Collectins in innate immune defense of pigs and chickens

Collectins in the innate immune defense of pigs and chickens / Astrid Hogenkamp

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Collectins in innate immune defense of pigs and chickens

Collectines in het aangeboren immuunsysteem van varkens en kippen

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter One

General Introduction

Rationale for this research

In 2005, the Dutch livestock consisted for the greater part of poultry and pigs. In the Netherlands, almost 93 million chickens were kept, of which approximately half was meant for consumption [1]. In the same year, more than 11.3 million pigs were kept, of which over 5.5 million were meant for consumption [1]. In this respect, one has to consider that these numbers represent the average number of animals present in that year, so the cumulative number of animals is quite a lot higher. Chickens are slaughtered when they are approximately six weeks of age, which means that the actual number of animals will be approximately 400 million. Applying the same calculation to the number of pigs, which are slaughtered when they are about 14 weeks old, the total number of pigs slaughtered is about 20 million. To compare: in 2005, the total population of The Netherlands was 16.4 million people [1]. In years preceding the outbreak of Foot and Mouth disease in 2001, and the outbreak of Avian Influenza A Virus (subtype H7N7) in 2003, the total numbers of animals were even higher. With a livestock this large, a number of considerations come to mind, such as economical factors, animal welfare, and food safety, all of which have to be regarded seriously in order to maintain a healthy agricultural sector. The use of food-additives for animal feed, especially antibiotics, is intricately involved in all three aspects, for reasons explained below.

Indications of beneficial effects on production as a cause of the use of antibiotics in the feed of pigs and poultry were reported as early as about fifty years ago [2, 3]. The biological basis for these effects (reviewed in [4, 5]) is multifaceted. As these antibiotic growth promotors (AGP) did not show the same results in germ-free animals [6, 7], the underlying mechanism must be focused on the microbiota present in the gastro-intestinal tract. Use of AGP will decrease competition for nutrients between the host and its microbiota. In addition, microbial metabolites that depress growth will be reduced [8, 9]. Furthermore, increased nutrient ingestibility has been associated with AGP-induced reduction of the gut wall and villus lamina propria [9-11]. Finally, a reduction in opportunistic pathogens and subclinical infection has also been linked to use of AGP.

Soon after the discovery of the benefits of AGP, it became apparent that their use in animal feed could lead to microbial resistance to these agents [12-14]. In spite of these reports, AGP were widely used for decades worldwide. More recently, epidemiological studies and feeding experiments have clearly shown that the use of different AGP will select for resistance among enterococci in poultry and pigs (reviewed in [15]). In 1999, the European Commission decided to phase out, and ultimately ban, the marketing and use of AGP in livestock feed. This measure was taken as a means to reduce the non-essential use of antibiotics in order to effectively address the problem of micro-organisms becoming resistant. The EU-wide ban entered into effect on January 1, 2006. However, pathogens in

poultry and pigs can still pose a considerable risk to both animal and public health [16-20], and the economic losses caused by infections can be considerable. Therefore, the search for safe alternative strategies to prevent infections was stimulated.

One approach to this challenge to defend animals from putative pathogens is to make use of what is already there: the immune system of the animal. An effective response from the adaptive immune system can swiftly eradicate an infection, but this system is dependent on prior exposure to the pathogen. When an animal is infected by a pathogen for the first time, it takes up to four or five days before antibodies can be detected in the plasma. In this respect, one has to take into account that early-weaned piglets and broiler chickens are immunologically naive. This poses a serious problem, since any delay in the host immune response can give the pathogen the opportunity to cause more damage to the animal and to spread to other animals.

Fortunately, this temporal gap is filled by the innate immune system, which provides a first line defense against invading pathogens. Like the adaptive immune system, the innate immune system relies heavily on its ability to distinguish self from non-self. However, whereas the adaptive immune system generates a tailor-made response to an invading pathogen, the effector mechanisms of innate immunity rely on the recognition of features that are common to many pathogens. These pathogen-associated molecular patterns (PAMPs) can be recognized by pattern recognition molecules at the site of infection, which is most often either an internal or an external epithelial surface. For example, the respiratory system provides a route of entry for airborne pathogens, the gastro-intestinal tract is host to billions of micro-organisms and insect bites or wounds allow pathogens to penetrate the skin.

An interesting group of pattern recognition molecules, the collectins, has been reported to be expressed in many epithelial surfaces. Exploiting the properties and functions of these proteins, which will be discussed in **Chapter Two**, may provide an alternative strategy towards preventing infections in pigs and poultry. In another study by Herías et al [21], the expression of Surfactant Protein D (SP-D) was localized to various tissues, including the gastro-intestinal tract. In **Chapter Three,** the investigations into the possible function of SP-D in the innate defense of the porcine gastro-intestinal tract will be discussed.

In chickens, the only collectin that had been previously described was chicken Mannan Binding Lectin. **Chapter Four** deals with the characterization and expression sites of three newly discovered chicken collectins, chicken Collectin 1 (cCL-1), chicken Collectin 2 (cCL-2) and chicken Collectin 3 (cCL-3), and one chicken lectin which was named chicken Lung Lectin (cLL). To investigate whether these (col)lectins are involved in the innate defense against invading pathogens, the expression levels of chicken collectins during viral and bacterial infections was studied. The results of this study are discussed in

Chapter Five. Chapter Six will discuss the characterization of recombinant cLL and the possible functions of this protein in chicken innate immunity.
The results of the studies described in this thesis are summarized and discussed in **Chapter Seven**.

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Chapter 2

Collectins - Interactions with Pathogens

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2.1 Introduction

The body is continually exposed to all kinds of microorganisms. The skin is home to many thousands of bacteria and the oral cavity contains over 700 different species of microbes. With every breath, we inhale all kinds of microorganisms, and our intestines are inhabited by approximately 1.10^{14} microbes. Bearing this in mind, it is obvious we must regulate our bacterial flora.

The immune defense system has the capability to generate a specific response, mediated by the adaptive immune system which is acquired in time through exposure to potential pathogens. However, when a pathogen is encountered for the first time, as is usually the case in early development, it takes up to four or five days before antibodies can be detected in the plasma. Even when the adaptive immune system has previously encountered a specific antigen, it can take up to three days for the adaptive immune system to produce sufficient amounts of antibodies directed specifically against an invading pathogen. Since many opportunistic pathogens are capable of colonizing tissue well within this time span, a direct response is required to safeguard the health of the host.

The innate immune system provides a first line defense against potential pathogens, bridging the interval between exposure to the pathogen and the specific response of the adaptive immune system. Both systems rely heavily on the ability to distinguish self from non-self, and the antigens that trigger a response in the adaptive immune system are usually complex and relatively large in size. Molecules that are composed of repeated structures, such as simple polysaccharides, do not offer enough characteristic epitopes for antibody attachment. In the innate immune system however, pathogen-associated molecular patterns (PAMPs) such as LPS from the Gram-negative cell wall, peptidoglycan and lipoteichoic acids from the Gram-positive cell wall are recognized by various different pattern recognition molecules. The latter include the Toll-like receptors and scavenger receptors. These molecules are associated with the cell which produces them, but there are also many pattern recognition molecules which are secreted into the extracellular space, such as most of the collectins, which play an important role in innate immunity.

Collectins belong to the calcium-dependent (C-type) lectin superfamily [1]. To date, several different types of collectins have been identified, and their expression has been localized to various tissues (**Table 1a-c**).

Mannan binding lectin (MBL), conglutinin and collectin-43 (CL-43) are all serum proteins which are mainly synthesised in the liver [2-9]. Thus far, the latter two proteins have only been identified in Bovidae, which is also true for collectin-46 (CL-46), a collectin expressed in bovine thymus and liver [10]. Surfactant Protein A (SP-A) and Surfactant Protein D (SP-D) are mainly expressed in the lungs [11-15], but they have also

Table 1a. Tissue localisation of collectins – tissues of endodermal origin				
	Human	**Rat**	**Mouse**	**Rabbit**
Lung	SP-A[a] [15] SP-D[a] [13] CL-L1[b] [23] CL-P1[b] [24]	SP-A[a] [301] SP-D[a] [302]	SP-A[a] [303] SP-D[a] [303]	SP-A[b] [304]
Esophagus	SP-D[a] [306]	-	SP-D[b] [307]	-
Stomach	SP-D[a] [306] CL-L1[b] [23] CL-P1[b] [24]	SP-D[a] [308]	SP-A[b] [307] SP-D[b] [309] CL-L1[b] [22]	-
Small intestine	SP-D[a] [306] CL-L1[b] [23] CL-P1[b] [24]	SP-A[a] [311]	SP-A[b] [307] SP-D[b] [307] CL-L1[b] [22]	-
Large intestine	SP-A[b] [306] SP-D[b] [306] CL-P1[b] [24]	SP-A[a] [311]	-	-
Liver	SP-D[a] [306] MBL[a] [312] CL-L1[b] [23] CL-P1[b] [24]	MBL[a] [313]	SP-A[b] [307] MBL[b] [3]	MBL[a] [314]
Gall bladder	SP-D[a] [306]	-	-	-
Pancreas	SP-A[b] [306] SP-D[a] [306]	-	SP-A[b] [307]	-
Thymus	SP-A[b] [306] CL-P1[b] [24]	-	-	-
Middle ear / Eustachian tube	-	-	-	SP-A[a] [318]
Placenta	SP-D[b] [306] CL-L1[b] [23] CL-P1[a] [24]	-	-	-

[a] expression observed at protein level, assessed by immunohistochemistry, Western Blotting and/or isolation of the protein. [b] expression only observed at mRNA level. - no expression observed / not measured

been localized to extrapulmonary tissues [16-21]. Recently identified collectins include collectin liver 1 (CL-L1), which is most highly expressed in liver, adrenal glands and placenta [22, 23], and collectin placenta 1 (CL-P1), a membrane-bound collectin which is expressed in vascular endothelial cells [24, 25]. Chicken Collectin 2 (cCL-2), homologue to mammalian Collectin 11, was recently discovered in our laboratory and may represent another unique class of collectins. Its expression was localized to various tissues [26]. To our knowledge, no tissue localization studies have been performed for Collectin 11 in other

Table 1a. Tissue localisation of collectins – tissues of endodermal origin - *continued*			
	Pig	**Chicken**	**Bovidia**
Lung	SP-D[a] [289] SP-A[a] [289]	SP-A[b] [26] cCL-1[b] [26] cCL-2[b] [26] cCL-3[b] [26]	SP-D[a] [305]
Esophagus	-	cCL-3[b] [26]	-
Stomach	SP-D[b] [310]	-	-
Small intestine	SP-D[b] [310]	cCL-1[b] [26]	conglutinin[b] [10] SP-D[b] [305]
Large intestine	SP-D[b] [310]	-	SP-D[b] [305]
Liver	MBL[b] [315] SP-D[b] [310]	cCL-1[b] [26] cCL-2[b] [26] cCL-3[b] [26] MBL[b] [5]	conglutinin[b] [316] CL-43[b] [317] CL-46[b] [10]
Gall bladder	-	-	-
Pancreas	-	-	-
Thymus	-	cSP-A[b] [26] cCL-1[b] [26] cCL-2[b] [26] cCL-3[b] [26]	conglutinin[b] [10]
Middle ear / Eustachian tube	SP-A[a] [17, 133] SP-D[a] [17, 133]	-	-
Placenta	-	-	-

[a] expression observed at protein level, assessed by immunohistochemistry, Western Blotting and/or isolation of the protein. [b] expression only observed at mRNA level. - no expression observed / not measured

species. cCL-1 and cCL-3 were found to be the avian homologues of CL-L1 and CL-P1, respectively [26]. The primary structure of a collectin is comprised of four domains; (1) a cysteine rich N-terminal domain, (2) a collagenous domain, (3) an alpha-helical neck domain and (4) a lectin or carbohydrate recognition domain (CRD), which mediates the calcium-dependent carbohydrate binding [27-31]. The basic structural unit of a collectin is a trimer, either composed of three identical polypeptides or a combination of two identical polypeptides and a closely related monomer (**Figure 1**). With the exception of CL-43, CL-L1 and the membrane-bound CL-P1, these trimers can multimerize to varying degrees, resulting in differently shaped multimeric forms. In the case of SP-D and conglutinin, trimers assemble into cruciform dodecamers which extend the spatial range of their CRDs

Table 1b. Tissue localisation of collectins – tissues of mesodermal origin*					
	Human	**Rat**	**Mouse**	**Pig**	**Chicken**
Heart	SP-D[b] [306] CL-P1[a] [24]	-	CL-P1[a] [24] SP-D[b] [307] SP-A[b] [307]	-	-
Skeletal muscle	CL-P1[b] [24]	-	CL-L1[b] [22]	SP-D[b] [310]	-
Mesentery	SP-A[a] [319] SP-D[a] [319]	SP-A[a] [319] SP-D[a] [319]	-	-	-
Urinary bladder	SP-D[a] [306]	-	-	-	-
Uterus	SP-D[a] [21] CL-P1[b] [24]	-	SP-D[a] [307]	-	-
Kidney	SP-D[a] [306] CL-P1[b] [24]	-	-	-	cCL-1[b] [26] cCL-2[b] [26] cCL-3[b] [26]
Prostate	SP-A[b] [306] SP-D[a] [20] CL-L1[b] [23]	-	-	-	-
Testes	SP-D[b] [306] CL-P1[b] [24]	-	CL-L1[b] [22] SP-D[b] [307] SP-A[b] [307]	-	-
Spleen	SP-D[b] [306] CL-L1[b] [23] CL-P1[b] [24]	-	SP-A[b] [307]	-	-

*Note that some species listed in Table 1a. have are not listed in this table. For these species, no record of collectin expression in tissues of mesodermal origin was found. [a] expression observed at protein level, assessed by immunohistochemistry, Western Blotting and/or isolation of the protein. [b] expression only observed at mRNA level. - no expression observed / not measured

considerably. SP-D can assemble into even higher order multimers, which are often referred to as fuzzy ball-like structures. SP-A and MBL will multimerize into octadecamers, in which the six trimers are arranged into a bouquet-shaped hexamer, resembling the complement protein C1q. Trimerization is thought to be initiated by the neck-domain. It contains several so-called heptad repeats, which are characterized by the presence of hydrophobic residues at every fourth and seventh position. This allows for the formation of a triple amphipathic alpha- helical coiled coil, in which the hydrophobic

Table 1c. Tissue localisation of collectins – tissues of ectodermal origin*				
	Human	**Mouse**	**Chicken**	**Bovidia**
Brain	SP-D[b] [306] CL-P1[b] [24]	-	-	-
Salivary gland	SP-A[b] [306] SP-D[a] [306] CL-P1[b] [24]	SP-D[b] [307]	cCL-3[b] [26]	SP-D [b] [305]
Mammary gland	SP-D[b] [306] CL-P1[b] [24]	-	-	conglutinin[b] [10] SP-D[b] [305]
Sweat glands	SP-D[a] [306]	-	-	-
Adrenal gland	SP-D[b] [306] CL-L1[b] [23] CL-P1[b] [24]	-	-	-

*Note that some species listed in Table 1a. have have not been listed. For these species, no record of collectin expression in tissues of ectodermal origin was found. [a] expression observed at protein level, assessed by immunohistochemistry, Western Blotting and/or isolation of the protein. [b] expression only observed at mRNA level. - no expression observed / not measured

residues of each monomer are wrapped in between the hydrophilic residues. The folding of the neck domains into this alpha-helical coiled coil aligns the collagen chains, allowing for a zipper-like folding of the collagen helix. The collagen domain is composed of repeating tripeptide motifs of Gly-X-Y, where X or Y can be any amino acid, but most frequently proline or hydroxyproline. The size of the collagen domain can vary considerably between different collectins and determines for a large part the molecule's shape and dimensions, thereby greatly affecting the function of the protein. In SP-A and MBL, the collagen-domain is interrupted, resulting in a kink that causes the trimers to angle away from the central core. After the collagen domain is properly folded, the trimers are stabilized by formation of disulfide bridges between the cysteines of the N-terminal domain, which are also believed to play a role in multimerization of the trimeric subunits.

As the affinity of a single CRD for carbohydrates is low, the basic requirement for carbohydrate recognition is for the collectins to trimerize, which allows for simultaneous and multivalent interactions with carbohydrates. C-type lectins have relatively broad monosaccharide specificity, but they can be divided into either mannose/glucose type or galactose type collectins. All collectins (with the exception of CL-P1) are of mannose/glucose type, [32, 33] recognising sugars such as D-mannose, N-acetyl-D-glucosamine, and L-fucose. A common feature of these sugars is the presence of vicinal, equatorial hydroxyl groups on the 3- and 4- position of the sugar ring of the hexoses. Binding of a collectin to a sugar occurs through the formation of a non-covalent bond

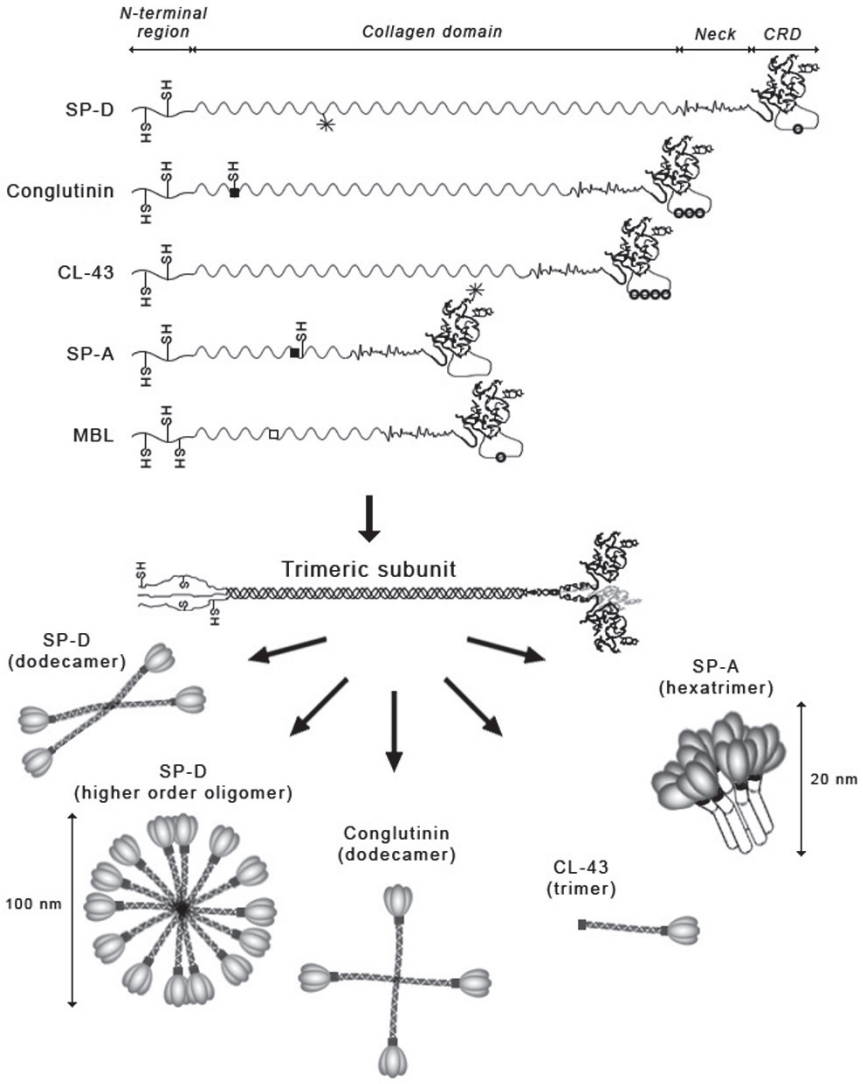

Figure 1. Primary and tertiary structures of various collectins.
The domain organization of the polypeptide chains of five well-characterized members of the collectin family are drawn to scale (top) All collectins depicted are from human, except conglutinin and CL-43, which are only found in Bovidae. The neck-CRD is illustrated as the main chain structure, as determined for human SP-D (adapted from [214]). Open circles on the CRD indicate the number of extra amino acid residues compared to SP-A that are present in loop 4 of the MBL crystal structure. Asterisks indicate conserved N-glycosylation sites; squares in the collagen domain represent incomplete (white) or interruption in (black) triplet sequence. Triple helix formation over the collagen domain results in the clustering of three CRDs. The trimer is the subunit which is assembled into oligomeric forms (bottom), except for CL-43, which is only present as a trimer.

involving the hydroxyl groups, whereby two coordination bonds between the sugar and the ligated calcium are formed [34, 35]. Together with four hydrogen bonds, which are established between the sugar and calcium ligands, this forms a tightly linked complex of calcium, protein and sugar. Galactosides are poorly recognized by collectins due to the axial conformation of the hydroxyl group on the 4-position of the hexose.

The clustering of the CRDs ensures that collectins can bind with high affinity to oligosaccharide-complexes, which are typically found on the surface of micro-organisms such as bacteria, viruses, fungi, and yeasts. In this chapter, an overview will be given of the various effects that collectin interactions with pathogens can have on the micro-organisms themselves, and how these interactions affect other components of the immune system.

2.2 Interactions with Gram-negative bacteria

Collectins have been shown to interact with many different Gram-negative bacteria (**Table 2**). The most important target for this interaction is lipopolysaccharide (LPS), which consists of a hydrophobic domain known as lipid A, KDO (3-deoxy-D-manno-oct-2-ulosonic acid) domains and additional core and O-antigen sugars [36]. Smooth LPS contains all of these domains, but LPS consisting of only lipid A and (progressively shorter) core oligosaccharides is referred to as rough LPS [37] (**Figure 2**).

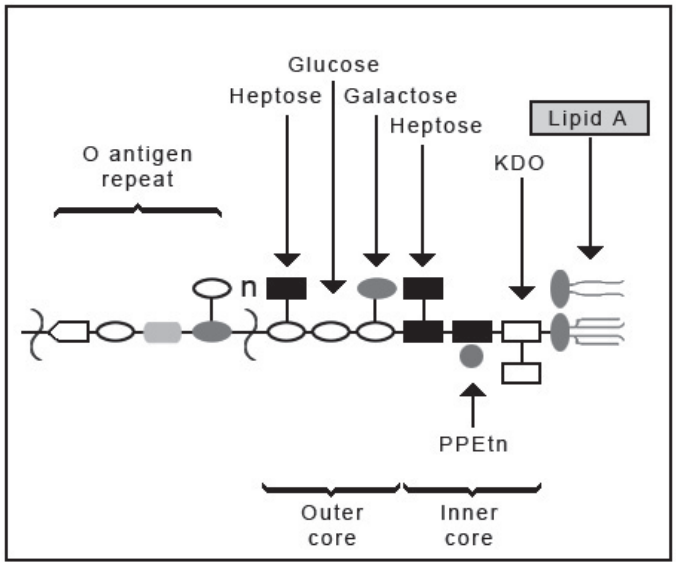

Figure 2. Schematic representation of lipopolysaccharide of *E. coli* K12.
KDO = 3 – deoxy – D – mannooctulosonic acid, P = phosphate, Etn = ethanolamine.
Adapted from [36] *by B. Gerrissen, 2006.*

Table 2. Interactions of collectins with Gram-negative bacteria			
Species	**Collectin**	**Observed effects –** *Observed effects in case of deficiency*	**References**
Escherichia coli	**SP-A**	Aggregation	[47]
		Increased uptake by neutrophils	[46]
		Increased killing by macrophages	[46]
		Increased membrane permeability	[49]
	SP-D	Aggregation	[47]
		Increased uptake by neutrophils	[47]
		Increased association with and antigen presentation by BMDCs	[48]
		Increased membrane permeability	[49]
Haemophilus influenzae	**SP-A**	Aggregation	[44]
		Increased killing by macrophages	[44]
		Decreased killing by macrophages	[52]
		Increased inflammation and inflammatory cell recruitment	[52]
	SP-D	*Increased inflammation and inflammatory cell recruitment*	[52]
	MBL	Binding	[54-56]
Klebsiella pneumoniae	**SP-A**	Binding	[56]
		Enhanced phagocytosis by macrophages	[56]
		Reduced RNA synthesis	[49]
	SP-D	Aggregation	[61]
		Enhanced internalization and killing by macrophages	[39]
		Decreased attachment to lung epithelial cells	[43]

Collectins were initially thought to be capable of binding to rough LPS only. Analysis of mutant forms of *Salmonella minnesota* and *Escherichia coli* LPS suggested that the glucose containing core oligosaccharide of LPS was sufficient for CRD dependent binding of SP-D [38]. SP-D is capable of binding *Klebsiella pneumoniae* LPS in a lectin-like manner [39] and binding studies showed that SP-A binds to the lipid A domain of LPS [40]. This

Table 2. Interactions of collectins with Gram-negative bacteria – continued			
Species	**Collectin**	**Observed effects –** *Observed effects in case of deficiency*	**References**
Pseudomonas aeruginosa	**SP-A**	Binding	[42]
		Enhanced uptake by alveolar macrophages	[67]
		Slower rate of clearance	[69]
		Decreased uptake by macrophages	[69]
	SP-D	Binding	[51]
		Aggregation	[42]
		Increased phagocytosis by macrophages	[51]
		Increased phagocytosis by monocytes	[42]
		Increased/decreased TNF-α production	[42]
		Inhibition of corneal cell invasion	[78]
		Slower rate of clearance	[68]
		More severe inflammation	[69]
	MBL	*Negative effect on lung function*	[81]
		Prevention of systemic spread after burn injury	[84]
Helicobacter pylori	**SP-D**	Aggregation	[87]
		Decrease in motility	[87]
Chlamydia trachomatis	**SP-A**	Aggregation	[89]
		Enhancement of phagocytosis by macrophages	[89]
	SP-D	Enhancement of phagocytosis by macrophages	[89]
		Inhibition of infection of endocervical cells	[21]
		Inhibition of infection of prostate gland epithelial cells	[20]
	MBL	Inhibition of infection of HeLa cells	[94]
Chlamydia ***pneumoniae***	**SP-A**	Aggregation	[89]
		Enhancement of phagocytosis by macrophages	[89]
	SP-D	Aggregation	[89]
		Enhancement of phagocytosis by macrophages	[89]
	MBL	Inhibition of infection of HeLa cells	[94]
		Association with increased risk of coronary artery disease	[101]
Chlamydia psittaci	**MBL**	Inhibition of infection of HeLa cells	[94]

binding appears not to be mediated by the CRD or the carbohydrate moiety present on the CRD of SP-A, since binding occurs in the presence of mannan and heparin. A deglycosylated form of SP-A is also capable of binding to LPS derived from *S. minnesota* R595 and the Rc mutant of *E. coli* O111:B4. When compared to SP-D and MBL, SP-A trimers display a conformational difference which gives them a more extensive hydrophobic surface. This difference could explain the high affinity of SP-A for lipid A as compared to the other collectins [41]. Since SP-A is incapable of binding to the wild-type strain of *E. coli* O111:B4 [40], it was suggested that the terminal oligosaccharide moiety may mask lipid A and thereby prevents the interaction of SP-A and lipid A. However, SP-A and SP-D are capable of binding both smooth and rough forms of *Pseudomonas aeruginosa* [42]. Also, SP-D selectively binds to smooth forms of LPS expressed by O-serotypes with mannose rich repeating units in their O-polysaccharides, whereas *K. pneumoniae* strains with galactose-rich repeating units in their O-polysaccharides are not bound, which is consistent with the known low affinity of SP-D for galactose [43]. In addition, SP-A can bind to *Haemophilus influenzae* via its major outer membrane protein P2 in a calcium-dependent manner [44]. The results of the latter study suggest that SP-A binds to *H. influenzae* via the C-terminal region of the molecule, but not necessarily the CRD. It remains unclear which part of the LPS is bound by MBL. It has been suggested that the three-dimensional structure of LPS makes an important contribution to the binding of MBL to Gram-negative bacteria, because the magnitude of MBL binding cannot be predicted from the identity of the LPS terminal sugar alone. MBL binds to core structures of LPS on the surface of *Salmonella typhimurium* and *Neisseria gonorrhoeae*, but addition of O-antigen (*S. typhimurium*) or sialic acid (*N.gonorrhoeae*) to the core abrogates the binding. Truncation of the LPS within the core leads to lower levels of MBL binding [45]. In most instances, binding of a collectin will lead to aggregation of the bacteria, and depending on the strain and the collectin, this can have various outcomes. Therefore, some of the more extensively studied microorganisms will be discussed below.

2.2.1 Escherichia coli

Surfactant proteins A and D interact with *E. coli* LPS, and both collectins are capable of aggregating *E. coli* J5 (a rough strain), although higher concentrations of SP-A are necessary to achieve maximal aggregation compared to SP-D. No aggregation is observed when *E. coli* O111 (a smooth strain) is incubated with either collectin. SP-A enhances the binding of *E. coli* J5 to rat alveolar macrophages, acting as an opsonin of *E. coli* J5, but SP-D does not. In addition, preincubating the bacteria with SP-A increases bacterial killing by macrophages [46]. SP-A and SP-D are also able to increase neutrophil uptake of *E. coli* K12 in a calcium-dependent manner. The ability to enhance the uptake is correlated with

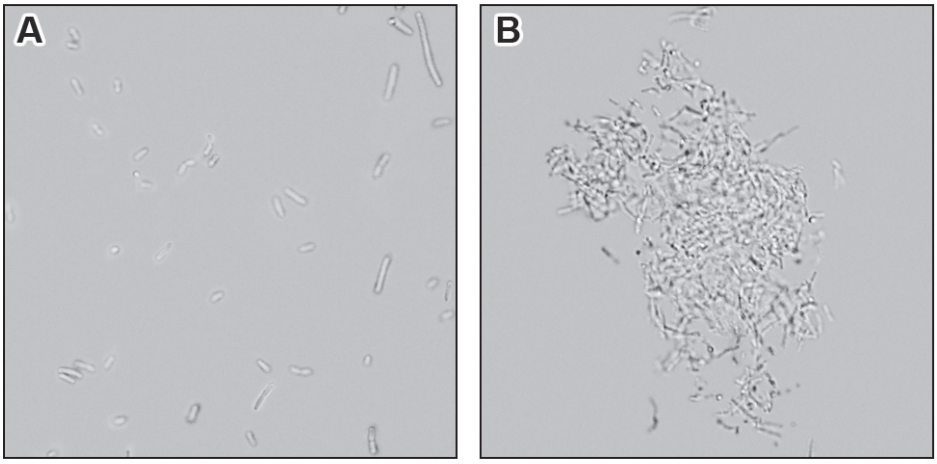

Figure 3. Aggregation of *E. coli* K12 by porcine SP-D.
(A) control, (B) *E. coli* K12 incubated with 25 µg/ml pSP-D.
By A. Hogenkamp, 2006.

the ability of both collectins to aggregate the bacteria (**Figure 3**), since the concentrations of SP-D and SP-A which induce aggregation correspond to those that induce increased uptake of *E. coli* by neutrophils. Also, trimeric preparations of SP-D are unable to aggregate or stimulate uptake of the bacteria by neutrophils. However, when neutrophils are preincubated with either SP-A or SP-D and then washed before the addition of *E. coli* K12, uptake is also increased, suggesting a direct interaction of the collectins with the neutrophils [47]. SP-D also enhances the association of *E. coli* HB101 with immature bone marrow-derived dendritic cells (BMDCs). Furthermore, SP-D enhances the antigen presentation by the BMDCs of an ovalbumin fusion protein expressed by *E. coli* HB101 to ovalbumin-specific MHC class II T cell hybridomas [48]. Taken together, these results show that the pulmonary collectins play an important role in enhancing the interactions of *E. coli* with immune cells. A recent study focused on the possible direct antimicrobial effects of the collectins, and it was found that the macromolecular synthesis of *E. coli* K12 is decreased when the bacteria are incubated with either SP-A or SP-D. Bacterial growth is also inhibited when bacteria are grown in the presence of SP-A or SP-D. These effects appear to be independent of aggregation since a reduction of bacterial growth is also observed in radial diffusion assays, and aggregation mediated by antibacterial antibodies does not affect macromolecular synthesis of *E. coli* K12. Furthermore, SP-A and SP-D increase bacterial permeability, as assessed by protein leakage from the bacteria and vital dye staining [49]. Collectin-mediated inhibition of RNA-synthesis was observed for most rough *E. coli* laboratory strains tested in this study, but not for smooth strains, suggesting a

protective function for the O-antigen. However, one clinical isolate *E. coli* with rough type LPS was affected by neither SP-A nor SP-D and partial restoration of the O-antigen of *E. coli* K12 did not affect the inhibition of RNA synthesis mediated by the collectins. Increased susceptibility to growth inhibition by SP-A and SP-D was observed for a mutant strain of *E. coli* K1 which did not express outer membrane protein A (OmpA) while the parental strain, a smooth variant, was relatively resistant. OmpA is required for structural integrity of the outer membrane and the generation of normal cell shape [50]. Taken together, the results from this study suggest that SP-A and SP-D may kill bacteria by interacting with the cell membrane.

2.2.2 Haemophilus influenzae

H. influenzae is aggregated and opsonized by SP-A [44], and the latter increases the association of type a H. influenzae with alveolar macrophages, resulting in increased killing of the bacteria. SP-D has only minimal effects on the phagocytosis of *H. influenzae* by alveolar macrophages in vitro [51]. Decreased killing of *H. influenzae* has been observed in mice lacking SP-A, but not in mice lacking SP-D, but deficiency of either SP-A or SP-D is associated with increased inflammation and inflammatory cell recruitment after infection [52]. Also, alveolar macrophages show a decreased uptake of bacteria in both SP-A and SP-D-deficient mice, in contrast to the findings of Restrepo and colleagues [51]. In SP-D-deficient mice however, macrophages are lipid laden [53] which may affect their ability to phagocytose bacteria. *H. influenzae* type b is bound by MBL, but non-type b is not [54], and heterogeneity in MBL binding was again demonstrated in a study by Shang et al. [55], who observed a marked variation in the binding of MBL to different isolates of *H. influenzae*. This emphasizes the need to examine multiple isolates of a specific bacterial strain to determine collectin binding to any particular strain, since it is possible for sugar arrays on the surface of bacteria to vary even within species.

2.2.3 Klebsiella pneumoniae

Collectins have also been shown to interact with *K. pneumoniae*. SP-A enhances the phagocytosis of *K. pneumoniae* by serving as an opsonin, binding to a specific Manα1Man-containing structure in the capsule of certain serotypes of K. pneumoniae, and potentially to SP-A receptors on macrophages. In addition, SP-A activates the macrophages, resulting in increased activity of the mannose receptor. Epidemiological studies indicate that among the serotypes of *Klebsiella* isolated, a higher frequency of *Klebsiella* serotypes bearing a capsule that lacked those mannosyl sequences, is recognized by the mannose receptor (and presumably by SP-A), suggesting that SP-A together with the mannose receptor provides an important defense mechanism against invading pathogens [56]. SP-A can also exert a direct effect by reducing RNA synthesis in *K. pneumoniae* [49].

However, *K. pneumoniae* exhibits phase variation in capsule formation during different stages of infection [57]. The pathogen reaches the lower respiratory tract by first colonizing the upper respiratory tract [58, 59]. To colonize the upper respiratory tract the pathogen needs to adhere to the epithelial cells [60] of the upper respiratory tract (or other mucosal surfaces). A capsule may interfere in this process in part by masking adhesins, thereby allowing unencapsulated phase variants to predominate at this site. SP-D can bind to unencapsulated phase variants of *K. pneumoniae*, and aggregates unencapsulated (but not capsulated) phase variants of *K. pneumoniae*. This is inhibited by the addition of maltose and by calcium depletion, strongly suggesting that the CRD mediates this interaction. Furthermore, purified LPS from the unencapsulated phase variant of *K. pneumoniae* also inhibits aggregation, but capsular polysaccharides do not [39]. This is consistent with the finding that SP-D can aggregate *Klebsiella* that express a high proportion of rough type LPS on their surface [61], and, as mentioned earlier, that SP-D has been found to selectively bind to LPS containing mannose rich O-antigens [43]. SP-D can inhibit the adhesion of unencapsulated *Klebsiella* serotypes to lung epithelial cells. This inhibition is mediated by the CRD and dependent on the degree of multimerization of the collectin, since trimeric subunits are inactive in this respect [43]. Coating *K. pneumoniae* with SP-D also enhances alveolar macrophage association with the unencapsulated bacteria, leading to increased internalization and killing of *K. pneumoniae*, and an increased nitric oxide production by the alveolar macrophages [39]. In addition, when macrophages or peripheral-blood monocytes are exposed to unencapsulated strains of *K. pneumoniae* coated with SP-D, the synthesis of proinflammatory cytokine mRNA is stimulated [62].

2.2.4 Pseudomonas aeruginosa

SP-D can bind to *P. aeruginosa*, which stimulates phagocytosis of the bacteria by alveolar macrophages in vitro. SP-D may function as an opsonin, since macrophage internalization of *P.aeruginosa* is increased by approximately 50% when the bacteria are preincubated with SP-D. Macrophages that are allowed to adhere to SP-D coated slides are less capable to internalize bacteria, suggesting that this adherence leads to a clustering of SP-D receptors on the basolateral surface of the macrophage [51]. This would leave the receptors inaccessible to interact with SP-D opsonized bacteria added to the apical side of the macrophage [51].

Although the receptor for SP-D was not identified in this study, it is possible that the calreticulin-CD91 (CRT-CD91) receptor complex is involved. When SP-A and SP-D bind to LPS, this causes a clustering of their collagen domains, which in turn can interact with the macrophage CRT-CD91 receptor complex. This interaction can induce phagocytosis, pro-inflammatory cytokine production and enhancement of adaptive immune responses [63]. Additionally, SP-D as well as SP-A can bind to CD14; SP-D binds to a carbohydrate moiety on CD14 whereas SP-A binds via its neck domain to a peptide

component of CD14. This binding alters LPS/CD14 interactions [64]. Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism; however, this pathway appears to be calcium-independent [65]. SP-A decreases the binding of Re-LPS to CD14, but the binding to LPS binding protein (LBP) is unaffected by SP-A. However, when SP-A, CD14 and LBP are all incubated together, SP-A reduces the ability of LBP to transfer Re-LPS to CD14, suggesting that SP-A may modulate Re-LPS responses by altering the competence of the LBP-CD14 receptor complex. Therefore, it is feasible that SP-A can alter the efficacy of phagocytosis by macrophages [66].
SP-D can aggregate both smooth and rough strains of non-mucoid *P. aeruginosa* [42], although this seems to be strain-dependent. SP-A binds (but does not aggregate) both smooth and rough strains of *P. aeruginosa* [42], but like SP-D this seems to depend on which isolate of *P. aeruginosa* is tested since Manz-Keinke and colleagues [67] reported no binding of SP-A to the bacteria.

SP-D is capable of stimulating phagocytosis of the bacteria by human monocytes [42], and SP-D-deficient mice have been shown to clear *P. aeruginosa* at a slower rate than wild-type mice [68]. SP-D has either a stimulating or a dampening effect on TNF-α production, depending on the strain of *P. aeruginosa* tested. This latter effect was demonstrated to be dependent on LPS serotype, suggesting that the effect of SP-D on cytokine production could reflect differences in presentation of different types of LPS to the target cells or to LPS-binding proteins present in serum [42]. Alternatively, this effect may be mediated by direct interactions with other molecules involved in LPS signal transduction, such as CD14.

SP-A-deficient mice are susceptible to *P. aeruginosa* infection, as bacteria are cleared from the lungs of SP-A-deficient mice at a much slower rate than from wild-type mice [69]. Alveolar macrophages show a decreased uptake of bacteria in SP-A-deficient mice, supporting earlier findings by Manz-Keinke and colleagues [67], who demonstrated that SP-A enhances uptake of *P. aeruginosa* by alveolar macrophages.

P. aeruginosa also causes a more severe inflammation in SP-A-deficient mice, and significantly higher concentrations of proinflammatory cytokines were also found in these mice. Increased levels of anti-inflammatory cytokines are also measured, but their concentrations are relatively low and the observed increase is not sufficient to modify the inflammatory response. The finding that the absence of SP-A leads to increased lung inflammation is supported by earlier studies, which demonstrated an SP-A mediated decrease in the release of TNF-α by LPS-stimulated macrophages [70] and *P. aeruginosa* stimulated human monocytes [42]. However, earlier studies indicated a stimulating effect of SP-A on the production of TNF-α, IL-1 and IL-6 by mononuclear cells [71] and TNF-α production was demonstrated to be stimulated by SP-A in macrophages and THP-1 cells, a human monocyte/macrophage cell line [72]. It remains to be determined whether the absence of SP-A directly accounts for these differences, or whether the severity of infection

and failure of early bacterial clearance are the main causes of altered levels of cytokines in SP-A-deficient mice.

However, the interaction between SP-A, SP-D and *P. aeruginosa* is not unidirectional, as the bacteria are capable of producing elastase which can cleave the collectins [73, 74]. This degradation will eliminate many of the collectins' normal immune functions, which may represent a novel strategy of *P. aeruginosa* to evade immune defenses. It may also explain why cystic fibrosis patients preferentially establish infections with *P. aeruginosa* and why mortality is often due to that organism despite the presence of other bacteria in the lung [75].

P. aeruginosa is an opportunistic pathogen and the lung is not its only playfield. The bacterium can also cause sight threatening corneal infections, which can arise after damage caused by contact lens wear or corneal injury. Two different types of *P. aeruginosa* have been isolated from infections, one of which invades epithelial cells [76] and another type which causes cytotoxicity [77].

SP-D is present in human tear fluid and in cultured human and mouse corneal cells, and mouse cornea in vivo [78]. SP-D inhibits rabbit corneal epithelial cell invasion by *P. aeruginosa*, but this effect is independent of aggregation since SP-D does not aggregate the bacteria at concentrations inhibitory to bacterial invasion. Growth or swimming motility of the bacteria are unaffected, so SP-D may protect the corneal cells from invasion by blocking receptors involved in entry of *P. aeruginosa* into cells. These findings show that the role of SP-D in innate immunity stretches further than the pulmonary environment, and implicate an important function for this collectin in defending epithelial surfaces against microbial pathogens.

Polymorphisms in the mbl-2 gene which lead to lower levels in serum concentrations of MBL have been implicated in a variety of infections [79]. Also, in patients with cystic fibrosis (CF), some variant *MBL* alleles have been shown to affect lung function adversely [80, 81], suggesting that MBL function is a potential modifier in CF lung disease. This adverse effect of lung function was mainly observed in patients with chronic *P. aeruginosa* infection [81] but did not seem to be due to MBL mediated clearance of *P. aeruginosa*, since MBL binds only very weakly to *P. aeruginosa* [54, 82] and no complement-activation was observed [82]. It was suggested that the effect of *P. aeruginosa* on lung function in MBL-deficient CF patients reflects a role for MBL in intercurrent infections with other organisms, or in the inflammatory process. However, other investigators have observed no correlation between MBL pathway deficiency and reduced lung function in CF patients. Also, MBL-deficient CF patients were shown to be colonized by *P. aeruginosa* to a lesser degree than MBL-sufficient CF patients [83]. The investigators speculated that this effect may be due to a favoured colonization by *Staphylococcus aureus* in MBL-deficient patients, an organism which is bound by MBL [54]. Since an impaired lung function was observed for MBL-deficient CF patients infected with *S. aureus*, it was

suggested that microbiological context should be taken into account when validating MBL as a prognostic predictor in CF. MBL also affects the susceptibility to *P. aeruginosa* after burn injury by preventing a systemic spread of the organism [84]. In a study by Moller-Kristensen and coworkers [84], both wild-type and MBL-deficient mice were resistant to a 5 % total body surface area burn or a subcutaneous infection with *P. aeruginosa* alone. However, when mice were inoculated with *P. aeruginosa* after burn injury all MBL-deficient mice died from septicemia, whereas only one-third of wild-type mice died. The authors suggested that MBL-deficiency in humans may be a premorbid variable in the predisposition to infection in burn victims.

2.2.5 Helicobacter pylori

The hypothesis that collectins may play an important role in defending supported epithelial surfaces against microbial pathogens is further supported by the finding that SP-D is expressed in the gastric mucosa, and that expression levels are increased in *Helicobacter pylori*-associated gastritis. Motility is an important factor in *H. pylori* virulence; it enables the organism to migrate to a suitable niche within the gastric mucosa, and inhibition of mobility can prevent colonization [85, 86]. *H. pylori* is bound and aggregated by SP-D, and motility is decreased by about 50 % [87]. Therefore, SP-D could have an important function in gastric innate immune defense.

2.2.6 Chlamydiae

Chlamydiae are intracellular bacterial pathogens which replicate in vesicles within the host cells. This allows them to evade many immunological defense pathways [88].

SP-A is able to aggregate both *C. trachomatis* as well as *C. pneumoniae*, but SP-D only aggregates *C. pneumoniae*, and does so to a lesser extent than SP-A [89]. This difference can probably be explained by differences in sugar binding affinity of SP-A and SP-D. SP-A preferentially binds to mannose and N-acetylglucosamine [90], and these sugars are abundant in the LPSs of *Chlamydiae* [91]. SP-D has a strong affinity to glucose-containing sugars but also binds mannose and other monosaccharides with weak affinity [90]. Both SP-A and SP-D enhance the phagocytosis of *Chlamydiae* into THP-1 cells. Increased phagocytosis is not observed when THP-1 cells are preincubated with collectins before addition of *Chlamydiae*, suggesting that SP-A and SP-D may act as bridges bringing aggregated bacteria and macrophages closer together for more efficient phagocytosis. The extent of aggregation of the bacteria by the two different collectins is also reflected by the pattern of uptake into THP-1 cells. Large numbers of bacteria are seen in some of the cells when *C. pneumoniae* was preincubated with SP-A, whereas preincubation with SP-D leads to the internalization of one or two bacteria into many cells. Interestingly, SP-A enhanced internalization also leads to a proportionally larger amount of viable bacteria in macrophages. Furthermore, macrophage viability is decreased, but it is unclear whether

macrophage cell death is directly caused by SP-A or by the larger numbers of bacteria phagocytosed by the macrophages [89]. In this context, it should be mentioned that *C. trachomatis* as well as *C. pneumoniae* can prevent programmed cell death in infected macrophages and epithelial cells [92, 93]. Since *Chlamydiae* are intracellular pathogens, premature macrophage cell death could be the means by which the host to rid itself of the infection.

More evidence for the role of collectins in the defense of extrapulmonary epithelial surfaces is found in recent studies which indicate that SP-D may also have a protective effect in the female and male reproductive tract. SP-D mRNA and protein are present in the epithelial cells of human cervical tissue, endometrium and oviduct, and SP-D can inhibit infection of an endocervical cell line by *C. trachomatis* [21]. SP-D mRNA and protein are also present in the epithelial cells of prostate glands, and SP-D has been shown to inhibit infection of a prostate epithelial cell line by *C. trachomatis* [20].

MBL has been shown to inhibit the infection of HeLa cells by *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* in the presence and absence of complement [94]. By binding to high mannose moieties of the major outer membrane protein (MOMP), MBL blocks the attachment of the organisms to HeLa cells.

Infection with *C. pneumoniae* has been associated with coronary artery disease [95-97] although those investigations have produced conflicting results [98]. *MBL* variant alleles have also been associated with an increased risk of artherosclerosis [99, 100]. A higher proportion of patients with coronary artery disease were found positive for antibodies directed against *C. pneumoniae* [101]. This difference was only seen in patients carrying variant *MBL* alleles, so the association of C. pneumoniae infection with the development of severe coronary artery disease was solely dependent on individual variations in the mbl-2 gene. It is not clear which mechanism underlies this finding, but it is possible that lower serum levels of MBL lead to a reduced clearance of *C. pneumoniae* or, since MBL has been shown to inhibit entry into cells, more organisms are able to infect host cells.

C. pneumoniae is also associated with the development of asthma [102-106]. However, this seems partially dependent on the modifying effects of MBL, since children who had been infected with *C. pneumoniae* had a higher risk of developing asthma if they carried *MBL* variant alleles, compared to children with a normal *MBL* genotype [107].

2.3 Interactions with Gram-positive bacteria

Lipoteichoic acid (LTA) and peptidoglycan (PepG) are two important cell wall components in Gram-positive bacteria. PepG can elicit excessive release of proinflammatory cytokines from immune cells [108], and LTA has been shown to mediate the attachment of Gram-

Table 3. Interactions of collectins with Gram-positive bacteria			
Species	**Collectin**	**Observed effects –** *Observed effects in case of deficiency*	**References**
Staphylococcus aureus	**SP-A**	Aggregation	[47]
		Increased attachment to and phagocytosis by macrophages	[115, 116]
		Increased phagocytosis by neutrophils	[47]
	SP-D	Aggregation	[47]
		Increased phagocytosis by neutrophils	[47]
	MBL	Binding	[55]
Streptococcus pneumoniae	**SP-A**	Aggregation	[47]
		Enhanced phagocytosis by macrophages	[125]
	SP-D	Aggregation	[47]
		Decreased uptake by macrophages	[52]
		Increased lung inflammation	[52]
Group B Streptococcus	**SP-A**	Binding	[124]
		Decreased association with macrophages	[123]
		Increased systemic spread	[123]
		Greater bacterial burden and lung inflammation	[123]
Alloiococcus otitidis	**SP-A**	Binding	[129]
		Increased phagocytosis by macrophages	[133]
	MBL	Binding	[129]
		Increased phagocytosis by macrophages	[133]

positive bacteria to cell walls [109]. LTA of *Bacillus subtilis* is bound by SP-D, but not by SP-A [110] although interaction of SP-A with LTA cannot be excluded since the binding of collectins was tested using LTA attached to a microtiter plate, which could render possible targets on LTA unavailable for SP-A binding. This would mean that SP-A and SP-D bind different parts of LTA. SP-D can also bind to PepG of *Staphylococcus aureus*, but SP-A binding to PepG has not been demonstrated [110, 111].

SP-A binds to the extracellular domain of Toll-like receptor 2 (TLR2) via the neck domain [111]. The extracellular domain of Toll-like receptor (TLR2) binds to PepG derived from *S. aureus* [112], acting as a cell signalling receptor for PepG [113, 114]. Coincubation of TLR2 with SP-A reduces the binding of sTLR2 to PepG, and PepG- induced nuclear factor-κB (NF-κB) activity is reduced in the presence of SP-A. Furthermore, SP-A significantly reduces the PepG-elicited TNF-α secretion by rat alveolar macrophages and U937 cells, a macrophage-like cell line [111].

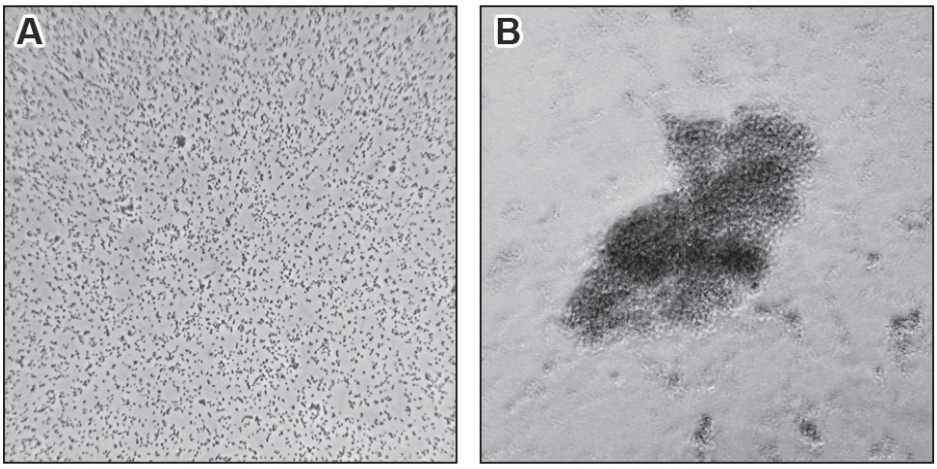

Figure 4. Aggregation of *S. aureus* by porcine SP-D.
(A) control, (B) *S. aureus* incubated with 25 µg/ml pSP-D. *By A. Hogenkamp, 2006.*

This indicates that direct interaction of SP-A with TLR2 alters PGN-induced cell signalling, resulting in a reduced inflammatory response. These findings indicate that collectins play an essential role in the interaction of the innate immune system with Gram-positive bacteria (**Table 3**). *Staphylococcus aureus* and *Streptococcus pneumoniae* have been intensively studied, and some of these investigations will be discussed below.

2.3.1 Staphylococcus aureus

Preincubation of rat alveolar macrophages with SP-A increases the phagocytosis of rat serum-opsonized *S. aureus* [115]. No enhancement of phagocytosis was observed for surfactant-opsonized bacteria, which contradicts the suggested opsonizing function of SP-A. However, since both *S. aureus* and the alveolar macrophages were continuously exposed to SP-A, an opsonic effect cannot be excluded. It is possible that the biological activity of SP-A in whole surfactant preparations differs from purified SP-A, but it seems unlikely that phagocytosis will be enhanced by inactivated SP-A [115]. SP-A mediates attachment of *S. aureus* to rabbit alveolar macrophages [116], but this does not lead to increased ingestion of *S. aureus*. Preincubation of the macrophages with SP-A does not increase attachment,whilst preincubating the bacteria prior to addition to the macrophages does. Since SP-A can also bind to rabbit alveolar macrophages it was suggested that SP-A acts as an opsonizing ligand for *S. aureus* [116]. The differences between the two studies might be explained by differences in SP-A preparations, or intrinsic differences in the response of alveolar macrophages from different animal species (i.e., rats and rabbits).

In another study, SP-A was demonstrated to bind to both monocytes and *S. aureus*. Furthermore, SP-A mediates the phagocytosis of the bacteria by monocytes via interaction with the C1q receptor (C1qR) present on these cells. However, SP-A does not stimulate the intracellular killing of *S. aureus* by monocytes, neither is the release of superoxide radicals stimulated by the presence of SP-A [117] which is in line with the dampening effect of SP-A on a PepG elicited inflammatory response described earlier.
SP-A can aggregate S. aureus (**Figure 4**). *S. aureus* phagocytosis by neutrophils is also enhanced by both SP-A and SP-D [47], although it must be noted that it is unclear whether any discrimination was made between neutrophil-associated *S. aureus* and intracellular bacteria in this study. *S. aureus* is bound and aggregated by SP-A and SP-D, and the level of aggregation seems to correlate with enhancement of bacterial uptake by neutrophils. MBL has also been demonstrated to bind to different clinical isolates of *S. aureus* [54], *S. haemolyticus* and *S. epidermidis* [55], which is probably mediated by MBL binding to β-1,4-linked *N*-acetylglucosamine (GlcNAc) present on PepG [118]. Interestingly, the PepG stimulated production of proinflammatory cytokines by U937 cells is reduced by MBL, while levels of chemokines are enhanced. Therefore, MBL may down-regulate macrophage-mediated inflammation while enhancing phagocyte recruitment [118].
It is not yet known if MBL causes this effect solely by blocking PepG interaction with the cells, but it is possible that MBL also interacts with macrophage cell surface receptors such as TLR2.

CL-P1, one of the more recently discovered collectins, has been shown to be able to bind and phagocytose *S. aureus* particles, thereby acting as a scavenger receptor [24]. This protein was independently identified by another group [119], which also demonstrated specific binding of this scavenger receptor, which they named SRCL, to *S. aureus*. However, an alternative splice variant of SRCL/CL-P1, SRCL II, was shown to be capable of binding to bacteria even though this variant lacked the CRD. No specific binding to *S. aureus* by SRCL/CL-P1 could be demonstrated in another study, but moderate binding to galactan and arabinogalactan was observed for a soluble form of the SRCL/CL-P1 CRD [120]. Since similar structural components are thought to be present in some bacterial cell walls [121, 122] the CRD of SRCL/CL-P1 may still recognize some bacteria. This may prove important since SRCL/CL-P1 has been shown to be expressed on vascular endothelial cells [24] and nurse-like cells [120], suggesting a role in antigen presentation for SRCL/CL-P1 in these immunocompetent cells.

2.3.2 Streptococcus pneumoniae

In vitro studies have shown that SP-A binds to type 25 *Streptococcus pneumoniae*, but in contrast to *S. aureus*, binding of SP-A does not increase association of *S. pneumoniae* to macrophages directly, even when immune opsonins are added to the bacteria [116]. However, fewer bacteria are associated with alveolar macrophages when SP-A-deficient

mice are intratracheally inoculated with group B streptococcus (GBS) [123]. Strain differences may account for these contrasting findings, but other factors present in the lung may also be of influence. Pulmonary infiltration of macrophages is not altered in SP-A-deficient mice [124] but bacterial burden, pulmonary inflammation and systemic dissemination of GBS are increased [123].

SP-A binds to GBS, and co-administration of GBS with exogenous SP-A restores bacterial clearance in SP-A-deficient mice to the same levels as in wild-type mice [124]. Oxygen radical production by alveolar macrophages is also reduced in SP-A-deficient mice infected with GBS compared to wild-type mice. These effects were countered by the co-administration of exogenous SP-A, although oxygen radical production was not restored completely. Macrophages isolated from SP-A-deficient mice were shown to generate less superoxide radicals, suggesting that the absence of SP-A may prevent normal activation of the macrophages. Furthermore, SP-A (but not SP-D) enhances phagocytosis of *S. pneumoniae* by alveolar macrophages. This effect seems to be independent of the (calcium-dependent) SP-A binding to the bacteria, since addition of EDTA has no effect on the uptake enhanced by SP-A [125]. Also, a mutant form of SP-A in which several amino acids in the CRD are replaced with corresponding amino acids of the SP-D CRD could still bind *S. pneumoniae* but failed to enhance phagocytosis.

This augmentation of phagocytosis appears to be regulated by a different mechanism from the Calreticulin/CD91 pathway (described by Gardai and colleagues [63]), since mutants forms of SP-A with intact collagen tails do not enhance phagocytosis, possibly indicating that for alveolar macrophages the CRD of SP-A is involved in enhancing phagocytosis. Scavenger receptor A (SR-A) has been shown to mediate opsonin-independent phagocytosis of Gram-positive bacteria by macrophages [126]. Interestingly, SP-A increases the cell surface localization of SR-A through stimulation of Casein Kinase 2 (CK2) activity. CK2 induced protein phosphorylation probably mediates increased cell surface localization of SR-A. It remains unknown how SP-A stimulates CK2 activity, but it is possible that an as of yet unidentified SP-A receptor is involved. SP-A probably does not bind to SR-A itself, since the SR-A ligands fucoidan and polyinosinic acids do not compete with SP-A for binding to rat bone marrow derived macrophages [127]. In the latter study, a 210-kDa protein (SP-R210) was purified, which was suggested to act as an SP-A receptor on alveolar macrophages and epithelial type II cells. However, it is unclear whether this protein is in some way involved in SP-A mediated SR-A recycling although it has been reported that a polyclonal antibody against SP-R210 is able to inhibit the SP-A dependent uptake of *Mycobacterium bovis* bacillus Calmette-Guerin by macrophages [128].

Overall, SP-D levels do not increase in SP-A-deficient mice infected with GBS, indicating that the lack of SP-A is not compensated by an increased expression of SP-D [123]. However, SP-D levels did seem to be decreased 48 hrs after infection in both wild-type and SP-A-deficient mice, as assessed by Western Blot analysis. Since this involved a

qualitative assessment, it is difficult to draw any firm conclusions, but it is possible that in the absence of SP-A more SP-D is bound to the bacteria than in the wild-type mice. When SP-D or SP-A aggregate bacteria, they can be disposed of through mucociliary clearance. This mechanism could account for lower levels of SP-D in SP-A-deficient mice.

Killing of GBS is not decreased in SP-D-deficient mice, although bacterial uptake by alveolar macrophages is similarly impaired as compared to SP-A-deficient mice [124]. This is strange, since in contrast to SP-A, SP-D does not enhance phagocytosis of *S. pneumoniae* by rat alveolar macrophages [125]. Bacterial killing in SP-D-deficient mice is associated with increased lung inflammation and increased production of oxidant. In addition, isolated alveolar macrophages from SP-D- and SP-A-deficient mice generate significantly more and less superoxide and hydrogen peroxide, respectively, than wild-type alveolar macrophages. Increased oxidant production may contribute to more effective bacterial killing in the lungs of SP-D-deficient mice [52]. These results do not clarify the exact role of SP-D in *S. pneumoniae* infection. SP-D, as well as SP-A, can bind and aggregate *S. pneumoniae*, and both collectins increase neutrophil phagocytosis of the bacteria [47]. Overall, these results suggest that SP-D functions to dampen the inflammatory response during *S. pneumoniae* infection, and that other phagocytes than alveolar macrophages are also stimulated by SP-D.

2.3.3 Alloiococcus otitidis

Another indication that collectins play an important role in the defense against Gram-positive bacteria is given by the finding that SP-A and MBL can bind *Alloiococcus otitidis* [129], a pathogen associated with otidis media with effusion [130, 131]. SP-A and MBL bind to *A. otitidis* in a calcium-dependent manner although the binding of SP-A appears to be in part mediated by its neck domain.

In addition, SP-A and MBL augment the phagocytosis of *A. otitidis* by macrophages. Involvement of SR-A was suggested since addition of fucoidan or poly I, which are ligands for SR-A [132], attenuate phagocytosis of *A. otitidis* in both the presence and absence of SP-A or MBL. In contrast to SP-A, MBL was shown to be present in human middle ear effusion, although various other studies have shown that SP-A is present in the porcine Eustachian tube epithelium [17, 133]. Animal specific expression might account for this difference.

2.4 Interactions with Mycobacteria

Mycobacteria belong to the family of mycobacteriaceae and are classed as Gram-positive bacteria due to their lack of an outer cell membrane, even though they do not retain the crystal violet stain when a Gram staining is performed. *Mycobacterium tuberculosis* is

Table 4. Interactions of collectins with Mycobacteria			
Species	**Collectin**	**Observed effects –** *Observed effects in case of deficiency*	**References**
Mycobacterium tuberculosis	**SP-A**	Binding	[139]
		Increased phagocytosis by macrophages	[140]
		Reduction of level of reactive nitrogen intermediates	[144]
		Increased inflammatory response by macrophages	[149]
	SP-D	Aggregation	[150]
		Reduced phagocytosis by macrophages and monocytes	[150]
		Reduction of intracellular growth	[151]
Mycobacterium avium	**SP-A**	Binding	[143]
		Increased phagocytosis by macrophages	[143]
	SP-D	Binding	[143]
		Increased phagocytosis by macrophages and monocytes	[143]
	MBL	Binding	[153]
		Increased phagocytosis by neutrophils	[153]
Mycobacterium bovis bacillus Calmette-Guerin [BCG]	**SP-A**	Enhanced uptake by macrophages	[128]
		Increased levels of TNF-α and nitric oxide	[144]
	MBL	Increased phagocytosis by neutrophils	[154]
Mycobacterium leprae	**MBL**	*Possible protection against infection*	[157]

an important cause of pulmonary disease, infecting nearly a third of the world's population [134]. These organisms live and replicate in macrophages [135, 136], which they enter by phagocytosis [137]. Interactions of collectins with mycobcteria are listed in **Table 4**.

Lipomannan (LM) and mannosylated lipoarabinomannan (ManLAM), two major mycobacterial cell-wall lipoglycans, have been identified as ligands for SP-A [138]. Specific binding of SP-A to *M. tuberculosis* has indeed been demonstrated [139]. SP-A levels are increased about threefold in bronchoalveolar lavage fluid (BAL) obtained from HIV-infected patients, and this leads to a higher level of phagocytosis of *M. tuberculosis* by alveolar macrophages [140]. The underlying mechanism involves SP-A mediated enhancement of cell surface expression of the mannose receptor. SP-A selectively enhances cell surface MR localization on these cells. This process involves both the N-linked carbohydrates present in the CRD [141, 142] and the collagen-like domain of SP-A,

since truncated mutant forms of SP-A were shown to be much less effective [141], and addition of type V collagen attenuates the enhanced attachment of *M. tuberculosis* to murine alveolar macrophages mediated by SP-A [139].SP-A also binds to *M. avium* in a lectin-like manner, and phagocytosis of the bacteria by rat alveolar macrophages and human monocyte-derived macrophages is
enhanced. This is mediated by enhanced cell surface expression of MR, as with *M. tuberculosis* [143]. Thus, SP-A may influence the uptake of mycobacteria in several ways. SP-A can bind to mycobacteria in a lectin-like manner. The carbohydrates present on the CRD of SP-A do not seem to influence this binding, neither does the collagen domain appear to play a role. However, if SP-A interacts with mycobacteria via its CRD, binding to the organisms would leave only the collagen domain of the protein to interact with macrophages. Since addition of collagen attenuates the SP-A mediated enhancement of MR cell surface expression on macrophages, the only logical explanation would be that SP-A targets a receptor present on the macrophages which in turn mediates MR expression.

Alveolar macrophages from SP-A-deficient mice have reduced MR expression compared to wild-type mice, which again underlines the assumption that the cell surface expression of MR is enhanced when macrophages are exposed to SP-A [141]. It is possible that in a healthy host, SP-A mediates the cell surface expression of MR via its N-linked carbohydrates and its collagen domain, but when the host becomes infected, the CRD will become "preoccupied" with bound mycobacteria, leaving only the collagen domain to interact with the macrophages.

SP-A affects the response to *M. tuberculosis* infection via its modulation of the inflammatory response. Murine alveolar macrophages, primed with interferon-γ (IFN-γ), generate higher levels of reactive nitrogen intermediates (RNI) when they are incubated with *M. tuberculosis*. In the presence of SP-A, the levels of RNI are suppressed. This effect was found to be dependent on the N-linked carbohydrates present on the CRD of SP-A [144]. It appears that SP-A attenuates the cytotoxic response of alveolar macrophages, which, in combination with the enhanced phagocytosis discussed earlier, contributes to the survival of *M. tuberculosis* in the macrophages.

SP-A enhances the uptake of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) by macrophages through interaction with SP-R210 [128], which is a specific SP-A-receptor [127]. In contrast to the findings of Pasula and colleagues [144], BCG-induced production of TNF-α and nitric oxide production is enhanced by SP-A [145].
In addition, SP-A enhances killing of ingested BCG; however, macrophages had not been activated by IFN-γ prior to exposure to BCG and SP-A. Since IFN-γ increases SP-A binding to rat bone marrow derived macrophages [146], Weikert and colleagues speculated that the differences found between their results and those of Pasula and colleagues [144] were due to the altered signalling mechanisms or routes of entry for SP-A mycobacterial complexes. However, IFN-γ stimulates nitric oxide production by murine alveolar

macrophages via a TNF-α-dependent mechanism [147]. Addition of *M. avium* bacilli to the macrophages enhances this response, while addition of *M. avium* to unstimulated macrophages fails to induce nitric oxide production. SP-A inhibits TNF-α secretion, thereby suppressing the nitric oxide production in macrophages stimulated both by IFN-γ alone and by IFN-γ plus *M. avium*. Hussain and colleagues [147] reasoned that the differences in inflammatory responses, mediated by SP-A, could be explained by the differential regulation of nitric oxide production by murine and rat alveolar macrophages. Murine alveolar macrophages require IFN-γ and microbial stimulation to induce nitric oxide production, whereas rat alveolar macrophages do not need prior IFN-γ stimulation [148]. Another explanation for the difference in the observed effects could be the different mycobacterial strains and/or different types of macrophages studied.

Interestingly, SP-A has been found to promote inflammation in the presence of infection by *M. tuberculosis* and inhibit inflammation in uninfected macrophages [149]. This way, areas of the lungs where no infection has been established yet, can be protected from damage caused by inflammatory responses. These findings could in part reflect the model proposed by Gardai and colleagues [63] who found that SP-A suppresses the release of proinflammatory cytokines by macrophages by binding to signal inhibitory regulatory protein α (SIRP α) with the CRD.
However, when the CRD of SP-A binds to LPS or necrotic cell debris, the collagen domain of SP-A binds to CD91/calreticulin, stimulating the production of proinflammatory cytokines.

Different results have been obtained in studies on SP-D in mycobacterial infection. SP-D can bind and aggregate *M. tuberculosis* in a lectin-like manner (but not the avirulent *Mycobacterium smegmatis*). Lipoarabinomannan (LAM) was identified as the major binding molecule for SP-D. Binding of SP-D to *M. tuberculosis* results in a reduced phagocytosis by human monocyte-derived macrophages and human alveolar macrophages [150]. This inhibition of phagocytosis is independent of aggregation of *M. tuberculosis* by SP-D, since SP-D inhibits phagocytosis even at concentrations below those necessary for aggregation. Furthermore, a collagen deletion mutant of SP-D consisting of the neck domain and the CRD, which does not cause aggregation, also reduces phagocytosis of *M. tuberculosis*. In addition, the intracellular growth of *M. tuberculosis* is reduced by SP-D. Binding of SP-D to surface-exposed LAM could block *M. tuberculosis* interaction with the mannose receptor on the macrophages, resulting in reduced phagocytosis. How this reduced interaction contributes to reduced levels of *M. tuberculosis* survival in the macrophages is not clear [151].

In contrast, SP-D increases phagocytosis of *M. avium* by rat alveolar macrophages and human monocyte-derived macrophages, most likely through enhancing the cell surface expression of MR [143]. SP-D-enhanced phagocytosis of *M. avium* is not inhibited by EDTA, but addition of the MR ligands LAM, zymosan or mannan attenuated the effect of

SP-D. It was shown that SP-D increased the cell surface localization of MR on monocyte derived macrophages, but the exact mechanism involved was not investigated further. SP-D binding to *M. avium* is calcium independent, and inhibited by addition of LAM. Surprisingly, the binding of SP-D to *M. avium* derived LAM is calcium-dependent, indicating that that other molecules on the surface of *M. avium* are bound by SP-D in the absence of calcium.

Involvement of the collagen domain, such as has been shown for SP-A [139, 141], appears to be unlikely for SP-D since a collagen deletion mutant also inhibited phagocytosis of *M. tuberculosis* [151]. Because of its cruciform structure, SP-D is more capable of binding to multiple ligands than SP-A, so the contrast between the findings of Kudo and colleagues [143] and those of Ferguson and colleagues [151] may be explained by the structural difference of LAM of *M. tuberculosis* and *M. avium* [152]. It is possible that the affinity of SP-D for ligands involved in increasing the MR cell surface localization is lower when compared to its affinity for LAM of *M. tuberculosis*, keeping the CRDs of the protein "preoccupied with binding to this mycobacterium, whereas the affinity of SP-D for LAM from *M. avium* may be lower, allowing some of the CRDs to interact with the macrophages.

Human recombinant MBL is capable of binding to *M. avium* in a calcium-dependent manner that can be inhibited by mannan. This binding is thought to occur via the terminal mannose residues of mannosylated LAM. Phagocytosis of *M. avium* by human neutrophils was shown to be enhanced when the mycobacteria were preincubated with MBL [153]. However, recombinant rat MBL binding to *M. avium* is calcium independent, and excess crude LAM does not inhibit the binding. Furthermore, MBL does not enhance the phagocytosis of *M. avium* by human monocyte-derived macrophages [143]. However, at higher concentrations of MBL, EDTA did have some effect on MBL binding to *M. avium*. The binding of MBL to LAM was not investigated, and therefore it cannot be excluded that MBL binds to LAM in a calcium-dependent manner, whilst also targeting other ligands on the surface of *M. avium* in the absence of calcium. The differences found concerning the enhancement of phagocytosis remain unclear, but it is likely that they can be ascribed to differences between cell types used for the assessment of phagocytosis (i.e. macrophages and neutrophils) and/or the source of MBL (i.e. human MBL and recombinant rat MBL).

Recombinant human MBL also mediates enhanced phagocytosis of BCG by human neutrophils [154]. In the absence of active complement, the phagocytosis of BCG is only slightly enhanced by MBL, and increasing concentrations of MBL also increase complement activation in the presence of BCG. Therefore, it is likely that complement receptors are involved in the MBL mediated phagocytosis of BCG, especially since complement receptors CR1 and CR3 have been shown to be involved in the phagocytosis of *M. tuberculosis* [155]. The use of serum-free media may also explain why MBL-

Table 5. Interactions of collectins with Mycoplasmas			
Species	**Collectin**	**Observed effects –** *Observed effects in case of deficiency*	**References**
Mycoplasma pneumoniae	**SP-A**	Binding	[167, 168]
		Growth inhibition	[167, 168]
	SP-D	Binding	[162]
		Possible inhibition of infection	[162]
	MBL	Binding	[163]
Mycoplasma pulmonis	**SP-A**	Binding	[164]
		Increased killing by macrophages	[164]
		Slower rate of clearance	[165]

mediated phagocytosis of *M. avium* into human monocyte-derived macrophages was not observed [143]. However, the absence of serum does not inhibit phagocytosis of *M. avium* by human neutrophils [153].

In patients with lepratomous/borderline-lepratomous leprosy, significantly higher levels of MBL have been demonstrated. This disease is caused by *M. leprae*, a mycobacterium which is avidly bound by MBL [156]. Interestingly, low serum levels of MBL were shown to confer protection against *M. leprae* [157]. Taken together, these studies suggest that MBL and the lung collectins are able to enhance phagocytosis, thereby aiding mycobacterial pathogens to infect the host, although multiple other factors have to be taken into account.

2.5 Interactions with Mycoplasmas

Mycoplasmas are members of a class named Mollicutes, members of which are characterized by their small genomes and a permanent lack of a cell wall [158]. The Mollicutes are thought to have diverged from the Streptococcus branch of the Gram-positive bacteria [159]. Mycoplasmas are the smallest self-replicating organisms that are capable of cell-free existence [160]. Most species of Mycoplasma are extracellular pathogens. They use their tip organelle, which has a high concentration of adhesins, to attach to the eukaryotic cell. There are also mycoplasmas which use their tip organelle to enter host cells [161]. The interactions of collectins with mycoplasmas are listed in **Table 5**. Both human and rat SP-D can bind to *M. pneumoniae*, and although binding of human SP-D is entirely calcium-dependent and susceptible to inhibition by carbohydrates, rat SP-D is still capable of binding to isolated *M. pneumoniae* membranes in the presence of EGTA. Protease-treatment of *M. pneumoniae* membranes revealed that the ligand for rat SP-D that requires calcium for binding is protease-resistant, whereas the ligand that is

bound in the absence of calcium is protease-sensitive. The major ligands for human SP-D in *M. pneumoniae* membranes are lipids, indicating that SP-D is likely to recognize *M. pneumoniae* in the alveolar environment, especially since binding of human SP-D is not inhibited by hydrophobic surfactant components [162]. It is not known what the role of SP-D in *M. pneumoniae* infection may be, but binding of the collectin to this pathogen may hamper infection of host cells.

Human MBL is capable of binding *M. pneumoniae*, *M. hominis* and *M. orale*. Furthermore, a disproportionate number of patients with mycoplasma infections was shown to have mutations in exon 1 of the *MBL-2* gene, which is known to result in lower plasma levels of MBL, indicating an important role for MBL in mycoplasma infection [163].

SP-A can bind to *Mycoplasma pulmonis* in a calcium-dependent manner, but addition of mannosyl-BSA has no effect on total binding of SP-A to mycoplasmas, suggesting that the N-linked carbohydrate in the CRD or the neck domain is involved in this interaction. SP-A mediates killing of *M. pulmonis* by IFN-γ activated murine alveolar macrophages isolated from mycoplasma resistant C57BL/6 mice, but SP-A alone does not affect survival of *M. pulmonis*. The addition of SP-A leads to an increase of nitric oxide production by alveolar macrophages resulting in increased mycoplasmal killing [164]. This effect is abrogated by addition of copper-zinc superoxide dismutase, implicating peroxynitrite generated by the alveolar macrophages as the toxic oxygen-nitrogen intermediate [165].

SP-A-deficient mice clear *M. pulmonis* at a slower rate than 129/J mice, a representative parent strain, and CH3 mice, which is a susceptible strain [165]. SP-A-deficient mice against a C57BL/6 background also have a decreased ability to clear *M. pulmonis* as compared to the C57BL/6 controls [166]. These differences are dose and time-dependent. Significantly decreased killing of mycoplasmas in SP-A-deficient mice is only seen at lower doses of *M. pulmonis* and in the early stage of infection. In addition, uninfected C57BL/6 mice have lower bronchoalveolar lavage nitrite and nitrate levels as compared with the SP-A-deficient mice, whereas infected C57BL/6 have higher levels of nitrite and nitrate than SP-A-deficient mice. Therefore, SP-A may help regulate nitric oxide production in response to a specific stimulus, suppressing nitric oxide production in the absence of bacteria, but increasing nitric oxide production during infection. Although higher levels of TNF-α were found after infection in the SP-A-deficient mice [166], they did not seem to correlate with the TNF-α dependent regulation of nitric oxide production found earlier [147].

Human and rat SP-A bind to *M. pneumoniae* with high affinity, in a calcium-dependent manner [167, 168]. For human SP-A, binding is thought to occur at least partially through interaction with a novel surface exposed 65-kDa hSP-A binding protein of *M. pneumoniae*, MPN372, which is 41% similar to the S1 subunit of *Bordetella pertussis* toxin. Recombinant MPN372 inhibited adherence of viable *M. pneumoniae* cells to hSP-A.

Table 6. Interactions of collectins with fungi and yeasts			
Species	**Collectin**	**Observed effects –** *Observed effects in case of deficiency*	**References**
Aspergillus fumigatus	**SP-A**	Aggregation	[171]
		Increased killing by macrophages and neutrophils	[171]
		Increased killing by polymorphonuclear cells	[174]
		Inhibition of allergen binding to IgE	[173]
		Inhibition of histamine release from basophils	[174]
	SP-D	Aggregation	[171]
		Increased killing by macrophages and neutrophils	[171]
		Increased killing by polymorphonuclear cells	[174]
		Inhibition of allergen binding to IgE	[174]
		Inhibition of histamine release from basophils	[174]
	MBL	Aggregation	[171]
		Increased killing by polymorphnuclear cells	[174]
Pneumocystis carinii	**SP-A**	Binding	[183, 184]
		Enhanced attachment to macrophages [rat SP-A]	[186]
		Reduced attachment to macrophages [human SP-A]	[187]
	SP-D	Aggregation	[193]
		Increased attachment to macrophages	[191]
		Slower rate of clearance	[194]
	MBL	Binding	[195]
		Stimulation of respiratory burst in neutrophils	[195]

The exact biological role of MPN372 has not been elucidated yet [168]. However, SP-A also binds to protease-treated *M. pneumoniae*, indicating that the ligand for SP-A has to be either a lipid or a highly protease resistant protein [167]. The protease resistance of MPN372 has not been tested, but it is likely that SP-A targets more than one ligand on the surface of mycoplasmas.

Interestingly, addition of SP-A to cultures of *M. pneumoniae* reduces growth of the organism, as assessed by colony formation, metabolic activity, and DNA replication, indicating that human SP-A has a direct bacteriostatic effect on *M. pneumoniae* [167]. These effects are reversed by the addition of dipalmitoylphosphatidylglycerol but it remains to be seen whether SP-A can still exert this influence in vivo.

Table 6. Interactions of collectins with fungi and yeasts – continued			
Species	**Collectin**	**Observed effects –** *Observed effects in case of deficiency*	**References**
Cryptococcus neoformans	**SP-A**	Binding	[202]
	SP-D	Aggregation	[202]
	MBL	Binding	[202]
	CL-43	Binding	[202]
Candida albicans	**SP-A**	Binding	[208]
		Reduced inflammatory cytokine release	[209]
	SP-D	Aggregation	[210]
		Growth inhibition and decreased hyphal outgrowth	[210]
		Inhibition of phagocytosis by macrophages	[210]
	MBL	Binding	[211]
		Increased survival rates of infected mice	[211]
Histoplasma capsulatum	**SP-A**	Inhibition of protein synthesis	[213]
		Increased cell wall permeability	[213]
		Slower rate of clearance	[213]
	SP-D	Inhibition of protein synthesis	[213]
		Increased cell wall permeability	[213]
		Slower rate of clearance	[213]

2.6 Interactions with fungi and yeasts

Fungi and yeast are eukaryotic organisms that can be very useful to man, for instance in the production of bread and beer (*Saccharomyces cerevisea*) or cheese (*Penicillium roqueforti*). However, there are several fungi and yeasts that can cause serious illness, especially in immunocompromised hosts. Collectins have been shown to interact with various fungi and yeasts (**Table 6**), and these interactions will be discussed below.

2.6.1 Aspergillus fumigatus

Aspergillus fumigatus is an airborne fungus, which causes a wide spectrum of clinical conditions, including allergic bronchopulmonary aspergillosis (ABPA) and invasive pulmonary aspergillosis (IPA) [169, 170]. Human SP-A, human SP-D, and, to a lesser degree, human MBL are capable of aggregating *A. fumigatus* conidia [171]. Interestingly, binding of human SP-A, but not human SP-D, is inhibited by hydrophobic surfactant components, which suggests that SP-A binding observed in vitro may sometimes be an overestimate relative to that occurring in vivo [172].

SP-A and SP-D bind to the purified allergens gp55 and gp45 in a carbohydrate-specific and calcium-dependent manner, probably by interacting with the carbohydrate residues since binding is inhibited by deglycosylating the allergens. SP-A and SP-D increase killing of *A. fumigatus* conidia by alveolar macrophages.

Additionally, the pulmonary collectins act as chemoattractants for neutrophils, which enhances the phagocytosis and killing of *A. fumigatus* [171].
For murine neutrophils, increased killing correlates with enhanced oxidative burst [173]. Human SP-A, SP-D and MBL also enhance *A. fumigatus* phagocytosis and killing by human polymorphonuclear cells [174] MBL induced killing is lower when compared to SP-A and SP-D, but is enhanced in the presence of complement. Both SP-A and SP-D inhibit the binding of *A. fumigatus* allergens to *A. fumigatus*-specific IgE from ABPA patients. Furthermore, histamine release by *A. fumigatus*-stimulated basophils isolated from ABPA patients was blocked in the presence of SP-A or SP-D [173].

ABPA is characterized by the presence of both type I and type III hypersensitivity reactions leading to increased levels of IgE, specific IgE, specific IgG, blood and pulmonary eosinophilia [175]. In a murine model of ABPA, administration of human SP-A, human SP-D and a truncated mutant form of SP-D consisting of the neck domain and the CRD led to a lowering of blood eosinophilia, pulmonary infiltration, and specific Ab levels [176]. Furthermore, the cytokine profiles in treated mice display a shift from a Th2 (pathogenic) to a Th1 (protective) response.

In a murine model of IPA, therapeutic administration of SP-D or MBL increases the survival rate of infected mice, while SP-A has no significant effect on mortality. SP-A and SP-D administration suppresses IgE levels and decreased pulmonary cellular infiltration by eosinophils and lymphocytes in ABPA-mice [174].

When *A. fumigatus*-sensitized mice are challenged SP-A protein levels decrease rapidly [177]. It is unclear what causes the drop in SP-A protein levels but since absolute levels of protein were measured in this study, it is possible that the dilution caused by extravasated plasma proteins leads to a lower SP-A concentration. Another explanation may be that binding to the fungus depletes free SP-A. The measured decrease in SP-A concentration is transient, and levels are restored after 72 hrs. The change in SP-A protein levels are not associated with changes in SP-A expression, since SP-A mRNA levels are actually increased after challenge with *A. fumigatus*. The lowered level of SP-A is associated with an influx of neutrophils and eosinophils, contradicting the earlier mentioned chemoattractant role of SP-A. IL-4 and IL-5 levels are also elevated, contributing to an allergic reaction. In vitro, SP-A inhibitedboth *A. fumigatus*-induced $CD4^+$ T cell proliferation as well as the release of IL-4 and IL-5 by these cells, which suggests that SP-A directly suppressed allergen-stimulated $CD4^+$ T cell function [177].

Lung resistance and airway hyperresponsiveness are significantly improved after treatment with SP-D in *A. fumigatus*-challenged mice [178]. Levels of eotaxin, a

chemoattractant for eosinophils, were shown to be reduced in mice treated with SP-D, correlating with lower eosinophil counts in BAL. A decreased histamine release is observed in lung slices when treated with SP-D during sensitization, suggesting that mast cells are less responsive to allergen-induced degranulation and mediator release after treatment with SP-D. Since this effect was not observed when SP-D was present only during the allergen challenge, it is assumed that the inhibition of histamine release is a result of interactions between SP-D and allergen-specific immunoglobulins [179].

2.6.2 Pneumocystis carinii

Pneumocystis carinii is an opportunistic fungus which often causes life-threatening pneumonia in the immunocompromised host [180]. This pneumonia is characterized by filling of the alveolar spaces with a foamy surfactant-like material which encases the *P. carinii* organisms [181]. *P. carinii* possesses a major surface glycoprotein complex called glycoprotein A (gpA). Differences in the relative molecular mass of gpA are related to the host species from which the organism is derived [182].

SP-A can bind to *P. carinii* in a lectin-like manner, and the carbohydrate moiety of gpA was identified as a binding site for SP-A [183, 184]. Alveolar macrophages have been shown to be necessary for optimal clearance of *P. carinii* [185]. Human SP-A enhances the attachment of *P. carinii* to rat alveolar macrophages and significantly increases phagocytosis. Increased attachment of *P. carinii* to alveolar macrophages appears to be in part mediated by the collagen domain of SP-A since addition of type V collagen reduced the SP-A mediated attachment, although a direct interaction of SP-A with the soluble collagen could not be excluded [186].
In contrast, SP-A reduces binding and phagocytosis of *P. carinii* by human alveolar macrophages in vitro. SP-A binding to *P. carinii* appears to interfere with its recognition by alveolar macrophages [187]. It is not clear how this difference between human and rat macrophages can be explained. In the rodent study, the assays to investigate SP-A mediated attachment were performed at 4°C instead of 37°C, which could lead to differences in the kinetics of SP-A binding. However, the assay to assess SP-A mediated phagocytosis was carried out at 37°C in both studies, therefore, species origin of the alveolar macrophages or differences in glycosylation patterns of *P. carinii* probably account for the conflicting observations.

Finally, SP-A levels are elevated in HIV-infected patients with *P. carinii* pneumonia [188] and an increase in both SP-A protein and mRNA levels has been demonstrated in glucocorticoid-immunosuppressed rats infected with *P. carinii* [189].

SP-D accumulates in the lung during *P. carinii* pneumonia [190], and rat SP-D is a major component of the alveolar exudate that characterizes *P. carinii* pneumonia [191]. SP-D was shown to be present on the surface of *P. carinii* organisms isolated from BAL. SP-D binds *P. carinii* gpA in a lectin-like manner [191, 192] and promotes aggregation of the

organism [193]. SP-D was found to augment binding of *P. carinii* to rat alveolar macrophages [191], although phagocytosis will probably be attenuated by SP-D mediated aggregation, since the size of the aggregates is considerably larger than the macrophages [193]. In the early stage of infection, clearance of *P. carinii* organisms is delayed in SP-D-deficient mice. The introduction of *P. carinii* into CD4-cell-depleted SP-D-deficient mice led to increased lung inflammation, elevated numbers of macrophages, neutrophils and lymphocytes in BAL, higher levels of nitric oxide, elevated expression of nitric oxide synthase and increased levels of 3-nitrotyrosine [194]. It has to be noted however that CD4 positive T-lymphocyte depletion was necessary in order to accomplish an infection in both the wild-type and the SP-D-deficient mice in this study, so it is difficult to ascribe the observed effects directly to the absence of SP-D. Nevertheless, from the results obtained in this and other studies, it seems likely that SP-D plays an important role in host defense against *P. carinii*.

Little is known about the interactions of MBL with *P. carinii*. MBL binds the organism, and can stimulate respiratory burst in neutrophils [195]. It is not known how MBL contributes to the defense against *P. carinii*, although it is possible that MBL binds to the organism when exudation of plasma occurs during infection.

2.6.3 Cryptococcus neoformans

Another pathogen which thrives in the immunocompromised host is *Cryptococcus neoformans*, a basidiomycete yeast-like fungus found mostly in bird droppings and decaying wood [196]. Infection with *C. neoformans* is initiated in the lungs with the inhalation of unencapsulated spores, but from there it can disseminate to other parts of the body, causing meningoencephalitis [197, 198]. An important factor in the virulence of *C. neoformans* is the ability to produce a large capsule, which functions as an antiphagocytic agent [199-201].

SP-A, SP-D, MBL and CL-43 have all been shown to bind acapsular *C. neoformans* in a lectin-like manner. Schelenz et al [202] observed no binding to the encapsulated form of *C. neoformans* by any of the collectins, and only SP-D was capable of aggregating acapsular spores. In contrast, Walenkamp et al [203] reported SP-A binding to acapsular spores and (to a lesser extent) encapsulated spores. EDTA only partially inhibited binding of SP-A to acapsular *C. neoformans*, so part of that appears to be calcium-independent. Attachment of acapsular spores to peripheral blood mononuclear cells was not enhanced by SP-A [203].

In contrast to those earlier reports [202, 203], SP-D has since been shown to bind both acapsular and encapsulated spores in a calcium-dependent manner, with highest affinity for acapsular *C. neoformans* [204]. SP-D binds to the capsular components glucuronoxylomannan (GXM) and mannoprotein 1, and aggregation of the acapsular spores was demonstrated to be inhibited by GXM [204]. It is known that *C. neoformans* sheds

GXM into its environment in large amounts [201], suggesting this shedding may provide C. neoformans with a mechanism to escape clearance mediated by SP-D.

2.6.4 Candida albicans

Candida albicans is a pathogen which can alter its cell morphology from yeast to hyphae, enabling this fungus to penetrate host tissues [205]. *C. albicans* can germinate in the respiratory tract after entering the lung via inhaled air [206], or via aspiration [207].

SP-A can bind to *C. albicans*, and phagocytosis of serum-opsonized *C. albicans* by alveolar macrophages is inhibited by SP-A [208]. SP-A down-regulates proinflammatory cytokine production evoked by *C. albicans* in alveolar macrophages and monocytes, but release of anti-inflammatory cytokines is not influenced by SP-A [209]. This was still observed in the presence of surfactant lipids or serum components, indicating that it is possible SP-A elicits this response in vivo, even under conditions of plasma leakage resulting from lung damage. Preincubating *C. albicans* with SP-A failed to inhibit cytokine release, indicating that the change in cytokine release is caused by an effect of SP-A on the macrophages themselves. Also, TNF-α release was unaltered when macrophages grown on a substrate of SP-A were exposed to *C. albicans*, suggesting the underlying mechanism may require internalization of SP-A [209].

C. albicans is bound and aggregated by SP-D in a lectin-like manner [210]. Interestingly, incubating *C. albicans* with SP-D leads to profound fungal growth inhibition and a decreased hyphal outgrowth. Decreased uptake of radiolabeled precursors is also observed, indicating that protein synthesis is inhibited. It is unclear whether this is a direct effect of SP-D, or if SP-D mediated aggregation leads to a reduced access to the precursors. Furthermore, SP-D inhibits phagocytosis of *C. albicans* by alveolar macrophages, which is probably due to the size of the aggregates [210].

MBL is also capable of binding to *C. albicans* cells and hyphae [211], but the pattern of binding to individual yeast cells is highly variable. This can be explained by changes in the cell surface of *C. albicans*, which becomes more hydrophilic when cells are grown at 37°C. Accordingly, *C. albicans* grown at 37°C was bound by MBL in a more uniform pattern. The putative ligand for MBL has not yet been identified. Interestingly, *C. albicans* yeasts and hyphae in infected tissue were bound by MBL, and administration of MBL increased survival rates in mice intravenously infected with *C. albicans*. This suggests that therapeutic administration of MBL may help to prevent disseminated candidiasis.

2.6.5 Histoplasma capsulatum

Histoplasma capsulatum is a facultative intracellular pathogen which can cause acute or chronic pneumonia. Microconidia enter the lung where they convert to yeast in the extracellular environment or in macrophages, which they enter via phagocytosis. Once

Table 7. Interactions of collectins with viruses			
Species	**Collectin**	**Observed effects –** *Observed effects in case of deficiency*	**References**
Herpes Simplex Virus	**SP-A**	Binding	[217]
		Increased phagocytosis by macrophages	[216]
	MBL	Binding	[218]
		Increased infection	[218]
	conglutinin	Binding	[218]
		Increased infection	[218]
Rotavirus	**SP-D**	Binding	[220]
		Haemagglutination inhibition and neutralization	[220]
	conglutinin	Binding	[220]
		Haemagglutination inhibition and neutralization	[220]
	CL-43	Binding	[220]
		Haemagglutination inhibition and neutralization	[220]
Respiratory Syncytial Virus	**SP-A**	Binding	[223]
		Reduction of viral infectivity	[223]
		Slower rate of clearance	[230]
	SP-D	Binding	[229, 230]
		Enhanced phagocytosis by macrophages and neutrophils	[230]
		Slower rate of clearance	[230]
Human Immunodeficiency Virus	**SP-D**	Binding	[238]
		Reduction of infectivity	[238]
	MBL	Binding	[248-250]
		Inhibition of DC-SIGN mediated transfer to T-cells	[247]
	conglutinin	Binding	[235]

inside the cell, *H. capsulatum* can replicate until the macrophage bursts, releasing a large number of yeast cells into the environment [212].

SP-A and SP-D can inhibit protein synthesis of *H. capsulatum*, and the collectins increase cell wall permeability, leading to protein leakage from the cells. Incubation of *H. capsulatum* with SP-A or SP-D kills the yeast, leading to lower colony counts when higher concentrations of collectin are used. However, when *H. capsulatum* is internalized by macrophages, the organism is protected from these effects. In addition, SP-A-deficient mice are more susceptible to *H. capsulatum* infection than wild-type mice, clearing the yeast at a slower rate [213].

Table 7. Interactions of collectins with viruses - continued			
Species	**Collectin**	**Observed effects –** *Observed effects in case of deficiency*	**References**
Hepatitis B Virus	**MBL**	Binding	[262]
		Possible involvement in progression of disease	[258-260]
Hepatitis C Virus	**MBL**	*Poor responsiveness to interferon treatment*	[268]
		Disease progression	[269]
		Association with chronic infection	[259]
Influenza A Virus	**SP-A**	Binding	[288]
		Inhibition of haemagglutination activity	[280]
		Increased uptake by macrophages	[293]
		Slower rate of clearance	[294, 295]
		Increased inflammation	[294]
	SP-D	Binding	[281, 282]
		Inhibition of haemagglutination activity	[280]
		Increased binding to neutrophils	[280]
		Increased respiratory burst responses by neutrophils	[280]
		Slower rate of clearance	[283]
		Increased inflammation	[287]
		Increased alveolar damage	[287]
	MBL	Binding	[272]
		Reduced virus infectivity	[272]
		Inhibition of viral expansion	[278]
	conglutinin	Aggregation	[274]
		Inhibition of haemagglutination activity	[274]
		Neutralization of virus infectivity	[272], [273]
	CL-43	Binding	[277]
		Inhibition of haemagglutination activity	[277]
		Increased uptake by neutrophils	[277]

It is not known how the collectins increase membrane permeability, but since the effect is calcium-dependent, it is possible that binding of calcium ions causes a conformational change in the CRD. This change could expose regions in the CRD capable of interacting with the cell membrane of pathogens. The CRD of SP-D contains large charged domains [214], which may interact with membrane phospholipids or with other surface molecules.

2.7 Interactions with viruses

Viruses have to infect host cells in order to replicate. This infection can have devastating effects, resulting in serious diseases, such as AIDS, hepatitis and influenza. Anti-viral therapy is difficult, as antibiotics have no effect on viruses and only a few anti-viral drugs are known. Collectins can interact with several different viruses (**Table 7**), and serve as a first-line defense against these invasive pathogens. Some prominent examples are described below.

2.7.1 Herpes Simplex Virus

Members of the herpesviridae family are double stranded DNA viruses which include herpes simplex virus (HSV) I and II. HSV-I infection is commonly described as a cold sore, but can also cause pneumonia in immunocompromised patients [215], while HSV-II causes genital herpes.

SP-A can act as an opsonin for HSV-I, increasing phagocytosis of SP-A-HSV-I complexes by rat alveolar macrophages [216]. The binding of SP-A to HSV-I, which may have a role in preventing virus entry into cells, is thought to occur via the N-linked sugar attached to the CRD of SP-A [217]. It is unclear how SP-A mediates the phagocytosis of HSV-I. It is possible that the collagen domain of SP-A interacts with the macrophages, but C1q does not stimulate phagocytosis although it contains a collagen domain similar to that of SP-A [216]. In contrast, human MBL and bovine conglutinin have been demonstrated to mediate HSV-II infection in mice [218]. Both MBL and conglutinin bind to immobilized HSV-II antigens in a calcium-dependent manner, which is inhibited by carbohydrates.

However, when either collectin was used to pretreat mice infected intravenously with HSV-II the following day, an increase in virus titres in the liver is observed. This suggests that any opsonising activity of MBL and conglutinin provides the virion with an alternative route of entry into susceptible cells. Both MBL-A and MBL-C have been shown to bind to HSV-II, triggering complement activation. MBL-A levels are increased in intraperitoneally HSVII infected mice, while MBL-C levels remained unaffected. MBL-A and -C double knockout mice clear HSV-II from the liver less efficiently than wildtype mice, and this correlates with compromised liver function. Since no differences in viral burden in the spleen or the brain are observed, it is possible that MBL-mediated protection is limited to preservation of liver homeostasis. Intraperitoneal administration of recombinant human MBL, before and throughout the infection, significantly lowers viral burden of the liver [219]. Due to major differences in experimental design, the contrasting results found in those studies are difficult to compare. Route of entry for both the virus and the collectins may be relevant, and the neutralizing effect of human MBL may be

diminished or even counteracted in some way when native MBL-A and MBL-C are present.

2.7.2 Rotavirus

Rotaviruses are unenveloped, double stranded RNA viruses that commonly cause viral gastroenteritis in infants and young animals. Conglutinin, CL-43 and bovine SP-D are capable of binding in a lectin-like manner to a bovine strain of rotavirus, and specific binding to the capsid glycoprotein VP7 was observed. Binding results in haemagglutination inhibition and neutralization of rotavirus infectivity, with CL-43 displaying the highest activity in both types of assay. Since CL-43 exists as a simple trimeric unit, this result suggests that oligomerization is not obligatory for antiviral activity against rotaviruses, at least not for that collectin [220].

2.7.3 Respiratory Syncytial Virus

Paramyxoviruses are single stranded RNA negative-strand viruses, which cause a wide variety of infections. Respiratory syncytial virus (RSV) causes bronchitis and pneumonia [221]. Its two integral envelope glycoproteins mediate cell attachment (G glycoprotein) and fusion of the virion with the cell membrane (F glycoprotein) [222].

SP-A, but not MBL, binds to the F glycoprotein of RSV in a calcium-dependent manner, which neutralizes virion infectivity [223]. Consistent with these results, *MBL* variant alleles are not associated with frequency or severity of RSV [224]. SP-A-deficient mice have been shown to clear RSV at a slower rate than wild-type mice and infiltration of neutrophils after RSV administration is more severe, consistent with an observed increase in macrophage inflammatory protein-2. Administration of human SP-A promoted viral clearance, and pulmonary infiltration by inflammatory cells is reduced to levels comparable to those of wild-type mice [225]. SP-A binding to the G glycoprotein has also been demonstrated [226] but increasing concentrations of SP-A increased the level of RSV infection in a human larynx carcinoma cell-line. Since administration of exogenous SP-A did not affect viral clearance in wild-type mice [225], attachment of SP-A to the G glycoprotein does not enhance infection in vivo.

During infection with another paramyxovirus, parainfluenza, mRNA-levels of ovine SP-A and SP-D are increased, and the increase in mRNA expression is accompanied with a decrease in parainfluenza virus replication [227]. No change in SP-A protein levels was observed in this study (SP-D protein levels were not measured), although it is possible that an increase in SP-A protein levels was not measured because more protein was utilized for binding to the virions. Similar effects were seen in cultured human alveolar type II epithelial cells, which express significantly more SP-A mRNA after RSV exposure, but no changes are measured in SP-D mRNA expression. Intracellular levels of SP-A proteins as

well as SP-A protein secretion are decreased in RSV-infected cells, suggesting that RSV may decrease SP-A mRNA translation as well as SP-A protein secretion [228].

Native human SP-D, as well as a recombinant trimeric form of SP-D consisting of the neck domain and the CRD, are capable of binding to the G glycoprotein of RSV in a calcium-dependent manner [229]. Recombinant rat SP-D has also been shown to bind to G glycoprotein, as well as F glycoprotein [230]. SP-D-deficient mice clear RSV from the lungs at a slower rate than wild-type mice. SP-D deficiency was associated with increased lung inflammation, higher levels of proinflammatory cytokines, and increased inflammatory cell recruitment after infection. Phagocytosis of RSV by alveolar macrophages isolated from SP-D-deficient mice is decreased compared to that of alveolar macrophages isolated from wild-type mice. In vitro, SP-D enhanced phagocytosis of RSV by alveolar macrophages (but not peritoneal macrophages) and neutrophils isolated from wild-type mice [230]. Trimeric SP-D decreased RSV infection in a human larynx carcinoma cell-line, and therapeutic administration of trimeric SP-D to mice infected with RSV leads to decreased RSV replication in the lung, suggesting that SP-D can block RSV-infection by coating the G glycoprotein of the virus [229].

2.7.4 Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS). HIV can enter host cells by means of its surface glycoproteins. The viral glycoprotein (gp) gp160 is cleaved by a host protease into gp120 and gp41, which assemble into trimers [231]. Gp120 generally binds to CD4, expressed by T cells, macrophages and dendritic cells. Binding of gp120 to CD4 allows gp41 to cause fusion of the viral envelope with the plasma membrane, allowing the viral genome and associated viral proteins to enter the cytoplasm [232]. Alternatively, HIV can enter dendritic cells by binding to DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) [233] which has been shown to be the major target for gp120 on monocyte-derived dendritic cells [234]. HIV infection leads to the depletion of $CD4^+$ Tcells, increased susceptibility to opportunistic infection, and eventually to death.

Although the focus has been on MBL (see below), other collectins have also been implicated in the innate defense against HIV. Conglutinin can bind to gp160, and it exerts a dose-dependent inhibition of gp160 binding to CD4 [235]. SP-A levels are increased in BAL isolated from HIV-infected patients, especially in patients with AIDS-related pneumonia [188] or *M. tuberculosis* infection [140]. As discussed earlier, it was observed that SP-A enhances attachment of the mycobacteria to alveolar macrophages [140, 236], and this is inversely correlated with peripheral blood CD4 lymphocyte counts. This could mean that elevated levels of SP-A during the progression of HIV represent an important risk factor to the development of an opportunistic pulmonary infection. The mechanism responsible is not known yet, but it is possible that HIV infection modulates the

expression of SP-A. It does not seem likely that SP-A itself propagates HIV-infection, since SP-A has no effect on uptake of HIV-1 into alveolar macrophages [237] and SP-A does not bind to gp120 [238].

SP-D binds to the HIV envelope via N-linked sugars present on gp120, and reduces infectivity of the virus. SP-D has a higher affinity for gp120 than MBL, and infectivity is reduced at a lower concentration than that required for MBL. This is not a result of differences in the CRD of these collectins, since a recombinant chimeric protein consisting of the CRD of MBL and the N-terminal domain and the collagen domain of SP-D inhibited infection to an even greater extent than native SP-D. A trimeric variant of the chimera did not bind to gp120 to a greater extent than MBL, suggesting that multimerization of SP-D is an important factor in its ability to reduce infectivity [238].

The role of MBL in HIV infection is unclear, with conflicting data reported many studies. MBL levels are elevated in HIV patients [239], but this finding does not correlate with either CD4 cell counts or complement activation. The course of infection does not seem to be influenced by MBL, as MBL levels do not correlate with length of survival, or duration of the period before development of full-blown AIDS [240]. However, homozygous carriers of variant *MBL2* alleles, known to modulate the basal serum level of MBL [16], appear to be at increased risk of HIV infection. Furthermore, MBL2 variant alleles are associated with shorter survival time after diagnosis of AIDS [241, 242]. Normal levels of MBL are measured in HIV positive patients whose infection does not progress into AIDS for a long time (so called long term non-progressors), suggesting a protective effect of wildtype MBL2 [243]. Moreover, a moderate protective effect against bacterial pneumonia was found for serum MBL >650 ng/ml [244].

Higher plasma virus load levels in patients with a MBL*B allelic variant have been reported [245], providing further evidence that gene polymorphisms should be taken into account when studying the course of the disease.

Results from yet another study showed that MBL levels and the capacity of MBL-mediated complement activation were increased in AIDS-patients [246]. In addition, MBL levels increased during highly active antiretroviral therapy, and this increase was associated with a good virologic response. MBL can inhibit DCSIGN-mediated transfer of infectious HIV from dendritic cells to T cells, most likely by preventing gp120 from interacting with DC-SIGN [247]. Furthermore, MBL is capable of binding to primary and passaged isolates of HIV via the mannosylated carbohydrates on gp120 [248-250], and MBL binding to gp120 leads to complement activation [33]. Sialic acid residues on the carbohydrate moiety of gp120 reduce the binding of MBL, which implies that modification of the high-mannose oligosaccharides in the Golgi apparatus may impair collectin-mediated defense against HIV [251, 252]. Indeed, decreased neutralization of passaged HIV by MBL has been demonstrated, although MBL efficiently bound and opsonized infectious passaged

HIV [253]. In spite of the conflicting results described, it is probable that MBL affects the course of the disease.

2.7.5 Hepatitis B virus

Hepatitis B Virus (HBV) is a retro-transcribing virus which belongs to the family of Hepadnaviridae. HBV is a relatively common cause of acute hepatitis, sometimes continuing as chronic liver disease and culminating in cirrhosis and/or hepatocellular carcinoma [254, 255].

Several studies have investigated whether MBL is relevant to HBV infection, but the results have been inconsistent. In some studies, no association between MBL variant alleles and susceptibility to chronic HBV infection [256] or carriage of HBV was found [257]. In contrast, MBL genotypes correlating with high levels of MBL have been associated with recovery from an HBV infection and those correlated with lower levels of MBL were associated with viral persistance [258-260]. Another study found that a mutation in the first exon of the MBL gene (codon 54), which leads to lower levels of functional MBL, is associated with enhanced susceptibility to acute HBV infection [261]. Recently, MBL has been shown to bind Hepatitis B surface antigen (HBsAg) in a lectin-like manner. Co-incubation of MBL with HBsAg and C4 leads to C4 deposition, indicating that MBL activates complement on HBsAg-MBL complexes [262]. This suggests that MBL plays an important role in the clearance of HBV. However, MBL serum levels and MBL gene polymorphisms were shown to be similar in HBsAg-carriers with minimal fibrosis, individuals who recovered spontaneously, and healthy controls [262]. In contrast, low MBL genotypes resulting in low MBL serum levels do associate with the occurrence of cirrhosis and hepatocellular carcinoma in HBsAg carriers [262]. This does not exclude a role for MBL in the acute phase of HBV infection. In humans, cytotoxic T cells help to clear HVB by killing infected hepatocytes [263]. However, noncytopathic mechanisms mediated by IFN-γ and TNF-α also play an important role in viral clearance [264]. Therefore, MBL may play a role in acute HBV infection by regulating cytokines such as TNF-α [265].

2.7.6 Hepatitis C virus

Hepatitis C Virus (HCV) is a single-stranded RNA positive-strand virus, belonging to the Flaviviridae. Infection can lead to chronic hepatitis, and ultimately to cirrhosis and/or hepatocellular carcinoma [266]. Homozygous carriage of variant alleles of codon 54 of the MBL gene seemed to predict a poor response to interferon treatment in patients with chronic HCV infection [267]. The authors hypothesized that HCV RNA would not be completely removed after interferon treatment, unlike in patients with normal levels of functional protein. This preliminary study was followed by a study in which 4 polymorphic loci of the MBL gene were studied in relation to interferon-resistant HCV infection [268]. The results of this latter study further supported the possibility that MBL gene

polymorphisms can explain why some patients are poor responders to interferon treatment. However, direct measurement of MBL serum levels was not performed and only the genotype was correlated to interferon treatment responsiveness. Interferon responders were shown to have a similar concentration of MBL to the non-responders, but Matsushita and colleagues speculated that interferon treatment may stimulate the (non-functional) MBL expression in non-responders to a level similar to that of the responsive patients. However, serum samples were obtained prior to the beginning of interferon treatment [268]. Despite the discrepancies within this study, results from another investigation [269] showed a possible association between possession of the codon-52 mutation (implying low to moderate MBL) and disease progression. Also, lower MBL levels have been associated with chronic HCV infection [259]. No significant associations between MBL levels and presence or absence of cirrhosis, rate of progression of the disease, severity of hepatitis or response to antiviral therapy were found in a more recent study [270]. These contrasting results are probably best explained by the more careful discrimination between groups in the latter study, in which partial responders to interferon treatment were excluded from the analysis.

2.7.7 Influenza A Virus

The orthomyxovirus Influenza A Virus (IAV) is a single-stranded, negative strand-RNA virus. It is a common cause of acute respiratory illnesses and may cause epidemics, even pandemics, of considerable morbidity and mortality. The virus attaches itself to susceptible cells via the SA-receptor present at the tip of its spike protein haemagglutinin (HA), which binds cell membrane glycolipids or glycoproteins containing N-acetylneuraminic acid. The virus is then engulfed by pinocytosis into endosomes, where the viral content is released into the cytoplasm [271]. The viral RNA is transported to the nucleus where new virion mRNA is synthesized and translated by host ribosomes in the cytoplasm. The viral membrane proteins form patches on the plasma membrane, where nucleocapsids are packaged and new virions are released through budding, killing the host cell in the process. These newly formed virions have surface glycoproteins that contain N-acetylneuraminic acid as a part of their carbohydrate structure, which makes them vulnerable to self-agglutination by the haemagglutinin. A major function of the viral neuraminidase (NA) is to remove these residues. Another important function of NA is to ensure release from the cell surface by cleaving sialic acids during the budding stage. In many studies the interactions of collectins with IAV have been investigated, and it is now clear that collectins play an essential role in the innate host defense against this virus. Bovine conglutinin was identified as a β-inhibitor of IAV and can bind to IAV via an N-linked carbohydrate at residue 165 of HA. By binding to this residue, conglutinin inhibits haemagglutination and neutralizes virus infectivity [272, 273]. Conglutinin aggregates IAV particles, acts as an opsonin for IAV and enhances human neutrophil hydrogen peroxide

production [274]. Conglutinin-treated IAV also causes less neutrophil deactivation. The inhibition of haemagglutination and enhancement of neutrophil respiratory burst responses by conglutinin is not dependent on its collagen domain. A trimeric recombinant form of the protein, which lacks the collagen domain, is still able to inhibit haemagglutination, while a similarly truncated form of SP-D shows a reduced haemagglutination inhibition [275]. Indeed, a chimeric protein consisting of the N-terminal domain and the collagen domain of SP-D, and the neck domain and CRD of conglutinin shows an increased potency to inhibit infectivity compared to wild-type recombinant SP-D [276], localizing these activities to the neck domain and/or the CRD of conglutinin.

Bovine collectin 43 (CL-43) is also capable of binding to IAV, but since this protein is expressed as a single subunit (trimer) it does not aggregate the virus. Haemagglutination is inhibited though, and the uptake of IAV by neutrophils is enhanced by CL-43 [277].

MBL can bind to IAV [272], inhibiting IAV infection, a property dependent on the lectin activity of the protein but independent of complement activation [278]. Furthermore, MBL can inhibit viral expansion by preventing viral spread from infected cells to neighboring uninfected cells. In contrast to most other collectins, these antiviral activities involve binding to both HA and NA [278]. Construction of chimeras demonstrated that antiviral activity is greatly enhanced when the neck domain and CRD of MBL are attached to the collagen domain of SP-D [279]. The antiviral activity of the chimera is also greater than that of SP-D. These findings offer an insight into how the different domains of the collectins could be used in the construction of possible agents in antiviral therapies.

SP-D potently inhibits haemagglutination, aggregates IAV (**Figure 5**), increases the binding of IAV to neutrophils and enhances neutrophil respiratory burst responses to the virus [280]. Binding has been shown to occur through interaction of the CRD with oligosaccharides present on HA [281, 282] and NA [282]. In mice, IAV infection leads to higher levels of SP-D in the lung [283]. Like conglutinin, preincubating IAV with SP-D causes reduced neutrophil deactivation, and all of these effects appear to be mediated by the lectin activity of the protein [280]. However, the protective effect of SP-D also relies heavily on the multimerization of the protein [29, 284-286]. Compared with wild-type mice, SP-D-deficient mice clear IAV from the lung at a slower rate. In addition, alveolar destruction is increased and the inflammatory response is enhanced. These effects are countered by the administration of exogenous SP-D. Less glycosylated strains of IAV are cleared more efficiently, corresponding with the binding properties of SP-D to carbohydrates on the viral surface [283]. Expression in SP-D-deficient mice of a fusion protein consisting of the N-terminal and collagen domains of SP-D and the neck and lectin domains of conglutinin can also restore IAV clearance, but lung inflammation was not normalized and damage to alveoli persisted [287]. This indicates that inflammatory effects in response to infection with IAV are regulated differently by SP-D and conglutinin. The

interaction of SP-A with IAV is different from that of most other collectins, since the binding is mediated by interactions between the HA receptor of IAV and sialic acid residues present on the N-linked carbohydrate moiety in the CRD [288]. Interestingly, porcine SP-D has been shown to bear a similar sialic acid-rich carbohydrate moiety on its CRD [289], which enhances its antiviral activity against IAV [290]. This characteristic is especially important for interactions with poorly glycosylated strains of IAV [291]. Since pigs can be infected with both avian as well as human IAV, and major variations in IAV glycosylation may occur, this finding could have important implications for the possible adaptation of avian IAV in pigs.

SP-A can inhibit haemagglutination [280], but not as well as SP-D or MBL [292]. It predominantly acts as an opsonin, resulting in an increased uptake of IAV by alveolar macrophages [293]. SP-A-deficient mice clear IAV at a slower rate than wild-type mice [294, 295], and this can be countered by administration of exogenous SP-A [294]. Uptake of IAV by macrophages does not seem to be affected by the absence of SP-A. The impaired IAV clearance in SP-A-deficient mice is associated with increased lung inflammation and decreased neutrophil myeloperoxidase activity. Furthermore, the absence of SP-A leads to a shift from a Th2 to a Th1 cytokine profile [294]. Recent reports have also implicated lung and salivary scavenger receptor glycoprotein 340 (gp340) in the host defense against IAV. This protein is present in BAL and saliva and is capable of inhibiting haemagglutination and infectivity of IAV. Similar to SP-A, gp340 interacts with IAV via the sialic acid receptor on HA [296]. It has been demonstrated that gp340 shows cooperative antiviral interactions, mostly through increased haemagglutination inhibition and enhanced viral aggregation [297]. Although gp340 has been shown to bind to SP-D [298], the increased antiviral activity does not involve gp340 binding. However, the source of gp340 influences the interaction with IAV. Although salivary and lung gp340 are identical in amino acid sequence, salivary gp340 from one donor was shown to have a greater density of α-(2,3)-linked sialic acids compared with salivary gp-340 from another donor, or several preparations of lung gp-340. Furthermore, by binding to the CRD of SP-D, salivary gp-340 can antagonize the haemagglutination inhibition and viral aggregation of SP-D [299].

Interestingly, preincubation of neutrophils with SP-D prior to exposure to IAV reduces the oxidant responses of these cells. SP-A and gp340 significantly reduce the ability of SP-D to promote a neutrophil oxidant response [300]. Furthermore, viral replication and host response are regulated by SP-D but not SP-A when IAV is glycosylated at a N165 on HA domain [281]. The net effect of these components is an increased phagocytosis of IAV by neutrophils while the respiratory burst is reduced. A schematic illustration of the interactions between the pulmonary collectins and IAV is given in **Figure 5**.

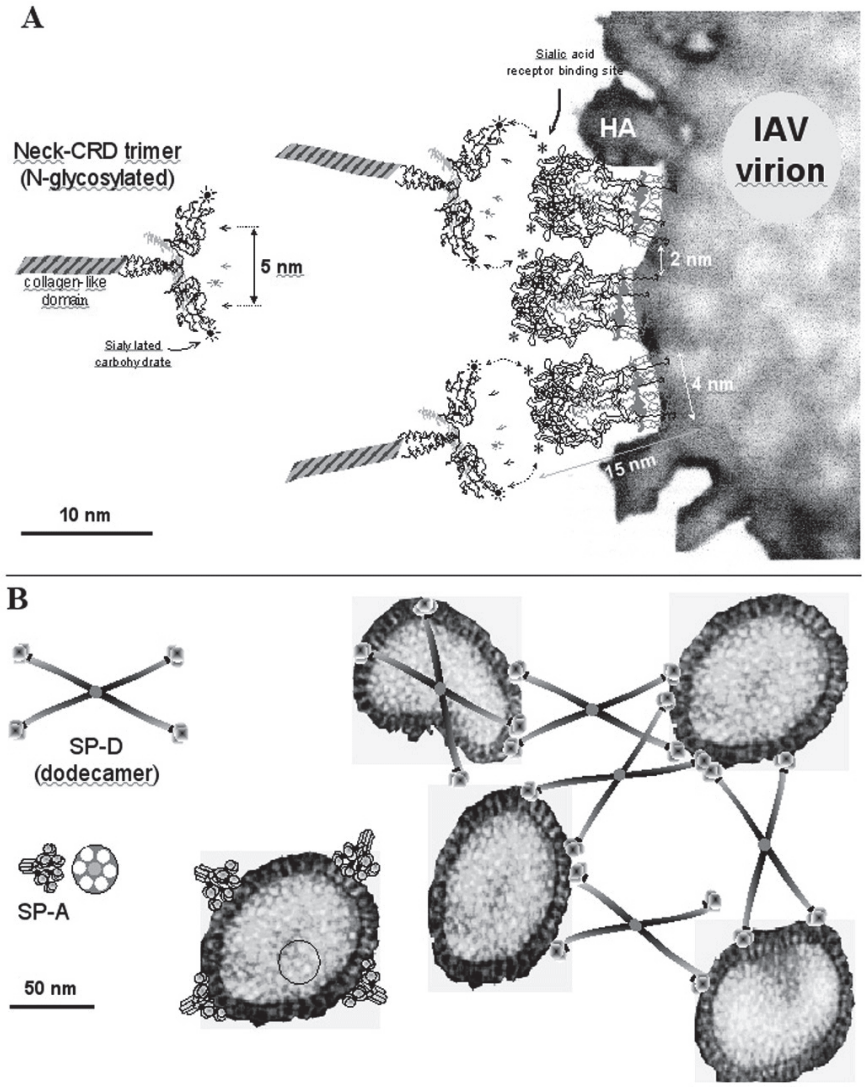

Figure 5. Schematic illustration of the interactions of SP-A and SP-D with Influenza A Virus.
(A) Size comparison of trimeric subunits relative to the size and distribution of HAs present on the surface of an IAV virion; CRDs and HAs are visualized as main structures (adapted from [214] and [320], respectively). Based upon relative dimensions, a single trimeric subunit can bind one, two or even three HA molecules, mediated by either the CRD binding sites (arrowheads) that may interact with sugar residues (not depicted) on the HA1 domain of HA, or by the sialylated N-linked carbohydrates present on SP-A and porcine SP-D with the SA-receptor on HA (asterisks). (B) Model of IAV interaction of a single SP-D molecule with various IAV particles. The octadecameric and relatively condensed organization of 18 CRDs with six or more HA molecules (black circle); the distribution of HA molecules on the IAV particle matches the spatial distribution of the six trimeric CRDs in SP-A. EM-images of IAV particles courtesy of Linda M. Stannard, University of Cape Town, South Africa. http://web.uct.ac.za/depts/mmi/stannard/emimages.html

Conclusions

Collectins can interact with a very broad range of pathogens. They can bind to Gram-negative bacteria (LPS), Gram-positive bacteria (PepG and LTA), mycobacteria (LM and ManLAM), mycoplasma (lipids), fungi (allergenic glycoproteins, GXM), and viruses (viral glycoproteins, HA receptor). Depending on the species and the pathogen involved, collectins can either help neutralize these pathogens directly through aggregation, or indirectly by modulating the phagocytic activity and/or inflammatory responses of immune cells such as macrophages, neutrophils and dendritic cells. Collectins are present in the lung and in serum, but they are also expressed in various other tissues exposed to the outside environment. Overall, these proteins should be regarded as very potent protectors in innate immunity.

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Chapter Three

Effects of Surfactant Protein D on Growth, Adhesion and Epithelial Invasion of Intestinal Gram-Negative Bacteria

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Abstract

Surfactant protein D (SP-D) interacts with various different microorganisms and plays an important role in pulmonary innate immunity. SP-D expression has also been detected in extrapulmonary tissues, including the gastro-intestinal tract. However, its function in the intestine is unknown and may differ considerably from SP-D functions in the lung. Therefore, the effects of porcine SP-D (pSP-D) on several strains of intestinal bacteria were studied by means of bacterial growth assays, colony-count assays, radial diffusion assays and differential fluorescent staining. Furthermore, the effect of pSP-D on the adhesion- and invasion-characteristics was investigated. All bacterial strains tested in this study were aggregated by pSP-D, but only *E. coli* K12 was susceptible to pSP-D-mediated growth inhibition. Bacterial membrane integrity of *E. coli* K12 was affected by pSP-D, but this did not lead to a reduced bacterial viability. Therefore, it is unlikely that pSP-D has a direct antimicrobial effect, and the observed effects are most likely due to pSP-D-mediated bacterial aggregation. The effects of pSP-D on bacterial adhesion and invasion were studied with the porcine intestinal epithelial cell line IPI-2I. Preincubation with pSP-D results in a several-fold increase in adhesion (*E. coli* and *Salmonella*) and invasion (*Salmonella*), but did not affect the IL-8 production induced by the bacteria. Results obtained in this study suggest that pSP-D promotes uptake of pathogenic bacteria by epithelial cells. This may reflect a scavenger function for pSP-D in the intestine, which enables the host to generate a more rapid response to infectious bacteria.

Introduction

Surfactant protein D (SP-D) is a member of the collectin family of proteins, so termed because they are collagenous C-type lectins [1]. SP-D is thought to play an important role in innate immunity by aiding in the first line defense against invading pathogens. It has been shown that SP-D can interact with Gram-negative bacteria [2-6], Gram-positive bacteria [7, 8], yeasts and fungi [9-12], mycobacteria [13, 14], mycoplasmas [15] and viruses [16-19]. This interaction is, in most cases, mediated by the binding of the carbohydrate recognition domain (CRD) to oligosaccharide-complexes [20-22], which are typically found on the surface of microorganisms. Depending on the microorganism, this interaction can lead to aggregation of the microorganism, neutralization by immune cells such as neutrophils or macrophages, or direct killing of the microorganisms. SP-D was originally found to be expressed in the lungs [23, 24], but its expression has also been detected in many extrapulmonary tissues [25, 26], including the gastro-intestinal tract [27-31]. This is interesting, since it is not known what function SP-D may have in this environment. In contrast to the lung, which has to remain sterile to function, the intestine is

inhabited by many microorganisms [32] and the structure and the function of the gastro-intestinal tract are highly dependent upon the presence of a resident microbiota [33, 34]. The gastro-intestinal tract is also the most important route of entry for foreign antigens, including pathogens [35-37]. The intestinal epithelium serves as an important physical barrier [38], and epithelial cells are also involved in innate immune defenses, producing mucus [39], defensins [40, 41], chemokines and cytokines [42]. It would be very interesting to know whether SP-D could be added to this list of protective agents present in the gut.

To investigate this possibility, we chose to examine porcine SP-D (pSP-D) for four reasons: (1) pSP-D has been shown to be present in the porcine gastro-intestinal tract [27-29], (2) enteric pathogens in pigs pose a considerable risk to both animal and public health [43-46], (3) the economic impact of losses in pig rearing caused by enteric pathogens is considerable [47], and (4) the pig could be very useful as a mammalian animal model, as genome analysis shows pig to be much more closely related to humans than are rodents. Furthermore, the great similarity between pig and human in the gastro-intestinal tract makes the pig an excellent model for gastrointestinal research [48].

In this study, we investigated the interactions of pSP-D with several intestinal pathogens isolated from pigs. In addition, the effect of pSP-D on the adhesion- and invasion characteristics of bacteria was studied using the porcine ileal epithelial cell line IPI-2I.

Materials and Methods

Bacterial strains and culture conditions

The following strains were used: *Escherichia coli* K12 [49], *Salmonella enterica* serotype Typhimurium DT 104 [50], *Salmonella* serotype Enteritidis strain 90-13-706 [51], Group B *Salmonella*, *Escherichia coli* K88 174, *Escherichia coli* K88 209, *Escherichia coli* K88 483, (clinical isolates, provided by Dr. Engeline van Duijkeren, Department of Infectious Diseases and Immunology, Clinical Infectiology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands), *Escherichia coli* RM200Q [52] and *Escherichia coli* RM201C [52] (a gift from Dr. Alison Weiss, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH). Overnight cultures were prepared freshly for every experiment by cultivation from a frozen stock at 37°C in Luria Bertani broth (LB) (Trypton (10 g/l), yeast extract (5 g/l), NaCl (10 g/l) in 10 mM Tris, pH 7.4).

Purification of porcine SP-D

pSP-D was purified as described earlier [53]. Lungs from 80 week-old pigs (Animal Laboratory, Utrecht University, Utrecht, The Netherlands) were lavaged three times with approximately 50 ml lavage buffer per kg body weight. Lavage buffer consisted of 20 mM

Tris-HCl (pH 7.4), 150 mM NaCl, and 10 mM ethylenediaminetetraacetic acid (EDTA) and was kept at 4°C. EDTA was added to prevent binding of pSP-D to ligands. After 1 hr, the bronchoalveolar lavage fluid (BALF) was centrifuged at 150 × *g* for 10 min to remove cells. The surfactant fraction was removed by centrifugation (20,000 × *g* for 2 h, 4°C). Subsequently, Tris-HCl (pH 7.4) was added to a final concentration of 50 mM. $CaCl_2$, and Tween-80, sodium azide, and phenylmethylsulfonylfluoride respectively were added to final concentrations of 15 mM, 0.1% (vol/vol), 0.02% (wt/vol), and 0.1 mM. The pH was readjusted to 7.4. Mannan-sepharose (Sigma, St. Louis, MO), equilibrated in 50 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, and 0.05% (vol/vol) Tween-80, was added to the recalcified BALF (1 ml bed volume of mannan-sepharose per liter BALF), and the mixture was stirred overnight at 4°C. Sepharose beads were packed into a column (8 × 30 mm) and washed with five bed volumes washing buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 500 mM NaCl, 0.05% Tween-80, pH 7.4). This washing procedure was repeated using washing buffer without Tween-80. pSP-D was eluted with a 50-mM Tris-HCl (pH 7.4) buffer containing 5 mM EDTA, and concentrated using Amicon Ultra centrifugal filter units with a molecular weight cutoff of 100 kDa (Millipore, Billerica, MA), after which the buffer was changed to 5 mM Tris, 150 mM NaCl, pH 7.4 by repetitive washing. Purified pSP-D and low-molecular weight filtrates (LMWF) from the concentration steps were stored in aliquots at -20°C. Recombinant rat SP-D (rrSP-D) was a kind gift from Dr. Jo Rae Wright (Department of Cellular Biology, Duke University Medical Center, Durham, NC). Rat SP-D (rSP-D) was a kind gift from Dr. Frank McCormack (Division of Pulmonary/Critical Care Medicine, University of Cincinnati College of Medicine, Cincinnati, OH). Unless stated otherwise, protein dilutions used in this study were prepared freshly using minimal medium (5 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, 0.1 g/l Trypton, pH 7.4; abbreviated MM-Ca^{2+}).

Electrophoresis and Western Blot Analysis

Proteins (0.1-1 µg/lane) were analyzed by SDS-PAGE as described by Laemmli [54] using 10% polyacrylamide gels. Protein bands were visualized by silver staining. For immunoblot analysis, proteins (0.1-1 µg/lane) or homogenates of pig intestinal mucosal scrapings (0.2-0.3 µg protein/lane) were transferred onto nitrocellulose membrane. Immunostaining was performed using polyclonal antibodies raised in rabbit against pSP-D, which had been affinity purified using a Sepharose-pSP-D column. Primary antibodies were detected by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Nordic Immunological Laboratories, Tilburg, The Netherlands).

Enzymatic Digestions

Collagenase

Purified pSP-D was incubated with collagenase (type VII from *Clostridium histolyticum*,

Sigma; 1 U/2 µg SP-D) in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 10 mM $CaCl_2$ for 16 hr at 37°C. After incubation, the collagenase-resistant fragment (CRF) was affinity purified using mannan-sepharose (0.5 ml bed volume per 0.5 ml incubation mixture). The matrix-bound CRF fraction was isolated as described for the isolation of intact pSP-D, with the exception of the use Microcon centrifugal filter units (MWCO 10 kDa) (Millipore, Billerica, MA) for concentration and washing.
Purified CRF was stored in aliquots at -20°C.

Endo-/N-glycosidase F
N-deglycosylation was performed using a mixture of Endoglycosidase-F and N-Glycosidase-F (NGF; from *Flavobacterium meningosepticum,* Roche Diagnostics GmbH). Purified pSP-D (10 µg/15 µl) was mixed with an equal volume of 0.1 M sodium phosphate (pH 7.0), 50 mM EDTA, followed by the addition of 0.5 U enzyme mixture. After incubation at 37°C for 16 h, deglycosylated pSP-D (pSP-D_{deglyc}) was concentrated and washed using Microcon centrifugal filter units (MWCO 100 kDa) (Millipore, Billerica, MA) and stored at -20°C.

Bacterial growth assays
Overnight cultures of bacteria were grown at 37°C in LB medium. Bacteria were inoculated into fresh LB-medium and grown to mid-log phase at 37°C, spun down, and resuspended in MM-Ca^{2+} at an OD_{620} of approximately 0.2 absorbance units, depending on the bacterial strain. This suspension was diluted 100 times, corresponding to 2 x 10^6 cfu/ml (colony forming units/ml). Of this suspension, a 10 µl aliquot was incubated in a honeycomb microplate (Oy Growth Curves AB Ltd., Finland) with either 10 µl of medium, 10 µl of LMWF, pSP-D (50 µg/ml), Bovine Serum Albumin (BSA) (Carl Roth GmbH, Karlsruhe, Germany) (40 µg/ml) or Polymyxin B sulphate (PMB) (Sigma, St. Louis, MO) (40 µg/ml). Additionally, standards of decimally diluted bacterial suspension (2 x 10^6 cfu/ml - 2 x 10^3 cfu/ml) were incubated in MM-Ca^{2+}. In the case of *E. coli* K12, bacteria were also incubated with rat SP-D (rSP-D) (50 µg/ml), recombinant rat SP-D (rrSP-D) (50 µg/ml), CRF (25 µg/ml), or pSP-D_{deglyc} (50 µg/ml).

After 3 hrs of incubation, either 10 µl MM-Ca^{2+} or 10 µl minimal medium containing 8 mM EDTA instead $CaCl_2$ (MM-EDTA) was added to the wells. The plates were left at room temperature for 1 hr, after which 170 µl LB (preheated to 30°C) was added. LB contains approximately 0.1 mM $CaCl_2$, which is insufficient to recalcify samples treated with MM-EDTA. The honeycomb microplates were placed in the Bioscreen C MBR analyzer system (Oy Growth Curves AB Ltd., Finland). To maximally discern between differences in growth curves, bacteria were grown at 30°C for 24 hrs, whilst being continuously shaken. Bacterial growth was monitored every 20 min with a broad band filter (405 nm to 600 nm).

Radial Diffusion Assay

Inhibition of bacterial growth was also investigated using a radial diffusion method [55]. Molten Biozym LE agarose (1.0%) (Biozym, Hameln, Germany) in buffer containing 10 mM Tris, 5 mM $CaCl_2$ and 1.0 % LB medium was mixed with mid-log phase *E. coli* K12 (final density 2×10^6 cfu/ml agarose) at a temperature of 40°C and allowed to harden by cooling. Agar composed of tryptone (40 g/l) (Oxoid, Hampshire, United Kingdom), yeast extract (20 g/l) (Becton Dickinson, Franklin Lakes, NJ), and agarose (1%) was layered on top. Wells were punctured in the agar to which either 5 µl of LMWF, BSA (1 mg/ml), PMB (1 mg/ml), pSP-D (0.5 mg/ml), pSP-D_{deglyc} (100 µg/ml), CRF (50 µg/ml), rSP-D (87 µg/ml) or rrSP-D (200 µg/ml) were added. After overnight incubation at 37°C, the plates were visually inspected for clearing around the wells.

Bacterial permeability assay

Bacterial membrane integrity was assessed by staining with the permeant fluorescent probe SYTO 9 and the impermeant fluorescent probe propidium iodide (*Bac*Light; Molecular Probes Europe BV, Leiden, The Netherlands). Mid-log phase *E. coli* K12 were spun down and resuspended in MM-Ca^{2+} at an OD_{620} of 0.1 absorbance units, corresponding to approximately $1x10^8$ cfu/ml. One hundred µl of bacterial suspension was incubated with either MM-Ca^{2+} (5 µl), LMWF (5 µl), pSP-D (50-150 µg/ml final concentration), rat SP-D (5 µg/ml final concentration), BSA (100 µg/ml final concentration) or PMB (25 µg/ml final concentration). After addition of the dye-mixture (SYTO9 : propidium-iodide = 1:1), fluorescence was measured at excitation and emission maxima of 485 nm and 530 nm for SYTO 9, and 485 nm and 630 nm for propidium iodide [56]. For fluorescence microscopy, bacteria were grown and incubated as described above. After addition of the dye-mixture, 10 µl of the bacterial suspension was trapped between a slide and coverslip and observed in a fluorescence microscope. However, since bacterial density was quite low for some of the control samples, bacteria were spun down and resuspended in approximately 20 µl MM-Ca^{2+} to facilitate observation.

Colony-count assay

Growth conditions and incubations were the same as those described for the bacterial growth assays. After 3 hrs of incubation at 37°C, samples were diluted 5000x in MM-Ca^{2+} or MM-EDTA. Of these diluted suspensions, 100 µl was spread on Trypton Soy Agar plates (Oxoid, Hampshire, United Kingdom). Plates were incubated overnight at 37°C and colonies were counted the following day.

Cell-culture

The IPI-2I cell line (ECACC 93100622) was established from the ileum of an adult boar (d/d haplotype) and immortalized by transfection with an SV40 plasmid (pSV3-neo) [57].

IPI-2I cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Cambrex Bio Science, Verviers, Belgium) supplemented with 1% (v/v) non-essential amino acids, 50 µg/ml gentamycin, 10 mM $NaHCO_3$, 1.7 mM glutamine, 0.024 U/ml Insulin, 25 mM HEPES, and 10% (v/v) FCS. IPI-2I cells were seeded into 24-well tissue-culture plates (2 cm^2 / well), with 5 x 10^4 cells/cm^2 and grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. In all experiments, IPI-2I cells were used between passages 6 and 26.

Adhesion and invasion experiments

IPI-2I cells were grown to confluency (5 days) in 24-well tissue-culture plates. The medium was changed to plain low-glucose DMEM (Imperial Laboratories, Hampshire, UK) at least 1 hr before the start of the experiment. Mid-log phase bacteria were spun down, resuspended and diluted in MM-Ca^{2+} to a bacterial density of 2 x 10^6 cfu/ml. Bacteria were incubated for 3 hrs at 37°C with either 25 µg/ml pSP-D, or a corresponding volume of MM-Ca^{2+}. After three hrs, warm plain low-glucose DMEM was added to the bacteria, and 0.5 ml of this suspension was added to each well.

In the adhesion assay, medium containing non-adhering bacteria was removed after 1 hr of incubation, and the monolayers were washed three times with warm plain low-glucose DMEM. The monolayers were lysed in 1% Triton-X100 in MM-Ca^{2+} or MM-EDTA at room temperature for 5 min in order to release the bacteria. The suspensions were serially diluted 10-fold and 100 µl of each dilution was plated on TSA-plates. Colonies were counted the next day. Numbers of cell-associated bacteria were calculated as total (adhering and intracellular) bacterial cfus.

In the invasion assay, medium containing non-adhering bacteria was removed after 1 hr of incubation, and the monolayers were washed once with warm plain low-glucose DMEM. To determine the number of bacteria that invaded the epithelial cells, extracellular bacteria were killed by incubating the IPI-2I cells for 2 hrs with 0.5 ml of 300 µg/ml colistin in warm plain low-glucose DMEM. Cells were washed three times with plain low-glucose DMEM, and finally lysed in 1% Triton X-100 in MM-Ca^{2+}. The number of intracellular bacteria was determined by colony-plating as described above.

ELISA

The IL-8 secretion of IPI-2I cells was determined in the medium using the human IL-8 Cytosets™ antibody pair kit containing matched, pretitered, and fully optimized capture and detection antibodies, recombinant standard and streptavidin-horseradish peroxidase (Cat. No. CHC1304) (Biosource Europe SA, Nivelles, Belgium). The assay was performed following the manufacturer's instructions.

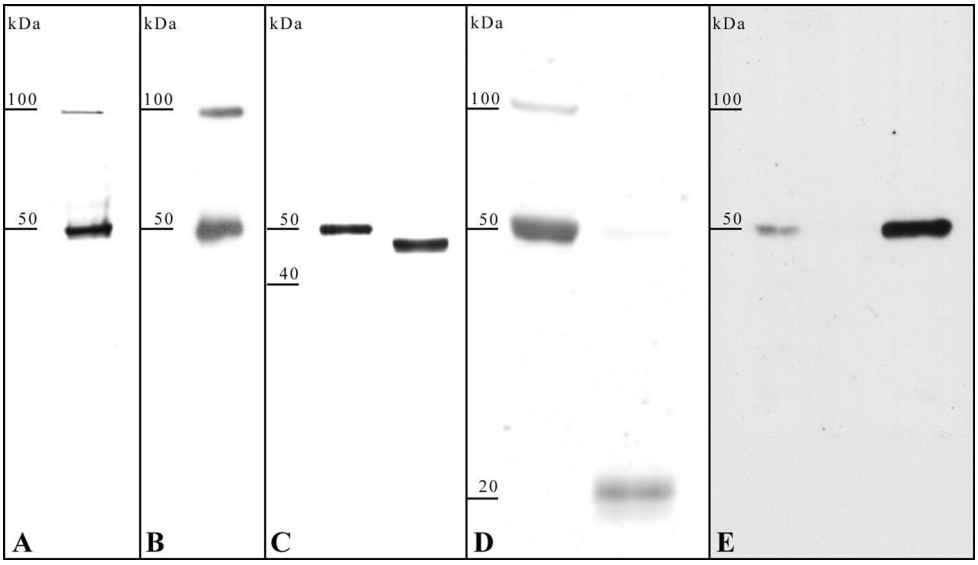

Figure 1. Purification and enzyme digestions of pSP-D.
pSP-D was purified from BALF using mannan-sepharose affinity chromatography, and concentrated by filtration. pSP-D content was analyzed by silver-staining (A) and immunoblotting (B). (C): pSP-D was N-deglycosylated using Endoglycosidase-F and N-Glycosidase-F. *Left lane: pSP-D, right lane: N-deglycosylated pSP-D*. (D): Collagenase-resistant fragments were produced by treating pSP-D with collagenase. *Left lane: pSP-D, right lane: collagenase-treated pSP-D.* (E): Immunoblot analysis shows that pSP-D can also be detected in porcine intestinal mucosal scrapings and, similar to pulmonary pSP-D, appears as a 50 kDa band. *Left lane: pSP-D in porcine intestinal mucosal scrapings, right lane: pSP-D isolated from BALF.*

Statistical Analysis
Statistical analysis was performed using SPSS version 12.0.1 for Windows. For experiments with multiple treatment groups, average data were analyzed by one-way analysis of variance followed by a Bonferroni test for significant difference, in which $P < 0.05$ was considered to indicate statistical significance.

Results

Purification and enzymatic treatment of porcine SP-D
pSP-D was purified from bronchoalveolar lavage from adult pigs as described earlier [53]. The average yield of the purification procedure was 1.0 – 2.0 mg pSP-D from BALF derived from one animal. The EDTA-eluted fraction of mannan-bound pSP-D released pure pSP-D (**Figure 1A** and **1B**). The minor band of approximately 100 kDa represents non-reducible dimeric SP-D [58]. pSP-D was treated with NGF (**Figure 1C**) which resulted in a

size shift of approximately 3 kDa, indicating that the N-linked carbohydrates had been sufficiently removed [53]. Furthermore, pSP-D was treated with collagenase to obtain the so-called collagenase resistant fragment (CRF) which comprises the neck domain and the CRD (**Figure 1D**). The presence of pSP-D was also demonstrated in porcine intestinal mucosal scrapings (**Figure 1E**).

Bacterial growth assay

In order to assess effects on bacterial growth, 10 µl aliquots of a 2 x 10^6 cfu/ml bacterial suspension of all bacterial strains were incubated with either MM-Ca^{2+}, LMWF, pSP-D, BSA, or PMB. In the case of *E. coli* K12, the effects of rSP-D, rrSP-D, pSP-D_{deglyc} and CRF were also investigated. A decimal dilution of the bacterial suspension was added as a standard of bacterial growth. After three hrs, MM-Ca^{2+} or MM-EDTA was added. Because collectin-mediated bacterial aggregation is dependent on the presence of Ca^{2+}, adding EDTA caused aggregates to disintegrate (as assessed by light microscopy, not shown). LB-medium was added and bacterial growth was monitored for 24 hrs using the Bioscreen C MBR. Of all the bacterial strains tested, only *E. coli* K12 appeared to be responsive to inhibition of growth by pSP-D, rSP-D and rrSP-D (**Figure 2A**). Bacteria treated with PMB did not grow at all (not shown) and no effect was observed when bacteria were treated with BSA (not shown). Incubation with LMWF obtained from the concentration of pSP-D did not affect bacterial growth (not shown), indicating that the observed effects can only be related to the presence of pSP-D. A significant increase in time to reach an $OD_{405-600}$ of 0.5 absorbance units was observed for bacteria treated with pSP-D, rSP-D and rrSP-D, indicating a reduced specific rate of growth. For pSP-D and rrSP-D, the increase in time was similar to that of 10 x diluted control samples. In the case of rSP-D this slow growth was even more pronounced, exceeding the time 100x diluted control samples needed to reach this density. No effect on growth rates was observed when bacteria were treated with CRF, suggesting that aggregation by pSP-D underlies the observed effects in pSP-D treated samples. However, pSP-D_{deglyc}-treated samples did not differ from the controls either, although pSP-D_{deglyc} was observed to be capable of aggregating *E. coli* K12 (as assessed by light microscopy, not shown). Additionally, the maximal bacterial density was reduced in all samples treated with pSP-D, rSP-D and rrSP-D (**Figure 2B**). The addition of EDTA did not abolish these effects, indicating that the lower bacterial densities measured during the experiment were not a result of aggregates interfering with measurements taken by the Bioscreen C. Taken together, these results suggest that pSP-D, rSP-D and rrSP-D are capable of inhibiting bacterial growth.

Radial Diffusion Assay

Radial diffusion assays were performed to examine whether the results obtained in the bacterial growth assays were caused independently of collectin-mediated aggregation of the

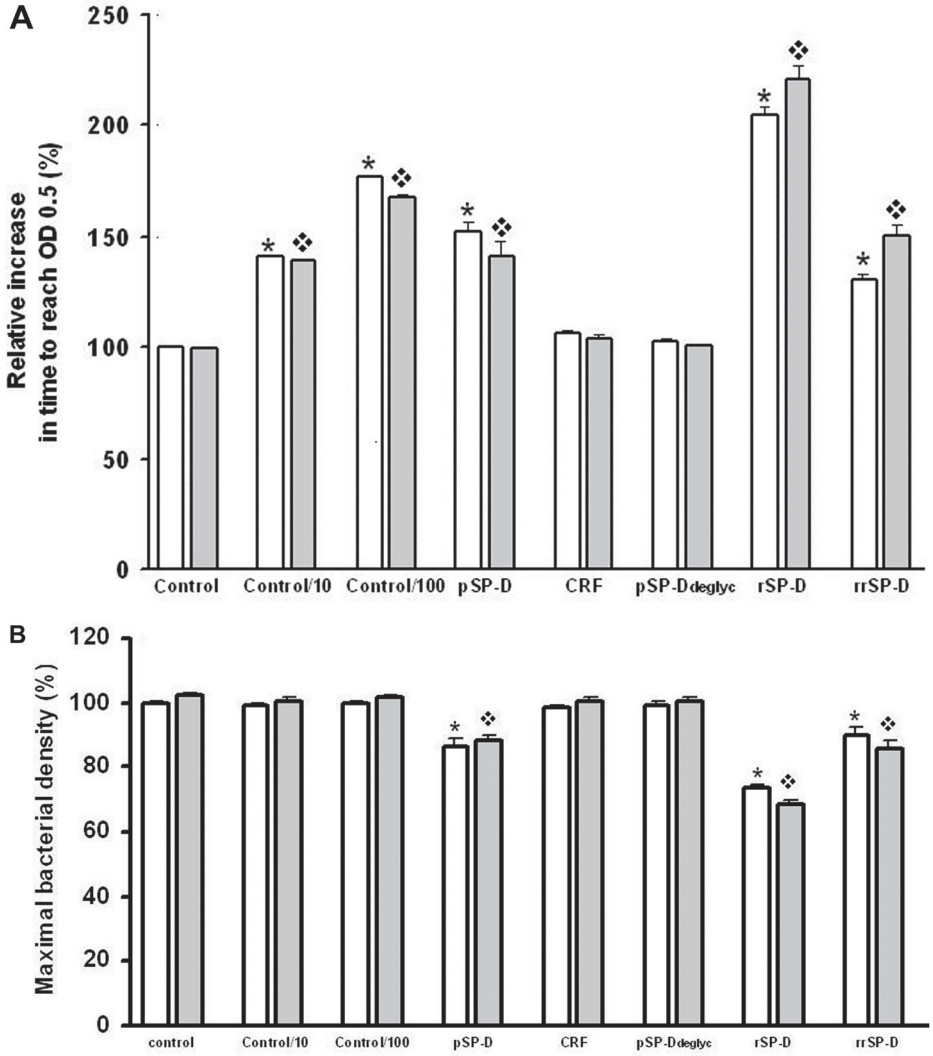

Figure 2. Incubation of *E. coli* K12 with pSP-D leads to bacterial growth inhibition.
(A) A significant increase in the time to reach an OD of 0.5 was observed when *E. coli K12* were treated with pSP-D, rSP-D and rrSP-D, indicating a reduction in specific growth rates. Values are given as a percentage of controls treated with MM-Ca^{2+} (mean: 450 min, SEM: 3.81). (B) A significant reduction in the maximal density achieved was observed in samples treated with pSP-D, rSP-D or rrSP-D. Values are given as a percentage of controls treated with MM-Ca^{2+} (mean = 0.9197, SEM = 0.0061). White bars: treated with MM-Ca^{2+}, gray bars: treated with MM-EDTA. * = significantly different from controls treated with MM-Ca^{2+}, ❖ = significantly different from controls treated with MM-EDTA. (p <0.05)

bacteria. Wells were punctured into agar containing mid-log phase *E. coli* K12 (2×10^6 cfu/ml), to which either pSP-D, pSP-D_{deglyc}, CRF, rSP-D rrSP-D, BSA or PMB was added. After overnight incubation at 37°C, no clearing around any of the wells was observed, except for those to which PMB had been added (not shown).*Bacterial membrane permeability*

Earlier studies have reported that collectins can directly increase bacterial and fungal membrane permeability [3, 12]. Therefore, bacterial membrane integrity was assessed by staining with the permeant fluorescent probe SYTO 9 and the impermeant fluorescent probe propidium iodide. SYTO9 is a green fluorescent nucleic acid stain capable of penetrating bacterial cell membranes of live and dead organisms. Propidium iodide is a red fluorescent nucleic acid stain which can only cross bacterial membranes if they are damaged, i.e., cells will stain red when their membranes have been damaged. Mid-log phase bacteria were incubated for 3 hr at 37°C with MM-Ca^{2+}, LMWF, pSP-D, rSP-D, BSA or PMB. After the incubation the dye mixture was added and cells were inspected for differential nuclear staining in a fluorescence microscope. No effects on membrane permeability were observed for any of the strains incubated with BSA or LMWF (not shown), and propidium-iodide staining was observed for all strains incubated with PMB (not shown). Bacterial aggregation was not observed in any of the samples incubated with MM-Ca^{2+} (**Figure 3**), LMWF, BSA or PMB (not shown). Staining with propidium-iodide was observed for pSP-D-treated-*E. coli* K12. All strains were to some extent aggregated by pSP-D (**Figure 3**) or rSP-D (not shown). Aggregation-patterns varied between bacterial strains; whereas both *E. coli* K12 and *S. typhimurium* DT 104 were aggregated into a few large clumps with very few free bacteria, the other strains were aggregated into numerous small clumps and a higher proportion of bacteria was not aggregated. However, red-to-green ratios of fluorescence emitted by collectin-treated *E. coli* K12 did not differ from those treated with MM-Ca^{2+}, LMWF or BSA (not shown).

Colony Count Assays

To quantify the effects of pSP-D on bacterial viability, colony count assays were performed. When bacteria are aggregated, they will form one colony on an agar plate, leading to lower colony counts. However, when the aggregates are disrupted, viable single bacteria released from the aggregates will form colonies, leading to higher colony counts. This effect was indeed observed when *E. coli* K12, *E. coli* K88 209 and *S. typhimurium* DT 104 were incubated with pSP-D (**Figure 4**). The finding that colony counts were restored to control values after addition of EDTA indicates that no bacteria were killed. Aggregation of the other strains was insufficient to cause lower colony counts. Incubation with rSP-D also led to lower colony counts for *E. coli* K12 and *S. typhimurium* DT 104 when bacterial suspensions were diluted in MM-Ca^{2+}. Dilution in MM-EDTA resulted in significantly lower colony counts for rSP-D-treated *S. typhimurium* DT 104 as compared to the control,

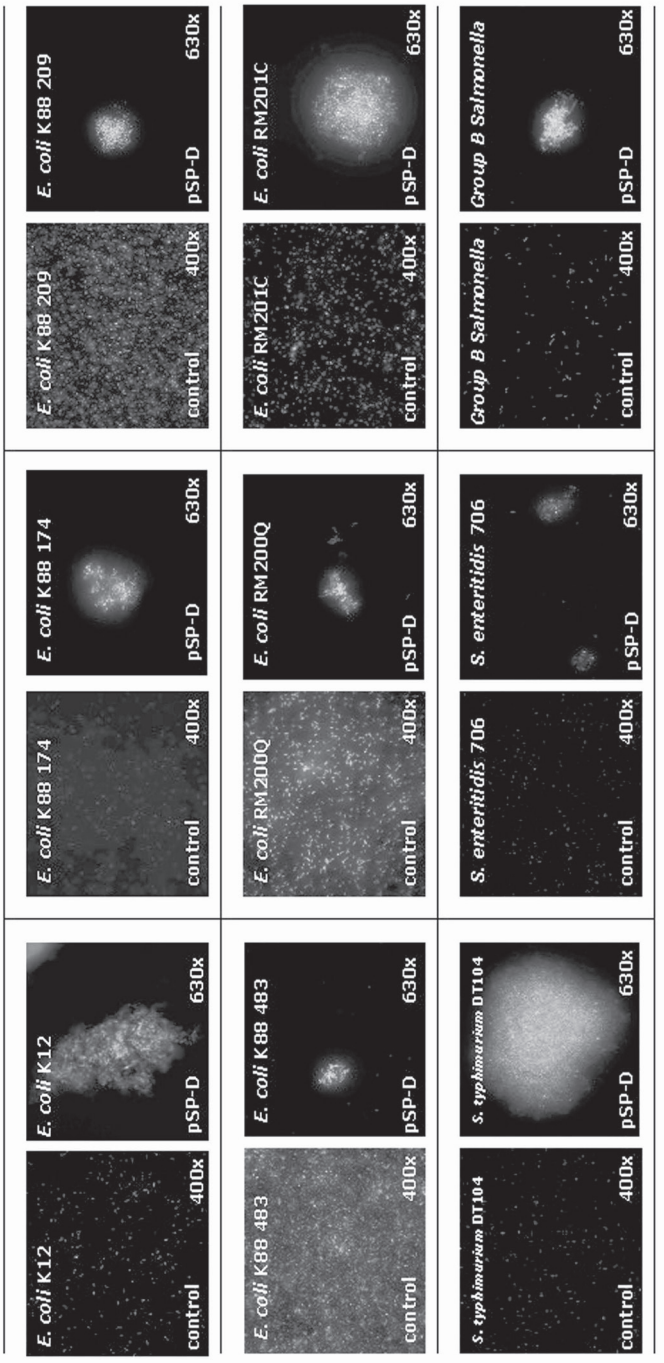

Figure 3. Bacterial permeability is increased in p-SP-D-treated *E. coli* K12, but not in other strains.

Bacterial membrane permeability was assessed by staining with the permeant fluorescent probe SYTO 9 and the impermeant fluorescent probe propidium iodide.
Red fluorescent staining was observed in pSP-D-treated *E. coli* K12 (upper left panel), indicating an increase in bacterial membrane permeability. pSP-D-treatment of other strains clearly leads to bacterial aggregation, but no effects on bacterial membrane permeability were observed. Similar results were observed for rSP-D (not shown).
(for colour print see page 173)

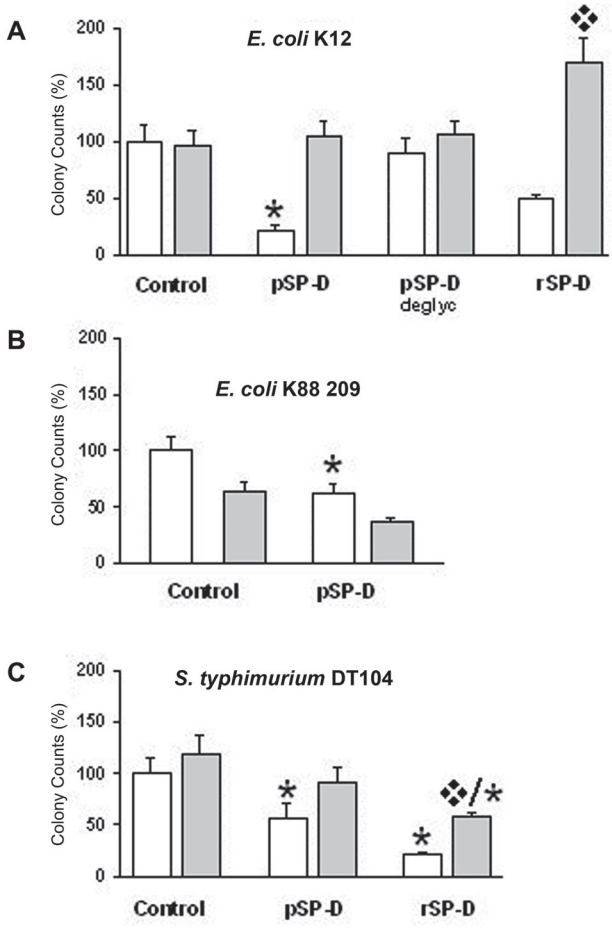

Figure 4. Bacterial survival is not significantly affected by pSP-D.
(A): Incubation of *E. coli* K12 with pSP-D or rSP-D led to significantly lower colony-counts in the presence of Ca^{2+}. No effects were observed for pSP-D_{deglyc}-treated samples. Disruption of bacterial aggregates by EDTA restored colony-counts to control values for pSP-D-treated bacteria, but significantly higher colony counts were observed for rSP-D-treated bacteria. (B): Incubation of *E. coli* K88 209 with pSP-D in the presence of Ca^{2+} significantly decreases colony counts. However, addition of EDTA also significantly affected bacterial viability, but between EDTA-treated samples no significant differences in colony counts were observed. (C): Significantly lower colony counts were observed for pSP-D or rSP-D treated *S. typhimurium* DT 104 in the presence of Ca^{2+}. Disruption of bacterial aggregates by EDTA restored colony-counts to control values for pSP-D treated bacteria, a significant decrease was observed for rSP-D treated bacteria. White bars: treated with MM-Ca^{2+}, gray bars: treated with MM-EDTA. Values are given as a percentage of controls diluted in MM-Ca^{2+} (*E. coli* K12: mean = 355, SEM = 51; *E. coli* K88 209: mean =432, SEM = 56; *S. typhimurium* DT104: mean = 136, SEM = 21). * = significantly different from controls diluted in MM-Ca^{2+}, ❖ = significantly different from controls diluted in MM-EDTA. (p <0.05)

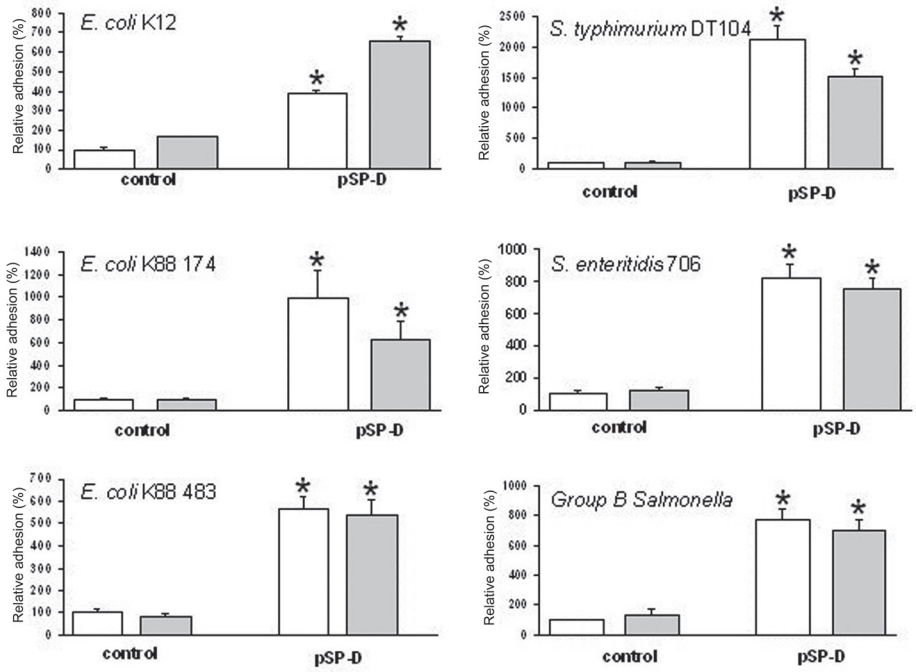

Figure 5. Bacterial adhesion to IPI-2I cells is significantly increased by pSP-D.
Pre-incubation of all bacterial strains tested in these assays was shown to increase the adhesion of bacteria to IPI-2I cells. Dilution in the presence of either Ca^{2+} or EDTA did not affect bacterial counts any further. White bars: treated with MM-Ca^{2+}, gray bars: treated with MM-EDTA. Values are given as a percentage of controls diluted in MM-Ca^{2+}. (*E. coli* K12: mean = 441, SEM = 40; *E. coli* K88 174: mean =62, SEM = 8; *E. coli* K88 483: mean =286, SEM = 49; *S. typhimurium* DT104: mean = 310, SEM = 24; *S. enteritidis* 706: mean = 55, SEM = 10; Group B *Salmonella*: mean = 105, SEM = 7). * = significantly different from controls treated with MM-Ca^{2+} (p <0.05)

indicating decreased bacterial survival. In contrast, dilution in MM-EDTA led to significantly higher colony-counts for rSP-D treated *E. coli* K12.

Adhesion and invasion-assays

To study the possible function of pSP-D in the gastro-intestinal tract, adhesion and invasion characteristics were studied using the IPI-2I cell-line, which do not express pSP-D (unpublished results, H. Hendriks et al.). Since serum-free medium was used during the experimental procedures, no interference of the serum collectin mannan binding lectin (MBL) should be expected. Low-glucose DMEM was used to avoid binding of sugars in the medium to pSP-D. For all strains tested, preincubation with pSP-D led to significantly higher numbers of bacteria adhering to the IPI-2I-cells (**Figure 5**), and the number of

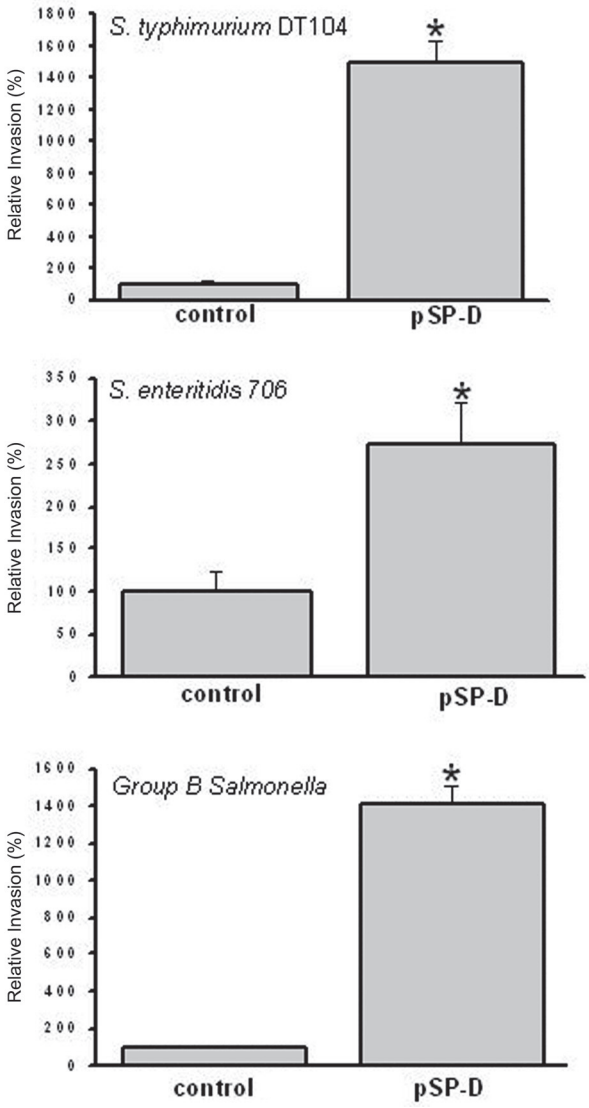

Figure 6. Bacterial invasion into IPI-2I cells is significantly increased by pSP-D.
Pre-incubation of *S. typhimurium* DT 104, *S. enteritidis* 706 or Group B *Salmonella* was shown to increase the invasion of bacteria into IPI-2I cells. Values are given as a percentage of control value (*S. typhimurium* DT104: mean = 24, SEM = 6; *S. enteritidis* 706: mean = 113, SEM = 26; Group B *Salmonella*: mean = 26.5, SEM = 8.5). * = significantly different from controls (p <0.05)

invading *Salmonella* was also significantly increased (**Figure 6**). Taken together these results suggest that pSP-D promotes uptake of bacteria by intestinal epithelial cells.

IL-8 secretion of IPI-2I cells

To investigate whether pSP-D has a stimulatory effect on the inflammatory response of IPI-2I cells, IL-8 production was measured. Although all bacterial strains elicited an increased IL-8 production, no significant differences in IL-8 production were observed for IPI-cells incubated with pSP-D-treated bacteria or controls (not shown).

Discussion

SP-D was originally found in the lung, and much of the research to date has focused on its function in the pulmonary environment. However, other studies have indicated that SP-D may also have a role in innate immunity in other tissues [5, 26, 59]. SP-D expression has also been localized to the porcine gastro-intestinal tract [27-29], which is a natural habitat for a large bacterial community and the most important route of entry for pathogenic bacteria. Therefore, we examined the effect of pSP-D on the growth rate and maximal bacterial density of the laboratory strain *E. coli* K12 and various bacterial strains isolated from the porcine intestine.

Of all the strains tested, *E. coli* K12 was most susceptible to pSP-D-induced effects, which included a significant decrease in the specific rate of growth and the maximal bacterial density (**Figure 2A** and **2B**) and an increase in membrane permeability (**Figure 3**). However, pSP-D did not affect bacterial viability (**Figure 4**). These data are in line with a mechanism in which pSP-D causes bacterial membrane damage which impairs growth but is not severe enough to kill *E. coli* K12. This mechanism appears to be at least partly dependent on aggregation, since no inhibitory effects were observed for CRF (**Figure 2A** and **2B**), which is unable to aggregate bacteria [60]. Furthermore, bacterial growth was not inhibited in the radial diffusion assay (not shown). It is possible that aggregation of the bacteria is a means of intensifying the interaction between the CRD and the bacterial membrane, which ultimately leads to impaired membrane integrity. Since this would affect the bacterial proton motive force, much of the energy normally invested in bacterial growth would now be needed to maintain a chemiosmotic potential, resulting in slow growth and lower bacterial densities.

Interestingly, incubation of *E. coli* K12 with pSP-D_{deglyc} in the bacterial growth assay had no effect on growth rates or maximal bacterial densities (**Figure 2A** and **2B**). Other studies have shown that the Asn-linked oligosaccharide in the CRD of pSP-D has a profound effect on the interactions of pSP-D with Influenza A Virus [61]. However, since CRF (which still contains the oligosaccharide moiety in its CRD) failed to affect bacterial growth (**Figure 2A** and **2B**), this oligosaccharide moiety cannot be solely responsible for mediating the inhibitory effects of pSP-D observed in this study. A possible explanation may be that pSP-D aggregates bacteria partially through self-recognition, as has already been described for SP-A [62], which is also N-glycosylated in its CRD [63]. Self-recognition involves one of the CRDs of a multimeric pSP-D molecule interacting with bacterial surface ligands, while another CRD of the same molecule recognizes the oligosaccharide moiety of another pSP-D molecule (or vice versa). This increases the number of interactions and, as a result, the extent of bacterial aggregation. *E. coli* K12 was

indeed aggregated by pSP-D_{deglyc} (as assessed by light microscopy, not shown), indicating that the CRD of pSP-D_{deglyc} is still capable of binding to the bacteria. However, results from the colony count assays showed that the extent of this aggregation was decreased as compared to pSP-D, leading to colony counts similar to those of control samples (**Figure 4**), supporting the hypothesis that aggregation by pSP-D is partly mediated by self-recognition.

Taken together, these results suggest that pSP-D is capable of reducing growth rates of *E. coli* K12 via an aggregation-dependent mechanism which involves increasing the bacterial membrane permeability without causing a significant reduction in bacterial viability. However, these effects appear to be strain-dependent, since the growth rate and maximal density of the other strains were unaffected by pSP-D. Although all strains were found to be aggregated by both pSP-D (**Figure 3**) and rSP-D (not shown), only the colony counts of pSP-D treated-*E. coli* K88 209 and *S. typhimurium* DT 104 were similarly affected by incubation with pSP-D as compared to *E. coli* K12. It has to be noted that addition of EDTA to *E. coli* K88 209 also significantly affected bacterial viability, but since pSP-D treated samples diluted in MM-EDTA did not differ significantly from their respective controls, it is unlikely that viability is affected by pSP-D. These results show that not all strains are aggregated to the same extent, and the extent of aggregation does not necessarily reflect the inhibitory effect of pSP-D on bacterial growth. It is possible that pSP-D exerts its inhibitory effect on specific targets on the bacterial membrane, which are available in *E. coli* K12 but may be absent or shielded from pSP-D in other strains.

Because Wu et al. [3] reported that rSP-D and rrSP-D inhibit bacterial growth of *E. coli* K12 by increasing bacterial membrane permeability, we included rSP-D and rrSP-D in some of our experiments to compare the effect of pSP-D to SP-D isolated from other species. Bacterial growth rate and maximal density were decreased for rSP-D- and rrSP-D-treated *E. coli* K12 in the bacterial growth assays (**Figure 2A** and **2B**), and an increased bacterial membrane permeability was observed for rSP-D treated *E. coli* K12 (not shown). However, incubation of *E. coli* K12 with rSP-D did not affect bacterial viability significantly, and addition of EDTA to rSP-D treated samples led to significantly higher colony counts, suggesting an *increase* in bacterial survival. In contrast, while no effects on growth rate or bacterial density were observed for rSP-D-treated *S. typhimurium* DT 104 and no increased bacterial membrane permeability was observed, bacterial viability significantly decreased for rSP-D treated samples. This latter observation is in accordance with the findings of Wu and colleagues [3], but it remains unclear why *E. coli* K12 is not similarly affected. Differences in experimental setup may explain these contrasting results.

A decreased growth rate could, in theory, keep bacteria from colonizing the intestine by increasing the time available to dispose of possible pathogens by peristalsis. We hypothesized that adhesion to and invasion into epithelial cells would be furthermore impaired when bacteria are aggregated by pSP-D. To test this hypothesis, pSP-D-treated

bacteria were added to IPI-I2 cells, and allowed to adhere for 1 hr. Cells were then lysed and diluted in minimal medium containing either calcium or EDTA. In contrast to our hypothesis, adhesion- and invasion characteristics observed in this study showed that preincubation with pSP-D led to significantly higher numbers of adhering (**Figure 5**) and invading bacteria (**Figure 6**). It is not clear what the mechanism behind this increased bacterial uptake is.

A possible explanation for the observed results with respect to the *in vivo* situation is a mechanism which enables the epithelial cells to discriminate between commensal bacteria and possible pathogens. For example, in the absence of CD14, TLR4-positive cells are unable to detect LPS unless it is in a complex with other bacterial virulence factors [64]. It is possible that aggregation by pSP-D contributes to the presentation of such stimulating factors, acting as a scavenger molecule. Another mechanism could involve the inflammatory responses which are elicited after invasion into an epithelial cell by a pathogen. Epithelial cells have been demonstrated to initiate a defensive program when flagellin, the structural subunit of bacterial flagella, is presented to the basolateral pole of model intestinal epithelia, pointing to a receptor which would initiate inflammatory responses once bacteria pass the epithelium [65]. Increased invasion mediated by pSP-D could, in theory, reduce the time it would take to elicit an inflammatory response, thereby contributing to the clearance of the infecting pathogen. Although incubation of the bacteria with pSP-D did not lead to changes in IL-8 production in this study, it is possible that expression and/or secretion of other cytokines can be stimulated by pSP-D *in vivo*.

In summary, these results show that while pSP-D is capable of reducing the growth rate of the laboratory strain *E. coli* K12, pathogenic intestinal bacteria remain unaffected. Although all strains are aggregated to some extent, bacterial viability is not significantly decreased by pSP-D. A direct, antimicrobial effect on *S. typhimurium* DT 104 was observed for rSP-D, but it is unclear what the underlying mechanism is since membrane permeability was not observed to be affected. Incubation of intestinal bacterial isolates with pSP-D led to an increase in adhesion and invasion. This may seem detrimental to the host, but it can also reflect a scavenger function for pSP-D in the intestine, which enables the epithelial cells to respond to infectious bacteria within a shorter time-span.

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Chapter 4

Characterization and Expression Sites of Newly Identified Chicken Collectins

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Abstract

Collectins are members of the family of vertebrate C-type lectins. They have been found almost exclusively in mammals, with the exception of chicken MBL. Because of their important role in innate immunity, we sought to identify other collectins in chicken. Using the amino acid sequences of known collectins, the EST database was searched and related to the chicken genome. Three chicken collectins were found and designated chicken Collectin 1, (cCL-1), chicken Collectin 2 (cCL-2), and chicken Collectin 3 (cCL-3), which resemble the mammalian proteins Collectin Liver 1, Collectin 11 and Collectin Placenta 1, respectively. Additionally, a lectin was found which resembled Surfactant Protein A, but lacked the collagen domain. Therefore, it was named chicken Lung Lectin (cLL). Tissue distribution analysis showed cCL-1, cCL-2 and cCL-3 are expressed in a wide range of tissues throughout the digestive, the reproductive and the lymphatic system. Similar to SP-A, cLL is mainly localized in lung tissue. Phylogenetic analysis indicates that cCL-1, cCL-2 and CL-3 represent new subgroups within the collectin family. The newly found collectins may have an important function in avian host defence. Elucidation of the role of these pattern-recognition molecules could lead to strategies that thwart infectious diseases in poultry, which could also be beneficial for public health.

Introduction

Collectins are collagenous proteins that are part of the Ca^{2+}-dependent (C-type) lectin superfamily [1]. The basic structural unit of a collectin is a trimer of three identical or two closely related polypeptide chains, which can multimerize to various degrees. The monomeric subunits can be characterized by four features. An N-terminal cysteine-rich domain stabilizes the trimeric subunits through the formation of disulfide bridges between the cysteines of the monomers. [2-6]. The N-terminal domain is followed by a collagenous domain with Gly-X-Y repeats, linked to a C-type carbohydrate recognition domain (CRD) via the neck domain. The neck domain forms an α-helical coiled coil, which can be recognized by a heptad repeat pattern with two hydrophobic residues at the first and the fourth position. The neck domain is thought to align the collagen chains and thereby initiate the trimerization of three monomers [7-9].

The collectin family includes mannan binding lectin (MBL) [10-13], conglutinin [14, 15], collectin 43 (CL-43) [16, 17], collectin 46 (CL-46) [18], pulmonary surfactant proteins A and D (SP-A and SP-D) [19-23], and the more recently characterized collectin liver 1 (CL-L1) [24, 25] and collectin placenta 1 (CL-P1) [26, 27]. Conglutinin, CL-43, and CL-46 have thus far only been described in *Bovidae*.

Table 1. Primers used to confirm predicted sequences of cLL, cCL-1, cCL-2 and cCL-3		
Gene	**Primers**	**Annealing temperature**
cLL Forward Primer cLL Reverse Primer	5' – AAC AGG GCT CTC CAC AGA AG – 3' 5' – ACA GAA TGG TCG CTG CTC TC – 3'	53°C
cCL-1 Forward Primer cCL-1 Reverse Primer	5' – AGG CAT TTC CTG AGA AGC TG – 3' 5' – GTG CCA GAC ATC TTT TGT GG – 3'	52°C
cCL-2 Forward Primer cCL-2 Reverse Primer	5' – GAG TCA GTG ACC TGT AAG TGC – 3' 5' – ATG GGC TAA CTT ATA CAA CCT GCT – 3'	55°C
cCL-3 Forward Primer 1 cCL-3 Reverse Primer 1	5' – GGC ATG GCC CGA CAG TAG – 3' 5' – CAA GGC TTT CTG TCC AGC TT – 3'	60°C
cCL-3 Forward Primer 2 cCL-3 Reverse Primer 2	5' – GAG GTG GAG AGT GAT CTG AAG AA – 3' 5' – TTC AAG TGT ATC ATT ATT AGC TTT TGC – 3'	60°C
cCL-3 Forward Primer 3 cCL-3 Reverse Primer 3	5' – GCT GCC AAC AAC TCA GCA T – 3' 5' – TTC GCT CTG TAG TGC CAT TG – 3'	60°C
cCL-3 Forward Primer 4 cCL-3 Reverse Primer 4	5' – CAC GGT CAG CTC ATC AAG AA – 3' 5' – CCG AAA TCT GCC TCT TTA TCC – 3'	60°C
cCL-3 Forward Primer 5 cCL-3 Reverse Primer 5	5' – ATG AAT GGA GGT GGC TGG AT – 3' 5' – CCC GGT AGC AAA CCT TAG TG – 3'	56°C

MBL, conglutinin, CL-43 and CL-46 are serum collectins synthesized by the liver [11, 15, 18]. SP-A and SP-D are mainly produced in the lungs by alveolar type II cells and Clara cells, but expression of both lung collectins has also been localized to extrapulmonary tissues [28-32]. CL-P1 is a membrane bound protein expressed in vascular endothelial cells [26]. CL-L1 is expressed mainly in liver, placenta and adrenal gland as a cytosolic protein, but it has been located to most tissues except skeletal muscle [24].

Thus far, the only described collectin in birds is MBL [13, 33, 34], which can activate the avian complement cascade despite the lack of MASP1-like enzymatic activity [35]. Although in some studies the presence of SP-A was indicated in chicken lung lavage and tissue [36, 37], SP-A was shown to be absent from chicken surfactant in a study by Bernhard and colleagues [38]. In 2001, Vitved and colleagues [39] described the nucleotide sequence of a gene designated chicken SP-A (GenBank accession number **AF411083**). The predicted amino acid sequence of this gene contains three Gly-X-Y repeats. So far, there have been no reports of other avian collectins.

Collectins are key molecules in innate immunity (reviewed by [40-42]). Via their CRD, they can bind to non-self glycoconjugates on the surface of live micro-organisms which can lead to aggregation, neutralization and lysis. Binding can also result in enhanced uptake of micro-organisms by various immune cells through opsonization, and, in the case of MBL, activation of the complement cascade via the lectin pathway [43, 44].

Collectins have been shown to interact with Influenza A Virus (IAV) [45-49]. In the light of recent outbreaks of avian influenza, increasing our knowledge of innate immunity in poultry could not only have an impact on animal health and welfare, but also

Table 2. Primers used for tissue distribution of cSP-A, cMBL, cLL, cCL-1, cCL-2 and cCL-3		
Gene	**Primers**	**Annealing temperature**
cSP-A Forward Primer	5' – GTC AGT GGT TAC AAA GAC AGC – 3'	57°C
cSP-A Reverse Primer	5' – AGT TCC TCC AGT CTC TTC AC – 3'	
cMBL Forward Primer	5' – ATT CAG TGT AGT GCT CCT GC– 3'	60°C
cMBL Reverse Primer	5' – CCA AAT CAG TTA TTT GTC GGT GC– 3'	
cLL Forward Primer	5' – GCT GAA AGA GGG ATA TCT CAA GC– 3'	53°C
cLL Reverse Primer	5' – TCA GCT GTT TTC CCA CTG GTA GC – 3'	
cCL-1 Forward Primer	5' – TTG ATG GAT GTG TTA AGC CGA GC – 3'	60°C
cCL-1 Reverse Primer	5' – ATG TCG GTG ACC AGG GAA TGC – 3'	
cCL-2 Forward Primer	5' – GAG AAA TGG GAG ACA AAG GAC – 3'	55°C
cCL-2 Reverse Primer	5' – CCT TCA CCA GCA GAT AAA TC – 3'	
cCL-3 Forward Primer	5' – ACA GAG GTG GAG AGT GAT CTG – 3'	57°C
cCL-3 Reverse Primer	5' – GGC TCT GTC TGT CAT CTA G– 3'	

be of great importance to public health. Therefore, the objective of this study was to search for new collectins in the chicken.

Materials and Methods

RNA extraction and cDNA synthesis

Total cellular RNA from various tissues from healthy female Ross 308 broiler chicken was extracted using TRIzol© (Invitrogen, Carlsbad, CA) and Magnalyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany). The cDNAs used as templates in PCR were synthesized using 1 µg DNase I treated RNA with M-MLV-RT and 500 µg/ml oligo dT12-18 primers (Invitrogen, Carlsbad, CA) in a 20 µl reaction volume with incubation at 37°C for 50 min.

Database search and primer design

The amino acid sequence of human, murine and porcine SP-D (GenBank accession numbers: AY216721, AF192134, and AF132496, respectively) were used to conduct a TIGR chicken expressed sequence tag (EST) database search (http://tigrblast.tigr.org/tgi/gggi), using a translated nucleotide basic local alignment search tool (tBLASTn). ESTs which had collectin-like characteristics were used in a BLAST-like alignment tool search (BLAT) of an annotated chicken genomic sequence database (UCSC Genome Bioinformatics Site; http://genome.ucsc.edu/) [50]. This revealed 4 genes predicted by NCBI's Gnomon, a program which predicts the gene structure in genomic DNA in a multistep fashion. To confirm the sequences of coding regions of these genes, primers were designed based on the sequences flanking the 5´- and 3´-ends as predicted by Gnomon (**Table 1**). In the case of the predicted gene XP_419148, resembling CL-P1, five primer pairs were used in order to amplify overlapping parts of its coding region.

Polymerase chain reaction and amplified DNA fragment isolation
To amplify the cDNA, a PCR reaction was performed using FastStart DNA Taq-polymerase (Roche Diagnostics Gmbh, Mannheim, Germany) and primers as specified in **Table 1**. Amplification comprised 2 min of initial denaturation at 95°C, followed by 40 cycles consisting of 95°C for 30s, 30s at respective annealing temperatures specified in **Table 1**, 72°C for 1 min and a final extension at 72°C for 7 min. PCR-products were analyzed by agarose gel electrophoresis and purified with a QIAEX agarose gel extraction kit (Qiagen, Valencia, CA).

Cloning and sequencing of the PCR products
The purified PCR fragments were ligated into a pGEM-T-Easy vector (Promega, Madison, WI). Ligated plasmids were transfected into *Escherichia coli HB101* cells by electroporation. Clones were selected by growth on Luria-Bertani broth (LB)-plates containing 100 µg/ml ampicillin. Positive clones were screened with PCR for correct product size and sequenced. Sequence reactions were performed using an ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA). All reactions were carried out in both directions using the T7 and Sp6 primer sites and separated on an ABI PRISM 3100 fluorescent DNA sequencer (Applied Biosystems, Foster City, CA).

Expression analysis
The tissue mRNA expression profiles were investigated by extracting total RNA from 45 different tissues from healthy ROSS 308 broiler chickens, one male and two females. Within 30 min after the animals were sacrificed, tissue samples were taken, rinsed with cold physiologic saline solution, snap frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted and DNase I treated as described above. cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. New primers were designed in order to amplify a shorter region of the cDNA, providing a PCR product less susceptible to degradation. Furthermore, two other sets of primers were designed for chicken SP-A and chicken MBL, in order to compare the expression distribution of the newly found genes to those of known chicken collectins. To assess the quality of the mRNA samples, chicken β-actin primers were used. These primers are summarized in **Table 2.** Amplification comprised 5 min of initial denaturation at 95°C, followed by 40 cycles consisting of 95°C for 30s, 30s at respective annealing temperatures specified in **Table 2**, 72°C for 1 min and a final extension at 72°C for 7 min. To ensure the specificity of the primers, PCR-products were purified, cloned and sequenced as described above. The resulting sequences proved to be identical to the Gnomon-predicted sequence of the amplicons.

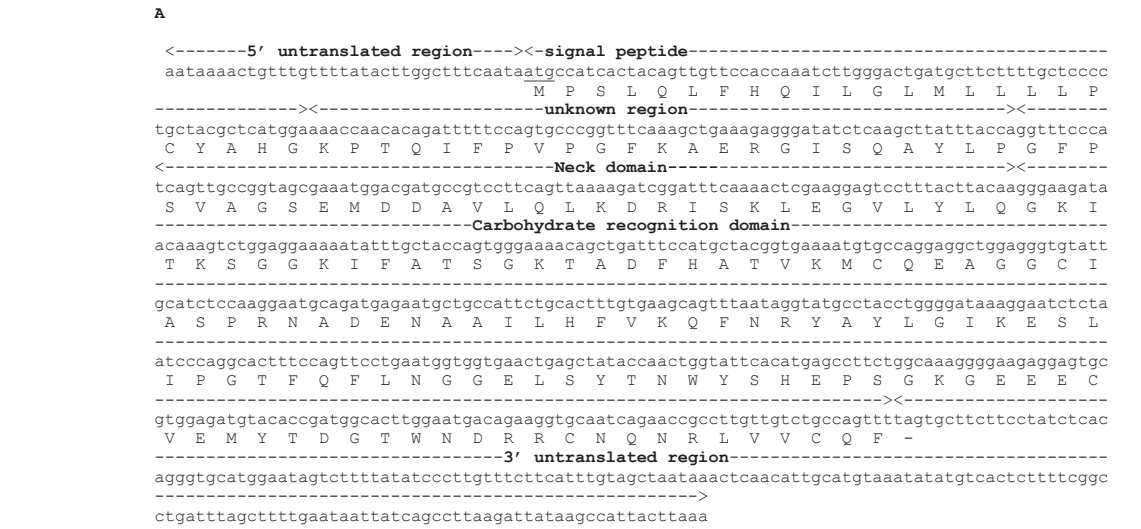

Figure 1. Nucleotide sequence of cLL cDNA and the predicted amino acid sequence. Start sites are underlined. UTR: Untranslated Region

Molecular phylogenetic analysis

The amino acid sequences of the CRDs of various collectins and those of the newly found chicken genes were aligned by hand. The alignment was used to generate a phylogenetic tree by the neighbor-joining method [51] as implemented in PAUP* 4.0b10 [52]. Also, a phylogenetic analysis was performed, using maximum parsimony (heuristic search option; TBR, ACCTRAN, MULPARS invoked, 100 replicates with 100 trees saved per replicate), as implemented in PAUP* 4.0b10. Due to large dissimilarities in amino acid sequences already in the ingroup, and the resulting difficulty to align the sequences and the absence of prior knowledge of C-type lectin evolution, no outgroups were included and trees are presented as unrooted. Support values for the phenetic and phylogenetic analyses were calculated by bootstrap resampling the datamatrix, using 1000 bootstrap replicates.

Results

Identification and cloning of chicken Lung Lectin, cCL-1, cCL-2 and cCL-3

Using the amino acid sequences of human, murine and porcine SP-D a TIGR chicken EST database search revealed several ESTs: TC199499, TC218505, TC202597, and TC189452, which had collectin-like characteristics. A BLAT search revealed four corresponding Gnomon predicted genes: (1) XP_421514, located on chromosome 6, which partly resembled SP-A, (2) XP_418393, located on chromosome 2 resembling CL-L1, (3) XP_426207, located on chromosome 3 and similar to collectin 11, and (4) XP_419148, located on chromosome 2, resembling CL-P1.

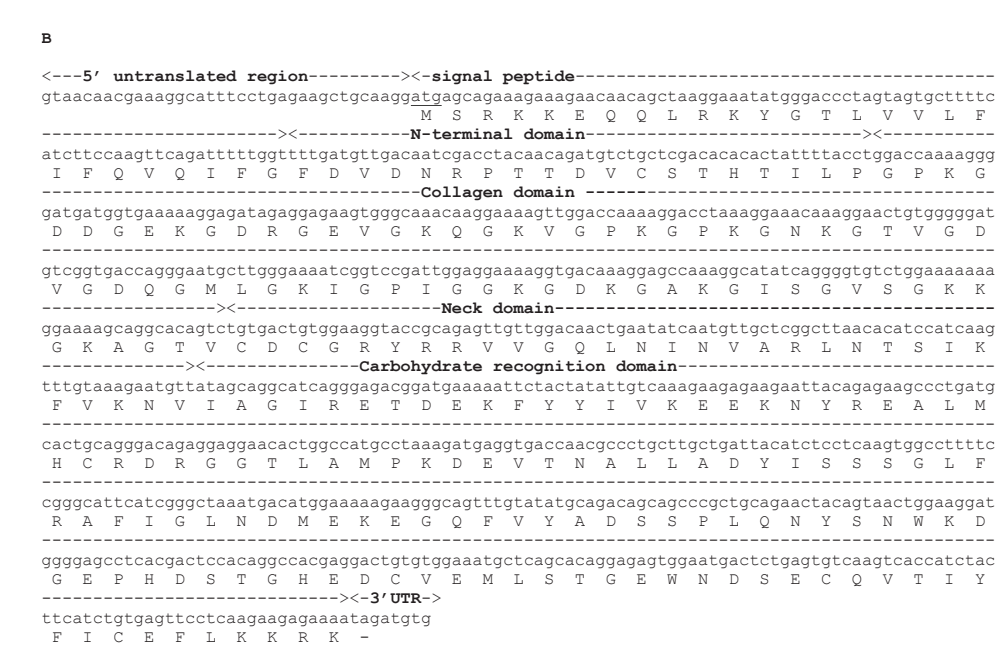

Figure 2. Nucleotide sequence of cCL-1 cDNA and the predicted amino acid sequence. Start sites are underlined. UTR: Untranslated Region

These genes were designated chicken Lung Lectin (cLL), chicken Collectin 1 (cCL-1), chicken Collectin 2 (cCL-2) and chicken Collectin 3 (cCL-3), respectively. Using the primers specified in **Table 1**, a single cDNA fragment of the expected size was amplified in each case. Because of its size, the primers for cCL-3 were designed on five overlapping fragments of its coding sequence. Comparison to human and murine CL-P1 showed the Gnomon-predicted coding region of cCL-3 missed part of its 5'- and 3'-ends. Using the sequence of hCL-P1 in a BLAST search, two ESTs (TC206150 and CR387231) were found. The sequence of TC206150 was used to design the forward primer of the first fragment of cCL-3 and CR387231 was used to design the reverse primer of the last fragment. All five fragments of cCL-3 were cloned and sequenced as described above. Resulting consensus sequences are shown in **Figure 1-4**. The cDNA sequences of cLL, cCL-1, cCL-2 and cCL-3 were submitted to GenBank (accession numbers **DQ129667**, **DQ129668**, **DQ129669** and **DQ157755**, respectively).

Sequence analysis

The mRNA sequence of cLL was identical to the sequence predicted by Gnomon. For cCL-1, the mRNA sequence was also found to be identical to the predicted sequence, with the exception of the first exon, which was revealed to start 42 nucleotides upstream from the

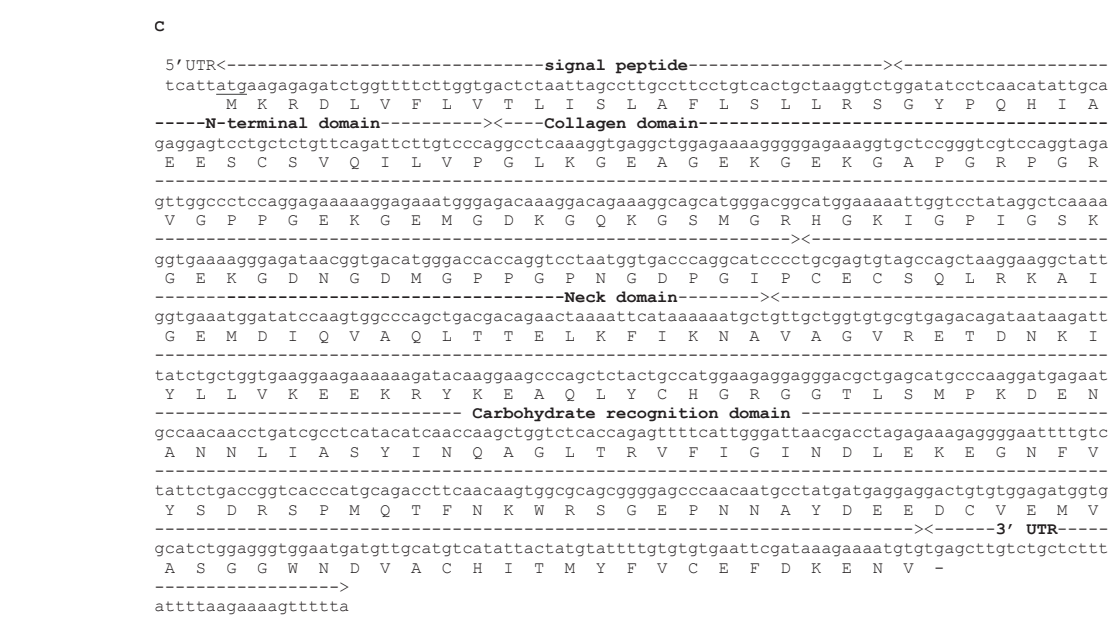

Figure 3. Nucleotide sequence of cCL-2 cDNA and the predicted amino acid sequence. Start sites are underlined. UTR: Untranslated Region

predicted start site of this exon. In the case of cCL-2, the sequence differed from the Gnomon-predicted sequence by only one nucleotide, resulting in a methionine instead of an isoleucine at position 79 in the amino-acid sequence. Sequence analysis of cCL-3 revealed an alternate start and stop site for this gene. In addition, two nucleotides were found to differ from the predicted sequence, resulting in an isoleucine instead of a methionine at the position 153 in the amino acid sequence and a silent mutation at position 155.

Structure of cLL, cCL-1, cCL-2 and cCL-3 deduced amino acid sequences and comparison with collectins in other vertebrates

To compare the overall domain organization of the predicted proteins, the basic structural features of cLL, cCL-1, cCL-2 and cCL-3 are shown together with other collectins in **Figure 5**. More specifically, protein sequence similarity for the different domains of cLL, cCL-1, cCL-2 and cCL-3 as compared to their interspecies homologues is shown in **Table 3-6**. The N-terminal domains of cCL-1, cCL-2 and cCL-3 contain one cysteine residue (see **Figure 2-4**), which could help stabilize a trimeric subunit. The size of the collagenous domains in cCL-1 and cCL-2 are comparable to those of SP-A and MBL in other species (**Figure 5**), but in contrast to the latter two the Gly-X-Y pattern is uninterrupted. In cCL-3, the predicted protein sequence shows a larger collagenous domain of 49 Gly-X-Y triplets. Overall, cCL-1, cCL-2 and cCL-3 are highly similar to their interspecies homologues. As for cLL, only the neck domain and the CRD are compared to those of SP-As, since this

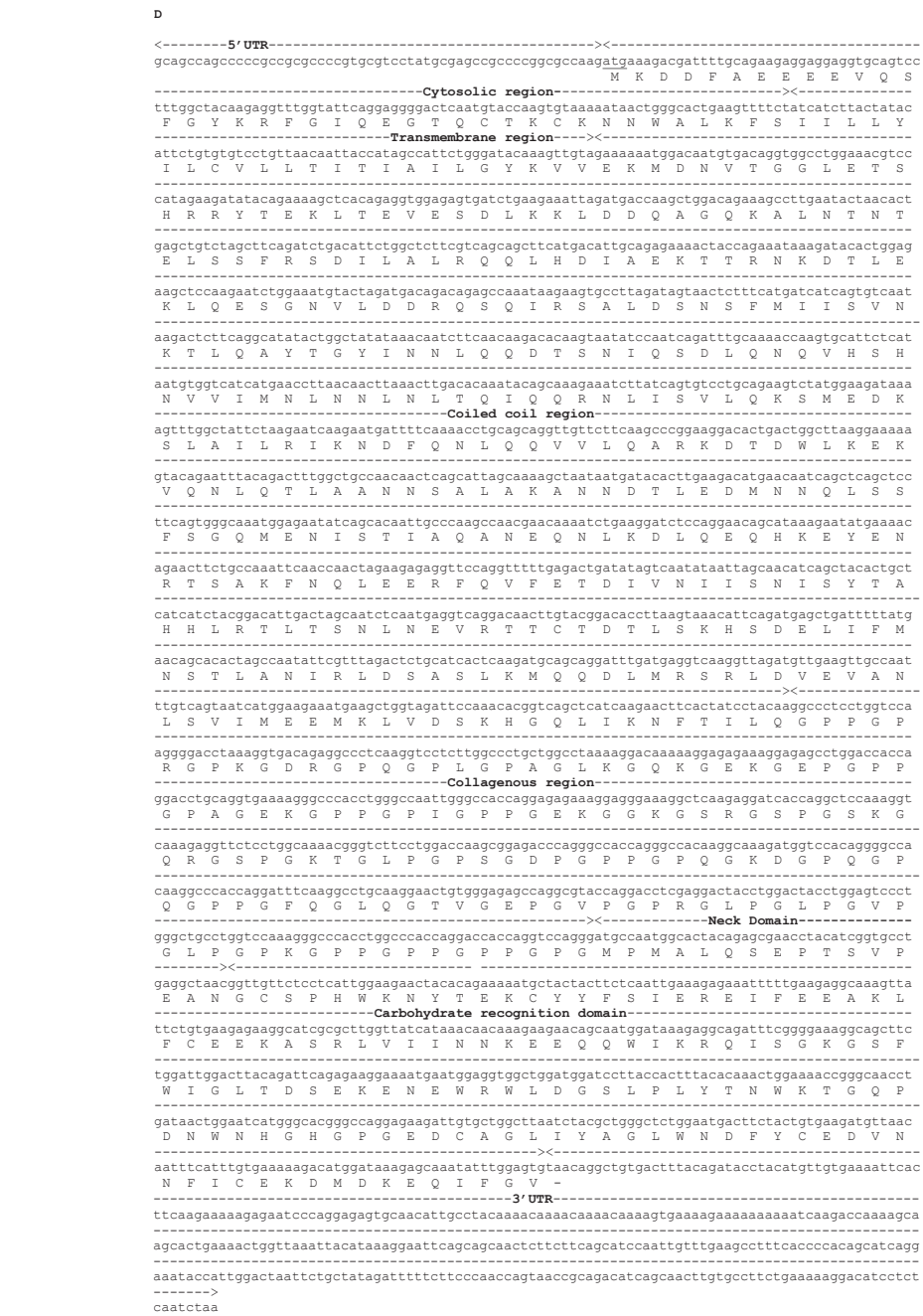

Figure 4. Nucleotide sequence of cCL-3 cDNA and the predicted amino acid sequence. Start sites are underlined. UTR: Untranslated Region

Table 3. Comparison of cCL-1 to CL-L1-proteinsequences. Sequence identities in percentages.				
	Human CL-L1	**Murine CL-L1**	**Rat CL-L1**	**Canine CL-L1**
Total corresponding region (277)	66%	63%	66%	68%
Signal Peptide (27)	33%	22%	26%	37%
N-terminal region (19)	63%	58%	58%	68%
Collagen domain (72)	71%	69%	72%	75%
Neck domain (30)	77%	80%	83%	83%
CRD (129)	73%	73%	75%	74%

Table 4. Comparison of cCL-2 to Collectin 11-proteinsequences. Sequence identities in percentages.				
	Human Collectin 11	**Murine Collectin 11**	**Rat Collectin 11**	**Xenopus Collectin 11**
Total corresponding region (271)	84%	86%	83%	83%
Signal Peptide (22)	59%	73%	68%	68%
N-terminal region (18)	61%	67%	67%	78%
Collagen domain (72)	90%	88%	82%	92%
Neck domain (30)	90%	93%	90%	80%
CRD (129)	86%	88%	88%	84%

Table 5. Comparison of cCL-3 to CL-P1-proteinsequences. Sequence identities in percentages.			
	Human CL-P1	**Murine CL-P1**	**Canine CL-P1**
Total corresponding region (735)	80%	79%	80%
Cytosolic region (39)	100%	100%	100%
Transmembrane domain (23)	87%	91%	91%
Coiled coil domain (380)	80%	79%	78%
Collagen domain (147)	84%	84%	83%
Neck domain (16)	63%	56%	81%
CRD (137)	73%	66%	72%

Table 6. Comparison of cLL to SP-A-proteinsequences. Sequence identities in percentages.				
	ChickenSP-A	**Human SP-A1**	**Murine SP-A**	**Porcine SP-A**
Neck domain (30)	33%	23%	37%	27%
CRD (120)	54%	45%	45%	39%

protein does not contain a collagen domain. Generally, the absence of cysteine residues in the N-terminal domain hampers stable trimeric subunit formation. However, the neck region, which contains a heptad repeat pattern, could provide sufficient stabilization for a trimer to form. In **Figure 6**, the helical wheel diagrams for cLL, cCL-1 and cCL-2 are

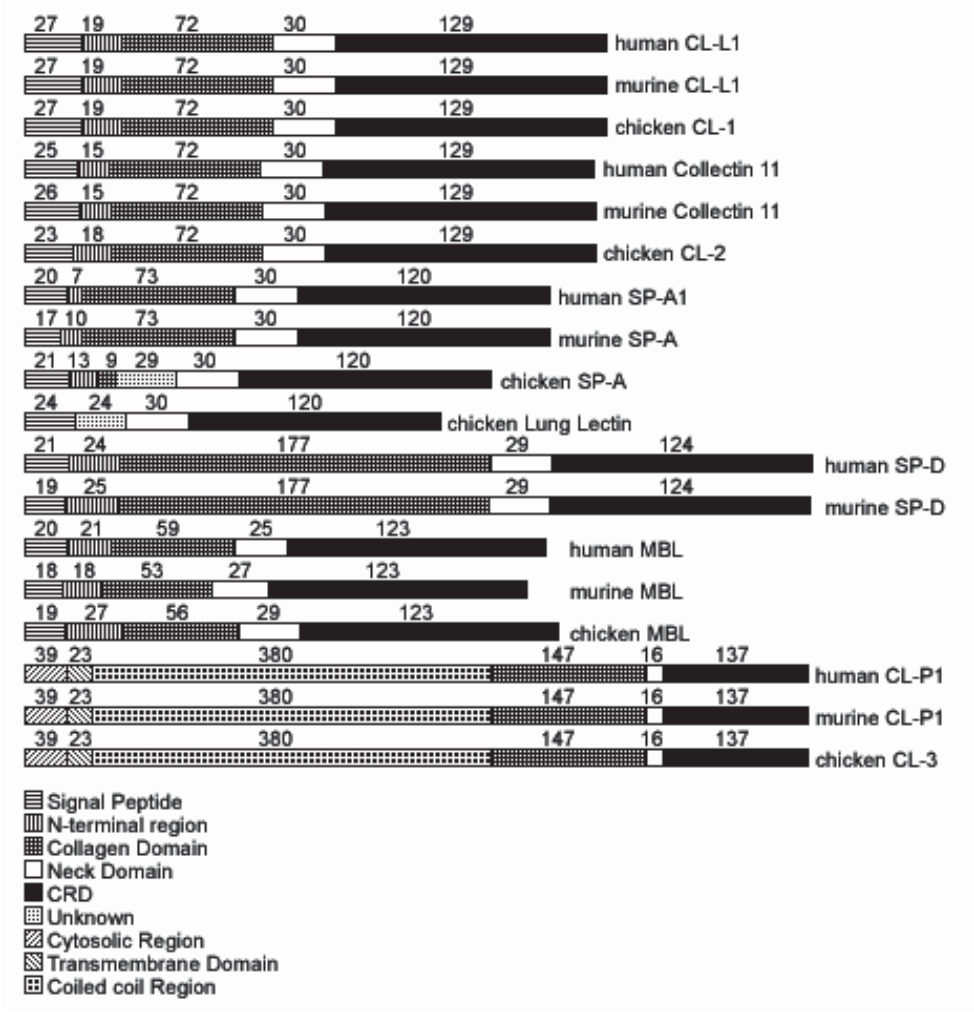

Figure 5. Comparison of overall domain organization of cLL, cCL-1, cCL-2, cCL-3 and some other collectins. The number of residues in the different domains / regions in the proteins is indicated above the sequence. All proteins are drawn to scale, with the exception of the CL-P1s and cCL-3, which are drawn on a 1:2 scale due to their size.

shown, which suggest that a similar structure is likely to exist in these proteins as well. The heptad repeat pattern is absent in the neck domain of chicken CL-3, similar to its human and murine counterparts.

Alignment of the amino acid sequences with those of other collectins shows that the CRDs of the predicted proteins contain most of the conserved residues of the C-type lectin motif [53] (**Figure 7**). Most of the collectins are mannose/glucose type C-type lectins. This specificity is determined by three residues (Glu-Pro-Asn) at positions

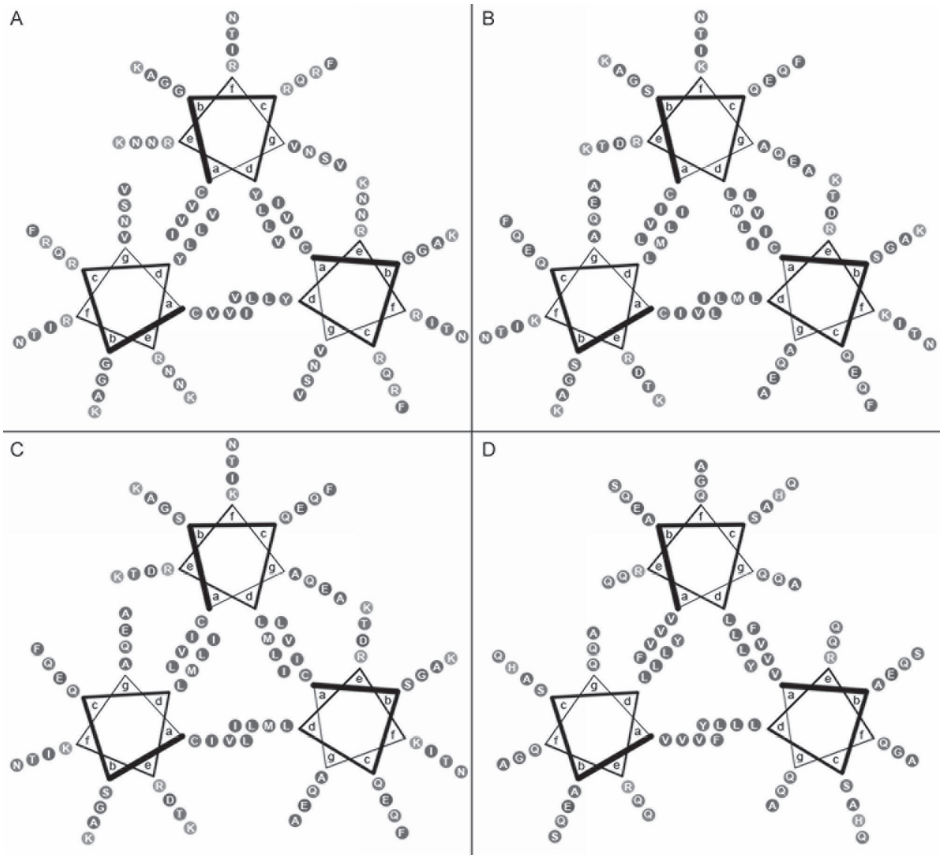

Figure 6. Helical wheel diagrams for (A) cLL, (B) cCL-1, (C) cCL-2 and (D) human SP-D. In the neck domain, a repeat of seven residues forms a heptad, the individual positions of the residues denoted by letters a to g. Residues at the first (a) and fourth (d) position are generally hydrophobic. Pink: Non-polar hydrophobic residues; Green: Polar, positively charged hydrophilic residues; Blue: Polar, uncharged residues; Red: Polar, negatively charged hydrophilic residues. (for colour print, see page174)

equivalent to Glu185 and Asn187 in MBL [54-56]. This motif can be observed in the predicted amino acid sequence of cCL-2. A preference for galactose over mannose is determined by a Gln-Pro-Asp motif, which is found in the predicted amino acid sequence of cCL-3. In the CRD of cLL however, a Glu-Pro-Ser motif is found, and cCL-1 deviates from its interspecies homologues in having a Glu-Pro-His motif.

Figure 7. Alignment of carbohydrate recognition domains. The CRDs of several collectins were aligned with the predicted amino acid sequences of cLL, cCL-1, cCL-2 and cCL-3. The sequence motif characteristic of C-type CRDs as described by Drickamer (1993) is shown below the alignment. Absolutely conserved residues are indicated by the one letter amino-acid code, while residues conserved in character are indicated by Φ, aromatic; θ, aliphatic; Ω, aromatic or aliphatic; O, contains oxygen; Z, either E or Q. Ligands for calcium 1 or 2 are denoted 1 or 2, respectively. Residues different from the motif are indicated by shading.

Tissue expression analysis

The expression of cLL, cCL-1, cCollectin11 and cCL-3 was analyzed in 45 different tissues from the gastro-intestinal tract, the reproductive tract, the respiratory system, the urinary tract, lymphatic tissues, liver, uropygial gland, skin, and cockscomb.

Results are shown in **Figure 8**. As expected, expression of chicken MBL mRNA was highest in liver, but reasonably high signals were seen in larynx, abdominal air sac, and infundibulum as well.

A lower expression level was found in thymus, and a faint signal could be observed in ovary and uterus. Chicken SP-A mRNA expression levels were highest in the respiratory system, but high expression was also observed in thymus and magnus, and a low expression signal was seen in liver. In addition, a faint signal was seen in the uterus. The tissue expression analysis for cLL showed a pattern similar to that of cSP-A, but expression of cLL was also observed in proctodeum, ovary, isthmus, Harderian gland, bursa, urethra and cockscomb. A high level of expression was observed for cCL-1 mRNA in lung tissue, but this gene has a more widespread expression pattern as compared to cLL. Weaker signals were observed in larynx, syrinx and cranial air sac. Furthermore, cCL-1 was expressed throughout the lower gastrointestinal tract in increasing levels starting from a faint signal in duodenum and ending with relatively high signals in proctodeum, coprodeum and urodeum. In the upper part of the gastrointestinal tract, expression was detected in tongue, crop, and mucosa of the crop. A ubiquitous expression of cCL-1in the reproductive tract of female animals was observed, as well as a strong signal in testis. Expression was also observed in thymus, spleen, bursa, kidney, urethra, liver, skin and cockscomb.

Expression analysis for cCL-2 mRNA revealed a tissue distribution pattern very similar to that of cCL-1, although expression levels in the respiratory system, lymphatic tissues and the gastro-intestinal tract were lower or absent, with the exception of the coprodeum and lung tissue where a strong signal was observed. In the reproductive system, cCL-2 expression could not be detected in infundibulum, magnum and isthmus. The tissue distribution pattern of cCL-3 mRNA was also similar to that of cCL-1, but a much stronger signal was observed in jejunum. In addition, cCL-3 mRNA expression was observed in the Harderian gland, and a low signal was seen in the proventriculus and the gizzard. In the respiratory tract, moderate expression was observed in trachea, syrinx, lung, and humurus.

Molecular phylogenetic analysis

The topology produced by neighbor-joining analysis is shown in **Figure 9**. Bootstrap values of the neighbor-joining analysis as well as the parsimony analysis are plotted onto the tree. Bootstrapping under the parsimony optimality criterion consistently gives lower

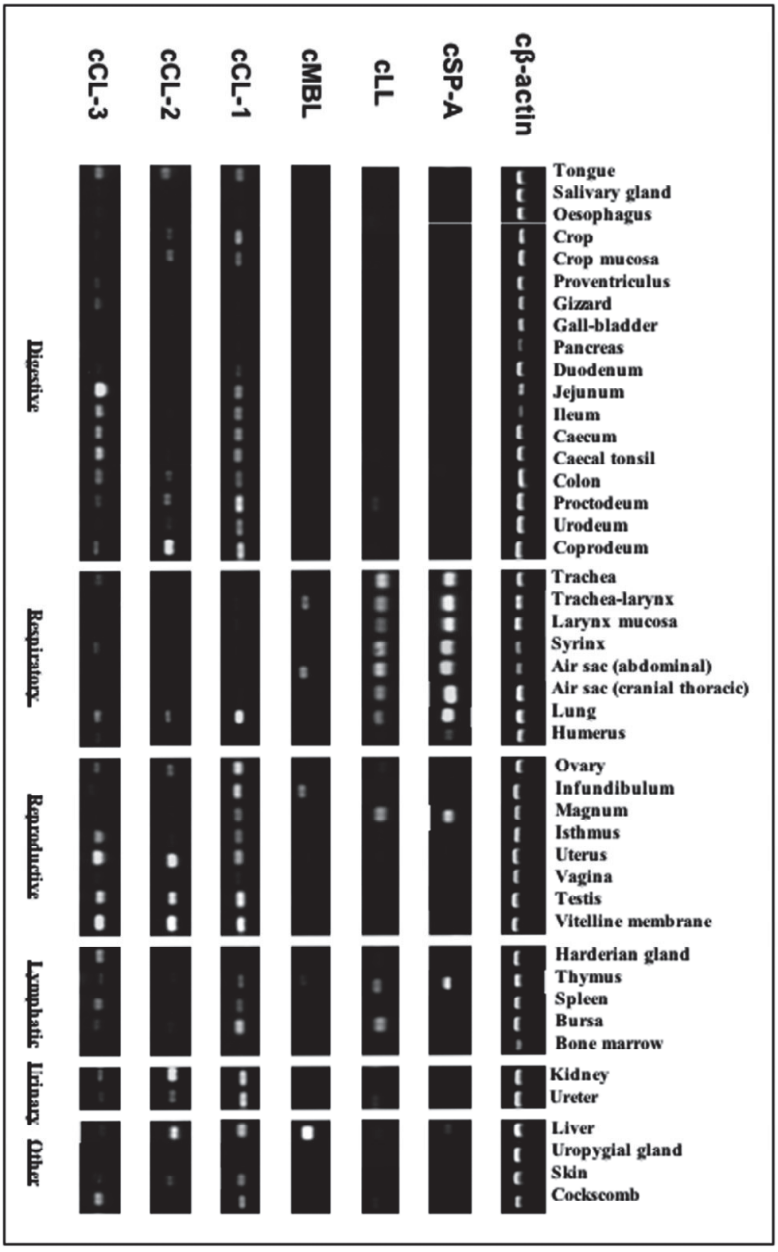

Figure 8. Tissue mRNA expression profiles for cMBL, cSP-A, cLL, cCL-1, cCL-2 and cCL-3. Transcripts were amplified by RT-PCR using total RNA from several tissues. Chicken β-actin was to assess the quality of the mRNA.

bootstrap values compared to neighbor-joining bootstrap values. Neighbor-joining has been reported to perform poorly for estimating evolutionary trees [52]. Moreover, neighbor-joining programs may give misleading bootstrap frequencies because they do not suppress zero-length branches and/or are sensitive to the order of terminals in the data [57].

Thus, focusing on maximum parsimony bootstrap values, the CRDs of SP-A and cLL are shown to be a well supported clade. Chicken LL is sister to cSP-A, and the CRDs share a more recent common ancestor than the common ancestor of the cLL / SP-A clade. As expected, the CRDs of cCL-1 and cCL-3 are placed in well supported clades together with the CRDs of their mammalian homologues CL-L1 and CL-P1, respectively. Also, cCL-2 and mammalian Collectin 11-CRDs are shown to be a well supported clade, indicating these proteins share a more recent common ancestor than the common ancestor of the CL-L1 / Collectin 11-clade.

Discussion

In this study, the EST database was screened to identify new collectins in chicken. Several interesting candidates were found, and the sequences of these ESTs revealed four Gnomon predicted genes, which we were able to clone and sequence. Three were designated cCL-1, cCL-2 and cCL-3, resembling mammalian Collectin Liver 1, Collectin 11 and Collectin Placenta 1, respectively.

The fourth sequence, cLL, was first named cSP-A2, since the amino acid sequence of its CRD is most similar to SP-As in other species. Like SP-A, it also contains a heptad repeat pattern which allows for the formation of an α-helical coiled coil, which is important for the formation of trimers. cLL lacks a collagenous domain, an essential characteristic needed in order to classify a protein as a collectin. Therefore, this protein was designated chicken Lung Lectin, since its mRNA expression pattern showed it to be mainly localized to the lungs. Another striking difference is the presence of a Glu-Pro-Ser motif in the CRD of cLL, where most other mannose type-collectins contain a Glu-Pro-Asn motif. However, it seems that some variation in this motif is permissible since most SP-As contain a Glu-Pro-Arg motif, whilst retaining their preference for mannose over galactose [56].

In contrast to cLL, the predicted amino acid sequence of cSP-A contains all four characteristics of a collectin. The N-terminal domain of this protein contains one cysteine residue, and the presence of three Gly-X-Y triplets could help stabilize a trimeric subunit. It is debatable however, whether three Gly-X-Y repeats are enough to constitute a functional collagen domain.

In humans, the MBL gene, the SP-A genes and the SP-D gene are clustered together on the long arm of chromosome 10. Interestingly, the cSP-A, cLL and cMBL are all located at the terminus of chromosome 6, syntenic to the collectin cluster on human

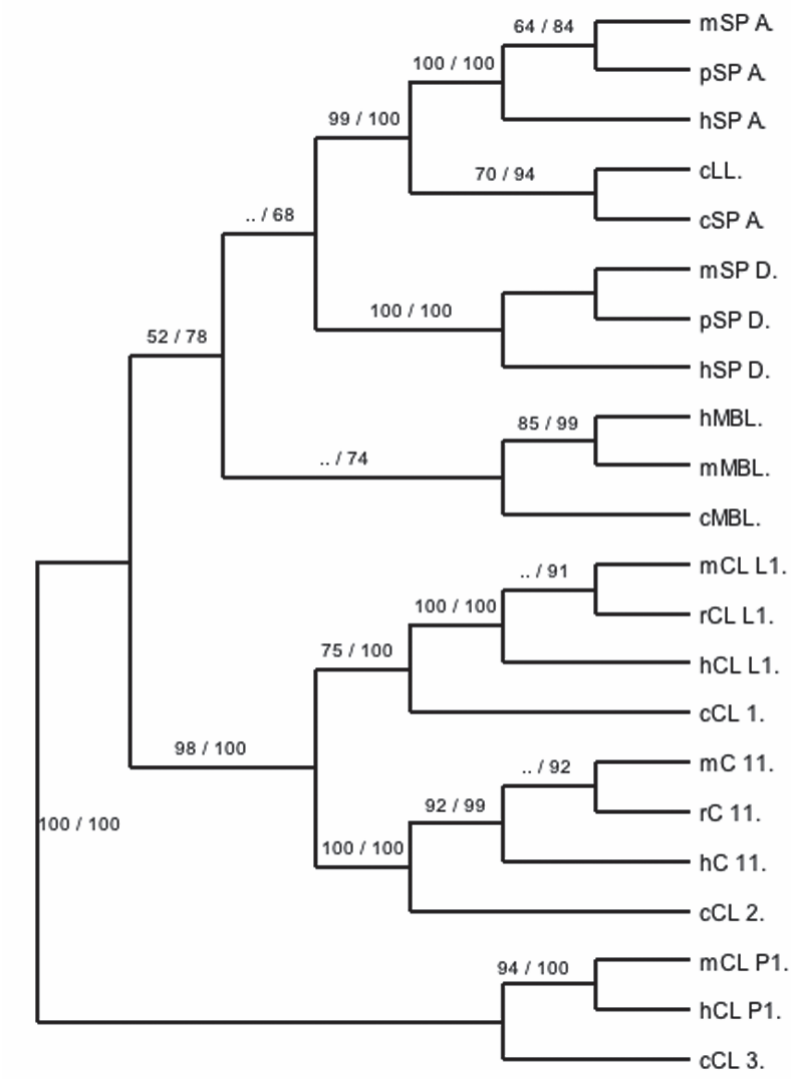

Figure 9. Neighbor-joining tree with maximum parsimony bootstrap values (before slash) and neighbor-joining bootstrap values (after slash).

chromosome 10. This finding provides further support to the notion that a collectin cluster is conserved in vertebrates. Phylogenetic analysis of the CRD shows that cSP-A and cLL share a common ancestral line with SP-As found in other species, but also that these

proteins are more similar to one another than to the mammalian SP-As, thus forming a separate clade within the "SP-A-clade" in the tree.

In this perspective, it is interesting to consider the difference between avian and mammalian lungs. In mammals, surfactant prevents the alveoli from collapsing at end expiration. In a study by Ikegami and colleagues [58] it was shown that the collagen domain of SP-A has a significant role in surfactant lipid association, tubular myelin formation and the protection of surfactant from protein inhibition. Also, deletion of the collagen domain was shown to convert the protein into an inhibitor of surfactant function.

In bird lungs, gas exchange occurs in capillaries instead of alveoli. Bernhard and colleagues [38] showed that surfactant isolated from bird lungs was composed primarily of dipalmityolphosphatidylcholine and SP-B, which was sufficient to maintain patency, and no SP-A like proteins were found. In other words, in chicken lungs there seems to be no need for a collagenous SP-A-like protein in order to keep the capillaries open, but it could very well be possible that cLL and cSP-A have a role in chicken lung immune defence.

Another more recently discovered collectin is CL-L1. In contrast to other collectins which are secreted into the extracellular space, human CL-L1 has been located to the cytoplasm of hepatocytes, where it is suggested to bind to internal ligands. Human CL-L1 does not multimerize to higher degrees but is present as a trimeric subunit [24].
As was also observed for cLL in the present study, both human and murine CL-L1 contain a Glu-Pro-Ser motif in their CRD. It was shown that recombinant murine CL-L1 had calcium-independent lectin activity, while human CL-L1 showed weak calcium-dependent activities for galactose, mannose, fucose and N-acetylglucosamine [24, 25]. However, no extensive monosaccharide preference studies were performed for these proteins, so the exact effect of substituting Asn by Ser is unknown. In this study, the chicken homologue of CL-L1 was identified and designated cCL-1. In contrast to its mammalian counterparts the CRD of cCL-1 contains a Glu-Pro-His motif. Since there are no other collectins known to contain this motif, it is unclear how it will affect the monosaccharide specificity of cCL-1.
Another interesting structural feature of human and murine CL-L1 is the presence of the four consecutive lysine residues at the C-terminus of the protein which are suggested to act as a nuclear localization signal (NLS). These NLSs are also found in yeast aminoacyl-tRNA synthetases [59] and at the C-terminus of human cytoplasmatic methionyl-tRNA synthetase [60]. In contrast to human and murine CL-L1, the C-terminus of cCL-1 consists of a Lys-Lys-Arg-Lys motif. Despite of this difference, the sequence still fits the proposed four-residue consensus sequence Lys-Arg/Lys-X-Arg/Lys for peptide-directed nuclear transport [61]. However, there is no experimental evidence for nuclear localization neither for human nor murine CL-L1. It would be interesting to see if these proteins are actually capable of moving to the nuclear compartment and if so, what their function there is.

cCL-1 was observed to be expressed in numerous tissues, consistent with the finding that human CL-L1 has an ubiquitous expression distribution. Murine CL-L1 is

suggested to be involved in embryonic development, showing expression in the amnion and the visceral yolk sac, and a dramatic increase in expression after the onset of organogenesis [25]. Interestingly, a high level of cCL-1 mRNA expression was also found in the vitelline membrane which surrounds the yolk. One could speculate that such a highly conserved protein present in so many different tissues must have an important function. It has been proposed that CL-L1s are part of a unique group in the collectin family [26]. Our data support this assumption, as the CL-L1 group is clustered together in one clade in the phylogenetic tree.

To date, there have been no publications on Collectin 11. The sequences of human and xenopus Collectin 11 have been submitted to GenBank (**BC000078** and **BC056052**, respectively), and a Collectin 11 gene has been predicted by Gnomon in various other species. In this study, the chicken homologue of this gene was designated cCL-2, and its predicted amino acid sequence shows that this protein is very likely to be a collectin with a monosaccharide preference for mannose over galactose. The gene structure of cCL-2 resembles that of the CL-L1 genes very closely (not shown), but the cCL-2 gene is located on chromosome 3 whereas the cCL-1 gene is located on chromosome 2, ruling out the possibility that either gene arose through a duplication of the other.

Together with the observation that cCL-2 forms a well supported clade with its mammalian homologues, this indicates that the Collectin 11 group may represent yet another unique class in the collectin family. The tissue distribution of cCL-2 mRNA resembles that of cCL-1, but further study is needed to establish whether or not cCL-2 is also localized in the cytoplasm. The sequence of human CL-P1 was first described by Ohtani *et al.* in 2001, and displays a unique feature; unlike all other collectins, this protein contains a transmembrane-domain which indicates that this protein could function as a transmembrane receptor. The predicted amino acid sequence of this protein shows that it is a type II membrane protein resembling scavenger receptor AI (SR-AI) [62]. It has a cytosolic domain containing an endocytosis motif (Tyr-Lys-Arg-Phe), which is also found in the asialoglycoprotein receptor [63]. Other structural features of CL-P1 include a coiled coil domain, a collagen domain and a CRD. Despite the absence of the heptad repeat pattern in the neck domain, CL-P1 can form oligomers due to the coiled coil structures located at the N-terminal side of the collagenous domain which contributes to the formation of trimers. The collagen domain has three polycationic regions containing the basic amino acids arginine or lysine. These polycharge islands are proposed to serve as binding sites for negatively charged ligands. The CRD of CL-P1 contains six cysteine residues and is homologous to the CRDs in the asialoglycoprotein receptor and the macrophage lectin 2. The chicken homologue of this protein was designated cCL-3. Since cCL-3 is highly homologous to human and murine CL-P1, it was not surprising to find cCL-3 clustered together with human and murine CL-P1 in the phylogenetic tree in a well supported clade.

The mRNA expression of cCL-3 was localized to various tissues, consistent with the finding that human CL-P1 mRNA was expressed in most tissues [26]. In the same study, immunohistochemical analysis using a chicken anti-human CL-P1 antibody showed that murine CL-P1 is present in murine vascular endothelial cells in the heart. Expression of hCL-P1 protein was also found in most vascular endothelial cells and in human heart sections. Together with the finding that CL-P1 can bind and phagocytose bacteria and yeast when transfected in Chinese hamster ovary cells, this indicates a putative role for CL-P1 in innate immunity in the vascular space. cCL-3 may have a similar function.

In summary, four chicken genes encoding three collectins and a lectin were found. The lectin cLL most resembles SP-As found in other species when the amino acid sequence of its CRD is compared. However, since this protein lacks a collagen domain its function may be different from that of the collectins. The collectins cCL-1, cCL-2 and cCL-3 are highly conserved between species, and their expression was found in various tissues. Further studies are needed to further elucidate the biological function of cLL, cCL-1, cCL-2 and cCL-3. It is noteworthy that although the initial EST database search was performed using the amino acid sequences of SP-Ds, no SP-D-like genes were found. Similarly, no chicken SP-A-like genes containing a collagen region of comparable size were found. It is possible that chicken analogues of the mammalian lung collectins do not exist, and it will be interesting to investigate the implications for innate immunity in chickens. The newly found chicken collectins and lectin may fulfill a role in avian host defence. It will be interesting to elucidate the exact biological functions of these proteins

Acknowledgements

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Chapter 5

Expression Levels of Chicken (Col)lectin mRNA During Infection with Influenza A Virus or Infectious Bronchitis Virus Superinfected with *Escherichia coli*

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Abstract

The innate immune system is dependent on the capability to quickly distinguish self from non-self. It relies for an important part on pattern recognition molecules, such as collectins, which are capable of binding to regular patterns of carbohydrates present on the surface of pathogens. Our laboratory previously reported the finding of chicken collectin 1 (cCL-1), chicken collectin 2 (cCL-2) and chicken Lung Lectin (cLL). In order to investigate whether these molecules are involved in the defense against invading bacteria and viruses, mRNA expression levels of cCL-1, cCL-2, cLL and chicken Surfactant Protein A (cSP-A) were determined. Samples from three infection models were analyzed; (1) Lung and spleen tissue samples from chickens inoculated with either a vaccine strain or a virulent strain of Infectious Bronchitis Virus (IBV) and subsequently superinfected with *E. coli*; (2) Tracheal organ cultures (TOC) infected with avian Influenza A Virus (IAV) subtype H9N2; (3) Lung tissue samples from chickens inoculated with avian IAV H9N2. In the first model, several significant differences were observed in mRNA expression levels of all genes investigated. In particular, cCL-2 mRNA expression was significantly increased in lung tissue isolated from chickens 3 hrs after inoculation with *E. coli* 506, while expression was reduced at 24 hrs *p.i.*. Furthermore, a clear reduction of cSP-A mRNA expression was observed in all groups inoculated with *E. coli* 506. In the second model, no differences in mRNA expression levels could be observed between control and the IAV-infected TOCs, but the preparation and/or culturing itself significantly reduced mRNA expression levels of cCL-1, cCL-2 and cLL, but not cSP-A, suggesting that under stressful conditions these genes may be downregulated. In the third model, cCL-2 mRNA expression was significantly lower in lung tissue samples isolated from chickens that had been infected with IAV compared to control samples. The mRNA expression levels of the other (col)lectins remained unaffected. Taken together, these results demonstrate that the mRNA expression levels of chicken (col)lectins can be altered during stress and after infection, and indicate that chicken (col)lectins could play a role in the attachment and entry of *E. coli* and IAV.

Introduction

In the early stages of life, the adaptive immune system is not yet fully developed. Although highly efficient in respect to raising an effective response, it requires prior contact with an invading pathogen in order to generate this specific response. Even when there has been prior contact with a pathogen, it can still take up to three days before a response is generated by the adaptive immune system. If this temporal gap were to be left unattended, the invading pathogen would have the opportunity to flourish and implications for the host could be very serious. Fortunately, the innate immune system provides a first line defense

against potential pathogens, bridging the interval between exposure to the pathogen and the specific response of the adaptive immune system. Pathogen-associated molecular patterns (PAMPs) are recognized by various different pattern recognition molecules associated with the innate immune system. Within this group of pattern recognition molecules, the calcium dependent (C-type) lectins [1] represent a family of proteins which are found throughout the animal kingdom. Within this family of proteins, the collagenous C-type lectins [2], commonly known as collectins have been shown to interact with a very broad range of micro-organisms (reviewed in **Chapter 2**, [3]), including Gram-negative bacteria [4-6] and viruses [7-10]. Until recently, only two collectins had been identified in chicken, Mannan Binding Lectin (MBL, [11]) and chicken Surfactant Protein A (cSP-A, [12]). Chicken MBL is capable of activating complement, and has been classified as a moderate acute phase reactant [13]. The nucleotide sequence of cSP-A was described in 2001 [12], but as the predicted amino acid sequence of this gene contains only three Gly-X-Y repeats instead of an extended collagenous domain, it is not clear whether cSP-A will have a similar function as its mammalian homologues. We recently reported the discovery of several new chicken collectins, including chicken Collectin 1 (cCL-1), chicken Collectin 2 (cCL-2), and a C-type lectin which was designated chicken Lung Lectin (cLL) [14], all of which are expressed in the chicken respiratory tract. Based on the described roles of SP-A, MBL, and Surfactant Protein D (SP-D) (**Chapter 2**, [3]) one could expect these new chicken collectins to have similar properties. However, the function of the mammalian counterpart of cCL-1, Collectin Liver 1 [15, 16], is not yet fully understood. Collectin Kidney 1 [17], homologue to cCL-2, has only recently been identified, and its biological function also still has to be elucidated. Based on the sequence of its carbohydrate recognition domain (CRD), cLL is most similar to SP-A [14] but lacks a collagenous domain which could have implications for its function. This protein is capable of binding mannose and trimannose in a calcium-dependent manner and may be involved in innate defense against Influenza A Virus (Chapter Six). Taken together, little is known about the putative functions of these proteins. In this study, samples derived from three different infection models were used to examine the effects of viral and bacterial infections on the mRNA expression levels of cCL-1, cCL-2, cLL and cSP-A in chicken lung, trachea and spleen. Results from these experiments show that the expression levels of these genes are altered during stress and after infection. In particular, cCL-1 and cCL-2 mRNA expression levels were found to be elevated in chicken lungs 3 hrs after inoculation with *E. coli* 506, while a significantly lower level of cCL-2 mRNA expression was observed 24 hrs after infection. This effect was not seen in chicken lungs isolated from chickens that had been previously inoculated with IBV. Furthermore, a significant reduction in cCL-2 mRNA levels was seen in chicken lung tissue at 1 and 3 days after inoculation with avian Influenza A Virus. The exact biological role of these proteins still needs to be elucidated, but results from this study indicate that they may play a role in the immune defense against *E. coli* and IAV.

Materials and Methods

Model 1. In vivo infection with Infectious Bronchitis Virus (IBV) superinfected with *E. coli*.

Animals

Eighteen day-incubated eggs originating from a Mycoplasma Gallisepticum-free broiler parent (Cobb) flock were obtained from a commercial hatchery and hatched at the research facility of the department of Farm Animal Health (Utrecht University). From day of hatch (day 1), broilers were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, The Netherlands) To diminish leg disorders and hydrops ascites, after 14 days of ad libitum supply, the feed was restricted on 'skip a day base'. Restriction was approximately 75% of ad libitum intake. Tap water was provided ad libitum throughout the experimental period.

Inocula

The IBV vaccine was obtained as commercial freeze-dried 1000 doses vial of H120 that contains per dose at least $10^{3.0}$ EID_{50} (egg infective dose 50%) (Nobilis® IB H120; batch number 041106A). The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, The Netherlands as freeze-dried vials containing $10^{8.3}$ EID_{50} /1.2 ml/vial. The virus had been passaged twice in SPF embryonated eggs. All IBV inocula were prepared in distilled water prior to use, and broilers received 1 dose/ml of H120 and $10^{4.6}$ EID_{50}/ml of IBV M41. The *Escherichia coli* strain 506 (O78; K80) is a gentamycin-sensitive strain isolated from a commercial broiler [18]. *E. coli* 506 was prepared as described [19]. Bacterial density of the *E. coli* culture was $10^{7.6}$ colony forming units (CFU)/ml.

Experimental design

At day of hatch (day 1) broilers were randomly divided into four groups of 30 chickens. Each group was housed in a separate isolator. Blood samples were taken from all chickens at 20 and 35 days of age. At 30 days of age all groups were inoculated oculo-nasally (in each eye and nostril, one droplet of 0.05 ml) and intratracheally (1 ml). Group 1 received IBV H120, group 2 IBV M41 and groups 3 and 4 received distilled water. At 35 days of age groups 1, 2 and 3 were intratracheally inoculated with 1 ml *E. coli* culture. Group 4 received 1 ml PBS-diluted glucose broth intratracheally.

Clinical and postmortem examination

Clinical signs resulting from IBV infection were determined 2 and 4 days after IBV

infection and at the time of sacrifice. All birds were individually scored. A bird was recorded as having signs of IBV infection if nasal discharge was observed after mild pressure on the nostril [19]. From each group, 5 broilers were electrocuted and bled at 3 hrs, 1 day and 7 days after *E. coli* inoculation. At each time point, colibacillosis lesions were scored macroscopically from all broilers. Lesion scoring was performed in the left and right thoracic airsac, pericardium and liver as described previously [18]. Lesion scores ranged from 0 to 3 per organ, leading to a maximum score of 12 per bird [19].

Model 2. In vitro tracheal organ cultures (TOC) infected with avian Influenza A Virus

Tracheal organ cultures (TOCs)
Tracheas of 18 day-old Cobb broiler embryos were aseptically removed and placed in warm culture medium containing DMEM (GIBCO; Invitrogen, Carlsbad, CA) with 2.38 g HEPES (Sigma, St. Louis, MO) and 100 units penicillin/100 µg streptomycin (GIBCO; Invitrogen, Carlsbad, CA) per ml. They were cut into 3 mm rings resulting in 6 TOCs per trachea. TOCs were transferred to a 12-well plate containing culture medium.

Virus
Avian Influenza A Virus, subtype H9N2, isolate A/Chicken/United Arab Emirates/99 was produced in eggs according to routine procedures. Virus was kindly provided by Intervet BV, Boxmeer, The Netherlands.

Experimental design
TOCs were divided randomly into two groups, infected and non-infected, containing 3 rings per group per well. TOCs were inoculated with 10^5 EID_{50} IAV in a total volume of 0.5 ml culture medium per well and incubated at 37°C, 5% CO_2. The control group was incubated in 0.5 ml culture medium. After 1 hr 1.5 ml culture medium was added and incubation continued. TOCs were harvested at 0, 3, 15 and 24 hrs post inoculation and stored in RNALater (Ambion, Austin, TX) at -80°C until further processing.

Model 3. In vivo infection with avian Influenza A Virus

Experimental design
One-day-old White Leghorns were housed under SPF conditions and all experiments were carried out according to protocols approved by the Intervet Animal Welfare Committee. Chickens were divided into 2 groups, infected and non-infected, containing 40 animals per isolator. One group of 14-day-old chickens was inoculated via aerosol spray with 20 ml $10^{7.7}$ EID_{50} IAV. The control group was inoculated via aerosol spray with 20 ml saline. Aerosol spray was made with an aerographer and compressor at a pressure of 1.5 Atm,

Table 1. Primers and probes used for RT-PCR*		
28S	**F**	5'- GGCGAAGCCAGAGGAAACT -3'
(X59733, [20])	**R**	5'- GACGACCGATTTGCACGTC -3'
	P	5'- (FAM) - AGGACCGCTACGGACCTCCACCA – (TAMRA) - 3'
cLL	**F**	5'- CTTACAAGGGAAGATAACAAAGTCTGG -3'
(DQ129667, [14])	**R**	5'- CATTCCTTGGAGATGCAATACACC -3'
	P	5'- (FAM) - CTCCTGGCACATTTTCACCGTAGCATGG – (BHQ) - 3'
cSP-A	**F**	5'- GGAATGACAGAAGGTGCAATCAG -3'
(AF411083, [13])	**R**	5'- GCAATGTTGAGTTTATTAGCTACAAATG -3'
	P	5'- (FAM) – CCGCCTTGTTGTCTGCCAGTTTTAGTGC – (BHQ) - 3'
cCL-1	**F**	5'- ATTGTCAAAGAAGAGAAGAATTACAGAG -3'
(DQ129668, [14])	**R**	5'- GAGGAGATGTAATCAGCAAGCAG -3'
	P	5'- (FAM) – CGTTGGTCACCTCATCTTTAGGCATGGC – (BHQ) - 3'
cCL-2	**F**	5'- GGGAGCCCAACAATGCCTATG -3'
(DQ129669, [14])	**R**	5'- GTAATATGACATGCAACATCATTCCAC -3'
	P	5'- (FAM) – TGCCACCATCTCCACACAGTCCTCCTC – (BHQ) -3'
GAPDH	**F**	5'- GTGGTGCTAAGCGTGTTATC -3'
(K01458, [44])	**R**	5'- GCATGGACAGTGGTCATAAG -3'
IAV	**F**	5'- GGTCAGACATTGCGAGTAAG -3'
(AF461530, [44])	**R**	5'- CTAGCAGGCACATTCCTCAG -3'

*Accession numbers for genes and references are given between brackets. *F: forward primer, R: reverse primer, P: probe, BHQ: Black Hole Quencher*

resulting in droplets with an average diameter of 50 µm. Chickens remained in the aerosol spray in a closed isolator (0.79 m3) for 10 min, after which the isolator was reventilated. At day 1 and 3 post inoculation chickens were killed (n=5 per time point per group) and lung tissue samples were isolated and stored in RNALater (Ambion) at -80°C. Lungs were divided into 4 equal parts; the quarter in which the bronchus enters the lung (cranial side) was used for RNA isolation.

Quantification of chicken (col)lectin mRNA expression

RNA extraction and cDNA synthesis

For samples derived from all three experimental models, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacturer's instructions. Purified RNA was eluted in 30 µl RNase-free water and stored at -80°C. cDNA was synthesized using iScript cDNA Synthesis Kit (Biorad, Hercules, CA). All samples were separately processed, but cDNA synthesized from RNA isolated from spleen tissue in the first experimental model was pooled.

Real Time Qualitative Polymerase Chain Reaction

A quantitative PCR reaction was set up for five genes: cLL, cSP-A, cCL-1, cCL-2 and 28S using the primers and probes depicted in **Table 1**. Probes were labeled with the fluorescent reporter dye FAM at the 5' terminus, and a black hole quencher (Eurogentec) at the 3' terminus, except for 28S which was labeled with FAM at its 5' terminus and TAMRA at the 3' terminus. All qPCR reactions were performed using two-step qPCR technology (Eurogentec), utilizing 5 µl cDNA per reaction in 25 µl in a MyiQ Single-Color Real-Time PCR Detection System (Biorad, Hercules, CA). For 28S, the following cycle protocol was used: 2 min at 50°C, 10 min at 95°C (denaturation); 40 cycles: 10s at 95°C and 60s at 59°C. For all other genes the cycle protocol was as follows: 2 min at 50°C, 10 min at 95°C (denaturation); 40 cycles: 15s at 95°C and 60s at 60°C. Results are expressed in terms of the threshold cycle value (Ct), the cycle in which the change in reporter dye passes a significance threshold (ΔRn). To generate standard curves, Log10 dilution series regression lines were made. In order to correct for variation in RNA preparation and sampling, Ct values for (col)lectin-specific product for each sample were standardized using the Ct value of 28S-specific product for the same sample. Using the slopes of the (col)lectin Log10 dilution series regression lines, the difference in input total RNA, represented by the 28S mRNA, was then used to adjust (col)lectin-specific Ct values as follows: Corrected Ct value = Ct + (Nt-Ct') * S/S', where Ct = mean (col)lectin of sample, Nt = experimental 28S median, Ct'= mean 28S of sample, S = (col)lectin slope, and S'= 28S slope [20]. Results are presented as 40-Ct values.

Viral load of TOCs in the experimental second model was assessed using iQ SYBR green supermix (Biorad). Primers for avian IAV and GAPDH detection (Invitrogen) are listed in Table 1, and were used at 400 nM concentration. Amplification and detection of specific products was achieved with the following cycle profile: 5 min at 95°C; 40 cycles: 10s at 92°C, 10s at 55°C and 30s at 72°C. In order to correct for variation in RNA preparation and Ct value of sampling, Ct values for IAV-specific product for each sample were standardized using the GAPDH-specific product for the same sample. 40-Ct values for IAV were calculated similarly to (col)lectin 40-Ct values described above.

Statistical analysis

Statistical analysis was performed using SPSS version 12.0.1 for Windows. Analysis of mean values between multiple groups was carried out using Levene's Test for Equality of Variances. When equality of variance was assumed, mean values between two groups were compared with an independent t-test. For experiments with multiple treatment groups, average data were analyzed by one-way analysis of variance followed by a Tukey test for significant difference. In all analyses a p value <0.05 was considered to indicate statistical significance.

Table 2. Macroscopical lesions.*			
	Time post inoculation with *E. coli* 506		
Group	**3 hrs**	**1 day**	**7 days**
PBS control	0^{A}	0^{A}	0^{A}
E. coli	0^{A}	1.6 ± 1.6^{B}	0^{A}
H120	0.5 ± 0.0^{A}	2.4 ± 1.5^{B}	5 ± 4.1^{B}
M41	0.5 ± 0.0^{A}	3.4 ± 0.9^{B}	1.5 ± 3^{B}

*Mean colibacillosis lesion (MLS) score is shown ± s.d., as calculated per group of five commercial broilers. Values within the same column and with different superscript differ significantly ($p < 0.05$).

Results

Model 1. In vivo infection with Infectious Bronchitis Virus (IBV) superinfected with *E. coli*

Clinical signs

Group 4, which received distilled water and PBS broth is referred to as PBS control group. Group 3 received distilled water and was inoculated with *E. coli* 506 on day 35, and is referred to as the *E. coli* group. Similarly, groups that had been previously inoculated with either IBV H120 or IBV M41 are referred to as the H120 group and M41 group, respectively. Nasal discharge was observed 2 to 9 days after inoculation with IBV. From 2-6 days post IBV inoculation, numbers of animals with nasal discharge were significantly higher in the M41 group (30 out of 30 birds) compared to the H120 group (3 out of 30 birds). Differences in macroscopical lesions, indicated by mean lesion scores (MLS) are shown in **Table 2**. Despite significant differences in nasal discharge, comparable clinical and macroscopical changes were observed in the H120 group and the M41 group after inoculation with *E. coli* 506. Furthermore, the *E. coli* group had recovered fully on day 7, whereas MLS were significantly higher in the superinfected groups.

mRNA expression levels of chicken (col)lectins

Expression levels of cCL-1, cCL-2, cLL and cSP-A in chicken lung tissue are shown in **Figure 1**. Expression levels of cCL-1 were elevated ($p < 0.05$) in the *E. coli* group 3 hrs post inoculation (*p.i.*) compared to the PBS control group, indicating an effect of the *E. coli* infection. Expression levels returned to control values 1 day *p.i.* In addition, a time dependent increase in cCL-1 mRNA expression ($p < 0.05$) was observed in the control group when expression levels at 1 day with *E. coli* 506 (1 day *p.i.*) were compared to 7 days

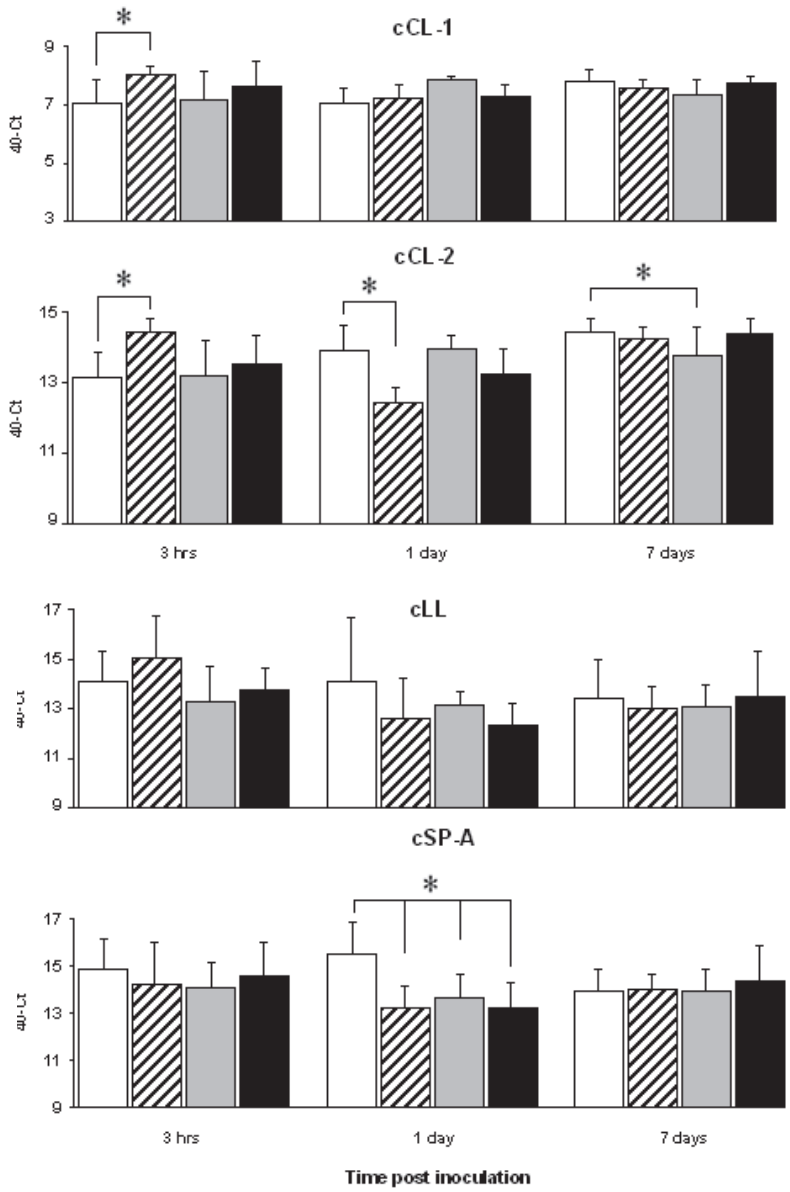

Figure 1. Results from Model 1. *In vivo* infection with Infectious Bronchitis Virus (IBV) superinfected with *E. coli*

Mean chicken (col)lectin mRNA expression levels in chicken lung tissue are shown at 3 hrs, 1 day and 7 days after inoculation with *E. coli* 506. 40-Ct values are given as mean ± s.d.

White bars: PBS control; Hatched bars: E. coli 506; Grey bars: H120; Black bars: M41+ E. coli 506.

* significant difference (p <0.05)

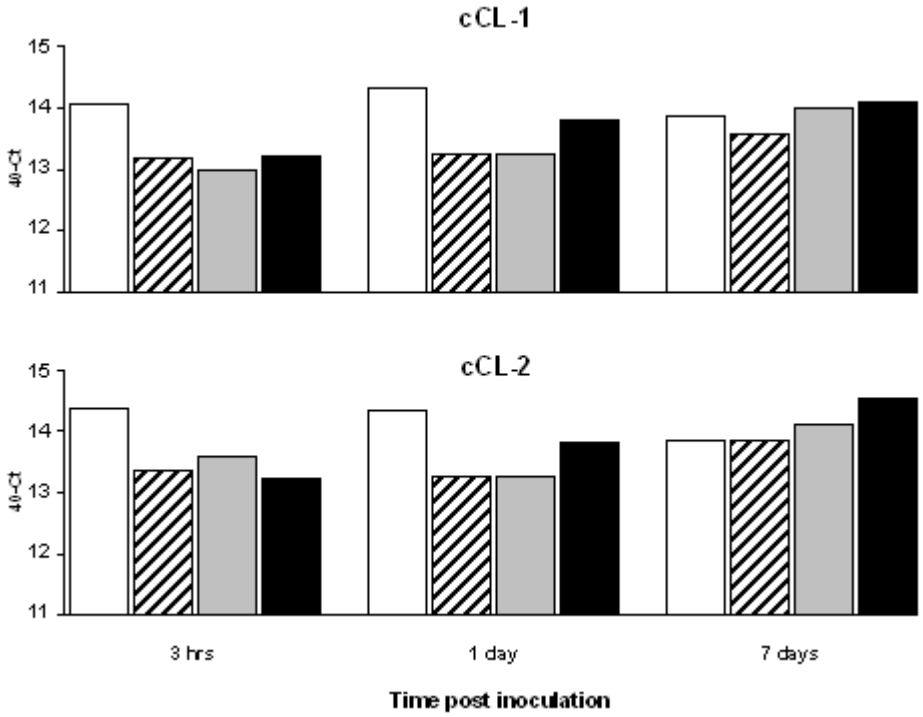

Figure 2. Results from Model 1. *In vivo* infection with Infectious Bronchitis Virus (IBV) superinfected with *E. coli*

Mean chicken (col)lectin mRNA expression levels in chicken spleen tissue are shown at 3 hrs, 1 day and 7 days after inoculation with *E. coli* 506. As cDNA samples were pooled, only mean 40-Ct values are shown. *White bars: PBS control; Hatched bars: E. coli 506; Grey bars: H120 + E. coli 506; Black bars: M41 + E. coli 506*

p.i. These results do not correlate to expression patterns observed in the spleen (**Figure 2**), as cCL-1 expression was decreased at 3 hrs and 1 day *p.i.* in all groups compared to the PBS control group.

Expression levels of cCL-2 mRNA in lung tissue were significantly ($p < 0.05$) increased in the *E. coli* group 3 hrs *p.i.* compared to the PBS control group, but this effect was reversed 1 day *p.i.*, where cCL-2 mRNA expression was significantly lower ($p < 0.05$). These *E. coli*-induced differences in cCL-2 mRNA expression were not observed in the IBV groups (both H120 and M41), although a significant reduction in cCL-2 mRNA expression was observed 7 days *p.i.* in the H120 group ($p < 0.05$). Furthermore, similar to cCL-1, a time-dependent increase in cCL-2 mRNA expression in the lung was observed within the PBS control group 3 hrs *p.i.* vs 7 days *p.i.* ($p < 0.05$). In the spleen, a slightly

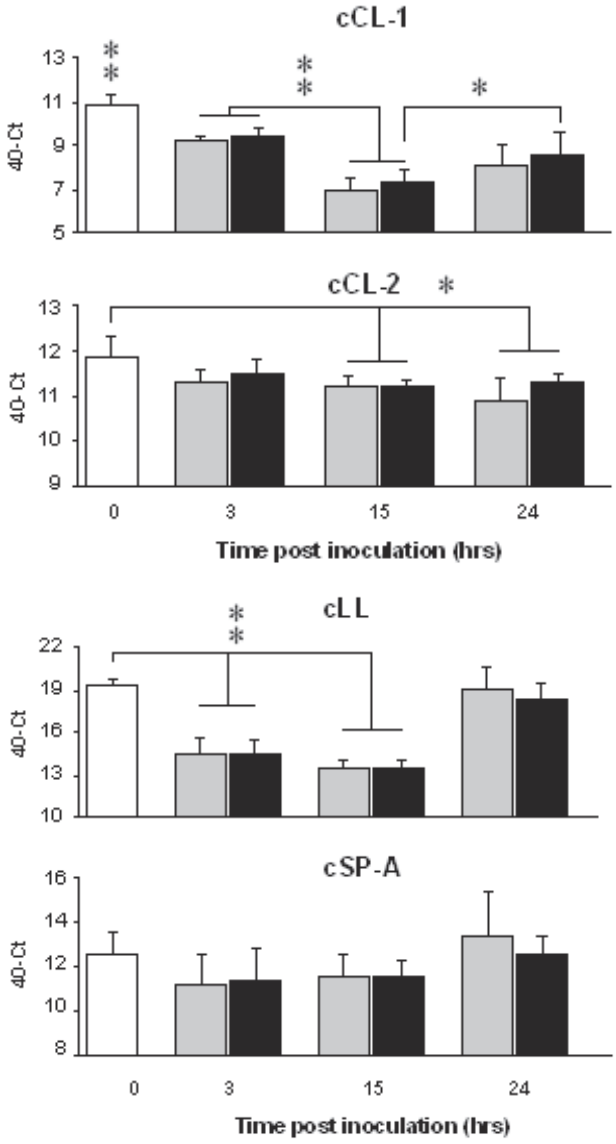

Figure 3. Results from Model 2. *In vitro* tracheal organ cultures (TOC) infected with avian Influenza A Virus.

Mean chicken (col)lectin mRNA expression in avian IAV-infected TOCs are shown. 40-Ct values are given as mean ± s.d. Results are shown for expression levels at time point 0 (start of the experiment), 3 hrs, 15 hrs, and 24 hrs *p.i. White bars: time point 0; Grey bars: PBS control; Black bars: infected TOCs*.
* significant difference $p < 0.05$, ** $p < 0.001$.

reduced cCL-2 mRNA expression was observed in all groups compared to the PBS control group at 3 hrs *p.i.* and 1 day *p.i.* After 7 days *p.i.*, cCL-2 mRNA expression levels had returned to values of the PBS control group. For cLL, no difference in mRNA expression in lung tissue was observed in any of the groups compared to the PBS control group. In the spleen, cLL mRNA expression differed between groups and between time points without showing an infection related expression pattern (data not shown). A significantly reduced mRNA expression of cSP-A in lung tissue was observed for all groups 1 day *p.i.* compared to PBS control values ($p < 0.05$). Similar to cLL, mRNA expression levels of cSP-A in spleen differed between groups and between time points without showing an infection related expression pattern (data not shown).

Model 2. In vitro tracheal organ cultures (TOC) infected with avian Influenza A Virus

Infection with Avian Influenza A Virus subtype H9N2

To check whether TOCs exposed to IAV had been infected by IAV, viral load was assessed by measuring mRNA expression of IAV-specific product. No signal was measured in uninfected samples, whereas in IAV-infected TOCs mean 40-Ct values at 3, 15 and 24 hrs *p.i.* were 4, 13 and 22, respectively.

Expression levels of chicken (col)lectins

Expression levels of cCL-1, cCL-2, cLL and cSP-A mRNA in TOCs infected with IAV were measured. Results are depicted in **Figure 3**. For all 4 genes examined, no significant effect of IAV-infection was observed compared to the control. However, there were some effects of the preparation of TOCs and/or culturing procedures on chicken (col)lectin mRNA levels. Mean mRNA expression levels of cCL-1 were significantly decreased after 3 hrs ($p < 0.001$) in both the infected and the uninfected group, and dropped even further after 15 hrs ($p < 0.001$, compared to both time points 0 and 3 hrs). This decrease in cCL-1 mRNA levels was sustained until at least 24 hrs after the start of the experiment, for both the infected and the uninfected groups. In the infected group cCL-1 mRNA expression levels were significantly higher at 24 hrs *p.i.* compared to 15 hrs *p.i.*, but still lower than time point 0. In the uninfected group, this increase in mean cCL-1 mRNA expression was also observed, but the difference was not significant. However, expression levels of cCL-1 mRNA did not differ significantly in the infected and uninfected TOCs at 24 hrs. Therefore, the increase in cCL-1 mRNA expression the infected group cannot be attributed to infection with IAV. A more pronounced effect was seen for cLL, where mRNA expression levels dropped dramatically ($p < 0.001$) after 3 hrs in both the uninfected and the infected groups. This decrease was sustained at 15 hrs ($p < 0.001$), but in contrast to cCL-1, cLL mRNA expression levels in both the infected and the uninfected group were completely restored at 24 hrs *p.i.*. cCL2 mRNA expression levels were not significantly decreased at 3 hrs *p.i.* in

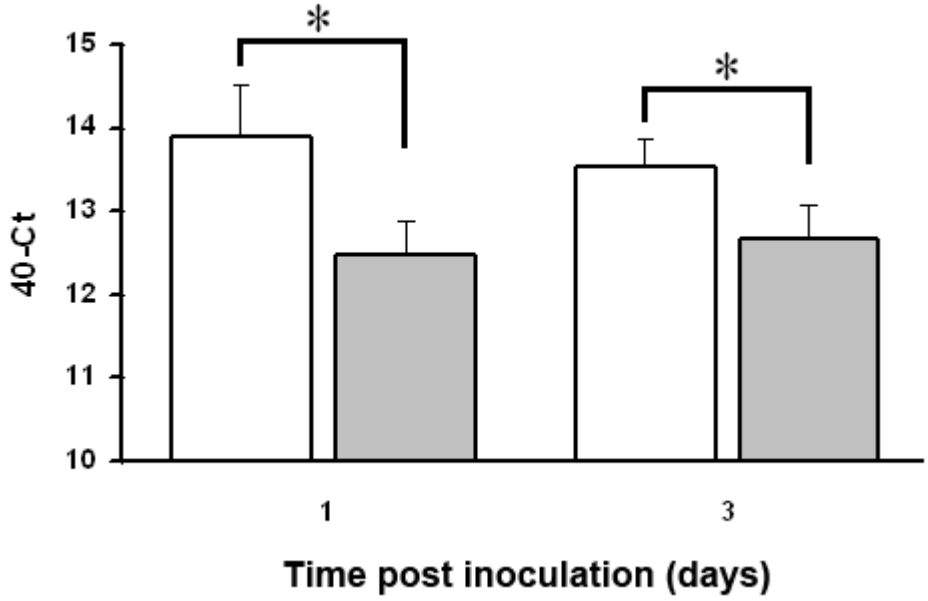

Figure 4. Results from Model 3. *In vivo* infection with avian Influenza A Virus
Mean cCL-2 mRNA expression in avian IAV infected chicken lungs. 40-Ct values are given as mean ± s.d. Results are shown for expression levels at 1 day and 3 days *p.i. White bars: PBS control; Grey bars: infected chicken lungs.* * significant difference, day 1 $p < 0.005$, day 3 $p < 0.02$

either the infected or uninfected group, but after 15 hrs *p.i.* cCL-2 mRNA levels were significantly ($p < 0.05$) reduced in both groups, and this reduction was maintained until 24 hrs after the start of the experiment ($p < 0.05$). cSP-A mRNA levels were not significantly affected in either the infected or the uninfected group during the experiment.

Model 3. In vivo infection with avian Influenza A Virus

mRNA expression levels of chicken (col)lectins
In the third model, lung samples were taken from chickens that had been spray-inoculated with IAV. Expression levels of cCL-1, cCL-2, cLL and cSP-A were measured (**Figure 4**). There were no significant differences in mRNA expression levels of cCL-1, cLL or cSP-A between infected or non-infected lungs.
However, cCL-2 mRNA expression was significantly lower in infected birds compared to uninfected controls on both 1 day ($p < 0.005$) and 3 days *p.i.* ($p < 0.02$).

Discussion

Recently, our laboratory reported the discovery of several new chicken collectins including cCL-1 and cCL-2 [14]. Furthermore, a C-type lectin was discovered which was designated chicken Lung Lectin (cLL) [14]. All of these genes and the previously discovered cSP-A [12] are expressed in the chicken respiratory tract. The aim of this study was to examine whether the mRNA expression of cCL-1, cCL-2, cLL and cSP-A is affected by infection by viruses and bacteria. For this purpose, samples from three infection models were analyzed for the expression of these genes.

In the first model, birds were inoculated with a virulent or vaccine strain of IBV and superinfected with *E. coli*. Clinical and macroscopical changes were comparable in both the vaccine and virulent virus groups, despite significant differences in numbers of animals with nasal discharge. Interestingly, mean colibacillosis lesion scores in *E. coli* and both IBV groups were similar at 1 day *p.i.*, whereas 7 days *p.i.* the *E. coli* group had fully recovered and both IBV groups still showed clinical signs, indicating that prior exposure to IBV results in prolonged colibaccilosis. This is in line with the findings of Matthijs et al. [19]. Changes in (col)lectin levels may affect attachment and entry of *E. coli* after previous exposure to IBV and might play a role in the enhanced susceptibility to viral/bacterial superinfections.

In this superinfection model, several changes in collectin expression profiles were observed. A significant increase in cCL-1 and cCL-2 mRNA expression in chicken lungs was detected in the *E. coli* group compared to the PBS control group 3 hrs *p.i.* (Figure 1). As a difference of 1 Ct-value should be interpreted as a Log2 difference, this increase corresponds to an approximately twofold increase in mRNA levels. This increase reflects an early response to *E. coli* 506 infection and coincides with the clinical effects in the *E. coli* group. Expression of cCL-1 and cCL-2 mRNA was not increased in the IBV-groups 3 hrs *p.i.*, and colibacillosis was prolonged in these groups. It is possible that cCL-1 and cCL-2 contribute to a reduction in systemic dissemination of the bacteria, and that IBV interferes with their mRNA expression. MBL affects susceptibility to Pseudomonas aeruginosa infection after burn injury by preventing a systemic dissemination of the organism [21]. Similarly, in SP-A-deficient mice, an infection with Group B Streptococcus infection leads to an increased systemic spread compared to wildtype mice [22].

In contrast to cCL-1, cCL-2 and cSP-A mRNA expression levels in lung tissue were significantly decreased at 1 day *p.i.*. A decrease in cCL-2 and cSP-A mRNA levels could reflect an *E. coli*-induced interference in the expression of these genes. A similar downregulation of other innate immune molecules such as defensins and Toll-like receptors has been described and could be essential to the pathogen's survival in the host [23-26]. However, for cSP-A, this decrease was observed in the *E. coli* group as well as the IBV

groups, suggesting that cSP-A expression is not affected by IBV. In contrast, for cCL-2 this decrease was only observed in the *E. coli* group. Furthermore, cCL-2 mRNA levels in the H120 group were also reduced 7 days *p.i.*, suggesting a delayed response in cCL-2 mRNA expression due to IBV infection.

Expression levels of cLL mRNA in lung tissue did not differ significantly, with the exception of a slight decrease at 1 day *p.i.* in the M41 group. However, no difference was found between the PBS control group and the M41 group, so the reason for this decrease cannot be attributed to the treatment. Therefore, it does not seem likely that cLL mRNA expression is affected by either IBV or *E. coli* infection.

Differences could not be analyzed statistically with respect to cCL-1 and cCL-2 mRNA expression in spleen tissue because cDNA-samples had been pooled. However, overall, the results show a decrease in cCL-1 and cCL-2 mRNA levels as a result of all treatments compared to the controls at 3 hrs and 1 day *p.i.*, and levels were restored to control values 7 days *p.i.* (Figure 2). This indicates that the observed changes in cCL-1 and cCL-2 mRNA expression are tissue specific. Expression of cLL mRNA in spleen did not show an infection related expression pattern, and similar results were observed for cSP-A mRNA expression.

Although it is obvious that *E. coli* 506 affects expression levels of all (col)lectins, the effect of IBV is less clear. However, whether these differences also reflect changes in the innate immune response remains to be elucidated.

In the second model, TOCs were infected with avian IAV, and the effects of infection on (col)lectin mRNA expression was monitored. Although no significant differences in mRNA expression levels as a result of IAV infection were observed for any of the (col)lectins, the preparation and/or culturing of the TOCs resulted in downregulation of all genes with the exception of cSP-A. The immediate drop in cLL and cCL-1 mRNA levels suggests an acute response to stress. Similarly, elevated SP-A and SP-D protein levels are associated with acute stress responses [27, 28]. Another possibility is that substances present in the medium influence the expression of these genes. As cCL-2 mRNA expression was downregulated after 15 hrs, it is less likely than cLL and cCL-1 to be affected by acute stress. However, culturing conditions may explain the results obtained for cCL-2 mRNA expression.

In the third model, expression levels of chicken (col)lectin were measured in lung samples taken from chickens that were spray-inoculated with avian IAV. No differences as a result of infection were observed for cCL-1, cSP-A or cLL, but a significant decrease in cCL-2 expression levels was seen on both day 1 and day 3, suggesting that IAV interferes with cCL-2 mRNA synthesis. A similar drop in expression was observed in the first experimental model. This downregulation could reflect an evasion strategy of the pathogen.

In most studies investigating the mRNA expression levels of collectins during bacterial and viral infections, an increased expression in response to infection was observed

[29-31]. Furthermore, a decreased clearing of bacteria or viruses has often been seen in SP-A or SP-D-deficient rodents [32-36], and this is frequently associated with increased inflammation and inflammatory cell recruitment after infection [22, 36-39]. Contrary to SP-A and SP-D, the chicken (col)lectins were either up- or downregulated depending on the experimental model and the time of measurement. A lower level of mRNA could indicate that expression is suppressed by the pathogen. This makes an interpretation of the observed effects difficult, especially in correlation with the clinical signs observed in the first experimental model. However, it is obvious that mRNA levels of chicken collectins are affected by pathogens.

In conclusion, the results obtained in this study show that mRNA expression of chicken collectins cCL-1, cCL-2, and cSP-A can be affected by both bacterial and viral infections. In addition, mRNA expression of cCL-1 and cLL appear to be acutely affected under stressful conditions. As pathogens in poultry can pose a considerable risk to both animal and public health [40-43], it would be very interesting to see how these changes relate to the effects chicken (col)lectins may have in chicken innate immune responses.

Acknowledgements

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Chapter 6

Structural and Functional Aspects of Recombinant Chicken Lung Lectin

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Abstract

Many proteins of the calcium dependent (C-type) lectin family have been shown to play an important role in innate immunity. They can bind to a broad range of carbohydrates, which enables them to interact with ligands present on the surface of micro-organisms. We previously reported the finding of a new putative chicken lectin, which was predominantly localized to the respiratory tract, and thus termed chicken Lung Lectin (cLL). In order to investigate the biochemical and biophysical properties of cLL, the recombinant protein was expressed, affinity purified and characterized. Recombinant cLL (rcLL) was expressed as four differently sized peptides, which is most likely due to post-translational modification. Crosslinking of the protein led to the formation of two differently sized high-molecular weight products, indicating that rcLL can form trimeric and possibly even multimeric subunits. rcLL was shown to have lectin activity, preferentially binding to α-mannose in a calcium dependent-manner. In addition, rcLL was used to investigate whether this protein could play a role in avian innate immunity. Preliminary results showed an inhibitory activity against infection of tracheal organ cultures by Avian Influenza A Virus, subtype H9N2. Furthermore, rcLL was shown to inhibit the haemagglutination-activity of a human isolate of Influenza A Virus, subtype H3N2. Taken together, these results show that rcLL has C-type lectin activity, as predicted from its sequence. In addition, anti-viral effects were observed for rcLL. Future research should elucidate the role of cLL in chicken innate immunity.

Introduction

The effectiveness of the innate immune system highly depends on the recognition of pathogens. For this purpose, the innate immune system relies on pattern recognition molecules which are capable of binding to regular patterns of carbohydrates present on the surface of pathogens. Within this group of pattern recognition molecules, the calcium dependent (C-type) lectins represent a family of proteins which are found throughout the animal kingdom [1]. These proteins share a structural homology in their carbohydrate recognition domains (CRD) but differ with respect to their carbohydrate specificity. We recently reported the discovery of a chicken lectin which was designated chicken Lung Lectin (cLL) due to its predominant expression in the chicken respiratory system [2]. cLL was identified as a C-type lectin, and although it could not be classified as a collectin due to its lack of a collagen domain, the sequence of its CRD was found to be most homologous to that of the collectin Surfactant Protein A (SP-A) (39% - 45% similarity, depending on the species) [2]. To our knowledge, no other C-type lectins with more structural similarities to cLL have been identified to date. Collectins and several other C-type lectins have been

Table 1. Primers used for PCR and Real-Time quantitative PCR (RT-qPCR)*		
cLL (DQ129667, [2])	**F**	5'- GGATCCAAACCAACACAGATTTTTCC-3'
	R	5'- GCGGCCGCAAACTGGCAGACAACAAG-3'
GAPDH (K01458, [27])	**F**	5'- GTGGTGCTAAGCGTGTTATC-3'
	R	5'- GCATGGACAGTGGTCATAAG-3'
IAV (AF461530, [27])	**F**	5'- GGTCAGACATTGCGAGTAAG-3'
	R	5'- CTAGCAGGCACATTCCTCAG-3'

*Accession numbers and references for genes are given between brackets.
F: forward primer, R: reverse primer

identified as important molecules in innate immunity. For example, SP-A plays an important role in innate defense against invading pathogens in the lung (reviewed in [3, 4]), including Influenza A Virus (IAV), while other lectins such as RegIIIγ [5], and its human homologue HIP/PAP [6] have been shown to have antimicrobial properties [7]. Tetranectin, a secreted C-type lectin
[8, 9] which has also been described in chicken (unpublished data, Accession Number **CAC20217**) has been shown to bind to plasminogen [10]. In this study, we expressed recombinant cLL (rcLL) to investigate structural and functional characteristics of this protein. The carbohydrate-specificity of rcLL was tested, and the putative protective role of rcLL were studied by investigating viral inhibition of Avian IAV, subtype H9N2. In addition, haemagglutination-inhibition and neutralization of viral infectivity of various IAV strains isolated from humans were tested.

Materials and Methods

RNA extraction and cDNA synthesis
Total cellular RNA from lung tissue from healthy female Ross 308 broiler chicken was extracted using TRIzol© (Invitrogen, Carlsbad, CA) and Magnalyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany Diagnostics GmbH, Mannheim, Germany). The cDNAs used as templates in PCR were synthesized using 1 µg DNase I treated RNA with M-MLV-RT and 500 µg/ml oligo dT12-18 primers (Invitrogen, Carlsbad, CA) in a 20 µl reaction volume with incubation at 37°C for 50 min.

Polymerase chain reaction and amplified DNA fragment isolation
To amplify cDNA of the mature peptide sequence of cLL, a PCR reaction was performed using FastStart DNA Taq-polymerase (Roche Diagnostics Gmbh, Mannheim, Germany), and cLL specific primers shown in **Table 1**. The forward primer contained the BamHI restriction site sequence, and cLL reverse primer contained the NotI restriction site sequence. Amplification comprised of initial denaturation at 95°C for 2 min, followed by

40 cycles consisting of 95°C for 30s, 49°C for 30s, 72°C for 1 min and a final extension at 72°C for 7 min. PCR-products were analyzed by agarose gel electrophoresis and purified with a QIAEX agarose gel extraction kit (Qiagen, Valencia, CA).

Cloning and sequencing of the PCR products

The purified PCR fragments were ligated into a pCR® 4-TOPO® plasmid vector (Invitrogen, Carlsbad, CA). Ligated plasmids were transfected into TOP10 Escherichia coli cells by heatshock. Clones were selected by growth on Luria-Bertani broth (LB)-plates containing 100 µg/ml kanamycin. Positive clones were screened with PCR for correct product size and sequenced. Sequence reactions were performed using an ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA). All reactions were carried out in both directions using the T7 and T3 primer sites and separated on an ABI PRISM 3100 fluorescent DNA sequencer (Applied Biosystems, Foster City, CA). cLL-inserts were isolated from positive clones using BamHI and NotI restriction sites incorporated into the sequence during PCR, and ligated into a pABC-cystatin-hisN vector with a cystatin signal peptide and an in-frame N-terminal His-tag.

Transfection

HEK293-EBNA cells (ATCC CRL10852) were grown in 90% Freestyle (GIBCO; Invitrogen, Carlsbad, CA) and 10% Ca^{2+}-free Dulbecco's modified Eagle's medium (DMEM) (GIBCO; Invitrogen, Carlsbad, CA), containing 5% FCS (Invitrogen), 1% pluronic (Sigma-Aldrich, St.Louis, CA), 10 mM HEPES, 4 mM L-glutamine, 200 U/l penicillin G, 0.1 mg/l streptomycin and 50 µg/ml geneticin. Cells were maintained in exponential growth using Erlenmeyer flasks at 120 rpm on an orbital shaker mounted in a Reach-In CO_2 incubator (Clean Air Techniek, Woerden, The Netherlands). HEK293-EBNA cells were transfected using DNA-PEI (Polysciences, Warrington, PA) according to Durocher et al [11]. Briefly, 24 hrs before transfection, cells were seeded at 2.5 x 10^5/ml in medium without FCS. The next day DNA-PEI cmplexes were formed by a 10 min incubation of plasmid DNA at 20 µg/ml with PEI at 40 µg/ml in Optimem (GIBCO; Invitrogen, Carlsbad, CA), 25 µl of this mixture was used for each ml of cell culture to be transfected. Small scale transfections (4 ml) were performed in 6 well plates, large scale transfections were performed in a Bioreactor (New Brunswich Scientific, Edison, NJ).

Purification of rcLL

The supernatant of transfected HEK293-EBNA cells was collected after 6 days and concentrated to a final volume of approximately 250 ml using a holow fiber column (molecular-mass cut-off 10 kDa, Amersham Biosciences, Uppsala, Sweden). Purification of rcLL was performed by affinity chromatography (adapted from [12]). Briefly, 1 ml (bed-

volume) mannan-sepharose (Sigma, St. Louis, MO), equilibrated in 50 mM Tris-HCl, 5 mM $CaCl_2$, and 0.05% (vol/vol) Tween-80, pH 7.4 was added to the supernatant and $CaCl_2$ was added to a final concentration of 5 mM. The mixture was stirred overnight at 4°C. Sepharose beads were washed with 25 ml washing buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 500 mM NaCl, 0.05% Tween-80, pH 7.4). This washing procedure was repeated with 25 ml washing buffer without Tween-80. rcLL was eluted with 50 mM Tris-HCl, 5 mM EDTA, pH 7.4. The eluted protein was concentrated using Amicon Ultra centrifugal filter units with a molecular weight cutoff of 10 kDa (Millipore, Billerica, MA), after which the buffer was changed to 5 mM Tris-HCl, 150 mM NaCl, pH 7.4 by repetitive washing. Purified rcLL was stored in aliquots at -20°C.

Electrophoresis and Western Blot Analysis

Proteins (0.1-1 µg/lane) were analyzed by SDS-PAGE as described by Laemmli [13] using 10% polyacrylamide gels. Protein bands were visualized by Coomassie staining. For immunoblot analysis, proteins (0.1-1 µg/lane) were transferred electrophoretically from the gels onto nitrocellulose membrane. Immunostaining was performed using mouse anti-His_6 monoclonal antibody (Roche Diagnostics Gmbh, Mannheim, Germany). Primary antibodies were detected by peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma, St. Louis, MO).

Enzymatic deglycosylation of rcLL

N-deglycosylation treatment of rcLL was performed using N-glycanase® PNGase F (ProZyme, Inc., San Leandro, CA) according to manufacturer's instructions. Briefly, 45 µl cLL (230 µg/ml) was mixed with a 10 µl incubation buffer (0.25 M sodium phosphate, pH 7.0) 2.5 µl denaturation solution (2% SDS and 1 M β-mercaptoethanol). The sample was heated to 100°C for 5 min, after which the sample was cooled and 2.5 µl detergent solution (15% NP-40) was added. 2 µl of N-glycanase (5 U/ml) was added, and after 16 hr of incubation at 37°C, samples were concentrated using Microcon centrifugal filter units (MWCO 10 kDa) (Millipore, Billerica, MA). Samples were immediately processed for further analysis. O-deglycosylation treatment was performed using 1 µl 0.5 mU/µl O-Glycosidase (Roche Diagnostics Gmbh, Mannheim, Germany) according to the protocol used for N-glycanase-PNG-ase. However, for O-glycosidase treatment the rcLL storage buffer was changed to phosphate buffer (pH 7.0) using Microcon centrifugal filter units (MWCO 10 kDa) (Millipore, Billerica, MA). Deglycosylation by both N-glycanase-PNG-ase and O-glycosidase was carried out similarly to O-deglycosylation, but in this case both enzymes were added simultaneously.

DIG-Glycan detection

To assess possible glycosylation of rcLL and enzymatically treated rcLL, the DIG-glycan detection kit (Roche Diagnostics Gmbh, Mannheim, Germany) was used according to the manufacturer's instructions. Briefly, samples containing rcLL were loaded onto gel and subsequently transferred from the gel onto nitrocellulose membrane. The presence of glycoconjugates was assessed by labeling oxidized sugars with DIG-0-3-succinyl-ε-aminocaproic acid hydrazide, which was subsequently detected using anti-digoxigenin-AP and staining with NBT/X-phosphate.

Bis (Sulfosuccinimidyl) suberate (BS)-crosslinking of rcLL

Crosslinking of rcLL using Bis (Sulfosuccinimidyl) suberate (BS^3) (Pierce, Rockford, IL) was performed according to the manufacturer's instructions. Briefly, the rcLL storage buffer was changed to 10 mM HEPES buffer, pH 7.5 containing 3 mM EDTA using Microcon centrifugal filter units (MWCO 10 kDa) (Millipore, Billerica, MA). Final concentration of rcLL was approximately 170 µg/ml. BS^3 (50 mM) was added to the protein solution and incubated at room temperature for 30 min. The reaction was stopped by adding SDS-PAGE sample buffer to the mixture, which was subsequently heated to 100°C and loaded onto the gel for further analysis.

MALDI TOF-TOF

After protein separation on 12% SDS-PAGE and fixation in 50% methanol and 7% acetic acid, rcLL was visualized using GelCode Blue Stain reagent (Pierce, Rockford, IL). The four visible bands were cut from the gel individually and subjected to in-gel tryptic digestion as previously described [14]. Subsequently, these bands were identified by matrix-assisted laser desorbtion/ionization (MALDI) TOF-TOF analysis. The samples, dissolved in 0.1% acetic acid, were concentrated using µC18-ZipTips (Millipore) and eluted directly on the target plates in 1 µl of a saturated solution of R-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile. Data were acquired on a MALDI TOF-TOF instrument (Applied Biosystems 4700 Proteomics Analyzer) in positive reflectron mode at a laser intensity of 3800 and a bin time of 0.5 ns.

Carbohydrate specificity

Polyacrylamide (PAA)-coupled glycoconjugates (~20% substitution; Lectinity, Lappeenranta, Finland) were coated (5 µg/ml) in 0.2 M sodium cacodylate buffer (pH 9.2) on NUNC maxisorb plates (NUNC, Roskilde, Denmark) overnight at 4°C. Plates were blocked with 1% ELISA-grade BSA (Fraction V, fatty acid free; Calbiochem, San Diego, USA) in TSM (20 mM Tris-HCL pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) and rcLL was added (10 µg/ml) for 2-3 hrs at 37°C in the presence or absence of 10 mM EDTA. After washing with TSM containing 0.1% Tween-20, 1 µg/ml anti-His_6 antibody

(Roche Diagnostics Gmbh, Mannheim, Germany) was added for 1 hr at room temperature. Binding was detected using a peroxidase-labeled anti-mouse IgG antibody (Jackson, West grove, PA, USA) and measured in a Benchmark Microplate Reader using the Microplate Manager software (both from Biorad, Hercules, CA).

Viral inhibition-assays

A. Testing the activity of rcLL against avian IAV, subtype H9N2

Tracheal organ cultures (TOCs)
Tracheas of 18 day-old Cobb broiler embryos were aseptically removed and placed in warm culture medium containing DMEM (GIBCO; Invitrogen, Carlsbad, CA) with 2.38 g HEPES (Sigma, St. Louis, MO) and 100 units penicillin/100 µg streptomycin (GIBCO; Invitrogen, Carlsbad, CA) per ml. They were cut into 3 mm rings resulting in 6 TOCs per trachea. TOCs were transferred to a 24-well plate containing culture medium.

Virus and immune serum
Avian IAV, subtype H9N2, isolate A/Chicken/United Arab Emirates/99 was produced in eggs according to routine procedures. Chicken immune serum was obtained from SPF chickens 5 weeks after vaccination with IAV. Virus and immune serum was kindly provided by Intervet BV, Boxmeer, The Netherlands.

Experimental design
TOCs were divided randomly and transferred to a 24-well plate (2 TOCs per well) containing culture medium. Culture medium was modified to infection medium by adding 3.5 mM $CaCl_2$ (Merck, Darmstadt, Germany). Pre-mixes of virus and rcLL were made by combining virus ($2*10^5$ EID_{50} IAV/ml infection medium) with rcLL (25 µg/ml), virus with chicken immune serum, and virus with infection medium as negative control. All premixes were made and treated similarly in all three experiments. All tubes were vortexed briefly and incubated for 1 hr at 37°C, 5 % CO_2. Subsequently, culture medium was removed and 0.25 ml premix per well was added to TOCs. Plates were incubated for 1 hr at 37°C, 5 % CO_2 after which 0.5 ml infection medium was added. TOCs were harvested at 4 hrs post inoculation and stored in RNALater (Ambion, Austin, TX) at -80°C.

Real-Time quantitative reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Purified RNA was eluted in 30 µl RNase-free water and stored at -80°C. cDNA was synthesized using iScript cDNA Synthesis Kit (Biorad, Hercules, CA).

Quantitative RT-PCR was performed with a MyiQ Single-Color Real-Time PCR Detection System (Biorad, Hercules, CA) using iQ SYBR green supermix (Biorad, Hercules, CA). IAV and GAPDH specific primers (Invitrogen, Carlsbad, CA) are shown in **Table 1**, and were used at 400 nM concentration. Amplification and detection of specific products was achieved with the following cycle profile: one cycle of 95°C for 5 min, 40 cycles of 92°C for 10s, 55°C for 10s and 72°C for 30s. Results are expressed in terms of the threshold cycle value (Ct), the cycle in which the change in reporter dye passes a significance threshold (ΔRn). To generate standard curves, GAPDH and IAV PCR-fragments were cloned and used to generate Log10 dilution series regression lines. In order to correct for variation in RNA preparation and sampling, Ct values for IAV-specific product for each sample were standardized using the Ct value of GAPDH-specific product for the same sample. Using the slopes of the IAV Log10 dilution series regression lines, the difference in input total RNA, represented by the GAPDH mRNA, was then used to adjust IAV-specific Ct values as follows:
Corrected Ct value = Ct + (Nt-Ct') * S/S', where Ct = mean IAV of sample, Nt = experimental GAPDH median, Ct'= mean GAPDH of sample, S = IAV slope, and S'= GAPDH slope [15]. Cycle thresholds values (Ct) are expressed subtracted from 40 (negative end point). Therefore, higher levels represent higher levels of IAV-specific mRNA.

B. Testing the activity of rcLL against human isolates of IAV
Virus preparations
IAV was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified on a discontinuous sucrose gradient as described previously [16]. Virus stocks were dialyzed against PBS, aliquoted, and stored at -70°C. A/Phillipines/82 (H3N2) (Phil), and its bovine serum β inhibitor-resistant variant Phil/BS, were provided by Dr. E. M. Anders (Department of Microbiology, University of Melbourne, Melbourne, Australia). A/Puerto Rico/8/34 (H1N1) (PR-8), which lacks the high-mannose glycans on the haemagglutinin molucule, was provided by Dr. J. Abramson (Department of Pediatrics, Bowman Gray School of Medicine, Wake Forest University, Winston Salem, NC).

Neutralization of infectivity
Madin-Darby canine kidney (MDCK) cell monolayers were prepared in 96-well plates and grown to confluence. These layers were then infected with a PBS^{++} (PBS with 1 mM calcium and 0.5 mM magnesium; Gibco BRL, Grand Island, NY) -diluted IAV preparation (Phil strain) which was preincubated for 30 min at 37°C in the presence or absence (control) of increasing amounts of rcLL. After exposure of the MDCK cells to the IAV or IAV/rcLL mixture for 30 min at 37°C, the cells were washed three times in serum-free DMEM (Gibco BRL, Grand Island, NY) containing 1% (w/v) penicillin-streptomycin and

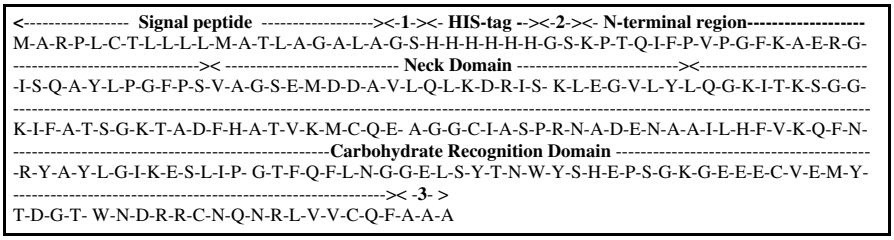

Figure 1. Predicted amino acid sequence of rcLL.

1: extra Gly-Ser-residues added after signal peptide; **2**: extra Gly-Ser-residues added as a result of inserting the BamHI restriction site into the sequence; **3**: extra Alanine-residues added as a result of inserting the NotI restriction site into the sequence

subsequently incubated for 7 hrs at 37°C. Next, the monolayers were washed, fixed, and FITC-labeled for IAV nucleoprotein as described previously [17], after which fluorescent foci were counted.

Haemagglutination-inhibition assay

Haemagglutination (HAA)-inhibition was measured by serially diluting collectin preparations in round-bottom 96-well plates (Serocluster U-Vinyl plates; Costar, Cambridge, MA) using PBS^{++} as diluent (25 µl per well). After adding 25 µl of IAV solution, giving a final concentration of 40 HAA U/ml or 4 HAA U/well, the IAV/rcLL mixture was preincubated for 15 min, followed by the addition of 50 µl of human erythrocyte suspension in PBS^{++}. The entire procedure was performed at room temperature. The minimal concentration of rcLL, required to fully inhibit the HAA caused by the virus, was determined by reading the plates after 2 hrs. HAA was detected as the formation of a pellet of erythrocytes.

Statistical analysis

Statistical analysis was performed using SPSS version 12.0.1 for Windows. Analysis of mean values between groups was carried out using Levene's Test for Equality of Variances. When equality of variance was assumed, mean values between groups were compared with an independent t-test, in which $p < 0.05$ was considered to indicate statistical significance.

Results

Production and characterization of recombinant cLL

Preliminary results from the small scale transfections (not shown) showed that rcLL was most effectively secreted using the mature peptide sequence ligated into the pABC-cystatin-hisN vector with a cystatin signal peptide and an in-frame N-terminal His-tag. Therefore,

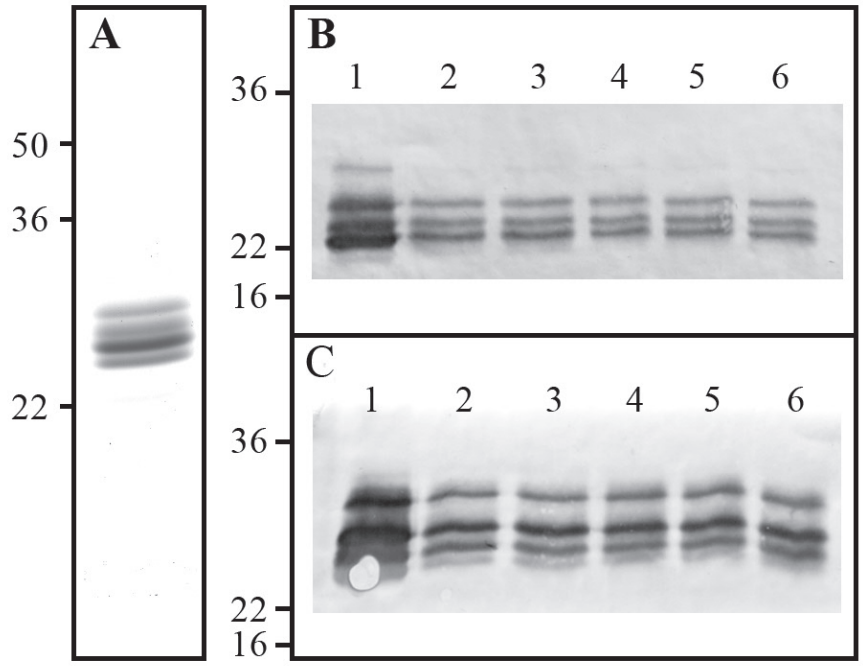

Figure 2. Production and structural characterization of rcLL.
(**A**) Coomassie staining of rcLL eluted from mannan-sepharose beads. (**B**) Western Blot-analysis of the eluted rcLL and enzymatically treated rcLL using anti-His6. (**C**) DIG-glycan detection for both Sham-treated and enzymatically treated rcLL. *(B&C): 1: untreated rcLL, 2: Sham-treated N-Glycanase-PNGase, 3: N-Glycanase-PNGase-treated rcLL, 4: N-Glycanase-PNGase and O-glycosidase-treated rcLL, Lane 5: O-glycosidase-treated rcLL, Lane 6: Sham-treatment O-glycosidase.*

this vector was selected for large-scale (1 liter) transfections. Affinity purification using mannan-sepharose yielded approximately 500-800 µg of rcLL per batch (**Figure 2a**). The predicted amino-acid sequence of rcLL is shown in **Figure 1**. However, four different bands varying in size between approximately 22 kDa and 27 kDa were observed. Western Blot analysis using anti-His_6 antibody showed a positive signal for all four bands (**Figure 2b**) in the untreated rcLL-sample (Lane 1). Results of the DIG-glycan detection are shown in **Figure 2c** and indicate that all of the 4 recombinant protein bands contained sugar moieties. Enzymatic treatment of rcLL did not result in a size shift (lane 2-6), indicating that the sugars are not N- or O-linked sugars. Crosslinking of rcLL resulted in the formation of two high-molecular weight bands of approximately 85 kDa and 160 kDa (**Figure 3**), suggesting that rcLL is capable of forming multimers, possibly trimers and hexamers based on the observed masses.

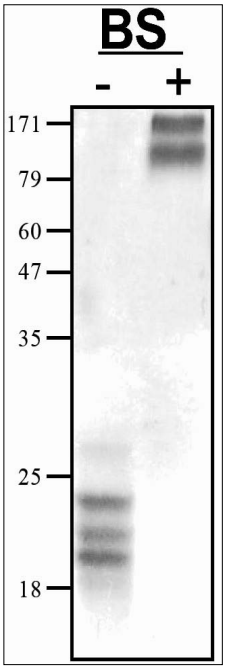

Figure 3. BS-crosslinking of rcLL.
Left lane: untreated rcLL; Right lane: crosslinked rcLL.

Analysis by MALDI-TOF-TOF

MALDI-TOF-TOF analysis was performed in order to investigate the cause of appearance of four bands. However, the spectra of the trypsin-digested protein of all four bands were similar, and could not explain the size difference observed on SDS-PAGE. Several peaks in the spectra could be assigned to sequences of rcLL, including the N- and C-terminus which were retrieved for all four bands (data not shown).

Carbohydrate specificity

Carbohydrate specificity of rcLL was tested by use of PAA-coupled glycoconjugates. rcLL was observed to preferentially bind to α-mannose-PAA and trimannose-PAA (Man3) (**Fig. 4**). Binding to α-mannose-PAA and Man3-Paa was also tested in the presence of EDTA, which significantly reduced the binding, indicating that rcLL binding to α-mannose and Man3 was calcium-dependent.

Viral inhibition-assays

A. Inhibitory activity of rcLL against avian IAV, subtype H9N2

In order to assess whether rcLL is capable of protecting TOCs from infection by Avian Influenza A Virus subtype H9N2, viral inhibition assays were carried out. Results of these tests are depicted in **Figure 5**. In experiment 1, inoculation of TOCs with a premix of IAV and rcLL from the first batch (1.1) led to a significant decline of IAV mRNA expression ($p < 0.05$), compared to the negative control (IAV-medium premix), suggesting a rcLL-mediated inhibition of entry of IAV into TOCs. In experiment 2, the second batch of rcLL (2.2) failed to induce this inhibition. The positive control, chicken immune serum, also failed to caused a significant decline in IAV mRNA expression in this experiment. Therefore, both rcLL batch 1 (3.1), and rcLL batch 2 (3.2) were retested in experiment 3, together with rcLL batch 3 (3.3). Due to the low amount of rcLL still available for testing, batch 1 was tested in duplicate, and batch 2 in triplicate. A decline of IAV mRNA expression was not found for samples treated with either rcLL batch 2 (3.2) and 3 (3.3). For rcLL batch 1, a decline in IAV mRNA expression was found in one of the replicates.

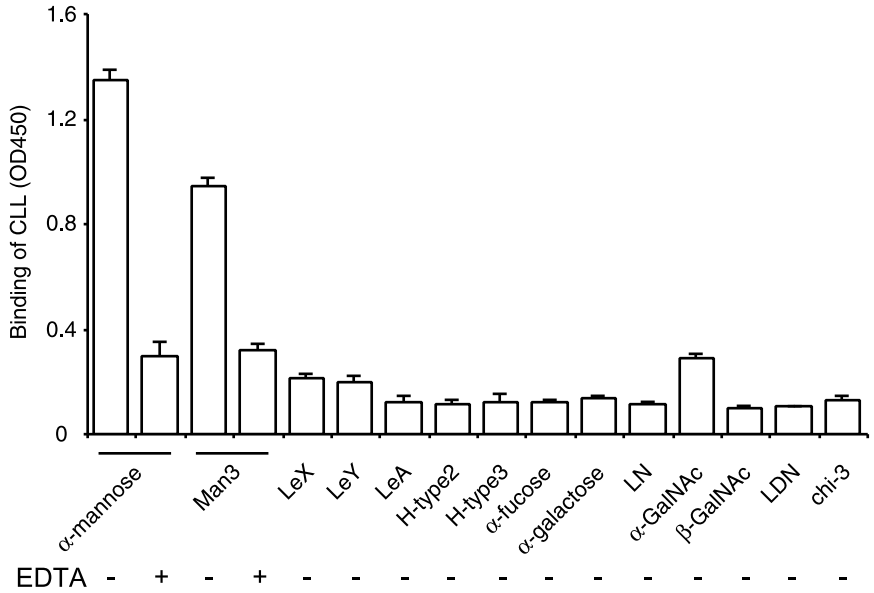

Figure 4. Carbohydrate binding specificity of rcLL.
rcLL binds to α-mannose and Man3 in a calcium-dependent manner. *Man3: trimannose; LeX: Lewis X; LeY: Lewis Y; LeA: Lewis A; H-type 2: Lewis H-type 2; H-type 3: Lewis H-type 3; LN: Gal-β-GlcNAc; LDN: GalNAc-beta-GlcNAc; chi-3: GlcNAc-GlcNAc-GlcNAc*

B. Inhibitory activity of rcLL against human isolates of IAV

Neutralization of infectivity

In order to investigate whether rcLL was capable of protecting MDCK cell monolayers from infection by Phil-strain, Phil/BS-strain or PR-8 strain IAV, virus preparations were preincubated with increasing concentrations of rcLL prior to adding them to the cells. No differences were observed in the number of fluorescent foci (data not shown).

rcLL mediated HAA-inhibition
In the HAA-inhibition assay, rcLL inhibited HA-activity of the Phil/BS strain at a mean concentration of 9.3 ± 2.3 μg/ml in three separate experiments. At this concentraion, erythrocytes were no longer agglutinated by Phil/BS. For Phil and PR-8, HA-activity was observed in one experiment at 64 and 32 μg/ml, respectively.

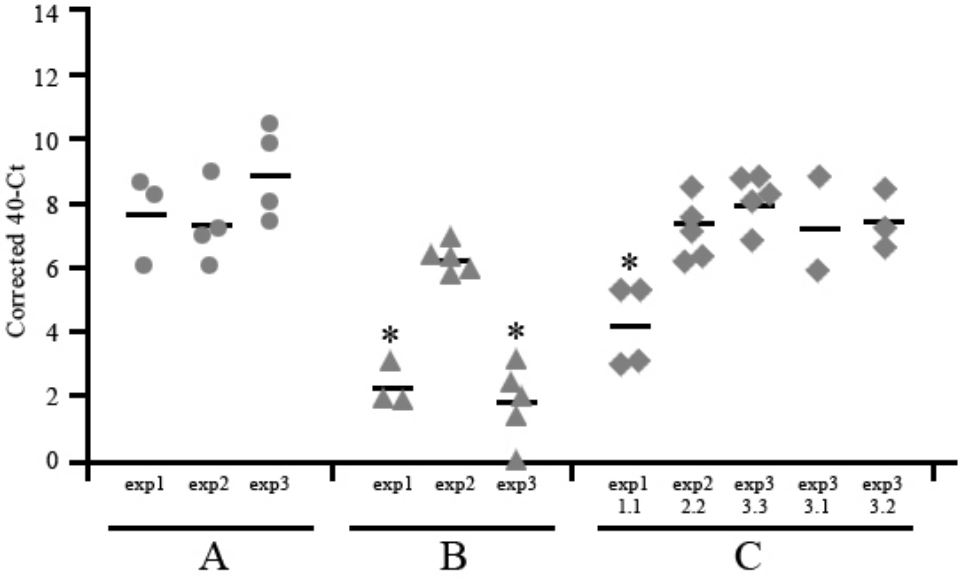

Figure 5. rcLL-mediated inhibition of Avian IAV, subtype H9N2.
Quantification of IAV mRNA in TOCs after inoculation with (**A**) IAV + medium (control), (**B**) IAV + chicken immune serum and (**C**) IAV + rcLL for 4 hrs. In the first experiment rcLL batch 1 (1.1) was used, in the second experiment rcLL batch 2 (2.2) was used, and in the third experiment rcLL batch 3 (3.3) was tested. In experiment 3 batch 1 (3.1) and 2 (3.2) were retested. * = significantly different from control within an experiment ($p < 0.05$). *Circles: IAV + medium (control); Triangles: IAV + Chicken immune serum; Diamonds: IAV + rcLL*

Discussion

We previously reported the finding of cLL which, based on sequence homology, was predicted to be a C-type lectin [2]. The sequence of its CRD is most similar to SP-A, but cLL does not contain a collagenous domain and therefore could not be classified as a collectin.
To our knowledge, this protein lacks further similarity to other C-type lectins and should therefore be regarded as a novel type of lectin [2].

In this study, recombinant cLL (rcLL) was used to further characterize structural and functional properties. It was found that rcLL appeared as four differently sized products on SDS-PAGE gel (**Figure 2a and b**). No potential GPI-modification sites were found in the sequence, and since the sequence of rcLL does not contain any potential N- or O-glycosylation sites, it is highly unlikely that differential glycosylation could account for the appearance of four products instead of one. However, a positive signal was observed for all bands using the DIG-glycan detection kit (**Figure 2c**). Aspecific DIG-labeling or staining

may explain this result. Accordingly, enzymatic treatment with N-Glycanase and/or O-glycosidase did not result in a size shift (**Figure 2b and c**), and no difference in intensity of DIG-glycan staining was observed between enzymatically treated samples. However, it cannot be excluded that rcLL contains sugar moieties that are insufficiently removed by enzymatic treatment.

It is likely that posttranslational modifications account for the size differences, since analysis of the four bands by MALDI-TOF-TOF resulted in similar spectra, and the C- and N-terminal parts of the protein could be retrieved for all four bands. This method is rather stringent, therefore it is possible that posttranslational modifications are removed during the procedure. Further analysis using 2D gel electrophoresis combined with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) may reveal what causes the differences in mass. As this concerns a recombinant protein, it would be very interesting to find out whether the same size differences are present in native cLL.

In mammalian species, collectin monomers are known to organize into higher order multimers [18, 19]. Crosslinking rcLL led to the formation of two high molecular weight products (**Figure 3**), suggesting that this protein is capable of forming trimeric subunits and higher order subunits. As the largest product observed in the crosslinking was ~160 kDa, corresponding to six monomeric subunits of rcLL, it is possible that rcLL is capable of forming hexameric multimers. In support of the assumption that rcLL can form trimers at the least, the presence of heptad repeats in the cLL-amino acid sequence could allow for the formation of trimeric subunits [2]. Furthermore, it is known that the carbohydrate-binding activity of C-type lectins is quite weak when they are in their monomeric form [20]. During the procedure of affinity-purifying rcLL by use of mannan-sepharose beads, the beads were washed twice with a relatively large volume of washing buffer. These conditions would most likely be too stringent if carbohydrate-binding by rcLL were weak, making it more likely that this protein is present as a trimer.

Analysis of the carbohydrate-specificity of rcLL revealed that the protein binds to α-mannose and Man3 in a calcium-dependent manner, but other carbohydrates tested (including galactose) were not bound by rcLL (**Figure 4**). It is possible that the presence of a Glu-Pro-Ser motif in the CRD of rcLL accounts for the relative specificity of rcLL. Most other mannose type-collectins contain a Glu-Pro-Asn motif [21] and most SP-As contain a Glu-Pro-Arg motif, whilst retaining their preference for mannose over galactose [22].

In order to investigate the functional properties of rcLL, the inhibitory activity against avian IAV subtype H9N2 was investigated. Incubating TOCs with a premix of avian IAV subtype H9N2 and rcLL led to a decline of IAV specific product (**Figure 5**), suggesting a rcLL-mediated inhibition IAV entry in TOCs. Unfortunately, this result was not reproducible using subsequent batches of rcLL. The negative control was in the same range in all experiments, indicating that viral entry was similar. However, the positive control failed to inhibit IAV in the second experiment. Repeating the assay with the first

batch led to a similar inhibition for one of the replicates, but the second replicate was at the same level as the control. It appears that small day-to-day differences can have an effect on the anti-viral activity of rcLL. However, the results from especially the first experiment indicate that rcLL could potentially inhibit IAV infection of TOCs.

In a second set of experiments, the activity of rcLL against human isolates of IAV was tested. In HAA-inhibition assays rcLL showed strong activity against the Phil/BS strain. Two other strains tested, Phil strain and PR-8, were less susceptible to rcLL inhibition. It is not yet clear what mechanism underlies this preference. The Phil/BS strain (subtype H3N2) differs from the parent Phil strain, in that the high-mannose oligosaccharide overlying the sialic acid receptor-binding site of the HA molecule is absent [23]. It is possible that this reveals targets for rcLL binding that are not available in the parent strain, resulting in increased binding. The PR-8 strain also lacks high mannose glycans on the HA molecule [24], but since this concerns a H1N1 subtype of IAV it is possible that differences in targets available on the HA molecule or the neuraminidase may result in decreased binding. It will be interesting to see what mediates rcLL binding to IAV, since binding of SP-A (to which cLL is most similar) is thought to occur via binding of the sialic acid receptor of the virus to sialylated N-linked oligosaccharide present in the CRD of SP-A [25]. It is possible that rcLL, similar to SP-D [26], binds to HA and neuraminidase in a C-type lectin-like manner, but the exact mechanism remains to be elucidated.

In summary, we successfully expressed chicken lung lectin in HEK293-EBNA cells. The purified protein proved to be a C-type lectin, as predicted from its sequence. Analysis of its carbohydrate specificity revealed that this protein has a high preference for binding α-mannose and trimannose. From the results of our preliminary tests, it appeared that rcLL potentially has an anti-viral activity against avian IAV. Furthermore, rcLL showed HAA-inhibition activity against the human IAV strain Phil/BS. Taken together, these results indicate that cLL could play an important part in the innate immune system of chickens.

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Chapter 7

Summary and General Conclusions

Introduction

Chickens and pigs make up the largest part of the EU livestock. Over the past decades, it has become increasingly apparent that the production of meat is not without any risk. In addition, it is more and more obvious that potentially pandemic diseases may originate from our own livestock. In this respect, pigs and chickens are of special interest, for reasons explained below.

Influenza A Virus is a prominent example of a viral pathogen. Wild aquatic birds are considered to be the natural reservoirs of all subtypes of Influenza A Virus (IAV) [1], and chickens can very easily be infected with IAV when they come into contact with these birds, for example during bird migration. Pigs can be infected by both avian and human influenza A virus. Adaptation of avian influenza to mammals may occur in pigs [1, 2], and co-infection of a pig with both avian and human influenza may lead to the emergence of new influenza strains into the human population [3].

However, there are various other diseases that can pose a serious risk to both animal and public health [4-9]. Therefore, keeping these animals healthy is not only a matter of preserving animal welfare, but also of great economical importance, and, not in the least, of importance for public health.

One approach to resolving this issue involves investigating the possibility to exploit the animals own innate immune system to increase their health. Young animals have not yet fully developed their adaptive immune system, as this requires prior contact with invading pathogens. The temporal gap between infection and the response generated by the adaptive immune system is filled by the innate immune system. Pattern recognition molecules such as collectins [10] play an important role in innate immune defense, because they are able to bind to a broad range of pathogens. Binding of a collectin to targets on the surface of a micro-organism is mediated by the carbohydrate recognition domain (CRD), which is located at the C-terminus of the protein [11-15]. This binding of a collectin is often followed by aggregation and/or destruction of the pathogen by various immune cells (**Chapter Two**).

Collectins play an essential role in innate immunity, and exploiting their characteristics could provide a means to increase animal health. The research presented in this thesis was focused on investigating the role of collectins in innate defense of pigs and chickens, and the findings are summarized below.

Summary of results

In **Chapter Three**, investigations into the role of Surfactant Protein D (SP-D) in the porcine gastro-intestinal tract are described.

Firstly, the interaction of porcine SP-D (pSP-D) with the laboratory strain *E. coli* K12 and bacteria isolated from the porcine intestine was studied. The results obtained in this study suggest that pSP-D is capable of reducing growth rates of *E. coli* K12 via an aggregation-dependent mechanism which involves increasing the bacterial membrane permeability without causing a significant reduction in bacterial viability. In addition, it was found that these aggregation-dependent effects appear to be partially mediated by self-recognition of pSP-D. However, these effects are species-dependent, as the growth rate and maximal density of other bacterial strains tested in this study remained unaffected.

Secondly, next to studying the direct effects of pSP-D on intestinal bacteria, the effect of pSP-D on bacterial adhesion- and invasion- characteristics was investigated. Surprisingly, preincubation of bacteria with pSP-D led to a significant increase in adhesion and invasion into the IPI-I2 cells. It is not yet clear what the mechanism behind these effects is, but it could reflect a scavenger function for SP-D in the intestine. Increased invasion mediated by pSP-D could, in theory, reduce the time it would take to elicit an inflammatory response, thereby contributing to the clearance of the infecting pathogen.

In **Chapter Four**, the discovery of several new chicken (col)lectins is described. In this study, the EST database was screened to identify new collectins in chicken. Four chicken genes encoding three collectins and a lectin were found. All genes were typed as C-type lectins based on the C-type-lectin motif which was present in the predicted sequence of their CRDs. The chicken collectins were designated chicken Collectin 1 (cCL-1), chicken Collectin 2 (cCL-2) and chicken Collectin 3 (cCL-3). They were found to be the chicken homologues of mammalian Collectin Liver 1 [16, 17], Collectin Kidney 1 [18] and Collectin Placenta 1 [19, 20], respectively, and their sequence was shown to be highly conserved. Tissue distribution analysis of mRNA expression showed that cCL-1, cCL-2 and cCL-3 are expressed in numerous tissues. The newly discovered chicken lectin was designated chicken Lung Lectin (cLL) because of its predominant expression in the chicken respiratory tract. Based on the predicted amino acid sequence of its CRD, cLL was found to be most similar to Surfactant Protein A. However, since cLL lacks a collagen domain it could not be classified as a collectin. Phylogenetic analysis of the CRD shows that chicken SP-A and cLL share a common ancestral line with SP-As found in other species, but these proteins are more similar to one another than to the mammalian SP-As. In addition, results from this study support the assumption that cCL-1, cCL-2 and cCL-3, together with their respective mammalian homologues CL-L1, CL-K1 and CL-P1 represent three new unique classes within the collectin protein family.

To investigate whether the newly found chicken (col)lectins are involved in avian innate immunity, the mRNA expression levels of cCL-1, cCL-2, cLL and cSP-A during bacterial and viral infections were investigated (**Chapter Five**). For this purpose, samples derived from three infection-models were analyzed.

In the first model, chickens were inoculated with Infectious Bronchitis virus (IBV) and subsequently superinfected with *E. coli* 506. Most prominent results from this experiment included an increase in cCL-1 and cCL-2 mRNA expression shortly after infection with
E. coli 506 compared to the control. This response was not observed in chickens previously infected with IBV. It is possible that an upregulation of cCL-1 and cCL-2 expression reflects an early immune response to *E. coli* infection, which is somehow disturbed by prior infection by IBV. Expression of cCL-2 was significantly lowered the following day. Downregulation of cCL-2 could reflect an evasion strategy of the pathogen. Similarly, cSP-A expression levels were significantly decreased 1 day after infection. However, this decrease was observed in all groups, suggesting that infection by IBV does not affect cSP-A expression.

In the second model, tracheal organ cultures (TOCs) were infected with Avian Influenza A Virus subtype H9N2. Although no differences in expression levels as a result of IAV-infection were observed for any of the (col)lectins, the preparation and/or culturing of the TOCs resulted in downregulation of all genes with the exception of cSP-A. Expression of cLL and cCL-1 mRNA was affected as soon as 3 hrs after the start of the experiment, suggesting a response to acute stress.

In the third model, chickens were spray-inoculated with avian Influenza A Virus subtype H9N2, and chicken (col)lectin expression levels were measured in lung samples. No differences as a result of infection were observed for cCL-1, cSP-A or cLL, but a significant decrease in cCL-2 expression-levels was observed on both day 1 and day 3, suggesting that IAV-infection results in a downregulation of cCL-2 mRNA.

Taken together, the results obtained in this study show that mRNA expression of chicken (col)lectins cCL-1, cCL-2, cSP-A and cLL can be affected by both bacterial and viral infections, and mRNA expression of cCL-1 and cLL is directly affected under stressful conditions.

In **Chapter Six**, structural and functional aspects of cLL were investigated. For this purpose, recombinant cLL (rcLL) was successfully expressed in HEK293-EBNA cells and affinity purified using mannan-sepharose beads. It was found that rcLL was expressed as four differently sized products, which is probably caused by post-translational modifications. Crosslinking rcLL led to the formation of two high molecular weight products, suggesting that this protein is capable of forming trimeric subunits and higher order subunits. rcLL was shown to bind to α-mannose and trimannose in a calcium-dependent manner, but other carbohydrates tested (including galactose) were not bound by rcLL.

In order to investigate the functional properties of rcLL, viral inhibition assays and haemagglutination-inhibition assays were performed. In the first set of experiments, inoculation of TOCs with a premix of avian Influenza Virus subtype H9N2 and rcLL led to

a decline of IAV specific product, suggesting an rcLL-mediated inhibition IAV entry in TOCs. Unfortunately, this result was not reproducible, which was most likely caused by small day-to-day differences. Since the results from the first experiment showed a clear inhibition, it cannot be excluded that rcLL inhibits entry of the virus into TOCs. In the second set of experiments, activity of rcLL against human isolates of IAV was tested. The infectivity of human isolates of IAV was not neutralized by rcLL. However, a clear inhibition of HAA-activity of the Phil/BS strain (H3N2) was found.

In summary, characterization of rcLL has revealed that this protein is capable of binding sugars, and, as predicted from its sequence, does so in a calcium-dependent manner. Furthermore, rcLL showed anti-viral activity against avian IAV in our preliminary tests, and HAA-inhibition of a human isolate of IAV was shown.

General conclusions

Surfactant Protein D in the porcine gastro-intestinal tract

The research presented in this thesis provides a first step in elucidating what the function of SP-D in the gastro-intestinal tract may be. Protein-expression of SP-D had been previously located to the gastro-intestinal tract [21] and epithelial cells (but not the mucin-producing Goblet-cells) appear to express this protein quite abundantly. The situation *in vivo* is not clear yet, but the most likely scenario is that SP-D is secreted into the extracellular space. If this protein is indeed capable as functioning as a scavenger-protein, as suggested by the results presented in Chapter Three, its location will most likely be at the surface of the epithelial cells. Surface-active phospholipid material, present as lamellated particles, has been shown to be present on the surface of gut epithelial cells [22, 23]. These surfactant-like particles (SLP) are very similar to pulmonary surfactant [24] and the presence of SP-A and SP-B has been associated to SLP in the colon [23, 25]. It may very well be possible that SP-D is also associated with these SLP, which would provide a good location for SP-D proteins to interact with invading pathogens. As the results obtained in this thesis suggest a scavenger function for SP-D, modulating its expression in the gastro-intestinal tract may enhance capability of the host's defense system to detect the presence of an invading pathogen, which could significantly benefit intestinal health.

Chicken (col)lectins and their role in avian innate immunity

As collectins have been shown to play an important part in innate immunity in mammals, it may not seem very surprising that similar proteins are present in birds. However, the mammalian and avian lineages diverged about 310 million years ago [26] and therefore the presence of these proteins reflects their importance in the evolution of innate immunity. Interestingly, although expression of SP-D has been demonstrated in many mammalian

species, we were unable to locate a potential candidate gene for the expression of chicken SP-D. If the chicken genome does not contain an SP-D gene, it is possible that the niche that SP-D occupies in mammalian innate host defense is otherwise occupied in chickens, and perhaps one of the (col)lectins described in **Chapter Four** will reveal itself as a potential candidate.

As shown in **Chapter Five**, expression of the (col)lectin genes cCL-1, cCL-2, cLL and cSP-A can be affected by bacterial and viral infections. In particular, a significant upregulation of both cCL-1 and cCL-2 in the initial stage of infection by *E. coli* 506 may reflect the involvement of these proteins in the acute innate immune response. Most other infection-related effects found in this study concerned a down-regulation of chicken collectin mRNA-expression, suggesting that the pathogens examined in this study interfere with the expression of these genes. This could reflect a means to evade immune responses mediated by these collectins.

The lectin cLL was further characterized in **Chapter Six.** In Chapter Four, cLL was shown to be homologous to SP-A. However, it does not contain a collagenous domain. It is possible that cLL is a chicken-specific innate defense protein. We successfully expressed rcLL, which was shown to have C-type lectin activity. Preliminary data suggest inhibitory activity of rcLL against avian Influenza A Virus, and rcLL displayed HAA-inhibitory activity against the human isolate Phil/BS.

Future directions

An increase in adhering and invading pathogens is not an effect one would expect from SP-D, a collectin that has been shown to fulfill a protective function in the defense against many different types of pathogens. Therefore, the most obvious question to be answered is what the downstream effects of SP-D mediated adhesion and invasion may be. Although we did not observe any differences in IL-8 secretion, this does not exclude the possibility that secretion of other cytokines is increased. Furthermore, results obtained with a cell-line are likely to differ from the situation *in vivo*, where other cells are also involved in the interaction with putative pathogens. For instance, immune cells which populate the lamina propria may be involved, and it would be interesting to see whether SP-D is capable of recruiting these cells in order to ward off further infection, or if SP-D is involved in antigen-presentation to these cells. In addition, the immunomodulatory effects of SP-D on these cells should be investigated.

When observing effects such as these, one is tempted to speculate that SP-D-specific receptors may be involved. To date, these have not been identified in epithelial cells, therefore, it would be very interesting to see whether these cells display SP-D-specific receptors on their surface.

Preliminary data from our lab have indicated that another porcine intestinal cell-line expresses SP-D, which may provide more insight into how adhesion and invasion are influenced by SP-D. Finally, the interaction of SP-D with other intestinal (Gram-positive) bacterial species and yeasts should be investigated.
Because not much is known about any of the newly discovered chicken (col)lectins described in this thesis, further research on the structural characteristics and functions of cCL-1, cCL-2, cCL-3 and cLL may reveal new insights in chicken innate defense. The experiments described in this thesis show that on an mRNA level chicken collectins are affected by viral and bacterial infection. Although investigating changes in the mRNA expression levels of chicken (col)lectins can provide further insight into (col)lectin function, it is even more important to determine changes in their protein levels in the chicken respiratory tract. Therefore, the production of antibodies directed at the new chicken (col)lectins should have a high priority. In analogy with the mammalian collectins, several aspects of innate immune functions of chicken (col)lectins should be investigated, including their ability to bind micro-organisms, their capacity to function as an opsonin, and their possible direct antimicrobial activity. Furthermore, it should be determined which cell types express these proteins, and whether these cells secrete chicken (col)lectins or if these proteins remain in the cytosol.

This latter question is especially important for cCL-1, as its mammalian homologue CL-L1 has been shown to be located in the cytosol of hepatocytes [16]. This is interesting, since most collectins are secreted, but the finding that the sequence of CL-L1 contains a C-terminally located nuclear localization signal (NLS) may explain this finding. As cCL-1 does not contain the exact NLS, future research should reveal whether this protein is secreted *in vivo*.

The mammalian homologue of cCL-2, Collectin Kidney 1 (CL-K1) has only recently been discovered [18] and its exact function still needs to be elucidated. Interestingly, CL-K1 was also found in human serum, but its exact function there is not yet known. It would be interesting to see if cCL-2 protein is also found in chicken serum.

The function of the membrane-bound Collectin Placenta 1 (CL-P1) [19, 20], the mammalian homologue CL-3, is not known yet. It was shown that it can help phagocytose bacteria and yeasts [20], which indicates that CL-P1 acts as a scavenger molecule. Expression of cCL-3 should elucidate whether this chicken protein has similar properties.

Finally, the structure of rcLL should be further analysed. The putative posttranslational modifications may be revealed using other techniques such as 2D-gel electroforisis in combination with LC-MS/MS. More importantly, the function of native cLL should be studied. Although results obtained with a recombinant protein should always be carefully interpreted, the results presented in this thesis indicate a potential role for this protein in the innate defense against IAV. This is very promising, and future research should elucidate the exact mechanism behind this inhibition.

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Nederlandse samenvatting

Waarom dit onderzoek?

Groeibevorderende antibiotica (GBA) werden sinds de jaren '50 toegevoegd aan het veevoer, omdat deze stoffen een positief effect hadden op de gewichtstoename van het vee. Het is nog steeds niet precies duidelijk hoe deze stoffen dit effect tot stand kunnen brengen, maar uit verschillende onderzoeken is een aantal werkingsmechanismes naar voren gebracht: (A) GBA zijn antibacterieel, zorgen ervoor dat er minder sub-klinische infecties optreden in het maag-darm kanaal van het dier. Het immuunsysteem van het dier niet hoeft te worden aangesproken om een beginnende infectie onschadelijk te maken, wat energie bespaart; (B) doordat er minder bacteriën zijn, komen er minder bacteriële stoffen vrij die de groei van het dier kunnen remmen; (C) het dier kan meer voedingsstoffen zelf benutten, omdat er minder voedingsstoffen worden verspild aan de groei van de bacteriën.

Samenvattend zorgen GBA er dus voor dat de dieren meer energie kunnen steken in de productie van vlees. Met een totaal aantal van ongeveer 20 miljoen varkens en 200 miljoen kippen dat jaarlijks in Nederlands geslacht wordt, is het niet moeilijk voor te stellen dat zelfs een kleine verbetering in het slachtgewicht van de dieren al voor grote winsten kan zorgen.

Het is in de EU sinds 1 januari 2006 echter verboden nog langer GBA toe te voegen aan het veevoer, omdat er steeds meer aanwijzingen waren dat het gebruik van deze stoffen leidt tot het ontstaan van bacteriën die resistent zijn tegen antibiotica. Dat zou tot gevolg kunnen hebben dat wanneer mensen geïnfecteerd raken, bijvoorbeeld door het eten van besmet vlees, er geen medicatie beschikbaar zou zijn om de infectie tegen te gaan.

Omdat GBA werden afgeschaft, werd het zoeken naar alternatieven door de EU gestimuleerd. Hierbij werd het waarborgen van het welzijn van de dieren voorop gesteld. Eén van de meest voor de hand liggende opties in deze situatie is om te kijken naar wat er al is, namelijk, het immuunsysteem van het dier zelf.

Het immuunsysteem

Het immuunsysteem kan ruwweg worden opgedeeld worden in twee delen, het verworven en het aangeboren immuunsysteem. Het verworven immuunsysteem zorgt, door het maken van antistoffen die specifiek gericht zijn tegen één bepaalde pathogeen, voor een zeer effectieve bestrijding van een infectie door pathogenen zoals bacteriën. Echter, voordat een dergelijke op maat gemaakte afweerrespons echt volledig is geactiveerd verstrijken er, in het meest gunstige scenario, een paar dagen.

De B-cellen die deze antistoffen maken zullen eerst moeten delen, zodat er genoeg B-cellen zijn om genoeg antistoffen te maken. In het minst gunstige scenario kan het zelfs een week duren voor het verworven immuunsysteem antistoffen produceert, en dit is

meestal het geval wanneer een pathogeen voor het eerst het lichaam binnendringt. In dit geval moeten de B-cellen zich eerst nog verder gaan specialiseren tot een B-cel die de juiste antistoffen kan maken. In jonge dieren (en mensen) is het laatste scenario vaak van toepassing, simpelweg omdat zij nog te kort leven om al veel pathogenen tegen te zijn gekomen.

De tijd tussen het binnendringen van een pathogeen, en het vormen van een specifieke afweer daartegen, wordt overbrugd door het aangeboren immuunsysteem. Net als het verworven immuunsysteem is het aangeboren immuunsysteem in staat om vreemde structuren te herkennen, zoals die op de celwand van een bacterie. Echter, in tegenstelling tot het verworven immuunsysteem, is het aangeboren immuunsysteem echter een stuk minder kieskeurig. Het herkent pathogenen, door middel van zogenaamde patroonherkennende moleculen, structuren die vaak op pathogenen voorkomen. Met andere woorden, waar het verworven immuunsysteem bacteriesoort A, subtype B herkent en daar dus heel specifiek mee afrekent, zal het aangeboren immuunsysteem alleen zien dat het om lichaamsvreemde structuur X gaat. Vaak wordt dit onderscheid gemaakt op basis van het patroon waarin bepaalde structuren, zoals suikergroepen, op de celwand van een bacterie zijn verspreid. Ook al is de laatste aanpak iets minder effectief, het aangeboren immuunsysteem heeft het voordeel dat het direct in actie kan komen.

Een ander voordeel is dat resistentie tegen deze patroonherkennende moleculen maar heel zelden zal kunnen optreden. De structuren die door patroonherkennende moleculen worden herkend zijn namelijk vaak van levensbelang voor pathogenen en kunnen dus niet worden aangepast om herkenning te voorkomen.

Veel patroonherkennende moleculen worden constant door het lichaam aangemaakt, en zullen dus ook aanwezig zijn op het moment dat er een pathogeen binnendringt. Er zijn veel verschillende soorten patroonherkennende moleculen, en collectines, waar dit proefschrift over gaat, zijn daar een belangrijk voorbeeld van.

Collectines

Collectines zijn eiwitten die een collageendomein en een C-type lectinedomein bevatten. C-type lectines zijn afhankelijk van de aanwezigheid van Calcium voor hun werking. De structuur van collectines is erg bepalend voor hun functie. De kleinste, functionele vorm van een collectine is opgebouwd uit drie monomeren, kettingen van aminozuren, die tezamen een trimeer vormen. Deze trimeren kunnen verder multimeriseren (groeperen) tot octadecameren (18 monomeren), zoals het geval is voor SP-A en MBL, of dodecameren (12 monomeren), zoals SP-D.

Een monomeer kan grofweg worden opgedeeld in vier domeinen; (1) een N-terminaal domein. Hierin bevinden zich meestal één of meerdere cysteïnes. Deze aminozuren kunnen, omdat ze een zwavelatoom bevatten, onderling bruggetjes slaan en zijn daarom erg bepalend voor hoe een eiwit zich opvouwt. (2) Het collageendomein, wat

per collectine erg kan variëren in lengte. (3) Een nekdomein, waarin zich enkele "heptad repeats" bevinden. Dit zijn delen van de monomeer die bepaalde aminozuren bevatten die elkaar aantrekken. Hierdoor vormen de drie monomeren een drievoudige alpha-helix, waarbij ze ongeveer als linten om een meipaal om elkaar heen zullen draaien. Deze heptad repeats zijn dus zeer belangrijk voor het vormen van trimeren. Tenslotte is er (4) het C-terminale lectinedomein. Het lectinedomein is het deel van het eiwit dat suikergroepen kan herkennen, en hiermee kunnen collectines binden aan onder andere bacteriën, schimmels en virussen. Als die binding eenmaal heeft plaatsgevonden, kunnen collectines pathogenen aggregeren (samen doen klonteren) waarna er in de meeste gevallen witte bloedcellen zoals neutrofielen of macrofagen worden gerekruteerd om de pathogenen onschadelijk te maken. Goed beschreven voorbeelden van collectines zijn Mannan Binding Lectin (MBL), Surfactant Protein A (SP-A) en Surfactant Protein D (SP-D). MBL wordt aangemaakt in de lever en wordt daarna uitgescheiden in de bloedbaan. Hier kan het, na binding aan bijvoorbeeld een bacterie, het complementsysteem activeren waardoor de bacterie onschadelijk gemaakt wordt. SP-A en SP-D zijn oorspronkelijk ontdekt in de oppervlakte-actieve stof (surfactant) in de long. De afgelopen decennia is steeds duidelijker geworden dat deze laatste twee eiwitten erg belangrijk zijn voor de aangeboren afweer in de long, waar zij binnendringende pathogenen onschadelijk maken. In **Hoofdstuk Twee** zijn de interacties die collectines kunnen aangaan met pathogenen beschreven.

Vraagstellingen binnen dit onderzoek

Het in dit proefschrift beschreven onderzoek heeft zich gericht op collectines in het varken en de kip.

Het onderzoek naar varkens-collectines heeft zich gericht op de rol van SP-D in de darm. Uit eerder onderzoek binnen onze onderzoeksgroep was al gebleken dat SP-D ook op veel andere plaatsen in het lichaam van het varken tot expressie wordt gebracht (wordt aangemaakt), waaronder de darm. Het was echter nog niet duidelijk welke rol SP-D zou kunnen spelen in de aangeboren afweer in deze omgeving. De longen moeten immers nagenoeg steriel zijn om goed te kunnen functioneren, terwijl de darm juist ontzettend veel micro-organismen bevat. De vraag die wij wilden beantwoorden was of SP-D ook in deze omgeving een rol zou kunnen spelen in de aangeboren afweer.

Het onderzoek naar kippen-collectines begon met het zoeken naar nieuwe collectines, omdat er tot dusver nog slechts twee kippen-collectines beschreven waren. Deze zoektocht in het genoom van de kip leverde een aantal goede kandidaat-genen op. Vervolgens is er gekeken naar de messenger (boodschapper) RNA (mRNA) expressie van deze genen in verschillende infectiemodellen, om te zien of de mRNA expressie van de kippen-(col)lectines beïnvloed zou worden door bacteriële en/of virale infecties. Messenger RNA is een soort kopietje van het DNA, wat cellen kunnen gebruiken als mal om de eiwitten die in het DNA zijn gecodeerd te gaan maken. Vaak betekent een verhoogde

mRNA-concentratie dat er op dat moment ook veel van het eiwit wordt aangemaakt. Een verandering in de mRNA expressie tijdens een infectie zou dan dus ook de betrokkenheid van het eiwit bij het bestrijden van de infectie kunnen weerspiegelen.
Tenslotte is één van deze kandidaat-genen tot expressie gebracht in een zogenaamd expressie-systeem, zodat structurele en functionele eigenschappen van het eiwit onderzocht konden worden. Hierbij is het DNA van dit kippengen is geïsoleerd en overgebracht in gekweekte humane cellen. Deze humane cellen brengen het kippengen tot expressie, en op deze manier kan binnen een relatief korte tijd een redelijke hoeveelheid van het eiwit verkregen worden. Een eiwit dat op deze manier wordt verkregen noemt men recombinant.

Samenvatting van de resultaten

In **Hoofdstuk Drie** is de rol van SP-D in het maag-darmkanaal van het varken onderzocht. Hiervoor is gekeken naar de directe interactie van varkens SP-D (pSP-D) met de laboratorium-stam *Escherichia coli* K12 en verschillende Gram-negatieve bacteriën die uit de varkensdarm waren geïsoleerd.

De gevonden resultaten wijzen erop dat pSP-D in staat is de groeisnelheid en de maximale bacteriële dichtheid van *E. coli* K12 te reduceren. Deze vermindering in groeisnelheid en bacteriedichtheid van *E. coli* K12 is afhankelijk van aggregatie door pSP-D, waarbij pSP-D ook de membraanpermeabiliteit van *E. coli* K12 verhoogt. Kortgezegd betekent dat dat deze bacteriën makkelijker stoffen naar binnen (en buiten) laten . Deze verhoging in membraanpermeabiliteit leidt echter niet tot verhoogde bacteriesterfte, iets wat eigenlijk wel verwacht zou kunnen worden omdat de bacteriën lek zijn geraakt.

De andere bacteriestammen die in dit onderzoek zijn getest, worden ook geaggregeerd door pSP-D, maar zijn niet gevoelig voor de negatieve effecten van pSP-D op groeisnelheid en bacteriedichtheid. Er werden ook geen effecten op de membraanpermeabiliteit waargenomen. Dit geeft aan dat aggregatie door pSP-D geen garantie is voor negatieve effecten op bacteriële groei.

Naast de directe effecten van pSP-D op deze bacteriën, is ook onderzocht of pSP-D mogelijk darmepitheelcellen zou kunnen beschermen tegen adhesie en invasie door deze bacteriën. In tegenstelling tot wat verwacht werd, bleek dat pSP-D zorgt voor een sterke *verhoging* in het aantal bacteriën dat zich aan de epitheelcellen hechtte en binnendrong.

Het is niet duidelijk welk mechanisme hier achter schuilt, maar het is mogelijk dat pSP-D er op deze manier voor zorgt dat het immuunsysteem snel geattendeerd wordt op de aanwezigheid van binnendringende pathogenen. Hierdoor zou, in theorie, de tijd tussen het binnendringen van een bacterie en de respons van het immuunsysteem verkort kunnen worden, wat eraan bij zou dragen om een infectie tijdig te onderdrukken.

In **Hoofdstuk Vier** wordt de ontdekking van drie nieuwe kippencollectines en een kippen C-type lectine beschreven. Hiervoor is er in het genoom van de kip gezocht naar kandidaat-genen, waarbij chicken Collectin 1 (cCL-1), chicken Collectin 2 (cCL-2),

chicken Collectin 3 (cCL-3) en chicken Lung Lectin (cLL) zijn gevonden. De eerste drie zijn verwant aan collectines die al eerder in zoogdieren zijn beschreven, respectievelijk Collectin Liver 1 (CL-L1), Collectin Kidney 1 (CL-K1) en Collectin Placenta 1 (CL-P1). cLL is het meest verwant aan SP-A, maar kan geen collectine genoemd worden omdat het geen collageendomein bevat.

Uit de analyse van de expressiepatronen van deze genen is gebleken dat het mRNA van cCL-1, cCL-2 en cCL-3 in zeer veel verschillende weefsels wordt aangemaakt (tot expressie komt). Expressie van het mRNA van cLL werd voornamelijk gevonden in het respiratoire systeem, vandaar ook de naam chicken Lung Lectin. De analyse van de voorspelde aminozuursequenties van de gevonden kippen (col)lectines liet zien dat deze genen erg geconserveerd zijn. Oftewel, tijdens miljoenen jaren evolutie is de volgorde van de aminozuren van deze soorten eiwitten voor een groot deel behouden gebleven. Uit de fylogenetische analyse van de gevonden kippen(col)lectines bleek dan ook dat deze allen bij hun respectievelijke homologen worden gegroepeerd in aparte clades. Dat wil zeggen dat alle gevonden genen, wanneer zij vergeleken worden met hun zoogdier-equivalenten, in evolutionaire termen in de juiste groep terecht komen. cLL en cSP-A delen, zoals verwacht, een gemeenschappelijke voorouder met de zoogdier-SP-A's.

In **Hoofdstuk Vijf** is de mRNA expressie van cCL-1, cCL-2, cLL en cSP-A tijdens bacteriële en virale infecties bestudeerd. Hiervoor zijn monsters afkomstig uit drie verschillende infectiemodellen geanalyseerd.

In het eerste model werden kippen geïnfecteerd met een vaccin-stam of een veld-stam van Infectious Bronchitis Virus (IBV), waarna er een superinfectie werd bewerkstelligd door de kippen te besmetten met *E. coli* 506 (de IBV-groepen). Hiernaast waren er nog twee groepen, waarvan er één helemaal niet werd besmet (de controle-groep), en één waarbij de kippen alleen met *E. coli* werden besmet (de *E. coli*-groep). Bij de analyse van de mRNA expressie van de vier kippen(col)lectines werden verschillende observaties gedaan. In de *E. coli*-groep werd kort na besmetting met *E. coli* 506 een stijging in mRNA expressie van cCL-1 en cCL-2 mRNA waargenomen. Dit effect werd niet waargenomen in de IBV-groepen. Het is mogelijk dat deze stijging in mRNA niveaus een respons is op infectie met *E. coli* 506, en dat deze respons wordt verstoord door eerdere infectie met IBV. Eén dag na besmetting met *E. coli* 506 was het mRNA expressie niveau van cCL-2 in de *E. coli*-groep significant verlaagd. Dit zou een strategie van *E. coli* 506 om een immunologische respons te omzeilen kunnen weerspiegelen. Een dergelijke verlaging van mRNA expressie werd ook waargenomen voor cSP-A, maar hierbij werden geen verschillen tussen de verschillende behandelingsgroepen geobserveerd, wat aangeeft dat de mRNA expressie van cSP-A niet wordt beïnvloed door IBV.

In het tweede infectiemodel werden embryonale trachearingetjes (TOCs), die werden geïsoleerd uit kippenembryos, geïnfecteerd met aviair Influenza A Virus (IAV), subtype H9N2 (een laag-pathogene variant van vogelgriepvirus). Er werden geen effecten

waargenomen op de mRNA expressieniveaus van de kippen(col)lectines als gevolg van de infectie. Het prepareren van de ringetjes en/of de kweekcondities bleek echter wel een negatief effect te hebben op de mRNA expressie van cLL, cCL-1 en cCL-2 in dit weefsel. Er werden geen significante verschillen waargenomen in de expressiepatronen van cSP-A. De mRNA expressieniveaus van cLL en cCL-1 waren al kort na de start van het experiment verlaagd. Dit suggereert een acute respons op de stress die het weefsel ondervindt van het prepareren en/of de kweekcondities.

In het derde model werden kippen geïnfecteerd met IAV subtype H9N2, waarna de mRNA expressieniveaus van de kippen(col)lectines in longweefsel werden bepaald. Er werden geen verschillen gevonden in mRNA expressieniveaus van cCL-1, cSP-A of cLL, maar de expressie van cCL-2 mRNA was op zowel de eerste als de derde dag na infectie met IAV significant verlaagd, wat aangeeft dat IAV de expressie van cCL-2 beïnvloedt.

De resultaten die in de verschillende infectiemodellen zijn gevonden geven aan dat de mRNA expressie van de kippen(col)lectines kunnen worden beïnvloed door bacteriële en virale infecties. Bovendien is aangetoond dat de mRNA expressie van cCL-1 en cLL wordt verlaagd onder stressvolle condities.

In **Hoofdstuk Zes** zijn de structurele en functionele eigenschappen van cLL onderzocht. Hiervoor is gebruik gemaakt van recombinant cLL (rcLL), wat succesvol tot expressie werd gebracht in HEK-293 EBNA cellen (menselijke embryonale niercellen).

Dit recombinante eiwit bleek uit vier peptiden (monomeren) van verschillende grootte te bestaan. Uit de resultaten van de massaspectrometrie-analyse, een techniek waarmee de grootte van moleculen bepaald kan worden, werd echter niet duidelijk wat de grootteverschillen zou kunnen veroorzaken, omdat er voor elk van de vier peptiden een vergelijkbaar piekenpatroon werd gevonden. Waarschijnlijk worden de grootteverschillen veroorzaakt door posttranslationele modificaties (veranderingen die optreden in de structuur van het eiwit nadat het is gemaakt). Om te kijken of rcLL in staat is om trimeren te vormen is er gebruik gemaakt van crosslinking. Met deze techniek kunnen peptiden die een interactie met elkaar aangaan, min of meer permanent aan elkaar vastgezet worden. Crosslinking van rcLL leidde tot de vorming van twee producten met een hoge moleculaire massa. De kleinste van deze twee was ongeveer drie keer zo groot als de afzonderlijke peptiden, en de grootste zes keer. Dit suggereert dat rcLL in staat is om trimeren te vormen, en wellicht zelfs multimeren. Suikerbindingsassays lieten ziet dat rcLL in staat is om op een calcium-afhankelijke wijze te binden aan α-mannose en trimannose, en dus, zoals voorspeld in Hoofdstuk Vier, een C-type lectine is.

Om de functionele eigenschappen van rcLL te bestuderen zijn er virale inhibitie-assays uitgevoerd, waarbij rcLL werd toegevoegd aan aviair IAV, subtype H9N2 (de preïncubatie-stap). Hierna werd dit virus (met of zonder rcLL) gebruikt om TOCs te infecteren. Wanneer een virus een cel infecteert, zet het zijn eigen DNA tussen het DNA van de gastheercel. Op deze manier gebruikt het virus de geïnfecteerde cel om veel kopieën

van zichzelf te maken. Als een cel geïnfecteerd is, zal er dus virus-specifiek mRNA in de cel aanwezig zijn. Met behulp van qPCR, een techniek waarmee de hoeveelheden mRNA in weefsel gemeten kunnen worden, is de hoeveelheid IAV-specifiek mRNA in de TOCs bepaald. Op deze manier kon het effect van rcLL op de infectie van TOCs door IAV bekeken worden.

In het eerste experiment werd een lagere hoeveelheid van IAV-specifiek product gevonden wanneer het virus was gepreïncubeerd met rcLL, wat een remming van infectiviteit suggereert. In vervolgexperimenten werd dit remmende effect van rcLL echter niet meer waargenomen. Waarschijnlijk hebben kleine verschillen in de opzet van de experimenten gezorgd voor de wisselende resultaten, maar omdat in het eerste experiment een sterke inhibitie van infectie door IAV werd gevonden kan een remmend effect van rcLL niet worden uitgesloten.

In een andere set experimenten met humane isolaten van IAV is gekeken of rcLL de infectie van MDCK cellen door de verschillende virus-isolaten kon remmen. Hierbij werden geen verschillen gevonden, rcLL-behandelde virus-isolaten infecteerden de cellen even goed als de niet behandelde virus-isolaten. Een andere manier om de effecten van rcLL op de activiteit van het virus te bepalen is een hemagglutinatie (HA)-assay. IAV is in staat rode bloedcellen samen te klonteren (hemagglutinatie), en de remming van deze activiteit door rcLL is vrij eenvoudig te meten. Er werd voor Phil/BS (H3N2) een duidelijke remming van HA-activiteit door rcLL geobserveerd, en voor de twee andere humane isolaten, Phil (H3N2) en PR-8 (H1N1), werd eenmaal HA-remming door rcLL waargenomen. Samenvattend geven de resultaten die in dit hoofdstuk zijn beschreven aan dat rcLL suikers kan binden op een calcium-afhankelijke wijze. Verder bleek uit onze voorlopige resultaten een remming van infectie van TOCs door aviair IAV, en is er een duidelijke remming van HA-activiteit van een humaan isolaat van IAV waargenomen.

Conclusies

Surfactant Protein D in het maag-darmkanaal van het varken

Het onderzoek dat in dit proefschrift is beschreven verschaft een eerste blik op de mogelijke functie van SP-D in de darm van het varken. Eerder onderzoek heeft al aangetoond dat SP-D tot expressie wordt gebracht in het maag-darmkanaal, en epitheelcellen lijken dit eiwit sterk tot expressie te brengen. Het is nog niet duidelijk hoe de situatie *in vivo* (in het dier zelf) is, maar SP-D zal meest waarschijnlijk uitgescheiden worden in het extracellulaire milieu. Als dit eiwit inderdaad in staat is om, zoals wordt voorgesteld in Hoofdstuk Drie, het immuunsysteem te attenderen op de aanwezigheid van binnendringende pathogenen, dan zal het apicale oppervlak van de epitheelcellen (het deel wat blootgesteld wordt aan de darminhoud) de meest logische locatie van SP-D zijn. In andere onderzoeken is waargenomen dat er een surfactant-achtig materiaal aanwezig is op

het oppervlak van darmepitheelcellen. In de dikke darm is de aanwezigheid van SP-A en SP-B in dit surfactant-achtige materiaal al aangetoond. Het is mogelijk dat SP-D, net als SP-A en SP-B, ook wordt geassocieerd met dit materiaal, waardoor zich SP-D op een goede locatie zou bevinden om een interactie aan te gaan met binnendringende pathogenen. Modulatie van de expressie van SP-D in de darm zou zo mogelijk een positief effect kunnen hebben op de werking van de darm, en daarmee de gezondheid van het dier.

Kippen(col)lectines en hun rol in de aangeboren afweer van de kip
Uit eerder onderzoek is het belang van collectines in de aangeboren afweer bij zoogdieren duidelijk aangetoond. Daarom is het misschien niet verwonderlijk dat deze eiwitten ook in de kip zijn gevonden. Echter, de evolutionaire wegen van zoogdieren en vogels scheidden zich ongeveer 310 miljoen jaar geleden. Dat deze eiwitten ook bij vogels kunnen worden gevonden (sterk geconserveerd zijn) geeft dus wel aan dat hun rol in de evolutie van het aangeboren immuunsysteem zeer belangrijk is. Daarbij is het interessant om te vermelden dat hoewel SP-D in veel zoogdieren is aangetoond, wij dit gen niet hebben kunnen localiseren in de kip. Het is mogelijk dat de rol die SP-D in de aangeboren immuniteit van zoogdieren speelt, in vogels wordt vervuld door een ander eiwit. Mogelijk blijkt één van de genen die in Hoofdstuk Vier is gevonden een goede kandidaat. Zoals aangetoond in Hoofdstuk Vijf kan de mRNA expressie van cCL-1, cCL-2, cLL en cSP-A worden beïnvloed door bacteriele en virale infecties. In het bijzonder zou de stijging in de mRNA expressieniveaus van cCL-1 en cCL-2 in de eerste stadia van infectie met *E. coli* 506 de betrokkenheid van deze genen bij de acute respons van het aangeboren immuunsysteem kunnen weerspiegelen. In overige resultaten van de infectiemodellen werden voornamelijk dalingen van de mRNA expressieniveaus waargenomen, wat aangeeft dat infectie door de pathogenen die in deze modellen werden bestudeerd de expressieniveaus van de kippen(col)lectines beïnvloedt. Het is mogelijk dat deze pathogenen op deze manier een respons van het aangeboren immuunsysteem omzeilen.
In Hoofdstuk Vier was al aangetoond dat cLL het meest verwant is aan SP-A, maar een collageendomein mist. Het is mogelijk dat dit eiwit een kip-specifiek immuuneiwit is. Verdere karakterisatie van dit eiwit met behulp van recombinant cLL, zoals beschreven in Hoofdstuk Zes, heeft laten zien dat rcLL C-type lectine activiteit heeft, een remmende werking zou kunnen hebben op aviaire IAV, en bovendien de HA-activiteit van het humane isolaat Phil/BS remt.

List of abbreviations

AGP	Antibiotic growth promotor
AIDS	Acquired Immunodeficiency Syndrome
BALF	Bronchoalveolar lavage fluid
BCG	Bacillus Calmette-Guerin
BLAST	Basic local alignment tool;
BLAT	BLAST-like alignment tool
BMDC	Bone marrow derived macrophage
BSA	Bovine Serum Albumin
cCL-1	chicken Collectin 1
cCL-2	chicken Collectin 2
cCL-3	chicken Collectin 3
C1qR	C1q receptor
cfu	colony forming unit
CD	Cluster of differentiation
CK2	Casein Kinase
CL-43	Collectin 43
CL-46	Collectin 46
CL-L1	Collectin Liver 1
CL-P1	Collectin Placenta 1
CRD	Carbohydrate Recognition Domain
CRF	collagenase resistant fragment
CRT-CD91	Calreticulin-CD91
DC-SIGN	Dendritic Cell-Specific ICAM-3-grabbing Nonintegrin
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EST	expressed sequence tag
GBS	Group B Streptococcus
gpA	Glycoprotein A
GXM	Glucuronoxylomannan
IAV	Influenza A Virus
IBV	Infectious Bronchitis Virus
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

IPA	Invasive pulmonary aspergillosis
HA	Haemagglutinin
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
LAM	Lipoarabinomannan
LBP	LPS binding protein
LM	Lipomannan
LMWF	low-molecular weight filtrate
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
ManLAM	Mannosylated lipoarabinomannan
MBL	Mannan Binding Lectin
MIP	Macrophage Inflammatory Protein
MM-Ca^{2+}	minimal medium containing calcium
MM-EDTA	minimal medium containing EDTA
MR	Mannose receptor
NLS	nuclear localization signal
PMB	Polymyxin B sulphate
PMN	Polymorphonuclear cell
pSP-D	porcine surfactant protein D
$pSP\text{-}D_{deglyc}$	N-deglycosylated pSP-D
rcLL	recombinant chicken Lung Lectin
RNI	Reactive Nitrogen Intermediates
rrSP-D	recombinant rat surfactant protein D
rSP-D	rat surfactant protein
RSV	Respiratory Syncytial Virus
SP-A	Surfactant Protein A
SP-B	surfactant protein B
SP-D	Surfactant Protein D
TLR	Toll Like Receptor
TOC	Tracheal Organ Culture
TNF	Tumor Necrosis Factor

Dankwoord

Jaaaaa, het is af!! Zonder de steun van een heleboel mensen was het er echter niet van gekomen. Daarom wil ik deze bladzijden graag gebruiken om iedereen te bedanken die op één of andere wijze heeft bijgedragen aan de totstandkoming van dit proefschrift, waarbij een aantal mensen in het bijzonder.

In de eerste plaats natuurlijk Henk, die als promotor altijd wel een interessante invalshoek (of drie, of acht) bedacht waarvanuit ik weer verder kon. Ik wil je graag hartelijk bedanken voor de enthousiaste begeleiding en het vertrouwen wat je me de afgelopen jaren hebt gegeven. Ik kon altijd wel even langslopen om de over gang van zaken te discussiëren, hoewel we het over de Uithof als leuke plek om te wonen nooit eens zullen worden. Wegens jouw zelf prachtig omschreven link naar Dzjengis Khan zal ik daar echter maar niet meer over beginnen.

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Albert, mijn kamergenoot en levende lab-encyclopedie! Als jij voor elke vraag van mij een euro had gehad zou je je eigen aankomende promotie al voor een goed deel kunnen financieren. Ik heb met jou als kamergenoot een hele leuke tijd gehad in 315, op wat liftmuziek na dan. Jouw enthousiasme voor het onderzoek heeft me er bij wat dipjes hier en daar vaak doorheen gesleept. Ik kijk nu al uit naar jouw promotie, en ben zeer vereerd dat je me daarvoor al paranimf hebt gevraagd. Ik ben alleen wel heel benieuwd hoeveel vragen er gesteld kunnen worden.

Leonie en Karlijn (de vleesmeisjes), ik vond het erg gezellig met jullie erbij! We blijven de borrelafspraken/miepsessies gewoon doorzetten. Voortaan netjes op dagen dat Karlijn ook kan! Alleen wordt dat nu wel om~~v~~..fietsen voor Leonie, als dat maar goed gaat, helemaal naar Lunetten. Peter Koolmees, ik hoop dat jij deze cafébezoeken ook zal blijven bijwonen, ik vond het altijd erg gezellig. Dat geldt ook voor Martin, wie ik nog wil bedanken voor het kritische commentaar op mijn schrijfsels. I-I-Ik vind de locatie van die pottenbakclub alleen nog steeds wel een beetje verdacht.

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de IHC, en zonder jou was het werk in het histologielab veel minder leuk geweest. Fons, zelfs na een overname van jullie afdeling door V&V kon ik altijd bij je terecht met mijn vragen, waarvoor veel dank. Peter Tooten, dank je voor alle hulp met de ELISA's en mijn vanaf nu voorgoed veranderde kijk op zand.

Hoa, without you the IPI's probably would not have lasted as long as they did. You were a great colleague and I really appreciate your help with everything. I wish you all the best when you are reunited with your family! Gertie en Betty, het Biacore-verhaal is uiteindelijk in de koelkast beland, maar ik heb toch veel aan jullie gehad. Gertie, je spons-imitatie ga ik echt nooit vergeten! Betty, zullen we het er maar op houden dat ik nu echt een grote meid ben en een corrigerende tik echt niet meer gaat helpen? Ook Stefanie (die wel weet hoe ze studenten moet inpakken) en Veronica (hope you're not too busy to come to my party?) en alle andere (ex)V&V-ers wil ik graag bedanken voor de leuke tijd op het lab.

Lieve vrienden en familie, ik ben jullie erg dankbaar voor jullie steun, geduld en oprechte interesse. Beppe, hierbij wil ik jou in het bijzonder bedanken. Femke & Merel, mijn paranimfen en beste vriendinnen, jullie hielden mij geregeld met beide benen op de grond door al mijn gezeur flink te relativeren. Femke, ik heb Willem de Ridder helemaal niet nodig, ik heb jou al. Merel, laten we de Flitz-Ekko combi vooral in stand houden, een avondje kletsen en dansen is niet alleen ontzettend leuk, het doet ook echt wonderen voor stress.

Lieve mamma, dankzij jou ben ik zo ver gekomen! Zonder jouw liefde en vertrouwen had ik stukken minder stevig in mijn schoenen gestaan. Het is wat later dan beloofd, maar ik hoop dat de Nederlandse samenvatting eindelijk eens wat meer licht werpt op wat ik nou gedaan heb.

Ben, lief, de laatste regels zijn voor jou. De afgelopen vier jaar waren niet altijd even makkelijk voor ons allebei, maar zéker nu we deze weg zonder kleerscheuren hebben afgelegd heb ik alle vertrouwen in onze toekomst samen. Dank je voor al je steun, je luisterend oor (zelfs als het over qPCR-en gaat), je hulp maar bovenal voor het feit dat je er altijd voor me bent.

Curriculum vitae

Astrid Hogenkamp werd geboren op 26 maart 1977 te Arnhem. Na het behalen van het VWO diploma in 1996 aan het Liemers College te Zevenaar, begon zij in hetzelfde jaar aan de studie Biologie aan de Universiteit Utrecht. Tijdens deze studies deed zij haar eerste onderzoekstage bij de leerstoelgroep Moleculaire Celbiologie, met als onderwerp de mogelijke betrokkenheid van HXT5 in de accumulatie van trehalose in Saccharomyces cerevisiae. De scriptie werd geschreven bij de leerstoelgroep Gedragsbiologie, en had als onderwerp de verwerking van sociale informatie in reactief en proactief agressieve kinderen. De tweede onderzoekstage werd, gecoördineerd vanuit de Wetenschapswinkel Biologie, uitgevoerd bij het IRAS, waarbij een verkennend onderzoek werd opgezet om de mogelijke blootstelling van omwonenden aan pesticiden gebruikt in de bloembollenteelt te onderzoeken. In september 2002 behaalde zij haar doctoraal examen. In december 2002 trad zij in dienst als assistent in opleiding (AIO) bij de onderzoeksgroep Molecular Host Defense, die inmiddels behoort tot het departement Infectieziekten en Immunologie, Faculteit Diergeneeskunde, Universiteit Utrecht. Onder leiding van Prof. Dr. Henk Haagsman en Dr. Edwin Veldhuizen werd het in dit proefschrift beschreven onderzoek verricht.

List of Publications

1. **Astrid Hogenkamp**, Veronica Herías, Peter C.J. Tooten, Edwin J.A. Veldhuizen. Henk P. Haagsman. 2007 Effects of Surfactant Protein D on growth, adhesion and epithelial invasion of intestinal Gram-negative Bacteria. Mol Immunol. 44(14): 3517-3527
2. Veronica Herías, **Astrid Hogenkamp,** Alphons J. van Asten, Monique H. Tersteeg, Martin van Eijk, Henk P. Haagsman. 2007 Expression sites of the collectin SP-D suggest its importance in first line host defense: Power of combining in situ hybridisation, RT-PCR and immunohistochemistry. Mol Immunol. 44(13):3324-3332.
3. **Astrid Hogenkamp,** Martin van Eijk and Henk P. Haagsman. 2007 Collectins – interactions with pathogens. In: Collagen-related lectins in innate immunity. Dave Kilpatrick (Ed.) Research Signpost, Kerala, India, pp 119-177.
4. Edwin J.A. Veldhuizen, Henno G.C.J.M.Hendriks, **Astrid Hogenkamp**, Albert van Dijk, Wim Gaastra, Peter C.J. Tooten, Henk P. Haagsman. 2006 Differential regulation of porcine beta-defensins 1 and 2 upon Salmonella infection in the intestinal epithelial cell line IPI-2I. Vet Immunol Immunopathol. 15; 114(1-2):94-102.
5. Henk P. Haagsman, **Astrid Hogenkamp**, and Martin Van Eijk. 2006 Collectin-mediated innate immune defense in the lung. Journal of Organ Dysfunction 2;4: 230-236
6. **Astrid Hogenkamp**, Martin van Eijk, Albert van Dijk, Alphons J. van Asten, Edwin J.A. Veldhuizen, and Henk P. Haagsman. 2006 Characterization and expression sites of newly identified chicken collectins Mol Immunol. 43(10):1604-16.

7. **Astrid Hogenkamp**, Manon Vaal, and Dick Heederik. 2004 Pesticide exposure in dwellings near bulb growing fields in The Netherlands: an explorative study. Ann Agric Environ Med.11(1):149-53
8. René Verwaal, Johannes W.G. Paalman, **Astrid Hogenkamp**, Arie J. Verkleij, C. Theo Verrips, Johannes Boonstra. 2002 HXT5 expression is determined by growth rates in Saccharomyces cerevisiae. Yeast., 15;19(12):1029-38

Colour Prints

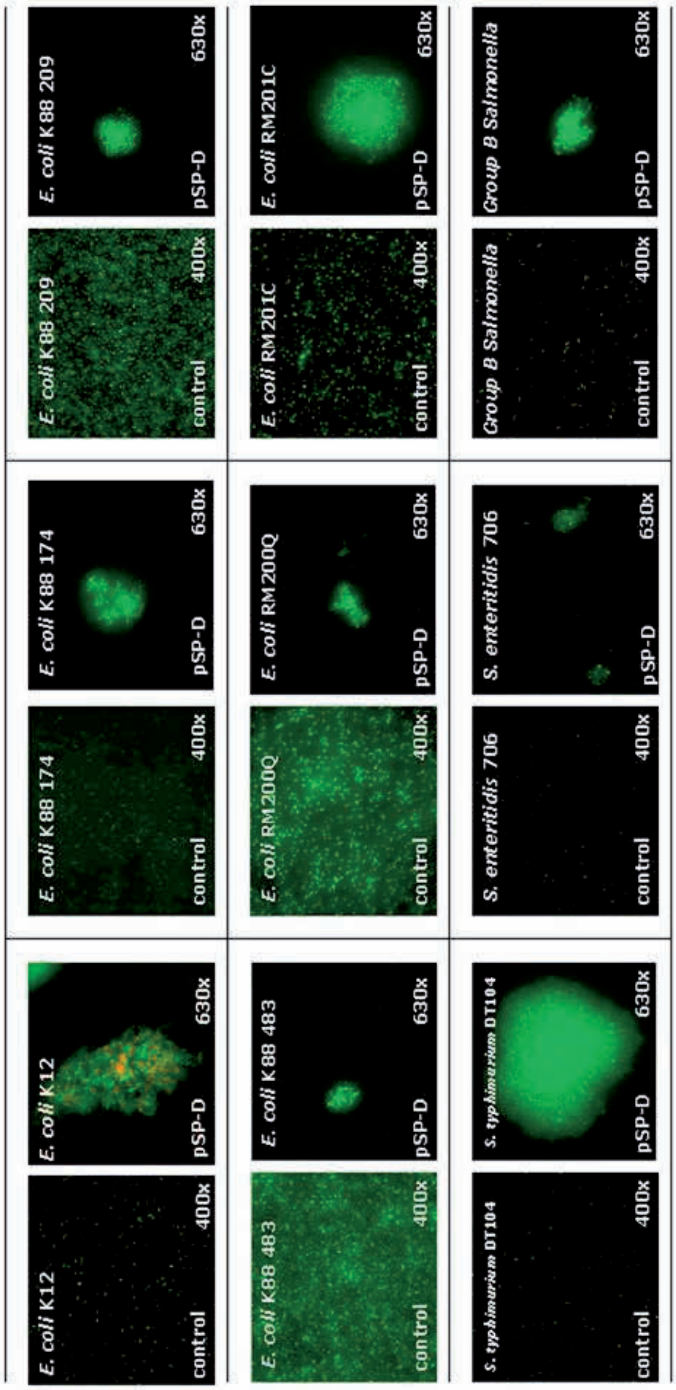

Figure 3. Bacterial permeability is increased in p-SP-D-treated *E. coli* K12, but not in other strains.

Bacterial membrane permeability was assessed by staining with the permeant fluorescent probe SYTO 9 and the impermeant fluorescent probe propidium iodide.
Red fluorescent staining was observed in pSP-D-treated *E. coli* K12 (upper left panel), indicating an increase in bacterial membrane permeability. pSP-D-treatment of other strains clearly leads to bacterial aggregation, but no effects on bacterial membrane permeability were observed. Similar results were observed for rSP-D (not shown).

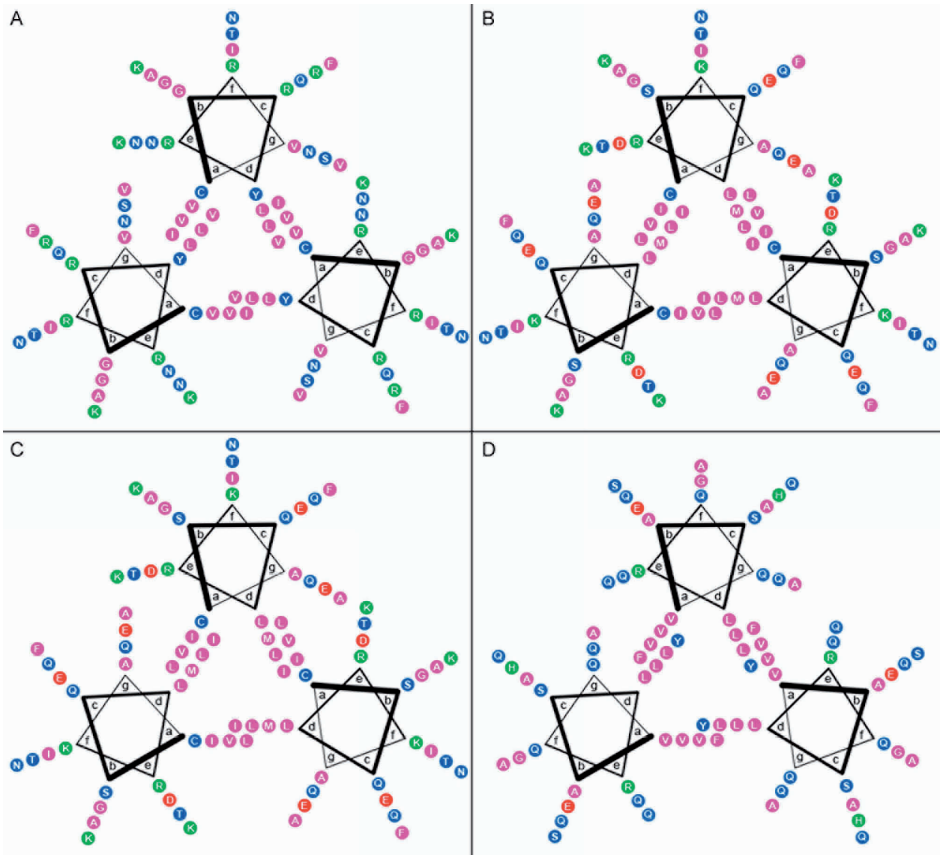

Figure 6. Helical wheel diagrams for (A) cLL, (B) cCL-1, (C) cCL-2 and (D) human SP-D. In the neck domain, a repeat of seven residues forms a heptad, the individual positions of the residues denoted by letters a to g. Residues at the first (a) and fourth (d) position are generally hydrophobic. Pink: Non-polar hydrophobic residues; Green: Polar, positively charged hydrophilic residues; Blue: Polar, uncharged residues; Red: Polar, negatively charged hydrophilic residues.