up-regulated the expression of bradykinin receptor B2R in endothelial cells (Debreczeni et al. 2019). Bradykinin is a pro-inflammatory peptide, which is released from kininogens primarily by the proteolytic action of kallikreins. In the blood, plasma kallikrein generates the majority of bradykinin by cleaving HK. Bradykinin is a strong activator of endothelial cells inducing vasodilatation, increasing vascular permeability and triggering the production of second-generation inflammatory mediators. In patients with hereditary angioedema (HAE), typicly caused by C1-inhibitor deficiency, the uncontrolled kallikrein-mediated release of bradykinin results in recurrent tissue swellings. However, we cannot exclude that other proteases can also contribute to this process. Since C1-inhibitor also plays a role in the regulation of MASP-1 activity, the bradykinin generating ability of MASP-1 might contribute to the initiation of HAE attacks and worsening the disease symptoms. MASP-2 also cleaves HK but it does not liberate bradykinin. Plasma kallikrein cleaves HK much more efficiently than MASP-1; the specificity constant of the kallikrein-mediated cleavage (1.5×10^5 M^{-1} s^{-1}) is 375-fold higher than that of MASP-1-mediated cleavage (4.0×10^2 M^{-1} s^{-1}). We should keep in mind, however, that during complement activation, the local concentration of active MASP-1 on a surface can be much higher than in the serum, consequently MASP-1 can cause local endothelial activation by cleaving PARs and HK during infection or oxidative stress. In case of C1-inhibitor deficiency, however, the effect of MASP-1 might be systemic, and it can contribute to the elevated bradykinin level in HAE patients.

Regulation of MASPs

The natural inhibitors of the mammalian blood proteolytic cascade enzymes are almost exclusively serpins. Serpins are suicide inhibitors that change their conformation during the inhibitory reaction and form irreversible covalent enzyme-inhibitor complexes with the target proteases. C1-inhibitor inhibits the proteases of the classical and the lectin pathway: C1r, C1s, MASP-1 and MASP-2 (Davis et al. 2010). It is also a physiological inhibitor of the contact system inhibiting factor XIIa, factor XIa and plasma kallikrein. Antithrombin, an important inhibitor of the coagulation pathway, also inhibits MASP-1 and MASP-2 (Fig. 6). Heparin (and other glycosaminoglycans) can facilitate the formation of the serpin-protease covalent complexes. We have shown that C1-inhibitor and antithrombin are equally efficient inhibitors of the lectin pathway in the presence of heparin (Paréj et al. 2013). The IC_{50} values, determining through C4 deposition in 100-fold diluted normal human serum, are 35.6 and 41.3 nM for the C1-inhibitor and the antithrombin, respectively. Since both MASP-1 and MASP-2 are essential for the lectin pathway activation, inhibition of either protease is enough for the attenuation of lectin

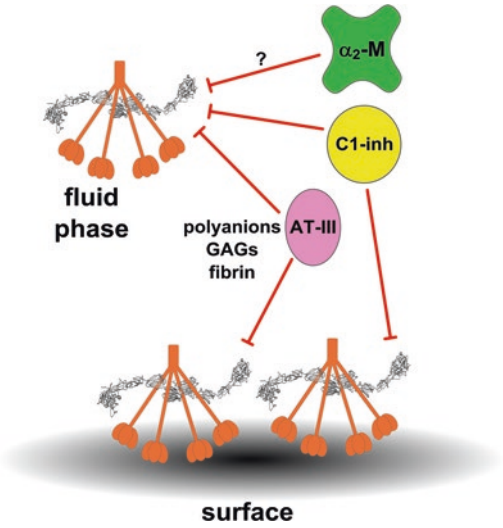

Fig. 6 Regulation of the lectin pathway. The major inhibitors of the lectin pathway are serpins. C1-inhibitor (C1-inh) is an efficient inhibitor of MASP-1 in the presence or absence of heparin. Antithrombin (AT-III) is even a better inhibitor, but only in the presence of heparin (or other glycosamino glycans (GAGs), polyanions, or fibrin). MASP-2 is very efficiently inhibited by C1-inhibitor, which is further enhanced in the presence of heparin. Antithrombin inhibits MASP-2 efficiently only in the presence of heparin. α_2-Macroglobulin (α_2M) is also a potential inhibitors of MASP-1 and MASP-2, but its inhibitory action has only been unequivocally proven in the fluid phase

pathway activation. Inhibition of both proteases at the same time confers an efficient prevention of lectin pathway activation. The second-order association rate constants (k_a) for the serpin-protease reactions were determined in the presence and absence of heparin. The MASP-1-C1-inhibitor reaction is not dependent on the heparin; the k_a values are approximately 6×10^3 M^{-1} s^{-1} in both cases (Dobó et al. 2009). The MASP-1-antithrombin reaction, however, is facilitated by heparin significantly; the k_a value increases from 1.4×10^3 M^{-1} s^{-1} to 4.0×10^4 M^{-1} s^{-1} in the presence of 50 μg/mL heparin. It seems that antithrombin in the presence of heparin is the best natural inhibitor of MASP-1. In the case of MASP-2, the effect of both serpins is heparin dependent. In the presence of heparin, the k_a for the MASP-2-C1-inhibitor reaction increases by an order of magnitude (from 1.6×10^5 M^{-1} s^{-1} to 1.3×10^6 M^{-1} s^{-1}), whereas the k_a for the MASP-2-antithormbin reaction increases even more, by two orders of magnitude (from 4.3×10^2 M^{-1} s^{-1} to 4.7×10^4 M^{-1} s^{-1}). The heparin concentration dependence of the rate of the MASP-2-antithombin reaction shows a bell-shaped curve, suggesting that heparin binds to both proteins and speeds up the formation of the Michaelis complex. The typical example of this "bridging" mechanism is the heparin potentiation of the thrombin-antithrombin reaction. In the case of the MASP-1-antithrombin reaction, however, the "bridging" mechanism does not apply; instead the concentration dependence (saturation curve) suggests an allosteric mechanism. The central role of the serpins in the regulation of the lectin

pathway is also indicated by the fact that significant levels of MASP-1-C1-inhibitor and MASP-1-antithrombin complexes were observed in resting normal human serum (Hansen et al. 2015). It may suggest, that a slow "tick-over" of the lectin pathway takes place in the blood, and the serpins prevent the overall activation of the system before danger signals emerge. On the other hand, it was shown that platelet activation and coagulation lead to the formation of MASP1-/2-serpin complexes, which could explain the presence of such complexes in the serum (Ekdahl et al. 2016).

Besides serpins, inhibitors with different mechanism of action may also contribute to the regulation of the MASPs. The canonical inhibitors are usually small, substrate-like proteins and make a tight but non-covalent complex with the target protease. Although the canonical inhibitors play a significant role in many physiological processes they have almost no influence on the blood cascade systems. The only exemption is tissue factor pathway inhibitor (TFPI), an important inhibitor of the extrinsic coagulation pathway, which was also shown to inhibit MASP-2 (Keizer et al. 2015). The IC_{50} value of TFPI for lectin pathway inhibition was 10 μM in 100-fold diluted serum. Taking into account that the plasma concentration of TFPI is 2.25 nM, it is unlikely that this weak inhibitory effect would play a considerable role in the regulation of lectin pathway activation.

Macroglobulins represent another type of protease inhibitors which are abundantly present in the blood. Like serpins, macroglobulins also undergo a major conformation change after the cleavage of the bait region by the target protease, and sequester the protease by forming a cage-like structure around it. A fraction of the protease molecules may form a covalent bond with the thioester domain of the macroglobulin, but this is not required for the inhibition: the cage will prevent the contact between the protease and its protein substrates. α_2-Macroglobulin (α_2-M) is an abundant inhibitor in the blood and may play a role in the regulation of the plasma cascade systems (Goulas et al. 2017). The published results concerning the lectin pathway regulation, however, are controversial (Fig. 6). Several reports showed that α_2-M forms complexes with MASP-1 and MASP-2. In fluid phase, α_2-M blocked the rMASP-1 mediated C2 cleavage, while it could not prevent C4 deposition on mannan-coated surface (Ambrus et al. 2003; Petersen et al. 2000). Another report claimed that α_2-M protected *Neisseria gonorrhoeae* from lectin pathway mediated killing, and α_2-M and C1-inhibitor acted synergistically to inhibit the lectin pathway on the surface of the bacteria (Gulati et al. 2002). We also demonstrated that α_2-M is able to sequester MASP-1 in the fluid phase, however, we were unable to detect any inhibitory effect on mannan-coated plates (Paréj et al. 2013). α_2-M is a huge tetrameric glycoprotein composed of 1451-residue-subunits. It is very likely that steric hindrance prevents α_2-M to make a contact with the protease in the MBL-MASP complexes immobilized on the activation surface. It is also possible that the rate of the α_2-M-MASP reaction is much slower than the rate of the C4 and C2 cleavage by the MASPs. It is plausible to presume that the role of α_2-M is not the immediate prevention of MASP-1 and MASP-2 proteolytic action during lectin pathway activation, but the slow removal of the activated proteases from the bloodstream. We should also remember that none of the above mentioned

inhibitors react with MASP-3. MASP-3 has no known physiological inhibitor. Like factor D, MASP-3 is regulated differently, maybe through its very narrow substrate specificity and through the self-inhibited structure of the processed protease. Further experiments need to prove these assumptions.

Conclusion and Perspectives

There is no doubt that MASPs are crucially important in developing an efficient innate immune response. MASP-1 and MASP-2 are indispensable for the activation of the lectin pathway, while MASP-3 is necessary for alternative pathway activation. A growing number of evidences suggest that MASP-1 also contributes to the alternative pathway in an activator-dependent manner. In addition to the complement activation, MASP-1 is also capable of activating endothelial cells directly, by cleaving PARs, and indirectly by liberating bradykinin from HK. The activated endothelial cells secret cytokines that attract neutrophil granulocytes, and their altered adhesion molecule pattern facilitates adhesion between neutrophils and endothelial cells. In this way MASP-1 forms a link between the humoral and cellular immune response. The cross-talk between the lectin pathway and the coagulation system has also been studied intensively in the recent years and the results point to the importance of the proteolytic activity of MASPs. Since MASPs are members of a larger proteolytic network, we can predict that further functions, beyond complement activation, will be discovered in the future. MASP-3 is an interesting candidate in this respect. It was shown that deficiency of MASP-3 results in the development of the 3MC syndrome, which manifests in serious developmental abnormalities (Rooryck et al. 2011; Sirmaci et al. 2010). It seems that MASP-3 plays a crucial role in embryogenesis, by cleaving a presently unknown substrate. Although it was reported earlier that MASP-3 can process insulin-like growth factor-binding protein 5 (IGFBP-5) (Cortesio and Jiang 2006), there is no evidence that it would be the key substrate in the developmental processes. Another interesting problem is the activation of MASP-3 in the blood. We know that MASP-3, like factor D, is present predominantly in the active form in the circulation, however, we do not know which protease is responsible for zymogen MASP-3 activation (Oroszlán et al. 2017). Several lines of evidence suggest that the activator protease is not a known member of the complement cascade, but it could rather be a more distant member of the blood's proteolytic network.

While the complement system is a key component of innate immunity, its uncontrolled, excess activation can cause self-tissue damage and contribute to the development of serious disease conditions, including ischemia reperfusion injury (myocardial infarction, stroke), age-related macular degeneration, and several neurodegenerative disorders (Dobó et al. 2018). Inhibiting the activity of the complement proteases is a therapeutic option in these diseases. Targeting MASP-1 or MASP-2 we can block the lectin pathway activation. MASP-2 seems to be a favorable target since its serum concentration is much lower, than that of MASP-1.

Both antibody and small-protein inhibitors have been developed and tested against MASP-2 (Schwaeble et al. 2011; Clark et al. 2018; Szakács et al. 2019). Long-term inhibition of MASP-3 can result in the attenuation of alternative pathway activity. MASP-1 is also a promising target, since it has multiple substrates connected to immune response and inflammation. It is very likely that new anti-complement drugs will be developed and applied in the clinical practice in the near future.

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Structures of the MASP Proteases and Comparison with Complement C1r and C1s

Christine Gaboriaud, Véronique Rossi, and Nicole M. Thielens

Several recognition proteins of the defence collagen family associate with proteases to initiate the complement cascade. The associated proteases, which are the subject of this review, mediate the proteolytic activation trigger. MBL-associated serine proteases (MASPs) mainly activate the lectin complement pathway (LP), while C1r and C1s activate the classical complement pathway (CP). This chapter will briefly introduce the current structural knowledge on these effector proteases and question what we know on MASPs structures, their common properties and how they differ from the C1r/s proteases. We will primarily focus on the structural comparison of the N-terminal domains, where the collectin binding sites are located and highlight novel aspects on their interaction with collagens. We also aim to highlight further molecular details associated to functional specificities, and to mention questions remaining open and needing further investigations. This chapter complements other reviews that describe the main general lines of complement activation mechanisms (Merle et al. 2015), the proposed structure-based scheme of the lectin pathway activation (Kjaer et al. 2013) and previous comparisons of LP and CP proteases (Gál et al. 2007; Pike and Wijeyewickrema 2013).

Common Protease Modular Structure and Mode of Interaction with Defence Collagens

MASP-1, MASP-2, MASP-3, C1r and C1s serine proteases share identical mosaic organization (Fig. 1). The enzymatic activity of their C-terminal serine protease (SP) domain is controlled by the five preceding modules: two CUB (for complement C1r/C1s, Uegf, Bmp1) modules separated by an epidermal growth factor

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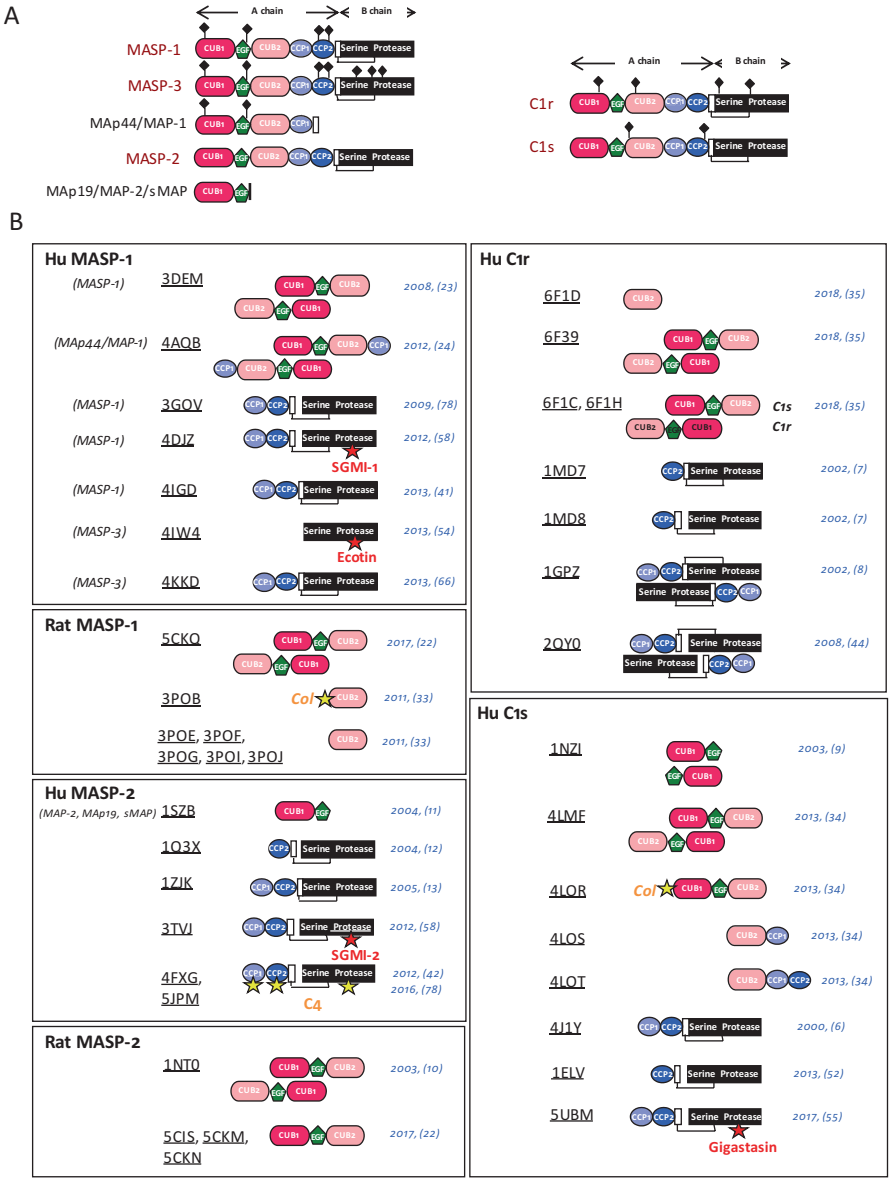

MASP-1, *Hu PO:48740-1,Rat PO:Q8CHN8;* ***MASP-3***, *Hu PO:48740-2;* ***MAp44/MAP-1***, *Hu PO:48740-3;* ***C1r***, *Hu PO: P00736*
MASP-2, *Hu PO:O00187-1, Rat PO:Q9JJS8;* ***MAp19/MAP-2/sMAP***,*Hu PO:O10187-2;* ***C1s***, *Hu PO: P09871*

Fig. 1 MASPs and C1r/s share identical mosaic structures. (**a**) Modular structure of the MASPs related proteins (left) and C1r/s (right). The serine proteases are activated by a specific cleavage between the A and B chains, which are held together by a disulphide bridge. The activation peptide at the N-terminus of the Serine Protease domain is shown in white. Glycosylation sites are shown with black diamonds. (**b**) This table lists the X-ray structure coordinates deposited in the Protein Data Bank, with their accession code and their schematic representation, such as head-to-tail dimers or stars indicating the presence of protein ligands (inhibitor in red, ligand/substrate in yellow). Enzyme active forms are indicated using a space separation between the two chains, since activation cleavage splits A and B chains. Corresponding references numbers are indicated

(EGF)-like module, and a pair of complement control protein (CCP) modules. LP and CP proteases are synthesized as single polypeptide chain proenzymes and become activated when a specific Arg-Ile bond is cleaved after the activation peptide in the SP domain, resulting in two chains, A and B, held together by a disulphide bridge (Fig. 1). As will be detailed later, the activation cleavage is mainly triggered when the associated defence collagen binds to a target surface.

Alternative splicing products also modulate LP activity. MASP-1 and MASP-3 are the products of a single *MASP1* gene. They contain identical A chains (except for the 15 C-terminal residues), while the B chains are encoded by different exons. In addition, alternative splicing of the *MASP1* and *MASP2* genes produces two non-enzymic proteins, called MAP-1 (also named MAp44) and MAP-2 (also named MAp19 or sMAP) (see Fig. 1 and refer to (Bohlson et al. 2019) for the latest update on complement nomenclature). MASP-1, MASP-3, C1r and C1s have four, seven, four and two glycosylation sites, respectively, whereas MASP-2 is not glycosylated (Fig. 1).

There are no X-ray crystal structures available for the full-length proteases, because of inter-domain flexibility. A dissection strategy has been setup early to unveil structure/function details of the CP and LP proteases (Gaboriaud et al. 2000; Budayova-Spano et al. 2002a, b; Gregory et al. 2003, 2004; Feinberg et al. 2003; Harmat et al. 2004; Gál et al. 2005). Two structurally independent functional regions have been identified in these complement proteases, an N-terminal interaction region (CUB1-EGF-CUB2) and a catalytic region comprising two CCP modules and the serine protease domain (CCP1-CCP2-SP). Therefore, the structural descriptions and comparisons in this review will mainly focus on these two independent functional domains. The X-ray crystal structures of several fragments within the interaction and catalytic regions have been solved for the LP and CP proteases (Fig. 1).

LP proteases are probably less flexible than CP proteases, and they stably associate with the prototype MBL collectin tetramer. The overall scheme of MBL/MASPs association, illustrated on Fig. 2a, and the main structural principles of the activation of the LP have been reviewed in details (Kjaer et al. 2013). As a main principle, MASPs associate as homodimers through the interaction domain (in green), which also mediates their association with various collectins (MBL, ficolins, CL-L1 (CL-10), CL-K1 (CL-11) or CL-LK). The protease binding sites are at the same position in the collectin collagen stems (orange), thus lying in a plane parallel to the carbohydrate surface recognized by the collectins. The height of this plane will be specific and constant for each defined collectin type. The catalytic domains (pink and grey) protrude out of the collagen cone in roughly the same plane, if one considers that the modular junctions are essentially rigid, as shown in Fig. 2a. Investigations of the MBL/MASP-1 complex, combining small-angle X-ray scattering (SAXS) and electron microscopy (EM) studies, further provided experimental observations in line with such a global scheme (Kjaer et al. 2015). As revealed recently (Sharp et al. 2019), similar interaction principles are observed for the interaction of the C1r and C1s proteases with C1q within the C1 complex (Fig. 2b), although the position of the C1s catalytic domain differs.

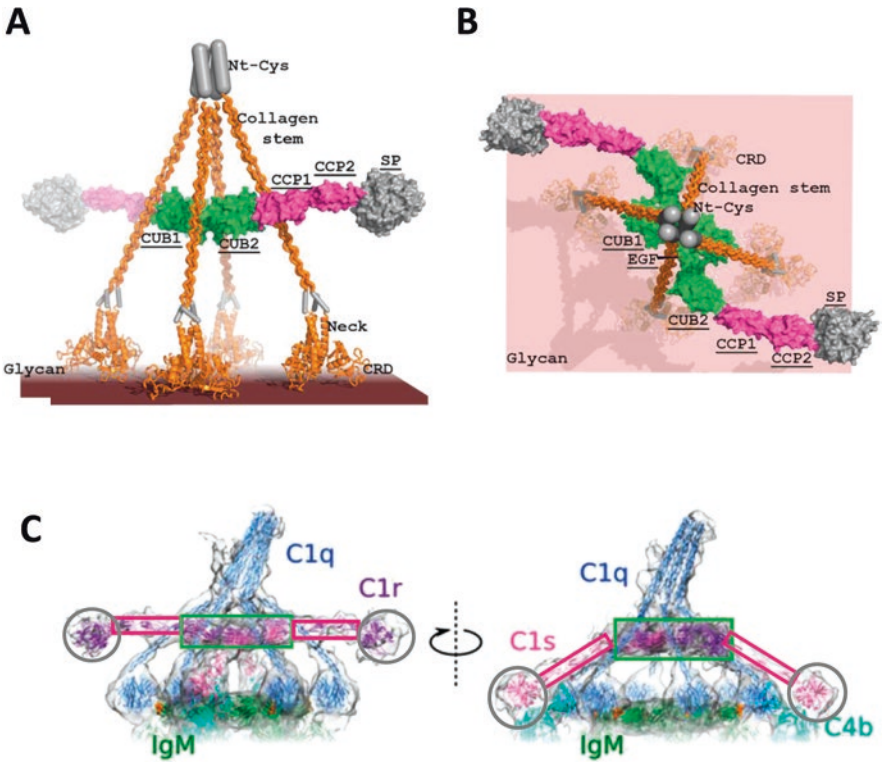

Fig. 2 Overall view of LP or CP activating complexes assembly. (**a**, **b**) Main global model of interaction between collectins (here MBL) and MASPs proteases. The N-terminal CUB1-EGF-CUB2 interaction domains (here in green) binds inside the cone defined by the collagen stems (orange helices), with the catalytic domains pointing outside the cone (pink + grey). The orientation and height of the catalytic Serine Protease domains (SP, grey) is defined by the association of the proteases within the collectin bound to a glycan surface, held by a rigid handle composed of CUB2-CCP1-CCP2 modules. Side (**a**) and top (**b**) views. Figure extracted from the review by Kjaer et al. (Kjaer et al. 2013) with publisher's permission. (**c**) Cryo-EM snapshot of IgM/C1/C4b mega complex structure. As labelled, the C1s and C1r proteases are shown in pink and purple, respectively; C1q and C4b in dark and light blue, respectively. The protease interaction domains lie in a plane parallel to the IgM binding platform (green rectangle). Two perpendicular views are shown, which highlight the different orientations of the catalytic domains of C1r (left) and C1s (right). (Figure adapted from Sharp et al. 2019)

As shown in Fig. 2, the interaction regions mediate protease homodimerization (MASPs) or heterodimerization (C1r/C1s). These regions are also involved in the protease interaction with the collagen-like region of collectins (LP proteases) or of C1q (CP proteases).

Central CUB1-EGF Interactions in LP and CP Proteases Assembly: A Marked Similarity

Early biochemical analyses demonstrated that a general feature of the CUB1-EGF regions of the MASPs is their ability to form Ca^{2+}-dependent homodimers (Thielens et al. 2001; Phillips et al. 2009), a property shared with the CUB1-EGF region of C1s (Thielens et al. 1990). Although the CUB1-EGF fragment of C1r appears as a momoner when analysed by gel filtration in the presence of calcium, it associates as a Ca^{2+}-dependent heterodimer with the corresponding fragment of C1s and with full-length C1s (Thielens et al. 1990, 1999; Busby and Ingham 1987).

The crystal structures of the CUB1-EGF segment of MASP-2 (MAp19) (Gregory et al. 2004) and of C1s (Gregory et al. 2003) each revealed a head to tail assembly. In both cases, the linear dimer is held by interactions between the CUB1 module of one monomer and the EGF module of its counterpart. The EGF Ca^{2+}-binding sites are centrally located (green circles in Fig. 3a) and stabilize this interface likely explaining the Ca^{2+}-dependence of the dimerization, even if the accessibility of the calcium ions to EDTA slightly differs between the different CUB1-EGF module pairs (Thielens et al. 1999, 2001). The dimer interface is stabilized by a combination of evenly distributed hydrophobic interactions and hydrogen bonds that are mostly conserved or substituted by similar residues in the MASP/C1r/C1s family (Gregory et al. 2003, 2004). The main structural features of the interaction domains which are common to the LP and CP proteases are illustrated in Fig. 3a, with a comparative focus on the area surrounding the calcium-binding sites in Fig. 3b–d.

The EGF-like module, with one major and one minor anti-parallel double-stranded β-sheets and three disulphide bridges, exhibits the characteristic EGF fold. As predicted from the occurrence of a consensus calcium-binding motif, each EGF module of the MASP/C1r/C1s family binds a calcium ion (Fig. 3b–d). It is coordinated in a conserved manner by seven oxygen ligands, including six homologous residues contributed by the EGF modules and a water molecule that also forms an H-bond with a conserved Gly residue in loop 4 of the CUB1 module (Fig. 3) (Gregory et al. 2003, 2004). Additional CUB1-EGF interactions provide further stabilization of the intramonomer CUB1-EGF interface (dark green in Fig. 3b–d). Conserved residues stabilizing the dimer interface are shown with light-green highlights and sticks (Fig. 3c, d). A particular feature of MASP-1/3 and C1r EGF lies in the presence of a cluster of charged residues in loop Legf (Fig. 3c), which is unusually large in C1r EGF and is mostly disorganized, in contrast with the corresponding loop in MASP-2 and C1s. The role of this charged, surface-exposed and flexible loop in potential interactions is currently unknown.

The CUB1 modules exhibit a typical compact ellipsoid structure. They differ from the canonical topology of spermadhesins, assembled from two five-stranded antiparallel β-sheets, by lacking the first two β-strands. Such a deletion is observed solely in the CUB1 module of the MASP/C1r/C1s family (Gaboriaud et al. 2011). Unexpectedly, unveiling the first X-ray structures human C1s and MASP-2 CUB1-EGF fragments revealed the presence of a Ca^{2+}-binding site on the distal edge of the

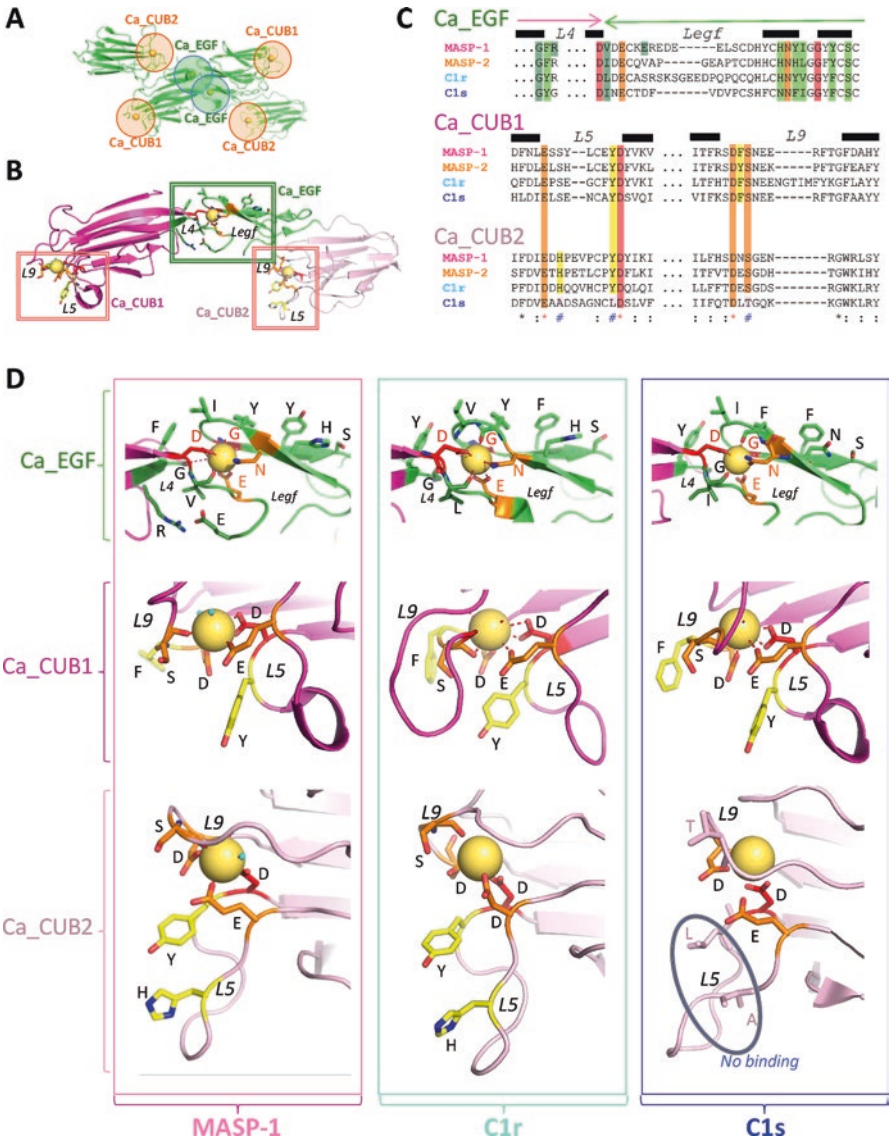

Fig. 3 Structural similarity of EGF, CUB1 and CUB2 calcium binding sites. (**a**) Overall relative spatial orientation of the calcium-binding sites in MASP1/3 CUB1-EGF-CUB2 dimer. (**b**) Relative position in CUB1-EGF-CUB2 monomer (MASP-1/3) of the zones around each calcium-binding site. (**c**) The corresponding conserved sequence features in MASPs and C1r/s of these zones around calcium-binding sites: Ca_EGF, Ca_CUB1 and Ca_CUB2. Ca_EGF is at the boundary between CUB1 and EGF, see arrows above defining the transition between the two modules. Conserved aspartic residues with a bidentate calcium binding are in red. Other conserved residues are shown in orange (calcium ligands), light green (conserved CUB-EGF dimer interface), dark green (intramonomer CUB1-EGF interface) or yellow (binding to collectin). Marked sequence difference in C1s CUB2 module, which does not take part directly in C1q binding, is highlighted in

(continued)

CUB1 module (Gregory et al. 2003, 2004). Coordination of the Ca^{2+} ion was found to involve three acidic residues (red and orange, Fig. 3b–d) that, together with a closely associated tyrosine residue, are conserved in a large proportion of the CUB repertoire and define a signature of Ca-binding CUB (cbCUB) modules (Gaboriaud et al. 2011). All CUB modules of the MASP/C1r/C1s family belong to this cbCUB domain subset (Fig. 3c, d). The Ca^{2+} ion is the central element of a network of interactions that stabilize the distal end of the CUB module. However this ion is exposed to the solvent and readily exchangeable, which likely explains why the Ca^{2+}-binding site was partly disordered and free in the X-ray structure of the CUB1-EGF-CUB2 fragment of rat MASP-2, crystallized in the absence of calcium (Feinberg et al. 2003). Recently published crystal structures of the same rat fragment confirmed the presence of Ca^{2+} ions in each of the CUB modules (Nan et al. 2017).

The currently available X-ray crystal structures (Fig. 1) of CUB domains, CUB1-EGF and CUB1-EGF-CUB2 fragments of human and rat MASP-1/3 and MASP-2, and of human C1r and C1s have further confirmed these similarities among the LP and CP proteases, especially for the central mode of calcium-dependent association of CUB1-EGF pairs (green circle in Fig. 3a), and for the structural motifs defining the three calcium-binding sites (Fig. 3c, d).

Similar Interaction Properties Mediate Protease Association with Defence Collagens

The CUB1-EGF fragment of MASPs has been shown to bind to MBL and ficolins in a Ca^{2+}-dependent manner. However, the affinity increases when the CUB2 module is also present, i. e. in the full-length proteases or in the CUB1-EGF-CUB2 fragments of MASPs (10^{-8} M versus 10^{-9} M range) (Teillet et al. 2008; Skjoedt et al. 2012). These observations suggested that the interaction of MASPs with MBL and ficolins involves a major contribution of the N-terminal CUB1-EGF module pair, but is strengthened by the following CUB2 module.

Mutational analyses of CUB1 and CUB2 modules identified homologous binding sites in MASP-1/3 and MASP-2 for MBL and ficolins, located in close vicinity of their Ca^{2+}-binding sites (Gregory et al. 2004; Teillet et al. 2008), in loops L5 and L9 (yellow and orange residues in Fig. 3b). On the collectin side, mutagenesis studies in the collagen-like regions of rat or human MBL and ficolins identified a

Fig. 3 (continued) light pink and marked by a blue #. CUB L5 and L9 loops, essential elements of the interaction with the recognition protein, are labelled. (**d**) Comparative views of the CUB1-EGF monomeric interface (Ca_EGF) as well as CUB1 and CUB2 calcium-binding sites in MASP1/3, C1r and C1s. Positions where conserved binding residues are missing in C1s CUB2 are displayed with pink sticks and highlighted with a blue circle. Same residue colour code as described above in (**c**). Figure drawn using PDB files 3DEM (MASP1/3), 6F1C (C1r and C1s CUB2), 1NZI (C1s CUB1 and EGF). Human MASP-1 is shown here as a representative example of MASPs structures which are very similar, as shown in Nan et al. (2017)

conserved lysine residue essential for the interaction with the MASPs (Wallis et al. 2004; Teillet et al. 2007; Girija et al. 2007; Lacroix et al. 2009). This information allowed to propose an initial model of the complex of a tetramer of MBL and the MASP-1/3 CUB1-EGF-CUB2 dimer, featuring four major homologous interaction sites involving the conserved lysine residue of the collagen-like region of MBL and acidic Ca^{2+} ligands of CUB1 and of CUB2 of the protease (Teillet et al. 2008). This fits with the more complete model later proposed in Fig. 2a (Kjaer et al. 2013).

Whereas it was initially proposed that the interaction of the C1s-C1r-C1r-C1s tetramer with C1q differed from that of the MASP dimers with MBL or ficolins (Gaboriaud et al. 2007), two 2009 studies (Phillips et al. 2009; Bally et al. 2009) provided evidence for similarities in the collagen binding sites of the initiating complexes of the CP and LP, despite different stoichiometries. Phillips et al. demonstrated *in vitro* cross-interaction of the subcomponents of the lectin and classical pathways, although cognate interactions were tighter than non-cognate interactions, the latter being more transient with faster dissociation rates (Phillips et al. 2009).

High affinity C1q binding sites in the C1r CUB1 and CUB2 modules and a lower affinity site in C1s CUB1 module were identified using site-directed mutagenesis of C1r and C1s (Bally et al. 2009). As observed previously for MASPs, these sites involve acidic residues also contributing Ca^{2+} ligands (red and orange in Fig. 3c, d). A refined model of the C1 complex was built, in which the CUB1-EGF-CUB2 of C1r and the CUB1-EGF of C1s interact through six binding sites with homologous lysines of the C1q stems, nicely reconciling the different stoichiometry of C1q and of its associated proteases (Bally et al. 2009).

As shown previously in Fig. 3c, d, several residues are conserved in the CUB1 and CUB2 modules of all these proteases, suggesting similarities in the interaction mechanism for the initiating complexes of LP and CP. Mutation experiments have shown that the Ca^{2+}-binding site not only plays a stabilizing role of the distal end of the CUB domain, but also provides two exposed acidic residues involved in the interaction with a lysine residue of the collagen-like triple helix. Different models of C1 and MBL-MASP complexes derived from modelling and binding studies proposed analogous interactions of C1q with the C1s-C1r-C1r-C1s tetramer and of tetrameric MBL with MASP dimers, with binding sites present on each CUB domain, except for C1s CUB2 (Wallis et al. 2010). Quite similarly, lysine attraction by the acidic calcium-binding residues was also observed for the binding between cobalamin-bound gastric intrinsic factor and the CUB6 module of cubilin, revealing a common mechanism for calcium-dependent CUB interactions (Gaboriaud et al. 2011; Andersen et al. 2010).

However, C1s CUB2 stands out as an exception regarding this common interaction scheme. Indeed, the mutation of the homologous acid residue in C1s CUB2 did not impact C1 formation, revealing that this module was not involved in C1q binding, although its calcium binding property is intact (Bally et al. 2009). We can observe that the conserved tyrosine and histidine residues in L5 (highlighted yellow) and the conserved serine residue in L9 (orange) are missing in C1s CUB2, thus suggesting their essential role for collagen binding (Fig. 3c, d). At this stage, we can also note that these loops L5 and L9 are significantly conserved within each CUB1 and CUB2 subsets (except for C1s CUB2), but significantly differ between CUB1 and CUB2.

Opposite Binding Orientations for CUB1 and CUB2 Modules with Collagen: A Structural View

X-ray structural analyses rapidly confirmed the common mode of interaction suggested above (Fig. 4). The first structure solved (Gingras et al. 2011) showed the CUB2 domain of rat MASP-1/3 in complex with a 27-residue synthetic collagen-like peptide containing a central lysine residue (to mimic the MASP binding site of MBL). The X-ray crystal structure of a complex of the same collagen-like peptide and the interaction region of human C1s (CUB1-EGF-CUB2 dimer) was solved later by the same team (Venkatraman Girija et al. 2013). The two structures confirmed the Ca^{2+}-dependency of the MASP-MBL interaction, with the side chain of a collagen lysine (K15 corresponding to Lys46 in rat MBL-A) contacting three residues involved in Ca^{2+} coordination (Fig. 4a, b). Intriguingly however, the fine structural comparison of the complexes of this same collagen peptide with MASP-1/3 CUB2 (Gingras et al. 2011) or C1s CUB1 (Venkatraman Girija et al. 2013) reveals a striking binding orientation difference, as illustrated on Fig. 4a, b, and with their superposition on Fig. 4e. This difference in orientation is observed despite the common interaction details described above. Does it reflect a different mode of interaction between MASPs and C1s, or between CUB1 and CUB2 modules? As mentioned before, CUB1 and CUB2 modules are conserved in the MASPs and C1r/C1s family (at the exception of C1s CUB2). But CUB1 and CUB2 readily differ in the size and composition of loops L5 and L9 (Fig. 3c, d), and these loops are involved in collagen binding (Fig. 4a, b). So we can hypothesize that different CUB1 and CUB2 relative orientations could be dictated by their differences in loops L5 and L9.

How Details of CUB-Collagen Interactions Explain the Differences in Orientation

CUB1 interaction details (Venkatraman Girija et al. 2013). In this structure, the side chain of lysine 15 mainly interacting with the CUB1 module of C1s comes from the **trailing** collagen strand (purple, Fig. 4c). It contacts the carboxylate groups of Glu45 and Asp98, and the hydroxyl and carbonyl groups of Ser100, three Ca^{2+}-coordinating residues of CUB1 (Fig. 4c). Additional contacts involve hydrogen bonds provided by Tyr52 of C1s (L5). Glutamic acid residues 48 (L5) and 102 (L9) clamp the two sides of the collagen helix. Since glutamic acid residue 48 interacts with the lysine 15 of the collagen middle strand (grey), we can note that two consecutive collagen lysines are recognized, at least in this specific C1s example. However, this secondary interaction may not be essential, since single lysine mutations of C1q can prevent C1 assembly (Bally et al. 2009). Of note, this glutamic acid 48 residue is conserved in CP but not in LP proteases.

CUB2 interaction details (Gingras et al. 2011). In that structure, the side chain of lysine 15 interacting with the CUB2 module of rat MASP-1/3 comes from the

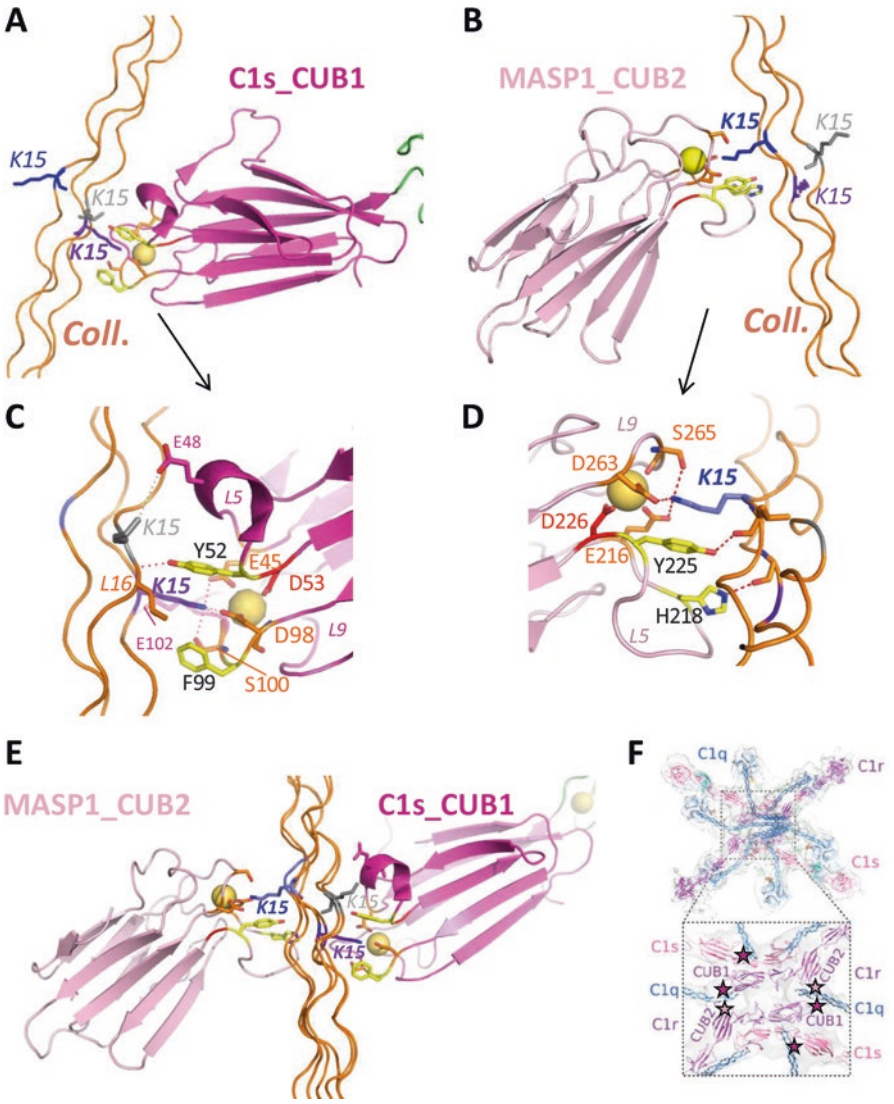

Fig. 4 Structural insight into the interaction of CUB modules with a MBL-like collagen peptide or with C1q. (**a**) C1s CUB1 (PDB code 4LOR, Venkatraman Girija et al. (2013)) and (**b**) MASP-1/3 CUB2 (PDB code 3POB, Gingras et al. (2011)) structures in complex with a MBL-like collagen peptide. The lysine residues of the common collagen ligand are shown as stick (dark blue, leading strand; grey, middle strand; purple, trailing strand). (**c**, **d**) Focus on interaction details of CUB1 (4LOR) and CUB2 (3POB) binding with collagen. Major residues are labelled. Same colour code as above and as in Fig. 3 for the CUB residues. Note that E48 in C1s (L5), which mediates direct electrostatic interactions with the middle collagen lysine, is not conserved in MASPs sequences. (**e**) The two structures above are shown after superimposition of the three essential lysines of the common collagen peptide (same colour code and stick representation as above in **c**, **d**). This superimposition suggests that one CUB1 and one CUB2 module can interact with the same collagen stem. (**f**) CUB1 and CUB2 modules from two C1r chains interact with the same C1q collagen stem in the context of the IgM/C1/C4b mega complex structure investigated by cryo-EM, as highlighted by stars (adapted from Sharp et al. 2019). C1s CUB1 interaction is also shown by star

leading collagen strand (dark blue, Fig. 4d). It interacts with the carboxylate groups of Glu216 and Asp263 and the hydroxyl group of Ser265 (orange), three Ca^{2+}-coordinating residues of CUB2. Additional interacting contributions come from residues in loop L5 (His218 and Tyr225 which occupy a groove in the collagen helix), which is stabilized by its close proximity to the collagen peptide. L5 and L9 loops thus occupy two consecutive grooves in the collagen helix.

The analysis of these two structures shed light on the role of loops L5 and L9 in collagen interaction. The residues involved in collagen binding are mostly conserved in the LP and CP proteases (except for C1s CUB2). But, as shown previously, the loops L5 and L9 significantly differ between CUB1 and CUB2 (Fig. 3). As discussed by Girija et al. (2013), the loop L9 in CUB2 would prevent binding in the CUB1 orientation, because of steric clashes. Thus, this difference in binding orientations may be a general feature of CUB1/CUB2 modules, acquired through evolution. Interestingly though, this difference in orientation fits with the constraint that CUB1 and CUB2 bind to two opposite collagen stems (Fig. 4a, b and global model in Fig. 2). Moreover, as illustrated on Fig. 4e, superimposition of the two X-ray structures opens the hypothesis that CUB1 and CUB2 modules could simultaneously bind to the same collagen stem. Such a possibility has not been explored in the initial models but is supported by the snapshot EM structure of the IgM/C1/C4b complex, where C1r CUB1 and CUB2 are observed to bind to the same collagen stem of C1q (Fig. 4f, Sharp et al. (2019)).

CUB1-EGF-CUB2 Homodimer (LP) Versus Heterodimer (CP) Interactions: Common Features and Differences

Comparing CUB-EGF-CUB sequences reveals strong similarities between LP and CP proteases. As mentioned before, CUB1-EGF head-to-tail dimer association is supported by tightly conserved sequences in the interaction domain of the LP and CP proteases. Sequence conservation further extends in the next EGF-CUB2 segment, as shown in Fig. 5a. Indeed, we could build a consensus sequence motif spanning: (a) the end of the EGF module, including residues involved in the CUB1-EGF dimer interface (boxed in green), (b) the three residues long linker (in grey) and (c) the beginning of the CUB2 module (in salmon). Remarkably, scanning sequence databases with this consensus sequence enables to retrieve most LP or CP protease homologs of different species (more than 800 sequences in translated EMBL). Importantly, scanning sequences with this motif does not retrieve other unrelated EGF-CUB containing protein. This, again, stresses how these proteases are similar.

However, by comparing different CUB1-EGF-CUB2 dimer structures from LP and CP proteases, we can observe striking differences in the relative CUB2 orientation, as illustrated in Fig. 5b–d. CUB1-EGF-CUB2 homodimers of LP proteases are structurally highly similar (Nan et al. 2017; Teillet et al. 2008). The C1s CUB1-EGF-CUB2 homodimer exhibits more flexibility but retains a similar overall shape,

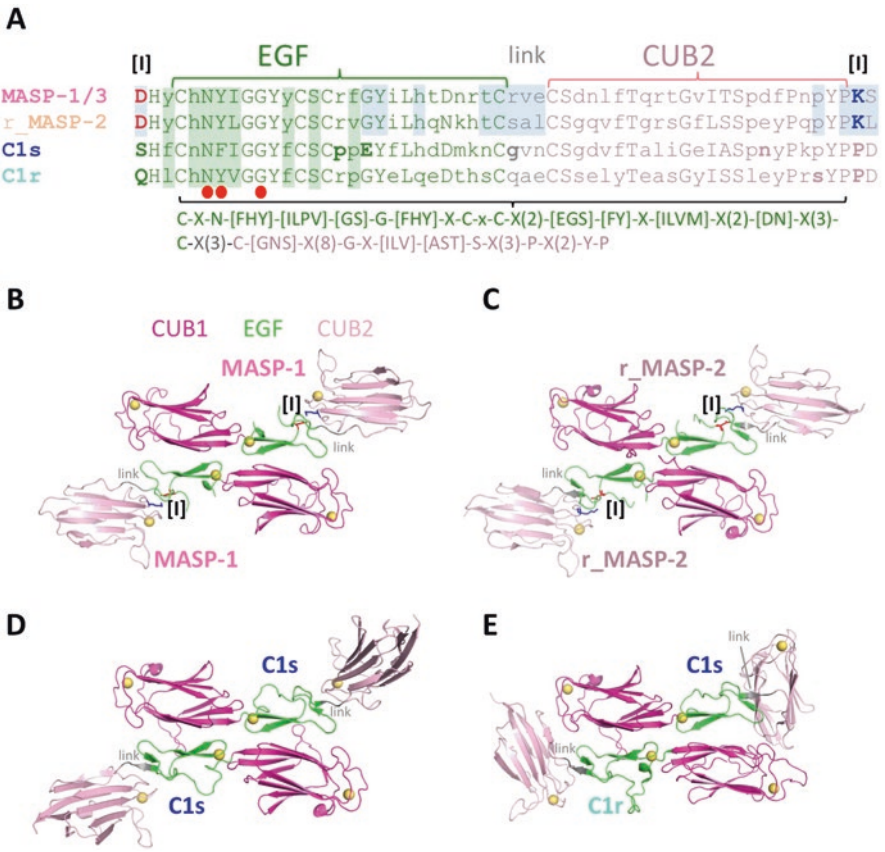

Fig. 5 Consensus and variations of the EGF-CUB2 interface in homo versus hetero-dimer contexts. (**a**) Strong sequence similarity between MASPs and C1r/s proteins in a segment spanning from the end of EGF (green) to the beginning of CUB2 (salmon), the three amino-acid long linker being displayed in grey. The consensus sequence motif shown below can be used to retrieve all MASPs or C1r/s homologs in other species. Label [I] indicates a conserved electrostatic interaction in MASPs (letter coloured red for aspartic acid and blue for lysine residues). The green highlights indicate conserved CUB1-EGF dimer interface residues. The light blue highlights show residues interacting at the EGF-CUB2 interface in MASPs. The red dots below the sequence are conserved calcium ligands. Majuscule letters show positions defined in the consensus sequence. Bold letters show significant differences between LP and C1r or C1s proteases; r_MASP2: rat MASP-2. (**b**) and (**c**) Homodimer CUB-EGF-CUB in MASP-1/3 (PDB code 3DEM, Teillet et al. (2008)) and MASP-2 (PDB code 5CIS), where the linker fragment is coloured in grey, and the two residues forming an electrostatic interaction [I] in red and blue, as in (**a**). The CUB1-EGF-CUB2 orientation is indicated. (**d**) Homodimer CUB1-EGF-CUB2 in C1s (PDB code 4LMF, Venkatraman Girija et al. (2013)). Same colour code. (**e**) Heterodimeric C1s/C1r CUB1-EGF-CUB2 association (PDB code 6F1C, Almitairi et al. (2018)). Same colour code. All dimers are shown with the same orientation of the CUB1-EGF central association

in the sense that CUB2 modules are not involved in the dimer interaction (Fig. 5b-d). In contrast, C1r-C1s heterodimers present a larger buried dimer interface, with additional contacts between CUB1 and CUB2 modules (Fig. 5e), which likely explains their preferential heterodimer association (Almitairi et al. 2018). These differences thus seem to correlate with homo versus heterodimer (C1r/C1s) association status.

Are these conformational differences related to fine sequence specificities? In the sequence alignment (Fig. 5a), the residues involved in the CUB1-EGF dimer interface (boxed in green) are far more conserved than the ones involved in the MASP EGF-CUB2 monomer interface (boxed in light blue). One ionic interaction (label I on Fig. 5a–c) is highlighted, because it is conserved in MASPs EGF-CUB2 interface, but not in C1r/s. As another striking difference, the three-residues long linker sequence (grey) starts with a glycine residue in C1s only (conserved in more than 150 species), which likely enhances the flexibility of its EGF-CUB2 junction.

Therefore, in contrast to the strong stability and similarity of the CUB1-EGF junction and interface, as described above, there is a significant flexibility at the EGF-CUB2 junction in the C1s molecule, as revealed by the variable and unusual position of the C1s CUB2 (Fig. 5d–e) in different X-ray structures. The recent cryo-EM study of the C1/IgM/C4b complex further confirmed this flexibility, since the C1s CUB2 module is seen there in another orientation (Sharp et al. 2019; Venkatraman Girija et al. 2013). Although probably less flexible than its C1s counterpart, the EGF-CUB2 junction also adopts a different conformation in C1r (Fig. 5e).

The above mentioned local sequence differences between MASPs and C1r/s are in line with the fact that MASPs mainly associate as homodimers whereas C1r and C1s interaction domains preferentially associate as heterodimers. In theory, heterodimer formation in MASP proteases is possible, but only via subunit exchange following EDTA dissociation. This concerns not only MASPs with identical interaction regions (MAp44/MAP-1, MASP-1 and MASP-3) (Rosbjerg et al. 2014), but also MASP-1, MASP-3 together with MASP-2 (Paréj et al. 2014). Some free MASPs heterocomplexes might be found in blood, but the majority of MASPs moieties are associated to collectin recognition molecules (Rosbjerg et al. 2014). Nevertheless, the main difference in favoured associations between CP and LP proteases, the wide versatility in the collectin recognition proteins and stoichiometries in complexes with MASPs, versus the strict C1 composition, really make a difference between LP and CP proteases.

How CCP1 and CCP2 Modulate LP and CP Catalytic Activities

The catalytic regions of LP and CP proteases, comprising the two CCP modules and the SP domain, mediate enzymatic activity. This activity implies a conformational transition in the C-terminal SP domain, to convert the proenzyme into an active state. As for coagulation proteases, activation of complement proteases proceeds though rapid cascade amplification with increased serum concentrations of the successive activation targets (C4/C2, C3). The activation of the LP or CP initiating complexes involves two successive steps. In the case of LP, MASP-1 auto-activates "on site", when its associated collectin pattern recognition molecule (PRM) binds to a target surface; activated MASP-1 will then activate MASP-2. The CP pathway proceeds quite similarly, with a first step of C1r auto-activation and C1s activation by activated C1r in a second step. These activations are triggered by C1 binding to an appropriate activating surface, the main prototype surface being IgM or hexameric IgG immune complexes platforms. For both LP and CP cascades, the next common step is C4 cleavage (by MASP-2 or C1s) and C2 cleavage (C2 being associated to C4b) to form the CP/LP convertase. One difference is that C1r and C1s are associated to the same C1q molecule, which is not the standard case for MASP-1 and MASP-2 in LP activating complexes.

From a biochemical point of view, the activation of LP or CP protease is driven by the specific proteolytic cleavage of a specific Arg-Ile bond in the N-terminal part of its SP domain, between the A and B chains, which remain attached through a disulphide bridge (see Fig. 1). From a structural standpoint, this cleavage is mediated by a partner SP domain, in an overall relative orientation similar to the one shown in Fig. 6a, impeded in the relative orientation shown in Fig. 6b.

Several structures corresponding to this catalytic region have been solved for the different C1r/s and MASPs proteases (Fig. 1). They correspond to different conformational states: active (activated/two chains) or proenzyme (zymogen). In order to get their X-ray structure, engineered point mutations have often been introduced to stabilize a specific conformational state (mutation of the active site, of the activation site, or introduction of patient mutations). In addition, stabilization was also achieved within complexes formed with inhibitors or cognate substrate (Fig. 1).

Fig. 6 (continued) exosite residues are shown in magenta. Other important residues discussed in the text are shown. (**d**, **e**) Focus on the active site details in the active (**d**) and proenzyme states (**e**). Same details as in (**c**). The aspartic acid at the bottom of the primary pocket (*D189*), the aspartic (*D194*) residue interacting with the new N-terminal extremity released by the activation cleavage (Nt) are labelled. K560 and R561 are essential residues in the C4 binding exosite (Pike and Wijeyewickrema 2013). In (**d**), the green segment and arginine side-chain show the position of the target C4 fragment inside the binding cleft (following superposition with the MASP-2/C4 complex, PDB code 5JPM, Croll and Andersen (2016)). The nomenclature of the loops A to E is the one defined by Perona and Craik (1995), with loops 1–3 mostly involved in the activating conformational transition

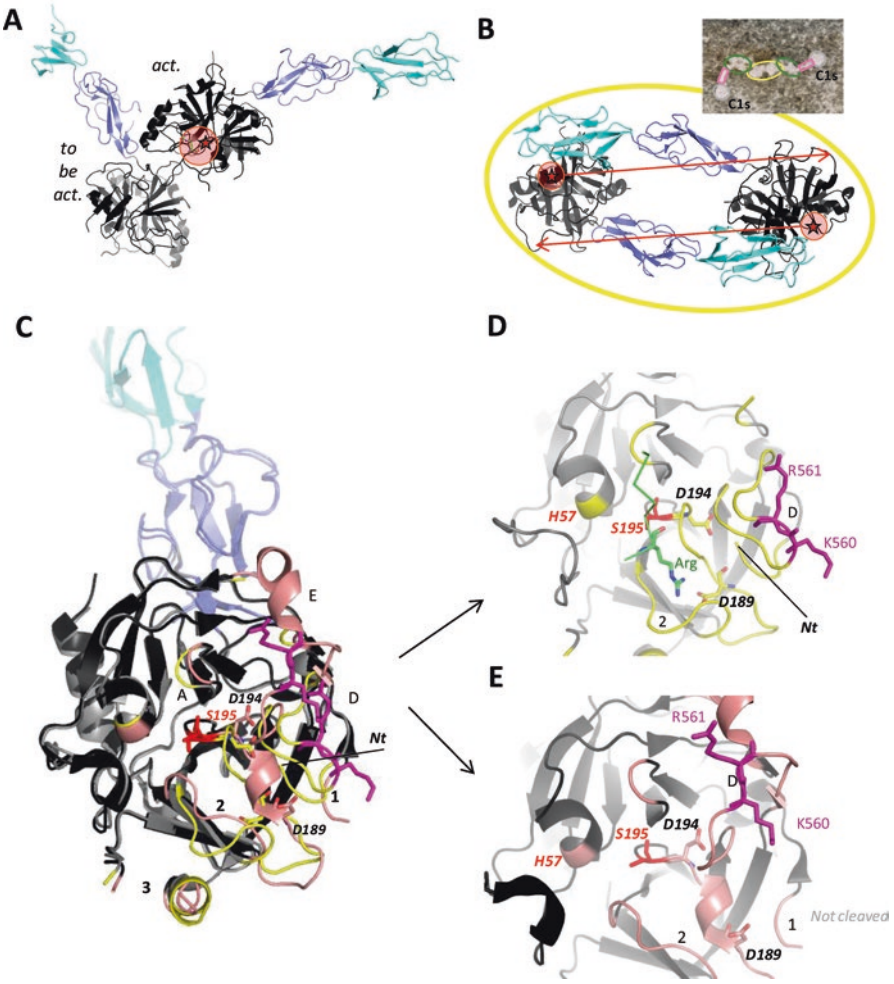

Fig. 6 SP domain activation: relative SP domain positions and activation conformational changes. (**a**) Relative overall orientation required for activating cleavage, where the bond to be cleaved (activation site) is close to the active site of the activating molecule (into the red circle). The SP domain to be activated (*to be act.)* and the activating SP domain (*act.*) are labelled. This C1r structure corresponds approximately to an enzyme product complex, since Arg446 of the cleaved activation loop (to be act.) sits in the S1 substrate-binding pocket of the other molecule (act.) (PDB code: 2QY0). (**b**) The head-to-tail dimeric structure of C1r catalytic domains, held by contacts between the SP domain and the CCP1 module of its partner (PDB code 1GPZ, major association also observed in 2QY0). In this orientation, the active site and activation sites are 90 Å distant (red arrows), at both ends of the dimer. This distance might help preventing unwanted autoactivation cleavage in the context of the C1s-C1r-C1r-C1s tetramer (Budayova-Spano et al. 2002b). A pioneer electron micrograph of the tetramer is shown above, the structure shown here corresponds to the central yellow circle, in a perpendicular view (Tschopp et al. 1980; Budayova-Spano et al. 2002b). (**c**) The activation conformational change is illustrated with the superimposed overview of active (yellow, 1ELV) and proenzyme (salmon, 4J1Y) states of C1s. The active serine (*S195*) is shown in red. The common backbone is shown in grey (active) and black (proenzyme). Two C4

CCP1-CCP2 as Elongated Arms or Handles

Evolutionary conserved rigid interaction between the SP domain and the preceding module can be detected at the sequence level, as initially illustrated with the examples of these complement and blood coagulation proteases (Gaboriaud et al. 1998). The presence of two CCP (or sushi) modules preceding the C-terminal SP domain is a specific hallmark of the MASPs/C1r/C1s proteases, which they share with haptoglobin, although the latter has lost catalytic activity (Tosi et al. 1989; Redmond et al. 2018).

The X-ray structure of C1s CCP2-SP fragment (Gaboriaud et al. 2000) first revealed that the CCP2 module stands perpendicular to the protease surface. Therefore, a structural role of a rigid handle was initially inferred. Sequence conservation (Gaboriaud et al. 1998) suggested that this property would mainly be conserved in the different CP and LP proteases, which was confirmed later. Little or moderate flexibility has been observed between CCP1 and CCP2, thus providing a handle extension. For example, CCP1-CCP2 lay rigidly in MASP-1 (Megyeri et al. 2013) and only moderate flexibility has been observed for C1s (Venkatraman Girija et al. 2013). From the functional standpoint, these elongated arms correctly position the C-terminal SP domain, both for the activation process and for the catalytic cleavage. As seen on Fig. 2, the position of C1s lying on C4 is quite different from that of MASP-1 in the MBL/MASP model, whereas the position of C1r looks similar to that of MASP-1. The CCP1-CCP2 arm will transmit and amplify displacements occurring in the preceding modules, in case of flexibility. This fits with the fact that C1s is highly flexible at the EGF-CUB2 junction preceding the CCP1-CCP2 modules (Fig. 4), with also limited flexibility at the CCP1-CCP2 junction (Venkatraman Girija et al. 2013) and CCP2-SP junction (Kidmose et al. 2012). The handle transmission is achieved through the CUB2-CCP1 connection, which is assumed to be rigid. Indeed, it is observed in a stable conformation in C1s, when comparing two different fragments containing this junction (Venkatraman Girija et al. 2013). It is also assumed to be rigid and conserved in MASP-1 and MASP-2, maintained by two ionic interactions and a central hydrophobic contact (Kjaer et al. 2013).

CCP1-CCP2 Include a C4 Binding Exosite in MASP-2 and C1s

What is the structural background explaining why MASP-2 and C1s can both cleave C4, and not the other proteases? The first piece of answer resides in the observation that both CCP1-CCP2 modules of C1s and MASP-2 play a role in properly positioning C4 before and after its cleavage. These two modules can be exchanged between MASP-2 and C1s, the ones in MASP-2 providing more catalytic efficiency (Rossi et al. 2005). The X-ray structure of the MASP-2/C4b complex clearly identified a negative patch at the CCP1 and CCP2 junction in MASP-2, which is involved

in binding an arginine patch in the C345C domain of C4/C4b (Kjaer et al. 2013; Kidmose et al. 2012). Mutational analyses further confirmed the essential role of several exosite residues in C4 deposition, mostly in MASP-2 CCP2 (E333, P340, D365, P368). Another negative patch at the CCP1-CCP2 junction is found in C1s, with a quite similar relative position towards C4b observed in the cryo-EM C1/IgM/C4b envelope (Sharp et al. 2019). Point mutations in C1s CCP1-CCP2 linker also revealed the importance of this sequence for C4 cleavage (Bally et al. 2005). This negative patch is not present in the other CP and SP proteases. An additional C4 binding exosite in the SP domain was also identified, as will be discussed later (Kjaer et al. 2013; Pike and Wijeyewickrema 2013; Kidmose et al. 2012).

CCP Modules Potentially Restricting Spontaneous Activation: A C1r Exception

Among the LP and CP proteases, only the catalytic regions in C1r can form head-to-tail dimers through interactions between the CCP1 of one monomer and the SP domain of its counterpart (cf. Figs. 1 and 6b, Budayova-Spano et al. (2002b) and Kardos et al. (2008)). Thus, in absence of C1q and in presence of calcium, C1r dimers are at the centre of C1s-C1r-C1r-C1s tetramers (Gaboriaud et al. 2014; Tschopp et al. 1980), this configuration being unique to the CP. As illustrated on Fig. 6b, this head-to-tail association of C1r catalytic domains prevents activation of one monomer by its counterpart, and the C1r SP domains are somewhat protected from unwanted activation, especially within the C1s-C1r-C1r-C1s tetramer. More details about the activation process will be described in the next section related to SP domains. This observation that C1r CCP1-CCP2 modules likely prevent spontaneous C1r activation sounds physiologically relevant since C1q and the C1r and C1s proteases are not always secreted by the same cells and circulate at high concentrations. How C1-inhibitor, which is assumed to bind to the C1 proteases tetramer further controls this unwanted activation of the proteases is currently not fully understood.

Structural Similarities in the C-Terminal Trypsin-Like SP Domain

Sequence and structural similarities assign the SP domain of MASPs and C1r/s to the largest S1A family of proteases, most commonly known as trypsin-like proteases (recently reviewed in Goettig et al. (2019)). Trypsin-like proteases are synthesized in a proenzyme state. They are activated on site through enzymatic cascade amplifications in the cases of complement and coagulation proteases. This involves a structural conversion which folds the active site for efficient binding and catalysis (Chakraborty et al. 2018).

Bovine chymotrypsin led to the very first X-ray structure solved in this family (Matthews et al. 1967). MASPs and C1r/s share therefore the chymo/trypsin-like fold and associated activation mechanism. In this family, the prototypic chymotrypsin serves as reference for the numbering scheme proposed by Hartley in 1970 for the SP domain (Hartley 1970), with the catalytic triad residues termed *His57*, *Asp102* and *Ser195*. In the simplest activation model early suggested for this proteases family, the new charged N-terminus, created by the activation cleavage, inserts into the activation pocket, attracted towards *Asp194* (as on Fig. 6c–e), which triggers concerted surface loop rearrangements.

The trypsin-like primary specificity, which dictates a preferential cleavage after an arginine or lysine, is another common structural feature shared by all the CP and LP proteases. This cleavage specificity is related to the presence of a conserved aspartic residue at the bottom of the primary substrate-binding pocket (noted *D189* on Fig. 6). This residue is only properly positioned in the active conformation, after the concerted rearrangements of loops 1–3, which are essential in shaping the enzyme activity (Fig. 6c–e).

As briefly mentioned before, C4 recognition by MASP-2 involves an additional exosite in the SP domain, which binds C4 sulfotyrosines (Kidmose et al. 2012). In this exosite, mutation of arginine residues 578 and 583 impaired C4 cleavage, further demonstrating their essential role (Drentin et al. 2015). Cumulating mutations in the CCP and SP exosites leads to severe reduction in C4 cleavage efficiency, suggesting cumulative functional effects of these distal exosites (Drentin et al. 2015). A similar exosite is found in C1s D loop, with residues K560 and R561 (Fig. 6d, e). Interestingly, the conformational switch converting C1s zymogen into an active enzyme is required to properly position these two exosite C4 binding residues (Fig. 6d, e, (Perry et al. 2013), reviewed in Pike and Wijeyewickrema (2013)). This remark holds true especially for arginine 583, which is displaced by 12 Å when comparing zymogen and active MASP-2 (Drentin et al. 2015). However, MASP-2 possibly exhibits higher plasticity than C1s. These overall structural similarities between C1s and MASP-2 readily explain why only these two enzymes can cleave the large C4 component.

Structural Differences Shaping Protease Specificity, an Opportunity to Design Specific Inhibitors

Even if they share a common trypsin fold, proteases of the S1 family have evolved different catalytic roles. The fine catalytic specificities are mainly defined by structural differences in the loops surrounding the active site (as initially described/reviewed by Perona and Craik (1995)). The length and conformation of these loops (Fig. 7) mainly differ in each of the LP and CP proteases, as well as their sequence details. Better understanding of how these loops shape substrate specificity is acquired through the structural analysis of protease/inhibitor complexes, because

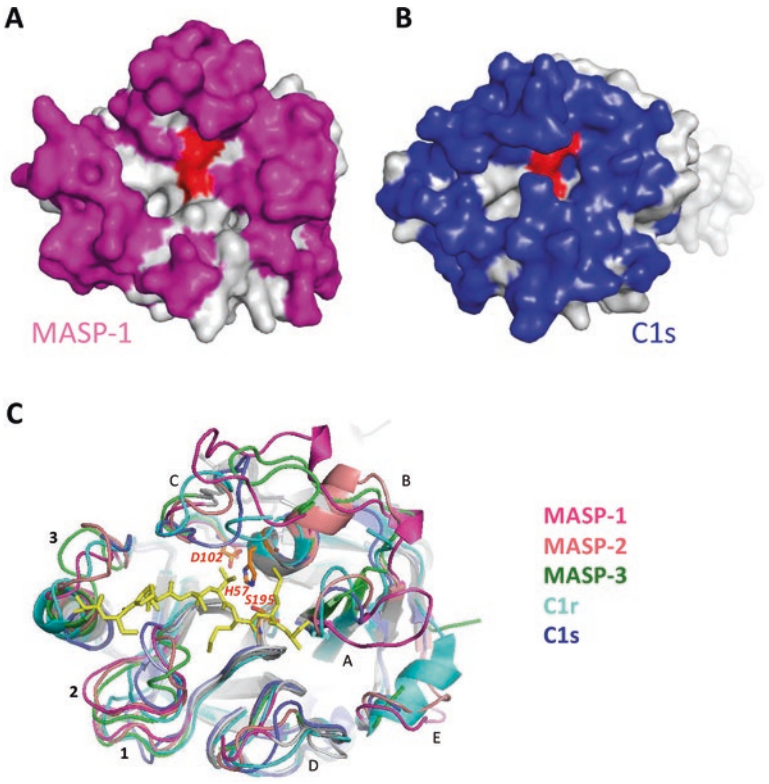

Fig. 7 Structural comparison of surface loops around the active site. (**a**, **b**) The active site (red) is surrounded by several loops, which define various interacting surfaces. A widely open active site was initially observed in digestive proteases, and this is only seen in MASP-1 (**a**), which fits with its more versatile enzymatic activity. In contrast, active site access is highly restricted in the C1s protease (**b**) and MASP-2, which mainly act on only two substrates, namely complement C4 and C2. More surface comparisons have been shown in Dobó et al. (2009). (**c**) Superimposition of LP and CP proteases showing the variation of length and conformation around the catalytic triad (orange sticks). The reference structure is the ecotin/MASP-3 complex (PDB code 4IW4), and thus the primary binding segment of ecotin (yellow) shows the position of the canonical substrate-binding site. Same loop labels as in Fig. 6, (**c**) from Gaboriaud et al. (2013)

these loops also shape differential inhibitor sensitivities. For example, inhibitors produced in various organisms have been described, such as gigastasin, a leech inhibitor for MASP-1, MASP-2 and C1s, or the bacterial pan-inhibitor ecotin (inhibiting MASP-2, MASP-3 and, to a lesser extent, MASP-1) (Gaboriaud et al. 2013; Pang et al. 2017; Nagy et al. 2019). X-ray structures of protease/inhibitor complexes have been solved in these two latter cases (Fig. 1). Gigastasin forms tight contacts with the C1s SP domain. These contacts span all subsites, plus the anion-binding exosite involved in C4 binding, through two sulfotyrosines near the gigastasin C-terminal terminus (Pang et al. 2017).

Reversely, taking into account the specific environment provided by each set of surface loops has enabled the design of highly specific inhibitors for MASP-1, MASP-2 and MASP-3, which can lead to promising therapeutic specific control of the different proteases. Structures of several enzyme/inhibitor complexes have been solved (Fig. 1). Structural details in these structures are often consistent with specific features of the designed inhibitors. For example, ecotin binds through hydrophobic residues in the S′ sites of MASP-3, a feature that is also found in the specific TFMI-3 inhibitor (Gaboriaud et al. 2013; Dobó et al. 2016a).

These specific inhibitors have already provided precious tools to decipher and deconvolute the functional role(s) of the MASP-1 and MASP-2 proteases (Héja et al. 2012a, b). They have also been used to demonstrate that MASP-3 is in fact the exclusive activator of pro-factor D in blood, making a strong connexion with complement alternative pathway activation (Dobó et al. 2016a).

MASP-1 SP domain is evolutionary distinct and supports a more versatile catalytic activity. One main structural basis supporting this difference is illustrated in Fig. 7, where MASP-1 clearly features a more open binding site. In that sense, MASP-1 is more close to trypsin than to highly specific coagulation or complement proteases. MASP-1 is a very potent activator of the LP pathway, already in its zymogen form and even more in its active state (Dobó et al. 2016b). One peculiar feature of MASP-1 is that *Asp189*, at the bottom of the primary specificity pocket, is already engaged in a salt bridge with an Arg side chain. Also the B loop in MASP-1 is highly extended, as in thrombin, which may constrict substrate specificity at this level (reviewed in (Dobó et al. 2016b)).

In contrast to MASP-1, access to the substrate-binding site is highly restricted in MASP-2, C1s and C1r by the surface loops surrounding the active site (Fig. 7). This explains why these proteases mainly act only on two substrates. Selective substrate recognition may also be further strengthened by a step of substrate-induced fit. This has been suggested from the structural plasticity observed in MASP-2 (Héja et al. 2012a), later confirmed in the case of its association to C4 (Kidmose et al. 2012). Analysis of the MASP-3/ecotin complex structure (4IW4, Fig. 1) also suggested allosteric conformational changes associated to ecotin binding to an extended MASP-3 exosite (Gaboriaud et al. 2013).

Structural Uncertainties Beyond Common Principles in the LP and CP Activation Mechanism

The simple activation scheme described previously, initially discovered on digestive proteases, has also evolved in the S1 proteases family. In the case of complement activation by LP and CP proteases, the associated collectin Pattern Recognition Molecule (PRM) plays a major role in triggering their activation only on site. For example, the major transactivation mechanism of LP activation proposes that juxtaposition of MBL-MASP complexes sitting on the same carbohydrate surface (as the

one shown in Fig. 2a) will drive protease contacts triggering LP activation (Kjaer et al. 2013; Degn et al. 2014). This process relies on the hypothesis that the SP domains are rigidly handled, as discussed before. Interestingly, this mechanism explains how activation is restricted to activating surfaces and how the recognition of these surfaces by the associated collectin controls the activation process. A similar transactivation mechanism has been proposed for the CP (Sharp et al. 2019; Mortensen et al. 2017), but uncertainties remain in this partly controversial issue. Further experimental evidences and details are needed to understand how the different activation events are topologically orchestrated in the context of various PRM-MASPs complexes or of C1 (Gaboriaud et al. 2014; Dobó et al. 2016b).

What about zymogen catalytic activity? MASP-1 zymogen is able to cleave MASP-1 and MASP-2, but active MASP-1 is far more potent on these activation cleavages (Megyeri et al. 2013; Dobó et al. 2016b). MASP-2 structural plasticity also led to the suggestion of a step of induced fit in its activation mechanism, with analogy to the "substrate-induced catalysis" proposed for the activation of complement factors D, B and C2 (Héja et al. 2012a).

Finally, we need to stress that some (auto-)activation schemes observed *in vitro* may not occur as such *in vivo*, where they can be mitigated by a lack of proximity or by physiological inhibitors. Since they share identical primary cleavage specificity (after Lys or Arg), CP, LP and other trypsin-like proteases can be indeed controlled by the same inhibitor. For example, C1-inhibitor is a main common physiological inhibitor of CP, LP but also of contact proteases. C1-inhibitor, in a similar way as the other members of the serpin family, acts as suicide substrate for C1r, C1s, MASP-1 and MASP-2. How C1-inhibitor binds to LP and CP activating complexes mainly remains an open question on the structural side. Some other inhibitors of the serpin family control the LP activation: anti-thrombin (for MASP-1 and MASP-2, Paréj et al. (2013)) and alpha2-macroglobulin (for MASP-1, Ambrus et al. (2003)). We also lack structural data on this ground, except that different sensitivities to inhibitors are modulated by surface properties that shape the function and specificity of each enzyme, as mentioned before.

LP and CP Protease Enzymatic Activities Out of the Complement System

MASP-1, with its wider substrate-binding site, as seen before, can in fact cleave a larger range of substrates (Gál et al. 2007). Therefore, active MASP-1 has been described as a promiscuous protease, able to boost coagulation (by acting on prothrombin, fibrinogen and coagulation factor XIII), and contributing to a powerful inflammatory reaction through activation of endothelial cells and bradykinin release (by acting on protease-activated receptors (PARs) and kininogen). More details can be found in (Dobó et al. 2016b; Garred et al. 2016).

Several mutations of the *MASP-1* gene that specifically affect MASP-3 SP domain are associated with a rare developmental disorder described as the 3MC syndrome. This suggests that MASP-3 is involved in other non-canonical functions, independent of the complement system, which remain to be deciphered. The structural impact of one of these mutations, G666[c197]E, has been investigated in MASP-3 zymogen form. The X-ray structure (4KKD, Fig. 1) provided evidence for a destabilizing effect on the active site, as expected, which further reinforced the hypothesis that an undefined MASP-3 enzymatic activity, abolished by this patient mutation, impacts the development in the 3MC syndrome (Yongqing et al. 2013). Other mutations in MASP-3 (Atik et al. 2015; Graul-Neumann et al. 2018) or in collectins CL-K1 and CL-L1 (Munye et al. 2017) have also been related to this 3MC syndrome, but structural and functional details are still elusive. However, these observations strongly suggest a crucial role for at least some of these lectin pathway molecules in development processes (Garred et al. 2016).

On another side, mutations were recently identified in *C1R* (mostly) and *C1S* genes in patients affected by the periodontal Ehlers-Danlos syndrome (Kapferer-Seebacher et al. 2016). Looking for a possible common molecular effect associated to the different patient mutations, molecular analyses led to the hypothesis that active C1s, or an active 40 kDa C1s fragment, may be one common pathological component (Gröbner et al. 2019; Bally et al. 2019). Because the identified patient mutations either alter the enzyme correct folding or the association with C1q, this CP protease activity is assumed to be free from the control of C1q. Consistently, complement has been shown to play a role in the dysbiotic transformation of the periodontal microbiota and in the inflammatory process that leads to the destruction of periodontal bone (Hajishengallis et al. 2013). However, additional effects in the case of the periodontal Ehlers-Danlos syndrome are expected because the symptoms differ from classical periodontitis (Kapferer-Seebacher et al. 2016). Non-canonical functions of these C1r and C1s proteases, out of the complement system, are likely to be involved in this and other diseases processes.

General Comments and Conclusion

Our current structural knowledge on the LP and CP proteases has dramatically increased during the past decades, starting from the resolution of the X-ray structure of C1s catalytic domain in 2000 (Gaboriaud et al. 2000). These homologous proteases share many structural similarities, as illustrated in Figs. 2, 3, 4 and 6. However, several differences can be highlighted when we compare LP and CP proteases, or each individual protease. On one hand, MASPs mainly associate as homodimers and bind to a wide variety of collectin molecules: MBL (which shows various oligomeric assemblies), the three ficolins and collectins (CL-K1, CL-L1 and CL-LK). On the other hand, the C1 stoichiometry is fixed and the physiological concentration of its components is higher. C1r and C1s probably briefly transit as a proenzyme tetramer before their association with C1q, in a configuration that can be interpreted

as a way to prevent self-activation, as described above. Such configuration is a distinctive feature of the CP proteases.

From an evolutionary point of view, C1r, C1s, MASP-2, MASP-3, as well as thrombin are more recent than MASP-1. The following two evolution marks support this idea: the AGY codon for the active serine (instead of TCN) and the absence of introns in the SP domain (Gál et al. 2007; Krem and Di Cera 2001). MASP-1 is thus atypical, with a wider spectrum of substrates in its active state (Dobó et al. 2014). Nevertheless MASP-1 activity is crucial in activating the LP (Dobó et al. 2016b). As briefly reminded in the last chapter section, recent studies of patient mutations in MASP-3, C1r, C1s have suggested further functional roles independent of the complement system. This opens the way to further structure/function studies associated to diseases.

Questions about the partial activity of proenzyme states as a function of protease plasticity have been addressed elsewhere (Gál et al. 2007, 2009; Dobó et al. 2016b). We would like to mention that a collapsed conformation in C1r zymogen structure further prevents substrate entry and involves another conformational transition towards the un-collapsed state (Gohara and Di Cera 2011). A different kind of collapsed proenzyme conformation has been observed for MASP-1 (Megyeri et al. 2013). MASP-2 was shown to partly cleave C4 in its proenzyme state. This is the subject of a minor controversial issue, related to the shift in the position of the two arginines of its C4 exosite in the SP domain, as shown in this review for the homologous case of C1s (Fig. 6) (Drentin et al. 2015). Nevertheless, such a tight control of C4 cleavage by C1s or MASP-2, which mainly requires that the proteases are activated, is in line with the strong need for complement activation only "on site", under the control of their associated recognition molecules.

The overall flexibility of these CP and LP proteases has limited their study by X-ray analyses to recombinant fragments. This has however provided insights into the relative flexibility of joints linking two successive modules, by comparing their positions in different structures. For example, the CUB1-EGF head-to-tail associations are remarkably conserved in all these CP and LP proteases, as detailed in section "Central CUB1-EGF Interactions in LP and CP Proteases Assembly: A Marked Similarity." MASP CUB2-CCP1 junction appears to be mainly rigid and mostly conserved in MASP-1 and MASP-3 (Kjaer et al. 2013). This CUB2-CCP1 is also likely rigid in C1s (Venkatraman Girija et al. 2013). As mentioned in section "CCP1-CCP2 as Elongated Arms or Handles," restricted flexibility at the CCP1-CCP2 junction further supports the initial notion that it can act as a handle or elongated arm. In contrast, far more flexibility has been observed at the EGF-CUB2 junction in the CP proteases, especially in C1s. Complementary studies using SAXS to analyse the soluble structure of full-length MASP-1, MASP-2 and of the C1 proteases tetramer, combined with rigid body modelling and negative stain EM allowed to propose low resolution models of the full-length protease assemblies, which mainly exhibit extended shapes (Kjaer et al. 2015; Nan et al. 2017; Tschopp et al. 1980; Mortensen et al. 2017). These experiments performed in solution provide however far less experimental values, which introduces a higher risk of over- or miss-interpretation. Within the current resolution revolution in cryo-EM techniques, cryo-electron

tomography has brought into the field the only current mid-resolution snapshot of the full-length C1r and C1s proteases within the IgM-C1-C4b giant complex (Fig. 2b, Sharp et al. (2019)), which represents a fantastic advance in our insight into these molecules. Further insights on this side are awaited, that will complete our understanding of the activation scenario of the CP proteases (as compared to LP), with the topological details of successive steps. How and where serpin inhibitors such as C1-inhibitor are engaged and control some of these steps further remain to be investigated.

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Biological Activities of SP-A and SP-D Against Extracellular and Intracellular Pathogens

Hadida Yasmin and Uday Kishore

Introduction

Air harbours enormous number of pathogens such as viruses, bacteria, fungi etc. Many of these pathogens can cause acute infections that are effectively cleared by the host immune system whereas some are able to establish persistent infection. The innate immune system constitutes the first line of host defence against these pathogens, where pulmonary surfactant proteins are considered to play important role in innate immunity in airways.

Pulmonary surfactant is a complex mixture of lipids (90%) and proteins (10%) that forms a thin film at the air-water interface of the alveoli reducing the surface tension at the alveolar interface, thus preventing alveolar collapse (Kishore et al. 2005). Of four surfactant proteins (SPs) (SP-A, SP-B, SP-C and SP-D), SP-A and SP-D belong to the collectin (collagen-containing C-type lectin) family. SP-A and SP-D can bind and agglutinate a wide range of microbial pathogens, and modulate host defence strategies for effective clearance of pathogens and regulating inflammatory processes of the lung.

SP-A and SP-D are hydrophilic proteins characterized by four domains consisting of: (1) N-terminal, cysteine-rich non-collagenous domain, which cross links monomeric subunits and helps in disulphide bond dependent oligomerization; (2) triple-helical Collagenous domain, which is involved in multimerising subunits, activation of immune system, binding site for the putative collectin receptors, Calreticulin/CD91; (3) trimerizing α-helical coiled-coil neck region, which is the

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nucleation point for refolding and (4) C-terminal C-type lectin domain or carbohydrate recognition domain (CRD), which binds to a range of carbohydrate, phospholipids and other self and nonself ligands. Each monomeric subunit can further assemble to yield multimers upto dodecamers in the case of SP-D. SP-A has a tulip-like appearance while SP-D can be visualised as its minimal cruciform structure under the electron microscopy (Fig. 1) (Crouch 1998; Nayak et al. 2012).

This chapter provides an overview of the various ligands on the pathogens that are recognised by SP-A and SP-D, and the effector mechanisms that are triggered by such recognition processes, which is aimed at clearing or restraining the invading pathogens at the mucosal surfaces at pulmonary as well as extrapulmonary sites.

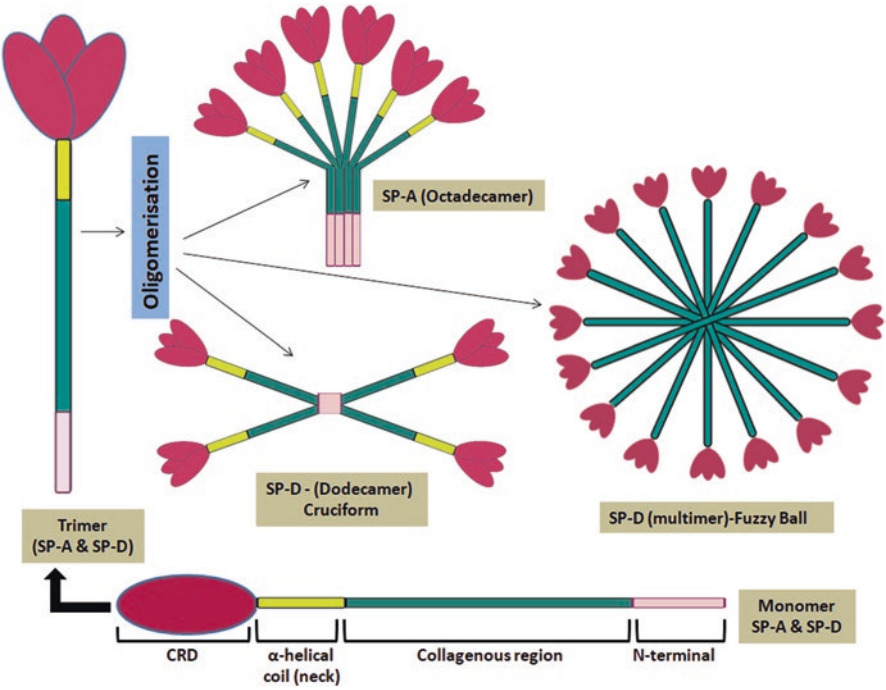

Fig. 1 SP-A and SP-D structure: The primary structure consists of four region N-terminal cysteine-rich region, long collagenous region, α-helical coiled neck region, and C-terminal carbohydrate recognition domain (CRD). This primary structure forms a trimer which can acquire bouquet-like structure assembled from six trimers in the case of SP-A. In case of SP-D, four trimeric units oligomerises to form cruciform dodecamer and also higher-order multimeric structures (fuzzy ball)

Protective Effects of SP-A and SP-D Against Viral Pathogens

Innate immune recognition of viruses is crucial for limiting several viral infections and its related pathogenesis. SP-A and SP-D act as soluble pattern recognition receptors (PRRs) in recognizing viral surface molecules and activating immune cells to facilitate the clearance of viral pathogens (Fig. 2).

Influenza A Virus

Influenza A virus (IAV) is associated with acute respiratory illness and represents an ongoing threat to human and animal health leading to substantial morbidity and mortality. Various Influenza pandemics were caused by H1NI (Spanish Flu) in 1981,

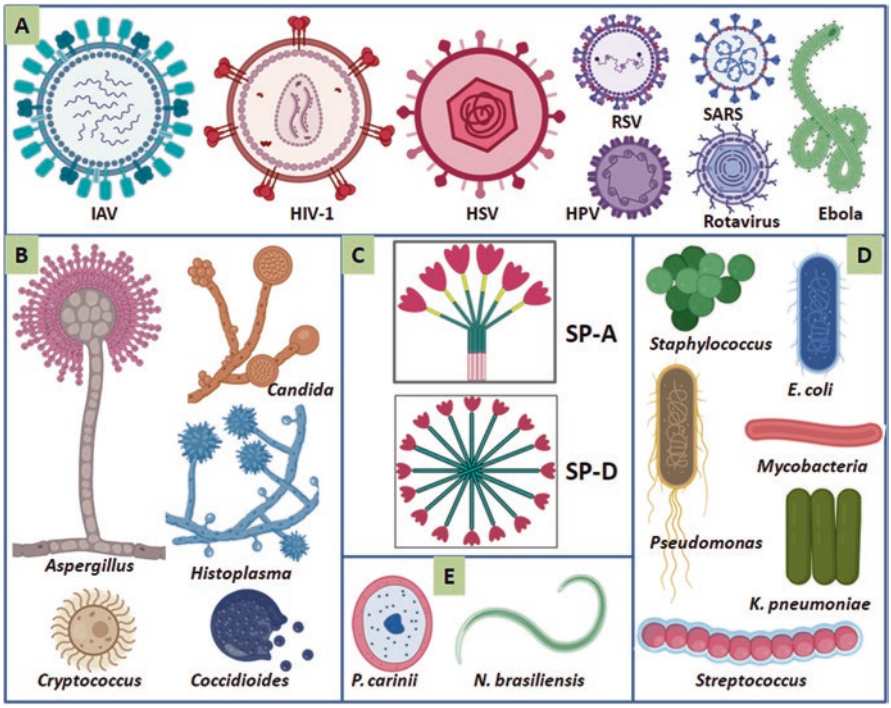

Fig. 2 SP-A and SP-D engage with range of pathogens, including (**a**) Viruses (Influenza A virus, Human Immunodeficiency virus-1, Herpes Simplex Virus, Respiratory syncytial virus, Human papilloma virus, SARS coronavirus, Rotavirus, Ebola) are shown here (**b**) Fungi viz. *Aspergillus, Candida, Histoplasma, Cryptococcus* and *Coccidioides* (**d**) Bacteria viz., *Streptococcus E. Coli*, Mycobacteria, *Pseudomonas, K. Pneumonia, Staphylococcus*, and (**e**) Parasites viz. *P carinii* and *N. brasiliensis*. Here octademamer of SP-A molecule and fuzzy ball multimeric SP-D molecule has been shown (**c**)

H2N2 (Asian Flu) in 1957, H3N2 (Hong Kong Flu) in 1986, and A(H1N1)pdm09 (Swine Flu) in 2009. (Taubenberger and Morens 2009; Tripathi et al. 2015; Hsieh et al. 2018a, b). The worst pandemic recorded was in 1918 killing up to 50 million people worldwide (Johnson and Mueller 2002), approximately 675,000 deaths in the US alone (Taubenberger and Morens 2006).

Most IAV subtypes possess two membrane-bound surface glycoproteins expressing N-linked oligosaccharides, Hemagglutinin (HA) and the neuraminidase (NA). HA attaches the virus to the cell with attaching terminal sialic acid residues on glycoproteins or glycolipids to initiate the infectious cycle, whereas NA cleaves terminal sialic acids to release virions (Kosik and Yewdell 2019). There are 16 HA and 9 NA in different subtypes of IAV circulating in humans and animals. When a subtype with a new HA or NA variant appears in the human population by genetic reassortment, it usually causes a pandemic because there is no pre-existing immunity against the new virus. HA and NA can contain mixture of branched structures terminating in the sugar mannose, complex branched structures terminating in galactose and/or *N*-acetyl-galactosamine (GalNAc) or even hybrid-type oligosaccharides (Basak et al. 1981; Ward and Dopheide 1981). Oligosaccharides attached to the globular head of the glycoprotein display considerable variations in number as well as location. These viruses have undergone antigenic drift possibly through addition of glycans to the HA (Sun et al. 2011; Abe et al. 2004). The numbers of N-linked glycosylation sites on the head of HA increases after their emergence in the human population in case of pandemic and seasonal H1N1 and H3N2 episodes (Tate et al. 2014).

Innate immunity plays an important role in mounting initial response against IAV infection. N-linked glycans present on the surface of IAV, are detected by soluble humoral factors of the innate immune system mounting anti-IAV activities against virions and virus-infected cells (Tate et al. 2014). SP-A and SP-D contribute to initial protection against IAV; SP-D seems to be the most potent due to its specific mode of binding to viral carbohydrates (Hartshorn et al. 1994). SP-A inhibits IAV by binding to HA in a calcium-dependent manner through its sialyted carbohydrates present in the lectin domain, and thus, is classified as ɣ-inhibitor (Benne et al. 1995; Hartshorn et al. 1997a, b). However, SP-D-mediated inhibition is via binding to high-mannose oligosaccharides on HA and is calcium-dependent, thus it is recognised as β-type inhibitor of IAV (Hartshorn et al. 1993a, b; White et al. 2004). SP-A exhibits greater hemagglutination activity against IAV subtypes with reduced number of glycosylation on HA molecule (Hartshorn et al. 1997a, b). Glycosylation of HA at N165 (glycosylation position at 165 amino acid residue) was found to be important for the neutralization of IAV by SP-D (Reading et al. 1997).

SP-A and SP-D show effectiveness in dealing with infectivity in a cooperative manner; SP-A, which is largely surfactant-associated (lipid associated) acts primarily at the surfactant interface, while SP-D, being largely a soluble (pulmonary secretions) molecule shows its potency in the fluid phase of airways (Hartshorn et al. 1994). The Ca^{2+}-dependent binding of SP-A to IAV strain A/X31 takes place through NA (Malhotra et al. 1994). Additionally, SP-A acts as an opsonin for the phagocytosis of IAV by alveolar macrophages. This opsonisation capability of SP-A was

due to its sialic acid residues, thus helping in the removal of virus (Benne et al. 1997). SP-D does not seem to act as an opsonin for the phagocytosis of IAV (Benne et al. 1997) but strongly neutralises and aggregates viral particles by binding to high mannose oligosaccharide residues near the sialic acid binding sites of HA, thus, inhibiting the attachment of IAV to host cells. These inhibitory effects are mediated by the calcium-dependent carbohydrate-binding property of SP-D on viral HA and NA (White et al. 2004). SP-D also has the ability to bind high mannose type II glycans on some IAVs (Qi et al. 2011).

Aggregation of IAV by surfactant proteins is an important neutralization mechanism that prevents viral particles from infecting target host cells, in addition to enhancing virion phagocytosis by macrophages to clear IAV more efficiently. The extended cruciform structure of SP-D helps in bridging interactions with multiple vial particles, leading to the formation of large viral aggregates (Brown-Augsburger et al. 1996). Aggregation of viral particles by SP-D possibly reduces the count of infectious viral particle and subsequently enhances clearance by mucociliary and phagocytic mechanisms. This viral aggregation by SP-D enhanced neutrophil binding of IAV and associated respiratory burst response against them (Hartshorn et al. 1994) (Fig. 3).

IAV infection can induce impaired responsiveness leading to dysfunction in respiratory burst, degranulation and intracellular bacterial killing by phagocytic cells, thereby increasing host's susceptibility to bacterial superinfections which is

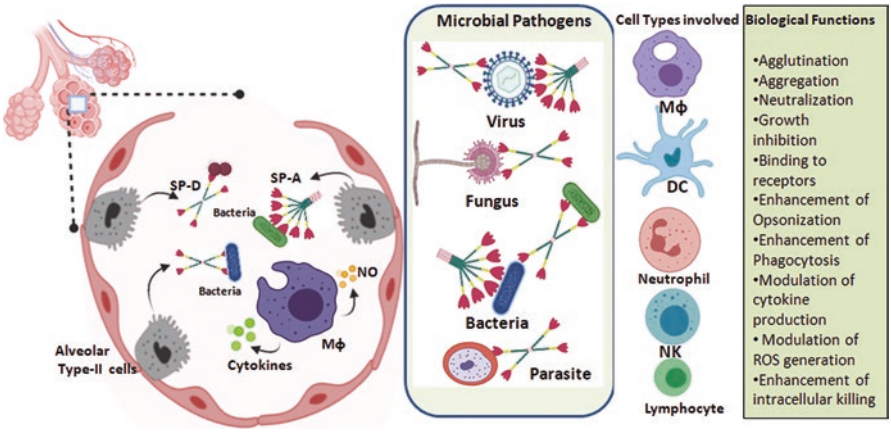

Fig. 3 Biological activities of SP-A and SP-D: Alveolar type-II cells in lungs secretes SP-A and SP-D which can bind, agglutinate and neutralize wide range of microbial pathogens including viruses, bacteria, fungus and parasites. SP-A (bouquet-like structure) and SP-D (cruciform structure) are capable of modulating host defence strategies for effective clearance of pathogens by cytokine production and ROS generation by effector cells such as macrophages (Mϕs), dendritic cells (DC), neutrophils, natural killer cells (NK) and lymphocytes. Both SP-A and SP-D are capable of enhancing opsonization, phagocytosis and eventually intracellular killing by innate immune cells such as alveolar macrophages (Mϕs) and dendritic cells (DC). This helps in clearing or restraining the invading pathogens at the mucosal surfaces at pulmonary as well as extrapulmonary sites

an important cause of morbidity and mortality during IAV epidemics (Kilbourne 1987). Hartshorn et al. have reported the protective effect of SP-D against bacterial superinfection *in vivo,* which was possibly due to opsonisation of the virus with SP-D (Hartshorn et al. 1994). SP-D strongly increased neutrophil respiratory burst response towards IAV *in vitro,* thus, demonstrating a proinflammatory response (White et al. 2005). In an experiment involving pre-incubation of neutrophils with SP-D, the H_2O_2 response to IAV was found to be reduced, whereas by preincubating IAV with dodecameric SP-D, H_2O_2 response to the virus increased quite strongly. This suggests that during preincubation with SP-D, possibly the inhibitory receptors of neutrophils were occupied by SP-D and thus, prevented the virus to bind with neutrophils. Thus, depending on whether SP-D is first incubated with IAV or neutrophils, SP-D can either increase or reduce respiratory burst responses of neutrophils upon exposure to IAV. Similarly, preincubation of IAV with SP-A increased neutrophil uptake of IAV and stimulated H_2O_2 generation. However, SP-A and SP-D together caused a reduction in H_2O_2 responses compared with SP-D alone (White et al. 2005). Human neutrophil peptides (HNPs) were subsequently found to bind SP-D and modify its interactions with IAV. Though HNPs were found not to inhibit HA activity of IAV but strongly interfered with neutralizing activity of SP-D by directly binding to its CRD. This binding of SP-D to HNP was not affected by the degree of multimerization of SP-D and was not calcium dependent (Hartshorn et al. 2006).

SP-D binding to pandemic IAV subtype (pH1N1) can modulate its replication in the lower respiratory tract (Hawgood et al. 2004). This was assessed by comparing chimeric IAV with HA segment of 1918 (H1N1), 1957 (H2N2), 1968 (H3N2) or 2009 (H1N1) with seasonal IAVs. HA of pandemic viruses showed lower binding for SP-D, whereas HA of seasonal influenza strain exhibited strong *in vitro* binding to SP-D with little lung pathology in the infected mice (Qi et al. 2011). The pandemic strains derived from zoonotic sources have fewer N-linked glycosylation sites (Bush et al. 1999; Hensley et al. 2009; Kash et al. 2006), which probably explains less SP-D binding, and hence, significant pathology in the lower respiratory tract. H1 and H3 subtypes that causes seasonal epidemics express more glycans on the head of their HA, bind SP-D efficiently, causing effective viral inhibition. The level of CCL2 and CSF3 chemokine expression were found to be lower with high SP-D binding activity thus exhibiting little lung pathology in infected mice (Qi et al. 2011; Hsieh et al. 2018a, b).

Human H1-containing IAV has two or more glycosylation sites, suggesting that the host specificity of IAV may also depend on the characteristics of HA glycosylation (Inkster et al. 1993). In a experiment where susceptibility of different H1N1 viruses (including strains of A(H1N1) pdm) were assessed towards the anti-viral activities of human SP-D, it was found that seasonal H1N1 viruses demonstrated variations in their sensitivity towards SP-D as the number and location of N-glycosylation sites on HA varied, whereas most A(H1N1) pdm viruses carried a single N-glycosylation site (Asn104) on the head of HA and found resistant to the antiviral activities of SP-D (Job et al. 2010). Thus, γ-inhibitors like SP-A may respond better in combating against strains that are resistant to SP-D (Stevens et al.

2004). Glycosylation on the HA appears to increase over time in strains that establish themselves in the human population (White et al. 2004). Interestingly, a recent study has shown that the anti-viral activity of isolated lectin domains of SP-D can markedly increase for seasonal strains of IAV by modifying specific residues around the saccharide binding pocket. The combined change of D325A (aspartic acid 325) along with R343V (arginine 343) in the neck and CRD regions of human SP-D showed neutralizing activity similar to full length SP-D dodecamers for seasonal IAV (Hartshorn et al. 2010; Crouch et al. 2011). At the same time, mutated versions of SP-D (D325A + R343V mutant neck and CRD; in the mutant neck and CRD region the penultimate mannose in the chain binds in the lectin site) showed enhanced binding to the reduced number of mannosylated glycans present on the HA of these strains, and thus, were able to inhibit pandemic IAV (Hsieh et al. 2018a, b). Pigs are considered to be important intermediates in the emergence of new IAV strains due to reassortment of viral genes derived from human, avian, or porcine influenza viruses. Hemagglutination inhibition activity by Porcine SP-D was found to be related to the terminal sialic acids (SAs) present on the N-linked oligosaccharide in the CRD region. The SA-mediated interaction of SP-D can be observed only in pigs as they have unique glycosylation profile of SP-D compared to ducks and swine viruses where there are no conserved glycosylation sites at the tip of their HA (Van Eijk et al. 2003). The carbohydrates of porcine SP-D is uniquely sialylated with α (2,6)-linked SA, in contrast to SP-A, which contains both α (2,3)- and α (2,6)-linked SAs on its N-linked carbohydrate as confirmed through lectin staining and by cleavage with linkage-specific sialidases (Van Eijk et al. 2004). Thus, an N-linked CRD glycosylation provides interactions with the SA-binding site of IAV and an enhanced interaction with IAV glycans were favoured by tripeptide loop (presence of a unique tripeptide extension of the long loop in the CRD of SP-D, referred to as "326^{GSS}") at the lectin-binding site. *N*-glycosylated neck-CRD fragment of porcine SP-D (RpNCRD) unlike the human analogue RhNCRD, demonstrated potent neutralizing activity against pandemic A/Aichi/68 (H3N2) (Van Eijk et al. 2018).

In 1957 pandemic, a novel H2N2 subtype was formed when H2 virus re-assorted with the circulating H1N1. Thus, considering the fact that a low pathogenicity avian influenza virus (LPAIV) subtype can re-assort leading to emergence of new pandemic, activities of two recombinant human SP-D forms against LPAIV strains (H2N1, H5N1, H6N1, H11N9) were assessed. It was found that these avian IAV strains, containing H2, H5, H6 and H11 were not susceptible to lung SP-D activity due to presence of predominantly complex glycans at the key glycosylation sites (Parsons et al. 2020).

A recombinant form of human SP-D (rfhSP-D), containing homotrimeric neck and CRD regions was used to test if rfhSP-D interfered with the ability of pH1N1 and H3N2 IAV subtypes to infection lung epithelial cell line (A549). rfhSP-D could inhibit IAV entry, down-regulate viral replication (M1) and associated pro-inflammatory response. mRNA levels of TNF-α, IFN-α, IFN-β and IL-6 were downregulated during the initial stage of IAV infection with rfhSP-D (Al-Ahdal et al. 2018). However, in similar assays, a recombinant fragment of human SP-A

composed of trimeric neck and CRD (rfhSP-A) enhanced the infection, as evident from enhanced viral replication (higher expression of M1 genes) as well as increased expression of TNF-α, IL-12, IL-6 and IFN-α (Al-Qahtani et al. 2019). Interestingly full length native SP-A was able to downregulate the expression of M1 genes, suggesting that a complete SP-A molecule is required for protection against IAV (Al-Qahtani et al. 2019). These two studies highlighted that SP-A and SP-D are quite distinct in their ability to negate IAV infection. SP-A seems to require its intact structure including collagen region as opposed to SP-D where rfhSP-D was found to be a self-sufficient entity in dealing with IAV infection.

Human Immunodeficiency Virus-1 (HIV-1)

HIV-1, which is responsible for acquired immunodeficiency syndrome (AIDS), remains a leading cause of global morbidity. SP-A and SP-D can be found at various mucosal locations such as lungs, oral cavities, gastro-intestinal tract, genitourinary tract as we all as in ovary, vagina and cervix etc. (Tino and Wright 1996; Madsen et al. 2000; Leth-Larsen et al. 2004; Nayak et al. 2012; Madhukaran et al. 2016); all are also important sites for HIV-1 transmission. Thus, the role of SP-A and SP-D in HIV-1 pathogenesis and transmission has been examined.

The glycosylated HIV-1 envelope protein, gp120, plays an important role in pathogenesis of AIDS.SP-D binds gp120 in a calcium-dependent manner; native dodecameric SP-D binds HIV-1 gp120 more strongly than native trimeric SP-D (Meschi et al. 2005a, b). SP-D possibly binds to the centre of the oligomerized gp120 molecule via glycans located in the V3 loop (Madsen et al. 2013). SP-D can agglutinate both gp120 and intact inactivated HIV Bal particles in the presence of calcium (Madsen et al. 2013). SP-A also binds with HIV-1 gp120 via high mannose oligosaccharides efficiently neutralize both R5 and X4 strains of HIV (Gaiha et al. 2008). Both SP-A and SP-D could inhibit infection of $CD4^+$ T cells by two different strains of HIV-1, BaL and IIIB (Gaiha et al. 2008; Madsen et al. 2013). SP-D enhanced binding to HIV-1 to immature monocyte-derived dendritic cells as well as transfer from DCs to T cells *in vitro* (Madsen et al. 2013).

Bronchoalveolar lavage of HIV-1-infected individuals showed increase level of SP-A, which was found to enhance the attachment of *Mtb* to alveolar macrophages. This possibly explains the increased risk of tuberculosis during HIV-1 infection (Downing et al. 1995). The ability of native human SP-D and rfhSP-D to bind gp120 was assessed in addition to viral inhibition in three different targets, Jurkat T cells, U937 monocytic cells and PBMCs. Both native SP-D and rfhSP-D inhibited HIV-1 entry efficiently and blocked CD4 and gp120 interaction. rfhSP-D also significantly suppressed HIV-1 induced cytokine storm and phosphorylation of kinases p38, AKT and Erk1/2 in HIV-1 induced immune activation *in vitro* suggesting the potential use of rfhSP-D for immunotherapy against viral infection (Pandit et al. 2014). Mucosal biocompatibility of rfhSP-D has been assessed *ex vivo* where it showed inhibition in HIV-1 transfer across the vaginal tissues and downregulation of NF-κB

and mTOR transcripts while the expression of tight junctions and cytoskeleton genes were upheld (Pandit et al. 2019).

A direct protein-protein interaction between rfhSP-D and dendritic cell-specific intercellular adhesion molecule-3-grabing non-integrin (DC-SIGN) through their C-type lectin domains has been observed (Dodagatta-Marri et al. 2017). SP-D and DC-SIGN showed competitive binding behaviour towards immobilized HIV-1 gp120, possibly suggesting SP-D and gp120 may occupy same sites on DC-SIGN as revealed through *in silico* analysis. rfhSP-D also inhibited cis-transfer of DC-SIGN-bound HIV-1 to T cells in culture.

Herpes Simplex Viruses (HSV)

Herpes viruses are large family of DNA viruses, which are known to cause lytic infection in permissive cells. HSV have co-evolved with human for million years; these viruses can establish a latent infection and persist in humans for the lifetime. Pathogenic effects occur when the host acquires genetic defects in the immune responses or if the viral load becomes too high (Kurt-Jones et al. 2017). Herpes simplex virus type 1 (HSV-1) is a typical human-restricted pathogen with higher frequencies in developing countries (Su et al. 2016) and is responsible for causing a lifelong latent infection in neurons; it can get reactivated causing lytic infection mostly in epithelial or mucosal cells (Nicoll et al. 2012; Roizman and Whitley 2013). Herpesvirus, compared to other enveloped viruses, needs the combined effort of multiple glycoproteins and multiple host receptors to infect, depending on the cell type (Watson et al. 2019). SP-A bindsHSV-1 infected Hep-2 cells which can be inhibited by heparin, but not by mannose polysaccharide. Heparin could also dissociate cell bound SP-A, suggesting the role of polyanionic oligosaccharide in SP-A-HSV-1 interaction (van Iwaarden et al. 1992). SP-A has been shown to act as an opsonin in the phagocytosis of HSV-1 by alveolar macrophages, suggesting its important anti-viral properties (van Iwaarden et al. 1990).

Respiratory Syncytial Virus (RSV)

Respiratory syncytial virus (RSV) is a major respiratory pathogen in infants and young children. RSV causes an upper respiratory tract infection that may progress to acute bronchiolitis or interstitial pneumonia (LeVine et al. 1999a, b). Almost every child suffers from a mild upper respiratory tract infection by RSV but morbidity and mortality are related to lower respiratory tract involvement only (Griese 2002). Heavily glycosylated G-protein present in the RSV envelope aids in attachment with the host cells. This G protein contains several sites for N-linked glycosylation and almost 30% of its amino acids are serine and threonine residues (Griese 2002).

The potential role of SP-A in RSV infection has been examined via SP-A-deficient (SP-$A^{-/-}$) mice. SP-$A^{-/-}$ mice had increased numbers of RSV plaque-forming units in their lungs than in SP-$A^{+/+}$ wild type mice. Infiltration of neutrophils and proinflammatory cytokines such as -TNF-α and IL-6 were also enhanced in lungs of SP-$A^{-/-}$ than in SP-$A^{+/+}$ mice after RSV administration. Thus, SP-A played important role in pulmonary clearance of RSV *in vivo* which was associated with an enhanced respiratory burst by the alveolar macrophage (LeVine et al. 1999a, b). Daily levels of surfactant proteins in bronchoalveolar lavage (BAL) fluid from ventilated infants with RSV infection and in a ventilated surgical patients (control) were investigated; concentrations of SP-A and SP-D per ml of BAL fluid were found to be significantly reduced in children with RSV infection, suggesting that the reduction of surfactant proteins may contribute to the respiratory failure in RSV patients (Kerr and Patron 1999).

G protein from RSV (human, A2 strain) interacts with both native and rfhSP-D via the CRD region of SP-D. rfhSP-D was able to inhibit viral replication in the lungs (Hickling et al. 1999). The binding of SP-A to RSV G-protein was found to be inhibitable by both EDTA and mannan, suggesting the involvement of carbohydrate moiety of the G-protein interacting through the carbohydrate recognition domain of the SP-A (Hickling et al. 2001).

Human Papillomavirus (HPV)

HPV is the most common viral infection of the reproductive tract. Most HPV infections get cleared by cell-mediated immunity within a year, but sometimes, it can result in persistent infection with an increased probability of progression into invasive cancers (Stanley 2010). In an attempt to assess the impact of SP-A during the early events of sexual HPV transmission, a study was conducted in wildtype C57BL/6 mice. SP-A-mediated opsonization of HPV16-PsVs (pseudovirions) and significantly increased HPV16-PsVs uptake by eosinophils, neutrophils, monocytes, and macrophages in the female reproductive tract (Ujma et al. 2019).

SARS Coronavirus (SARS CoV)

Severe acute respiratory syndrome (SARS) outbreak in 2003 attributed to pulmonary infection with a novel coronavirus (SARS-CoV) infecting more than 8000 individuals and caused approximately 10% mortality (LeDuc and Barry 2004). SARS-CoV infects human hosts through the respiratory system and it interplays with the host innate immune system in the lung alveoli. The spike protein (S-protein) that interacts with the host and shows high degree of glycosylation was found to interact with SP-D (Leth-Larsen et al. 2007). The effect of S-protein binding to macrophages and DCs was also investigated. Plasma SP-D levels were significantly

elevated in SARS-type pneumonia (Wu et al. 2009). SP-A and SP-D were found to bind with HCoV-229E (a common non-SARS human CoV) and pre-treatment of HCoV-229E with SP-A or SP-D inhibited viral infection of 16HBE, bronchial epithelial cells. SP-D showed better effectiveness in inhibiting infection of 16HBE cells whereas SP-A was found more effective at inhibiting infection of alveolar macrophages (Funk et al. 2012).

Other Viruses

Vaccinia virus that is principally transmitted between humans by aerosol droplets, interacts with SP-D directly through A27 viral protein which lacked glycosylation. When challenged with the virus, SP-$D^{-/-}$ mice incurred greater mortality compared to SP-$D^{+/+}$ wild type mice, suggesting SP-D participating in host defense (Perino et al. 2013).

Ebola virus binds human as well as porcine SP-D through its glycoprotein. This interaction enhanced pseudoviral infection in pulmonary cells (A549) suggesting the possible role of SP-D in enhancing viral spread (Favier et al. 2018). In case of pulmonary infection mediated by adenovirus, SP-A showed enhancement in viral clearance inhibiting lung inflammation (Harrod et al. 1999).

Rotaviruses are non-enveloped viruses having a glycoprotein VP7 which forms the smooth surface of the virion from where VP4, an outer capsid protein, protrudes as spikes. Bovine SP-D was able to bind with VP7 glycoprotein of rotavirus strain NCDV and displayed neutralizing activity that was dependent upon glycosylation of VP7 (Reading et al. 1997).

Bacterial Pathogens

Despite the fast-acting intracellular signalling mechanisms induced by PRRs, microbial pathogens have evolved countermeasures to thwart innate immunity in order to survive and proliferate in the host. It is now clear that evolution has selected a conserved set of anti-microbial peptides as well as Pattern Recognition Receptors (PRRs) that initiate signals as a first line of defence against invading pathogens. If a bacterial pathogen is able to successfully evade destruction by anti-microbial peptides, most host organisms have evolved a second line of defence centred on microbial recognition of PAMPs by PRRs and the subsequent production of cell-intrinsic immune mechanisms and/or recruitment of immune cells. In response to these challenges, many bacterial pathogens have modified the molecular structure of their PAMPs, thereby avoiding immune detection through stealth and evasion. For example, lipopolysaccharide (LPS) is a ubiquitous component of Gram-negative bacteria cell wall, and is composed of diverse O-antigen side chains that are anchored to the outer leaflet of the bacterial envelope by Lipid A. Importantly, Lipid A is directly

recognized by the mammalian TLR4-MD2-CD14 PRR complex to activate innate immune signalling pathways.

Gram Positive Bacteria

There are several Gram-positive bacteria which can cause respiratory distress in adults leading to pulmonary inflammation, like *Staphylococcus aureus* and *Streptococcus pneumonia* (Ewig and Torres 1999; Goel et al. 1999). Pneumococci are one of the leading causes of septicemia, meningitis, and lower respiratory tract infections in humans, where *S. pneumoniae* produces two hemolysins contributing to the pathogenicity (Navarre and Schneewind 1999). Group A streptococci are responsible for pharyngitis, impetigo, rheumatic fever, and acute glomerulonephritis, whereas Group B streptococci can cause neonatal sepsis and meningitis in developed countries (Navarre and Schneewind 1999).

The cell wall of Gram-positive bacteria is composed of a peptidoglycan (PepG) macromolecule that is attached with several other accessory molecules such as teichoic acids, teichuronic acids, polyphosphates, or carbohydrates. About 40% weight of the bacterial cell wall comprises of multiple layers of cross-linked PepG (Shockman and Barrett 1983). PepG and lipoteichoic acid (LTA) are capable of inducing inflammatory response and can also initiate septic shock (De Kimpe et al. 1995). SP-A was found to bind with a wide range of Gram-positive bacteria such as *Staphylococcus aureus* (van Iwaarden et al. 1990; Kuan et al. 1992a, b; Greertsma et al. 1994; McNeely and Coonrod 1994a, b; Manz-Keinke et al. 1992), Group A *Streptococcus* (Ohmer-Schröck et al. 1995), Group B *Streptococcus* (LeVine et al. 1997, 1999a, b), *Streptococcus pneumonia* (Kuronuma et al. 2004; Sano et al. 2007) mostly either with the PepG or with LTA via CDRs and also enhanced uptake by phagocytosis. SP-D could bind with *Bacillus subtilis*, *Staphylococcus aureus*, Group B *Streptococcus* and *Streptococcus pneumonia* (van de Wetering et al. 2001; Hartshorn et al. 1998; Stepherd 2002; Jounblat et al. 2004). Hartshorn et al. (1998) have shown that SP-A and SP-D both were able to increase calcium-dependent uptake of *Streptococcus pneumoniae*, and *Staphylococcus aureus* by neutrophils. The aggregation capability was influenced by the degree of multimerization of SP-D (Hartshorn et al. 1998). The N-terminal and/or collagen domains of SP-D contribute to the enhanced bacterial binding and aggregating activities since multimeric structure was found to be important for SP-D efficacy (Hartshorn et al. 2002). rfhSP-D (consisting of the head and neck regions of the native molecule) could bind with several strains of *Streptococcus pneumoniae* where the strength of binding varied between different capsular serotypes, but was not able to enhance killing of pneumococci by human neutrophils (Jounblat et al. 2004).

SP-A gene-deficient (SP-A$^{-/-}$) mice showed increased susceptibility to airway challenge of group B streptococci, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (LeVine et al. 1997, 1998, 1999a, b). Although SP-A binds to both *Staphylococcus aureus* and enhances its phagocytosis by monocytes, it fails to

stimulate intracellular killing and production of reactive oxygen intermediates (Greertsma et al. 1994). SP-A can also enhance scavenger receptor A (SR-A)-mediated uptake of *Streptococcus pneumonia* (Kuronuma et al. 2004). It can also bind di-saturated phosphatidylglycerol of *Mycoplasma pneumonia* and inhibit its bacterial growth *in vitro* (Piboonpocanun et al. 2005).

Gram-Negative Bacteria

Gram-negative bacteria contain lipopolysaccharide (LPS), a cell wall-resident PAMPs, which allows PRR-containing phagocytes to recognize bacterial invasion and mount innate immune responses. LPS consists of a hydrophobic membrane anchor portion (lipid A) of relatively conserved core oligosaccharide coupled to a distal polysaccharide (O-antigen) that extends from the bacterial surface (Raetz and Whitfield 2002). O-specific chain consists of up to 50 repeating oligosaccharide units formed of 2–8 monosaccharide components which differs between strains. *Haemophilus influenzae*, for example, contains O-antigen structures that closely resemble human glycosphingolipids due to the presence of *N*-acetylneuraminic acid or L-fucose (Moran et al. 1996). Lipid A portion is responsible for the immunostimulatory activity of LPS (Matsuura 2013).

SP-A and SP-D bind LPS of Gram-negative bacteria and enhance their phagocytosis by alveolar macrophages (Pikaar et al. 1995). Lipid A moiety of smooth strains of Gram-negative bacteria contains O-antigen whereas the rough strains lack the O-antigen (Van Iwaarden et al. 1994). Binding of SP-A to LPS of *E. coli* appears dependent on Ca^{2+} but it is not affected by mannan and heparin, or by deglycosylation of the SP-A. SP-A associates via the lipid A moiety of rough LPS, but not with smooth LPS (Van Iwaarden et al. 1994). Lectin blot analysis demonstrated specific binding of SP-D to LPS from several strains of enteric Gram-negative bacteria including *E. coli.* SP-D can agglutinate *E. coli* in a calcium- and carbohydrate-dependent manner (Kuan et al. 1992a, b).

Klebsiella pneumoniae is an anaerobic, Gram-negative bacterium indigenous to the oral cavity and intestinal tract; however, it often causes severe respiratory and urinary tract infections. *K. pneumoniae* strains of the K2 capsular serotype are usually highly virulent in mice; the capsule is recognized by mannose receptor (MR) present on macrophages (Kabha et al. 1995). SP-A acts as an opsonin and enhances phagocytosis of K21a (low virulent, capsule containing Man α1 Man sequences) serotypes of *K. pneumoniae* by alveolar macrophages via MR (but not of K2) (Kabha et al. 1997).

The binding of native human SP-D purified from lung lavage as well as a recombinant fragment of human SP-D (rfhSP-D) composed of trimeric neck and CRD regions, to bind LPS from various Gram-negative bacteria (*E. coli, K. pneumoniae* and *Ps. aeruginosa)* has been examined by Kishore et al. (1996a, b). rfhSP-D was able to bind to the LPS similar to native SP-D (Kishore et al. 1996a, b). SP-A is also able to aggregate *Hemophilus influenza* type A and induce phagocytosis by

macrophages (McNeely and Coonrod 1994a, b). SP-A brings about NO-mediated killing of *M. pulmonis* by alveolar macrophage (Hickman-Davis et al. 1998). In addition, SP-A deficiency modifies surfactant aggregate content and lowers the inhibition resistance of LA surfactant *in vitro* compared with experiments involving congenic normal mice (uninfected B6 SP-A$^{-/-}$ versus B6 mice) (Hickman-Davis et al. 2007). Bacteriostatic effect of SP-A on *Mycoplasma pneumoniae* was found to be mediated by binding to its surface disaturated phosphatidylglycerols (Ledford et al. 2009).

Mycobacteria

Mycobacteria range from environmental, non-pathogenic species, to opportunistic pathogens that can infect immuno-compromised hosts (Saelens et al. 2019). Mycobacterium genus includes strict pathogens, potential or opportunistic pathogens, and non-pathogenic, saprophytic species. These are the causative organisms for most important diseases including tuberculosis (TB), leprosy, Buruli ulcer, and pulmonary non-tuberculous mycobacterial (NTM) disease (Forbes et al. 2018).

Mycobacterium tuberculosis

Tuberculosis caused by *Mycobacterium tuberculosis* (*Mtb*) is an ancient disease which co-evolved compatibly with humans. TB has now emerged as a major global health concern affecting almost one third of global population (Ferluga et al. 2020). Alveolar macrophages are the initial sites of infection with *Mtb* and innate immune arm in the lungs plays an important role in controlling the inhaled pathogen. SP-A enhances phagocytosis of the virulent *Mtb* Erdman strain by alveolar macrophages (Gaynor et al. 1995). SP-A shows high affinity binding to attenuated *Mtb* strain (H37Ra) with a K_d value of 1.9×10^{-9} M in a calcium dependent manner and also enhances adherence of bacilli to mouse alveolar macrophages (Pasula et al. 1997). Deglycosylated SP-A exhibits minimal binding to *Mtb* (Pasula et al. 1997) and does not enhance the adherence of *Mtb* to monocytes (Gaynor et al. 1995), indicating the importance of sugar moieties during the interaction. In spite of SP-A being helpful in aggregation and phagocytosis of *Mtb*, SP-A appears to suppress reactive nitrogen intermediate production, a likely mechanism through which *Mtb* possibly counteracts the cytotoxic response of alveolar macrophages (Pasula et al. 1999). Sidobre et al. (2000) have identified mycobacterial lipoglycans as putative ligands for human SP-A, which requires both the terminal mannose residues and the aglycone moiety for optimal binding. In addition, lipomannan and mannosylated lipoarabinomannan (ManLAM) are also SP-A ligands (Sidobre et al. 2002). Cell surface molecule, Apa (alanine- proline-rich antigenic) glycoprotein, was found to be another potential adhesion molecule on *Mtb* that can interact with human SP-A (Ragas et al. 2006).

SP-D has also been shown to bind and agglutinate virulent *Mtb* Erdman strain and reduce uptake of bacilli by human macrophages (Ferguson et al. 1999; Ferguson and Schlesinger 2000). This binding of SP-D to *Mtb* is calcium- and sugar-dependent. SP-D shows minimal binding to the avirulent *M. smegmatis*. Lipoarabinomannan (LAM) is a major surface lipoglycan of *Mtb* (Schlesinger et al. 1994) and the binding of SP-D to Erdman lipoarabinomannan seems to be mediated by the terminal mannosyl oligosaccharides (Ferguson et al. 1999).

The collagen region of SP-D seems to be required for enhanced binding to *Mtb* and is essential for agglutination (Ferguson et al. 2002). Dodecameric SP-D, but not rfhSP-D, causes agglutination of *Mtb*, confirming that the multivalent nature of SP-D is essential for agglutination. SP-D binds and masks the terminal mannose caps of Man LAM of *Mtb.* It is also capable of limiting the intracellular growth of bacilli inside the macrophages by enhancing phagosome-lysosome fusion (Ferguson et al. 2006).

During inhalation, respiratory pathogens are exposed to shear forces as they travel to the terminal airways. Interaction of SP-A and SP-D with virulent (H37Rv) and attenuated (H37Ra) *Mtb* strains has thus been investigated under shear conditions to mimic the dynamic lung microenvironment (Hall-Stoodley et al. 2006). SP-A binds both strains well nearly 4–5 times better under shear conditions, compared to static conditions and BSA control (Hall-Stoodley et al. 2006). Covalently surface-immobilised SP-D binds virulent *Mtb* and *Mtb* ManLAM-coated beads feebly and agglutinates bacilli poorly, compared to when *Mtb* is pre-incubated with soluble SP-D, which causes efficient bacterial agglutination, highlighting the importance of SP-D conformation in its biological functioning (Hall-Stoodley et al. 2006). In a study to examine the effect of SP-A on MR expression on human monocyte-derived macrophages, SP-A was found to specifically regulate surface expression of functional MR, without altering complement receptor (CR) expression. Monocyte-derived macrophages cultured on an SP-A substrate demonstrated enhanced pinocytosis of mannose BSA and phagocytosis of *Mtb* lipoarabinomannan-coated microspheres (Beharka et al. 2002). Antibodies against the SP-A-binding neck domain (α-SP-R210n) also inhibited *Mtb* induced proliferation of lymphocytes and secretion of IFN-γ and TNF-α is possibly through enhanced production of IL-10 and TGF-β1 (Samten et al. 2008).

Mycobacterium avium

Nontuberculous mycobacteria (NTM) such as *Mycobacterium avium* are slowly growing pathogens in natural and artificial environments. NTM may result in colonization, infection, and causing diseases that can be detected in the respiratory and gastrointestinal tracts or on the skin of healthy individuals (Griffith et al. 2007; Brown-Elliott et al. 2012; Forbes et al. 2018). *M. avium* complex (MAC) includes two species, *M. avium* and *M. intracellulare*. Pulmonary infection caused by the MAC can occur in immunocompetent hosts; disseminated infections usually occur in people living with HIV-1. The most common presentations of MAC lung

infections in immunocompetent hosts are TB-like apical fibrocavitary disease or interstitial nodular infiltrates and bronchiectasis (Griffith et al. 2007; Brown-Elliott et al. 2012). SP-A and SP-D bind *M. avium* in a calcium-dependent and independent manner, respectively (Kudo et al. 2004). The mutated form of SP-A (E195Q, R197D) show decreased binding to *M. avium* but can still stimulate phagocytosis similar to wild-type SP-A. SP-A and SP-D could enhance MR-mediated phagocytosis of *M. avium* by macrophages (Kudo et al. 2004). SP-D can agglutinate *M. avium*, involving CRD region (Ariki et al. 2011). The binding of SP-A strongly inhibits the growth of *M. avium* in culture. SP-D binds *M. Avium* surface in clusters whereas SP-A almost covers the entire bacterial surface as observed under scanning electron microscopy (Ariki et al. 2011). SP-A suppresses NO production by *M. avium*-stimulated alveolar macrophages through inhibition of TNF-α production (Hussain et al. 2003).

Mycobacterium bovis BCG

The live, attenuated BCG strain of *M. bovis* is used for TB vaccination. The effects of functional (in exon, non-synonymous) polymorphisms of SP-D on the interaction between SP-D and *M. bovis* BCG have been investigated by Hsieh et al. (Hsieh et al. 2018a, b). It appears that residue 11 Met (92T) is likely to cause susceptibility to TB as in comparison to SP-D 92C (amino acid residue 16, Threonine). SP-D 92T (amino acid residue 16, Methionine) which exhibits reduction in binding to *M. bovis* BCG, inhibiting phagocytosis and aggregation, and inhibition of intracellular growth (Hsieh et al. 2018a, b). SP-A enhances BCG-induced inducible NO synthase protein level, and subsequent production of TNF-α and NO in rat macrophages (Weikert et al. 2000).

Fungi

Fungal pathogens can cause life-threatening infections in immunocompetent as well as immunocompromised individuals. *Aspergillus fumigatus* and *Candida albicans* yeasts can cause opportunistic infections during immune suppression, as observed in patients receiving treatment against AIDS (Kauffman and Carver 1990). Furthermore, fungal infections are often persistent and not easy to treat as it is difficult to target them without affecting host cells.

Aspergillus fumigatus

Aspergillus fumigatus is a ubiquitous airborne fungus, which is responsible for allergic bronchopulmonary aspergillosis (in immunocompetent individuals), invasive pulmonary aspergillosis (affecting highly immunocompromised subjects) and a range of sub-acute and chronic forms of pulmonary aspergillosis (Madan and Kishore 2020).

Madan et al. have conducted *in vitro* and *in vivo* experiments to establish the protective role of SP-A and SP-D against allergic and invasive aspergillosis (Madan et al. 1997a, b). SP-A and SP-D bound and agglutinated *A. fumigatus* conidia and enhanced its uptake and killing by alveolar macrophages and neutrophils (Madan et al. 1997a). In another study where culture filtrate allergens and various purified glycosylated and non-glycosylated allergens of *A. fumigatus* were assessed, both SP-A and SP-D could bind to allergens and purified glycosylated allergens in a carbohydrate-specific and calcium-dependent manner but were unable to bind with the deglycosylated allergens (Madan et al. 1997b), suggesting that the binding was mediated through their CRD region with the carbohydrate residues on the allergen. Both the surfactants were also able to inhibit *A. fumigatus* allergen-induced histamine that was released from the basophils of allergic patients (Madan et al. 2001). Rat BAL fluid containing SP-D has been shown to inhibit binding of condia to the extracellular matrix proteins and A549 lung epithelial cells (Yang et al. 2000). Reduction in the conidia binding was observed with pre-treatment of epithelial cells and extracellular matrix proteins with SP-D (Ordonez et al. 2019).

SP-D shows reduced binding to *kre6* yeast mutant (cell wall comprising about 50% less β(1→6)-glucan than the wildtype) compared to the wild type, confirming that β(1→6)-glucan is a fungal ligand for SP-D (Allen et al. 2001). SP-D has been found to bind with *A. fumigatus* dormant conidial surface melanin pigment and galactomannan (GM) as well as galactosaminogalactan (GAG), two cell-wall polysaccharides. SP-D showed calcium- dependent binding with GM and GAG recognised by its CRD region, whereas SP-D binding was calcium-independent for melanin requiring collagen region (Wong et al. 2018). Human monocyte-derived macrophages (MDMs) show efficient phagocytosis towards SP-D- opsonised conidia and could subsequently induce the production of pro-inflammatory cytokines. MDMs cultured with SP-D-opsonized conidia produced significantly higher TNF-α, IL-6 and IL-8 than the control groups, unstimulated MDMs and when co-cultured with un-opsonized conidia (Wong et al. 2018).

Murine models of ABPA, when intranasally treated with SP-A and SP-D, demonstrated reduction in *A. fumigatus*-specific IgE and IgG levels, peripheral and pulmonary eosinophilia, and Th2 cytokine response (Madan et al. 2001). However, SP-D (and rfhSP-D) was considerably more effective in ameliorating the allergic features compared to SP-A. SP-$D^{-/-}$ gene deficient mice exhibited intrinsic hypereosinophilia and showed several-fold increase in the levels of IL-13 and IL-5 and reduction in the IFN-γ to IL-4 ratio following *A. fumigatus* allergen challenge. Intranasal administration of SP-D or rfhSP-D downregulated pulmonary

eosinophilia and specific IgG and IgE antibodies in ABPA murine models (Madan et al. 2005a). Reduction in the bronchial hyper-responsiveness, bronchial eosinophilia and in Th-2 cytokines due to exogenous SP-D treatment were found possibly due to reduction in eotaxin level in the lungs (Erpenbeck et al. 2006).

In a murine model of invasive pulmonary aspergillosis, treatment with SP-D or rfhSP-D reduced the mortality by about 85% compared to untreated groups (Madan et al. 2010), concomitant with higher production of TNF-α, IFN-γ and MIP-1α (Singh et al. 2009). *SP-D*$^{-/-}$ mice challenged intranasally with wildtype conidia or melanin ghosts (hollow melanin spheres) displayed reduction in pro-inflammatory cytokines in the lung compared with wildtype mice. SP-D was found to bind with melanin present on the dormant *A. fumigatus* conidial surface, facilitating conidial phagocytosis and also inhibiting ROS quenching capacity of melanin (Wong et al. 2018).

Candida albicans

Candida albicans is a commensal opportunistic fungus present on the skin and in mucosal tissues that causes candidiasis during immunosuppressive conditions. SP-D was found to bind to *C. albicans* yeast which was inhibited in the presence of EDTA and mannan (Ordonez et al. 2019). Incubation of *C. albicans* with SP-D results in the inhibition of hyphal outgrowth as well as phagocytosis by alveolar macrophages (van Rozendaal et al. 2000). *C. albicans* infection of a human airway epithelial cell line, Calu3, increased synthesis ofIL-8 and IL-6 significantly and infection decreased by neutrophils in the presence of SP-D; SP-D had no significant effect on the *C. albicans*-induced oxidative burst (Ordonez et al. 2019).

Histoplasma capsulatum

Histoplasma capsulatum is a dimorphic fungal pathogen; its inhalation results in a flu-like illness in most cases. However, but some instances, it can cause more serious pneumonitis or a chronic cavitary pulmonary infection (Deepe 1999). SP-A and SP-D treatment results in increased yeast permeability, and enhanced entry into pulmonary macrophages. However, SP-A and SP-D do not seem to inhibit the growth of macrophage-internalized *H. capsulatum* (McCormack et al. 2003).

Cryptococcus neoformans

Cryptococcus neoformans is a soil-dwelling organism that obtains its nutrition from digesting material in the environment and secretes a range of enzymes to degrade host molecules (Almeida et al. 2015). This fungal pathogen primarily affects immunocompromised individuals through inhalation of spores and may spread to the central nervous system causing life-threatening meningitis and is relatively common in AIDS patients.

SP-A and SP-D bind acapsular *C. neoformans* in a calcium-dependent manner (Schelenz et al. 1995). SP-D binds quite efficiently the acapsular form (but not the capsular form) and aggregates them. Assembly of glucuronoxylomannan (GXM) in the capsule probably lowers the affinity for SP-D in the capsular form preventing aggregation (van de Wetering et al. 2004). The binding ligand for SP-D are GXM and mannoprotein 1 (MP1) components of the cryptococcal capsular components (van de Wetering et al. 2004).

Interestingly, SP-D seems to facilitate infection of pathogenic fungus *C. neoformans in vitro* and *in vivo* (Geunes-Boyer et al. 2009a, b, c, 2012). SP-D bind and protects *C. neoformans* cells from macrophage induced H_2O_2-induced oxidative stress (Geunes-Boyer et al. 2012). *C. neoformans* infection appears to be facilitated by the presence of endogenous SP-D in wild-type mice influencing fungal burden in the lungs and faster dissemination to the CNS than in SP-$D^{-/-}$ mice (Geunes-Boyer et al. 2009a, b, c). SP-$D^{-/-}$ mice seem resistant to fungal infection; however, exogenous SP-D treatment renders mice susceptible (Geunes-Boyer et al. 2012). SP-D increases susceptibility to *C. neoformans* infection by augmenting *C. neoformans*-driven pulmonary IL-5 and eosinophil infiltration in lungs (Geunes-Boyer et al. 2012).

In an experiment where SP-A and SP-D double knock-out mice, humanized SP-D transgenic (hTG SP-D), and wild-type (WT) mice were treated with or without p38 inhibitor prior to intratracheal injection with *C. neoformans*, p38 MAPK phosphorylation level was found significantly higher in double knock-out mice than in the WT mice. This level came to normal following phosphorylated p38 (p-p38) inhibitor treatment in the double knock-out mice. Transgenic SP-D expression in the hTG SP-D mice also showed decrease in p38 level and showed enhanced *in vivo* phagocytic activity of *C. neoformans*. Thus, lack of SP-A and SP-D seems to influence higher phosphorylated p38 leading to enhanced phagocytic activity of the alveolar macrophages (Abdel-Razek et al. 2016).

Other Fungi

Coccidioides posadasii is a highly virulent soil fungus that causes coccidioidomycosis (Valley fever) in many arid regions of the Americas (Kollath et al. 2019). Both SP-A and SP-D bind to Coccidioidal antigens but no significant changes were

observed in the amounts of SP-A and SP-D in BALF after 5 days of intranasal challenge with *C. posadasii* (Awasthi et al. 2004).

S. cerevisiae, an ubiquitous ascomycetous yeast, is a common colonizer of mucosal surfaces and part of the normal flora of the gastrointestinal tract, the respiratory tract, and the vagina (Salonen et al. 2000). Fungemia the most important clinical syndrome caused by *S. cerevisiae* and has also been described in immunosuppressed patients. It can also cause pneumonia, empyema, liver abscess peritonitis, vaginitis, esophagitis, urinary tract infection, cellulitis etc. (Munoz et al. 2005). SP-D was found to bind and aggregate S. *cerevisiae*, which was further being inhibited by EDTA (Allen et al. 2001).

Parasite

Pneumocystis carinii

Pneumocystis carinii, an extracellular protozoan capable of causing diffused pneumonia in immunocompromised hosts, is a major infection in patients with AIDS. The infection presents as non-productive cough, shortness of breath, fever and bilateral interstitial infiltrates. Pneumocystosis-related surfactant changes have been reported in both humans and corticosteroid-treated experimental models (Aliouat et al. 1998; Prevost et al. 1997). SP-A was found to bind *P. carinii*; its level markedly increased in the infected pneumonia patients with AIDS in lower respiratory tracts (Phelps and Rose 1991; Zimmerman et al. 1992).

O'Riordan et al. reported SP-D as a major component of the alveolar exudates that typify *P. carinji* pneumonia and is capable of binding to the surface of *P. Carinji* organisms through saccharide-mediated interactions with gpA present on the surface of the organism (O'Riordan et al. 1995). With increasing concentrations of calcium SP-D binding to gpA was enhanced, whereas manganese and magnesium cations had negligible effect. SP-D exhibited maximum binding at pH 7.4, whereas inhibited significantly at pH 4. SP-D interactions with *P. Carinii* gpA was found to be facilitated by dodecameric and higher order forms of SP-D (Vuk-Pavlovic et al. 2001).

P. carinii pneumonia was also found to be associated with raised levels of alveolar SP-D where synthesis and secretion of SP-D increased with acute injury and epithelial activation (Atochina et al. 2003). The transgenic mouse model with overexpression of SP-D (SP-D OE) was used to understand the role of SP-D in the pathogenesis, where the transgenic mice showed about 30–50 fold greater SP-D level than the wild-type. The SP-D OE animals showed significant higher levels of TNF-α and macrophage inflammatory protein-2 in BLF throughout the period of infection. And as both the SP-D OE and WT were deficient of CD4 lymphocytes, the study suggests that SP-D possibly facilitates the development of *Pneumocystis* infection in an immunosuppressed mouse model (Vuk-Pavlovic et al. 2006).

Nippostrongylus brasiliensis

Nippostrongylus brasiliensis is a natural parasite of rat, closely related to human hookworm and is primarily used as an important model for studying host's parasite immune response. Thawer et al showed that with *N. brasiliensis* infection, SP-D concentrations increased in the lung. rfhSP-D could bind to L4 parasites to enhance their killing by alveolar macrophages. *N. Brasiliensis* infection of SP-D$^{-/-}$ mice resulted in profound impairment of host innate immunity and ability to resolve infection (Thawer et al. 2016). With prior treatment of rfhSP-D, the number of IL-13 producing type 2 innate lymphoid cells (ILC2) was enhanced and increased production of the type 2 cytokines IL-4 and IL-13 (Thawer et al. 2016).

Perspectives

It is clear that SP-A and SP-D have important roles to play in recognising a wide range of pathogens and clearing them via various mechanisms detailed in this chapter. A number of target ligands are already known; few other are yet to be discovered. The two surfactant proteins also modulate adaptive immune response, thus acting as a pro-active link between innate and adaptive immunity. There are several receptor candidates for collagen regions; however, in most cases, it is the CRD region that binds to the pathogen surface. The knock-out mice have given sufficient information about the pathogen susceptibility. However, the SP-D$^{-/-}$ mice yields lung phenotypes that are already leaky, dysregulated and inflammatory. This can cause a significant bias in the pathogen challenge model. A number of gene polymorphisms and alteration in the SP-A and SP-D protein levels have been noted in a range of pathological conditions; however, they are yet to become a clinically robust biomarker. The properties of rfhSP-D remain intriguing and elusive since presence of collagen region, and oligomeric state of SP-D, has been reported to be paramount in its efficiency. However, the recombinant fragment composed of neck and CRD region of human SP-D seems to have potent therapeutic effects in vitro, in vivo and ex vivo.

As it is evident from the literature review, studies about the effects of SP-A and SP-D on various pathogens are limited in some cases. Thus, there is a greater need to have a concerted effort in pursuing studies with emerging pathogens. A number of parasitic diseases need to be looked at in terms of the roles these two mucosal proteins can play outside lungs.

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Surfactant Protein D: A Therapeutic Target for Allergic Airway Diseases

Iesha Singh, Nazar Beirag, Uday Kishore, and Mohamed H. Shamji

Introduction

Allergic airway diseases are multifactorial and influenced by genetic, immune and environmental components (Allinne et al. 2019; Polverino et al. 2018). These diseases are growing challenges to global health (Pawankar 2014). The pattern of pulmonary inflammation such as rhinorrhoea, airway hyperresponsiveness and airway obstruction are exhibited as clinical features in asthma and allergic rhinitis (Jeffery and Haahtela 2006). Allergic airway disease is largely established by the overexpression of IgE generated in response to allergens that are innocuous to non-allergic individuals (Kratzer and Pickl 2016; Voskamp et al. 2020).

Peripheral blood mononuclear cells such as Dendritic cells (DCs), T and B lymphocytes, and granulocytes like mast cells and eosinophils, are critical in allergic reactions through the secretion of an array of mediators with airway constrictive and pro-inflammatory consequences (Figs. 1 and 2) (Méndez-Enríquez and Hallgren 2019). Immune hyperresponsiveness toward specific environmental allergens can lead to airway remodelling and pulmonary tissue damage (Palm et al. 2012; Kuruvilla et al. 2019). The current novel therapeutic regimens for airway allergic diseases, apart from allergen- immunotherapy, are mainly biologics that block allergic mediators such as cytokines and cellular receptors, such as Omalizumab, Dupilumab, mepolizumab, and Benralizumab (Staubach et al. 2018; Hellings et al.

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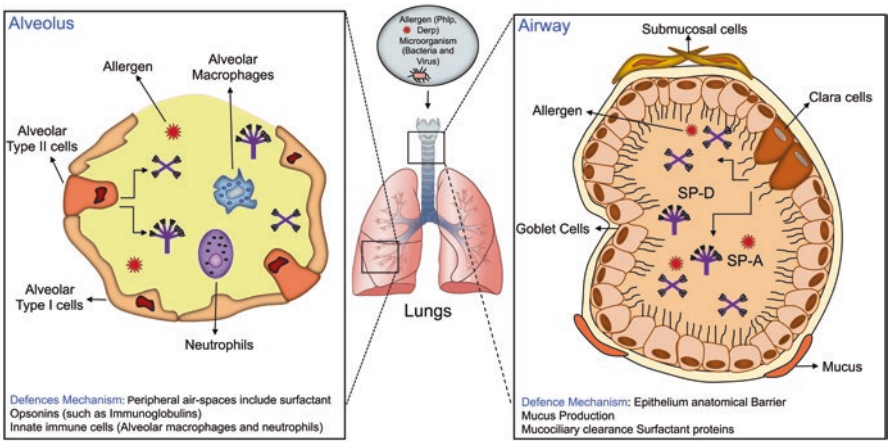

Fig. 1 Lungs are constantly challenged with Allergens and microorganisms leading to the production of several defense mechanisms. This includes formation of anatomical epithelium barrier, mucus production and mucociliary clearance surfactant protein. In airway surfaces Clara cells stimulate SP-A and SP-D production after allergen exposure. Small particles might reach to the alveolar gas-exchange region of the lungs. Defense mechanisms get activated and peripheral airspace starts secreting surfactants, other opsonins and innate immune cells, i.e., macrophages and neutrophils

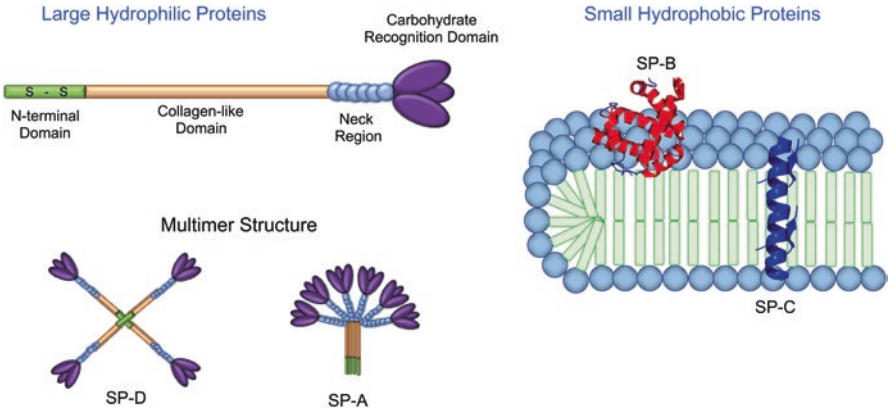

Fig. 2 (**a**) hydrophilic surfactant proteins SP-A and SP-D are multimeric proteins where single subunit monomer contains lectin domain (CRD), neck region and a collagen like domain. (**b**) Trimeric subunits come together to form oligomeric structure form through non-covalent bond to forms octamers (18 monomers) for SP-A, and dodecamers (12 monomers) for SP-D (**c**)

2017; Trischler et al. 2017; Fitzgerald et al. 2016). However, concerns have been raised about long-term side effects, prediction of treatment responses, the ability of these agents to promote tolerance induction, and the high financial cost (Breiteneder et al. 2019). Therefore, novel inhibitors of allergen-specific IgE and Th2 cytokines are much needed as an alternative therapeutic modality with lower costs and a

broader scope of application. Studies have shown the pivotal role of surfactant protein D (SP-D) in modulating immune hyperresponsiveness in the lung; this infers its importance to be considered an alternative therapeutic candidate in hindering pulmonary allergic reactions (Schleh et al. 2012; Sorensen 2018; Carreto-Binaghi and Taylor 2016; Winkler and Hohlfeld 2013). This chapter will highlight the immunomodulatory effects of SP-D in type I mediated hypersensitivity focusing on its impact on immune cells that fuel allergic airway reactions.

Surfactant Protein D: Molecular Structure and Function

Initial purification and characterization of SP-D in the late 80s and early 90s paved the way for the determination of the structure and the immune functions of SP-D (Persson et al. 1989; Possmayer 1988; Malhotra et al. 1990, 1992). SP-D protein is a member of collectin family of innate carbohydrate pattern recognition molecules; it is calcium-dependent C-type lectin, and has multimeric structure (Holmskov et al. 1994). It is synthesised by airway epithelium, mainly type II pneumocytes and non-ciliated Clara cells (Crouch et al. 1992). It is hydrophilic with 43 kDa molecular weight; its monomer structure is composed of four regions: a short N-terminal region attached to a collagen-like domain, followed by an α-helical coiled-coil neck region, and C-type lectin or carbohydrate recognition domain (CRD) (Fig. 3) (Håkansson et al. 1999). The collagen region can form triple-helical structures, and by virtue of trimerizing capability of neck region, the C-terminal CRD region forms

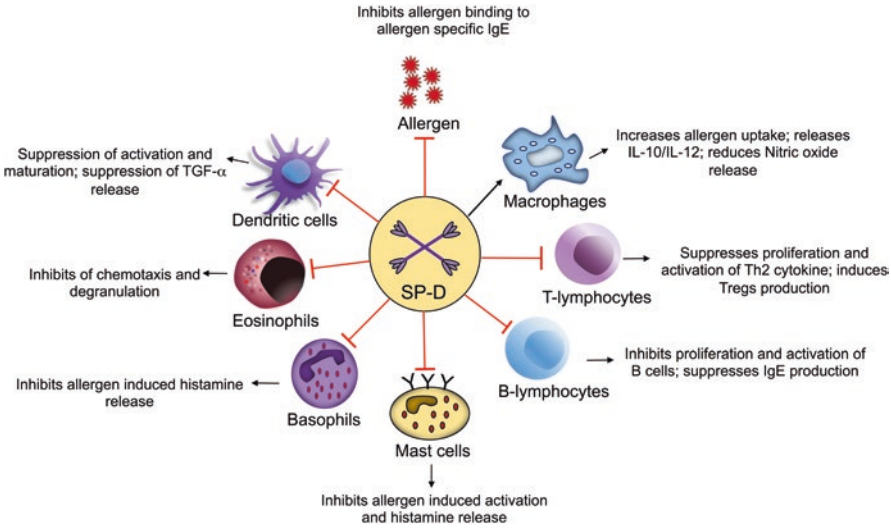

Fig. 3 Overview of the immunological functions of surfactant protein D and its interaction with other immune cells involved in allergic airway inflammation

a trimeric structure. This subunit can further oligomerise due to N-terminal region cross-linking to yield dodecamers. The homotrimeric CRD region mediates binding to various ligands on pathogens/allergens that exhibiting carbohydrate and charge patterns, whereas the collagen region binds to putative receptors on effectors cells such as macrophages (Kishore et al. 2006). SP-D multimerization is required for enhancing CRD binding affinity of to its ligands (Wright 2005). Interestingly, in the late 90s, a functional recombinant fragment of human SP-D (rfhSP-D) comprised of trimeric CRD and α-helical coiled neck region was expressed in E. coli and subsequently characterised for potential therapeutic applications via *in vitro*, *in vivo* and *ex vivo* allergic models (Kishore et al. 1996, 2006; Wang et al. 1996).

Surfactant Protein D: Immunomodulatory Mechanisms in Allergic Airway Inflammatory Events

The idea of immunomodulation by SP-D in allergy stems from the observations by Wang et al. who showed that SP-D (purified from human lung lavage) as well as rfhSP-D can bind to house dust mite extracts (*Dermatophagoides pteronyssinus; Der p*) and purified native *Der P1* in a calcium- and carbohydrate-dependent manner, thus, inhibiting *Der p*- IgE complex formation *in vitro* (Wang et al. 1996). Likewise, SP-D and rfhSP-D bound to *Aspergillus fumigatus* allergens and reduced histamine release from sensitised basophils derived from allergic bronchopulmonary aspergillosis (ABPA) patients (Madan et al. 1997). These findings suggested that SP-D binding to allergens can suppress early phase of inducing allergic airway reactions.

Protection by SP-D Against Allergic Eosinophilia

Infiltration of lung tissues with eosinophils is a sign of allergic airway inflammation ((Felton et al. 2014). This process involves eosinophilic differentiation and migration from the bone marrow to the lungs as a result of Th2 derived cytokines, particularly IL-13 and IL-5 (Esnault and Kelly 2016). Activation of eosinophils gives rise to the release of pro-inflammatory cytokines and cytotoxic proteins, including eosinophilic peroxidase (EPO), major basic protein (MBP) and reactive oxygen species (Acharya and Ackerman 2014). This leads to more destruction in the airway tissues and increases the tone of inflammatory allergic events (Yousefi et al. 2018).

SP-D has been shown to bind to eosinophils via its CRD and CD32 and block chemotaxis release and degranulation of Eosinophil Cationic peptide (Von Bredow et al. 2006). In addition, Madan et al. have demonstrated that SP-D and rfhSP-D reduced eosinophilia, allergen-specific IgE and IL-2, IL-4, and IL-5 levels in

pulmonary hypersensitivity murine models induced by *A. fumigatus* allergens (Madan et al. 2001). Similarly, Singh et al. found that rfhSP-D significantly reduced specific IgE, Il-4 and IL-5 levels, and peripheral eosinophilia in pulmonary mice models triggered by *Der p* (Singh et al. 2003). Furthermore, a study by Mahajan et al. demonstrated *ex vivo* that rfhSP-D increased apoptosis of activated eosinophils derived from allergic patients, without affecting eosinophils from healthy donors (Mahajan et al. 2008). Additionally, *A. fumigatus* allergen sensitized mice treated with SP-D (or rfhSP-D) showed a reduction in eosinophilia and eotaxin level, and consequently lower airway hyperresponsiveness (Erpenbeck et al. 2006). Thus, SP-D has a crucial role in regulating eosinophilia in allergic airway inflammation.

Allergen Uptake and Antigen Presentation Modulation

SP-D can effectively bind to allergens and enhance the uptake of allergens by alveolar macrophages, an important step in modulating the allergic airway inflammation ((Haczku 2008). Alveolar macrophages and Dendritic cells (DCs) play a crucial role in allergic pulmonary inflammation driven by T lymphocytes (Moser and Murphy 2000; Novak et al. 2004; Desch et al. 2013). DCs are accountable for inducing activation and differentiation of T lymphocyte in allergic airway reactions (Matzinger 1994). SP-D has been shown to bind to immature DCs in a carbohydrate and calcium-dependent manner (Brinker et al. 2001). Hansen et al. showed that SP-D can attenuate antigen presentation by DCs in the lung (Hansen et al. 2007). Additionally, SP-D interferes with DC maturation and TNF-α secretion in the pulmonary mice model (Hortobágyi et al. 2008). Therefore, SP-D may alter the allergen-antigen presentation by DCs to T lymphocytes which inhibits the intensification of airway allergic events (Fig. 4).

Alveolar macrophages have a crucial role in maintaining mucosal immune tolerance in the lung (Macaubas et al. 2003). Liu et al. have examined the role of SP-D on activated alveolar macrophages during allergic pulmonary inflammation in Derp-sensitised mice where it worked via blocking Nitric Oxide (NO) and TNF-α productions (Liu et al. 2005). Additionally, elevated expression of allergen-induced TLR4 in AMs of SP-D null mice has been reported (Schaub et al. 2004). These results indicated that SP-D can suppress inflammatory mediators in alveolar macrophages in allergic airway inflammation. Consistent with these observations, SP-D treatment in an allergic murine model increased levels of IL-10, IL-12, and IFN-γ in bronchoalveolar lavage fluid and reduced goblet cell hyperplasia. When alveolar macrophages were cultured in the presence of SP-D and allergen together, heightened levels of IL-10, IL-12, and IFN-γ were produced, suggesting alveolar macrophages being a target for SP-D actions against the development of airway hyperreactivity and inflammation (Takeda et al. 2003).

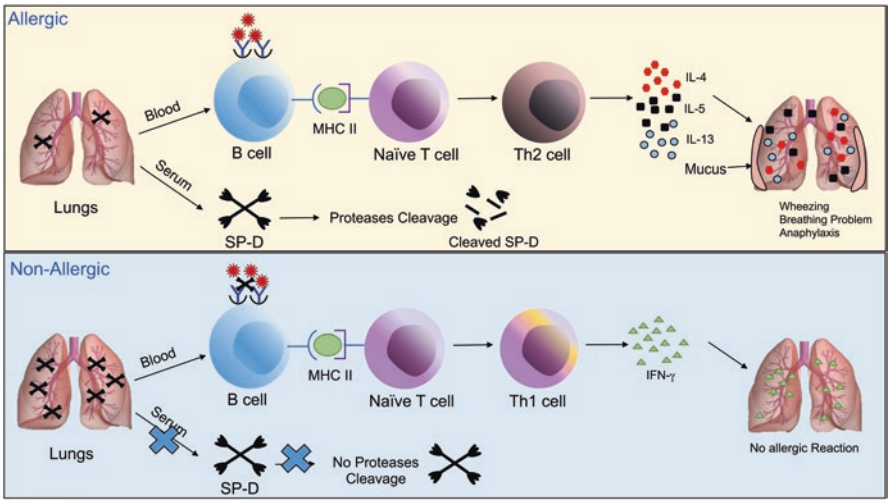

Fig. 4 Role of SP-D in healthy and allergic lungs

Modulation of Lymphocytes Mechanisms in Allergic Pulmonary Reactions

Inhalation of allergens in atopic patients induce inflammatory events involving mainly allergen-specific IgE and degranulation of mast cell and eosinophils; these events are orchestrated by B and T lymphocytes including IL-4, IL-5, and IL-13. Asthmatic patients have a high population of activated pulmonary T lymphocytes ($CD4^+$) characterised by elevated $CD25^+$ and $CD69^+$ expression (Corrigan et al. 1993). Genetically manipulated mice with Th2 cytokines deficiency have shown an absence of pulmonary allergic reaction features (Hamelmann et al. 2000). Thus, SP-D null mice show persistent activation of T lymphocytes in the lung in response to exogenous antigens (Fisher et al. 2002).

SP-D inhibits T lymphocyte proliferation in response to antigenic and mitogenic activation (Borron et al. 1998; Vass et al. 2004). SP-D has been shown ex vivo to inhibit histamine release and lymphocyte proliferation in asthmatic patients induced by phytohemagglutinin (PHA) and Der p (Wang et al. 1998). Moreover, high level of CTLA4 (a negative regulator of T-lymphocytes) has been reported in the presence of SP-D *in vitro* and *in vivo* (Lin et al. 2010). These results underline the potential role SP-D in regulate T-lymphocyte activation and proliferation expression in airway allergy.

B cells appear to be convenient target for SP-D in allergic inflammation. B lymphocytes play a crucial role in allergic airway inflammation (Ghosh et al. 2012), via production of allergen-specific IgE and secretion of IL-4, which induces Th2 differentiation (De Vooght et al. 2013; Harris et al. 2000). B lymphocyte null mice treated with cockroach allergens show low levels of Th2 cytokines (Lindell et al.

2008). In addition, allergen presentation by B lymphocytes results in T cell expansion, Th2 polarization and more allergen-specific IgE synthesis (Linton et al. 2003; Crawford et al. 2006). B lymphocytes are also involved in eosinophilic pulmonary inflammation (Drake et al. 2015).

Recently, an *ex vivo* study by Qaseem et al. using allergic rhinitis patients' samples revealed that rfhSP-D suppressed basophil and B, and T lymphocytes activations. rfhSP-D bound to B lymphocytes in a calcium- and carbohydrate-dependent manner through the CRD region. Furthermore, rfhSP-D hindered allergen-IgE complexes from binding to CD23 (FcεRII). rfhSP-D also blocked IgE synthesis by B cells despite the presence of IL-4, IL-21, and CD40L (Qaseem et al. 2017). These results put together the immune functions of SP-D in modulating granulocytes and lymphocytes in airway allergic reactions. CD23 is low affinity receptor for IgE mainly on B lymphocytes which is involved in allergen-specific IgE upregulation though the interaction with CD21 (complement receptor 2) (Conrad et al. 2007). The study of Qaseem et al. revealed SP-D reduced CD23 expression on B cells.

Surfactant Protein D Expression in Allergic Airway Diseases

SP-D levels are elevated in nasal tissue of patients with chronic rhinosinusitis (Ooi et al. 2007). High serum levels of SP-D have reported in allergic patients following allergen challenge (Koopmans et al. 2004). In addition, asthmatic patients showed high SP-D levels in the bronchial alveolar lavage fluid as compared with non-asthmatic controls (Cheng et al. 2000). Murine models with acute lung allergic reactions show high levels of SP-D in the pulmonary tract (Wang and Reid 2007). Thus, SP-D may serve as a biomarker for the severity of allergic immune response (Hartl and Griese 2006).

Significance and Future Direction

Research in unravelling various mechanisms of protective effects of SP-D against airway allergic diseases has highlighted the therapeutic potential of rfhSP-D. This small fragment of human SP-D seems to bind allergens, inhibit IgE-allergen interaction, suppress basophil activation, modify allergen presentation, suppress proliferation of allergen-stimulated B and T lymphocytes, induce Th2 to Th1 polarisation, and suppress IgE synthesis by primed B cells. The data so far in the field clearly point towards logical clinical trials.

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Surfactant Protein D in Immune Surveillance Against Cancer

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Introduction

Pulmonary surfactant protein D (SP-D) is a multi-functional, pattern recognition molecule involved in resistance to pathogen challenge and pulmonary inflammation including allergy (Madan et al. 1997a, b; Borron et al. 2002; Brinker et al. 2001; Takeda et al. 2003; Pandit et al. 2016). The immunomodulatory properties of SP-D are reliant on its domains: the C-terminal globular region, also called as carbohydrate recognition domains (CRDs), and the collagen-like region (CLR) connected through an α-helical neck region (Kishore et al. 2006; Waters et al. 2009). SP-D directly interacts with monocytes/macrophages and enhance or suppress inflammatory mediator production depending on the binding of either of these two domains (Gardai et al. 2003; Ledford et al. 2014). Experiments using murine models of allergy and pulmonary hypersensitivity showed that SP-D (or a recombinant fragment of human SP-D containing trimeric neck and CRD region;

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rfhSP-D) treatment reversed hypersensitivity response by lowering blood and pulmonary eosinophilia, specific IgE levels and a shift in cytokine profile from Th2 to Th1 type in the spleen cell culture (Madan et al. 2001; Singh et al. 2003). This was further confirmed by the negative regulation of allergic eosinophilic pulmonary inflammation and airway function with intratracheal administration of SP-D in the ovalbumin-induced murine models of lung allergy (Takeda et al. 2003). The eosinophilic inflammation of the airways has been directly correlated with the severity of asthma (Winqvist et al. 1982; Duncan et al. 2003; Bousqeut et al. 1990). SP-D knock-out (SP-$D^{-/-}$) mice showed elevated peripheral and pulmonary eosinophilia. Reconstituting the levels of SP-D by intranasal administration in the SP-$D^{-/-}$ mice reduced eosinophilia considerably. The susceptibility of SP-$D^{-/-}$ mice to allergen sensitization was consistent with severe pulmonary eosinophilia (Madan et al. 2005). These observations implicated a direct interaction of SP-D with the eosinophils.

Eosinophil Leukemic Cells

Interaction of SP-D with Human Eosinophilic Leukemic Cell Line

Direct interaction of SP-D with human eosinophils inhibited eotaxin triggered chemotaxis and eosinophil cationic protein (ECP) degranulation stimulated by Ca^{2+} ionophore in the eosinophils derived from healthy donors (von Bredow et al. 2006). Our group also showed a CRD and dose dependent binding of SP-D (and rfhSP-D) to human eosinophils (Mahajan et al. 2008). SP-D resulted in a significant increase in oxidative burst and CD69 expression in eosinophils derived from symptomatic allergic asthmatics; in addition, SP-D treatment induced apoptosis in these activated eosinophils *in vitro*. The viability of eosinophils from healthy donors was not affected following SP-D or rfhSP-D treatment. However, eosinophils from healthy donors, following priming with IL-5, showed apoptosis with rfhSP-D treatment (Mahajan et al. 2008). AML14.3D10 cell line, an advanced differentiated eosinophilic leukemic cell line, exhibits autocrine activation of the intracellular IL-3/GM-CSF/IL-5 signaling pathways (Baumann and Paul 1998; Paul et al. 1997). The interaction of SP-D with AML14.3D10 cells was examined in view of the ability of SP-D to selectively induce apoptosis in the sensitized eosinophils. Native SP-D, purified from the lung lavage obtained from alveolar proteinosis patients, as well as rfhSP-D, both showed dose and calcium dependent binding to AML14.3D10 cells. This binding was inhibited in the presence of cellular debris (known to interact with CRD of SP-D) suggesting the involvement of CRD region of SP-D in binding to AML14.3D10 cells (Mahajan et al. 2013).

Cell Cycle Arrest of Leukemic Cells

The hypotonic propidium iodide (PI) assay was used for cell cycle analysis by flow cytometry. The assay is based on measurement of DNA content by staining with PI. The rfhSP-D induced nuclear changes in the AML14.3D10 cells and led to accumulation of cells in the G2 phase. There was more than 20-fold increase in the G2 population of cells suggesting G2/M cell cycle arrest (Mahajan et al. 2013). The treatment also increased the sub G1 peak, i.e., the presence of fragmented DNA, suggestive of the cell apoptosis. The constant presence of rfhSP-D was required to induce cell apoptosis and the sustained downstream events leading to the cell death (Mahajan et al. 2013).

Induction of Apoptosis

rfhSP-D treatment for 24 h caused significant G2/M cell cycle arrest in AML14.3D10 cells; however the cell viability was not significantly affected at this time point. Thus, Annexin V-FITC assay (Zhang et al. 1997) was used to allow the direct evaluation of Phosphatidylserine (PS) externalization, a very early stage marker of apoptosis; a significant increase in the annexin-V positive cells was observed following treatment of AML14.3D10 cells with rfhSP-D at 48 h (Mahajan et al. 2013). Other assays including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as well as Trypan blue dye exclusion also showed a dose- and time-dependent decrease in the viability of rfhSP-D treated AML14.3D10 cells. NAD(P)H-dependent enzymatic reduction of MTT to MTT-formazan by Mitochondrial succinate dehydrogenase indicates well-being of the cells with respect to respiration and mitochondrial level (Mosmann 1983). The mitochondrial respiration efficiency of AML14.3D10 cells started to decline significantly by 48 h. At 72 h, nearly half of rfhSP-D-treated AML14.3D10 cells showed decrease in viability by MTT assay. At this time point, the rfhSP-D treated cells started to show DNA fragmentation, suggesting the progression of cells towards apoptosis (Riccardi and Nicoletti 2006). The trypan blue staining demonstrated that ~20% cells had lost the membrane integrity by 72 h. Thus, analysis of rfhSP-D treated cells by different assays at various time points such as release of intracellular oxidative burst, G2/M cell cycle arrest, increased levels of activated p53, cleavage of caspase-9 and PARP, and PS externalization suggest the sequential induction of events observed during rfhSP-D induced apoptosis in AML14.3D10 cell line (Mahajan et al. 2013) (Table 1).

Table 1 Sequential events on rhSP-D (10 μg/mL) treatment of eosinophilic leukemic cells (AML14.3D10 cell line)

Increased oxidative burst	Decrease in HMGA1 level	Increased p53 phosphorylation	Subsequent observations/ outcomes →	G2/M cell cycle arrest	MTT assay	Annexin-V FITC assay	Sub-G1 peak (DNA fragmentation)	Trypan blue staining
Induced at 24 h on treatment of AML14.3D10 cells with SP-D			Time points↓ (h)					
+	++	++	24	+	+	—	−	−
—	++	—	48	++	++	+	+	±
+	—	—	72	+++	+++	—	+++	++

Key:
+: Results positive
++/+++: Further increase in results
−: Results negative
±: Low yet significant levels
—-: Results not observed

Deciphering the Proteome and Phosphoproteome of SP-D Treated Eosinophilic Leukemic Cells

SP-D has immunomodulatory effects on a range of immune cells, including B- and T-lymphocytes, macrophages, dendritic cells and eosinophils (Singh et al. 2017; Lin et al. 2010; Brinker et al. 2001; von Bredow et al. 2006; Mahajan et al. 2008). The large-scale molecular changes initiated by SP-D in a human cell were thus studied, for the first time, using proteomics approach. Comparative analysis of rfhSP-D (10 μg/mL for 48 h) treated AML14.3D10 cells showed a total of 134 proteins with a three-fold or more change in the expression (Mahajan et al. 2014). The important observations on proteomic profile of rfhSP-D treated AML14.3D10 cells included increased expression of oxidoreductases and stress-related molecules, and decreased expression of survival related proteins such as high-mobility group A1 (HMGA1) (Mahajan et al. 2014). In addition, the mitochondrial anti-oxidant defense system was found compromised in the rfhSP-D treated cells. There was a decreased expression of Ubiquinol-cytochrome c reductase (complex III of ETC), Peroxiredoxin 3 isoform b and Mitochondrial matrix superoxide dismutase. This is likely to cause mitochondrial dysfunction, and hence, triggering of the intrinsic pathway of apoptosis (Turrens 2003).

Elucidation of Apoptotic Mechanisms

An important mechanism by which SP-D contributed to anti-leukemic activity was the reduced expression of survival related proteins, HMGA1, an oncogenic transcription factor that is over-expressed in various high-grade malignancies (Chiefari et al. 2013; Pierantoni et al. 2007; Hillion et al. 2008; Fedele et al. 2005). Inhibition of HMGA1 expression has been shown to block phenotype transformation in many cancer cells (Scala et al. 2000). HMGA1 is known to inhibit the function of p53 family members, i.e., oncosuppressors, in cancer cells (Pierantoni et al. 2007). p53 is a tumor suppressor protein and plays a major role in cellular response to DNA damage or other genomic aberrations. Activation of p53 may lead to either cell cycle arrest and DNA repair, or apoptosis (Chen 2016; Wang and El-Deiry 2007). The decreased expression of HMGA1 in the rfhSP-D treated cells correlated with an increased level of p53 activation, i.e., phosphorylation of p53 at Ser15 (Mahajan et al. 2013). Another protein, heterogeneous nuclear ribonucleoprotein K (hnRNP K), known to be induced by stress, was found to be upregulated. hnRNP K is required for the induction of p53 target genes (Moumen et al. 2005; Mahajan et al. 2014). An increased level of p21 was also observed in rfhSP-D treated AML14.3D10 cells. The increased levels of activated p53 and p21 expression are known to inactivate cyclin B-cdc2 complex that regulates G2/M transition and leads to either DNA repair or apoptosis (Stewart et al. 1995; Bunz et al. 1998). The rfhSP-D treated cells also showed an increased Tyr15 phosphorylation of cdc2, suggesting activation block of cdc2 (Li et al. 2011).

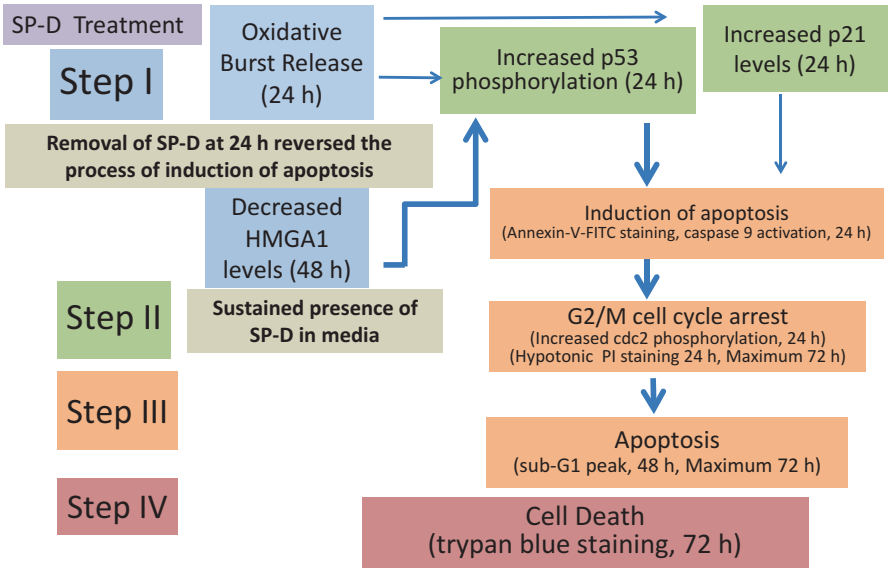

Fig. 1 Predicted steps of SP-D induced apoptosis of AML14.3D10 cells. Treatment with rfhSP-D leads to oxidative stress and reduction in the levels of HMGA1. Consequently, there is increased phosphorylation of p53 (Ser15) and p21 levels. Cdc2 phosphorylation contributes to inhibition of Cdc2 leading to arrest of cells in G2/M phase followed by apoptosis and cell death

Treatment of AML14.3D10 cells with rfhSP-D resulted in the activation of caspase-9, a hallmark of the intrinsic pathway of apoptosis (Elmore 2007). Once initiated, caspase-9 goes on to cleave procaspase-3 or procaspase-7, which in turn, cleaves several cellular targets, including Poly (ADP-ribose) polymerase (PARP), a well-known marker of apoptosis (Walsh et al. 2008). Although, PARP was found to be cleaved in the rfhSP-D treated cells, cleavage of caspase-7 was not observed (Fig. 1). This indirectly suggests that an intermediate activation of caspase-3 may be leading to the cleavage of PARP.

Validation of Anti-Cancer Activity of SP-D in Other Cancer Cells

HMGA1 is over-expressed in various high-grade malignancies, including AML (acute myeloid leukemia), ALL (acute lymphoid leukemia) and Burkitt's lymphoma (Fusco and Fedele 2007). We found that the cell lines with reported increased HMGA1 expression, AML (AML14.3D10 cell and THP-1 cell line), ALL (Jurkat and Raji) and human breast epithelial cell line (MCF-7) demonstrated a significant decrease in the viability of cells on treatment with rfhSP-D, although, rfhSP-D

treatment did not affect the viability of PBMCs isolated from healthy donors. The study indicated that rfhSP-D specifically exerted its apoptotic effect on the cancer cell lines.

Pro-apoptotic Effects of rfhSP-D on Pancreatic Cancer Cell Lines

In view of the above-mentioned observations, rfhSP-D has been examined for its importance in pancreatic cancer. The fluorescence analysis revealed that rfhSP-D binds evenly in clusters on the cell membrane of three pancreatic cancer cell lines, Panc-1, MiaPaCa-2 and Capan-2. No CY5 fluorescence was detected in the untreated controls suggesting the rfhSP-D binding observed in the treated cell lines was protein-specific. To determine whether rfhSP-D influences the cellular morphology and epithelial-to-mesenchymal transition (EMT) phenotypic expression, we treated a highly invasive pancreatic cancer cell line (e.g., Panc-1) with exogenous rfhSP-D (Kaur et al. 2018a).

rfhSP-D Induces Morphological Alterations in the Pancreatic Cell Line Panc-1

The optimal dose was determined by observing the effects of rfhSP-D on cell morphology and cell division of Panc-1 cells incubated with 0, 5, 10, and 20 μg/mL. Images of colonies of 10–15 cells were taken at 0, 6 and 24 h. Panc-1 cells, treated with rfhSP-D (5 μg/mL) and untreated cells, acquired spindle type cell morphology, reduced cell-cell contact, and continued to divide in a time dependent manner. No such alterations were seen in Panc-1 cells treated with rfhSP-D (10 and 20 μg/mL) and they appeared to be static. However, cell morphology at 10 μg/mL overcome these static effects by 24 h but not at 20 μg/mL dose. Interestingly, some dead cells were also seen at 20 μg/mL as compared to the other dose conditions. Subsequently, possible effects of rfhSP-D on EMT induction in Panc-1, MiaPaCa-2 and Capan-2 cells were investigated (Kaur et al. 2018a).

rfhSP-D Suppresses the Invasion Ability/Capacity in Pancreatic Cancer Cell Lines

The effects of rfhSP-D (20 μg/mL) on the invasion were analysed by incubating the pancreatic cancer cells in the upper surface of the matrigel chamber pre-coated with extracellular matrix proteins and serum containing media as a chemo-attractant in

the bottom surface for 22 h. Both high grade Panc-1 (50%) and MiaPaCa-2 (65%) cell lines, treated with rfhSP-D (20 μg/mL), showed significantly reduced invasion in the matrigel; however, almost no invasion occurred in low-grade Capan-2 (Kaur et al. 2018a) since Capan-2 is a non-invasive cell line.

rfhSP-D Reduces the Expression of EMT Markers

EMT induction is characterized by morphological alterations, enhanced motility, reduced cell-cell contact (Ellenrieder et al. 2001), and upregulation of mesenchymal markers, such as Vimentin (Maier et al. 2010), Snail (Peinado et al. 2003), and Zeb1 (Wellner et al. 2009). Most pancreatic cancer cells overexpress TGF-β, which suppresses immune surveillance and facilitates the escape, migration and increased resistance to anti-tumor immune responses (Sun et al. 1994; Beauchamp et al. 1990; Reiss 1999). rfhSP-D (20 μg/mL) treatment significantly downregulated the gene expression of TGF- β in the treated Panc-1 and MiaPaCa-2 at 12 h whereas no difference was seen in Capan-2 as analysed by qPCR and western blot. Fluorescence microscopy analysis revealed that TGF-β expression at 24 h diminished considerably within the cytoplasm of the treated Panc-1 and MiaPaCa-2 cell lines. During TGF-β induced EMT pathway, Smad2/3 are phosphorylated in the cytoplasm, followed by translocation into nucleus; however, Smad2/3 staining appeared very weak in the cytoplasm of the rfhSP-D treated Panc-1 and MiaPaCa-2 cell lines. Furthermore, gene expression of key markers of EMT, regulated by TGF-β such as Vimentin, Zeb1 and Snail, was also downregulated in all cell lines treated with rfhSP-D at various times ranging between 1 h and 12 h. Fluorescence microscopy and flow cytometry analysis also confirmed a significant decrease (~50%) in the cytoplasmic presence of these proteins in the treated as compared to untreated cells. Interestingly, blocking TGF-β via neutralizing antibody reduces the expression of EMT markers in a similar fashion as rfhSP-D. Interestingly, the effect was even more prominent when rfhSP-D and rabbit anti- human TGF- β were added together (Kaur et al. 2018a). Similarly, visual assessment of cell proliferation and migratory capacity of ovarian cancer cells, SKOV3, following treatment with rfhSP-D (10 μg/mL) for 24 h also revealed inhibition of growth as compared to the untreated cells (Kumar et al. 2019). These observations suggested that rfhSP-D interfered with EMT, therefore, we investigated the static affects occurred due to cell cycle arrest.

rfhSP-D Induces Cell Cycle Arrest in G1 Phase

Panc-1, MiaPaCa-2, Capan-2 cell lines treated with rfhSP-D (20 μg/mL) for 24 h were subjected to DNA quantitation using DNA binding dye, PI, to determine whether the cytostatic effect seen was due to growth arrest. rfhSP-D treatment inhibited the DNA synthesis during G1 phase in treated Panc-1 (68%) and

MiaPaCa-2 (50%) as compared to untreated Panc-1 (3%) and MiaPaCa-2 (2%) cells, respectively. The untreated cells for all cell lines as well as treated Capan-2 remained unaffected as cell cycle into next S and G2 phase continued (Kaur et al. 2018b).

rfhSP-D Induces Apoptosis in Pancreatic and Ovarian Cancer Cells

The fluorescence microscopy revealed that the cell membrane was no longer intact and the propidium iodide bound to DNA in the rfhSP-D (20 μg/mL) treated pancreatic cancer cell lines, Panc-1, MiaPaCa-2 and Capan-2 as compared to untreated cells, where no fluorescence was detected, indicating that cells were undergoing apoptosis at 48 h. The quantitative flow cytometry analysis was carried out by measuring the Annexin V/FITC binding to phosphatidylserine (PS), a cell membrane phospholipid, which is externalized during early apoptotic stage and PI, a DNA stain, passes through the porous cell membrane into the nucleus. It showed that rfhSP-D induced apoptosis in ~70% of Panc-1 and MiaPaCa-2 cells and ~43% in Capan-2 as compared to ~80% unstained, viable cells in the untreated samples at 48 h. A significant percentage of Panc-1 cells (~43%) and some MiaPaCa-2 and Capan-2 (~12%) were positive for PI alone, suggesting that these cells were either dead or in late apoptotic stage (Kaur et al. 2018b). The quantitative and qualitative analysis of ovarian cancer cells, SKOV3 treated by rfhSP-D (20 μg/mL) for apoptosis induction revealed similar trends as seen in pancreatic cancer cells. Approximately, 68% cells underwent apoptosis at 48 h of treatment (Kumar et al. 2019). Then, activation of apoptosis pathway was determined by assessing key markers of both intrinsic and extrinsic apoptosis pathway.

rfhSP-D Activates Apoptosis via Extrinsic Pathway in Pancreatic and Ovarian Cancer Cells

The treatment with rfhSP-D (20 μg/mL) activated cleavage of caspase 8 and 3, in addition to upregulation of pro-apoptotic gene, Fas at 12 and 24 h in all the cell lines. Moreover, both TNF-α and NF-κB mRNA expression levels showed a significant up-regulation in all the rfhSP-D treated cell lines at 12 and 24 h. Fluorescence microscopy of Panc-1, MiaPaCa-2 and Capan-2 cell lines showed that NF-κB was translocated to the nucleus at 24 h, which was not seen in the untreated cells (Kaur et al. 2018b). TNF-α and NF-κB are crucial factors in the apoptotic pathway and they can regulate Fas expression (Fulda and Debatin 2006). Therefore, it appeared that apoptosis occurred via extrinsic pathway as upregulated TNF-α binds to TNF type I receptor (TNFR1), which is internalized and forms a complex with

TNFR1-associated DEATH domain (TRADD) (complex I), stimulating the upregulation of NF-κB. Then, a complex II is formed upon binding of complex I to Fas-Associated protein with Death Domain (FADD), which is formed when Fas is activated. NF-κB upregulation promotes Fas upregulation as it acts as a transcription factor for Fas Subsequently, Complex II activates downstream caspase cascade, which causes the cleavage of caspase 8 followed by effector caspase 3 cleavage, which brings about apoptosis. These findings indicated that cell death is likely to occur via TNF-α/Fas-mediated apoptosis pathway (Kaur et al. 2016; Liu et al. 2012; Ashkenazi and Dixit 1998). Intrinsic markers such as caspase 9 and pro-apoptotic gene, Bax, remained unaffected. Moreover, the survival pathway such as mTOR is often deregulated in pancreatic cancer (Semba et al. 2003) and its activation is associated with poor prognosis (Kennedy et al. 2011). Upon treatment with rfhSP-D (20 μg/mL), mRNA expression of mTOR was downregulated in Panc-1 and MiaPaCa-2 cell line at 12 h, however, no difference was seen in Capan-2. In addition, fluorescence analysis revealed significant decrease in the cytoplasmic levels and an increased accumulation of mTOR in the nucleus of the treated cells in comparison to the untreated cells, where it has been shown to be present in its inactive form in previous studies (Betz and Hall 2013). Interestingly, ovarian cancer cells, SKOV3, also appeared to undergo apoptosis via extrinsic pathway as pro-apoptotic gene Fas and TNF-α were upregulated and survival pathway mTOR was downregulated (Kumar et al. 2019).

Anti-prostate Tumor Effects of SP-D

SP-D Expression in Prostate and Correlation with Gleason Score

Although the lung remains the major site of SP-D synthesis, its presence has been reported in non-pulmonary human tissues, including trachea, brain, testis, salivary gland, heart, prostate gland, kidney, and pancreas (Madsen et al. 2003). Elevated levels of SP-D were observed at inflamed sites in the prostate, manifesting protection against bacterial infection (Oberley et al. 2005). Testosterone withdrawal showed upregulation of TLR4 pathway and improved SP-D-mediated bacterial clearance in rat prostate cells (Quintar et al. 2012; Oberley et al. 2005). Differential SP-D protein expression in the glandular structures of inflamed malignant and non-malignant human prostate tissues has also been reported. A significant correlation between lower expression of SP-D and increased Gleason score and prostate tumor volume has been noted earlier (Kankavi et al. 2014). A low level of SP-D, a known anti-inflammatory molecule, may contribute to the development and/or progression of the human prostate cancer (Kankavi et al. 2014).

SP-D expression in LNCaP (androgen dependent) tumor cells was significantly lower in comparison to DU145 and PC3 (androgen-independent) tumor cells and

primary prostate epithelial cells. Furthermore, treatment with Dihydrotestosterone (DHT) upregulated levels of SP-D transcripts in Primary epithelial cells, LNCaP, but not in PC3 tumor cells, suggesting that SP-D expression was regulated by androgens (DHT) in an androgen-dependent cancer (Thakur et al. 2019). Similarly, SP-D expression was weaker in seminoma compared to normal testicular tissue that may contribute to reduced immunomodulatory and rheology processes in germ cell tumor (Beileke et al. 2015).

SP-D Binds Prostate Tumor Cells and Induces Apoptosis via Intrinsic Pathway

A significantly higher calcium dependent binding of rfhSP-D was observed with the androgen independent prostate cancer cells (DU145 and PC3) than the androgen-dependent prostate cancer cells (LNCaP). Primary prostate epithelial cells showed comparatively less binding to rfhSP-D than any of the prostate cancer cells, suggesting an involvement of certain interacting cell membrane proteins that are upregulated or differentially expressed on prostate cancer cells (Thakur et al. 2019). rfhSP-D caused a dose- and time-dependent reduction in the viability of prostate cancer cells (LNCaP, DU145 and PC3) irrespective of their androgen sensitivity. This effect was also observed in primary prostate cancer epithelial cells isolated from the metastatic PCa patients. Various attributes of apoptosis such as PS externalization, mitochondrial dysfunction and DNA fragmentation were evident in the rfhSP-D treated prostate cancer cells (Thakur et al. 2019). Anti-prostate cancer activity of rfhSP-D via induction of apoptosis in tissue explants from metastatic prostate cancer patients has also been demonstrated.

Mechanisms Involved in Anti-prostate Cancer Activity of rfhSP-D

p53 pathway plays a crucial role in the transmission of pro-apoptotic signals (Gottlieb et al. 2002). rfhSP-D treated LNCaP ($p53^{+/+}$, androgen dependent) cells showed significant upregulation in phosphorylated p53 (Thakur et al. 2019). rfhSP-D treatment led to a decreased level of Bcl2, with a concomitant increase in Bax, cytochrome c and cleavage of caspase 7, confirming induction of intrinsic apoptosis pathway (Thakur et al. 2019).

PC3 cells, a p53 null and highly metastatic prostate cancer cell line, also showed significant apoptosis following treatment with rfhSP-D, which suggested involvement of a p53 independent mechanism of apoptosis. Among 25% prostate cancer cases, diallelic deletion of the Phosphatase and tensin homolog (PTEN) gene and the associated increase in Akt phosphorylation correlates with hormone refractory

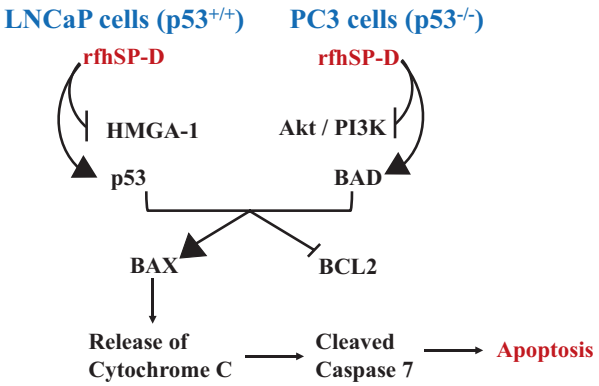

Fig. 2 Proposed mechanisms for rfhSP-D mediated apoptosis in androgen dependent (LNCaP) and independent (PC3) prostate cancer cells. rfhSP-D (20 μg/mL) treatment for 24 h upregulates p53 and downregulates pAkt, resulting in upregulation of Bad, Bax and release of cytochrome c leading to cleavage of caspase 7 in prostate cancer cells. SP-D interaction with some key molecules like HMGA1, CD14, SIRPα and EGFR has been reported previously and may be relevant as part of the proposed mechanisms of p53 and Akt

prostate cancer (Sircar et al. 2009). A significant downregulation of phosphorylated Akt was observed in both rfhSP-D treated PC3 ($p53^{-/-}$, androgen independent) and LNCaP cells (Thakur et al. 2019). Decreased levels of activated Akt may lead to decreased levels of phosphorylated Bad (Bcl-2 associated death promoter). Dephosphorylated Bad interferes with interaction of activated Bcl2 with Bax. Thus, an increased release of Bax triggers apoptosis (Ruvolo et al. 2001; Oltvai et al. 1993). Our studies suggested that besides activation of p53 pathway, rfhSP-D also inhibited Akt-PI3K pathway leading to Bax mediated apoptosis. Thus, this study unraveled PI3K/Akt, an anti-apoptotic pathway, as a novel target of rfhSP-D mediated anti-prostate cancer activity (Thakur et al. 2019) (Fig. 2).

Conclusions and Perspectives

A great advantage associated with the anti-cancer activity of SP-D (especially a recombinant fragment of human SP-D, i.e., rfhSP-D) is induction of apoptosis by simultaneous targeting of multiple cellular signaling pathways including transcription factors, tumor cell survival factors, protein kinases, resulting in the efficient and selective killing of cancer cells (Table 2). SP-D has been shown to inhibit the proliferation, migration and invasion of A549 human lung adenocarcinoma cells by binding to N-glycans of epidermal growth factor receptor (EGFR) via its CRD region, and thus, interfering with EGF signaling (Hasegawa et al. 2015). In UV treated apoptotic Jurkat T cells, SP-D enhanced membrane and nuclear blebbing, suggesting involvement of SP-D in induction of apoptosis (Djiadeu et al. 2017). rfhSP-D induced apoptosis in pancreatic adenocarcinoma cells via Fas-mediated pathway in

Table 2 Multiple signaling pathways targeted by rfhSP-D in various cancer cells

Cancer	Source of cancer cells	Targeted pathways	References
Eosinophilic leukemia	AML14.3D10	Intrinsic apoptosis, p53 upregulated	Mahajan et al. (2008)
Lung	A549 cells	Reduced viability, EGF signaling inhibited by binding to EGFR	Hasegawa et al. (2015)
Pancreatic	Panc-1, MiaPaCa-2, and Capan-2	Extrinsic apoptosis, induction of Fas	Kaur et al. (2016)
Pancreatic	Panc-1, MiaPaCa-2, and Capan-2	Inhibited invasion, reduced TGF-β	Kaur et al. (2018a, b)
Ovarian	SKOV3 cells	Extrinsic apoptosis, induction of Fas and TNF-α	Kumar et al. (2019)
Prostate	LNCaP, PC3, DU145, Primary tumor explants/cells isolated from biopsies of metastatic prostate cancer patients	Intrinsic apoptosis, p53 and pAkt pathways	Thakur et al. (2019)

a p53-independent manner (Kaur et al. 2016). Exogenous treatment of SKOV3 cells (an ovarian cancer cell line) with rfhSP-D led to increased caspase 3 cleavage and induction of pro-apoptotic genes, Fas and TNF-α (Kumar et al. 2019). Recently, Kaur et al. reported that rfhSP-D can suppress the invasive-mesenchymal properties of highly aggressive pancreatic cancer cells by inhibiting TGF-β expression in a range of pancreatic cancer cell lines via Smad2/3 signaling (Kaur et al. 2018a).

Bioinformatics analysis of SP-D presence/levels in normal and cancer tissues was performed to assess if SP-D can serve as a potential prognostic marker for human lung, gastric, breast, and ovarian cancers. Cancer tissues with significantly higher levels of SP-D compared to their normal tissue counterparts are more susceptible to SP-D-mediated immune surveillance mechanisms via infiltrating immune cells (Mangogna et al. 2018). In view of these poignant evidences, SP-D is likely to act as an integral component of the human innate immune surveillance against cancer cells.

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Testicular Immune Privilege: A Recently Discovered Domain of Collectins

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Introduction

Infertility refers to, 'a disease of reproductive system defined by the failure to achieve clinical pregnancy after 12 months or more of regular unprotected sexual intercourse' (WHO 2010). Nearly 15% of the world's population suffers from either primary or secondary infertility. About 40–50% of all cases of infertility are attributed to 'male factor', which is seen as an alteration in sperm count and/or motility and/or morphology (Kumar and Singh 2015). Increasing evidences point to a global decline in human sperm quality over a past few years (Aitken 2013; Sengupta et al. 2017). A range of factors that can lead to male infertility include systemic or localized infections and inflammation, auto-antibodies against sperm antigens, anatomical or genetic abnormalities, hormonal imbalances, trauma, surgical stress, gonadotoxins, and lifestyle practices (Durairajanayagam 2018; Katz et al. 2017). Idiopathic infertility (infertility due to unknown causes) encompasses almost 30–40% cases of male infertility (Jungwirth et al. 2015), while nearly 15% cases of male factor infertility are attributed to inflammatory or autoimmune conditions (Bachir and Jarvi, 2014).

Mammalian testis is a primary site of *spermatogenesis* and *steroidogenesis*, the key events that are crucial for male fertility. Spermatogenesis, confined to the seminiferous tubular compartment of the testis, is a process of morphological and functional differentiation of spermatogonial germ cell into the haploid male gamete, the

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spermatozoon. Synthesis of a steroid hormone, *testosterone*, responsible for maintaining spermatogenic process as well as secondary sexual male characteristics, takes place in the interstitial compartment interspersed between the tubules of the testis (Ravindranath et al. 2003).

The prominent cell component of the testicular interstitium is clusters of structurally heterogeneous androgen-producing Leydig cells. In addition, the immune cell population of the testis containing macrophages, lymphocytes, dendritic cells and mast cells is restricted to interstitium under physiological conditions (Hedger 1997).

Immune privilege is an evolutionary adaptation, where antigen specific adaptive immune responses are tightly controlled to protect auto-antigenic tissues in the body. Examples of such sites include brain, eye, pregnant uterus and testis (Hedger 2007). The immune privileged nature of the testis is of utmost importance for the *protection of male fertility*. The process of spermatogenesis in the testis commences after the onset of puberty i.e. long after the establishment of immune self-tolerance. Thus the developing spermatogenic cells expressing several neo-antigens are potentially auto-immunogenic. Therefore the mechanisms of tolerance are clearly important for preventing testicular autoimmunity post-puberty (Hedger 2012).

Innate Immune Responses in the Testis

Microbial pathogens that invade testis via circulating blood and/or the retrograde ascend of genitourinary tract are usually eliminated. This indicates that testis is capable of mounting normal inflammatory responses. These pro-inflammatory innate immune responses are initiated via pattern recognition receptors (PRRs) expressed on testicular cells. Several TLR (Toll-like receptor) members are expressed by testicular cells (Bhushan et al. 2008). TLR functions in murine Sertoli cells have been extensively studied (Winnall et al. 2011; Wu et al. 2008). Notably, TLR 3-mediated antiviral responses in mouse Sertoli, Leydig, and germ cells have been reported (Wang et al. 2012; Shang et al. 2011). TLR 11-initiated innate immune responses have been demonstrated in male mouse germ cells (Chen et al. 2014). RIG-I-Like receptor mediated innate antiviral responses have also been shown in mouse testis (Zhu et al. 2013). NOD 1 (Nucleotide-binding oligomerization domain-containing protein 1) and NOD 2 mRNAs were detected in some testicular cells, including Sertoli cells and germ cells (Lupfer and Kanneganti 2013). Murine and human testes also express several defensin molecules which may contribute to the innate anti-microbial defences (Com et al. 2003).

Systemic and localized infection result in an activation of inflammatory immune responses in the testis. Pro-inflammatory cytokines, reactive oxygen species and glucocorticoids produced in response to inflammation have considerably negative effects on the hypothalamic–pituitary–Leydig cell axis and seminiferous epithelium, thus adversely affecting the testicular functions of steroidogenesis and spermatogenesis (Guazzone et al. 2009). Such effects have been extensively studied

using bacterial lipopolysaccharide (LPS) to induce systemic or localized inflammation in experimental animal models (Liew et al. 2007).

Infection and inflammation can directly affect the seminiferous epithelium. The inflammatory mediators produced upon LPS challenge in the testis affect mitosis and meiosis of the spermatogenic cells, organization of the Sertoli cell cytoskeleton and intercellular junctions, and numerous Sertoli cell activities throughout the cycle of the seminiferous epithelium (O'Bryan and Hedger 2008).

Asymptomatic chronic infection of the male reproductive tract caused by bacterial and viral pathogens [*Chlamydia trachomatis, uropathogenic Escherichia coli, Neisseria gonorrhoeae*, human papillomavirus (HPV), hepatitis B virus (HBV), herpes simplex virus (HSV), human immunodeficiency virus (HIV), and mumps virus etc.] is also associated with an increase in seminal cytokines through the activation of humoral and cellular immune mechanisms (Naz et al. 1994). These cytokines at the site of infection are associated with increased levels of reactive oxygen species (ROS), which damage spermatozoa via lipid peroxidation. LPS also decreases sperm motility and viability (He et al. 2017). *In vitro* studies using human sperm have suggested that LPS affects human sperm motility via a decrease in intracellular cAMP levels (Li et al. 2016).

The innate immune system and the intrinsic testicular functions of spermatogenesis and steroidogenesis are intricately linked by a network of complex interactions. Therefore, in the testis a tight regulation of adaptive immune responses to safeguard developing sperm, and innate immune responses against pathogens, is crucial to protect fertility.

Factors Governing Testicular Immune Privilege

Presence of physical blood-testis-barrier, local immunosuppressive milieu, and systemic immune tolerance together contribute to the maintenance of immune privileged status of the testis. Figure 1 depicts a schematic representation of an array of factors involved in preserving testicular immune privilege.

Blood–Testis-Barrier

Majority of spermatogenic antigens lie behind blood–testis barrier, which is formed by highly specialized tight junctions between adjacent Sertoli cells. Together with local immune suppression, it provides an immune privileged microenvironment for the completion of spermatogenesis (Li et al. 2012; O'Bryan and Hedger 2008; Fijak and Meinhardt 2006).

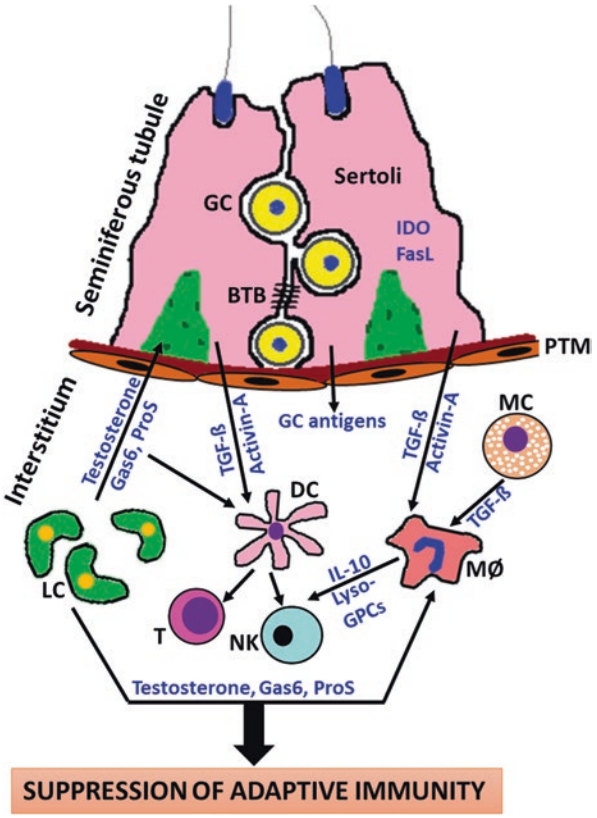

Fig. 1 Schematic representation of an array of immunosuppressive molecules secreted by testicular cells. These factors collectively create an immune microenvironment which suppresses adaptive immunity in the testis. (GC, germ cells; BTB, blood testis barrier; PTM, peritubular myoid cells; LC, Leydig cells; DC, dendritic cells; MC, mast cells; T, T lymphocytes; NK, natural killer cells; MΦ, macrophages)

Testicular Macrophages

Testicular macrophages (TM) comprise the largest leukocyte population of interstitium. Several studies have reported that the interstitial fluid surrounding TM has immunosuppressive properties, influencing the phenotype of TM. TMs locally produce small amounts of corticosterone, which suppresses the basal expression of pro-inflammatory genes. Compared with peritoneal macrophages, TMs exhibit lower constitutive expression of TLR pathway-specific genes. In TMs stimulated with LPS, the NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) signalling pathway is blocked due to the lack of IkBa (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) ubiquitination. Instead MAPK (microtubule associated protein kinase), AP-1 (Activator protein 1), and

CREB (cAMP response element binding) signalling pathways are induced in stimulated TM, which lead to production of pro-inflammatory cytokines such as TNF-α, although at much lower levels than in peritoneal macrophages (Wang et al. 2017; Bhushan et al. 2015).

Altered Expression of Major Histocompatibility Antigen

Reduced expression of major histocompatibility (MHC)—antigens on the seminiferous epithelium can aid in limiting the antigen-specific immune response. Additionally, expression of HLA-G and HLA-E, the immunoregulatory non-classical MHC antigens, which inhibit T cell and NK cell activity, has been found in the rhesus monkey testis (Ryan et al. 2002; Slukvin et al. 1999) and on human spermatogenic cells (Fiszer et al. 1997) respectively.

Cellular and Soluble Factors Mediating Active Immunosuppression

Deletion or inactivation of antigen-specific T cells is a characteristic feature of testicular immune privilege. Fas ligand (FasL), which triggers activation-induced cell death of activated T cells, is expressed by Sertoli cells. This could be responsible for local suppression of activated adaptive immune responses (Bellgrau et al. 1995; Ju et al. 1995). In addition, Sertoli cells also express high levels of transforming growth factor (TGF)- β1, inhibitors of complement [serine protease inhibitor G1 (SERPING1), SERPINE1, decay accelerating factor 2 (DAF2/CD55B) and CD55], inhibitors of granzyme B (serpina3n) and ligand B7-H1, which have direct inhibitory activity against T cells (Doyle et al. 2012; Lee et al. 2007; Sipione et al. 2006; Dal Secco et al. 2008).

The tryptophan-metabolising enzyme indoleamine 2, 3 dioxygenase (IDO), is responsible for inhibiting T cell mediated autoimmune response, and stimulating regulatory T cell (Treg) functions in pregnant uterus (Mellor and Munn 2004). Inhibition of IDO expression has been shown to abolish the ability of porcine Sertoli cells (SC) to protect pancreatic islet allografts in a co-transplantation model in mice as the protective effect of SC was strictly dependent on efficient tryptophan metabolism in the grafts, leading to TGF-β-dependent induction of auto-antigen specific Treg cells (Fallarino et al. 2009).

In addition, lyso-glycerophosphatidylcholines (lyso-GPCs) present in testicular interstitial fluid possess T-cell inhibitory activity *in vitro* (Foulds et al. 2008). Tyro3 family of receptor tyrosine kinases, consisting of Tyro3, Axl and Mer (TAM), and their ligands—the growth arrest-specific gene 6 (Gas6) and Protein S, negatively regulate innate immune responses (Rothlin et al. 2007). TAM receptors and Gas6

are constitutively expressed in the mouse testis (Wang et al. 2005). The transcription factor Ets-variant gene 5 (ETV5) is essential for spermatogonial stem cell (SSC) self-renewal. Recipient ETV5 knock-out mice displayed abnormal blood-testis-barrier and increased interstitial inflammation and tubular involution after transplantation (Morrow et al. 2007).

Immunoregulatory Cytokines, Peptides, Prostanoids and Hormones

Several cytokines with known immunoregulatory or immunosuppressive activities are constitutively expressed in the testis. These include TGF-β1, TGF-β2, TGF-β3, activin A, IL-10 (Barakat et al. 2008; O'Bryan et al. 2005), β-endorphin, α-melanocyte stimulating hormone (Bhardwaj et al. 1996; Grabbe et al. 1996), prostanoids (Winnall et al. 2007), prostaglandins of the D, E and I series and thromboxane A (Hata and Breyer 2004). Sertoli cell specific androgen receptor knock-out mice show impaired testicular immune privilege, indicating that testosterone functions locally in maintaining immune privilege. These mice exhibit defective Sertoli cell tight junctions, increased testicular IgG levels, infiltration of macrophages, neutrophils, plasma cells and eosinophils in the interstitium, and presence of autoantibodies against germ cell antigens in the serum (Meng et al. 2011). Experimental autoimmune orchitis (EAO) is an immunological infertility model induced in rodents by subcutaneous injection of syngenic testicular germ cells. This causes an infiltration of immune cells in the interstitium, inflammation, irreversible damage to seminiferous epithelium, germ cell apoptosis and disruption of spermatogenesis (Rival et al. 2008). In rats undergoing EAO, androgen replacement caused reduction of pro-inflammatory cytokines by testicular immune cells (Fijak et al. 2011).

Major Secreted Collectins: SP-A, SP-D and MBL in Male Reproductive Tract

Though SP-A and SP-D are mainly synthesized by alveolar type II cells and Clara cells, their extra-pulmonary expression has been demonstrated in rodent and human tissues suggesting systemic roles of these proteins (Fisher et al. 1995; Motwani et al. 1995). Several studies have reported the presence of collectins in the male reproductive tract of human, rodents and stallion (Beileke et al. 2015; Kankavi et al. 2008, 2006; Oberley et al. 2007; Madsen et al. 2003). Presence of SP-A and SP-D in the genital tract of stallion including, prepuce, prostate, testis, and seminal vesicle has been considered to be important in the immune surveillance and active barrier defense mechanism at these sites (Kankavi et al. 2006). In addition, Oberley and colleagues reported the presence of SP-D transcript and protein throughout murine

male reproductive tract including testis, epididymis, vas deferens, prostate, and coagulating gland. Their study also revealed that the infection of rat prostate with *Escherichia coli* significantly up-regulated levels of SP-D 24 and 48 hr. post-infection. Further, elevated prostatic SP-D mRNA levels were demonstrated in castrated mice compared to the levels in sham-castrated animals (Oberley et al. 2007).

SP-A and SP-D have been localized on human spermatozoa by means of immunofluorescence staining (Kankavi et al. 2008). SP-A was found in the mid piece, tail, and sometimes at the equatorial region of spermatozoa while SP-D was present in the tail and acrosome. Thus SP-A and SP-D are novel sperm-associated proteins with a potential role in mammalian sperm maturation. In a recent report by Beileke and colleagues, localization of SP-A and SP-D has been demonstrated in spermatogonia, spermatocytes, Sertoli cells and Leydig cells of healthy human testis (Beileke et al. 2015).

In rodents, two forms of MBL, MBL-A and MBL-C, exist which are the products of two related, but uncoupled, genes. In testis, only the MBL-A gene was found to be expressed (Wagner et al. 2003). Table 1 summarizes the localization of collectins in the male reproductive tract.

Table 1 Expression and localization of collectins in the male reproductive tract

Collectin	Protein localization	Transcript expression
SP-A	Mice testis, head and tail of sperm (Rokade and Madan 2016)	Mice testis (Rokade and Madan 2016)
	Human testis and prostate (Beileke et al. 2015)	Human testis and prostate (Beileke et al. 2015)
	Stallion testis, smegma, seminal fluid, prepuce, prostate, seminal vesicle (Kankavi et al. 2006)	Mice testis (Akiyama et al. 2002)
	Mid piece, tail, equatorial region of human sperm (Kankavi et al. 2008)	–
SP-D	Mice testis, head and tail of sperm (Rokade and Madan 2016)	Mice testis (Rokade and Madan 2016)
	Rat prostate (Oberley et al. 2007)	Human testis and prostate (Beileke et al. 2015; Madsen et al. 2000)
	Human testis and prostate (Beileke et al. 2015; Madsen et al. 2000)	Mice testis, epididymis, prostate (Oberley et al. 2007; Akiyama et al. 2002)
	Mice testis, epididymis, prostate (Oberley et al. 2007)	–
	Stallion testis, smegma, seminal fluid, prepuce, prostate, seminal vesicle (Kankavi et al. 2006)	–
	Tail and acrosome of human sperm (Kankavi et al. 2008)	–
MBL	Mice testis, connecting piece and tail of sperm (Rokade and Madan 2016)	Mice and human testis (Rokade and Madan 2016; Seyfarth et al. 2006; Wagner et al. 2003)

Expression and Localization of SP-A, SP-D and MBL in the Testis

Fine-tuning the regulation of autoimmune reactions against spermatogenic antigens and inflammatory immune responses against pathogens is critical to safeguard male fertility. SP-A, SP-D and MBL are known to regulate inflammatory immune responses, modulate the activity of other immune cells and restore immune homeostasis in the lung (Mahajan et al. 2013; Wang et al. 2011; Liu et al. 2010; Ip et al. 2009; Pastva et al. 2007; Kishore et al. 2002).

Analysis of the levels of SP-A, SP-D and MBL-A in the testis of pre-pubertal, pubertal and post-pubertal adult mice showed a developmentally regulated expression of these proteins in the testis with significant increase in their expression both at transcript and protein levels after the onset of puberty. Immunohistochemical analysis revealed no immunoreactivity for SP-A and SP-D proteins, while very weak immunoreactivity for MBL-A in the testis of pre-pubertal mice. A marked increase in the expression of all three collectins was evident in the testis of post-pubertal mice. We found the localization of SP-A, SP-D and MBL-A proteins in Leydig cells, myoid cells, Sertoli cells and germ cells, mainly the round spermatids and tails of elongated spermatids of adult testis (Rokade and Madan 2016). Intense staining for all the three proteins was observed in the interstitial spaces of the testis (Fig. 2).

Because collectins are soluble proteins, they can be acquired from peripheral circulation. Thus, to identify their testicular source, germ cells, Sertoli cells, myoid cells and Leydig cells were isolated to >90% purity and assessed for the expression of these proteins in purified cell populations by real time RT-PCR and immunofluorescence staining. Germ cells were found to express SP-A, SP-D and MBL-A while myoid cells expressed only MBL-A. We also demonstrated their expression on murine caudal sperm using immunofluorescence staining. SP-A and SP-D were found to be localized on sperm head and tail while a distinct staining for MBL-A was observed on the connecting piece and tail of spermatozoa (Fig. 3).

Together these findings establish a developmentally regulated expression of SP-A, SP-D and MBL-A in murine testis, and their localization on caudal sperm, hinting at the involvement of these proteins in male fertility.

Hormonal Regulation of SP-A, SP-D and MBL in the Testis

Earlier studies have suggested hormone -regulated expression of collectins in various tissues. Estrogen and progesterone positively regulate the expression of SP-D in the mouse uterus. Uteri of estrogen-treated mice showed four-fold increase in SP-D transcript levels while co-administration of estrogen and progesterone led to nine-fold increase in SP-D levels. However, treatment with progesterone alone significantly reduced the uterine SP-D expression (Kay et al. 2015). Glucocorticoids have

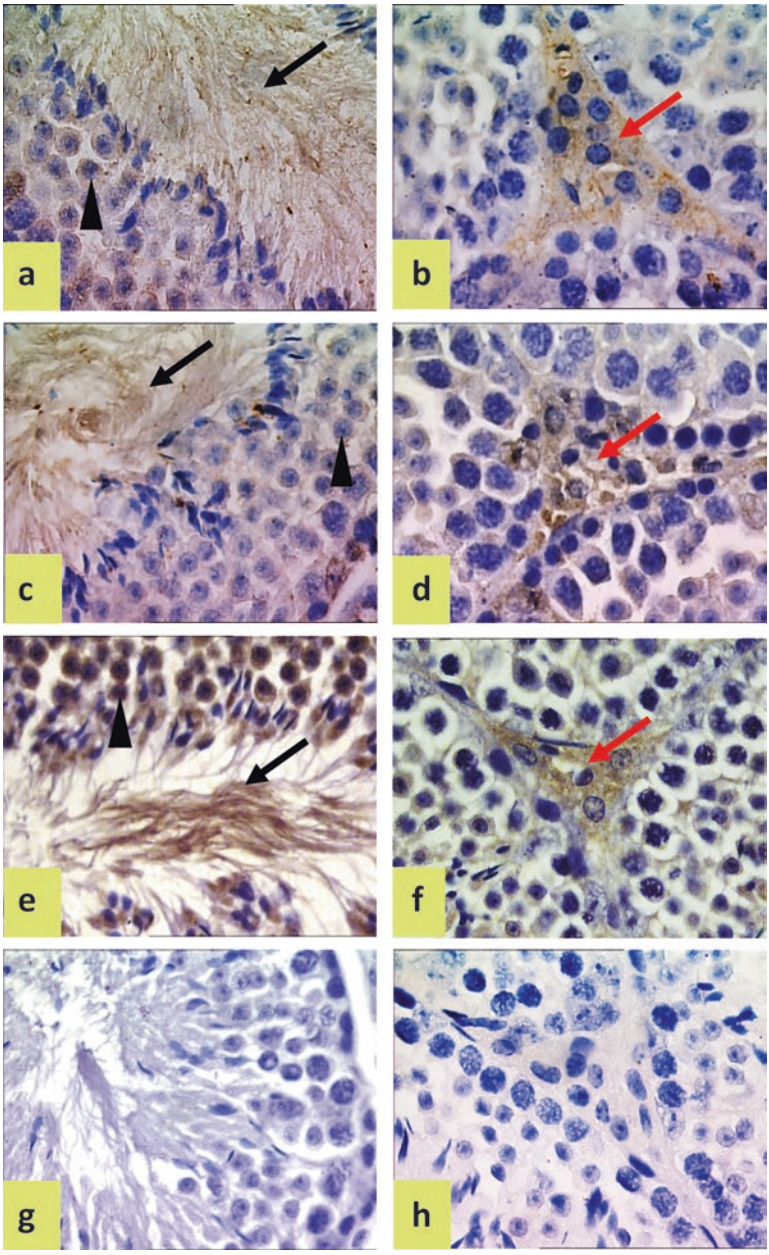

Fig. 2 Immunohistochemical localization of SP-A (**a**, **b**), SP-D (**c**, **d**), and MBL-A (**e**, **f**) in adult murine testis. Image (**g**, **h**) represents negative control sections. Red arrow indicates staining in interstitial compartment; black arrow indicates staining on spermatid tail while black arrow head indicates staining on round spermatids. Data are representative of five independent experiments (Magnification = 100×)

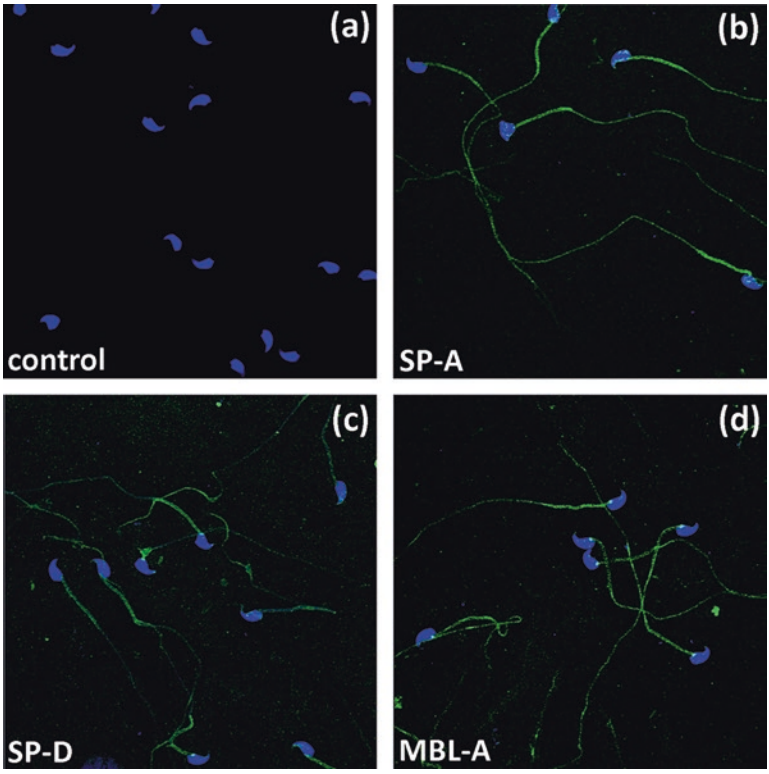

Fig. 3 Localization of collectins on adult murine caudal sperm. (**a**) Isotype control stained with normal rabbit IgG. (**b**) Localization of SP-A (green) on sperm head and tail. (**c**) Localization of SP-D on sperm head and tail. (**d**) Localization of MBL-A on sperm connecting piece and tail. Data are representative of five independent experiments (Magnification = 63×)

both stimulatory and inhibitory effects on SP-A expression in fetal human lung tissue (Mendelson et al. 1991; Liley et al. 1988). Accumulation of SP-D in lung tissue is increased following the exposure of fetal rats to glucocorticoids *in utero* (Mariencheck and Crouch 1994). Human hepatocyte cell line HuH-7 was exposed to growth hormone (GH), hydrocortisone, insulin-like growth factor (IGF)-1, insulin, interleukin (IL)-6 or thyroid hormones (T3 or T4). After 3 days the concentration of MBL in the culture supernatants was determined and the amount of mRNA for MBL was measured. GH, IL-6, T3 and T4 significantly increased MBL synthesis in a dose-dependent manner, while hydrocortisone, insulin and IGF-1 had no effect (Sørensen et al. 2006).

To test the effect of testosterone on the expression of collectins, a murine testosterone suppression model was developed using combined treatment with GnRH-antagonist acyline and androgen receptor (AR) antagonist flutamide (Panneerdoss et al. 2012). Successful suppression of testosterone production in the testis was evaluated by characteristic histological changes (impaired spermatogenesis,

sloughing of germ cells from seminiferous epithelium and atrophied tubules), and by measuring the testicular weight, and serum and intra-testicular testosterone levels. A significant down-regulation of SP-A, SP-D and MBL-A transcripts was observed in the testosterone suppression model, suggesting a positive regulation of collectins by testosterone in the testis. Further *in silico* analysis showing presence of steroid hormone responsive elements in the promoter region of murine SP-A, SP-D and MBL-A gene confirmed our findings (Rokade and Madan 2016).

Interestingly, earlier study by Oberley and colleagues has demonstrated an up-regulation of SP-D transcript in rodent prostate gland upon testosterone withdrawal following castration (Oberley et al. 2007). Castration induces increase in the infiltrating inflammatory cells in prostate (Meng et al. 2011). Since levels of SP-D are known to increase in response to inflammation (Oberley et al. 2007), we speculate that castration induced inflammation of prostate might have caused an up-regulation of SP-D expression rather than the absence of testosterone.

Lipopolysaccharide Modulates Expression of Collectins in the Testis

Systemic high dose lipopolysaccharide (LPS) challenge in mice is known to induce inflammatory immune response in the testis and reduces sperm viability and motility. Significant up-regulation in the expression of testicular SP-A and SP-D post LPS challenge (Rokade and Madan 2016), suggested that SP-A and SP-D may be integral to the control of inflammatory responses in order to sustain the normal physiological functions of testis.

Interaction of SP-D with Testicular Cells

The binding of SP-D to a broad range of cell surface proteins has previously been well characterized. This includes, binding through its CRD to Gp-340, a molecule purified from broncho-alveolar lavage (BAL) of alveolar proteinosis patients (Holmskov et al. 1997), myeloperoxidase, an intracellular defense molecule of neutrophils, which becomes exposed on the outer membrane of the cell undergoing apoptosis and defensins, an anti-bacterial cationic protein of neutrophils (Jakel et al. 2010). Moreover, SP-D is also known to bind decorins, a proteoglycan present in the amniotic fluid (Nadesalingam et al. 2003). Binding of SP-D to various classes of immunoglobulins including IgG, IgM, IgE and secretory but not serum IgA through its CRD domain has also been reported (Nadesalingam et al. 2005).

Further interaction of SP-D with LPS binding protein, CD14 has been documented (Sano et al. 2000). SP-D also binds to TLR-2 and TLR-4, which are involved in peptidoglycan and LPS induced inflammatory immune signalling (Yamazoe et al.

2008). Other binding partners for SP-D include calreticulin-CD91 complex (Vandivier et al. 2002; Ogden et al. 2001) and SIRP-α (Gardai et al. 2003).

To unravel the regulatory role of SP-D in controlling testicular inflammation and protecting sperm motility, we examined the binding of a recombinant fragment of human SP-D (rfhSP-D, composed of a homotrimeric neck and CRD region) with isolated testicular cells. Our results indicated dose dependent binding of rfhSP-D to testicular cells. The identity of the particular receptors of SP-D on the testicular cell surface is yet to be determined. Testicular cells are known to express various TLRs, which could be the potential receptors for SP-D. Binding of SP-D to testicular cells via its CRD region could be of relevance in the regulation of inflammatory immune signalling (Rokade et al., unpublished data).

SP-D Restores Testicular Immune Privilege Affected by LPS

LPS is a primary pathological factor associated with chronic inflammatory diseases and male reproductive dysfunction (Collodel et al. 2015). Several *in vitro* studies using native SP-D or rfhSP-D have identified the mechanisms of inhibitory actions of SP-D on LPS induced inflammation. SP-D inhibits LPS mediated inflammation through direct binding to LPS receptor CD14 (Anas et al. 2010; Hoyt 2000; Sano et al. 2000). The interaction of SP-D with extra-cellular domains of TLR-2 and TLR-4 via its C-terminal domain has also been reported, which has been implicated with its role in inhibiting LPS induced pulmonary inflammatory responses (Yamazoe et al. 2008). SP-D binds to a complex of recombinant soluble forms of TLR-4 and MD-2 with high affinity and down-regulates the TNF-α secretion and NF-kappa B activation elicited by both rough and smooth LPS, in alveolar macrophages and TLR4/MD-2-transfected HEK293 cells. Cell surface binding of both serotypes of LPS to TLR4/MD-2-expressing cells was attenuated by SP-D. The LPS induced production of monocyte chemoattractant protein-1 is also inhibited by SP-D (Hu et al. 2012). Notably, the exogenous administration of rhSP-D (synthesized by the transfection of Chinese hamster ovary cells with a complementary DNA encoding full-length human SP-D, purified as a mixture of dodecamers and multimers) was shown to protect LPS and LTA (lipoteichoic acid) induced lung injury in mice (Ikegami et al. 2007).

In view of the ability of SP-D to interact and interfere with LPS mediated inflammatory response, the effect of exogenous rfhSP-D treatment on LPS challenged wild type (WT) mice has been assessed (Rokade et al. 2017). The histological analysis of testis from 24 h post- LPS challenged mice showed vacuolization and an increase in the intercellular gaps in seminiferous epithelium due to disruption of cell-to-cell contact. Although the testis from rfhSP-D treated and LPS challenged mice also showed the presence of intercellular gaps and vacuoles in seminiferous epithelium, the number of tubules showing such features was low in rfhSP-D treated group. Analysis of transcript levels of cytokines viz., TGF-β1, IL-10 and TNF-α in the testis revealed that LPS challenge resulted in a pro-inflammatory response

evident by up-regulation of TNF- α and down-regulation of TGF- β1 levels compared to the control group. Treatment with exogenous rfhSP-D in LPS challenged mice significantly increased the levels of all three cytokines compared to the control and LPS alone group with a predominance of anti-inflammatory cytokine milieu. LPS challenge led to a significant decrease in the caudal sperm count and percentage of total sperm motility, while it increased the percentage of static sperm in caudal sperm suspension compared to the saline control group. Treatment with rfhSP-D restored the total sperm count and motility and reduced the percentage of static sperm within 24 h of LPS challenge.

Consistent with the involvement of SP-D in controlling inflammation, we observed a protective effect of rfhSP-D against LPS induced inflammatory response in the testis and sperm motility, thus highlighting the beneficial properties of SP-D in male infertility caused by inflammation (Rokade et al. 2017).

Fertility Characterization of SP-D Gene Knockout Mice

Mice deficient in SP-D gene have long been utilized to decipher the *in vivo* functions of SP-D in various tissues (Zhang et al. 2015; Lambertsen et al. 2014; Lemos et al. 2011). The role of SP-D in regulating pulmonary physiology and immune homeostasis has been well characterized. SP-D knockout (SP-D$^{-/-}$) mice exhibit exaggerated lung inflammation with increased levels of pro-inflammatory cytokines, metallo-proteinases and oxidant species (Korfhagen et al. 1998; Wright 2005; Yoshida et al. 2001). Lungs of SP-D$^{-/-}$ mice display delayed clearance of *Pneumocystis carinii* infection, increased inflammation, and altered nitric oxide metabolism (Atochina et al. 2004). SP-D$^{-/-}$ mice were also shown to be susceptible to invasive pulmonary aspergillosis and pulmonary hypersensitivity (Madan et al. 2010).

Recently SP-D$^{-/-}$ mice were found to have issues with female fertility. These females exhibited altered uterine immune profile with elevated levels of inflammatory cytokines, increased number of pro-inflammatory monocytes/macrophages and lower FOXP3 levels during the pre-implantation period (Kay and Madan 2016).

Under physiological conditions, SP-D$^{-/-}$ male mice were seen to exhibit significantly reduced weight of the testis and epididymis. In addition, a reduced degree of protein tyrosine phosphorylation was observed in the capacitated sperm from SP-D$^{-/-}$ mice. However, mating studies did not reveal any significant defect in fecundity (Rokade et al. 2017). Moreover, characterization of the immune status of the testis of SP-D$^{-/-}$ mice revealed that lack of SP-D significantly alters the immune milieu of the testis by increasing the levels of immune-regulatory molecules viz., TGF-β, Serpina3n, and IL-10, leading to reduced immune cell activation. In view of an overall increase in testicular immunosuppressive milieu, we found a reduced severity of inflammatory reaction in the testis of SP-D$^{-/-}$ mice following LPS challenge, reaffirming immunosuppressive microenvironment within the testis of the SP-D knockout mice (Rokade et al. 2017).

Conclusion and Future Perspective

Multiple immunological factors act together to protect auto-antigenic spermatozoa and regulate inflammatory immune responses against infections. Therefore, the predominance of immunosuppressive milieu in the testis of SP-D$^{-/-}$ mice could be a mechanism to compensate for the roles played by SP-D to preserve fertility. The current data seem to suggest an involvement of SP-D in regulating testicular immune privilege and male fertility. Further a detailed understanding of the putative roles of SP-D in male fertility is remains to be elucidated preferably involving the use of testis-specific knock-down model, restricted mating trials and *in vitro* fertilization assays.

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Collectins in Regulation of Feto-Maternal Cross-Talk

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Introduction

Implantation of embryo in the mother's womb involves biological trade-offs, or even conflicts, between factors that improve the maternal health and those that support the fetal development and growth. This has led to evolutionary adaptations in mammals resulting in an effective cross-talk at the feto-maternal interface. A diverse range of hormonal and immunological factors of maternal and embryonic origin at the feto-maternal interface modify maternal immune system in order to protect the conceptus from attack by the mother's immune system (Robinson and Klein 2012).

Pregnancy requires profound changes in the maternal immune system as the mother needs to harbor the semi-allogenic fetus. It has been suggested that a significant decline in the adaptive immune response during pregnancy enables the pregnant body to tolerate the fetal allograft. This may compromise maternal host-defense mechanisms making the mother more susceptible to infections (Mor and Cardenas 2010). During pregnancy, infections can gain access to gestational tissues and maternal endometrium via the maternal circulation, by ascending into the uterus from the lower reproductive tract, or by descending into the uterus from the peritoneal cavity (Espinoza et al. 2006). Intrauterine infections can significantly influence the pregnancy outcome. Bacterial and viral infections have been strongly associated with several pregnancy complications such as preterm labor (Goldenberg 2000;

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Lamont 2003; Espinoza et al. 2006), preeclampsia and intrauterine growth restriction (IUGR) (Arechavaleta-Velasco 2002; Hsu and Witter 1995; Adams Waldorf and McAdams 2013; von Dadelszen and Magee 2002; Mor and Cardenas 2010).

The innate immune system by means of its ability to distinguish 'non-infectious self' (mother, placenta, and fetus) from 'infectious non-self' (bacteria, virus, etc.), represents the first line of immune defense against pathogens (Janeway and Medzhitov 2002). An evolutionary conserved system of pattern recognition is one of the ways by which innate immune system confers the host defense. Pattern recognition receptors (PRRs), an integral component of the innate immunity, can recognize and bind to highly conserved 'pathogen-associated molecular patterns' (PAMPs) and endogenous stress proteins or 'damage-associated molecular patterns (DAMPs)' (Takeuchi and Akira 2010). This interaction results in the activation of PRRs and generation of an inflammatory response. There are different PRRs, including the well-defined family of cell surface Toll-like receptors (TLRs), scavenger receptors and C-type lectins, humoral mannose binding lectin (MBL), surfactant proteins, and cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).

Collectins, calcium-dependent C-type lectins belonging to a family of evolutionarily conserved mammalian pattern recognition molecules, are integral to immunomodulation and host defense. Surfactant protein A (SP-A), surfactant protein D (SP-D) and mannose binding lectins (MBL) are the three well characterized members of the collectin family (Wright 2005; Stuart et al. 2005). Both SP-A and SP-D are secreted by the alveolar epithelium, non-ciliated bronchiolar cells and other mucosal surfaces exposed to the external environment (Wright 2005), whereas MBL is secreted by hepatocytes to serum (Howard et al. 2018). The primary structure of collectins mainly consists of four regions: (i) a cysteine-containing N-terminus (required for disulfide-dependent oligomerization of monomeric subunits); (ii) A triple-helical collagen region composed of repeating Gly-X-Y triplets (associated with maintaining the molecule's shape, dimension, stability and oligomerization); (iii) α-helical coiled coil neck region (involved in protein trimerization); and (iv) a globular structure at the C-terminus comprising a C-type lectin or carbohydrate recognition domain (CRD) (which mediates calcium dependent ligand-binding to pathogens, carbohydrates and phospholipids etc.) (Kishore et al. 2006). SP-A and SP-D are large oligomeric structures. The monomeric subunits assemble into trimers at the neck region which further oligomerize to form large multimeric structures. Six such trimers oligomerize to form the octadecameric bouquet shaped structure of SP-A (650 kDa) and MBL (228–304 kDa), four trimers form a cruciform structure of 520 kDa for SP-D (Vieira et al. 2017; Holmskov et al. 2003; Turner 1996). SP-A and SP-D, as well as MBL, bind to a broad spectrum of pathogens including viruses, bacteria, fungi, allergens, apoptotic cells and enhance their uptake by innate immune cells such as macrophages, monocytes, neutrophils and dendritic cells (DCs). Clearance of pathogens by the collectins is achieved by multiple mechanisms, including opsonization and aggregation of the pathogens, in addition to regulating the cell-surface-receptor expression (Vieira et al. 2017; Holmskov and Jensenius 1993). MBL activates the lectin complement pathway, releases cytokines and coagulation factors during infection and tissue injury in

co-operation with three MBL-associated serine proteases (MASPs 1, 2 and 3) (Matsushita and Fujita 1992). The evidences available so far clearly signify that these soluble PRRs not only recognize and eliminate the pathogens but they are at the junction of both innate and adaptive immunity. Collectins regulate inflammation, host-pathogen interactions, immune tolerance and thus, offer immune protection at multiple levels. Also, it is fascinating that the collectins bring about both pro-inflammatory and anti-inflammatory functions based on their interaction with candidate receptors, SIRP-α and calreticulin/CD91 (Gardai et al. 2003). Importantly, there are increasing evidences highlighting the role of these soluble proteins in novel functions related to maintenance of pregnancy (implantation, vascular remodeling, and placenta formation) and parturition (Fig. 1).

We discuss here the expression profile of collectins at the feto-maternal interface, hormonal regulation and functional relevance of these molecules at different stages of pregnancy. In addition, this chapter will reveal the role of collectins in various pregnancy complications and their potential to serve as early disease prediction markers in pregnancy.

Expression and Localization of Collectins at the Feto-Maternal Interface and in Gestational Tissues and Maternal Serum

The expression of SP-D at the feto-maternal interface was first reported by Leth-Larsen and colleagues. The authors demonstrated immunostaining for SP-D in the cytoplasm of villous and extravillous trophoblast, interstitial and vascular trophoblasts of both early and late gestational placenta (Leth-Larsen et al. 2004). Both SP-A and SP-D were shown to be present in the chorio-decidual layer of the late pregnant uterus (Miyamura et al. 1994), the human fetal membranes, amniotic epithelium and chorionic membrane (Han et al. 2007), trophoblast of the late normal placental villi, early human placenta (Sati et al. 2010). Cytotrophoblasts and mesenchymal cells have been shown to express MBL during early pregnancy (Kilpatrick et al. 1995; Yadav et al. 2016). Synthesis of SP-A, SP-D and MBL by the human first trimester and term placental and decidual tissues has also been demonstrated (Yadav et al. 2014; Yadav et al. 2016). Madhukaran et al. reported the expression of SP-A and SP-D in early human decidua specifically on stromal cells and extracellular trophoblast (EVT) (Madhukaran et al. 2016). Expression of collectins by the early and late gestational tissues suggests plausible role of these soluble PRRs in the cellular processes and other signaling pathways relevant for the placental development, immune tolerance and thereby establishment and maintenance of pregnancy.

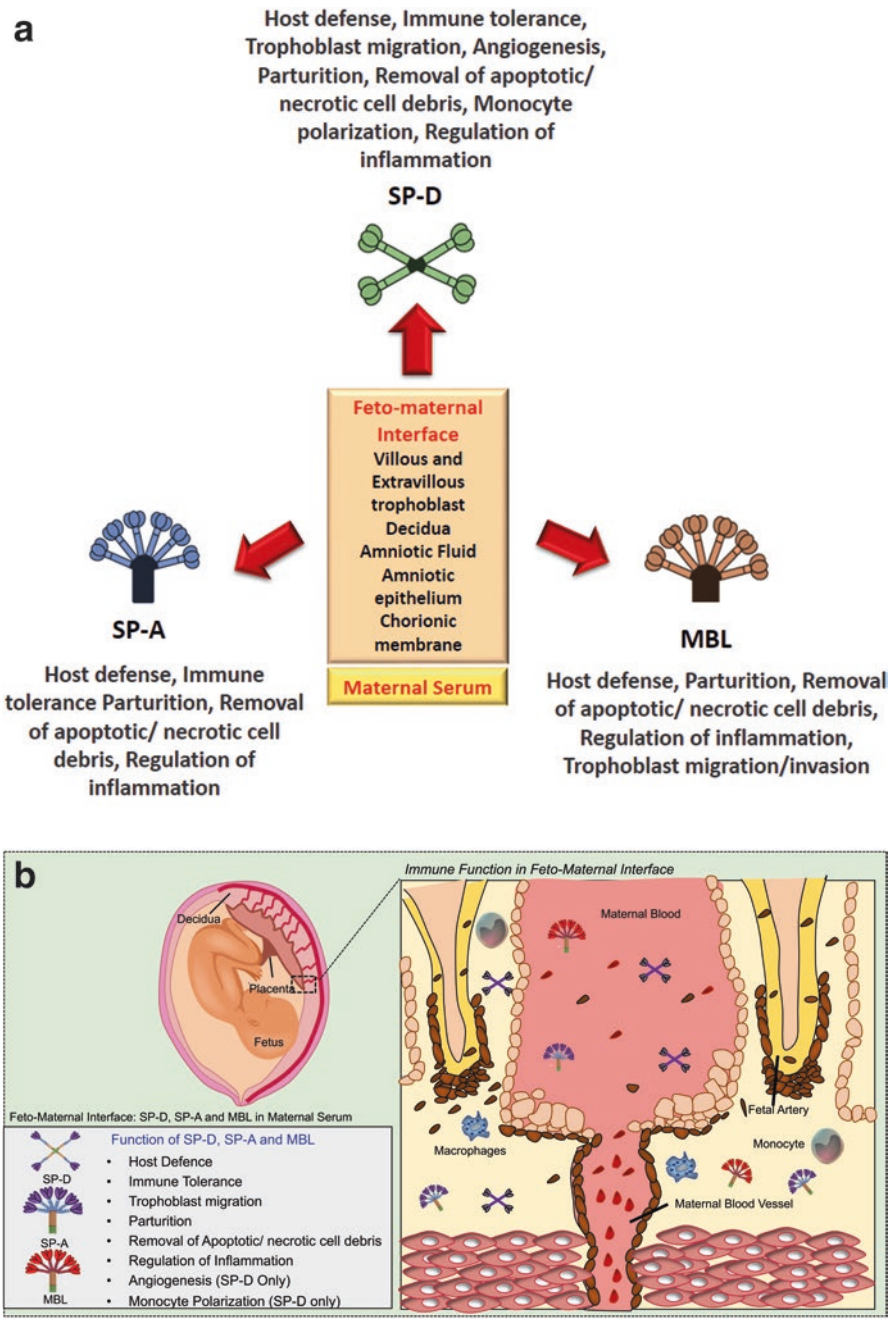

Fig. 1 (**a**, **b**) Proposed functions of SP-A, SP-D and MBL in pregnancy. SP-A, SP-D and MBL expressed by feto-maternal interface and maternal serum have been associated with numerous functions in pregnancy. Among the functions sumarized in the figure the role of SP-A, SP-D and

(continued)

Fig. 1 (continued) MBL in parturition and modulation of immune response have been adequetly adressed while others xare proposed functions and need abound experimental investigation. Recently, in our study we reported significant inhibition of trophoblast migration/proliferation in the presence of recombinant human SP-D (Kale et al. 2020a). Aletred angiogenic [vascular endothelial growth factors, placental growth factors, matrix metalloproteinases, tissue inhiibitor of matrix metalloproteinases], hormone synthesizing genes [Aromatase, Catechol-o-methyltransferase] and inflammatory molecules [TNF-α, IL-10] at feto-maternal interface of SP-D gene deficient mice indicates importance of SP-D for regulation of immune and angiogenic response essential for immune tolerance and placental development (Data unpublished). MBL have been shown to inhibit C1q mediated trophoblast migration of extravillous trophoblast cells isolated from first trimseter placental tissue (Agostinis et al. 2012). Treatment of peripheral blood mononuclear cells (PBMCs) of severe early onset PE with recombinant human SP-D polarized the pro-inflammatory M1 monocytes to immunoregulatory M2 phenotype, decreased pro-inflammatory cytokine response and retained anti-inflmmatory response suggests their role in modulation of sysetemic immune response in pregnancy (Data unpublished)

Expression Profile of Collectins in the Amniotic Fluid

Amniotic fluid (AF), being a rich source of various nutritive factors and antimicrobial peptides, provides protection and support to the developing fetus (Underwood et al. 2005). In view of the presence of numerous innate immune factors, AF performs a significant role in the host defense (Underwood et al. 2005). Fetal lungs have been shown to contribute to the AF levels of surfactant proteins previously (Condon et al. 2004). SP-A levels were detected in the human amniotic fluid at mid-pregnancy (15 to 19th weeks of gestation) and its concentration increased significantly at term (Pryhuber et al. 1991; Chaiworapongsa et al. 2008). Human AF SP-A levels increased from <3 μg/mL at 30 weeks gestation to >24 μg/mL at the term (Snyder et al. 1988). Also, the amniotic fluid SP-A concentration in the women at term in labor was shown to be significantly lower than that in the women not in labor (1.2–10.1 μg/mL vs. 2.2–15.2 μg/mL) (Chaiworapongsa et al. 2008). In contrast to SP-A, SP-D levels were detected in human AF at 26 weeks of gestation and its concentration increased gradually, reaching up to 3 μg/mL at term (Miyamura et al. 1994). Similar to SP-A and SP-D, the AF levels of MBL increased with advancing gestational age (Malhotra et al. 1994). MBL levels before and after 35 weeks of gestation were 304 μg/mL and 1070 μg/mL, respectively (Malhotra et al. 1994). Levels of SP-A in the umbilical cord blood of newborn babies delivered by the spontaneous labor between 36 and 38 weeks (4.8–50.2 ng/mL) were two-fold higher compared to those delivered through cesarean section i.e., with no labor (2.7–21.7 ng/mL) (Cho et al. 1999). Increasing levels of collectins in AF with the advancing gestation implicate their potential to serve as potential biomarkers to evaluate fetal lung maturity.

Collectins in the Umbilical Cord

The concentrations of SP-D in the umbilical cord blood and capillary blood in premature infants were twice as high as in mature infants. This increase in SP-D levels has been attributed to the capillary leak and an increased release of SP-D from epithelial cells due to inflammatory response (Dahl et al. 2006). Low levels of MBL in the umbilical cord blood are associated with increased risk of respiratory infections whereas, the high levels are associated with increased risk of respiratory morbidity in infants of asthmatic patients (Schlapbach et al. 2009).

Levels of Collectins in the Maternal Serum

Although, there were adequate evidences to show AF levels of collectins, limited reports were available on maternal serum levels of the collectins in pregnancy. A modest increase in serum MBL levels was reported in the third trimester of normal pregnant women (Kilpatrick 2000). Serum levels of MBL increased in the pregnancy as early by 12th weeks of gestation and dropped significantly post-pregnancy (Van de Geijn et al. 2007). Recently, we determined the profile of collectins in the maternal serum during pregnancy and post-partu. We observed that the maternal serum levels of SP-A and SP-D declined significantly at 8–12 weeks of gestation and remained constant till 32 weeks of gestation compared to non-pregnant women. However, post-partum, a significant increase was observed in the SP-A levels, whereas SP-D levels increased gradually. On the contrary, maternal serum MBL levels increased significantly in pregnancy compared to post-partum (Kale et al. 2020a) (Fig. 2). The gestational age-dependent changes in the serum and AF levels of collectins suggest distinct roles of these proteins at different stages of pregnancy and parturition in addition to their role in the host-defense.

Collectins in the Induction of Parturition (Term and Preterm Labor)

The cellular and molecular mechanisms that promote parturition at term are complex and multifactorial. Parturition is associated with an elevated local and systemic inflammatory response, induced by the infiltration of maternal and fetal tissues by neutrophils and macrophages (Thomson et al. 1999). This inflammatory signal activates pro-inflammatory transcription factors, nuclear factor-κB (NF-κB), which enhances the expression of contraction-associated protein (CAP), connexin-43 (CX43), the oxytocin receptor (OXTR), and cyclooxygenase 2 (COX-2) that transform the quiescent myometrium to a contractile state. Growing body of evidences

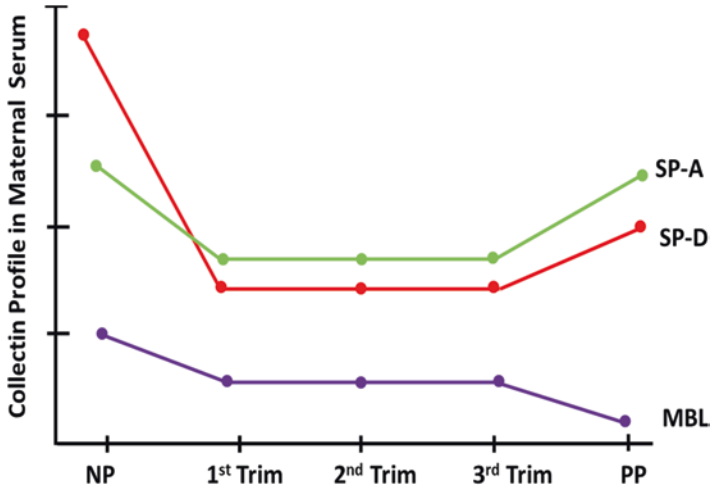

Fig. 2 Collectin profile in maternal serum. The figure is graphical representation of the SP-A, SP-D and MBL profile in maternal sera collected progressively from pregnant women (n = 30) in the first trim: (8–12 weeks), second trim: (20–24 weeks), third trim: (28–32 weeks), PP: Post-partum (within 2 weeks of Parturition) and NP: Non-pregnant women (Mid-Luteal Phase). During normal pregnancy SP-A and SP-D levels are downregulated in the first trimester compared to non-pregnant women and maintained consistently till 32 weeks of gestation and increased post-partum. Both, SP-A, and SP-D have been associated with anti-tumor function (Mitsuhashi et al. 2013; Mahajan et al. 2013). Similarity of the immune micro-environment at feto-maternal interface and tumor progression (Holtan et al. 2009), also significant inhibition of trophoblast cell migration/proliferation in presence of the recombinant human SP-D (Kale et al., 2020b) suggests relevance of decreased SP-A, SP-D levels for placental development. Increased levels of SP-A and SP-D post-partum implicates their important role in parturition induction. In contrast to SP-A and SP-D, levels of MBL show gradual decrease in the first trimester when compared to non-pregnant women and increase compared to post-partum. Consistently maintained collectins in pregnancy is essential to ensure balance between immune tolerance and host-defense function as their abnormal levels have been associated to pregnancy disorders

suggests that the signal for inflammatory stimulus required for the initiation of parturition arises from fetus (Condon et al. 2004; Mitchell et al. 1984).

Role of SP-A and SP-D in Parturition

Condon et al. 2004 reported that the SP-A is detected at 17 dpc in murine AF and its levels increased markedly towards term. The AF SP-A further promoted the migration of fetal amniotic fluid macrophages in murine uterus and enhanced the production of pro-inflammatory cytokines (IL-1β), through stimulation of NF-κB and induced parturition. Intra-amniotic injection of SP-A at 15 dpc caused preterm parturition at 16–17 dpc, whereas the injection of SP-A antibody delayed parturition by 24 h, suggesting that the activation of fetal AF macrophages by SP-A and their

subsequent migration into the maternal myometrium are key events for spontaneous parturition in mice (Condon et al. 2004). Consistent with the report by Condon et al., delayed parturition and lower levels of both pro-inflammatory and anti-inflammatory activation markers (IL-1β, IL-6, ARG1, YM1), CAP genes, connexin-43, and oxytocin receptor were observed at 18.5 dpc in the myometrium of TLR2, SP-A and SP-D gene deficient mice (Montalbano et al. 2013). Salminen et al. 2011 demonstrated that the administration of LPS to rSP-A mice (mice transgenically overexpressing rat SP-A) modified the inflammatory cytokine response and the expression of the pattern recognition receptors in fetal and gestational tissues. Furthermore, in rSP-A mice, the maternal LPS challenge increased TNF-α concentration and expression in the AF and fetal membranes, respectively. Similarly, mice overexpressing rat SP-D (rSP-D) showed significantly increased levels of TNF-α, IL-10 in the AF and fetal serum and the expression of IL-10 was noted in placenta after LPS challenge (Salminen et al. 2011; Salminen et al. 2012). These results suggested that both SP-A and SP-D in the fetal and gestational tissues contribute to inflammatory processes related to parturition and modulate the levels of intrauterine inflammatory mediators involved in the preterm birth.

While there are strong indications that SP-A and SP-D are involved in the parturition, Agrawal et al., suggested that SP-A suppressed the preterm labor and reduced inflammation in response to TLR2 ligands in mice (Agrawal et al. 2013). The discrepancy in these observations has been attributed to the different routes of administration of SP-A. Consistent with this finding, expression of SP-D transcripts was found to be reduced near parturition in mice and the presence of SP-A and SP-D significantly decreased TNF-α production by LPS challenged decidual macrophages (Madhukaran et al. 2016). The study suggested that the presence of SP-A and SP-D in decidua may be critical for protection of fetus against infection while the decreased SP-D transcripts near term at 19.5 dpc could be an indirect way to upregulate PGE2, cyclooxygenase enzyme-2 (COX2) and increased myometrial sensitivity to estrogen in association with parturition (Madhukaran et al. 2016).

The concentration of AF SP-A decreased during the spontaneous labor in humans; no fetal macrophages were found in the myometrium after labor (Chaiworapongsa et al. 2008; Lee et al. 2010; Kim et al. 2006). No significant difference was observed by Han et al. in the expression level of SP-A1 transcripts in chorioamniotic membranes between term with labor and term without labor (Han et al. 2007), suggesting that the mechanisms of labor in mice and humans are different. In contrast to these reports, Lee et al. 2010 investigated a potential role for SP-A in the human pregnancy and parturition by examining SP-A expression patterns in the AF and amnion. The results showed that the high molecular weight oligomeric SP-A was increased in AF with advancing gestation. Interestingly, these oligomers were more abundant in the placental amnion (amnion overlying the placental disc) before labor at term, while they increased primarily in reflected amnion during the labor (amnion of the extraplacental chorioamniotic membranes). SP-A significantly and selectively inhibited Prostaglandin F2α (PGF2α) production without affecting the production of other inflammatory mediators and angiogenic factors (including IL-6, IL-8, TNF-α, MMP-3, MCP-1, IL-1β, PGE2, sFlt-1 and VEGF) at the high

dose of 100 μg/mL, suggesting its key role in the decidual activation and onset of labor (Snegovskikh et al. 2011).

Human myometrial cells expressed SP-A and the treatment of SP-A triggered cellular signaling events leading to protein kinase C zeta (PRKCZ), MAPK1/3, and RELA (NF-κB p65 subunit) activation, PTGS2 (cyclooxygenase 2) induction, and cytoskeleton reorganization in the cultured human myometrial cells (Verdugo et al. 2008). These signaling pathways are vital for the myometrium to develop contractile activity at the end of pregnancy (Verdugo et al. 2008). In line with this report, Sotiriadis et al. showed that the treatment of human myometrial cell line (ULTR) with recombinant SP-A and SP-D induced contractility-associated protein (CAP) genes and pro-inflammatory cytokines, thus, shifting the uterus from a quiescent to a contractile state (Sotiriadis et al. 2015). We reported a significant increase in the transcripts and protein of SP-A and SP-D in the term human placenta of women undergoing spontaneous labor (Yadav et al. 2014). Further, treatment of placental tissue explants with native and recombinant human SP-D showed a significant increase in the labor associated cytokines, IL-1α, IL-1β, IL-6, IL-8, IL-10, TNF-α and MCP-1, implicating that SP-D plausibly contributes to the pro-inflammatory immune milieu of feto-maternal tissues at term (Yadav et al. 2014). Increased maternal serum SP-D and SP-A levels were observed at the term and after 2 weeks post-partum in one of our recent study (Kale et al. 2020a), thus, supporting the relevance for SP-A and SP-D in the pathways regulating parturition.

Genetics plays an important role in the risk of preterm birth. In addition, incidence of preterm deliveries is higher among mothers with a history of previous such deliveries. Common polymorphism Met31Thr of the gene encoding SP-D influencing the concentration, oligomerization and binding properties of SP-D was associated with the spontaneous preterm birth (SPTB) in preterm infants of families with recurrent SPTBs in northern Finnish population (Karjalainen et al. 2012). Future investigations into association of polymorphisms in the SP-A and SP-D genes and the maternal serum levels of these proteins during parturition may be useful to predict the preterm birth.

Involvement of MBL in Parturition

Although the role of MBL in parturition is still far from clear, a significant increase in maternal serum MBL levels during pregnancy and its sharp decrease after parturition (Van de Geijn et al. 2007) implicates its plausible association with the parturition and onset of labor. In a recent study, low MBL levels have been associated with the preterm birth and low birth weight (Koucký et al. 2018). However, Wang et al. 2017 reported no significant difference in the second trimester MBL levels of Taiwanese mothers with preterm and term deliveries (Wang et al. 2017). Besides preterm birth, low MBL concentrations have been considered a significant risk factor for spontaneous abortion, idiopathic recurrent late pregnancy loss, unexplained

recurrent miscarriages, chorioamnionitis, and preeclampsia (Christiansen et al. 1999, 2002, 2009; Kruse et al. 2002; Van de Geijn et al. 2007; Holmberg et al. 2008). MBL levels of ≤0.1 μg/mL are considered a clinically-significant risk factor for spontaneous abortion (Kilpatrick et al. 1999). In a recent study, Canda et al., reported decreased MBL immunoreactivity in the decidua and villous trophoblast of first time pathologic first trimester human miscarriage, suggesting decreased lectin pathway activity (Canda et al. 2018).

Serum/plasma levels of MBL are genetically determined. Bodamer et al. determined the five common MBL2 polymorphisms (codon 52, 54, 57; promoter −550, −221) in DNA samples isolated from blood of infants born prematurely and normally (Bodamer et al. 2006). The results of the study indicated that the frequency of the codon 52 polymorphism was significantly higher in the pre-term group (10.8%) compared to the term group (4.9%). Furthermore, the authors reported that carriers of genotypes (O/O) likely conferring deficient MBL plasma levels were more common in the group of premature birth (9.8% vs. 2.9%), while the promoter −550 C/C genotype was underrepresented in the pre-term birth group (24.5% vs. 39.2%). The study suggested that the fetal MBL2 genotype might be an additional genetic factor contributing to the risk of premature delivery (Bodamer et al. 2006). Hartz et al. reported that the infants with deficiency of MBL2 born between 32 and 36 weeks of gestation are at a higher risk of infection (Hartz et al. 2017). In contrast, no significant association was reported between MBL 2 promoter variants (−550 C > G and −221 G > C) and structural MBL variants with missense mutation (R52C, G54D, G57E) in exon 1 contributing to the low levels of MBL with unexplained recurrent pregnancy loss or miscarriage (Baxter et al. 2001; Berger et al. 2013). Single nucleotide polymorphisms (SNPs) in the promoter region of the MBL2 gene (H/L or X/Y) have been shown to modify basal levels of MBL in serum (Madsen et al. 1998). The individuals with high MBL genotype ((H/L) YA/ (H/L) YA and (H/L) YA/LXA) are associated with high MBL serum levels, the intermediate MBL genotype, (LXA/LXA and (H/L) YA/O); and the individuals with low MBL genotype (LXA/O and O/O) are associated with the intermediate and lowest MBL serum levels respectively (Frakking et al. 2006). Van de Geijn et al. determined the association of MBL genotype in 157 nulliparous women with parturition and gestational age. The authors reported that the maternal high MBL production genotype is associated with the premature birth (before 29 weeks of gestation) (~13%) compared to those with the intermediate and low MBL serum genotype groups (3%). The study postulated that during pregnancy the MBL-associated inflammation caused by higher MBL activity may contribute to an early delivery (Van de Geijn et al. 2008).

Hormonal Regulation of Collectins in Female Reproductive Tract and Pregnancy

During menstrual cycle, maternal endometrium undergoes cyclic changes in morphology and function under the influence of two primary steroid hormones, estradiol and progesterone. These changes are crucial for uterine receptivity, implantation and sustenance of the pregnancy (Su and Fazleabas, 2015). On the other hand, results of a number of clinical studies have suggested that the sex hormones influence the expression of innate immune proteins in the reproductive tract making it susceptible to infections by many pathogens (Sonnex 1998).

Transcript and protein expression of both SP-A and SP-D have been reported in the female vagina, uterus, ovary, cervix and oviduct (Akiyama et al. 2002; Oberley et al. 2004; Leth-Larsen et al. 2004). SP-A is reported to be expressed in the vaginal stratified squamous epithelium and cervico-vaginal lavage of both pre-and post-menopausal women (MacNeill et al. 2004). Similarly, MBL was shown to be expressed in endometrium and basal layer of the vaginal epithelium and cervico-vaginal lavage (Bulla et al. 2010). Importantly, expression of collectins in the female reproductive tract (FRT) has been shown to be regulated by ovarian steroid hormones.

Variations in the Expression of Collectins During Menstrual Cycle

Expression of SP-D in the epithelium of the endometrial glands is increased towards the secretory phase of regular menstrual cycle compared to the proliferative phase (Leth-Larsen et al. 2004). Expression of SP-D in the uterus of ovariectomized mice is positively regulated by estradiol, and negatively regulated by progesterone; expression of SP-D is increased nine-fold after administration of both estradiol and progesterone (Kay et al. 2015). In addition, in the cycling mouse uterus, SP-D has been shown to be hormonally regulated, with peak levels present in the estrogen dominated estrus phase, which then decreased in progesterone dominated diestrus phase (Kay et al. 2015). Indeed, Oberley et al. showed that administration of progesterone to mice in the diestrus phase makes them more susceptible to chlamydial infection, whereas it is difficult to infect the female reproductive tract with Chlamydia during the estrous stage of the reproductive cycle when estradiol levels are high (Oberley et al. 2007, Ito et al. 1984) suggestive of involvement of SP-D in innate defense against chlamydia. Using immunohistochemistry, SP-A expression within the glandular and stromal cells has been observed in the maternal endometrium during the early proliferative phase (day 6–7) of the menstrual cycle. The levels of MBL in the vaginal epithelial cells vary in a cycle-dependent manner with greater changes occurring in the secretory phase of the menstrual cycle. This change, which is associated with progesterone, implicates the protective role of MBL in the

female genital tract (Bulla et al. 2010). These findings strongly suggest that cyclic variations in SP-A, SP-D and MBL expression are hormonally regulated and affect innate host defense against pathogens in the FRT.

Regulation of Collectin Expression During Pregnancy

Until now, there were no adequate evidences to show hormonal regulation of collectin expression during pregnancy. Kay et al. reported a decreased expression of uterine SP-D at early stages of pregnancy when progesterone levels were much higher and estradiol was at basal level (Kay et al. 2015). Recently, we reported that the systemic levels of SP-A and SP-D are decreased and consistently maintained till 32 weeks of gestation in normal pregnancy. Importantly, our study indicated that the ratio of progesterone to estradiol rather than hormones alone, influences the systemic levels of collectins during pregnancy (Kale et al. 2020a). The relevance of decreased SP-D levels in pregnancy was further substantiated with an *in vitro* assay showing significant inhibition in HTR-8/SVneo (first trimester trophoblastic cell line) cell migration and proliferation with the treatment of a recombinant fragment of human SP-D (rfhSP-D) composed of trimeric CRDs.

Collectins in Implantation and Spontaneous Abortion

Implantation, which is essential for pregnancy initiation, involves opposition and adherence of the blastocyst to uterine luminal epithelium, followed by migration and invasion of trophoblastic cells through maternal spiral arteries present into the endometrial decidua (Kim and Kim 2017). Both the arms of the immune system under the regulation of ovarian hormones are modulated to regulate this process and substantiate a protective environment in maternal decidua to prevent fetal demise. However, any impairment may lead to implantation failure which can cause infertility, abortions, placental insufficiency and other complication related to pregnancy (Kim and Kim 2017).

SP-D in Spontaneous Abortions in Humans and Mice

Spontaneous abortion (SA) is the most common complication in the first trimester affecting 15% pregnancy (Cohain et al. 2017). Dysregulated immunoregulatory mechanisms at the feto-maternal interface and in the maternal circulation have been associated with SA previously (Ticconi et al. 2019; Calleja-Agius et al. 2012). As mentioned above, collectins are expressed by the luminal and glandular epithelium as well as stromal cells of the maternal endometrium (Kay et al. 2015). We reported

several novel findings using pregnant SP-D$^{-/-}$ knock-out female mice crossed with SP-D$^{+/+}$ males, such as (i) fertility defects with smaller litter size and increased pre-implantation embryo loss; (ii) extended estrous cycles with alterations in ovarian hormone profile and uterine expression of hormone responsive genes; and (iii) skewed uterine immune milieu towards inflammation (Kay and Madan 2016). Consistent with our observation, reduced liter size was reported earlier also in SP-A and SP-D double knockout mice (Montalbano et al. 2013).

In another study, we analyzed the expression of collectins in the inflamed human gestational tissues of SA and in 13.5 dpc placental tissues from resorption survived embryos of murine model (CBA/J X DBA/2J) of SA. We reported a significant downregulation in SP-A transcripts and upregulation in the SP-D transcripts in placental and decidual tissues of SA group compared to healthy pregnant women. Significant upregulation was observed in SP-A and SP-D transcripts at 13.5 and 14.5 dpc in the placental tissues of viable embryos from CBA/J X DBA/2J mated females wherein the inflammation was regulated compared to control (CBA/J X Balb/c) (Yadav et al. 2016). Recently, we reported a significant downregulation in the SP-D levels and P4 (Progesterone)/E2(Estradiol) ratio at 8–12 weeks of gestation in the women with a subsequent missed abortion (MA)/asymptomatic SA compared to normal healthy pregnant women (Kale et al. 2020a), suggesting their potential to act as early screening biomarkers for identification and preventative management of MA.

MBL and Pregnancy Loss

Higher expression of MBL has been reported in the uterine lumen of patients with unexplained infertility (Bulla et al. 2009). In addition, expression of MBL-A was detected at implantation site as early as 3.5 d of pregnancy in abortion-prone mice mating, whereas its deficiency prevented the pregnancy loss (Petitbarat et al. 2015). Similarly, we reported increased transcripts of MBL in placental tissues at 14.5 dpc in viable embryos of abortion-prone mice. These reports suggest that activation of the lectin pathway is accompanied by implantation failure both in human and mice. In contrast to this, genetic polymorphisms contributing to low maternal serum MBL levels have been associated with recurrent pregnancy losses and poor pregnancy outcome (Cieslinski et al. 2017; Kruse et al. 2002; Christiansen et al. 2009). In a recent study, Canda et al. reported decreased lectin pathway activity in tissues from first-time pathologic human miscarriage (Canda et al. 2018).

Together, these evidences suggest that collectins may perform a balancing act to regulate both local and systemic immune response during pregnancy. Optimal levels of these proteins are essential for a healthy pregnancy outcome whereas any dysregulation may lead to an unfavorable pregnancy outcome.

Preeclampsia and Gestational Hypertension: Role of Collectins in the Placental Development

Hypertensive disorders of pregnancies—preeclampsia (PE) and gestational hypertension, contribute inordinately to maternal and fetal morbidity and mortality. PE is characterized by hypertension, proteinuria, edema, hepatic and renal dysfunction after 20 weeks of pregnancy (Duhig et al. 2018). The disease has worldwide incidence of about 2–8% (Steegers et al. 2010). The WHO predicts the incidence of PE to be seven times higher in developing countries than developed (2.8% vs. 0.4% of live births) (WHO 2005). Although the precise etiology of the disease is still unknown, PE is considered as a disease with the placental origin. Impaired angiogenesis, increased oxidative stress, hormonal and immunological alterations in the early placental microenvironment may contribute to the placental defects in PE (Escudero et al. 2014; Hertig et al. 2010; Cornelius 2018). Intriguingly, most of these functions dysregulated in PE are governed by the collectins. While there are adequate reports showing association of SP-A, SP-D and MBL with SA and preterm birth, role of SP-A and SP-D in PE has been largely unexplored until recently whereas reports on MBL are scarce.

Involvement of SP-A and SP-D in Preeclampsia: Human and Murine Studies

Recently, we determined the systemic and placental profile of collectins before and after the disease onset and association of collectins with steroid hormones in pregnancy. It was observed that collectins are differentially expressed in the serum and placenta of PE women. SP-A and SP-D decreased significantly prior to onset (i.e. at 10–20 weeks) in women with severe early onset PE (EOPE), whereas both collectins increased significantly after the onset (i.e. at 28–32 weeks) in sera and term human placental tissue of these women. Moreover, the shift in SP-A and SP-D during the disease progression was found to be regulated by P4/E2 ratio. This transposition in SP-A, SP-D and P4/E2 levels in severe EOPE appears fascinating; however, the exact transition point is still not known. Weekly collection of serum samples between 20 and 32 week of gestation may facilitate determination of the transition point of SP-A and SP-D in severe EOPE. Most importantly, decreased SP-A, SP-D and P4/E2 ratio at 10–20 week of gestation were found to be potential risk factors for severe EOPE (Kale et al., 2020b). In corroboration, screening of SP-D gene deficient pregnant mice showed a significant defect in the placental development and elevation in mean arterial pressure. Additionally, we also observed significant decrease in the transcripts of several angiogenic markers, such as vascular

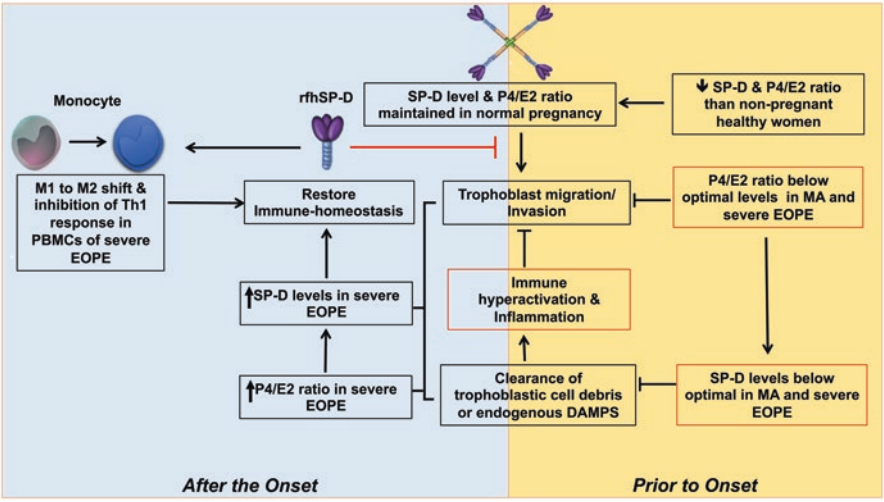

Fig. 3 Hypothetical model of role of SP-D in pregnancy and severe EOPE. During normal pregnancy SP-D levels are decreased in the first trimester and consistently maintained similar to that of P4/E2 ratio. As SP-D is potent anti-tumor molecule and shown to inhibit trophoblast migration/proliferation (Kale et al. 2020b), decreased SP-D may promote trophoblast migration/invasion during normal pregnancy. Decreased P4/E2 ratio below optimal prior to onset in severe early onset PE (EOPE) and/or missed abortion (MA) might affect trophoblast invasion directly or indirectly by decreasing the levels of SP-D. Further, loss of SP-D or levels of SP-D below optimal though would favor placental development but would also affect clearance of trophoblastic debris, a process critical to regulation of immune response in pregnancy. Impaired clearance of trophoblastic debris may lead to immune dysregulation which eventually affect placental development. Defective placental development might lead to elevated P4/E2 ratio, exaggerated inflammatory response and thereby, elevated SP-D after the onset to polarize monocyte to M2 phenotype and restore immune homeostasis

endothelial growth factor (VEGF), placental growth factor (PLGF), Matrix metalloproteinases 2 (MMP2), catechol-o-methyltransferase (COMT) and increase in tissue inhibitor of matrix metalloproteinases 2 (TIMP2) at 15.5 dpc in the placenta of SP-D gene deficient mice (Data unpublished). With an *in vitro* study using a recombinant fragment of human SP-D (rfhSP-D), we provided a proof of concept that elevated levels of SP-D after the onset in severe EOPE women could be essential for polarization of monocytes to immunomodulatory M2 phenotype and restore cytokine balance in peripheral blood mononuclear cells of severe EOPE women (Data unpublished). Observations from our studies provided an important insight to the plausible roles of SP-A and SP-D in the placental development and regulation of immune response in PE for the first time. Figure 3 shows a hypothetical model depicting plausible roles of SP-D in pregnancy and PE.

MBL Serum Levels in Pregnant Mothers

Significantly increased maternal serum MBL levels have been observed in the third trimester of women with PE (Kale et al. 2020b; Than et al. 2008; Celik and Ozan 2008; Agostinis et al. 2012). Furthermore, the increased MBL levels in PE patient sera have been shown to strongly inhibit the interaction of extravillous trophoblast (EVT) with C1q interfering with the process of EVT adhesion to and migration through decidual endothelial cells (DECs), suggesting that the increased level of MBL in PE may inhibit endovascular invasion of trophoblast cells (Agostinis et al. 2012). These observations warrant future studies defining the roles of collectins in placental development and associated hypertensive pregnancy disorders, such as gestational hypertension, eclampsia and HELLP (hemolysis, elevated liver enzymes, and a low platelet count) syndrome.

Gestational Diabetes: Metabolic Roles of Collectins?

Gestational diabetes mellitus (GDM), defined by glucose intolerance, is the most common metabolic disorder during pregnancy. GDM affects 3–17% of all pregnancies. Although the precise mechanisms underlying GDM are still elusive, a close immune–metabolic relationship have been documented (Wojcik et al. 2016). Zhao et al. performed genomic expression profiling of blood and placenta of GDM positive women of the Chinese ethnicity. The authors reported GDM-dependent alterations in the expression of numerous immune-related genes both in blood and placenta, supporting the notion of a link between immune system and GDM (Zhao et al. 2011).

Wojcik et al. reported increased SP-D mRNA levels in the blood leukocytes of hyperglycemic GDM patients (Wojcik et al. 2016). GDM is also associated with an increased risk of neonatal respiratory distress syndrome (RDS). Transcripts of SP-A mRNA were decreased in the fetal lungs of rat model of diabetic pregnancy, while insulin treatment led to a substantial increase in the SP-A mRNA levels in fetal lungs (Moglia and Phelps 1996). The study provided an important evidence that glucose metabolism dysfunction and/or inflammation during pregnancy can lead to alterations in the expression of surfactant proteins.

Several genetic factors have also been implicated in the pathogenesis of GDM. Megia et al. reported that women carrying low plasma levels of MBL and G54D mutant allele for MBL2 have a higher risk for developing GDM and having heavier infants. Thus, G54D mutation may lead to sustained release of inflammatory cytokines (TNF-α), known to down-regulate the insulin sensitivity and the development of GDM (Megia et al. 2004). Müller et al. suggested that the presence of MBL2 variants, leading to increased inflammatory response, may cause inflammatory damage to the pancreatic β-cell function, thereby affecting insulin secretion rather than insulin action (Müller et al. 2010).

Conclusions

The microenvironment at the feto-maternal interface creates a tolerogenic niche to facilitate the development of the semi-allogeneic fetus; perturbations in the feto-maternal immune cross-talk may give rise to various pregnancy complications. The presence of collectins in both early and late placental tissues can alter mother's immune response to the allogenic fetus, and thus, is relevant in mediating feto-maternal interactions. Consistently maintained AF and maternal serum levels of collectins during the pregnancy, and their altered levels in the early first trimester and at term and/or at post-partum, signifies gestational stage-dependent role of these proteins in pregnancy. Inhibition of trophoblast cell migration and proliferation in the presence of rfhSP-D signifies the relevance of decreased maternal serum levels of SP-D during the first trimester in the establishment of immune tolerance and placental development. Most importantly, altered serum levels of SP-A, SP-D and P4/E2 ratio at early stages of pregnancy in women with subsequent MA and severe EOPE in our studies (Kale et al. 2020a, b) highlights their potential to act as predictive biomarkers. We think that the maternal serum levels of SP-A, SP-D and P4/E2 ratio along with current prediction model such as combination of serum biomarkers [PLGF, sFLT1] and maternal risk factors [e.g. Mean Arterial Pressure (MAP), and uterine-artery Doppler Pulsatility Index (PI)] may improve the prediction rate for women at risk of EOPE (O'Gorman et al. 2017)**.** This would facilitate counseling of such high-risk pregnant women during early stages of pregnancy and also to administer potential preventive interventions such as low dose aspirin (Rolnik et al. 2017). However, future studies involving large multi-centric cohorts are needed to validate these speculations. As parental genotype may determine the circulatory levels of immune proteins in pregnancy, collectin polymorphisms along with their maternal serum levels need further investigations. In addition, studies with *in vitro* and *in vivo* model systems would provide better insight into the role of collectins in immune tolerance, implantation, angiogenesis, trophoblast invasion, tissue remodeling and removal of apoptotic cells, processes that critical for placental development.

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Bovine Collectins: Role in Health and Disease

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Introduction

The management of infectious diseases in cattle (*Bos taurus*) is of key importance for the economic well-being and maintenance of the agricultural and food industries worldwide, affecting both cattle breeding, meat and milk production. Common infectious diseases include mastitis, bovine respiratory disease (RBD), bovine tuberculosis, gastrointestinal disease, which account for significant economic burden and some other bovine zoonoses (Miles 2009; Abernethy et al. 2013; APHA 2020; Mcdaniel et al. 2014). Furthermore, the prevalence of infectious diseases is also a major cause of increased agricultural antibiotic use, which increases the risk of anti-microbial resistance (Prescott et al. 2012). Therefore, a greater emphasis should be placed on understanding the immunobiology of *Bos taurus*, particularly genetic deficiencies that contribute to infectious disease susceptibility but also immunological mechanisms that could potentially augment protection against these diseases without the requirement of anti-microbials.

As in all mammals, the bovine innate immune system is the first-line defence against pathogens and a major component of this are a group of proteins called collectins. Collectins are a family of proteins that have calcium (Ca^{2+}) dependent activity and similar structures characterised by a carbohydrate recognition domain (CRD) linked to collagen-like region through an alpha-helical coiled-coil neck region. They have diverse roles in innate immune defence and normal physiology. The bovine collectins comprise of two lung proteins: surfactant protein A (SP-A) and surfactant protein D (SP-D), a number of serum proteins, mannan-binding lectin (MBL), conglutinin (CGN), collectin of 43 kDa (CL-43), collectin of 46 kDa (CL-46) as well as two recently discovered collectins: collectin liver 1 (CL-L1) and

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collectin placenta 1 (CL-P1). The collectins are present in all mammals, except for CGN (CGN), which seems to be unique to *Bovidae* (Lu et al. 1993a).

The collectins can form several higher order structures. The N-terminal regions have 1–3 cysteine residues that can form disulphide links, cross-linking the collagen regions, which are comprised of three polypeptide chains and can be further organised into large oligomeric structures composed of a maximum of six subunits (Hansen and Holmskov 2002). Collectins are soluble pattern recognition receptors (PPRs) specialised at recognising pathogen-associated molecular pattern (PAMPs) of carbohydrate moieties such as lipopolysaccharide (LPS) that are present on microbial surfaces (Janeway 1989; Hansen and Holmskov 1998; Murugaiah et al. 2020). Recognition and binding occur via the CRD regions and can lead to pathogen agglutination, neutralization and/or clearance by opsonization of the microbe and recruitment of phagocytes for clearance (Kishore et al. 2006; Murugaiah et al. 2020). Mice engineered to be genetically deficient in SP-A or SP-D showed increased susceptibility to microbial infections (Levine and Whitsett 2001). Collectins have been found in mammalian species, with CGN, CL-43 and CL-46 predominantly found in Bovidae. It appears that the latter have evolved separately in Bovidae from other mammalian species, but share structural similarities to SP-D, indicating that they may have key roles in health and disease in cattle and related species (Hansen and Holmskov 2002; Gjerstorff et al. 2004a).

The study of bovine collectins and their specific role in bovine infectious disease has been neglected. In comparison, collectin immunobiology is well understood in human and other model systems (e.g., mice) (Murugaiah et al. 2020). In this review, the current knowledge on bovine collectins will be summarised but also gaps in current knowledge will be discussed with respect to findings in human and other mammalian species. It is hoped that this review will be a trigger for new studies on bovine collectins, leading to new approaches for bolstering the immune resilience and defence in cattle.

Genomic Characteristics of Bovine Collectin Family Members

A total of ten collectin genes have been identified in Bovidae, from cattle and other herbivore species. MBL1 and MBL2 encode for MBL-A and MBL-C proteins respectively, SFTPA1 and SFTPD encode for SP-A and D respectively, CGN1 encodes for CGN, CL-43 encodes for collectin 43 (CL-43), CL46 encodes for collectin 46 (CL-46), COLEC10 encodes for liver collectin (CL-L1), COLEC11 encodes for kidney collectin (CL-K1) and COLEC12 encodes for placental collectin (CL-P1) (Hansen and Holmskov 2002; Gjerstorff et al. 2004a; Fraser et al. 2018).

The majority of the bovine collectin genes in *Bos taurus* (SFTPA1, SFTPD, CGN1, MBL1, CL43 and CL46) are located on a single locus on chromosome 28, suggesting evolutionary conservation (Fig. 1). In contrast, the remaining bovine collectin genes are found on separate chromosomes: COLEC11 on chromosome 8, COLEC10 on chromosome 14, COLEC12 on chromosome 24 and MBL2 on

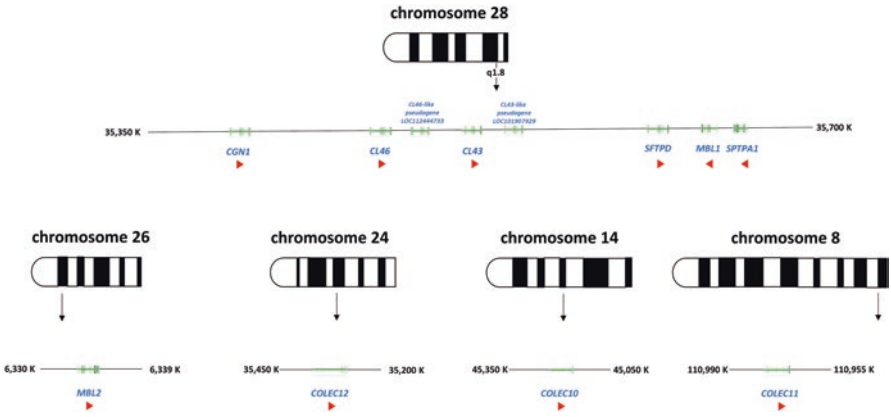

Fig. 1 *Bos taurus* collectin gene loci. Schematic representation of the chromosomal locations of the bovine collectins. The Ensembl accession numbers for each gene are as follows: SFTPA1 (ENSBTAT00000031298); SFTPD (ENSBTAG00000046421); MBL1 (ENSBTAT00000001165); MBL2 (ENSBTAG00000007049); CL43 (ENSBTAG00000047317); CL46 (ENSBTAG00000048082); CGN1 (ENSBTAG00000006536); COLEC10 (ENSBTAG00000017343); COLEC11 (ENSBTAG00000016225) and COLEC12 (ENSBTAG00000007705). (Data taken from Gjerstorff et al. 2004a; Gjerstorff et al. 2004b; and Fraser et al. 2018). Arrows denote direction of transcription

chromosome 26 (Fig. 1). Analysis of the genomic organisation of the collectin genes in *Bos taurus* suggest that they evolved from gene duplication events of a progenitor bovine SP-D gene after *Bovidae* separated into a distinct evolutionary family. Furthermore, CGN has thus only been found in Bovidae species and in addition to cattle (*Bos taurus*), it has also been found in sheep, goat, antelope, bison and several other species (Lu et al. 1993a; Hughes 2007; Mohan et al. 2015; Souza et al. 2018; Barik et al. 2020). Evolutionary analysis of the CRD regions of the additional collectins show that CL-43 and CGN are most similar and probably evolved from a prototype gene that in turn arose from an ancestral SP-D gene for CL-46 and a CGN/CL-43 prototype collectin. In *Bos taurus*, CGN, CL-43 and CL-46 genes are found on distal region of chromosome 28, which is analogous to collectin locus in humans that is located on chromosome 10 (Gallagher et al. 1993; Hansen et al. 2002a, 2003), and comprises the genes encoding MBL (MBL-C), SP-D, SP-A1 and SP-A2 (variants for SP-A) and MBL-A (Sastry et al. 1989; Kolble et al. 1993; Hoover and Floros 1998). Several repetitive and transposon like elements have been observed in CL-43 and CGN genes which may have contributed to gene duplication events at this locus and seems further evolved in the *Bovidae* (Hansen and Holmskov 2002). Other regulatory elements (e.g., promoter regions) of the CL-43 and CGN1 display high sequence similarity to SP-D, suggesting a conservation of the transcriptional control mechanisms governing the expression of these genes (He et al. 2000; Hansen et al. 2002b, 2003).

Diversity, Evolution and Molecular Characteristics of Family Members

The six bovine collectins, whose genes reside on chromosome 28 (SP-A, SP-D, CGN, MBL-A, CL-43 and CL-46), all show interesting structural similarities. Comparing SP-D, CL-43 and CL-46 reveals that they all have an extended collagen-like region of between 35 and 46 nm in length and are longer (by 15 and 25 nm) than the collagen-like region in SP-A and MBL-A. Furthermore, bovine SP-D, CGN and CL-46 has 171 amino acid residues in its collagen-like region, which is 6 fewer amino acids when compared to human SP-D. The bovine collectins also contain two conserved cysteine residues in the N-terminus of the protein, whilst SP-D and CL-43 also have an N-linked glycosylation motif at the start of their collagen-like regions (Lim et al. 1993; Lu et al. 1993b; Crouch et al. 1994; Hansen et al. 2002a; Hansen and Holmskov 2002). Structural studies reveal that the collectins are synthesised as single monomer polypeptides comprising a common CRD, coiled-coil neck, collagenous domain and N-terminal cysteine region (Fig. 2). The collectins then form homotrimeric subunits composed of these three polypeptide chains, except CL-LK which is a heterotrimeric composed of two CL-K1 and 1 CL-L1 polypeptides (Hansen et al. 2016). Most of the collectins can also form higher oligomeric structures with SP-D, CGN, and CL-46 forming cruciforms of four of the subunits, whilst SP-A, MBL-A, MBL-C and CL-LK form tetramer, pentamer or hexamer bouquet structures (Nayak et al. 2012). CL-43 and CL-P1 exit as a monomers, whilst CL-K1 and CL-L1 form dimeric structures (Crouch et al. 1994; Holmskov et al. 1995; Lim and Holmskov 1996; Hansen and Holmskov 1998; Hansen et al. 2016; Paterson et al. 2019) (Fig. 2). CL-P1 also processes a transmembrane domain and an extended α-helical coiled-coil region (Hansen et al. 2016). A comparison of the coding regions of the genes reveals that four exons of similar size encode most of the collagen-like region (Hansen and Holmskov 2002). There are slight differences in size and number of the four exons among the collectins, accounting for some of the structural differences seen. For example, SP-D in human, rat and mouse have collagen regions of 177 amino acid residues in length, compared to bovine SP-D, CGN and CL-46 which have six less amino acid residues (Hansen and Holmskov 2002).

A comparison of the CRDs from the bovine collectins revealed conserved residues among all family members, with CL-43, Cl-46 and CGN clustering together with SP-D confirming their evolutionary ancestry (Fig. 3). CL-P1 was the most divergent of the family, as it has a number of domains (e.g., transmembrane/intracellular domain), showing its unique property of being a membrane bound collectin and possibly a scavenger receptor (Fig. 3) (Ohtani et al. 2001). A comparison of the CRD from CGN from several species of Bovidae revealed a hide degree of conservation in the domain, suggesting the importance of this collectin to ruminant immunobiology (Fig. 4). The CRD of *B. taurus* CGN clustered together with that from *B. bison* and *B. indicus* × *B.* taurus and are closely related (Fig. 4). It would be interesting to further investigate the properties of these closely related CGNs

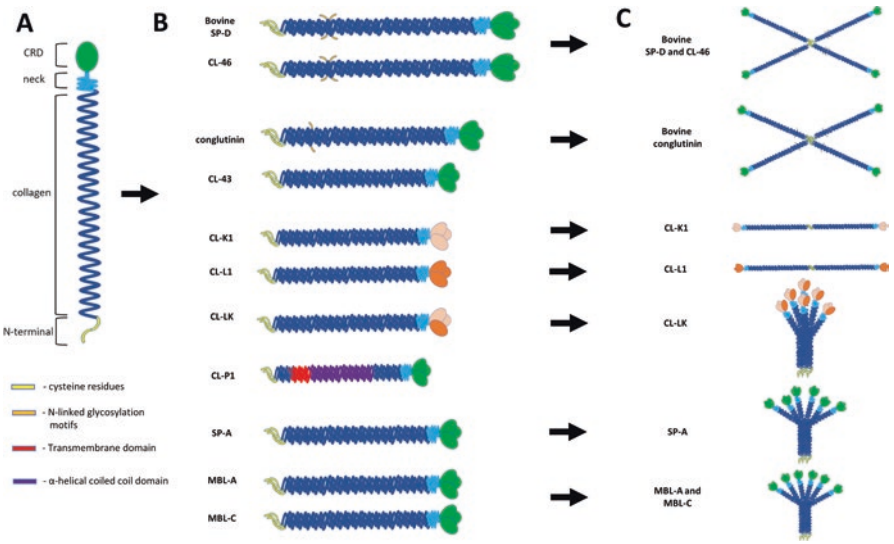

Fig. 2 Molecular structural characteristics of the bovine collectins. (**A**) Domain organization of collectin polypeptide monomer chain showing the carbohydrate recognition domain (CRD), coiled neck region, collagen domain and N-terminal domain. Each collectin has variations on this basic structure such as length and modifications. (**B**) Trimers of the bovine collectins are each built up by the association of three monomer polypeptide chains with the collagen regions intertwining to form a collagen triple helix. Individual collectins vary in length and modification (e.g., glycosylation). All the bovine collectins are soluble, except for CL-P1 which has a transmembrane/intracellular domain at its N-terminus and an α-helical coiled-coil domain. CL-LK is the only collectin to form a heterotrimer comprising of 2 CL-K1 and 1 CL-L1 monomers. (**C**) Collectins can form higher oligomeric structures. SP-D, CL-46 and conglutinin can form dodecamer cruciforms, comprising of four trimers. CL-K1 and CL-L1 are can form dimeric forms. MBL-A, MBL-C, SP-A and CL-LK can all from pentameric of hexameric bouquet-type structures

experimentally, since apart from cattle, the other Bovidae's CRDs that have been studied come from wild animals that have a diverse life-cycle and are not under managed breeding. Therefore, their innate immune markers, e.g., CGN may have undergone a natural selection process, altering the molecule's property, perhaps making it more effective. From the data, it appears that any changes to the CGN CRD from wild Bovidae are small and subtle, but this may enhance and widen the carbohydrate binding properties of CGN (Fig. 4).

Genetic Variation and Associations with Disease Susceptibility

Several insertion/deletion (InDels), single nucleotide polymorphisms (SNPs) and other genetic variations in the collectin genes are associated with susceptibility to infectious diseases in a number of species. However, in the bovine collectin genes, the nature and consequence of mutations has yet to be been explored fully, with only

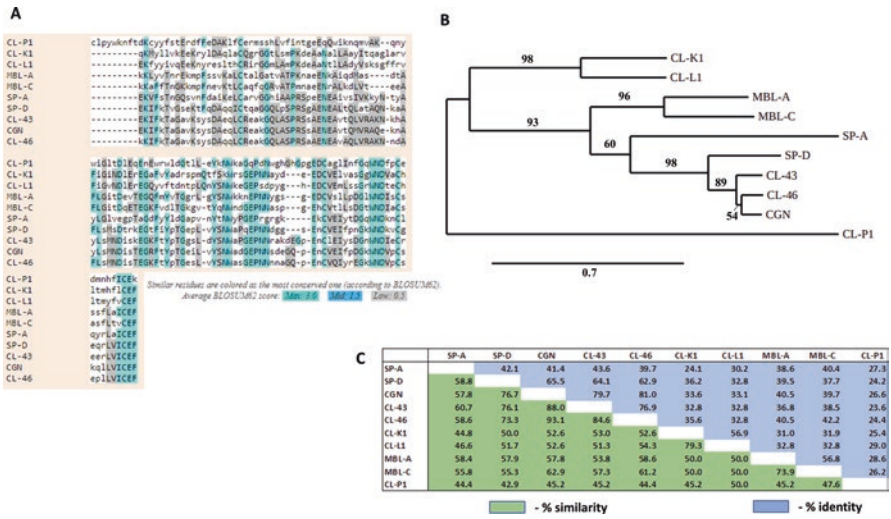

Fig. 3 Diversity and evolution of the CRD of the bovine collectins. (**A**) Protein sequences for the carbohydrate recognition domain (CRD) of each bovine collectin was selected (using the NCBI annotation positions) from amino acid motif EKIF to the CEF motif at the C-terminus. Sequences were aligned on the phylogeny.fr site (www.phylogeny.fr) using MUSCLE, with GBlocks curation. Conserved residues are shown in capitals and colours. (**B**) Phylogenetic tree of the CRD of the bovine collectins was done on the phylogeny.fr site and was based on the BioNJ algorthim with the Jones-Taylor-Thornton matrix with a gamma-distribution parameter of $a = 2.09$ (based on 118 amino acid sites). Numbers on the branches are percentages of 1000 bootstrap samples supporting a given branch. (Jones et al. 1992; Edgar 2004; Dereeper et al. 2008; Fraser et al. 2018). (**C**) Percentage similarity and identity matrices for bovine collectin CRDs were compiled using MatGAT (Campanella et al. 2003). Protein accession numbers are as follows: SP-A (NP_001071306.2), SP-D (NP_851369.1); CGN (NP_783630.2); CL-43 (NP_001002237.1); CL-46 (NP_001001856.1); CL-K1 (NP_001069771.1); CL-L1 (NP_001192642.1); CL-P1 (NP_001095313.1); MBL-A (NP_001010994.1) and MBL-C (NP_776532.1)

a few studies mainly describing genetic variability in bovine MBL genes. Mutations in the bovine MBL genes are associated with mastitis and milk production (Wang et al. 2011, 2012). Three SNPs were identified in the MBL1 from Chinese native cattle that were linked to mastitis resistance and increased milk production (Wang et al. 2011). Another study identified four SNPs in exon 1 of MBL2 in Chinese Holstein cattle and Luxi yellow cattle linked to mastitis and altered complement activity (Wang et al. 2012). Mastitis is commonly measured from the somatic cell score (SCS), which analyses the inflammatory cell content of milk. Other studies have also found mutations in the MBL1 and MBL2 genes of cattle and their links to SCS and complement activity (Liu et al. 2011; Zhao et al. 2012; Yuan et al. 2013). In other animals, mutations in the porcine MBL2 gene are linked with lower levels of MBL-C and are associated with pulmonary, gastro-intestinal infections and septicaemia (Lillie et al. 2007), whilst a SNP in porcine MBL1 is linked with lower levels of MBL-A (Juul-Madsen et al. 2011). Interestingly, polymorphisms at the MBL2 locus of water buffalo (*Bubalus bubalis*) have been reported to correlate with

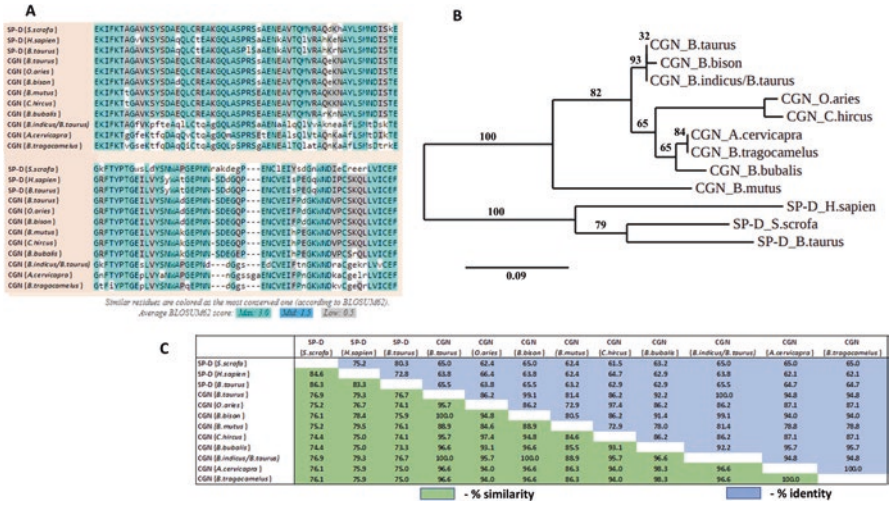

Fig. 4 Diversity and evolution of the CRD of conglutinin from different Bovidae species. (**a**) Protein sequences for the carbohydrate recognition domain (CRD) of each conglutinin collectin was selected (using the NCBI annotation positions) from amino acid motif EKIF to the CEF motif at the C-terminus. Sequences were aligned on the phylogeny.fr site (www.phylogeny.fr) using MUSCLE, with GBlocks curation. Conserved residues are shown in capitals and colours. (**b**) Phylogenetic tree of the CRD of the bovine collectins was done on the phylogeny.fr site and was based on the BioNJ algorthim with the Jones-Taylor-Thornton matrix with a gamma-distribution parameter of $a = 2.09$ (based on 118 amino acid sites). Numbers on the branches are percentages of 1000 bootstrap samples supporting a given branch (Jones et al. 1992; Edgar 2004; Dereeper et al. 2008; Fraser et al. 2018). (**c**) Percentage similarity and identity matrices for conglutinin CRDs were compiled using MatGAT (Campanella et al. 2003). Protein accession numbers are as follows: SP-D (S. scrofa) (NP_999275.1); SP-D (H. sapien) (NP_003010.4); SP-D (B. taurus) (NP_851369.1); CGN (B. taurus) (NP_783630.2); CGN (O. aries) (AFH75399.1); CGN (B. bison bison) (XP_010832460.1); CGN (B. mutus) (ELR45583.1); CGN (C. hircus) (XP_005699317.2); CGN (B. bubalis) (XP_006064838.1); CGN (B. indicus × B. taurus) (XP_027386536.1); CGN (A. cervicapra) (AGK36277.1); CGN (B. tragocamelus) (ADU25247.1)

resistance to *Brucella abortus* infection and susceptibility (Capparelli et al. 2008). In the porcine host, three SNPs within gene MBL1 were also found to correlate with disease susceptibility (Lillie et al. 2006b). Furthermore, in humans, MBL2 SNPs have been also been observed to increase susceptibility to several bacterial, viral, and parasitic infections, as well as ischemia–reperfusion injury, hepatitis, cystic fibrosis and autoimmune conditions such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Eisen and Minchinton 2003; Holmskov et al. 2003; Takahashi et al. 2005). In cattle, polymorphisms in the bovine MBL or other collectin genes and susceptibility to bovine tuberculosis is unknown, but studies in human tuberculosis are intriguing. Normal or increased levels of MBL seem to be associated with common infection with *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Garred et al. 1994, 1997), whilst approximately 30% of healthy persons have mutations associated to MBL deficiency and are associated

with susceptibility to tuberculosis and other inflammatory conditions in some ethnicities (Takahashi and Ezekowitz 2005; Thiel et al. 2006; Goyal et al. 2016).

Further insight into the consequences of genetic variability in the bovine collectins was provided in a recent study utilising next-generation sequencing (NGS) to analyse genetic variations in bovine collectins and ficolins from 120 cattle, 80 of which had infection and 40 that were apparently healthy (Fraser et al. 2018). This study found short nucleotide variants (SNVs) in the bovine collectin genes, with 74 variants that were associated with infectious disease (Fraser et al. 2018). Most of these genetic variants seem to be clustered in a 29-KB region upstream of the collectin locus on bovine chromosome 28 (Fraser et al. 2018). Using computational analysis, this study also predicted SNVs in the exons, introns, putative transcription binding factor sites that were likely to alter expression, structure and function of some collectin proteins, revealing potential susceptibility markers for bovine infections (Fraser et al. 2018). Potential deleterious mutations resulting in the following amino acid changes were identified in collagen-like domains of MBL2 (Pro42Gln), SFTPD (Pro132Ser), CGN (Arg173His), CL46 (Pro185Leu) and in CL43 (Thr117Ala and Gln185His), and in the CRDs of CGN (Glu302Asp) and SFTPD (Ala288Gly) (Fraser et al. 2018). The significance of these mutations has yet to be determined experimentally, but all these collectins have been associated with infectious disease susceptibility. For example, low plasma level of CGN has been implicated in increased risk of respiratory infection in cattle (Holmskov et al. 1998), whilst elevated level of CL-46 in calves has been found to be associated with resistance to parasitic infection (Li et al. 2011) as well as the associations of MBL mutations with mastitis (mentioned above). The concentration of genetic variation upstream of the collectin locus on chromosome 28 could also be indicative of important regulatory elements that control the expression of these collectins (Fraser et al. 2018). The above findings show the potential of expanding these genome-wide association studies on the bovine collectin genes to gain further insights into the genetic susceptibility to the major infectious diseases (e.g., bovine tuberculosis, RBS and mastitis) among different cattle breads.

Tissue Distribution, Physiological Role, and Protection Against Antimicrobial Infection

The ability of collectins to distinguish between self and non-self is dependent on the types of sugar moieties on glycoconjugates on the cell surface. These are essentially either monosaccharide or disaccharide in nature. Collectin CRDs generally favour binding of mannose-like ligands or L-fucose (Iobst and Drickamer 1994). SP-D prefers maltose, whilst CGN and CL-46 prefer N-acetylglucosamine and CL-43 prefers D-mannose (Lu et al. 1992; Holmskov et al. 1993; Hansen et al. 2002a; Paterson et al. 2019). These sugars are commonly found on microbial surfaces but are rare in self-glycoproteins (Drickamer and Taylor 1998). Moreover, microbial

targeting and clearance is facilitated by some collectins (e.g., SP-D, CGN, CL-46) higher order multimeric forms (e.g., cruciform) which mediate bridging interactions between different ligands, as opposed to trimeric subunit forms (e.g., SP-A, MBL and CL-43) (Hartshorn et al. 1993a). An overview of the literature to date is presented below on the bovine collectins and their roles in health and disease in the *Bovidae* or extrapolated from studies in other mammalian systems.

Surfactant Protein A and D

The composition of pulmonary surfactant consists of 90% phospholipid and 10% protein. This protein proportion contains four types of surfactant proteins (SP-A, SP-B, SP-C and SP-D). SP-B and SP-C are hydrophobic in nature and crucial for the physiological maintenance of the alveolar membrane surfaces. Conversely, SP-A and SP-D are hydrophilic proteins and have an important role in immunity in the lung. In mammals, SP-A and SP-D have been studies most extensively in human and mouse, but there are limited studies on the specific role they play in the Bovidae. We will review the general properties of SP-A and SP-D and what is currently known about their specific role in the Bovidae.

SP-A is mainly found in the alveoli and is synthesised by type II alveolar cells and the non-ciliated bronchial epithelial Clara cells (Voorhout et al. 1992; Nayak et al. 2012). Unlike SP-D, extrapulmonary expression of SP-A is limited to only a few tissues including in the human and rat small intestine the murine uterus, fetal membranes, human prostate, amniotic fluid, thymus and salivary gland (Van Iwaarden et al. 1990; Miyamura et al. 1994; Lin et al. 2001; Madsen et al. 2003) (Table 1). In bronchoalveolar lavage fluid (BALF) obtained from Holstein steers calves, the approximate SP-A concentration was 35 ng/mL whereas the serum concentration was 7.8 ng/mL (Eberhart et al. 2017).

SP-D is also predominately found in the pulmonary alveoli surfaces and is also synthesised by type II alveolar cells, non-ciliated bronchial epithelial Clara cells and serous cells of the trachea-bronchial glands (Crouch et al. 1992; Voorhout et al. 1992; Wong et al. 1996; Nayak et al. 2012). Unlike SP-A, SP-D is synthesised in a wide variety of tissues in mammals, including the trachea, placenta, prostate, small intestine brain, heart, kidneys, testis, salivary gland, and pancreas (Murugaiah et al. 2020) (Table 1). Low level of SP-D has also been detected in spleen, uterus, adrenal and mammary tissues (Fisher and Mason 1995; Madsen et al. 2000; Hogenkamp et al. 2007). SP-D is also present in the epithelial cells of various tissues (parotid, lacrimal and sweat glands, intra-hepatic bile ducts, gall bladder, esophagus, pancreas, urinary tract and fetal membranes (Miyamura et al. 1994; Madsen et al. 2000; Brauer et al. 2007). SP-D has been detected in alveolar macrophages, but is not synthesised in these cells, but rather internalize into the phagolysosomal compartment, where it is broken down (Dong and Wright 1998). Similar to the above mammalian studies, the expression of bovine SP-D has been observed in the lungs, mammary glands and gastrointestinal tract (Gjerstorff et al. 2004b). In humans, the

Table 1 Origin of bovine collecting and their tissue distribution

Collectin	Tissue of origin	Tissue of presentation	Comments
SP-A	Type II alveolar cells and the non-ciliated bronchial epithelial Clara cells	Alveolar space, mucosal surfaces, thymus? Uterus? prostate?	SP-A is limited to the lungs (alveoli), but there may be extrapulmonary expression of SP-A in other tissues in Bovidae which has been noted in other mammals. Role in antimicrobial innate immunity including opsonization, neutralization and clearance
		Amniotic fluid? salivary gland?	
SP-D	Type II alveolar cells, non-ciliated bronchial epithelial Clara cells and serous cells of the trachea, intestinal mucosa? thymus? prostate? mammary gland	Alveolar space, mucosal surfaces, semen, udder, trachea? placenta? prostate? small intestine? Serum?	SP-D expression has been detected in many non-pulmonary tissues in mammals and has been observed in the bovine lung and udder suggestion an important role in protection from mastitis
MBL	Liver and small intestine (rumen?) udder?	Serum	Bovidae have two different MBL proteins (A and C). Key antimicrobial collectin and has a broad role in innate immunity including opsonization, neutralization and complement activation
Conglutinin, CL-43 and CL-46	Liver	Serum	Unique to Bovidae and may have a role in antimicrobial defence and controlling general inflammatory response, e.g., in pneumonia, gastroenteritis and mastitis
CL-P1	Unknown in Bovidae. Placenta? Endothelial cells	Endothelial cells?	CL-P1 is the only membrane bound collectin, with some unique structural aspects. Role unknown in Bovidae but may be involved in complement activation
CL-K1	Unknown in Bovidae	Serum	Role unknown in Bovidae, but forms dimers and a heterotrimeric complex with CL-L1. Phylogenetically closely related to CL-L1
CL-L1	Unknown in Bovidae. Liver?	Unknown but ubiquitous in other mammals	Role unknown in Bovidae, but forms dimers and a heterotrimeric complex with CL-K1. Phylogenetically closely related to CL-K1. Possibly expressed in bovine liver and may be involved in embryonic development

concentration of SP-D in BALF ranges between 0.1 and 0.9 mg/mL and makes up about 0.5% of the protein content in the alveoli (Hartshorn et al. 1994; Honda et al. 1995). The serum concentration in healthy humans is approximately 66 ng/mL (Honda et al. 1995). In healthy calves, the mean BALF and serum concentration was determined to be 22 ng/mL and 9 ng/mL, respectively (Eberhart et al. 2017). The levels of SP-D in the bovine host seems to be significantly lower than in humans. It is not clear why this is the case but could be an indication of a greater role being played by other bovine collectins in the lungs (e.g., CGN).

Both SP-A and SP-D are well known to have anti-microbial properties and are significantly elevated in the mammalian infected lung and in response to allergens and hypoxia (Atochina et al. 2001; White et al. 2001; Kasper et al. 2002). The lung and serum concentrations of SP-A has not been shown to fluctuate significantly upon infection, particularly in bovine respiratory disease (BRD) (Eberhart et al. 2017). However, gross examination of cattle infected with tuberculosis showed increased immunostaining for SP-A in the type II pneumocytes surrounding the granulomas observed in the tissue (Beytut 2011). Similar findings were also reported for SP-A in the human lung infected with tuberculosis (Stanton et al. 2003). Both SP-A and SP-D bind to several microbes including viruses, bacteria, fungi and parasites and has been comprehensively reviewed recently (Murugaiah et al. 2020). SP-A and SP-D facilitate clearance of microbes by direct lysis, agglutination or neutralization and/or subsequent opsonization by phagocytes (Holmskov et al. 2003; Wu et al. 2003). SP-D gene knockout mice (SP-D$^{-/-}$) have elevated pulmonary macrophages and enhancement pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), inhibition of lymphocyte proliferation and reactive oxygen species (ROS) in the alveoli, suggesting that SP-D has an anti-inflammatory effect in lung immunity and infection (Botas et al. 1998; Korfhagen et al. 1998; Ikegami et al. 2000; Borron et al. 2002). SP-D is, therefore, likely to play a key role in regulating the local immune response in tissues that are particularly exposed to microbes by balancing the processes for microbial clearance with that of organisms, by a combination of enhancing the microbial elimination and reducing inflammation. This type of immune regulation is particularly key in the bovine udder, where control of mastitis is critical for the optimum stimulation of milk secretion pathways and high yielding milk production in dairy cattle. Bovine SP-D is expressed in the udder and has a higher expression than in the bovine lung, when compared to the of SP-D expression of human mammary tissue versus lung tissue (Madsen et al. 2000; Gjerstorff et al. 2004b). This suggests an important role for bovine SP-D in controlling infection in the udder. It is also intriguing that polymorphisms in the bovine SP-D gene have been found which may be linked with disease susceptibility (e.g., mastitis) (Gjerstorff et al. 2004b). Furthermore, in the bovine gastrointestinal system, regulation of the immune response against pathogens is also of key importance as ruminants need the symbiotic relationship with microbes for normal health. It is therefore likely that the bovine collectins (e.g., SP-D and CGN) play a key role in modulating the immune response against microbes in these tissues.

Numerous studies on the interaction of SP-A and SP-D with microbes have been reported in human and other mammals, but there are little specific studies in the

Bovidae (Murugaiah et al. 2020). However, studies on Gram-positive and Gram-negative bacteria, mycobacteria, and common respiratory viruses can be extrapolated to the Bovidae, where similar molecular mechanisms and outcomes may be seen.

SP-A and SP-D both bind to many Gram-negative and Gram-positive bacteria, particularly known respiratory pathogens, e.g., *Klebsiella pneumoniae, Pseudomonas aeruginosa*, mycobacteria, *Mycoplasma pneumoniae, Haemophilus influenzae, Legionella pneumophila* and *Streptococcus pneumoniae*, among others (Murugaiah et al. 2020). Several consequences from these interactions have been observed, including aggregation, enhanced uptake and clearance by phagocytes and even direct effects on bacterial growth (Pikaar et al. 1995; Murugaiah et al. 2020). SP-A and SP-D bind to different sites on the LPS ligand in Gram negative bacteria (SP-A: lipid A; SP-D: O-antigen and terminal oligosaccharides) (Kuan et al. 1992; Van Iwaarden et al. 1994). Furthermore, SP-A is also able to bind to capsular polysaccharides of *Klebsiella*, outer membrane protein (OMP) of *H. influenzae*, but does not bind to peptidoglycan (Mcneely and Coonrod 1994; Kabha et al. 1997; Murakami et al. 2002). SP-A also aggregates and enhances phagocytosis of *H. influenzae* and *K. pneumoniae* by macrophages (Mcneely and Coonrod 1994; Kabha et al. 1997). SP-D also binds to cell membrane lipids of *M. pneumoniae* (Chiba et al. 2002). SP-A and SP-D can also inhibit the growth of *M. pneumoniae*, *Escherichia coli*, *K. pneumoniae*, *Enterobacter aerogenes* and *P. aeruginosa* by compromising bacterial membrane integrity, increasing permeability or inhibiting metabolic pathways (Van Iwaarden et al. 1994; Wu et al. 2003; Piboonpocanun et al. 2005).

Of particular interest is respiratory infections with mycobacteria, where bovine tuberculosis continues to be a severe cause of morbidity and mortality of cattle in the UK and worldwide (Miles 2009; Abernethy et al. 2013; APHA 2020). The interaction of mycobacteria with bovine SP-A and SP-D has not been characterised, but there are numerous studies investigating these surfactant proteins in human and rodents infected with *M. tuberculosis* and *M. bovis* BCG bacilli. SP-A can binds to *M. tuberculosis* Apa glycoprotein on its surface (Ragas et al. 2007), whilst SP-D binds to Gram-positive ligands lipoteichoic acid, peptidoglycan and mycobacterial lipoarabinomannan (LAM) (Ferguson et al. 1999; Van De Wetering et al. 2001; Kudo et al. 2004). Both SP-A and SP-D can bind and agglutinate *M. tuberculosis*, but interestingly, they have opposing downstream effects by macrophages. SP-A enhances phagocytosis by macrophages mediated by upregulation of expression of the mycobacterial receptor, mannose receptor on the macrophage (Beharka et al. 2002). In fact, both SP-A and SP-D can facilitate phagocytosis without the need for direct microbial binding, by enhancing the expression of such cell surface phagocytic receptors on the macrophage (Beharka et al. 2002; Kudo et al. 2004). In contrast, SP-D inhibits phagocytosis by macrophages by blocking the interaction of LAM with mannose receptor (Ferguson et al. 1999, 2002). Binding of CGN to *M. bovis* BCG also results in inhibition of phagocytosis by macrophages, and thus, a similar mechanism has been proposed (Mehmood et al. 2019). However, SP-A$^{-/-}$, SP-D$^{-/-}$, and SP-A/D$^{-/-}$ knockout mice infected with *M. tuberculosis* could still

clear infection, questioning the relevance of the rodent model to human and bovine tuberculosis (Lemos et al. 2011).

Both SP-A and SP-D can also modulate the downstream adaptive response against pathogens. SP-A inhibits TNF-α secretion, via a toll-like receptor 2/NF-κB mediated mechanism (Murakami et al. 2002). SP-A and SP-D can also modulate the phagolysosome by promoting reactive oxygen and nitrogen intermediates enhancing intracellular killing, particularly in the case mycobacterial infection. SP-A enhances the killing of intracellular *M. bovis* BCG by increasing nitric oxide (NO) production and enhancing the pro-inflammatory cytokines, e.g., TNF-α (Weikert et al. 2000). But curiously, in alveolar macrophages primed with IFN-γ and infected by mycobacteria, SP-A decreased intracellular NO by inhibiting TNF-α secretion and nuclear factor-kappa B (NF-κB) activation (Pasula et al. 1999; Hussain et al. 2003). SP-A and SP-D can also directly bind to cell surface PRRs, such as Toll-like receptors (TLRs) and CD14, influencing the inflammatory response (Borron et al. 2000; Sano et al. 2000; Murakami et al. 2002). In tuberculosis, SP-A has been shown to promote both inflammation in the presence of infection and suppresses inflammation in uninfected macrophages, and thus probably controlling the inflammatory response against host-mediated tissue damage in the lung (Gold et al. 2004).

SP-A and SP-D can also bind and influence the outcome of viral infection enhancing viral phagocytosis and neutralisation (Murugaiah et al. 2020). Bovine SP-D is able to bind to bovine rotaviruses (Nebraska calf diarrhoea virus strain), targeting the VP7 glycoprotein (Reading et al. 1998). Bovine SP-D was also able to inhibit hemagglutination of rotavirus and neutralise infectivity; neutralization of rotavirus by bovine SP-D was dependent on the glycosylation of VP7 (Reading et al. 1998). Studies in animal models has also shown the importance of collectins in protection against viral infection. In studies with SP-A$^{-/-}$ and SP-D$^{-/-}$ knockout mice that were infected with Influenza A virus (IAV), both collectins are protective, but this is also dependent on the glycosylation of hemagglutinin (HA) and neuraminidase (NA) antigens (Levine and Whitsett 2001; Levine et al. 2002; Hawgood et al. 2004). Furthermore, in mice, it seems SP-D plays a greater role in IAV protection as it enhances the clearance of infection, whereas SP-A does not (Levine et al. 2001; Hawgood et al. 2004). SP-A binds to IAV mannose residues on HA and NA resulting in viral neutralisation and inhibition of release of viral particles from infected cells (Malhotra et al. 1994; Benne et al. 1995). SP-D also strongly inhibits IAV hemagglutination and promotes viral aggregation and neutralisation (Hartshorn et al. 1994). SP-A and SP-D also inhibit NA enzymatic activity, but this is much stronger with SP-D, but these interactions also seem to be dependent on the viral strain (Reading et al. 1997; Tecle et al. 2007; Job et al. 2010). Recombinant truncated forms of SP-A (rfhSP-A) and SP-D (rfhSP-D), composed of the α-helical neck and CRD domains have also been studied in IAV infection. rfhSP-A promotes IAV infection, replication and enhancement of the pro-inflammatory response, whereas full-length SP-A inhibits IAV infection and dampens the pro-inflammatory response, demonstrating that the full-length SP-A molecule is required for IAV protection (Al-Qahtani et al. 2019). In a similar study, rfhSP-D-was able to inhibit IAV entry, down-regulate key viral factors (M1) and dampen the pro-inflammatory

response (Al-Ahdal et al. 2018). These studies using rfhSP-A and rfhSP-D have provided greater understanding of the IAV infection process and the possible application of rfhSP-D in anti-viral therapy. SP-D also enhances IAV uptake by neutrophils and virus-induced respiratory burst, but SP-A (and mucins and gp-340) inhibit this effect, reducing the ability of SP-D to facilitate neutrophil viral clearance (White et al. 2001). SP-A is also able to bind to herpes viruses (Herpes Simplex virus type 1 (HSV-1), Cytomegalovirus), enhancing phagocytosis of the virus by macrophages (Van Iwaarden et al. 1991, 1992; Weyer et al. 2000). SP-D activity against herpesviruses has not been reported. SP-A and SP-D are also able to bind to Respiratory Syncytial virus (RSV) and are important for neutralising infectivity and enhancing viral clearance (Ghildyal et al. 1999; Hickling et al. 1999; Levine et al. 1999; Sano et al. 1999, 2000). SP-D is able to bind to glycosyl moieties on viral spike protein (S-protein) of the severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV). Increased serum levels of SP-D have also been reported in SARS patients (Leth-Larsen et al. 2007; Wu et al. 2009). Furthermore, both SP-A and SP-D bind to other coronavirus strains (HCoV-229E) and inhibit viral infection of human bronchial epithelial (16HBE) (Funk et al. 2012). It is unknown whether these collectins have any effect on novel coronavirus SARS-CoV-2, which is responsible for the COVID-19 pandemic, but the above data seems to suggest that SP-A and SP-D may play a protective role. In another recent emerging viral disease, SP-D has been shown to bind Ebola virus glycoprotein and enhance viral infection, suggesting a role in pathogenesis (Favier et al. 2018). In mice, SP-D has been observed to have a protective role against Vaccinia virus infection (Perino et al. 2013). SP-A has also been reported to enhance clearance of adenovirus infection in the lungs while dampening the immune response (Harrod et al. 1999).

Several studies have reported interesting interactions of SP-A and SP-D with Human Immunodeficiency virus-1 (HIV-1). SP-A and SP-D bind to the HIV-1 gp120 viral glycoprotein and inhibit direct viral infection of $CD4^+$ T cells (Meschi et al. 2005; Gaiha et al. 2008), but in dendritic cells (DCs), SP-A has the opposite effect, increasing HIV infectivity, and thus facilitating transfer of the virus from DC to $CD4^+$ T cells (Gaiha et al. 2008). The truncated rfhSP-D is also able to bind to HIV-1 gp120 and prevent infection of Jurkat T cells, U937 monocytic cells and PBMCs, but importantly also suppresses the viral-induced cytokine response from these cells (Pandit et al. 2014). In a follow-up study, rfhSP-D was also found to bind to dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) receptor, modulating the binding of HIV-1 and its transfer to $CD4^+$ T cells (Dodagatta-Marri et al. 2017). rfhSP-D has also been reported to block infection of HIV-1 across the vaginal epithelium (Pandit et al. 2014). As reported above with studies in IAV, rfhSP-D has a potential prophylactic and therapeutic potential against HIV-1 infection.

There have also been several studies showing the anti-fungal activity of SP-A and SP-D (Murugaiah et al. 2020). To date, the bovine SP-A and SP-D have not been shown to interact with fungal pathogens that infect the Bovidae. There is considerable scope here for further investigation as several fungal species (e.g., Candida and Aspergillus) are responsible for mastitis, abortion, otitis externa,

gastrointestinal infections, and pneumonia (Seyedmousavi et al. 2018). In particular, a common fungal disease in cattle is Ringworm caused by *Trichophyton verrucosum*, but no studies have been conducted on its possible interaction with collectins. An overview of SP-A and SP-D antifungal activity in other mammals will be presented and extrapolated to Bovidae where appropriate.

Both SP-A and SP-D can bind to the conidia of *Aspergillus fumigatus* and inhibit conidia infectivity and promote enhancement of phagocytosis and intracellular killing by neutrophils and alveolar macrophages (Madan et al. 1997). SP-A and SP-D can also inhibit the growth of *Histoplasma capsulatum*, but no effect is seen on aggregation or phagocytosis (Mccormack et al. 2003). SP-A has also been reported to bind to *Cryptococcus neoformans* (both encapsulated and non- encapsulated yeast form), but no enhancement of phagocytosis of the acapsular form was observed either (Walenkamp et al. 1999). SP-A and SP-D can also bind to *Coccidioides posadasii* and the fungus is also able to inhibit the expression of SP-A and SP-D in the lungs, possibly facilitating pathogenesis (Awasthi et al. 2004). SP-D can bind to *Candida albicans*, agglutinating the fungus and directly inhibiting its growth (Van Rozendaal et al. 2000; Awasthi et al. 2004), whilst SP-A is also able to bind to *C. albicans* and interfere with its attachment to alveolar macrophages, inhibiting phagocytosis and the pro-inflammatory response (Rosseau et al. 1997). This may be relevant in Bovidae, as candidiasis is reported in several conditions in ruminants (Seyedmousavi et al. 2018). SP-D also binds to *Saccharomyces cerevisiae* (Allen et al. 2001a, b). The opportunistic fungus Pneumocystis can infect several mammals in a species-specific manner and has been reported in calves and sheep (Settnes and Henriksen 1989). The interaction of bovine SP-A and SP-D with *Pneumocystis* in the Bovidae has not been reported, but studies in other mammalian species suggests they may play an important role in this opportunistic infection. Both SP-A and SP-D are able to bind to *Pneumocystis* via its major surface glycoprotein, resulting in aggregation and attachment to the alveolar epithelium in Pneumocystis pneumonia, but this does not increase its phagocytosis (Zimmerman et al. 1992; O'Riordan et al. 1995; Mccormack et al. 1997a, b; Vuk-Pavlovic et al. 2001). SP-D mediated aggregation of *Pneumocystis* may inhibit phagocytosis by macrophages and facilitate persistence of the fungus within the lung (Vuk-Pavlovic et al. 2001). In pneumocystis pneumonia, there is also an increase in the expression of SP-A and SP-D in the lungs (Phelps et al. 1996; Aliouat et al. 1998; Atochina et al. 2001; Qu et al. 2001). SP-A enhances attachment of *Pneumocystis* to rat alveolar macrophages *in vitro* (Williams et al. 1996) but reduces phagocytosis of the fungus in human alveolar macrophages *in vitro* (Koziel et al. 1998). Thus, enhanced levels of SP-A in *Pneumocystis* pneumonia may facilitate pathogenesis, whilst SP-D may contribute to fungal clearance.

Parasitic infections are also fairly common in cattle and other Bovidae species. This can have implications on general health, reproduction, growth, productivity of meat and milk and may result in death in severe infections. Parasitic diseases of cattle are commonly caused by protozoa, helminths (roundworms, tapeworms and flukes). To date, very little has been reported on the interaction of bovine collectins and parasitic infections of cattle. Some of these diseases are also important zoonotic

infections (Mcdaniel et al. 2014), so further studies are needed to determine whether collectins may play a role in bovine parasitic infection and transmission to humans. In general, there are also a limited number of functional studies on the role of SP-A and SP-D in protozoal and helminth infections in other mammalian species. In mice, it has been reported that there are increased serum levels of SP-D in renal and cerebral tissues, in animals experimentally infected with *Plasmodium berghei*, compared to controls (Cahayani et al. 2016). SP-D has also been reported to bind to the surface of *Schistosoma mansoni* larval stages, but it remains unknown what effect this has on the parasite (Van De Wetering et al. 2004a, b). However, SP-D may be essential for protection against helminth infection, since a study using an experimental model system showed that *Nippostrongylus brasiliensis*-infection of SP-D$^{-/-}$ knockout mice caused severe susceptibility to parasitic disease, whilst treatment with rfhSP-D enhanced parasite clearance and anti-parasitic immune responses (Thawer et al. 2016). SP-D was also able to directly bind to *N. brasiliensis* larvae and enhance its killing by alveolar macrophages (Thawer et al. 2016).

In ruminants, serum collectins such as SP-D and CGN may likely play a role in gastrointestinal and systemic infections from parasites. However, specific data on the involvement of collectins and parasitic infections is lacking, but gauging from studies in other mammals, much could be learned.

Mannan-Binding Lectin

Bovidae, as in most mammals, have two MBL genes (MBL1 and MBL2), which encode the MBL-A and MBL-C proteins, respectively (Loveless et al. 1989; Gjerstorff et al. 2004a). In mammals, MBL-A and MBL-C are serum proteins that are mainly synthesised by the liver and small intestine (Ezekowitz et al. 1988; Sastry et al. 1991; Hansen et al. 2000) (Table 1). In mice, mRNA expression of MBL genes was also detected in various other tissues (Shushimita et al. 2015), and this may also be these case for Bovidae, particularly the rumen and udder. The normal serum concentrations of bovine MBL-A and MBL-B have not been clearly defined but are estimated to be 0.8–7.4 μg/mL (Wang et al. 2012). MBL is a key anti-microbial collectin and has a broad role in innate immunity including opsonization, neutralization and complement activation (Van De Wetering et al. 2004a). The CRD of MBL selectively targets microbes by binding to cell-surface to mannose and N-acetylglucosamine residues and subsequent activation of MBL-associated serine proteases (MASPs) 1 and 2, leading to complement activation via the Lectin pathway, inducing phagocytic and inflammatory responses (Thiel et al. 1997; Matsushita et al. 2001; Sorensen et al. 2005).

Mastitis is a major disease of dairy cattle and is characterised by inflammation of the mammary gland brought on by infection of the udder. This decreases the quality and quantity of milk production, reduction in lactation, and in severe cases, early culling, resulting in a significant economic burden (Miller et al. 1993; Seegers et al. 2003). The main pathogens involved in mastitis are bacteria such as *E. coli*,

Staphylococcus aureus, *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Streptococcus agalactiaeare*, although some fungi may also rarely be involved. Host resistance to mastitis is a known trend and there has been a concerted effort to identify immune response genes that may play a role in this and will be favourable traits for selective breeding (Oviedo-Boyso et al. 2007; Swanson et al. 2009). The MBL genes have been implicated as playing a probable role in mastitis susceptibility in cattle. Furthermore, In pigs and mice, MBL-A is able to bind target bacteria (Lillie et al. 2006a, b; Phaneuf et al. 2007). Mutations in both MBL1 and MBL2 genes are also suggestive of susceptibility of animals to infection (Shi et al. 2004; Lillie et al. 2005, 2007; Capparelli et al. 2008), leading to lower serum levels of MBL and likely compromising innate immune responses (Madsen et al. 1995; Capparelli et al. 2008).

There is little data on the specific activity of bovine MBL-A and MBL-C proteins, but much can be extrapolated from studies of these MBL proteins in other mammals. In addition to complement-dependent activity, MBL also processes complement-independent functions with the ability to inhibit bacterial adhesion (Jack et al. 2005). MBL also acts as an opsonin to enhance bacterial uptake (Kuhlman et al. 1989; Polotsky et al. 1997; Jack et al. 2005). There also seems to be a spectrum of binding of MBL to various bacterial species, with potent binding observed with *S. aureus, Streptococcus pyogenes, Listeria monocytogenes* and non-encapsulated *Neisseria meningitidis* (Levitz et al. 1993; Van Emmerik et al. 1994; Neth et al. 2000), moderate binding observed in *E. coli*, *Haemophilus influenzae* and *Klebsiella* species, and no binding with *Pseudomonas aeruginosa*, *Enterococcus* species and *Streptococcus pneumoniae* (Levitz et al. 1993; Van Emmerik et al. 1994; Neth et al. 2000). Some bacteria have evolved mechanisms to overcome MBL binding, through capsule modifications and sialylation of LPS (Jack et al. 2005; Krarup et al. 2005). MBL is able to bind to several Gram-positive and Gram-negative bacterial ligands, including LPS (*E. coli*, *Salmonella*), peptidoglycan, teichoic acid and lipoteichoic acid (*S. aureus*) (Kuhlman et al. 1989; Polotsky et al. 1996; Nadesalingam et al. 2005a, b; Kurokawa et al. 2016), LAM (*M. avium*) (Polotsky et al. 1997), and mannosylated lipoarabinomannan (ManLAM) (*M. tuberculosis*, *M. bovis*, *Mycobacterium kansasii*, *Mycobacterium gordonae* and *Mycobacterium smegmatis*) (Bartlomiejczyk et al. 2014), and Ag85 (*M. tuberculosis*) (Swierzko et al. 2016). MBL, through opsonization enhances uptake and intracellular killing of *Salmonella* by phagocytes (Kuhlman et al. 1989) and can also inhibit flagellar function, impairing motility which is an important virulence factor (Xu et al. 2016). MBL can also enhance the phagocytosis of mycobacteria and *N. meningitidis* by phagocytes (Polotsky et al. 1997; Jack et al. 2001). MBL can interact with TLR2 in detecting *S. aureus,* and thus, modulate inflammation (Nauta et al. 2003; Ip et al. 2008). All of the above studies suggest that the bovine MBL proteins are likely to have similar activity against these common environmental bacterial pathogens.

There are no reports of activity between bovine MBL and bovine viral pathogens. Undoubtedly, MBL proteins will be involved since that there is considerable data of MBL activity on viral pathogens in other mammalian systems. It would be

particularly prudent to study collectin activity with important viral pathogens of cattle, e.g., those involved in bovine respiratory disease (RBD), such as infectious bovine rhinotracheitis virus (type 1 bovine herpesvirus (BHV1)), parainfluenza virus type 3 (PI3), bovine respiratory syncytial virus (BRSV), as well as bovine viral diarrhoea virus (BVDV), or blue-tongue disease or foot and mouth disease. Nevertheless, MBL has been shown to interact with several human viral pathogens including IAV, where binding of MBL results in the inhibition of viral infectivity (Hartley et al. 1992; Hartshorn et al. 1993b; Reading et al. 1995, 1997). MBL binds to IAV HA and NA antigens (Kase et al. 1999). MBL is able to neutralise HIV-1 *in vitro* targeting gp120 and gp41, and target HIV-infected $CD4^+$ T cells and monocytes and inhibiting reverse transcriptase activity (Ezekowitz et al. 1989; Saifuddin et al. 2000; Teodorof et al. 2014). MBL can activate complement upon binding to gp120 (Haurum et al. 1993). MBL is also able to bind to hepatitis B virus, hepatitis C virus, Ebola virus, flaviviruses such as Dengue and West Nile virus and herpes simplex virus type 1 (Gadjeva et al. 2004; Ji et al. 2005; Brown et al. 2007, 2010; Avirutnan et al. 2011; Fuchs et al. 2011).

Bovine MBL proteins have not been reported to have activity against bovine fungal pathogens. However, there are numerous studies of MBL proteins in other mammals having a significant role in infection and pathogenesis in several relevant fungal pathogens to the bovine host. These include most prominently *Aspergillus* and *Candida* species. MBL is able to bind to *A. fumigatus* (Neth et al. 2000), *B. dermatitidis* (Koneti et al. 2008), *C. albicans* (Kitz et al. 1992; Neth et al. 2000; Ip and Lau 2004; Van Asbeck et al. 2008), *C. parapsilosis* (Van Asbeck et al. 2008), *Pneumocystis* and *C. neoformans* (Chaka et al. 1997; Van Asbeck et al. 2008). MBL binding has been shown to result in fungal aggregation, enhancement of phagocytosis, and complement deposition (Ip and Lau 2004; Kaur et al. 2007; Van Asbeck et al. 2008).

Bovine collectins have not been explored with respect to protozoal and helminth pathogens. Apart from some bovine diseases, Bovidae species can also be reservoirs for zoonotic infections to humans. However, studies are summarised below on MBL from other mammalian species. MBL can bind to the malarial parasite *Plasmodium falciparum* and can activate complement lectin pathway (Klabunde et al. 2002; Garred et al. 2003; Korir et al. 2014). MBL also binds to *Trypanosoma cruzi* and *Leishmania* parasites (Green et al. 1994; Ambrosio and De Messias-Reason 2005; Cestari Idos et al. 2009). In helminth infections, MBL binds to *Schistosoma mansoni* cercariae as well as adult worms and is able to activate the lectin pathway and subsequent complement cascade (Klabunde et al. 2000).

Conglutinin

Conglutinin (CGN) was the first collectin to be discovered in mammals and is uniquely present in Bovidae (Ehrlich and Sachs 1902; Davis 3rd and Lachmann 1984; Hansen and Holmskov 2002). Although initially found in cattle (*Bos taurus*),

CGN has also recently been identified in goat, sheep and other grazing members of the Bovidae genus (Dec et al. 2011). CGN has closely evolved from bovine SP-D, as indicated by its similarity in structure, which facilitates its ability to bind to microbial surface glycoconjugates (N-acetylglucosamine, mannose and fucose) in a Ca^{2+} dependent manner (Loveless et al. 1989; Reid and Turner 1994; Hansen and Holmskov 2002). CGN is predominantly found in the bovine serum and is primarily secreted by the liver at an approximate concentration of 12 μg/mL (Lu et al. 1993a; Holmskov et al. 1998). It is unknown whether CGN is produced locally, e.g., bovine mucosa, but CGN has been detected in splenic, tonsillar, lymphatic and thymic macrophages and dendritic cells (Holmskov et al. 1992) (Table 1). CGN serum levels are heritable, with low calf mortality correlating with high maternal CGN serum levels (Holmskov et al. 1998). CGN has been observed to have anti-microbial properties, but its exact role in the bovine host remains to be properly elucidated. Acute infections, such as pneumonia and metritis are linked with low serum levels of CGN, suggesting predisposition to infection (Ingram and Mitchell 1971; Holmskov et al. 1998). CGN enhances the respiratory burst and reactive oxygen species in bovine granulocytes facilitating enhanced phagocytosis (Dec et al. 2012). Direct binding of CGN to bacterial and viral pathogens has been observed. CGN binds to *Escherichia coli* and *Salmonella typhimurium* (Friis-Christiansen et al. 1990; Friis et al. 1991) and to Gram-negative ligands LPS and peptidoglycan (Wang et al. 1995). CGN has also recently been shown to bind to *Mycobacterium bovis* BCG and other Gram-positive bacteria (Mehmood et al. 2019). CGN can also interact with components of the complement system. CGN binds to C1q receptor and can inhibit the dissociation of Bb from the C3-convertase C3bBb (Malhotra et al. 1990; Tabel 1996). CGN has the unique property of being able to bind iC3b, targeting mannose residues on the iC3b α-chain, but not C3, C3b or C3c (Laursen et al. 1994). Thus, CGN is able to target iC3b bound on cells, resulting in agglutination, such as iC3b-bound erythrocytes (Lachmann and Muller-Eberhard 1968). Furthermore, CGN is able to target and bind to iC3b-bound *E. coli* and facilitate enhancement of the respiratory burst of phagocytes (Friis et al. 1991). CGN is a key component of bactericidal activity in bovine serum (Ingram 1982), and *in vivo*, CGN is able to protect mice experimentally infected with *S. typhimurium* (Friis-Christiansen et al. 1990). In the bovine host, CGN serum levels are lowered during infection, as demonstrated by CGN consumption during *Babesia bovis* infection (Goodger et al. 1981; Ingram 1982). A recombinant truncated fragment of CGN (composed of α-helical neck and the CRD regions only) is able to bind to *M. bovis* BCG and inhibit its uptake by macrophages, with and without prior complement deposition, altering the macrophage inflammatory response (Mehmood et al. 2019). This CGN recombinant fragment also showed bacteriostatic and bactericidal activity against of M. bovis BCG (Mehmood et al. 2019). Thus, CGN seems to target the mycobacterium in two distinct ways: (1) via bacterial lipoarabinomannan (LAM), and (2) in the presence of complement deposition, iC3b (Mehmood et al. 2019). Furthermore, CGN inhibition of mycobacterial uptake by macrophages is probably the result of (1) blocking interaction of mycobacterial LAM with macrophage mannose receptor, and (2) blocking iC3b interaction with macrophage complement

receptors CR3 and CR4 (Mehmood et al. 2019). This study shows important complement-dependent and complement-independent mechanisms of CGN and its involvement in the key interface of host-pathogen interactions in mycobacterial infection and could provide novel avenues for study in bovine tuberculosis, a major infection of cattle in the UK.

CGN has been shown to bind to viral pathogens. CGN binds to the HIV-1 glycoprotein gp160 and inhibits its interaction with the host cell CD4 receptor (Andersen et al. 1991). CGN also acts as an opsonin for IAV, binding to viral hemagglutinin causing viral aggregation and enhanced viral uptake by phagocytes (Hartshorn et al. 1993a). CGN has been shown to inhibit IAV hemagglutination and infectivity and inhibit IAV-mediated neutrophil suppression whilst conversely enhancing neutrophil respiratory burst responses to the virus (Hartshorn et al. 1993a). Interestingly, the CRD of CGN, CL-46 and CL-43 have a greater innate antiviral activity against IAV than human SP-D CRD (Hartshorn et al. 2010a). Moreover, CGN was shown to have a higher inhibitory effect against IAV, than bovine SP-D or CL-43 (Reading et al. 1998). CGN can bind to herpes simplex virus type 2 (HSV-2) and mediate enhancement of infection in mice (Fischer et al. 1994). CGN has been shown to inhibit hemagglutination and infectivity of Newcastle disease virus (Reading et al. 1998), whilst a recombinant truncated fragment of sheep (*Ovis aries*) conglutinin (containing neck and CRD region) was able to reduce bovine herpes virus type I *in vitro* (Mohan et al. 2015). Similar recombinant truncated forms of conglutinin from other Bovidae have also been studied, including goat (*Capra hircus*), buffalo (*Bubalus bubalis*) and nilgai (*Boselaphus tragocamelus*) and were found to have similar reactivities to the full length CGN (Ramesh et al. 2019; Barik et al. 2020).

There are a few studies demonstrating CGN interaction with fungal ligands, which include binding to zymosan and other glycoproteins and polysaccharides from *Saccharomyces cerevisiae* (N-acetylglucosamine, mannose, mannan (Strang et al. 1986; Loveless et al. 1989; Lim and Holmskov 1996). Similarly, the direct binding of CGN to parasites/protozoa is unknown but an early study did report the serum levels of CGN *in vivo* correlating with the severity of Trypanosoma infection (Ingram and Soltys 1960).

CL-43 and CL-46

Like CGN, collectins CL-43 and CL-46 are predominantly serum proteins. CL-43 is synthesised in the liver; the average serum concentration is 21 μg/mL and is structurally similar to CGN and bovine SP-D, and has a preferential ligands similar to that of MBL (Holmskov et al. 1993). CL-43 is in a trimeric structure in the serum and does not form higher order multimeric forms (Holmskov et al. 1995). CL-46 is mainly expressed in the thymus and liver but was also detected in mammary gland and digestive system tissues, although its average serum concentration is not known (Hansen et al. 2002a) (Table 1). It is suggested that CL-46 may be involved in the clearance of apoptotic thymocytes, and there are thymus-related cis-elements

present in its promoter region which may be indicative of T cell mediated CL-46 expression (Hansen et al. 2002a). The carbohydrate preference of the CRD of CL-46 is similar to CGN, suggesting potential iC3b targeting in the thymus, such as the clearance of apoptotic T cells (Uwai et al. 2000) and the development of central tolerance.

The physiological role of CL-43 and CL-46 has not been fully elucidated in the bovine host, but there are a few studies showing their anti-microbial activities *in vitro*. CL-43 has been reported to bind to *E. coli* strain K12, enhancing uptake by phagocytes (Hansen and Holmskov 2002). CL-43 also exhibited significantly higher hemagglutination and inhibition activity against IAV than SP-D, but unlike multimeric forms, CL-43 cannot induce viral or bacterial aggregation (Hartshorn et al. 2002). Although CL-43 did not enhance IAV-induced neutrophil H_2O_2 production, it did strongly enhance neutrophil uptake of IAV, without the need for aggregation (as seen with SP-D) (Hartshorn et al. 2002). CL-43 (like CGN and bovine SP-D) can also bind to bovine rotavirus (Nebraska calf diarrhoea virus), via the VP7 glycoprotein resulting in hemagglutination and neuralisation (Reading et al. 1998). Furthermore, CL-43 showed the highest activity against the virus (Reading et al. 1998). For fungi, CL-43 is can bind to non-capsular forms of *Cryptococcus neoformans in vitro*, inducing agglutination but not with the encapsulated form of the yeast (Schelenz et al. 1995). CL-43 has also been shown to bind to immobilized yeast mannan (Holmskov et al. 1996). For CL-46, the only report of anti-microbial activity is on IAV, where a recombinant fragment (a trimeric neck and CRD) induced aggregation of the virus and strongly enhanced its uptake by neutrophils (Hartshorn et al. 2010b).

CL-L1, CL-K1 and CL-P1

The specific role of these lesser known collectins in the Bovidae is unknown. Nearly all of understanding of the biological role of CL-L1, CL-K1 and CL-P1 has come from human and murine studies. CL-L1 and CL-K1 are present in the blood, forming complexes with mannose-associated serine protease (MASPs) of the lectin complement pathway (Hansen et al. 2010; Axelgaard et al. 2013; Henriksen et al. 2013a; Ma et al. 2013). CL-K1 gene transcripts have been observed mainly in the liver, small intestine, placenta, pancreas, kidney and adrenal, thymus, spinal cord tissues (Keshi et al. 2006) (Table 1). Serum levels of human CL-L1 are approximately 3 μg/mL and for CL-K1 approximately 0.3 μg/mL (Hansen et al. 2010; Yoshizaki et al. 2012; Axelgaard et al. 2013). Another recent study showed the median serum concentration of CL-L1 to be 1.87 μg/mL and CL-K1 to be 0.32 μg/mL (Bayarri-Olmos et al. 2015). For CL-P1, it is in the human umbilical cord plasma at approximately 55–121.4 ng/mL but has not been detectable in adult plasma (Ma et al. 2015). The tissue distribution and serum concentrations of CL-L1, CL-K1 and CL-P1 have yet to be published in the Bovidae. However, there is unpublished data on the Gene Expression Omnibus (GEO) repository showing putative expression of COLEC10,

COLEC11 and COLEC12 in various tissues (NCBI 2020). Circulating CL-L1 and CL-K1 can both form heterotrimers called CL-LK, analogous to C1q globular heads (with a ratio of 2 CL-K1: 1 CL-L1), that can lead to enhanced complement activation via interaction with MASP-2, compared to homotrimers alone (Wong et al. 2008; Axelgaard et al. 2013; Henriksen et al. 2013a; Hansen et al. 2016). Mutations in the COLEC11 gene have been linked with the developmental defect syndrome 3MC (Mingarelli, Malpuech, Michels and Carnevale syndromes) (Carnevale et al. 1989; Rooryck et al. 2011). Serum concentration variability of CL-L1, CL-P1 and CL-K1 and its link with disease is not well understood but increased CL-K1 levels have been linked with the disseminated intravascular coagulation (DIC) condition in humans (Takahashi et al. 2014).

CL-L1, CL-K1 and CL-P1 can bind to various microbes. CL-K1 binds to bacterial and fungal ligands, as well as apoptotic cells (Keshi et al. 2006; Hansen et al. 2010). Murine CL-K1 is able to bind to IAV and reduce its infectivity (Hansen et al. 2010; Henriksen et al. 2013a). CL-K1 also binds DNA and via complement, may be involved in clearance of extracellular DNA, seen from apoptotic cells, neutrophil extracellular traps and biofilms (Henriksen et al. 2013b). CL-K1 can bind to *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *M. tuberculosis* (Keshi et al. 2006; Hansen et al. 2010; Troegeler et al. 2015). CL-P1 can also bind to *E. coli* and *S. aureus* (Ohtani et al. 1999; Jang et al. 2009). Both CL-L1 and CL-K1 can activate the lectin pathway of complement (Henriksen et al. 2013a), whilst CL-P1 can activate the alternative and classical pathways of complement (Roy et al. 2016). The role of CL-L1, CL-K1 and CL-P1 *in vivo* in the bovine host is unknown, but their average serum concentrations seem to be well below that observed for MBL, SP-A, SP-D and CGN, suggesting that these collectins may be more important in local clearance of pathogens and homeostasis. Furthermore, it is still unknown whether CL-L1, CL-K1 and CL-P1 can act as opsonins directly independent of complement deposition. CL-L1 prefers D-mannose, N-acetylglucosamine, D-galactose, L-fucose and D-fructose and binding requires Ca^{2+} (Ohtani et al. 1999; Axelgaard et al. 2013), whilst, CL-K1 prefers L-fucose, D-mannose and N-acetyl mannosamine (Ohtani et al. 1999; Hansen et al. 2010). Interestingly, CL-LK (complex of CL-L1 and CL-K1) has been shown to be a PRR for *M. tuberculosis*, targeting mannose-capped lipoarabinomannan (ManLAM) on the bacterium in Ca^{2+} dependent manner, but not to the related *M. smegmatis* because of a the lack of mannose caps on its LAM (Troegeler et al. 2015). Furthermore, the levels of CL-LK in serum of human tuberculosis patients are lower, compared to controls suggesting that it may be useful as a biomarker for the disease (Troegeler et al. 2015). It would therefore be prudent to examine the correlation of CL-L1, CL-K1, and CL-P1 in *Bos taurus* during mycobacterial infection to ascertain whether these may be useful biomarkers for bovine tuberculosis as well.

Concluding Remarks and Future Directions

The bovine collectins constitute a key component of the innate immune system of cattle, but much of our understanding of their specific biological role in the Bovidae remains unknown. Collectins have been shown to be involved extensively in protection from pathogens and maintenance of homeostasis in several mammalian systems (e.g., human and mice) and similar studies should be conducted in the bovine host with relevant bovine pathogens and other pathological conditions that affect cattle. This is a significant component of bovine immunity that remains unexplored and could be relevant to combatting a number of infectious diseases of cattle, e.g., mastitis, bovine tuberculosis and other bovine respiratory disease and gastrointestinal infections (Fig. 5). Of particular importance is the use of genomic technologies that can be used to further elucidate genetic diversity of bovine collectin genes and other immune markers, to identify traits linked with major diseases of cattle. Such data will allow selective breeding of pathogen-resistant animals and give other insights in how collectins and innate immunity can be bolstered to protect against cattle infection and the reliance of anti-microbial therapy.

Bovine MBL, SP-A and SP-D have been shown to play important roles in bovine innate immunity, but much remains unknown. What remains intriguing is CGN, CL-43 and CL-46, which have evolved from an SP-D ancestral gene, are unique in the Bovidae compared to other mammals. This evolutionary separation is perhaps

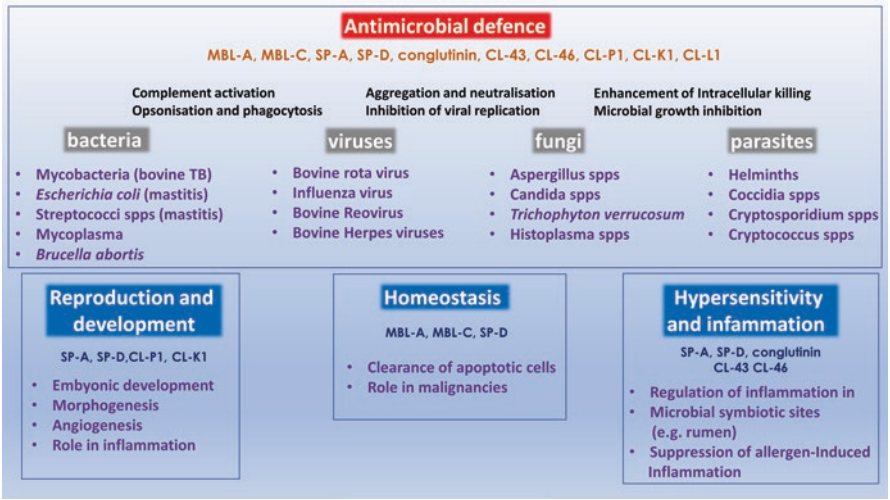

Fig. 5 Putative functions and antimicrobial interactions of the bovine collectins. Summary of the diverse roles of collectins, in antimicrobial defence, reproduction and development, homeostasis and hypersensitivity and inflammation. Note: most of these functions have not been reported in *Bos taurus*, but based on studies in other mammals, the bovine-relevant pathogens and other biological factors that are likely to interact with the bovine collectins in a similar manner. Please consult main body of the review for more details

due to the specific biology of ruminants, which have a high level of microbial symbiosis and thus need a balance of the inflammatory response to prevent tissue damage. These additional collectins may have evolved in this regard to target pathogens in an efficient manner, whilst having anti-inflammatory properties to facilitate microbial symbiosis and prevent tissue damage. Much has still to be learned about the interactions of bovine collectins, (especially CGN, CL-43 and CL-46) with bovine pathogens and in order to understand pathogenesis and susceptibility to infection. Of promise is the use of truncated recombinant forms (CRD and neck region) of some collectins (SP-A, SP-D and CGN) that has shown their potential in antimicrobial prophylaxis or therapy against IAV and tuberculosis (Al-Ahdal et al. 2018; Al-Qahtani et al. 2019; Mehmood et al. 2019). It is hoped that this review will encourage renewed focus and resources in studying bovine collectins more fully, a probable critical arm of bovine innate immunity.

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