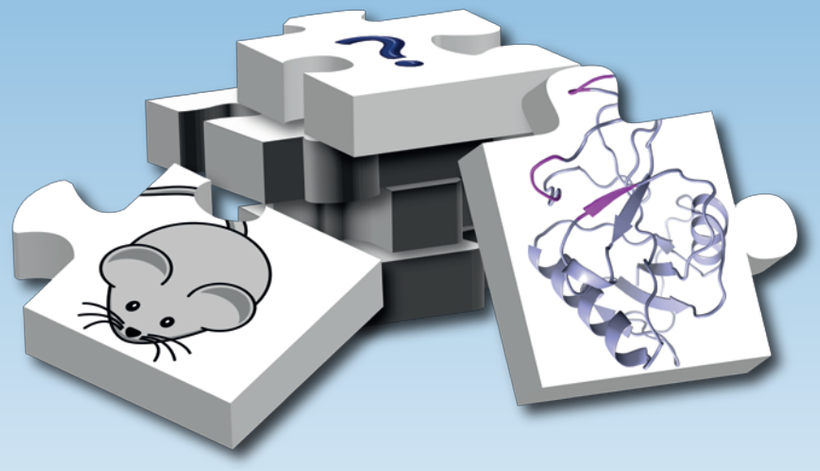
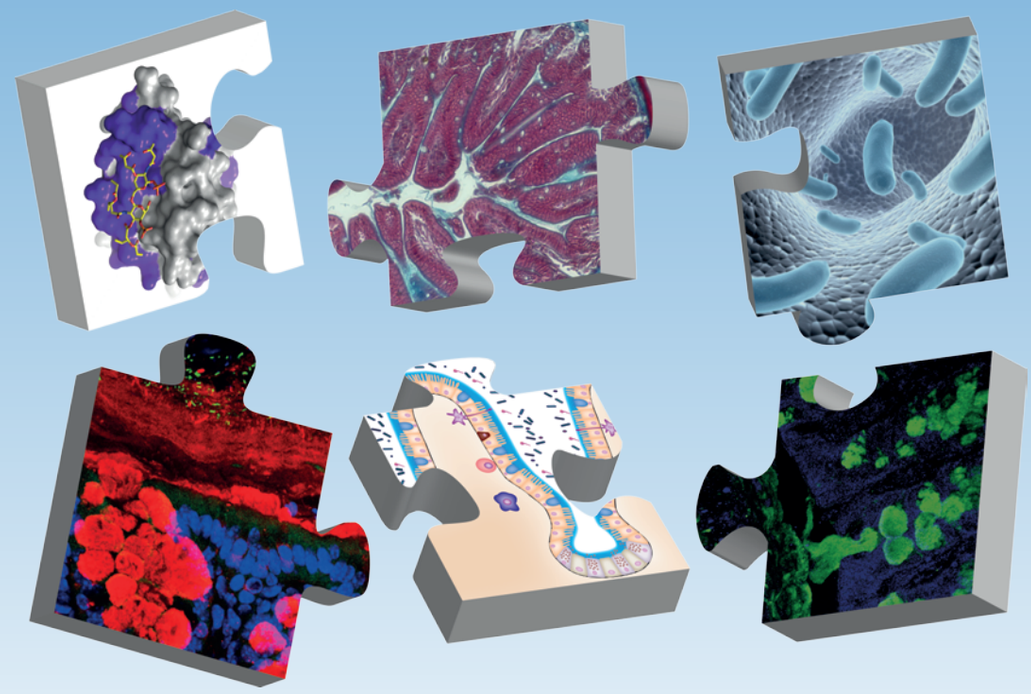


RegIII proteins as gatekeepers of the intestinal epithelium Linda M.P. Loonen 2013

# RegIII proteins as gatekeepers of the intestinal epithelium

Linda M.P. Loonen



# RegIII proteins as gatekeepers of the intestinal epithelium

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# RegIII proteins as gatekeepers of the intestinal epithelium

**Linda M.P. Loonen**

**Thesis**
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Voor mijn ouders

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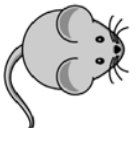

# Chapter 1

# General introduction

Linda M.P. Loonen

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## INTRODUCTION

The function of the mammalian gastro-intestinal (GI) tract is the digestion and absorption of food. Most of this digestion and absorption takes place in the small intestine, which includes the duodenum, jejunum and ileum. The large intestine or colon comprises the ascending, transcending and descending colon and functions to reabsorb water and salts from the undigested food matter, and to excrete waste material from the body. The GI tract is inhabited by trillions of bacteria that perform most of the digestion of complex carbohydrates that cannot otherwise be utilized by the host for nutrition. This intestinal community contains eukarya, archea and bacteria, collectively known as the microbiota comprising at least 500 to 1000 species.[1] The total number of microbial cells outnumbers the total amount of host cells by around 100 fold. Bacterial numbers increase from the proximal GI tract to the distal colon starting with $10^3$ to $10^5$ bacteria per ml of luminal contents in the stomach and duodenum to approximately $10^7$ to $10^{12}$ per ml in the ileum and colon.[2] The microbiota composition varies between individuals, and is also dependent on the intestinal site.[3] In humans, the composition changes a lot in the first 12 months of life, but then settles into a more adult composition.[4] In mammals and other metazoans these host-microbe relationships have evolved over millions of years to become largely mutualistic and symbiotic in nature.[5] The symbiotic benefits are diverse and include the competitive exclusion of pathogens, as well as participation in energy and nutrient metabolism, and tissue development and repair. In contrast, pathogens have by definition the capacity to cause harm to the host and may spread from the mucosa to other tissues in the body.

In healthy adults, the intestinal microbiota is dominated by 2 phyla, the Gram-negative Bacteroidetes and the Gram-positive Firmicutes.[6] Other phyla that are less abundant are Proteobacteria, Verrucomicrobia, Tenericutes, Deferribacteres and Fusobacteria.[6-10] The bacteria that reside in the outer layer of the mucosa are mainly commensal mucin-degrading bacteria that enzymatically degrade glycans and use the breakdown products as nutrients. *Akkermansia muciniphila* is one of the few isolated and characterized mucin degrading bacterial species.[11] Despite high species diversity and differences between individuals there appears to be a common 'functional core' of bacteria, which is shared among most of the community.[12, 13]

The coevolution of mammals with their intestinal symbionts has resulted in several adaptations of the mucosal tissue which maintains tolerance to microbiota, while maintaining effective defences against infection with opportunistic and obligate pathogens. The necessity to regulate immune responses to commensal organisms is evident from research on inflammatory bowel diseases (IBD) where loss of tolerance to the microbiota is a key aspect that stimulates loss of epithelial integrity and chronic inflammation.[14]

The development of an individual's immune system is partly shaped by the microbio-

ta.[15] In germ-free (GF) mice for example, the architecture of the mucosal epithelium is underdeveloped including the lymphoid tissues associated with the epithelium,[16] with smaller and fewer Peyer's patches (PP), fewer intraepithelial lymphocytes,[17] reduced antibody production and diversity,[18, 19] and reduced expression of some antimicrobial peptides.[20-22] Colonisation of these animals with a standard mouse microbiota restores these differences to levels found in conventionally reared mice.

The microbiota also makes a major contribution to host defence through their competition with pathogens. GF mice for example are more susceptible to infections by enteric pathogens.[23] Commensal as well as pathogenic bacteria have to compete for nutrients and binding to host surface receptors in the GI tract. Thus, colonizing commensal bacteria can inhibit colonization by pathogens, a phenomenon called 'colonization resistance'.[24, 25] For example, mice that have been treated with antibiotics to reduce the number of commensal bacteria were more susceptible to infection with enteric pathogens.[24]

Lastly, the microbiota contributes to host physiology and metabolism by fermenting otherwise non-digestible components of the host diet. Short-chain fatty acids (SCFA), the end products of bacterial fermentation, can contribute up to 5–15% of the total caloric requirements of humans.[26] A contribution of the microbiota to host energy harvest is evident in GF mice, which require a greater caloric intake than conventional mice to sustain a normal body weight.[27]

**The multi-layered defences of the intestinal tract: physical barriers**

The mucosa of the intestinal tract consists of different layers (Fig. 1.1). The outside layer is the serosa, which surrounds 2 layers of smooth muscle tissue, orientated in circular and longitudinal direction to facilitate peristalsis and movement of food through the intestine. Underneath the muscles there is a layer of submucosa, which contains connective tissue, blood and lymphatic vessels and nerves, followed by the muscularis mucosae which is a thin muscle layer separating the mucosa from the submucosa. The mucosa exists of the lamina propria (LP), connective tissue, containing immune cells, blood and lymph vessels, and nerves. A single layer of epithelial cells forms the last layer, forming a boundary between the mucosal compartment and the lumen of the intestine. The small intestine contains finger-like structures, so called villi, to increase the intestinal surface for more nutrient uptake. In this way the surface is increased to around 200 square metres, about the size of a tennis court.

The small intestinal epithelium absorbs nutrients from the digested food stream on one hand, and on the other hand acts as a barrier to the intestinal microbiota and opportunistic pathogens. Epithelial cells form a physical protective barrier between the luminal

content and the underlying LP. Tight junction (TJ) proteins are found near the apical surface of epithelial cells and function to keep bacteria and macromolecules outside the body, but allow diffusion of fluids, electrolytes and small molecules through the paracellular space. These proteins are composed of transmembrane proteins (occludins and claudins) and intracellular scaffold proteins (ZO-1, -2 and -3). Several specialized mucosal defence mechanisms exist to maintain the integrity of the single layer of epithelial cells and are discussed in more detail below. The epithelium contains 4 different cell types (Fig. 1.2) that all originate from intestinal stem cells. The stem cells that are at the bottom of the crypts are alternated with Paneth cells,[28] that can be recognized by the large granules in the cytoplasm that contain anti-microbial factors such as lysozyme, defensins, phospholipases and RegIII proteins. Paneth cells are replaced every 30 days and are only found in the crypts of the small intestine; they do not migrate up in the villus. Higher up in the crypt reside enterocytes, enteroendocrine cells and goblet cells, which all migrate to the top of the villus where they undergo apoptosis. The rapid turnover of epithelial cells in the intestine means that the intestinal epithelium is regenerated every 2 to 5 days in humans.

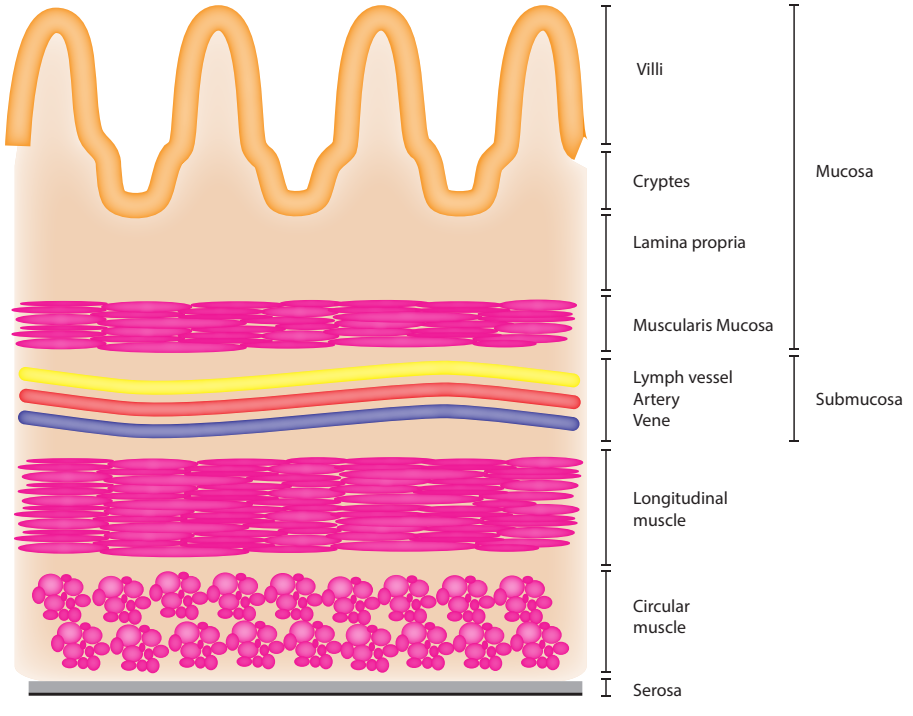


**Figure 1.1.** Schematic representations of the different layers in the intestine.

Enteroendocrine cells (less than 1% of the epithelial cell population[29]) secrete hormones

including serotonin and peptides in response to signalling by compounds in the lumen, particularly nutrients. The hormones secreted by the enteroendocrine cells play a role in controlling physiological and homeostatic functions in the digestive tract, like the modulation of host energy metabolism, mucus secretion and intestinal motility.[29] The enterocytes are the most abundant cells present in the intestine and possess microvilli on the apical surface, known as the "brush-border", which promotes the digestion and absorption of nutrients from the lumen. The apical surface of the epithelium is covered by the glycocalyx of membrane bound mucins that play a role in preventing microbial adhesion and colonization.

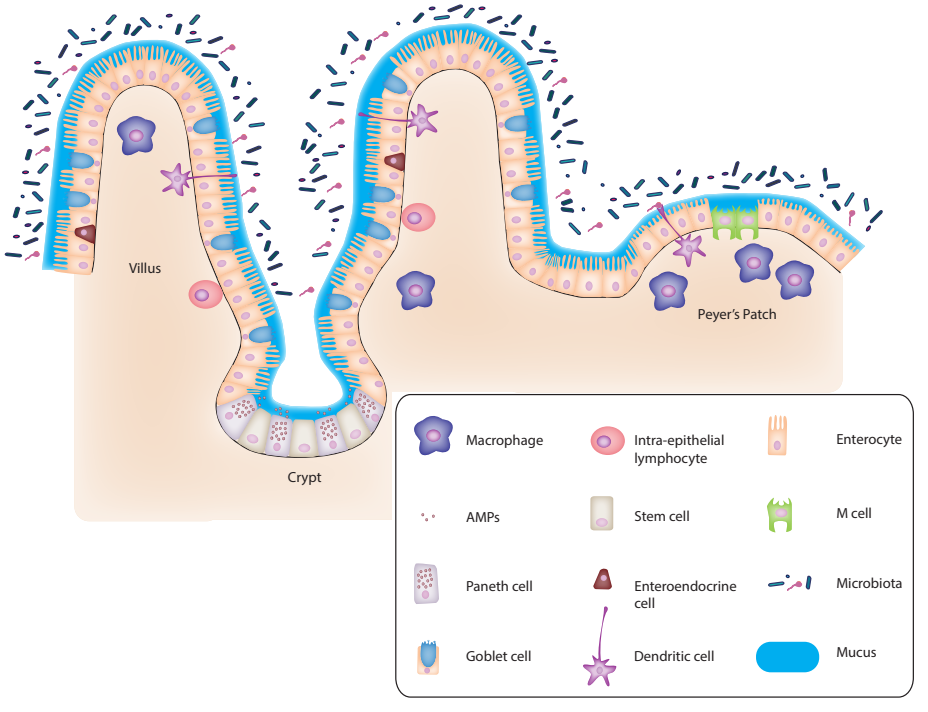


**Figure 1.2**. Schematic representation of the ileal epithelium with the different cell types present.

Goblet cells in the epithelium secrete mucin polypeptides that are glycosylated and polymerized to form a mucus gel overlaying the epithelium. This layer is protective, preventing the bacteria present in the gut from being in contact with the epithelial cells. Mucus also serves as a lubricant, facilitating the movement of the luminal content through the intestine. Furthermore, this layer provides carbohydrates that can be used as a food source by mucus degrading bacteria. The mucus layer is continuously produced and removed by peristaltic flushing. In the colon, the mucus barrier is thicker than in the ileum and consists of a dense layer attached to the cells and a less dense mucus layer above it that is in contact with the microbiota and luminal contents. The

inner layer is almost devoid of bacteria, whereas the outer, loose mucus layer contains bacteria.[30] In the ileum, the mucus layer is much thinner and it is not known whether the ileal mucus layer is stratified as is the mucus layer in the colon, or whether the mucin composition differs between small intestine and colon.

The vast majority of mucus in the human intestine comprises Muc2, which is a large (approx. 5 MDa), highly glycosylated protein (Fig. 1.3).[31] Like other mucins, Muc2 contains a PTS (proline, threonine and serine) protein core to which the glycan GalNAc (N-acteylgalactosamine) binds.[32, 33] A complex mixture of glycan epitopes is made by extension and branching of the GalNAc residues to a series of other glycosyltransferases.[34, 35] Muc2 is polymerized through the N- and C-termini to form large net-like polymers which form a transparent gel-like structure.

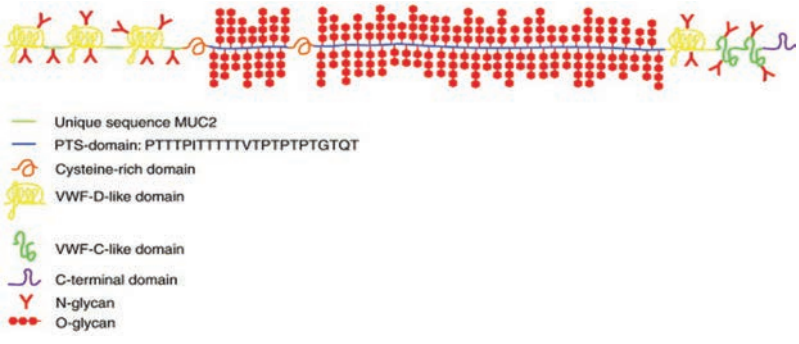


**Figure 1.3.** Figure from Dekker *et al*.[36] Schematic representation of a Muc2 molecule.

A diminished mucus layer is associated with different diseases, including colitis and IBD.[37-39] The protective role of mucus is most evident from studies in Muc2 knockout mice, which start to develop colitis after weaning, leading to severe pathology in the colon and premature death.[40] Although the absence of a mucus layer causes colitis by microbial induced inflammation, the microbiota seems to be important for the thickness of the mucus layer, as GF mice have a thinner inner mucus layer than conventionally housed mice.[30]

## The multi-layered defences of the intestinal tract: innate and adaptive immune barriers

*Mucosal-associated lymphoid tissue and secretory IgA*

Specialized lymphoid structures, including Peyer's Patches (PP) and isolated lymphoid follicles permit the sampling of luminal antigens via specialized microfold (M) cells in the follicular epithelium.[41] In addition, non-specific secretory IgA (sIgA) produced by

activated B cells and transcytosed across the enterocytes into the lumen via the polymeric IgA transporter, limits bacterial association and penetration with the intestinal epithelial cells by causing agglutination of bacteria in the intestinal lumen. [42-45] SIgA can also interfere with assembly of intracellular viruses in the golgi apparatus during transcytosis[46] and remove potential inflammatory antigen complexes from the LP, by binding during transport via the polymeric IgG receptor. Humans secrete several grams per day of sIgA into the intestinal lumen highlighting its importance to mucosal protection.

| Receptor | Localization | Adaptor molecule | Ligand | Ligand origin |
|---|---|---|---|---|
| TLR2 | Cell surface | TIRAP MyD88 | LTA<br>Lipoproteins | Bacteria<br>Fungi |
| TLR2/1 | Cell surface | TIRAP MyD88 | Triacyl lipopeptide | G- bacteria |
| TLR2/6 | Cell surface | TIRAP MyD88 | Diacyl lipopeptide | G+ bacteria<br>Mycoplasma |
| TLR3 | Cellular compartment | TRIF | dsRNA | Viruses |
| TLR4 | Cell surface | TIRAP MyD88<br>TRAM TRIF | LPS | G- bacteria |
| TLR5 | Cell surface | MyD88 | Flagellin | Bacteria |
| TLR7 | Cellular compartment | MyD88 | ssRNA | RNA viruses |
| TLR9 | Cellular compartment/Cell surface | MyD88 | CpG DNA | DNA viruses<br>Bacteria |
| TLR11 | Cell surface | MyD88 | Profilin<br>Uropathogenic bacterial components | Toxoplasma<br>Uropathogenic bacteria |
| NOD1 | Cell cytoplasm | RIP2 | Meso-DAP | G- bacteria |
| NOD2 | Cell cytoplasm | RIP2 | MDP | Bacteria |

**Table 1.1**. Microbial ligands and adapter proteins of TLRs and NOD receptors. Abbreviations used in this table: TIRAP, Toll/interleukin-1 receptor (TIR) domain–containing adapter protein; MyD88, Myeloid differentiation primary response gene 88; TRIF, TIR-domain-containing adapter-inducing interferon-β; TRAM, TRIF related adaptor molecule; RIP2, receptor interaction protein 2; LTA, lipoteichoic acid; dsRNA, double stranded RNA; LPS, lipopolysaccharide; ssRNA, single stranded RNA; CpG, C phosphate G; DAP, diaminopimelic acid; MDP, muramyl dipeptide; G-, gram negative; G+, gram positive

*Innate signalling and regulation of the intestinal barrier*

Dendritic cells (DCs) play a key role in the induction of tolerance and immunity. They reside in the PP and can sample luminal antigen in the LP by extending dendrites through the epithelial tight junctions.[47] Epithelial and immune cells such as DCs in the LP recognize microbes through common structures on microorganisms often referred to as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) via pathogen recognition receptors (PRRs). The best characterized family of PRRs is the Toll-like receptor family (TLR), which are expressed on cells of the myeloid and lymphoid lineages as well as on non-immune cells such as enterocytes and

fibroblasts.

Innate recognition of microbes and microbial-associated molecular patterns by host PRRs plays a pivotal role in the maintenance of intestinal homeostasis. These families of receptors are involved in immune cell activation, production of cytokines and chemokines as well as regulation of production of components of the gut barrier such as antimicrobials, mucin and TJ proteins.

Mammalian PRRs includes members of the family of TLRs, nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors which induce cellular responses in immune and other cells through cellular signalling pathways. The molecular structures recognized by the known human TLR and NOD and their cellular location are shown in Table 1.1.

TLRs participate in innate signalling on the cell surface and in endosomes whereas the NLRs, of which there are more than 20 family members, recognize intracellular ligands.[48] NOD1 and NOD2 detect the bacterial peptidoglycan fragments meso-DAP and MDP (Fig. 1.4); the specificities of most of the other NLRs remain uncharacterized.[49-51]

TLRs recruit a set of adaptor proteins containing Toll/Interleukin-1 receptor (TIR) domains that trigger downstream signalling cascades leading to the activation of transcription factor nuclear factor-kappa light-chain-enhancer of activated B cells (NF-κb), which controls the induction of pro-inflammatory cytokines and chemokines that are instrumental in induction of inflammatory and adaptive immune responses against pathogens.[52-55] Furthermore, NF-κb activation is linked to increased expression of some epithelial defensins and Reg proteins (Fig. 1.4). In the mucosal epithelium this serves to recruit phagocytes including neutrophils, macrophages and DCs to the site of invading pathogens. DCs play a crucial role in the induction of adaptive immune responses via the presentation of antigens and activation of naïve T cells in the peripheral lymph nodes. It became evident from different studies that TLRs control pathways involved in tissue repair and growth and that a basal level of TLR signalling from the luminal microbiota may indeed contribute to intestinal homeostasis.[56] Further evidence for the role of TLR signalling in the regulation of homeostatic mechanisms came from studies on the intestinal epithelial cells (IEC)-specific deletion of components of the Iκβ kinase (IKK) complex, required for NF-κb activation. These knockout mice developed severe chronic intestinal inflammation.[57] Several studies suggest that steady-state recognition of the normal microbiota by PRRs does occur and it contributes to homeostatic mechanisms and influences gene expression in IECs.

*Homeostasis of innate signalling in the mucosa*

PRR signalling is a crucial component of the innate defence against pathogens, but continued induction of inflammatory responses at the mucosal surface from contact with the resident microbiota would be pathological. To cope with these somewhat opposing

functions several adaptations of PRR signalling have evolved in the intestinal mucosa.[58] Low expression of TLRs on the apical surface of the epithelium has been suggested as a mechanism to regulate inflammatory responses at the mucosal surface.

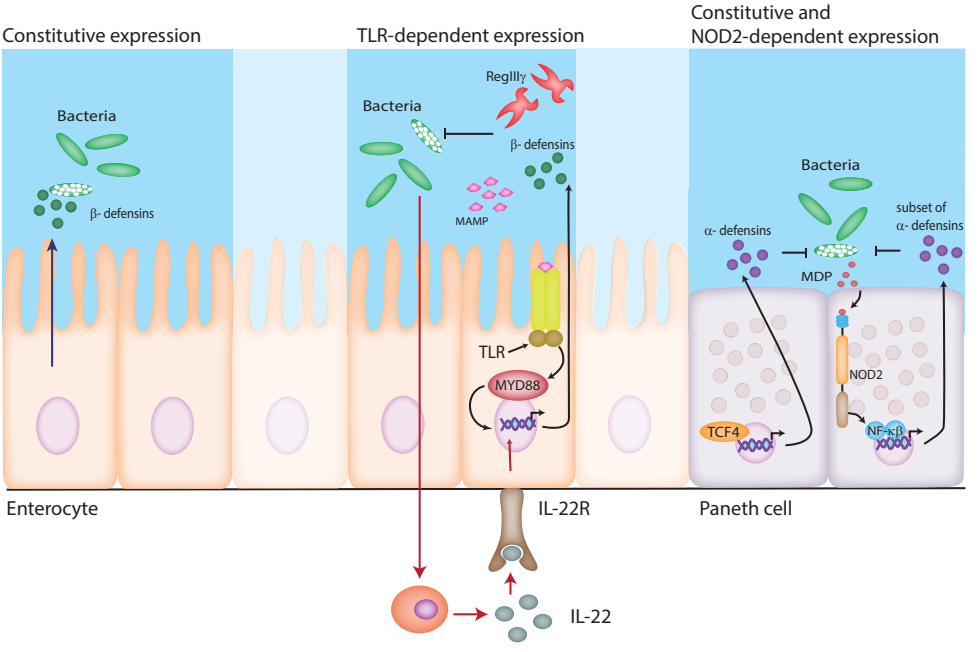


**Figure 1.4**. The regulation of antimicrobial peptide expression. Constitutive expression of β-defensins by enterocytes, TLR dependent expression of β-defensins and TLR dependent expression of RegIIIγ via MyD88-dependent signalling and IL-22, and NOD2 dependent expression of a subset of α-defensins via the MDP and the NF-κb pathway, or constitutive α-defensins production via TCF4 signalling.

The expression, distribution and cellular localization of PRRs on IEC play a role in host discrimination of commensals and pathogens in steady state conditions. Additionally, the response to surface expressed TLRs in IEC may be dependent on the polarity of the signalling. Moreover, the specificity of cellular responses after binding and activation of a PRR is governed by the differential recruitment of the adapter proteins MyD88, MAL, TRIF, and TRAM, that form a complex with the C-terminal domains of different TLRs.[59] Once the epithelial barrier is breached however, the initial inflammatory response may up-regulate PRR and co-receptors to overcome the mechanisms that are in place to dampen PRR signalling at the mucosal surface under steady state conditions.

The current view of TLR expression in the intestine is not comprehensive with respect to tissue localization and *in vivo* studies. Intestinal cell lines have been frequently used to study PRR localization and signalling but the results can differ between cell lines and

between *in vivo* and *in vitro.*[60, 61]

*Regulation of PPR Signalling by Microbes*

The relationship between humans and their intestinal microbiota is largely symbiotic in nature and over eons of co-evolution multiple adaptations appear to have developed to help maintain this peaceful co-existence. On the other hand, bacterial and viral pathogens have also evolved multiple mechanisms to subdue recognition by PRRs and avoid innate immunity.[62]

Short chain fatty acids such as butyrate are the main end product of fermentation for some species of gut commensals. Apart from being an important energy source for intestinal epithelial cells, butyrate plays role in the maintenance of homeostasis in the colon.[63] Butyrate can inhibit the activation of NF-κb, probably via histone deacetylation and suppression of cellular proteosome activity.[64] Other reported anti-inflammatory effects of butyrate include inhibition of the interferon (IFN)-γ production and/or signalling[65, 66] and the up-regulation of peroxisome proliferator-activated receptor *gamma* (PPARγ).[67-70]

NF-κb pathway inhibitors may also come from intestinal pathogens. *Yersinia* for example can inhibit NF-κb pathway at the level of IκB phosphoprylation whereas *Salmonella* spp. block ubiquitination and degradation of Iκβ to prevent translocation of NF-κb into to the nucleus.[71] Recently, an uropathogenic *Escherichia coli* strain CFT073 was shown to prevent activation of the NF-κb pathway via secretion of TcPC, which inhibits binding of MyD88 to TLRs.[72] This mechanism contributes to the persistence and spread of the pathogen by preventing recognition by the innate immune system and subsequent induction of adaptive immune responses. It is tempting to speculate that other pathogens and even commensal bacteria may possess similar mechanisms to avoid immune recognition and elimination from the intestinal tract.

*Antimicrobial defensins*

The epithelial cell barrier is reinforced by the mucus layer and a range of antimicrobial peptides and proteins that prevent intimate contact with the commensal and pathogenic bacteria. Paneth cells secrete a diverse range of peptides and proteins with antimicrobial properties, referred to as antimicrobial peptides (AMPs). A major group of these AMPs in mammals are defensins, which are highly basic, small antimicrobial peptides of 2-6 kDa with a β-sheet structure and six cysteine residues, which are disulfide linked.[73, 74] Defensins show antimicrobial activity against Gram-positive and Gram-negative bacteria, certain fungi, protozoa and enveloped viruses.[75, 76] There are 3 subgroups of defensins; α-defensins, β-defensins and θ-defensins, based on structural features of the gene, such as the connectivity of three cysteine linkages, and precursor and mature peptides.[77, 78] All subgroups are produced in vertebrates, but θ-defensins are only produced in non-human primates. Alpha defensins, produced by Paneth cells in a primarily constitutive way, are most abundant in the small intestine.[77] The propeptides of α-de-

fensins need processing to produce a mature, active peptide. Matrix metalloprotease (Mmp)-7, produced by mouse Paneth cells, is essential for processing the α-defensins in mice (also called cryptdins), but in humans trypsin is responsible for defensin processing.[77] Mice express more than 6 α-defensins and numerous cryptdin-related sequence (CRS) peptides which are not found in humans.[79, 80] Humans produce 2 known Paneth cell α-defensins, HD5 and HD6 as well as 6 β-defensins (HBD), produced by epithelial cells, such as enterocytes, in the intestine. Human β-defensin-1 is constitutively expressed in enterocytes, whereas the expression of human β-defensin-2 (HBD-2) and human β-defensin-3 (HBD-3) are induced by microbial products and inflammatory cytokines.[81-85] Inducible expression of HBD-2 and RegIII proteins (another antimicrobial) in enterocytes has been shown to be dependent on TLR or MyD88-dependent signalling (Fig. 1.4).[86, 87] The bactericidal activity of defensins is obtained through interaction with the outer membrane of the bacteria. By the entrance of the defensin molecule in the bacterial membrane, attracted by local electrostatic fields, and the interposing between the phospholipid groups of the membrane, the defensin displaces the lipids, thereby weakening the membrane. This is called the carpet wormhole mechanism and eventually leads to the formation of pores and cell death (Fig. 1.5).[75, 77, 88]

Another class of AMPs are the cathelicidins, which are not produced by Paneth cells but are produced by enterocytes on top of the colonic crypts. Cathelicidins are cationic peptides that kill fungi and both Gram-positive and Gram-negative bacteria by binding to the bacterial membranes via electrostatic interactions, creating transient pores in the membrane resulting in destruction.[89]

Other antimicrobial factors produced by Paneth cells include, lysozyme, secretory phospholipase A2, angiogenin-4 and hepatocarcinoma-intestine-pancreas/pancreatic-associated protein (HIP/PAP).[73, 74] The latter, highly similar to RegIIIγ (Regenerating gene III gamma) in mice, belongs to the C-type lectin superfamily based on cDNA sequence homologies,[90, 91] and shows inducible expression in the pancreas and intestine.[22] C-type lectins are proteins that have a C-type lectin-like domain (CTLD) and bind to carbohydrates, often in a calcium dependent manner (C-type).[92, 93] Binding of these lectins to specific sugar structures mediates biological events, such as cell-cell adhesion and innate immune responses to potential pathogens.[94] Reg proteins, more specifically RegIII proteins, have shown great relevance in homeostasis and disease, both in human but also in animal models. Although the RegIII proteins belong to the C-type lectin family, their binding capacity is not calcium-dependent.[95, 96]

*RegIII proteins*

Reg proteins were discovered by Keim and co-workers in 1984[97] as proteins heavily upregulated in pancreatitis in rats. It was therefore named pancreatitis-associated protein (PAP), and during acute disease PAP accounts for about 5% of the secreted protein, but returns to undetectable levels when the pancreas had recovered.[98] Independently from

them, Terazono *et al*. found a gene that they named reg1 (*re*generating *g*ene) implicating that this gene might be involved in island regeneration in the pancreas in rats.[99] In 1992, Lasserre *et al*.[90] found the PAP mRNA in humans to be upregulated in liver tumors, where after they named it HIP (hepatocarcinoma-intestine-pancreas).

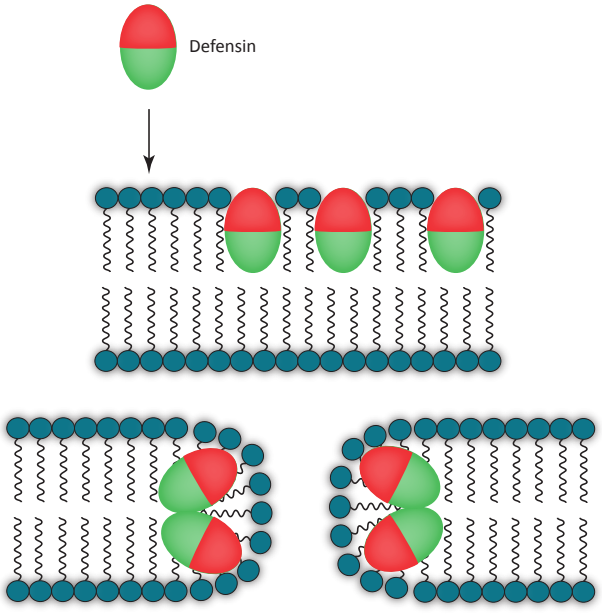


**Figure 1.5**. The carpet-wormhole model of action of defensins. Most defensins are amphipathic molecules that have clusters of positively charged amino-acid side chains (red) and hydrophobic amino-acid side chains (green). This allows them to interact with microbial membranes, shown schematically with their negatively charged phospholipid headgroups (blue) and hydrophobic fatty acid chains (black). In the top panel, electrostatic attraction and the transmembrane bioelectric field pull the peptide molecules towards and into the membrane. As peptide molecules accumulate in a 'carpet', the membrane is strained and the peptides transition into another arrangement (shown in the lower panel) that lowers the strain but results in the formation of membrane 'wormholes' or pores. For defensins, the specific arrangement of peptide molecules in the pores is not yet known. Adapted from Ganz *et al.*[88]

Other studies showed the presence of homologous proteins in humans[99, 100] and mice[101] and in other organs than pancreas.[101] Narushima and co-workers later showed the presence of 3 different RegIII genes in mice,[102] and suggested that all the mouse RegIII genes (classes I, II and III) diverged from a common ancestral gene. Type III Regs further diverged into RegIIIα, RegIIIβ, RegIIIγ and RegIIIδ. All RegIII proteins, except RegIIIδ, are produced in the intestine. RegIIIβ and RegIIIγ are the most similar to one another sharing 70% similarity.[102, 103] In 2001, a fourth Reg family member was found in human intes-

tine, and named RegIV.[104] The Reg nomenclature is quite puzzling as many genes were named after their first discovered function, only later to be discovered that genes were the same. See table 1.2 for a comprehensive overview of homologues genes.

| | *Homo sapiens* | *Mus musculus* | *Rattus norvegicus* |
|---|---|---|---|
| I | RegIα (PSP/PTP) | RegI | RegI |
| | RegIβ | | |
| II | | RegII | |
| III | RegIIIγ | RegIIIα | RegIII (PAPII) |
| | HIP/PAP (RegIIIα) | RegIIIβ (Reg2/PAPI) | PAPI (Reg2) |
| | | RegIIIγ (PAPIII) | PAPIII |
| | | RegIIIδ | |
| IV | RegIV | RegIV | RegIV |

**Table 1.2.** The nomenclature of the different Reg proteins. Between brackets the names that are also used for the same protein. This table is based on Lieu[105], Hunt[106] and Graf[107]

The expression of RegIIIγ and several other antimicrobial factors was shown to be induced in GF mice following colonization with conventional microbiota and also after weaning when the complexity and density of the microbiota rapidly increases.[22] Studies in NOD2$^{-/-}$ and MyD88$^{-/-}$ mice revealed that NOD2 regulates Paneth cell expression of α-defensins but not RegIIIγ and *vice versa* for the MyD88$^{-/-}$ mice (Fig. 1.4).[86, 108, 109] Paneth cells were shown to sense bacteria and induce the expression of several antimicrobial factors directly through MyD88 signalling pathways, using a mouse transgenic model in which MyD88 was expressed specifically in Paneth cells on a MyD88$^{-/-}$ background.[86] In this model MyD88 dependent expression of Paneth cell antimicrobials was shown to limit mucosal uptake of commensal and pathogenic bacteria into the mesenteric lymph nodes but had no effect on luminal numbers of bacteria.

Early studies on PAP1 showed that it aggregates bacteria, suggesting a possible protective role of PAP1 in bacterial infection.[110] Later studies indeed showed that intestinal colonization by bacteria,[22] infection[111] or inflammatory stimuli driven by mucosal inflammation and damage, as in IBD[112, 113] were triggers for expression of RegIII proteins. The anti-inflammatory function of rat PAP1 was shown in multiple studies. Vasseur *et al*.[114] showed that the anti-inflammatory response to PAP1 shared features with the response to interleukin (IL)-10, as both inhibit the activation of neutrophils and macrophages, inhibit IL-6 and TNFα expression and block translocation of NF-κb. Later, it was

shown by the same group[115] that PAP1 inhibits the inflammatory response by blocking NF-κb activation through a STAT3 (signal transducers and activators of transcription) dependent mechanism. Closa and co-workers[116] even suggested PAP1 to be a new anti-inflammatory cytokine, as it shares similarities in the mode of action with cytokines, like signalling via the JAK/STAT3/SOCS3 pathway. Both RegIIIγ and RegIIIβ were shown to be upregulated by IL-22 treatment in colonic tissue *ex vivo,* and to be upregulated in the colon of mice infected with *C. rodentium*, but not in *C. rodentium* infected IL-22$^{-/-}$ mice.[117]

*In vitro* studies with recombinant and refolded RegIIIγ and human HIP/PAP showed that RegIIIγ is not glycosylated[95] and that RegIIIγ and HIP/PAP have a bactericidal role towards Gram-positive bacteria through binding to peptidoglycan (PGN).[22] The *in vitro* bactericidal activity of RegIIIγ or HIP/PAP is inconsistent with other publications, where recombinant HIP/PAP from humans and rats was not bactericidal, but induced bacterial aggregation.[118, 119]innate immunity, lectin-like protein, N-terminal proteolytic processing, pancreatitis-associated protein (PAP *In vivo* studies showed that MyD88-mediated signalling increased the production of RegIIIγ after infection[109] and later it was shown that the microbiota is in closer contact to the epithelium in RegIIIγ$^{-/-}$ mice,[120] suggesting that RegIIIγ helps to minimize bacterial contact with the epithelium. Strikingly, exogenous mouse RegIIIγ was also found to have a protective role in IL-22$^{-/-}$ mice infected with *C. rodentium*, a gram-negative bacterium.[117]

Recombinant and refolded RegIIIβ has been proposed to kill Gram-negative bacteria and a number of Gram-positive bacteria.[121, 122] The bactericidal effect is growth phase dependent and in the case of Gram-negative bacteria, involves RegIIIβ binding to the lipid A anchor of lipopolysaccharide (LPS).[122] A RegIIIβ$^{-/-}$ mouse model was developed to look at the function of RegIIIβ during liver damage and showed that RegIIIβ is important in the protection against liver failure.[105] Furthermore, this model was used in an infection study where it was shown that RegIIIβ protects against infection with the Gram-negative bacterium *Yersinia pseudotuberculosis*, which was TLR2-dependent.[123]

**Infection models: mode of invasion of Salmonella enteritidis and Listeria monocytogenes**

Enteric bacterial pathogens are a substantial cause of foodborne infection. The host has many mechanisms to avoid infection by pathogens, such as stomach acid, mucus layers, secretion of antimicrobial proteins, including RegIII proteins, and the innate and adaptive immune system, described above. In healthy individuals, an intestinal infection usually results in a self-limiting gastroenteritis, but in immunocompromised persons, infants and elderly people infection with a pathogen might result in serious disease that the infected person is not able to contain and clear.

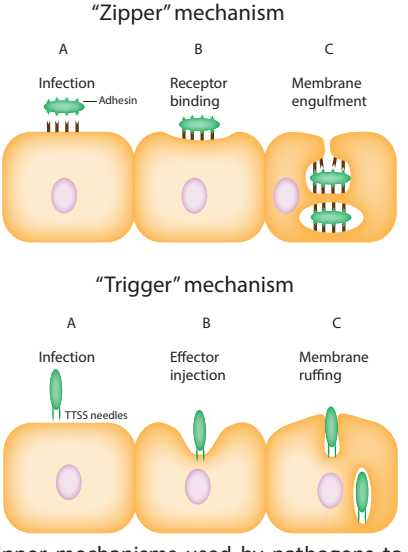


**Figure 1.6.** The trigger and zipper mechanisms used by pathogens to invade non-phagocytic cells. Adapted from Cróinín[126]. The zipper mechanism uses bacterial surface proteins (adhesins) (A) that bind receptors on the host cell membrane (B), triggering a signalling cascade that reorganizes the actin cytoskeleton to internalize the bacterium (C). The trigger mechanism employs the bacterial type III secretion system (T3SS)(A) to deliver proteins across the host plasma membrane (B) to directly interact with the cellular components that regulate actin dynamics (C).

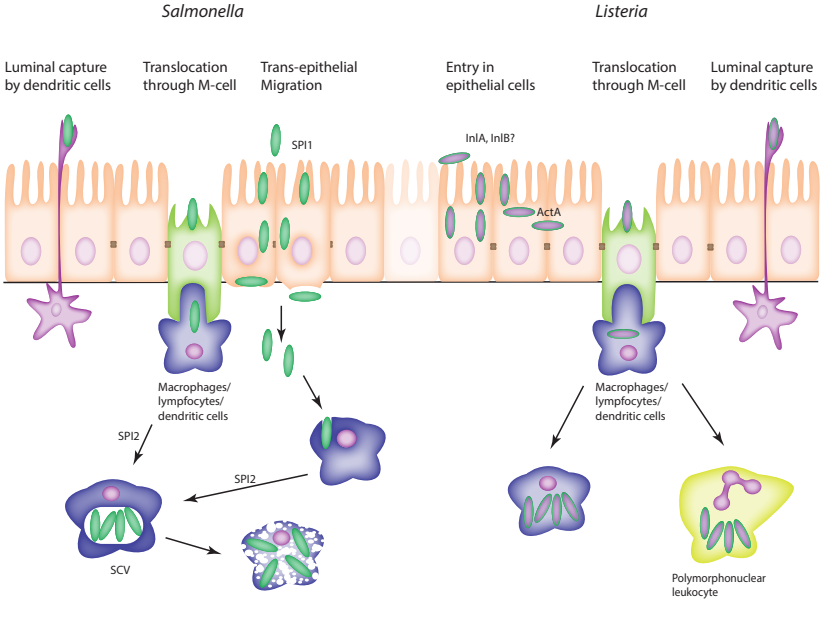


**Figure 1.7.** Adapted from Cossart *et al*.[125] The invasive strategies of enteroinvasive *Salmonella* and

*Listeria*. After the bacteria have crossed the epithelial barrier, they must survive being attacked by macrophages. The 2 bacterial species considered here have solved this issue differently: *L. monocytogenes* are phagocytosed but escape into the cytoplasm, and thus avoid being killed in lysosomal compartments. *Salmonella* remodel their phagosomes, thus avoiding its transition to a lysosome and creating an intracellular niche that allows their efficient replication. Having crossed the epithelial barrier and circumvented the threat of phagocytosis, *L. monocytogenes* disseminate systemically, possibly inside circulating monocytes and DCs. *Salmonella* may enter IECs through their basolateral pole in a TTSS- dependent manner. Alternative routes of invasion involve IECs directly, away from the FAE. In particular, invasion by *L. monocytogenes* is mediated by internalin (InlA) and possibly InlB. In addition, *Salmonella* are able to dislocate the brush border cytoskeleton and cause an apical entry ruffle. A third process of translocation may involve DCs crawling between IECs or sending pseudopods to capture luminal bacteria and retract in a subepithelial position. *Salmonella* are able to translocate in this way, possibly followed by systemic diffusion of *Salmonella*-loaded DCs.

In this thesis, two different pathogens, *Salmonella enterica* and *Listeria monocytogenes,* were used to study the effect of the absence of RegIIIβ or RegIIIγ during infection. Although these pathogens have different modes of infection, they both invade and translocate from the gut lumen to the extra-intestinal organs, like spleen and liver. Infection by enteric bacterial pathogens starts with adhesion to host cells, colonization and sometimes cellular invasion, intracellular multiplication and finally dissemination to other tissues or persistence can take place.[124] Adhesion to host cells may be mediated through pili, also called fimbriae, which are adhesive hair-like organelles that protrude from the bacterial surface. In addition to pili, a broad range of different bacterial nonpolymeric adhesins exist which recognize diverse elements of host-cell surfaces, including components of the extracellular matrix like collagens, adhesive glycoproteins such as fibrinogen and fibronectin, and integral host membrane adhesion receptors like integrins, cadherins and selectins.[124] After adhesion the bacterium has to be taken up by or actively enter the host cell, which can occur via different mechanisms. The pathogens used in this thesis have a different way of invading the host cells: *Salmonella* employs a so-called trigger mechanism, whereas *L. monocytogenes* uses a "zipper" mechanism (Fig. 1.6).[125]

The pathogenic species of *Salmonella* studied in this thesis is a serovar (serotyping variant) of *Salmonella enterica* subspecies *enterica* known as *S. enterica enterica* serovar *enteritidis* which for reason of brevity is referred to in this thesis as *Salmonella enteritidis*. This enteric pathogen is a Gram-negative, flagellated, facultative intracellular enteric bacterial pathogen that can colonize a range of animal hosts.[127, 128] *S. enteritidis* is equipped with a sophisticated repertoire of virulence factors that will allow it to penetrate the intestinal epithelium, mainly at the distal ileum,[129] after which it can disseminate systemically to colonize the spleen and the liver.[127] *Salmonella* can invade the host intestine via different mechanisms (Fig. 1.7). A major route of invasion is through M cells in the follicle associated epithelium (FAE) overlying the Peyer's Patches (PP), which

transport antigens and bacteria from the lumen to the LP. Another entry mechanism is via invasion of non-phagocytic enterocytes through the virulence-associated type 3 secretion system (TTSS) encoded by the *Salmonella* pathogenicity Island 1 (SPI-1). Besides that, *Salmonella* can also invade the body via dendritic cells, which intercalate between epithelial cells, or by paracellular pathways following disruption of TJs.[130, 131] After passing the epithelium by one of these mechanisms the bacteria encounter the basal membrane, where the invaders are taken up by phagocytic immune cells such as macrophages or dendritic cells. *Salmonella* then utilizes a second TTSS, encoded by *Salmonella* pathogenicity Island 2 (SPI-2), to survive and replicate within an endosomal or phagosomal vacuole, called the *Salmonella* containing vacuole (SCV).[128, 132, 133] In this SCV *Salmonella* can replicate to high numbers before it leaves the cell to infect new cells, ultimately causing systemic spread to other organs like spleen and liver.

*Listeria monocytogenes,* the other bacterial pathogen studied in this thesis, is a Gram-positive, facultative intracellular bacterial pathogen that infects macrophages and non-phagocytic cells. It is able to traverse three human barriers: the intestinal barrier, the blood-brain barrier and the feto-placental barrier. *L. monocytogenes* utilizes surface molecules internalin (Inl)A and InlB and their cellular receptors E-cadherin and Met, a receptor tyrosine kinase, to trigger cytoskeletal rearrangements and promote bacterial engulfment.[124, 125, 134, 135] E-cadherin is a key component of adherent junctions, which are situated below tight junctions making them inaccessible to *L. monocytogenes* present in the intestinal lumen. However, it was recently shown that E-cadherin around mucus-expelling goblet cells and around enterocytes being extruded from the tip of the villus is accessible to *L. monocytogenes* in the lumen.[136, 137] Once taken up by epithelial cells *L. monocytogenes* escapes from the vacuole using the membrane toxin listeriolysin and phospholipases and moves through the host-cell cytoplasm using continuous actin assembly at one pole of the bacterium. This process is driven by expression of the surface protein ActA at one pole of the cells.[138] From the mucosal sites of infection *L. monocytogenes* can spread to the liver and spleen where it replicates in phagocytes using the virulence mechanisms described above (Fig. 1.7). Binding of *L. monocytogenes* to InlA is specific for human E-cadherin but in mice infection occurs through M-cell mediated uptake into Peyer's patches.[138, 139] To develop mouse models that reflect natural routes of infection a transgenic mouse was constructed that expresses human E-cadherin.[135] Another approach has been to express a mutant form of InlA that recognizes mouse E-cadherin in *L. monocytogenes*.[140]

## Aims and outline of this thesis

The overarching goal of this thesis was to find out more about the protective role of RegIIIβ and RegIIIγ in the intestine. Additionally we investigated whether these proteins

might be candidate biomarkers of intestinal health and have a protective role in colitis and bacterial infection. At the outset of this thesis, murine RegIIIγ has been reported to be bactericidal for Gram-positive bacteria, whereas the role of murine RegIIIβ had not yet been investigated *in vitro*.

Initially, we used Muc2 knock out ($^{-/-}$) mice to investigate the effect of a reduced or absent mucus layer on expression and localization of RegIIIβ, RegIIIγ and Ang4 (Chapter 2) in the small intestine and the colon. The aims were to study the consequence of Muc2 deficiency on inflammation and to analyse the effect of weaning, when the density of the microbiota increases, by *in situ* hybridization, qPCR, histology and immunohistochemistry. In Chapter 3, we investigated this Muc2$^{-/-}$ model in more detail, to gain more insights into the role of mucus in the ileum. The age-dependent changes in morphology and temporal gene expression patterns in the ileum and proximal colon were compared in wild type (WT) and Muc2$^{-/-}$ mice at 3 different time points by micro-array analysis, qPCR, and histology.

In chapter 4 we expressed recombinant RegIII proteins and characterized their potential bactericidal properties *in vitro*. We also investigated binding of RegIII proteins to different species of bacteria and the influence of proteolytic processing by trypsin on their activity. Additionally, we sought to generate structural models of RegIII proteins binding to reported ligands in order to gain further insights into potential ligand binding sites and the amino acid residues conferring ligand specificity. It was difficult to produce large quantities of RegIII proteins in soluble form without resorting to refolding of insoluble inclusion bodies produced in *E. coli,* and these proteins lacked bactericidal activity. Therefore, we performed infection studies in knockout mice to further elucidate the role of these proteins in infection (Chapters 5 and 6).

An infection study was performed in RegIIIβ$^{-/-}$ mice in Chapter 5 to learn more about the protective mechanism of RegIIIβ in the intestine and whether this is specific for Gram-negative pathogens. Mice were orally infected with either a Gram-positive pathogen (*Listeria monocytogenes*) or a Gram-negative pathogen (*Salmonella enteritidis*), after which the translocation of the pathogens to different organs was examined. Additional *ex-vivo* experiments were performed to gain insights into the mechanisms involved in the protective function of RegIIIβ. In Chapter 6 we performed a comparable infection study in a newly constructed RegIIIγ$^{-/-}$ mouse model. The aim of this study was to determine the *in vivo* contribution of RegIIIγ to protection of the mouse mucosa from infection with the same pathogens as used in Chapter 5, *Listeria monocytogenes* and *Salmonella enteritidis*. We first generated and characterized a RegIIIγ$^{-/-}$ mouse, including histological and immuno-histochemical investigations of the mucus and spatial compartmentalization of bacteria in the intestine. Additionally, we investigated the effects of RegIIIγ deficiency in infection using microarray gene expression, qPCR and measurements of bacterial translocation and serum biomarkers.

Finally, Chapter 7 completes this thesis with a general discussion and conclusions on the obtained results, with recommendations and perspectives for future studies.

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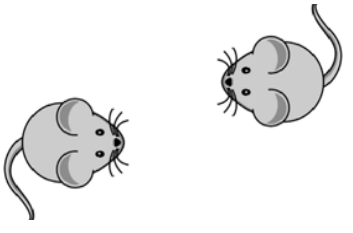

# Chapter 2

# Mucin Muc2 deficiency and weaning influences the expression of the innate defense genes RegIIIβ, RegIIIγ and Angiogenin-4

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**ABSTRACT**

**Background**: Mucin Muc2 is the structural component of the intestinal mucus layer. Absence of Muc2 leads to loss of this layer allowing direct bacterial-epithelial interactions. We hypothesized that absence of the mucus layer leads to increased expression of innate defense peptides. Specifically, we aimed to study the consequence of Muc2 deficiency ($Muc2^{-/-}$) on the expression of regenerating islet-derived protein III beta (RegIIIβ), regenerating islet-derived protein III gamma (RegIIIγ), and angiogenin-4 (Ang4) in the intestine shortly before and after weaning.

**Methods**: Intestinal tissues of $Muc2^{-/-}$ and wild-type (WT) mice were collected at postnatal day 14 (P14, i.e. pre-weaning) and P28 (i.e. post-weaning). RegIIIβ, RegIIIγ, and Ang4 expression was studied by quantitative real-time PCR, Western-blot, *in situ* hybridization, and immunohistochemistry.

**Results**: RegIIIβ and RegIIIγ were expressed by diverging epithelial cell types; namely enterocytes, Paneth cells, and goblet cells. Additionally, Ang4 expression was confined to Paneth cells and goblet cells. Expression of *RegIIIβ*, *RegIIIγ*, and *Ang4* differed between WT and $Muc2^{-/-}$ mice before and after weaning. Interestingly, absence of Muc2 strongly increased *RegIIIβ* and *RegIIIγ* expression in the small intestine and colon. Finally, morphological signs of colitis were only observed in the distal colon of $Muc2^{-/-}$ mice at P28, where and when expression levels of *RegIIIβ*, *RegIIIγ*, and *Ang4* were the lowest.

**Conclusions**: Expression of RegIII proteins and Ang4 by goblet cells point to an important role for goblet cells in innate defense. Absence of Muc2 results in up-regulation of *RegIIIβ* and *RegIIIγ* expression, suggesting altered bacterial-epithelial signaling and an innate defense response in $Muc2^{-/-}$ mice. The inverse correlation between colitis development and *RegIIIβ*, *RegIIIγ*, and *Ang4* expression levels might point toward a role for these innate defense peptides in regulating intestinal inflammation.

## INTRODUCTION

The mucus layer that covers the intestinal epithelium forms a physical barrier against bacteria and is thereby an important component of the innate defense. The mucin MUC2 is the structural component of the colonic mucus layer. Interestingly, particular bacteria can use the glycan-chains of mucins as a nutrient source or bind to the mucins as a foothold for colonization. Previously, it has been demonstrated that the intestinal mucus layer exists of two separate layers.[1, 2] The inner layer is densely packed, firmly attached to the epithelium, and devoid of bacteria. In contrast, the outer layer is colonized by bacteria, and has a less firm structure. Breaches in this protective mucus layer allow for direct contact between bacteria and the epithelial cells,[3] which leads to an inflammatory response. Muc2$^{-/-}$ mice lacking the mucus layer develop colitis spontaneously.[1, 4]

In human intestinal inflammatory diseases such as ulcerative colitis and necrotizing enterocolitis (NEC), the synthesis of MUC2 mucin is decreased,[5-9] which might lead to increased bacterial-epithelial interaction. Bacteria are known to play a key role in the development of colitis as the development of colitis in genetically engineered rodent models of inflammatory bowel diseases (IBD) such as Il-10 deficient mice and HLA-B27 transgenic rats is not observed when these animals are maintained under germ-free conditions.[10-12] However, colonization of Il-10 deficient mice and HLA-B27 transgenic rats with normal enteric microbiota leads to severe and chronic colitis. IBD and NEC are not caused by specific intestinal bacterial species, but altered microbial profiles might be involved in the pathogenesis of these diseases. In the pathophysiology of IBD and NEC, a dysbiosis of the microbiota is strongly implicated.[13, 14]

The composition of the microbiota is shaped in part by specific epithelial proteins, *e.g.* defensins and antimicrobial C-type lectins.[15] Some antimicrobial proteins, such as most α-defensins, are expressed constitutively and do not require bacterial signals for their expression.[16] However, expression of a subset of bactericidal proteins is, at least partly, controlled by recognition of microbe associated molecular patterns by pattern recognition receptors expressed by the epithelial cells.[17] For example, expression of the antimicrobial C-type lectin regenerating islet-derived protein III gamma (RegIIIγ, also called HIP/PAP in humans), is up-regulated in the small intestine and colon after bacterial reconstitution of germ-free mice.[18, 19] It has recently been demonstrated that RegIIIγ restricts bacterial colonization of the intestinal epithelial surface and consequently limits activation of adaptive immune responses by the microbiota.[20] In this respect, the mucus layer is of great importance as it functions as a mesh that retains bactericidal proteins such as RegIIIγ, and also molecules with immunologic properties such as sIgA.[21] Interestingly, RegIIIγ$^{-/-}$ mice exhibited a marked increase in numbers of mucosa associated Gram-positive bacteria.[20] Expression of the bactericidal peptide angiogenin-4 (Ang4), the orthologue of human ANG, is induced upon colonization with *Bacteroides thetaio-*

*taomicron*, an anaerobe Gram-negative microbe that belongs to the normal mouse and human microbiota.[22] Furthermore, in conventionally raised mice the expression of RegIIIγ and Ang4 increases substantially after weaning,[19, 22] when the complexity of the microbiota increases, and during experimental intestinal infection.[23-25] Regenerating islet-derived protein III beta (RegIIIβ), RegIIIγ and HIP/PAP appear to be important in inflammatory diseases and intestinal injury as their expression is increased in IBD patients and in dextran sulfate sodium models of mouse colitis.[18] Finally, RegIIIβ$^{-/-}$ mice show impaired clearance of Gram-negative bacteria *Yersinia pseudotuberculosis*[26] and *Salmonella enteritidis*.[27] Yet, both unchallenged RegIIIβ$^{-/-}$ and RegIIIγ$^{-/-}$ mice do not show gross morphological changes in the intestines.

The aim of this study was to investigate the effect of the mucus layer on RegIIIβ, RegIIIγ, and Ang4 expression and localization in the small intestine and colon using the Muc2$^{-/-}$ mouse as a model. With this approach we aimed to i) study the consequence of Muc2 deficiency, i.e. absence of a protective mucus layer and, ii) analyze the effect of weaning (i.e., transfer from breast milk to pelleted food), when the density and complexity of the microbiota increases significantly. This study demonstrates that the expression of the innate defense genes *RegIIIβ*, *RegIIIγ*, and *Ang4* differed between wild-type (WT) and Muc2$^{-/-}$ mice before and after weaning. Additionally, it highlights a new role for goblet cells in host innate immunity by demonstrating that they can produce the bactericidal peptides RegIIIβ, RegIIIγ, and Ang4.

## METHODS

### Animals

Muc2$^{-/-}$ mice were bred as previously described.[4] All mice were housed in the same specific pathogen-free environment with free access to standard rodent pellets (Special Diets Services, Witham, Essex, England) and acidified tap water in a 12-hour light/dark cycle. All animal experiments were reviewed by and performed with approval of the Erasmus MC Animal Ethics Committee (approval number: EUR 1074), Rotterdam, the Netherlands. WT and Muc2$^{-/-}$ mice were tested negative for *Helicobacter hepaticus* and norovirus infection.

### Experimental setup

Wild-type (WT) and Muc2$^{-/-}$ littermates were housed together with their birth mothers until weaning at the age of 21 days. After weaning, male WT and Muc2$^{-/-}$ mice remained housed with their littermates. Male WT and Muc2$^{-/-}$ mice were sacrificed at the postnatal ages of 14 days (P14) and 28 days (P28). Intestinal tissues were excised and either fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), stored in RNAlater® (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) at -20°C, or frozen in liquid nitrogen and stored at -80°C.

**Quantitative real-time PCR**

Total RNA was prepared using the RNeasy midi-kit (Qiagen, Venlo, the Netherlands). Total RNA (1.5 µg) was used to prepare cDNA using a standard protocol. The mRNA expression levels of *RegIIIß*, *RegIIIγ*, *Ang4* and Lysozyme type P (*lysozyme-P*), as well as the 'housekeeping' gene β-Actin (*Actb*) were quantified using real-time PCR analysis based upon the intercalation of SYBR® Green on an ABI prism 7900 HT Fast Real Time PCR system (PE Applied Biosystems) as previously described.[4] All primer combinations were designed using OLIGO 6.22 software (Molecular Biology Insights) and purchased from Invitrogen. An overview of all primer sequences is given in Table 2.1.

| gene | Forward primer | Reverse primer |
|---|---|---|
| ***RegIIIß*** | TGG GAA TGG AGT AAC AAT G | GGC AAC TTC ACC TCA CAT |
| ***RegIIIγ*** | CCA TCT TCA CGT AGC AGC | CAA GAT GTC CTG AGG GC |
| ***Ang4*** | TTG GCT TGG CAT CAT AGT | CCA GCT TTG GAA TCA CTG |
| ***Lysozyme-P*** | CAG GGT GGT GAG AGA TCC | AAG CGA GGA AGT GTG ACC |
| ***Actb*** | GGG ACC TGA CGG ACT AC | TGC CAC AGG ATT CCA TAC |

**Table 2.1:** Primer sequences for quantitative real-time PCR

**Western-blot analysis**

Jejunal and distal colonic samples were homogenized in 500 µl HIS buffer (50 mM Tris/HCl pH 7.5, 5 mM EDTA pH 8.0, 1% Triton X-100, 10 mM iodacetamide, 100 µg/ml soy bean trypsin inhibitor, 10 µg/ml pepstatine A, 10 µg/ml leupeptin, 1% (w/v) aprotinin and 1 mM PMSF). Total protein concentration was quantified using the bicinchoninic acid assay (Pierce assay, Perbio Science, Etten-Leur, the Netherlands). Twenty µg of total protein was denatured at 95°C for 5 min in Laemmli loading buffer and subjected to 12% (w/v) SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membranes (Protan BA 83, 0.2 µm) and the blots were blocked for 1 h at room temperature in 5% (w/v) non-fat dry milk (Campina Melkunie, Eindhoven, the Netherlands) dissolved in phosphate-buffered saline containing 0.1% (v/v) Tween-20 (PBST). Blots were incubated overnight at 4°C with anti-β-Actin antibody (1:10,000 in PBST, Abcam, ab6276) or with the custom made primary antibodies against RegIIIβ and RegIIIγ (1:20,000 in PBST, Eurogentec, Seraing, Belgium). These antibodies were generated in rabbits against the synthetically produced peptides, using the peptide sequences GEDSLKNIPSARISC (RegIIIβ) and MIKSSGNSGQYVC (RegIIIγ). The chosen peptide sequences correspond to unique sequences within the respective RegIII proteins, and allow for differentiation between the RegIIIβ and RegIIIγ proteins. Serum from immunized rabbits was affinity purified using the respective peptides. Selectivity and cross reactivity of the generated antibodies for the RegIII proteins were checked by ELISA. Finally, blots were incubated with the secondary antibody goat-anti-rabbit IRDye® 800CW (1:20,000, Li-cor, Westburg, Leusden, the Netherlands) for RegIIIβ and RegIIIγ, and goat-anti-mouse IRDye®680CW (1:20,000, Li-cor) for β-Actin. Signals were detected with the Odyssey scanner (Li-cor). Serial dilution series of the protein samples

were analyzed to ensure that the quantification of each protein by its cognate antibody was performed in the linear range of this technique. Expression of each protein is expressed relative to the expression of β-Actin.

**Histology**
Tissue fixed in 4% (w/v) paraformaldehyde in PBS was prepared for light microscopy, and 4-μm-thick sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff's (PAS) staining to study morphological changes and detect goblet cells, respectively. To detect differences in mucosal thickness in the colon, 10 well-oriented crypts were chosen per intestinal segment and measured using calibrated Leica Application Suite software, version 3.2.0 (Leica Microsystems BV, Rijswijk, the Netherlands).

**Immunohistochemistry**
Four-micrometer-thick sections were prepared for immunohistochemistry as described previously[28] using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and the staining reagent 3,3'-diaminobenzidine. The antigens were unmasked by heating the sections for 20 min in 0.01 M Tris/HCl (pH 9.0) supplemented with 0.05% (v/v) EGTA at 100°C. Expression of RegIIIβ was detected using a commercial anti-mouse RegIIIβ antibody (1:500 diluted in PBS containing 1% bovine serum albumin and 0.1% Triton X-100**,** R&D Systems Europe Ltd., Abingdon, United Kingdom, AF5110) and with the above described custom-made antibody against RegIIIβ (1:25,000 in PBS). RegIIIγ was detected using the above described custom made antibody against RegIIIγ (1:25,000 in PBS). Expression of angiogenin-4 was detected using an anti-human-angiogenin antibody (1:50 diluted in PBS containing 1% bovine serum albumin and 0.1% Triton X-100, R&D Systems Europe Ltd., Abingdon, United Kingdom, AF265). To identify goblet cells HA1 antibody, which is specific for Muc4 was used as described previously.[29] As the commercial and the custom-made RegIIIβ antibody gave similar staining patterns only data obtained with the commercial RegIIIβ antibody are shown.

**Probe preparation**
Digoxigenin-11-UTP-labelled RNA probes were prepared according to the manufacturer's instructions (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) using T3 and T7 RNA polymerase. Gene fragments of *Ang4*, *RegIIIβ* and *RegIIIγ* were amplified, using the primers listed in Table 2.2, and cloned in pBluescript II SK.

***In situ* hybridization (ISH)**
Non-radioactive ISH was performed according to a previously described method [30]. The digoxigenin-labeled hybrids were detected by incubation with anti-digoxigenin (Fab, 1:1000 in TBS/1% BA + 1% v/v sheep serum, Roche) conjugated to alkaline phosphatase for 2.5 h at room temperature. Thereafter, sections were washed in 0.025% (v/v) Tween in Tris-buffered saline (pH 7.5). For staining, sections were layered with detection buffer (0.1 M Tris/HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5) containing 0.33 mg/ml 4-nitroblue tetrazolium chloride, 0.16 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, 8% (v/v) poly-

vinyl alcohol (*Mw* 31,000–50,000, Aldrich Chemical, Milwaukee, WI, USA), and 1 mM levamisole (Sigma). The color reaction was performed overnight in the dark and was stopped when the desired intensity of the resulting blue precipitate was reached. Finally, sections were washed in 10 mM Tris/HCl pH 9.5 containing 1 mM EDTA, washed in distilled water, and mounted with Aquamount improved (Gurr, Brunschwig, Amsterdam, the Netherlands).



| Gene | Forward primer | Reverse primer | Amplified product (bp) |
|---|---|---|---|
| ***RegIIIβ*** | TGG GAA TGG AGT AAC AAT G | ATG TGA GGT GAA GTT GCC | 146 |
| ***RegIIIγ*** | CAA TCA CTG TGG TAC CCT G | GAT TTT CTC CTT CTC TGG C | 229 |
| ***Ang4*** | CCA GCT TTG GAA TCA CTG T | CTA TGA TGC CAA GCC AA | 151 |

**Table 2.2:** Primer sequences for probe preparation for ISH

**Statistical analysis**

All data are expressed as median. Statistical significance was assessed using the Mann-Whitney U test (Prism, version 5.00; GraphPad software, San Diego, CA). The data were considered statistically significant at $P < 0.05$.

## RESULTS

**Clinical symptoms and intestinal morphology**

At P14, when the mice received breast milk, there were no significant differences in body weights between Muc2$^{-/-}$ and WT mice (Fig. 2.1A). However, at P28, when mice had been transferred from breast milk to solid food, the body weights of Muc2$^{-/-}$ mice were significantly lower than that of WT mice ($P = 0.0108$). Clinical signs of colitis like rectal bleeding, bloody stools or rectal prolapse were not observed in Muc2$^{-/-}$ mice at P14 nor at P28. Morphological signs of colitis were only observed in the distal colon of Muc2$^{-/-}$ mice at P28, but not in the distal colon at P14, neither in the proximal colon and small intestine at P14 or P28 (Fig. S2.1). More specifically, at P28 the distal colonic tissue from Muc2$^{-/-}$ mice showed increased crypt lengths, (Fig. 2.1B and Fig. S2.1K and S2.1L) and flattening of the epithelial cells (Fig. 2.1C).

**Localization of RegIIIβ, RegIIIγ, and Ang4 mRNAs and proteins in the small intestine**

We first determined the expression pattern of the RegIII proteins in the small intestine of WT and Muc2$^{-/-}$ mice by immunohistochemistry. Interestingly, we did find differences in the localization of the RegIII proteins, although there were no major differenc-

es in small intestinal morphology between WT and Muc2$^{-/-}$ mice. Specifically, RegIIIβ and RegIIIγ were not expressed in the duodenum of WT mice at P14 (Fig. 2.2 and 2.3). However, the jejunum and ileum of WT mice clearly express RegIIIβ and RegIIIγ at this time point. At P28 RegIIIγ was still expressed in the jejunum and ileum of WT mice, but the expression was weak. RegIIIβ expression at P28 was also weak but present in each region of the small intestine of WT mice. In sharp contrast, in Muc2$^{-/-}$ mice the RegIII proteins were strongly expressed in the entire small intestine from duodenum till ileum (Fig. 2.2 and 2.3) at P14 as well as P28.

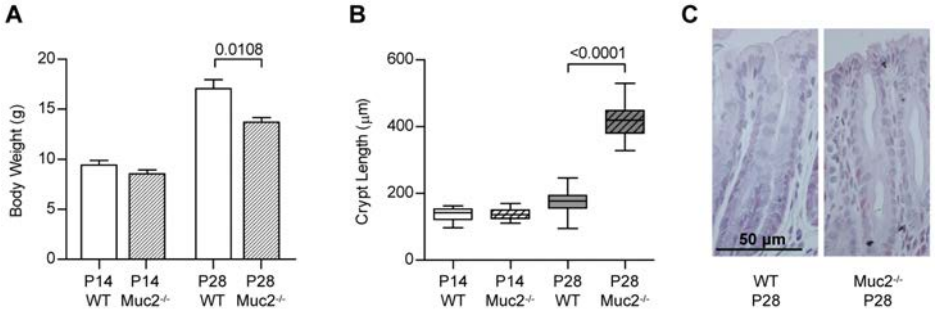


**Figure 2.1.** Clinical symptoms and intestinal morphology. Body weights (A) and crypt lengths in the distal colon (B) of WT and Muc2$^{-/-}$ mice at P14 and P28. Crypth length values are depicted as box-and-whiskers diagrams (maximum value, upper quartile, median, lower quartile and minimal value, respectively) *P* values are indicated when body weights/crypt lengths differ statistically between groups. (C) H&E staining of distal colonic tissue of Muc2$^{-/-}$ mice at P28.

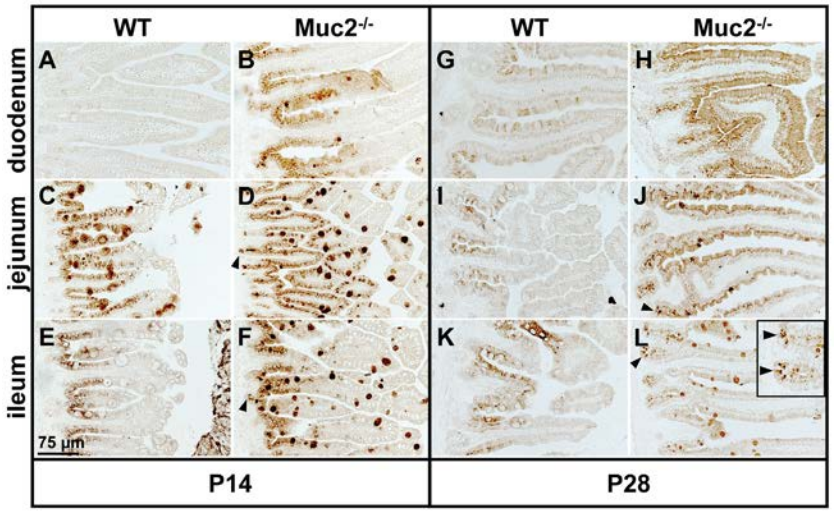


**Figure 2.2.** Localization of RegIIIβ protein in the small intestine. Immunohistochemical staining of RegIIIβ in the duodenum, jejunum, and ileum of WT (A, C, E, G, I, K) and Muc2$^{-/-}$ mice (B, D, F, H, J, L) at P14 (A-F) and P28 (G-L). Bar represents 75 µm. Arrowheads indicate RegIIIβ-positive Paneth cells.

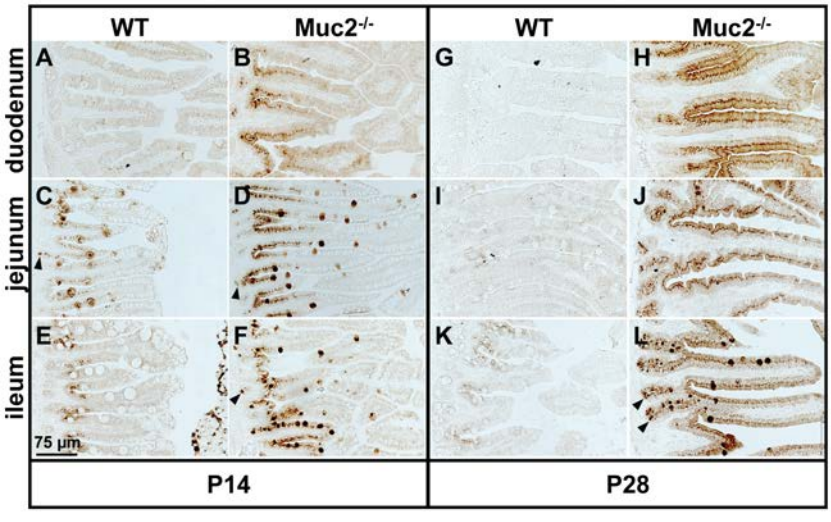


**Figure 2.3.** Localization of RegIIIγ protein in the small intestine. Immunohistochemical staining of RegIIIγ in the duodenum, jejunum, and ileum of WT (A, C, E, G, I, K) and Muc2$^{-/-}$ mice (B, D, F, H, J, L) at P14 (A-F) and P28 (G-L). Bar represents 75 µm. Arrowheads indicate RegIIIγ positive Paneth cells.

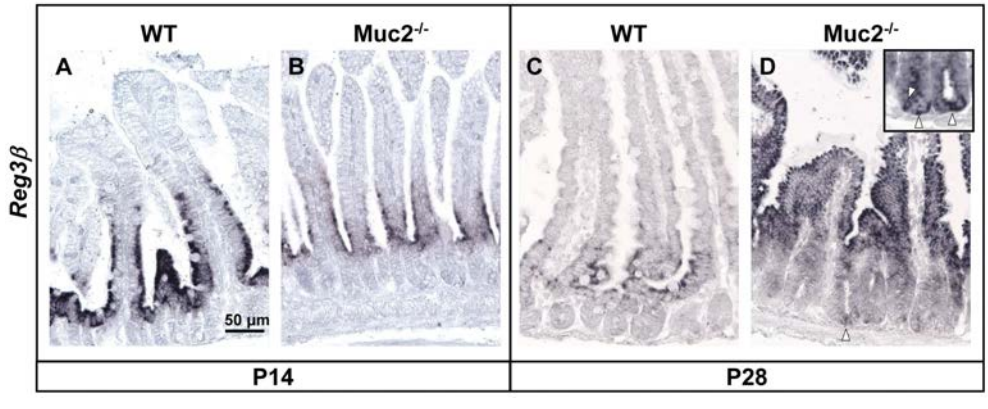


**Figure 2.4.** Localization of *RegIIIβ* mRNA in the jejunum. ISH for RegIIIβ mRNA in the jejunum of WT and Muc2$^{-/-}$ mice at P14 (A & B) and P28 (C & D) (arrowheads in insert in panel D indicate RegIIIβ mRNA-positive cells at the bottom of the crypts in Muc2$^{-/-}$ at P28). Bar represents 50 µm.

To determine the expression pattern of RegIII mRNAs and proteins in more detail we focused on the jejunum. In both, WT mice and Muc2$^{-/-}$ mice, *RegIIIβ* mRNA was detected by ISH and observed in epithelial cells at the base of the villi at P14 (Fig. 2.4A and B). In contrast, at P28 the expression pattern of *RegIIIβ* mRNA remained limited to the

villus base in WT mice, but had extended to the upper part of the villi in Muc2$^{-/-}$ mice (Fig. 2.4D). Interestingly, in Muc2$^{-/-}$ mice *RegIIIβ* mRNA was also observed in epithelial cells at the base of the crypts (Fig. 2.4D). *RegIIIγ* mRNA was observed in epithelial cells at the crypt bottom till the tips of the villi in WT and Muc2$^{-/-}$ mice at P14 and P28 (data not shown).

Immunohistochemical analysis revealed that in WT as well as Muc2$^{-/-}$ mice enterocytes were RegIIIβ- and RegIIIγ-positive (P14 and P28) (Fig. 2.2 and 2.3). Moreover, in WT mice at P14 and in Muc2$^{-/-}$ mice at P14 and P28, RegIIIβ- and RegIIIγ-positive cells had a 'bell/goblet' shape suggesting that these cells could be goblet cells. Staining of serial section of the jejunum with the goblet cell marker Muc4[29] or PAS demonstrated that the bell/goblet-shaped RegIIIβ- and RegIIIγ-positive cells in WT and Muc2$^{-/-}$ mice were indeed goblet cells (Fig. S2.2). Furthermore, in WT mice at P14 as well as in Muc2$^{-/-}$ mice at P14 and P28 Paneth cells at the bottom of the crypts were RegIIIγ-positive (Fig. 2.3). Yet, RegIIIβ-positive Paneth cells at the bottom of the crypts were only observed in Muc2$^{-/-}$ mice, but not in WT mice (Fig. 2.2).

Finally, small intestinal *Ang4* mRNA was localized in Paneth cells of both WT and Muc2$^{-/-}$ mice at both ages studied (Fig. S2.3, showing *Ang4* mRNA in Muc2$^{-/-}$ mice at P14). Yet, at P14, *Ang4* mRNA was not only observed in the Paneth cells but also at the apical side of the villus enterocytes in both types of mice. Ang4 protein expression patterns were similar to the *Ang4* mRNA expression patterns with expression localized to Paneth cells at P14 and P28, and to enterocytes along the villi at P14 in both WT and Muc2$^{-/-}$ mice (Fig. 2.5). Moreover, the abundance of Ang4-positive Paneth cells increased from P14 to P28 in both types of mice.

**Localization of RegIIIβ, RegIIIγ, and Ang4 proteins in the colon**

We also determined the expression pattern of the RegIII and Ang4 proteins in the colon of WT and Muc2$^{-/-}$ mice by immunohistochemistry. Both RegIIIβ and RegIIIγ were undetectable in the distal colon of WT and Muc2$^{-/-}$ mice at P14 or P28 (data not shown). However, in the proximal colon of WT and Muc2$^{-/-}$ mice these RegIII proteins were expressed within the epithelial cells in the crypt epithelium as well as the surface epithelium at both time points investigated (Fig. 2.6). Interestingly, in the proximal colon of WT mice RegIIIβ and RegIIIγ were only expressed by enterocytes, whereas in Muc2$^{-/-}$ mice these proteins were expressed by enterocytes as well as putatively by goblet cells, as based on the 'goblet' shape of the RegIIIβ- and RegIIIγ -positive cells in the Muc2$^{-/-}$ mice. Analysis of serial sections stained with the goblet cell marker Muc4 or with PAS revealed that these RegIIIβ- and RegIIIγ -expressing cells with a goblet shape were indeed goblet cells (data not shown). The abundance of cells with the typical bell/goblet shape seemed to decrease progressively from P14 to P28 in the proximal colon of the Muc2$^{-/-}$ mice.

Ang4 was hardly detectable in the distal colon of WT and Muc2$^{-/-}$ mice (data not shown).

However, in the proximal colon Ang4 was clearly expressed within the crypts and surface epithelium of both WT and Muc2$^{-/-}$ mice at P14 and only in WT mice at P28, where it was expressed by goblet cells (Fig. 2.7B and 2.7C). Detection of *Ang4* mRNA by ISH demonstrated that Ang4 was indeed expressed by goblet cells in the proximal colon of WT mice (Fig. 2.7A).

**Quantitative analysis of *RegIIIβ*, *RegIIIγ*, *Ang4*, and lysozyme-P mRNA levels**

We next analyzed the mRNA expression levels of RegIIIβ, RegIIIγ, Ang4, and lysozyme-P in the intestinal tissues of WT and Muc2$^{-/-}$ mice. In the jejunum *RegIIIβ* and *RegIIIγ* mRNA levels were comparable between WT and Muc2$^{-/-}$ mice at P14 (Fig. 2.8A and 2.8B). In contrast, at P28 *RegIIIβ,* and *RegIIIγ* mRNA levels significantly increased in the Muc2$^{-/-}$ mice, whereas these remained stable in the WT mice. The mRNA levels of *Ang4* and *lysozyme-P*, genes which in the small intestine are synthesized by Paneth cells, were comparable between the jejunum of WT and Muc2$^{-/-}$ mice at P14 as well as P28 (Fig. 2.8C and 2.8D). Remarkably, from P14 to P28 mRNA levels of both *Ang4* and *lysozyme-P* significantly increased in the jejunum of both types of mice.

A comparison of *RegIIIβ*, *RegIIIγ* and *Ang4* mRNA levels within colonic tissue of WT mice and Muc2$^{-/-}$ mice revealed six important findings (Fig. 2.9). 1) In WT as well as Muc2$^{-/-}$ mice, higher *RegIIIβ*, *RegIIIγ*, and *Ang4* mRNA levels were found in the proximal colon compared to the distal colon at P14 and P28. 2) During aging from P14 to P28, *RegIIIβ*, *RegIIIγ*, and *Ang4* mRNA levels in the proximal colon increased in WT as well as Muc2$^{-/-}$ mice. 3) Muc2$^{-/-}$ mice had significantly increased *RegIIIβ* and *RegIIIγ* mRNA levels in the distal colon at P14 and P28 compared to WT mice, a similar trend was also observed in the proximal colon. 4) In the distal colon, *RegIIIβ* and *RegIIIγ* mRNA expression levels were hardly detectable in WT mice. 5) *RegIIIβ*, *RegIIIγ*, and *Ang4* mRNA levels in the distal colon of Muc2$^{-/-}$ mice seemed to decrease during aging from P14 to P28. 6) Colonic expression levels of *RegIIIβ*, *RegIIIγ*, and *Ang4* mRNA showed considerable mouse to mouse variation.

**Quantitative analysis of RegIIIβ and RegIIIγ protein levels**

Expression of RegIIIβ and RegIIIγ protein was studied by Western-blot analysis. At P14 protein expression levels were below the detection limit in the jejunum of WT as well as Muc2$^{-/-}$ mice (data not shown). At P28, expression of both RegIIIβ (Fig. S2.4A) and RegIIIγ (Fig. S2.4B) was significantly increased in Muc2$^{-/-}$ mice compared to WT mice, in which RegIIIβ and RegIIIγ protein levels remained undetectable. These data correlate with the jejunal RegIIIβ and RegIIIγ mRNA expression levels as presented in Fig. 2.8A and B. Expression of RegIIIβ and RegIIIγ protein in the distal colon was not detectable by Western-blot (results not shown).

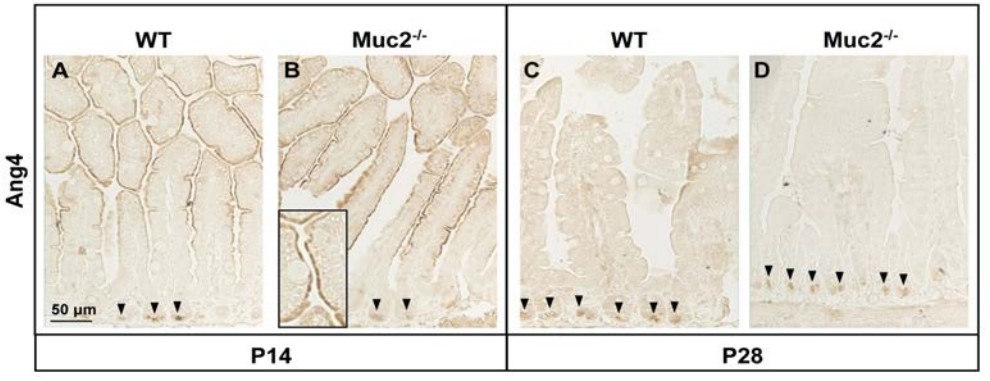


**Figure 2.5.** Localization of Ang4 protein in the jejunum. Localization of Ang4 in the jejunum of WT and Muc2$^{-/-}$ mice at P14 (A & B) and P28 (C & D). Arrowheads indicate Ang4-positive Paneth cells, and the insert in panel B shows enterocyte-specific Ang4 expression at P14. Bar represents 50 µm.

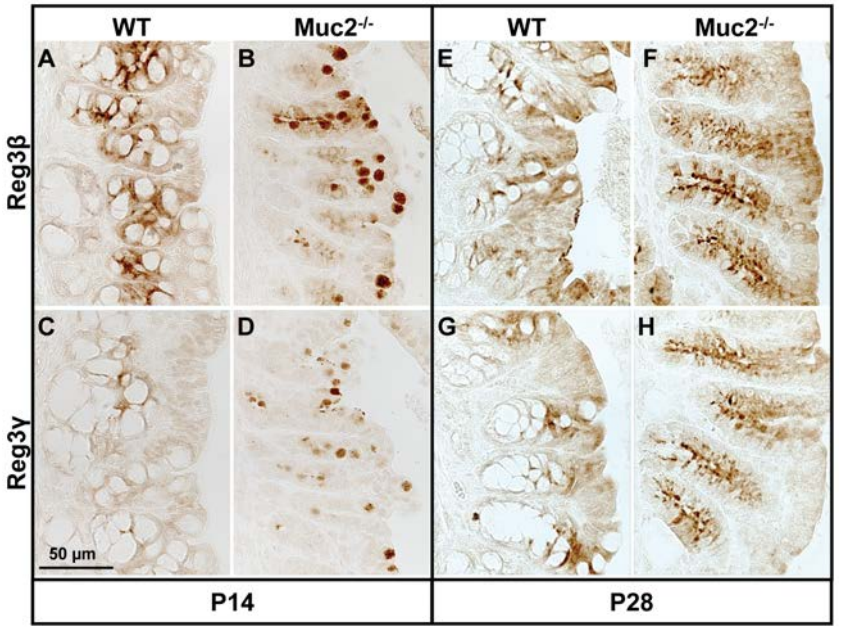


**Figure 2.6.** Localization of RegIIIβ protein in the proximal colon. RegIIIβ (A, B, E, F) and RegIIIγ (C, D, G, H) expression in the proximal colon at P14 (A-D) and P28 (E-H). Note, in WT mice RegIIIβ and RegIIIγ were only expressed by enterocytes, whereas in Muc2$^{-/-}$ mice these proteins were expressed by enterocytes and putative 'goblet' cells. Bar represents 50 µm.

## DISCUSSION

In the present study we examined the expression pattern and localization of RegIIIβ, RegIIIγ and Ang4 in intestinal tissue of WT and Muc2$^{-/-}$ mice at the age of 14 days and post weaning at 28 days. We demonstrated that the expression of these genes, which play a role in innate defense and shaping of the bacterial community in the intestine, differs between WT and Muc2$^{-/-}$ mice before and after weaning. These changes might be related to alterations in the composition of the microbiota during this time frame and in Muc2$^{-/-}$ mice also to the altered interactions between bacteria and the intestinal epithelial cells in the absence of the intestinal mucus layer.

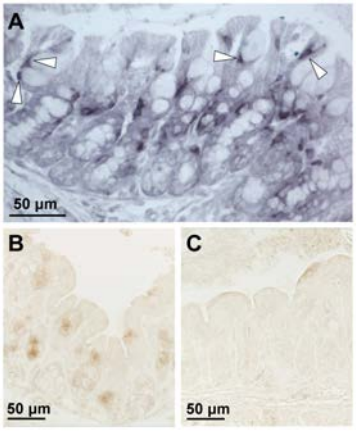


**Figure 2.7.** Goblet cell-specific expression of *Ang4* mRNA and protein in the proximal colon. Localization of *Ang4* mRNA in goblet cells of WT mice by ISH (A) at P28. Localization of Ang4 protein by immunohistochemistry in WT (B) and Muc2$^{-/-}$ (C) mice at P28. Arrows indicate *Ang4* mRNA in goblet cells. Bar represents 50 µm.

Although there were no morphological changes in the small intestine and proximal colon of Muc2$^{-/-}$ mice, these mice did show major differences in the expression pattern and localization of the C-type lectins RegIIIβ and RegIIIγ compared to WT mice. Specifically, in WT mice the RegIII proteins were only clearly expressed at P14 in the jejunum and ileum. Moreover, expression of the RegIII proteins was weak or below detection level in this type of mice at P28. In contrast, in Muc2$^{-/-}$ mice these RegIII proteins were strongly expressed in the entire small intestine from duodenum till ileum at P14 as well as P28. The differences in RegIII protein expression pattern within WT mice between P14 (i.e., before weaning) and P28 (i.e., after weaning) suggests that bacterial colonization, which is known to change during the weaning period, is highly likely to influence/

regulate RegIIIβ and RegIIIγ expression, which is supported by the findings of other authors.[18, 19] Along the same line, differences in RegIII protein expression between WT mice and Muc2$^{-/-}$ mice might point to a difference in the composition of the microbiota between these mice and thus that mucins, in particular mucin Muc2, also influence the composition of the microbiota.

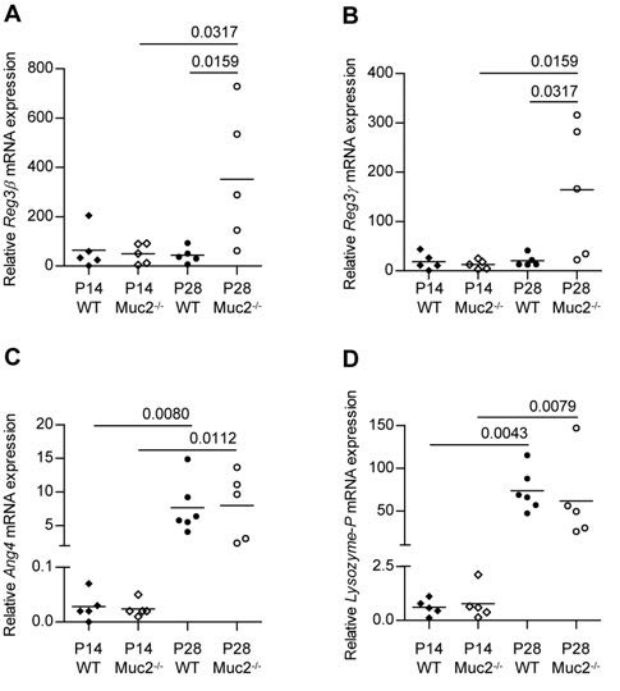


**Figure 2.8.** *RegIIIβ, RegIIIγ, Ang4* and *lysozyme-P* mRNA levels in the jejunum. Small intestinal expression of *RegIIIβ* (A), *RegIIIγ* (B), *Ang4* (C) and *lysozyme-P* (D) mRNA expression. The relative mRNA expression levels were normalized to *Actb* and expressed as median. *P* values are indicated when expression levels between groups differ statistically. Groups are depicted as: WT, P14 ♦; Muc2$^{-/-}$, P14 ◊; WT, P28 ●; and Muc2$^{-/-}$, P28 ○.

It was previously shown that RegIIIγ and Ang4 are expressed in small intestine and localized in Paneth cells[19, 22] and *RegIIIβ* mRNA was shown in colonic goblet cells and columnar cells.[18] Our immunohistochemical analysis revealed that in the small intestine of WT mice and Muc2$^{-/-}$ mice RegIIIγ and Ang4 are indeed expressed by Paneth cells. Yet, in Muc2$^{-/-}$ mice Paneth cells also express RegIIIβ. These data suggest that in the absence of Muc2, and thus in the absence of a mucus layer, Paneth cells increase their innate defense capacity by expressing RegIIIβ. Even more importantly, we also demonstrated that

during initial colonization RegIIIβ and RegIIIγ are not exclusively expressed by Paneth cells, but also by enterocytes and goblet cells in the small intestine and proximal colon of WT and Muc2$^{-/-}$ mice. Additionally, goblet cells in the proximal colon also appeared to synthesize Ang4. Besides secreting Relmβ, which is suggested to have an immune effector function,[31-33] goblet cells were until now not known to play a role in innate defense responses via the secretion of bactericidal proteins. Our demonstration that RegIIIβ, RegIIIγ, and Ang4 are expressed by goblet cells highlights a new and important role for goblet cells in innate defense and in helping to shape the bacterial community. Overall, the spatial RegIIIβ and RegIIIγ expression is remarkable in the sense that these proteins are expressed in at least 3 different epithelial cell lineages within the intestine, namely goblet cells, enterocytes, and Paneth cells.



Focusing on RegIII expression levels, we demonstrate that small intestinal expression of *RegIIIβ* and *RegIIIγ* mRNAs and proteins were increased in Muc2$^{-/-}$ mice compared to WT mice at P28 just after weaning. These data imply that loss of a protective mucus layer as in Muc2$^{-/-}$ mice leads to an increased innate defense response, probably as a result of increased epithelial-bacterial interactions and altered bacterial colonization as weaning is known to alter the composition of the microbiota. Small intestinal expression levels of *Ang4* mRNA were also increased after weaning in WT as well as Muc2$^{-/-}$ mice. We additionally showed that expression levels of Ang4 in the small intestine resemble lysozyme-P levels over time. As it is known that Paneth cell development occurs after birth in mice, with a complete constitution of the Paneth-cell lineage from the age of 3 to 4 weeks,[34] the increased expression of *lysozyme-P* and *Ang4* mRNAs at P28 compared to P14 is most likely due to increased Paneth cell numbers during development from P14 to P28.

Expression levels of *RegIIIβ*, *RegIIIγ*, and *Ang4* mRNA in the proximal colon were considerably higher compared to expression levels in the distal colon of WT and Muc2$^{-/-}$ mice at both time points investigated. An explanation for this could be that an intrinsic program encoded in the epithelial cells controls the segmental expression of the studied innate defense molecules. On the other hand, altered expression of the *RegIII* and *Ang4* genes might be related to changes in the composition of the microbiota as demonstrated for *RegIIIγ* in a simplified model where germ-free mice are sequentially colonized with *Bacteroides thetaiotaomicron* and then *Bifidobacterium longum.*[35] Specifically, colonization of germ-free mice with *Bacteroides thetaiotaomicron* induced *RegIIIγ* expression, but this was lowered by the subsequent introduction of *Bifidobacterium longum.* Thus the observed differences in *RegIIIβ*, *RegIIIγ*, and *Ang4* gene expression levels between the proximal and distal colon might be related to differences in the composition of the microbiota in these parts of the intestine. Indeed, it has been shown that the composition of mucosa-associated bacterial species may differ up to 4% between the right colon (i.e. proximal colon) and left colon (i.e. distal colon).[36, 37]

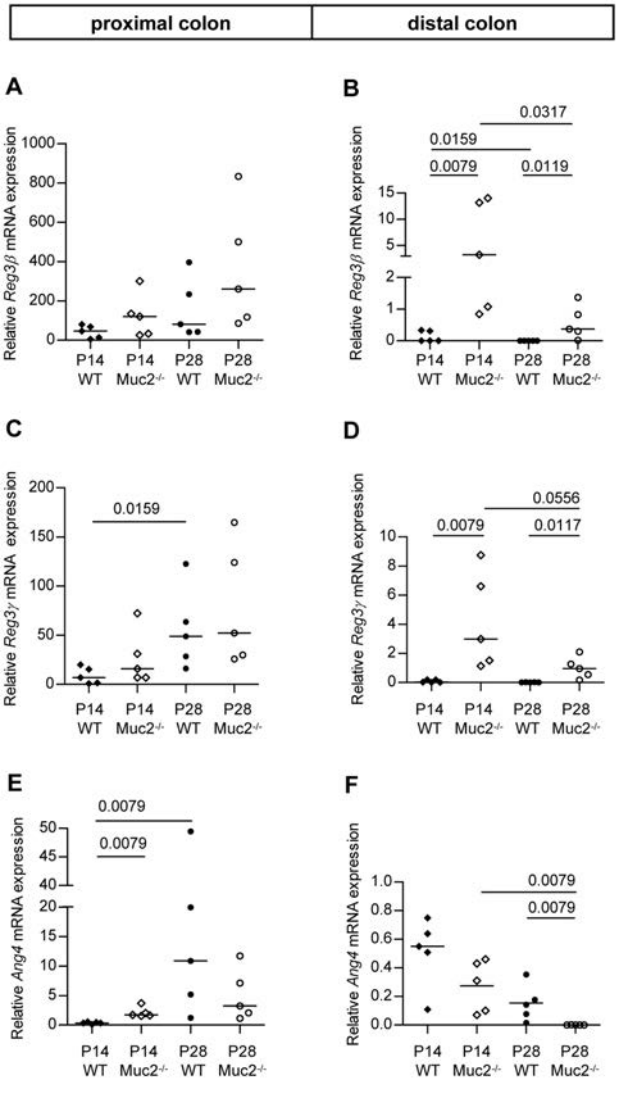


**Figure 2.9.** *RegIIIβ, RegIIIγ* and *Ang4* mRNA levels in the colon. Expression of *RegIIIβ* (A,B), *RegIIIγ* (C,D), *Ang4* (E,F) mRNA in colonic tissue of WT and Muc2$^{-/-}$. Proximal and distal colonic expression levels are shown in the left and right panels, respectively. The relative mRNA expression levels were normalized to *Actb* and expressed as median. *P* values are indicated when expression levels between groups differ statistically. Groups are depicted as: WT, P14 ♦; Muc2$^{-/-}$, P14 ◊; WT, P28 ●; and Muc2$^{-/-}$, P28 ○.

Regardless of the mechanisms of *RegIII* gene and *Ang4* gene regulation, there seems to be an inverse correlation between *RegIIIβ*, *RegIIIγ*, and *Ang4* gene expression levels and the location of colitis in Muc2$^{-/-}$ mice. Namely, morphological signs of colitis are only observed in the distal colon of Muc2$^{-/-}$ mice at P28, where and when the expression levels of RegIIIβ, RegIIIγ, and Ang4 were the lowest, but not in the proximal colon, where RegIIIβ, RegIIIγ, and Ang4 levels were the highest. These findings could imply that RegIII proteins and/or Ang4 regulate intestinal inflammation directly or indirectly. Interestingly, studies from Folch-Puy *et al.* indicate that PAP-I (also known as HIP, p23, or Reg2 protein) directly inhibits the inflammatory response by blocking NF-κB activation through a STAT3-dependent mechanism.[38] When RegIII proteins and/or Ang4 indeed limit intestinal inflammation one might even speculate that the distal colon is more prone to develop colitis than the proximal colon because *RegIIIβ*, *RegIIIγ*, and *Ang4* expression levels are lower in the distal colon than in the proximal colon. However, the inflammatory modulating capacities of the RegIII and Ang4 proteins still remain to be proven.

Expression levels of *RegIIIβ* and *RegIIIγ* mRNAs were consistently higher in Muc2$^{-/-}$ mice compared to WT mice in both the proximal and distal colon. This is most likely due to increased commensal bacterial-epithelial interactions in Muc2$^{-/-}$ mice compared to WT mice, which has been demonstrated before.[1] Given that the glycans on mucins are a nutrient source for bacteria,[39] loss of Muc2 is likely to influence the composition of the microbiota. Differences in the composition of the colonic microbiota between WT and Muc2$^{-/-}$ mice might in their turn also influence RegIII and Ang4 protein expression.

After weaning, expression levels of *RegIIIβ*, *RegIIIγ*, and *Ang4* mRNAs in the proximal colon were increased in WT and Muc2$^{-/-}$ mice. Cash et al. showed that *RegIIIγ* mRNA expression increased during the weaning period in the small intestine of conventionally raised mice, but not in germ-free mice.[19] The same accounts for Ang4 expression in the small intestine.[22] It is known that the density and complexity of the microbiota increases significantly after weaning. This can be explained by the food source itself, serving as a substrate for specific bacteria, but also by the loss of protective factors that are present in mother's milk but not in plant-based chow *e.g.* sCD14, sTLR2, TGFβ, IL-10, and lactoferrin.[40-43] Anyway, increased expression of RegIIIβ and RegIIIγ at P28 in WT as well as Muc2$^{-/-}$ mice might be regarded as an innate response to alterations in the number and composition of the microbiota that are related to the weaning process.

In summary, this study demonstrates that RegIIIβ and RegIIIγ can be expressed in diverging cell lineages, namely enterocytes, Paneth cells, and goblet cells. This study also highlights a new role for goblet cells in host innate immunity by demonstrating that they can produce the bactericidal peptides RegIIIβ, RegIIIγ, and Ang4. Additionally, absence of Muc2 resulted in strong up-regulation of *RegIIIβ* and *RegIIIγ* mRNAs in the small intestine and colon, suggesting altered bacterial-epithelial signaling in Muc2$^{-/-}$ mice, lead-

ing to increased innate defense capacity. Alterations in *RegIII* and *Ang4* gene expression were related to weaning from mother's milk, which is known to alter the composition of the microbiota. Therefore an important role for bacteria in regulation of *RegIII* and *Ang4* gene expression is suggested. Furthermore, morphological signs of colitis were observed in the distal colon of $Muc2^{-/-}$ mice, where expression levels of *RegIIIβ*, *RegIIIγ*, and *Ang4* mRNAs were the lowest, but not in the proximal colon where expression levels of these genes were the highest. These findings might point toward a role for RegIII proteins and/or Ang4 in regulating intestinal inflammation.

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## SUPPORTING INFORMATION

Colour figures can be found online or in the pdf version.

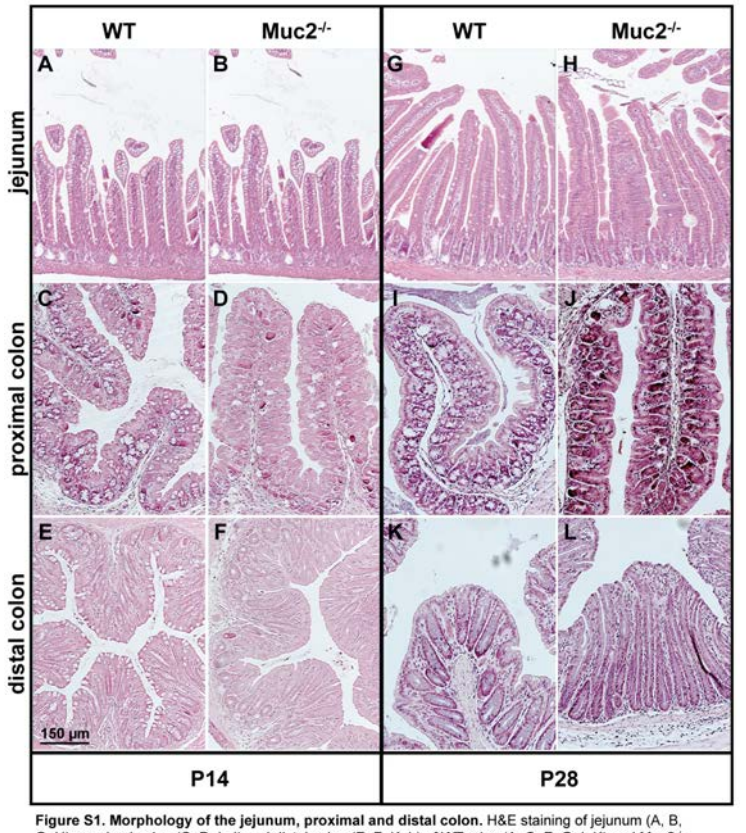



**Figure S1. Morphology of the jejunum, proximal and distal colon.** H&E staining of jejunum (A, B, G, H), proximal colon (C, D, I, J) and distal colon (E, F, K, L) of WT mice (A, C, E, G, I, K) and Muc2$^{-/-}$ mice (B, D, F, H, J, L) at P14 (A-F) and P28 (G-L). Bar represents 150 µm.

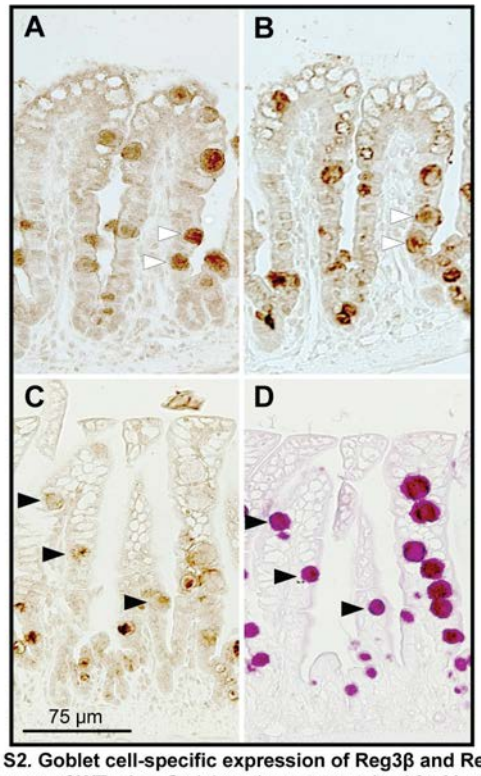



**Figure S2. Goblet cell-specific expression of Reg3β and Reg3γ in the jejunum of WT mice.** Serial sections were stained for Muc4 (A) Reg3β (B), Reg3γ (C) and periodic acid-Schiff's (PAS) (D). Co-localization of Reg3β and Muc4 is shown by white arrowheads and co-localization of Reg3γ and PAS staining is shown by black arrowheads. Bar represents 75 µm.

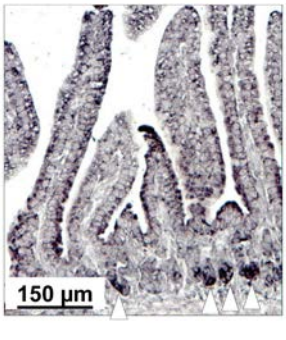



**Figure S3. *Ang4* mRNA expression by jejunal enterocytes and Paneth cells.** Localization of *Ang4* mRNA at P14 in small intestine of Muc2$^{-/-}$ mice. Arrowheads indicate *Ang4* mRNA-positive Paneth cells.

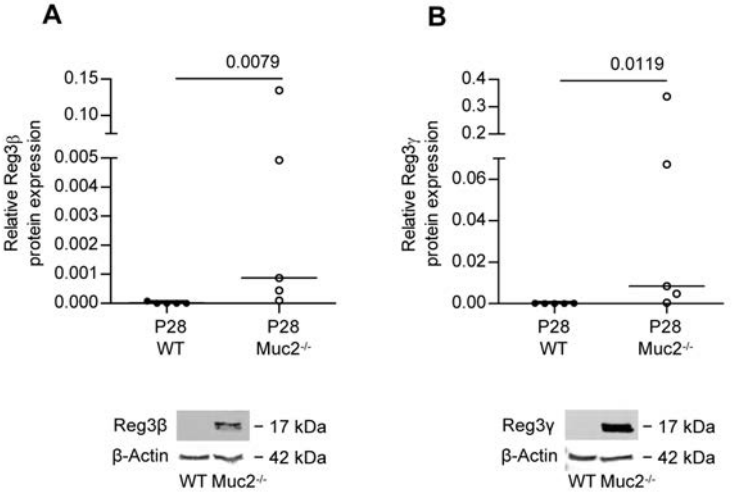
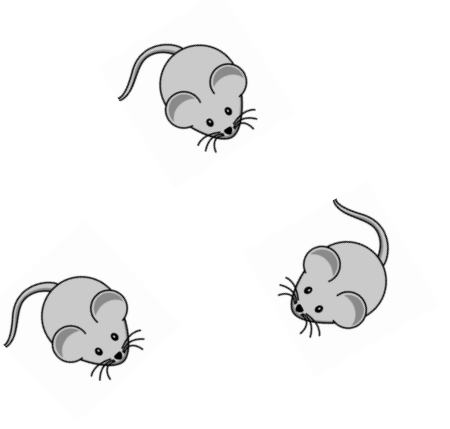



**Figure S4. Reg3β and Reg3γ protein levels in the small intestine.** Small intestinal expression of Reg3β (A) and Reg3γ (B) protein. Photomicrographs depict representative examples of Reg3β and Reg3γ expression in WT and Muc2$^{-/-}$ mice, and corresponding β-Actin expression. The relative protein levels were normalized to β-Actin and expressed as median. *P* values are indicated when expression levels between groups differ statistically. Groups are depicted as: WT, P28 ●; and Muc2$^{-/-}$, P28 ○.



# Chapter 3

# Homeostatic mechanisms preventing inflammation-mediated mucosal damage in the ileum of Muc2-deficient mice

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**ABSTRACT**

Muc2$^{-/-}$ mice develop spontaneous colitis after around 4 weeks of age but the effects of lack of mucus in the ileum have not been previously reported. We investigated morphological and gene expression changes in the proximal colon and ileum in Muc2$^{-/-}$ mice from before weaning (2 weeks) after weaning (4 weeks) and at 8 weeks of age. Lack of mucin resulted in mucosal tissue damage in colon at weeks 4 and 8 whereas in the ileum we observed signs of hyperplasia at week 8. In the proximal colon, network biology approaches showed expression of innate and adaptive immune pathways associated with colitis, increasing with time. Based on expression of IBD-related genes, the colitis observed in the Muc2$^{-/-}$ model was similar to that observed in the DSS-model, where mucus barrier function is also compromised. In contrast to the colon, many immune signalling pathways were down-regulated in the ileum at weeks 2, 4 and 8. Nevertheless, RegIIIβ and RegIIIγ were significantly upregulated in the ileum, suggesting their proposed antimicrobial and/or anti-inflammatory activities might be related to the suppression of immune pathways and avoidance of immune-mediated damage. Furthermore, we showed that RegIIIβ could specifically bind to mucin and fucosylated glycans *in vitro*, which may serve to inhibit bacterial binding to membrane bound mucins on the epithelium and also enable RegIIIβ to be retained in the secreted mucin.

## INTRODUCTION

A key element of the mammalian intestinal strategy for maintaining homeostasis with the microbiota is to minimize contact between luminal microorganisms and the intestinal epithelial cell surface. This separation between bacteria and epithelia is accomplished by a physical barrier that consists of mucus, antimicrobial proteins and IgA, secreted into the lumen.[1, 2] Mucus in the intestine, primarily composed of glycosylated mucin 2 (Muc2), is secreted by goblet cells in the epithelium. In the mouse colon two distinct layers can be distinguished; a stratified inner layer which is attached to the epithelium and largely devoid of bacteria, and a less dense outer mucus layer that is accessible for commensal microbes. Both mucus layers have essentially the same mucus composition suggesting the outer mucus layer arises from proteolytic cleavage and volumetric expansion of the inner layer. The density and stratified organization of the inner mucus layer is proposed to prevent penetration by bacteria thereby minimizing contact between bacteria and the epithelium.[3] In the small intestine, the mucus layer is thinner than in the colon, of uniform density and has a propensity to detach from the epithelium in histological procedures.



Increased contact between microbiota and epithelia is a feature of inflammatory bowel disease (IBD).[4] Mice with gene knockouts for Toll like receptor (Tlr)5, interleukin 10 (IL-10) and Slc9a3 (Nhe3) develop spontaneous colitis and the colonic mucin was shown to be more penetrable to fluorescent beads and bacteria.[5] In these knockout mouse models, some bacteria were seen in contact with the colonic epithelial surface, which was not observed in wild-type (WT) mice.[5] In humans, the colonic mucus in biopsy samples from ulcerative colitis (UC) patients was highly penetrable to fluorescent beads, whereas biopsies from healthy individuals and most of the UC patients in remission had a mucus layer that was seemingly impenetrable to beads.[5] The diminished barrier functionality of the mucus layer in colitis may result from the structural changes in the glycoprotein core and/or the sulphation and sialylation of mucins oligosaccharide residues, which have been reported in IBD patients.[6] Additionally, endoplasmic reticulum stress and a defective unfolded protein response have been proposed to play a role in mediating functional changes in mucus secretion and its functional properties.[7] The results, taken together with earlier studies showing increased association of bacteria with the epithelium in UC patients than in healthy persons,[8] suggests that at least in UC, defects in the organization of the inner mucus layer allow bacteria to reach the epithelium in larger quantities than normal. Excessive bacterial contact with the epithelium is known to trigger inflammatory responses through recognition of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) of the innate immune system, including TLRs and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and increasing epithelial permeability.[9] The influx of bacteria and their products across the epithelium increases the inflammatory response, resulting in the influx of immune cells, further loss of epithelial integrity and activation of adaptive

immune responses. All the above listed symptoms are characteristic for mouse colitis and for human IBD, which includes both UC and Crohn's disease (CD).[4] In human IBD, clinical pathology is associated with the altered transcription of 32 common genes.[10] Many of these genes are also differentially expressed in mouse models of colitis that share pathologic features with either CD or UC.

An abnormal composition and decreased complexity of the microbiota are common features in IBD patients.[4, 11] This has led to the identification of pathobionts such as the adherent invasive *E. coli* (AIEC), that in IBD patients increase in abundance, invade and persist intracellularly within epithelial cells and macrophages.[12] The genetic polymorphisms associated with IBD compromise the innate mechanisms that normally combat these pathogens.

The protective properties of mucus are evident in Muc2$^{-/-}$ mice, which develop spontaneous colitis after about 4 weeks, due to the lack of a mucus layer in the intestine.[13] In these mice, bacteria are found in the colonic crypts and in direct contact with the epithelial cells, something that is not observed in WT mice. The inflammatory responses occurring in Muc2$^{-/-}$ mice prior to the development of colitis have been studied in 2 and 4 week old mice.[14, 15] Distinct phases were observed in colitis development, which might be related to the expansion of the microbiota after weaning, and/or loss of protective factors in the mother's milk. The most notable changes observed in Muc2$^{-/-}$ mice were the exacerbation of inflammatory gene expression after weaning and a decline in regulatory T cells.[14] Muc2$^{-/-}$ mice do not develop ileitis but it is not known if there are adaptations in the host that compensate for lack of mucus layer in the small intestine, for instance increased production of antimicrobial peptides.

None of the previous studies in Muc2$^{-/-}$ mice mentioned above have investigated the effects of the lack of Muc2 in the ileum in detail, or the phenotype of heterozygote (Hz) mice, which are anticipated to secrete less Muc2. Our research interest was to investigate how the intestine responds to a reduction in mucus production over time as it might provide valuable information on homeostatic mechanisms and early indicators of gut barrier dysfunction and colitis. A further aim was to investigate the ileal responses to a mucus barrier defect as it might reveal differences between the ileum and colon with respect to homeostatic mechanisms, including regulation of innate responses and antimicrobial gene expression. Hereby we focussed mainly on RegIII proteins, as we have previously shown that RegIIIγ and RegIIIβ are upregulated in the intestine of Muc2$^{-/-}$ mice,[16] which might have a protective role in the intestine. We were also interested to analyse the expression mouse orthologues of a panel of known human IBD related genes in the Muc2$^{-/-}$ ileum and colon.

## MATERIALS AND METHODS

### Animals

Muc2$^{-/-}$ mice were bred as previously described[13] on a 129SV genetic background. All mice were generated from breeding Hz (Muc2$^{+/-}$) mice and genotyped as previously described.[17] Mice were housed together in a specific pathogen-free environment with *ad libitum* access to standard rodent pellets (Special Diets Services, Witham, Essex, England) and acidified tap water in a 12-hour light/dark cycle. The Erasmus MC Animal Ethics Committee approved all the animal experiments described in this paper (Rotterdam, The Netherlands).

### Experimental set up

Groups of WT, Hz and Muc2$^{-/-}$ littermates were housed together with their birth mother until weaning at the age of 21 days and sacrificed at 14, 28 and 56 days post-natally. Small intestinal and colonic tissues were excised immediately and either fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), stored in RNAlater (Qiagen, Venlo, The Netherlands) at -20°C, or frozen in liquid nitrogen and stored at -80°C.



### Histology

Tissue fixed in 4% (w/v) paraformaldehyde in PBS was prepared for light microscopy, and 4 µm-thick sections were stained with hematoxylin and eosin (H&E) to study histological changes. To detect differences in mucosal and epithelial thickness in the ileum, 10 well-oriented villi were chosen per intestinal segment and their length was measured.

### Pas/Alcian Blue staining

Five micron-thick paraffin sections of ileum and colon tissue were attached to polysine-coated glass slides (Thermo scientific, Germany). After overnight incubation at 37˚C**,** slides were deparaffinised using a series going from xylene to distilled water with decreasing ethanol steps. Sections were stained in 3% Alcian Blue (8GX Acros Organics, New Jersey, USA) for 35 min, rinsed in running tap water for 2 min and then rinsed in distilled water. The sections were submerged in periodic acid 0.5% for 10 min and rinsed for 1 min in distilled water. After this washing step the sections were incubated in Schiff's reagents (Merck, Germany) for 45 min and washed in freshly prepared $SO_2$ water (10 ml of 10% $K_2S_2O_5$ (Merck, Germany), 10 ml of HCl (1 mol/L) and 180 ml of distilled water) for 3 times 2 min, followed by a washing step in tap water for 5 min. After this step the sections were submerged 2 times for 3 min in 100% ethanol followed by submersion in xylene for 3 times 5 min, and then finally mounted in DPX mounting reagent (BDH Gurr Certistain, England) and air-dried overnight at 37˚C.

### Muc2 staining

Paraffin sections of both ileum and colon were cut at 5 µm and attached to polysine-coated slides (Thermo scientific). After overnight incubation at 37˚C, slides were

deparaffinised as above. An antigen retrieval step was performed by heating the sections for 20 min in 0.01 M sodium citrate (pH 6.0) at 100˚C. Sections were washed for 3 h with 3 changes of PBS. A blocking step to reduce non-specific binding was included using 5% goat serum (Invitrogen, Life technologies Ltd, Paisley, UK) in PBS with 0.1% Triton X-100 for 30 min at room temperature. Muc2 expression was detected by incubating the sections with custom designed anti-Muc2 antibody[3] diluted 1:500 in PBS containing 1% goat serum and incubated overnight at 4˚C. After primary incubation, sections were washed 3 times in PBS for 10 min, and the staining was visualized using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'–diaminobenzidine (DAB) as staining reagent. Finally, sections were mounted in DePeX and stored at room temperature.

**RNA isolation, cDNA synthesis and qPCR**

Total RNA was isolated using the RNeasy kit (Qiagen, Venlo, the Netherlands) with a DNase digestion step according to the manufacturer's protocol. One µg of RNA was reverse transcribed using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's protocol.

For qPCR 5 µl cDNA (1:20 diluted from cDNA synthesis mixture) was used, together with 300 nM forward and reverse primer, 6.25 µl 2x Rotor-Gene SYBR Green PCR kit (Qiagen), and demineralized water up to a total volume of 12.5 µl. QPCR was performed (2 min 95˚C, then 40 cycles of 15 sec at 95˚C, 1 min at 60˚C, and a final step of 2 min at 60˚C) on a Rotorgene 2000 real-time cycler (Qiagen). (see Table 3.1 for qPCR primer sequences).

| | Forward primer | Reverse primer |
|---|---|---|
| *Gapdh* | GGTGAAGGTCGGTGTGAACT | CTCGCTCCTGGAAGATGGTG |
| *Hprt* | GTTAAGCAGTACAGCCCCAAA | AGGGCATATCCAACAACAAACTT |
| *Muc2* | ACCTGGGGTGACTTCCACT | CCTTGGTGTAGGCATCGTTC |
| *Reg3γ* | TTCCTGTCCTCCATGATCAAAA | CATCCACCTCTGTTGGGTTCA |
| *Reg3β* | ATGCTGCTCTCCTGCCTGATG | CTAATGCGTGCGGAGGGTATATTC |
| *FUT2* | AGTCTTCGTGGTTACAAGCAAC | TGGCTGGTGAGCCCTCAATA |

**Table 3.1**. QPCR primer sequences

The raw data was analysed using the Rotorgene Analysis Software V5.0. Changes in transcript levels were calculated relative to the housekeeping genes according to the following equation:

$$\text{Ratio} = (E_{\text{reference}})^{\wedge} Ct_{\text{reference}} / (E_{\text{target}})^{\wedge} Ct_{\text{target}}$$

Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and hypoxanthine phosphoribosyltransferase (Hprt) were incorporated in all qPCR experiments as internal reference

genes; results were similar following normalization to either gene. Expression of the housekeeping genes, *Gapdh* and *Hprt*, was not different between WT, Muc2$^{-/-}$ and Hz mice. Reactions lacking reverse transcriptase or template were included as controls in all experiments and no amplification above background levels was observed for these controls. The melting temperature and profile of each melting curve was checked to ensure specificity of the amplification product. The PCR product of each template was also checked by sequencing.

**Transcriptome analysis**

RNA quantity and quality was assessed on the total RNA obtained from ileum and colon using spectrophotometry (ND-1000, NanoDrop Technologies, Wilmington, USA) and the Bionanalyzer 2100 (Agilent, Santa Clara, CA, USA), respectively. RNA was judged as being suitable for array hybridisation only if samples showed intact bands corresponding to the 18S and 28S ribosomal RNA subunits, displayed no chromosomal peaks or RNA degradation products, and had a RIN (RNA integrity number) above 8.0.



The Ambion WT Expression kit (Life Technologies Ltd, Paisley, UK) in conjunction with the Affymetrix GeneChip WT Terminal Labelling kit (Affymetrix, Santa Clara, CA, USA) were used for the preparation of labelled cDNA from 100 ng of total RNA without rRNA reduction. Labelled samples were hybridised on Affymetrix GeneChip Mouse Gene 1.1 ST arrays, provided in plate format. Hybridisation, washing and scanning of the array plates was performed on an Affymetrix GeneTitan Instrument, according to the manufacturer's recommendations. Detailed protocols can be found in the Affymetrix WT Terminal Labelling and Hybridisation User Manual.

Quality control (QC) of the datasets obtained from the scanned Affymetrix arrays was performed using Bioconductor[18] packages integrated in an on-line pipeline.[19] Various advanced quality metrics, diagnostic plots, pseudo-images and classification methods were applied to ascertain only excellent quality arrays were used in the subsequent analyses.[20] An extensive description of the applied criteria is available upon request. The more than 825.000 probes on the Mouse Gene 1.1 ST array were redefined according to Dai *et al*.[21] utilising current genome information. In this study, probes were reorganised based on the Entrez Gene database, build 37, version 1 (remapped CDF v13). Normalised expression estimates were obtained from the raw intensity values using the Robust Multi-array Analysis (RMA) pre-processing algorithm available in the Bioconductor library affyPLM using default settings.[22] Differentially expressed probe sets were identified using linear models, applying moderated t-statistics that implemented empirical Bayes regularization of standard errors.[23] The moderated t-test statistic has the same interpretation as an ordinary t-test statistic, except that the standard errors have been moderated across genes, i.e. shrunk to a common value, using a Bayesian model. To adjust for both the degree of independence of variances relative to the degree of identity and the relationship between variance and signal intensity, the moderated

t-statistic was extended by a Bayesian hierarchical model to define an intensity-based moderated T-statistic (IBMT).[24] IBMT improves the efficiency of the empirical Bayes moderated t-statistics and thereby achieves greater power while correctly estimating the true proportion of false positives. P-values were corrected for multiple testing using a false discovery rate (FDR) method proposed by Storey *et al.*[23] Only probe sets with a fold-change (FC) of at least 1.2 (up/down) and FDR < 0.05 were considered to be significantly regulated.

**Glycan microarray binding assays**

Glycan microarray experiments were performed in the Biofunctionals Nanomaterials Unit from CICbiomaGUNE (Donostia, Spain). Chemoenzymatic synthesis of 5-aminopentyl glycans structures and glycan array preparation was performed as described previously.[25, 26] Briefly, 50 µM glycan solutions in 300 mM sodium phosphate buffer (pH 8.5), were spatially arrayed on NHS activated glass slides (Nexterion® H, Schott AG, Germany) employing a robotic non-contact spotter sciFLEXARRAYER S11 (Scienion AG, Berlin, Germany). After printing, the slides were placed in a 75% humidity chamber (saturated NaCl solution) at 25˚C for 18 h. The unreacted NHS groups were quenched by placing the slide in a 50 mM solution of ethanolamine in 50 mM sodium borate buffer (pH 9.0) for 1h. The slides were then washed with PBS, nanopure water and dried in a slide spinner.

RegIIIβ protein was labelled with Hilyte PlusTM 555 protein labelling kit (AnaSpec, Freemont, USA) following the instructions of the manufacturer. A solution (100 µL) of RegIIIβ-555 protein (50 µg/mL in 25 mM TrisHCl, 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2, pH=8.5) was applied to the glycan array and the incubation was performed in the dark at 4˚C for 18 h. The slide was washed with water and bound fluorescent protein was measured in an Agilent G265BA microarray scanner (Agilent Technologies, Santa Clara, USA). Fluorescence quantification was achieved by ProScanArray® Express software (Perkin Elmer, Shelton, USA).

**Statistics**

All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). Data shown are the means and the standard errors of the means (SEM). QPCR data were analysed with the non-parametric Mann-Whitney test. Differences were considered statistically significant when $P<0.05$.

## RESULTS

**Intestinal expression of Muc2**

Before the onset of colitis, at approximately 4 weeks of age, Muc2 production in mice was quantified and visualized in the proximal colon and ileum of $Muc2^{-/-}$, Hz and WT

mice (Fig. 3.1). The amount of Muc2 RNA was highly variable between mice in each group (Fig. 3.1A and B). No transcription of *Muc2* could be measured in ileum or colon of Muc2$^{-/-}$ mice as expected. In the ileum of Hz mice *Muc2* transcription was intermediate to WT and Muc2$^{-/-}$ at all weeks of age. Expression of *Muc2* was not significantly different between Hz and WT in the colon at 4 and 8 weeks, possibly due to the influence of inflammatory processes and up-regulation of *Muc2* transcription as a protective mechanism. Muc2 was visualized in the proximal colon using immunohistochemistry and PAS-Alcian Blue staining (Fig. 3.1C). In Hz mice fewer Muc2 stained goblet cells were observed than in WT mice and no goblet cells containing mucus were observed in the Muc2$^{-/-}$ mice. QPCR analysis and histology results together showed that there is less Muc2 production in both the ileum and colon of Hz mice compared to WT mice.

**Changes in the intestinal morphology in Muc2$^{-/-}$ and Hz mice**

No morphological differences in colon were observed between WT and Hz at 2, 4 and 8 weeks (not shown), whereas Muc2$^{-/-}$ mice displayed a marked change in the architecture of the colon epithelium at week 4 and 8 compared to WT (Fig. 3.2). As reported in previous studies on Muc2$^{-/-}$ mice there were histological signs of colitis in the colon from 4 weeks of age onwards.[13, 15] We observed altered crypt architecture, increased crypt length and mild infiltration of immune cells in the lamina propria (LP) of Muc2$^{-/-}$ mice (Fig. 3.2). No differences in epithelial morphology were observed in the ileum of Muc2$^{-/-}$, Hz and WT mice at 2 weeks of age, but from 4 to 8 weeks of age elongation of the villus length was apparent in Muc2$^{-/-}$ mice compared to WT (Fig. 3.3).

**Altered gene expression in the proximal colon of Muc2$^{-/-}$ mice**

Muc2$^{-/-}$ mice showed altered gene expression at all ages compared to WT mice. Most differentially expressed genes were found from week 2 to 4 comparing Muc2$^{-/-}$ to WT mice (Fig. 3.4). To gain more insights into the pathways affected in Muc2$^{-/-}$ mice, a gene set enrichment analysis (GSEA)[27] was performed and the results were visualised as networks using the Enrichment Map plugin[28] that is part of Cytoscape 2.8.[29]

The networks in figure 3.5 show the major cellular processes and pathways that were differentially induced (nodes coloured in more intense shades of red depending on strength of induction) or repressed (nodes coloured in more intense shades of blue depending on strength of repression) in Muc2$^{-/-}$ mice compared to WT at week 4 compared to week 2 (Fig. 3.5A), week 8 compared to week 2 (Fig. 3.5B) or week 8 compared to week 4 (Fig. 3.5C). From week 2 to week 4 there was an induction of diverse immune pathways involving innate signalling, B and T cell activity, antigen processing, viral response, and IgA production, compatible with immune activation and inflammatory responses in the mucosa. The increased induction of pathways involved in cell cycle and mitosis may indicate increased immune or epithelial cell turnover. The only pathway down-regulated at week 4 in Muc2$^{-/-}$ mice was involved in neurotransmitter and G protein-couples receptor (GPCR) activity. When comparing week 8 to week 2 (Fig

3.5B), similar pathways were increased in Muc2$^{-/-}$ mice with the addition of induced expression of genes linked to *Mip-2* (macrophage inflammatory protein 2, a chemokine attractant for macrophages and neutrophils) and *interleukin* (*IL)-12* signalling pathways. GSEA of pathways differentially expressed in Muc2$^{-/-}$ mice vs WT between week 4 and 8 showed repression of GPCR signalling and metabolic pathways (Fig. 3.5C).

In Hz mice, 333 genes were differentially expressed (241 upregulated, 92 down-regulated) in the colon compared to WT and these were observed only at week 4. Two genes were down-regulated in Hz mice at week 2 and no genes were differentially expressed at week 8.

Heatmaps were generated for a selection of immunity genes of which only those differentially expressed in proximal colon of Muc2$^{-/-}$ vs WT at week 2, 4 and 8 are shown in figure 3.6. This included any of differentially expressed genes for TLRs, *Nod1, Nod2*, chemokines (e.g. *granulocyte macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein (MCP)-1, MCP-3, macrophage inflammatory protein (Mip)-1alpha, Mip-1beta* and *Regulated on Activation, Normal T cell Expressed and Secreted (RANTES)*), cytokines (e.g. *IL-22, IL-10, IL-6, IL-12*, and inflammatory *tumour necrosis factor (Tnf)-α* and *IL-1* $\beta$ ), antimicrobial (poly)peptides and components of the NF-κb pathway. Additionally general T cell markers such as *CD3e* and subset markers for T helper cells (*CD4*), Tregs (*Foxp3*) and Th17 (*ROR* $\gamma$ $\delta$ ) were included. In Muc2$^{-/-}$ mice transcription of several TLR genes was upregulated (*Tlr 4, 7, 8, 9, 12, 13*), except *Tlr2* and *Tlr5*, which were strongly repressed at week 4 and 8. Antimicrobial *RegIIIβ* and *RegIIIγ* were strongly upregulated, but beta-defensin (*Defb)37* was down-regulated. The up-regulated *Tnf*, *CD3e, CD4* and *CD8* suggest an increased infiltration or activity of T cells.

**Altered gene expression in the ileum of Muc2$^{-/-}$ mice**

Compared to the proximal colon, there were more differentially expressed genes at week 4 in the ileum between Muc2$^{-/-}$ and WT mice (Fig. 3.7). At week 8 the number of genes differentially expressed in Muc2$^{-/-}$ mice compared to WT mice was similar to that of the colon. The networks representing the pathways that were significantly modulated based on GSEA using the differentially expressed ileal genes as GSEA input were different to the colon. In the ileum, diverse pathways had been repressed at weeks 2, 4 and 8, including pathways involving TLR, immune, and chemokine signalling. In the ileum of the Muc2$^{-/-}$ mice, pathways involved in mitosis, cell cycle control and oxidative phosphorylation had been induced at all weeks compared to WT. At week 8, but not week 4, adaptive immune response pathways had been repressed compared to week 2. At both week 4 and 8, peroxisome proliferator-activated receptor (PPAR) $\alpha$ and lipid metabolism pathways were repressed compared to week 2. In general there were more pathway changes over time in the ileum than in the colon. In the ileum of Hz mice there were only 14 genes differentially expressed (9 increased, 5 decreased) compared to WT and these were all observed only at week 4. At week 8 and week 2 no genes were

differentially regulated in ileum of Hz mice compared to WT.

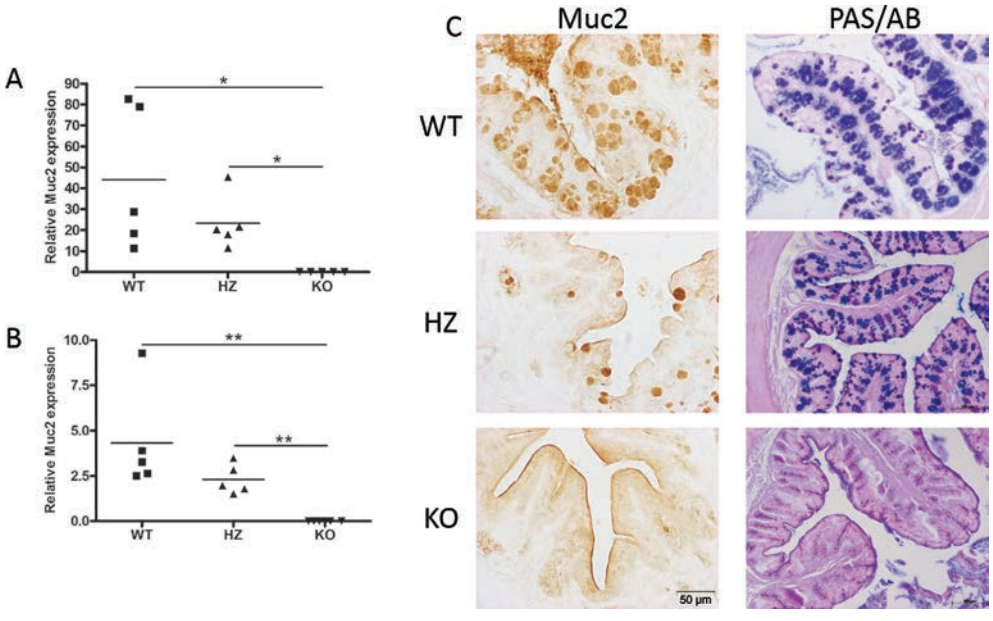


**Figure 3.1.** Relative Muc2 expression determined by qPCR in colon (A) at week 2 and ileum (B) at week 2. * p<0.05, ** p<0.01 C: representative pictures of Muc2 specific staining and PAS/AB staining of mucins in colon of wild-type (WT) Muc2 knockout (KO) and heterozygote (Hz) mice at 8 weeks of age.

Of all the immunity genes selected for heatmap analysis in colon tissue (Fig. 3.6), only 12 were differentially expressed in the ileum of Muc2$^{-/-}$ vs WT at all time points. As in the colon, ileal expression of *Tlr5* was repressed at weeks 2, 4 and 8. The gene encoding *Iκ β* , the inhibitor of NF-κb, was induced at weeks 2, 4 and 8, whereas the gene encoding the NF-κb transcriptional activating protein (*Nκap*) was down-regulated, compatible with the attenuation of inflammatory signalling and immune activation shown in the GSEA network maps (Fig. 3.8). Similar trends in gene expression were observed in Hz and Muc2$^{-/-}$ mice although the magnitude of the changes was greater in the Muc2$^{-/-}$ mice.

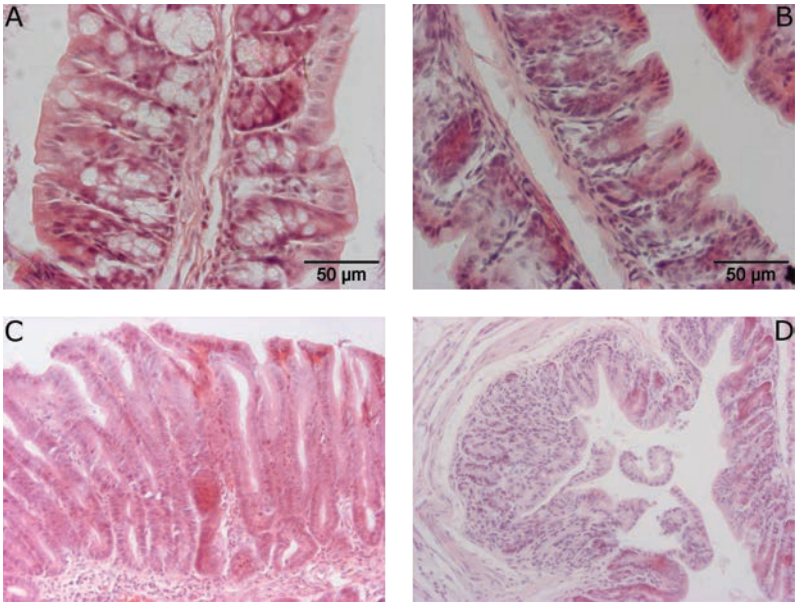


**Figure 3.2.** H&E staining of proximal colon in wild-type (A) and Muc2 knockout (C) mice at 4 weeks of age, and staining of proximal colon in WT (B) and KO (D) mice at 8 weeks of age.

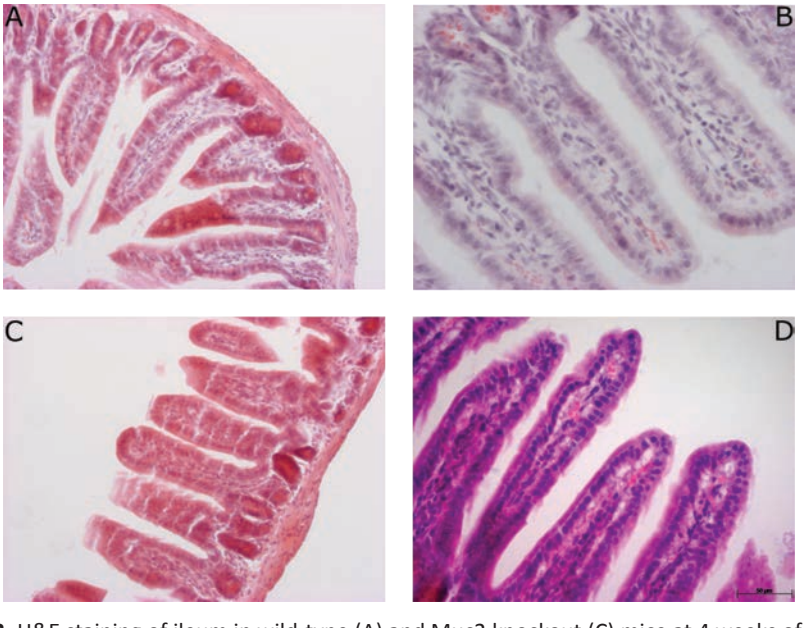


**Figure 3.3.** H&E staining of ileum in wild-type (A) and Muc2 knockout (C) mice at 4 weeks of age, and staining of ileum in WT (B) and KO (D) mice at 8 weeks of age.

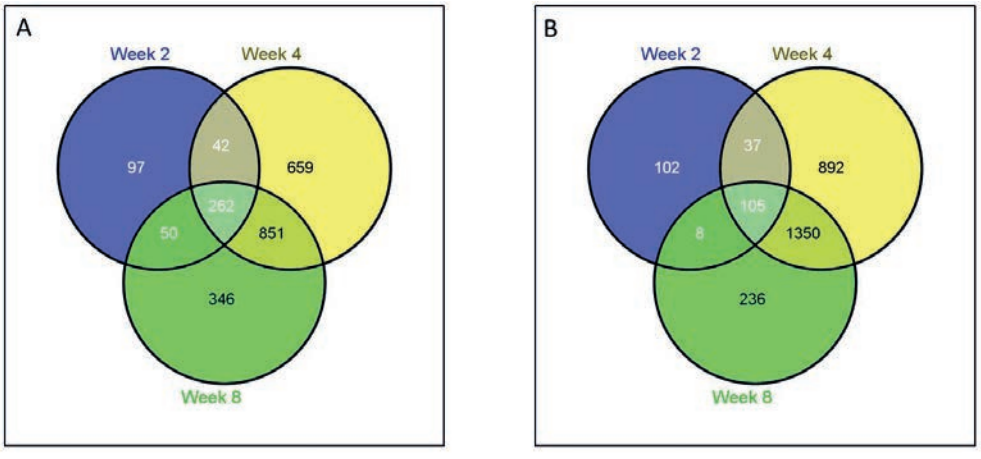


**Figure 3.4.** Venn diagram of the number of genes up-regulated (A) and down-regulated (B) in the proximal colon of Muc2 knockout (KO) mice compared to wild-type (WT) mice (p value < 0.05) at weeks 2, 4 and 8.



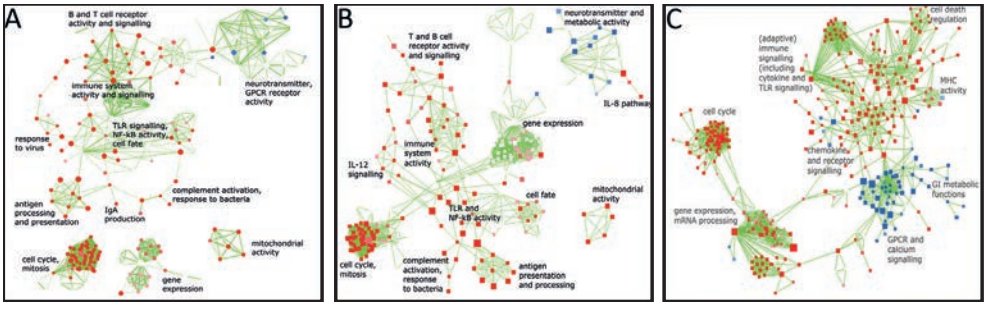


**Figure 3.5**. Network representation of gene set enrichment analysis (GSEA) profile of up-regulated (red) or down-regulated (blue) pathways in the proximal colon of Muc2 knockout (KO) mice compared to wild-type mice (WT) at A) week 2 versus week 4, B) week 2 versus week 8 and C) week 4 versus week 8.

### Differential expression of mouse orthologues of human IBD-related genes in the colon of Muc2$^{-/-}$ and Hz mice

The clinical colitis symptoms that develop in the Muc2$^{-/-}$ mice display similarity to the clinical IBD symptoms in humans. We thus investigated the expression of 32 genes en coding cytokines and their receptors, TLRs, T cell markers and antimicrobials, which were previously shown to be significantly altered in IBD.[10] Seven of the 32 IBD-related genes were differentially expressed in the colon of Muc2$^{-/-}$ mice at week 2 compared to WT mice (Table 3.2). At week 4, 13 IBD-related genes were upregulated, and 14 were upregulated at week 8, concomitant with tissue damage. The main differences in ex-

pression of IBD-related genes in Muc2$^{-/-}$ mice at week 4 and 8 were *matrix metalloprotease* (*Mmp)3, Mmp9, Cxcl10, IL-22ra2* and *IL-22ra1,* which were all upregulated except for *IL-22ra1* at week 8. Relative expression of the receptor for IL-22 (*IL-22ra1*) was increased at week 4 and week 8 compared to week 2 whereas the *IL-22ra2* gene, encoding a soluble antagonist of IL-22ra1, was significantly down-regulated at week 4 and at week 8 compared to week 2 (Table 3.2). At week 2, only 2 IBD genes were differentially expressed in Hz mice compared to WT; this increased to 5 genes at week 4, and 4 genes at week 8. Notably, the differentially expressed IBD-related genes in Hz mice at week 8 were different to those at week 4. Similar to the Muc2$^{-/-}$ mice, the Hz mice showed up-regulation of the genes encoding Th1 cytokines, lymphotoxin B *(Ltb), Ccl22, Reg3*, and *tissue inhibitor of metalloproteinase* (*Timp)1* at week 8 (Table 3.2).

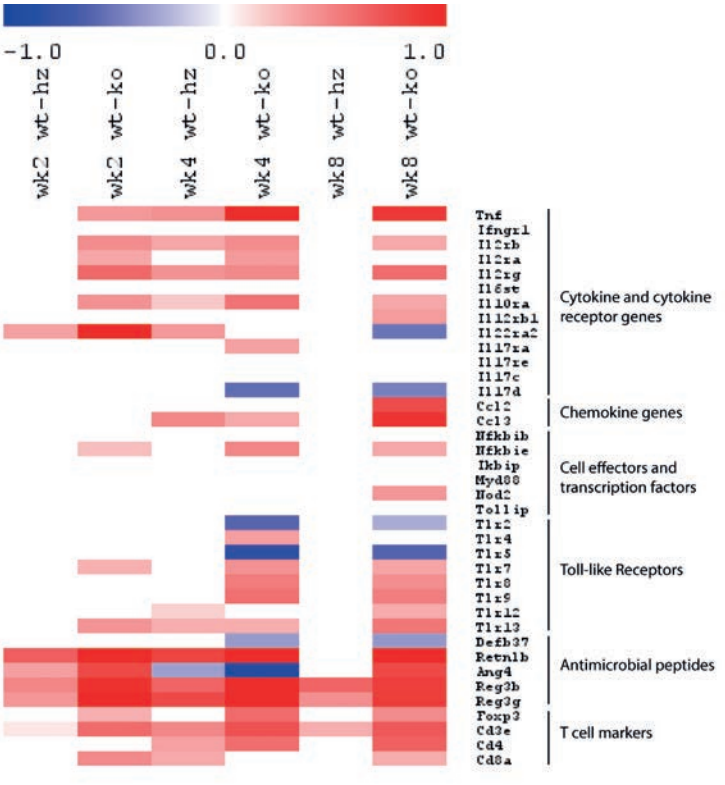


**Figure 3.6**. heat map of immunity related genes differentially expressed in proximal colon, using genes differentially expressed in Muc2 knockout (KO) versus wild-type (WT) mice with week 8 as a reference.

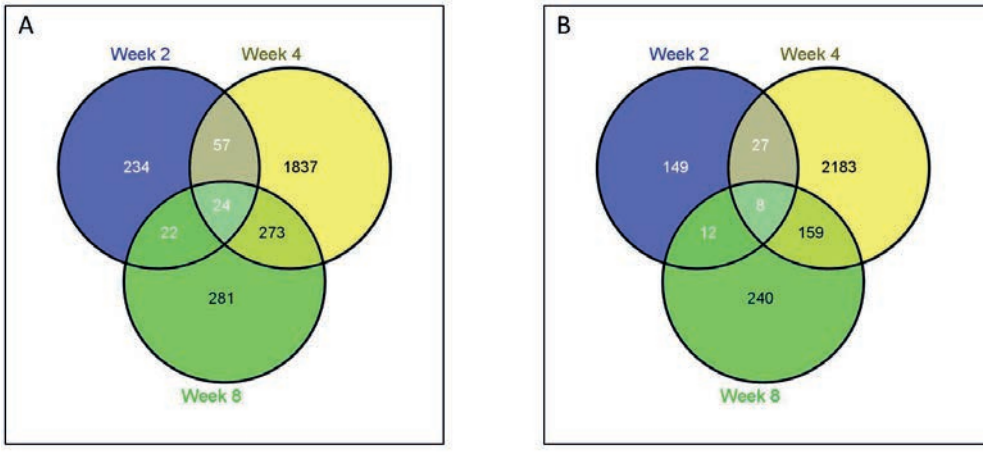


**Figure 3.7.** Venn diagram of the number of genes up-regulated (A) and down-regulated (B) in the ileum of Muc2 knockout (KO) mice compared to wild-type (WT) mice (p value < 0.05) at weeks 2, 4 and 8.



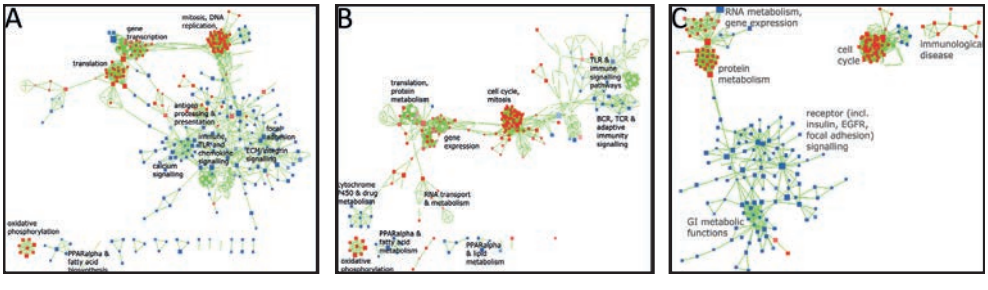


**Figure 3.8.** Network representation of gene set enrichment analysis (GSEA) profile of up-regulated (red) or down-regulated (blue) pathways in the ileum of Muc2 knockout (KO) mice compared to wild-type mice (WT) at A). week 2 versus week 4, B). week 2 versus week 8 and C). week 4 versus week 8.

**Differential expression of human IBD-related genes in ileum of Muc2$^{-/-}$ mice**

In general, fewer IBD-genes were differentially expressed in the ileum of Muc2$^{-/-}$ mice than in the colon (Table 3.3). Two of the 3 upregulated genes at week 2 were the same as those upregulated in the colon at week 2, namely *Mmp7* and *RegIIIγ*, which are involved in antimicrobial defence. At weeks 4 and 8 the overlap with differentially expressed IBD genes in the colon was less. Five genes differentially expressed in the ileum of Muc2$^{-/-}$ mice at weeks 4 and 8 were also differentially expressed in the colon (*Timp1, Ccl3, IL-22ra1, IL-6ra* and *Ltb*; Table 3.3). Fewer genes were differentially expressed in the heterozygous Hz mice than in the Muc2$^{-/-}$ mice compared to WT (Table 3.3).

| Gene | Name | Genes regulated in IBD | | | | | |
|---|---|---|---|---|---|---|---|
| | | Week 2 | | Week 4 | | Week 8 | |
| | | HZ | KO | HZ | KO | HZ | KO |
| Cytokine and cytokine receptor genes: | | | | | | | |
| Tnf | Tumor necrosis factor | NS | NS | NS | 2.03 | NS | 1.84 |
| Ifngr1 | Interferon-γ receptor 1 | NS | NS | NS | NS | NS | NS |
| Ifngr2 | Interferon-γ receptor 2 | NS | NS | NS | NS | NS | NS |
| Ltb | Lymphotoxin β | NS | 1.38 | NS | 1.33 | 1.24 | 1.46 |
| Il6ra | Interleukin 6 receptor | NS | NS | NS | NS | NS | NS |
| Il6st | Interleukin 6 signal tranductor | NS | NS | NS | NS | NS | NS |
| Il16 | Interleukin 16 | NS | NS | NS | NS | NS | NS |
| Il18r1 | Interleukin 18 receptor 1 | NS | NS | NS | NS | NS | NS |
| Il22ra1 | Interleukin 22 receptor 1 | NS | NS | NS | 1.26 | NS | NS |
| Il22ra2 | Interleukin 22 receptor 2 | 1.27 | 2.19 | 1.30 | NS | NS | -1.43 |
| Chemokine and chemokine receptor genes: | | | | | | | |
| Ccr2 | CCR2 | NS | NS | NS | 1.75 | NS | 1.38 |
| Ccl3 | MIP-1α | NS | NS | 1.37 | NS | NS | 1.89 |
| Ccl5 | RANTES | NS | 1.28 | NS | NS | NS | NS |
| Ccl7 | MARC (hu:MCP-3) | NS | NS | 1.37 | NS | NS | NS |
| Ccl11 | Eotaxin | NS | NS | NS | -1.57 | NS | NS |
| Ccl17 | TARC | NS | NS | NS | NS | NS | NS |
| Ccl20 | MIP-3 | NS | -1.43 | NS | NS | NS | NS |
| Ccl22 | | NS | 1.48 | NS | 1.46 | 1.42 | 1.81 |
| Cxcr3 | CXCR3 | NS | NS | NS | NS | NS | NS |
| Cxcl10 | IP-10 | NS | NS | NS | NS | NS | 1.62 |
| Gene involved in tissue remodeling: | | | | | | | |
| Mmp3 | Stromelysin 1 | NS | NS | NS | NS | 1.25 | 1.55 |
| Mmp7 | Matrilysin | 1.51 | 2.19 | NS | 1.28 | 1.30 | 1.25 |
| Mmp9 | Gelatinase B | NS | NS | NS | 1.45 | NS | NS |
| Mmp14 | Membrane type 1-MMP | NS | NS | NS | 1.31 | NS | 1.20 |
| Timp1 | Tissue inhibitor of metalloproteinase 1 | NS | 1.13 | 1.34 | 1.32 | NS | 1.74 |
| Regenerating islet-derived genes: | | | | | | | |
| Reg3g | Regenerating islet-derived 3 gamma | NS | 2.41 | 1.72 | 2.25 | NS | 1.82 |
| Multidrug resistance gene: | | | | | | | |
| Abcb1a | ATP-binding cassette, subfamily B (MDR/TAP), member 1A | NS | NS | NS | -1.56 | NS | -1.53 |
| Gene involved in epithelial metabolism and biosynthesis: | | | | | | | |
| Ptgs2 | Prostaglandin-endoperoxide Synthase 2 (COX-2) | NS | NS | NS | 1.20 | NS | 1.49 |

**Table 3.2.** A comparison of the differentially expressed genes, involved in IBD, in the colon of Muc2$^{-/-}$ (KO) and heterozygote (HZ) mice. Up- or down-regulated genes compared to WT, at different timepoints. Ns = non-significant.

| Gene | Name | Genes regulated in IBD | | | | | |
|---|---|---|---|---|---|---|---|
| | | Week 2 | | Week 4 | | Week 8 | |
| | | HZ | KO | HZ | KO | HZ | KO |
| Cytokine and cytokine receptor genes: | | | | | | | |
| Tnf | Tumor necrosis factor | NS | NS | NS | 1.88 | NS | NS |
| Ifngr2 | Interferon-γ receptor 2 | NS | NS | NS | NS | NS | NS |
| Ltb | Lymphotoxin β | NS | NS | NS | 2.36 | NS | NS |
| Il6ra | Interleukin 6 receptor | NS | NS | NS | NS | NS | -1.30 |
| Il6st | Interleukin 6 signal tranductor | NS | NS | NS | NS | NS | NS |
| Il16 | Interleukin 16 | NS | NS | NS | NS | NS | NS |
| Il18r1 | Interleukin 18 receptor 1 | NS | NS | NS | NS | 1.36 | NS |
| Il22ra1 | Interleukin 22 receptor 1 | NS | NS | -1.30 | NS | NS | -1.24 |
| Il22ra2 | Interleukin 22 receptor 2 | NS | NS | NS | NS | NS | NS |
| Chemokines and chemokine receptor genes: | | | | | | | |
| Ccr2 | CCR2 | NS | NS | NS | NS | 1.79 | NS |
| Ccr7 | CCR7 | NS | NS | NS | NS | NS | NS |
| Ccl2 | JE (hu: MCP-1) | NS | 1.22 | NS | NS | 1.25 | 1.25 |
| Ccl3 | MIP-1α | NS | NS | 2.08 | 1.89 | NS | NS |
| Ccl5 | RANTES | NS | NS | NS | NS | NS | NS |
| Ccl7 | MARC (hu: MCP-3) | NS | NS | 1.34 | NS | NS | NS |
| Ccl11 | Eotaxin | NS | NS | NS | NS | NS | NS |
| Ccl17 | TARC | NS | NS | 2.01 | 2.92 | NS | NS |
| Ccl20 | MIP-3α | NS | NS | NS | NS | NS | NS |
| Cxcr3 | CXCR3 | NS | NS | NS | NS | NS | NS |
| Cxcl1 | KC (hu: GROα) | NS | NS | NS | NS | NS | 1.22 |
| Cxcl10 | IP10 | NS | NS | 1.90 | NS | NS | NS |
| Tissue remodelling factors and antimicrobial petides: | | | | | | | |
| Mmp3 | Stromelysin 1 | NS | NS | NS | 1.34 | NS | NS |
| Mmp7 | Matrilysin | NS | 2.78 | NS | NS | NS | NS |
| Mmp9 | Gelatinase B | NS | NS | 1.42 | NS | NS | NS |
| Mmp14 | Membrane type 1-MMP | NS | NS | NS | NS | NS | NS |
| Timp1 | Tissue inhibitor metalloproteinase 1 | NS | NS | 1.42 | 1.51 | NS | 1.38 |
| Regenerating islet-derived genes: | | | | | | | |
| Reg3g | Regenerating islet-derived 3 gamma | NS | 2.22 | NS | NS | NS | NS |
| S-100 family genes: | | | | | | | |
| S100a10 | S-100 calcium binding protein A10 | NS | NS | NS | NS | NS | NS |
| S100a13 | S-100 calcium binding protein A13 | NS | NS | NS | 1.57 | NS | 1.31 |
| S100a4 | S-100 calcium binding protein A4 | NS | NS | NS | 1.70 | NS | NS |
| S100a5 | S-100 calcium binding protein A5 | NS | NS | NS | NS | NS | NS |
| S100a6 | S-100 calcium binding protein A6 | NS | NS | NS | 1.69 | NS | 1.31 |
| S100b | S-100 calcium binding protein B | NS | NS | NS | NS | NS | NS |
| Multidrug resistance genes: | | | | | | | |
| Abcb1a | ATP-binding cassette, subfamily B (MDR/TAP), member 1A | NS | NS | -1.4 | -1.68 | -1.23 | -1.90 |
| Genes involved in epithelial metabolism and biosynthesis: | | | | | | | |
| Ptgs2 | Prostaglandin-endoperoxide Synthase 2 (COX-2) | NS | NS | NS | -1.61 | NS | NS |

**Table 3.3.** A comparison of the differentially expressed genes, involved in IBD, in the ileum of Muc2$^{-/-}$ (KO) and heterozygote (HZ) mice. Up- or down-regulated genes, compared to WT, at different timepoints. Ns = non-significant.

**Differential expression of RegIII proteins and Fut2 in Muc2$^{-/-}$ and Hz mice**

An increase in the relative transcript amounts of both RegIII genes was measured in the colon of Muc2$^{-/-}$ mice at weeks 2, 4 and 8 by qPCR, yet failed to reach statistical significance due to high inter-individual variation in expression levels (Fig. 3.9 and Fig. 3.10). The relative expression of RegIII genes was higher in the ileum than in the colon and increased in Muc2$^{-/-}$ mice compared to WT or Hz. Interestingly, increased amounts of RegIIIγ and RegIIIβ transcripts were present in Hz mice compared to WT mice and Muc2$^{-/-}$ mice at week 4. In week 8, the relative expression of RegIIIγ and RegIII in the ileum was significantly increased in Muc2$^{-/-}$ mice compared to WT mice.

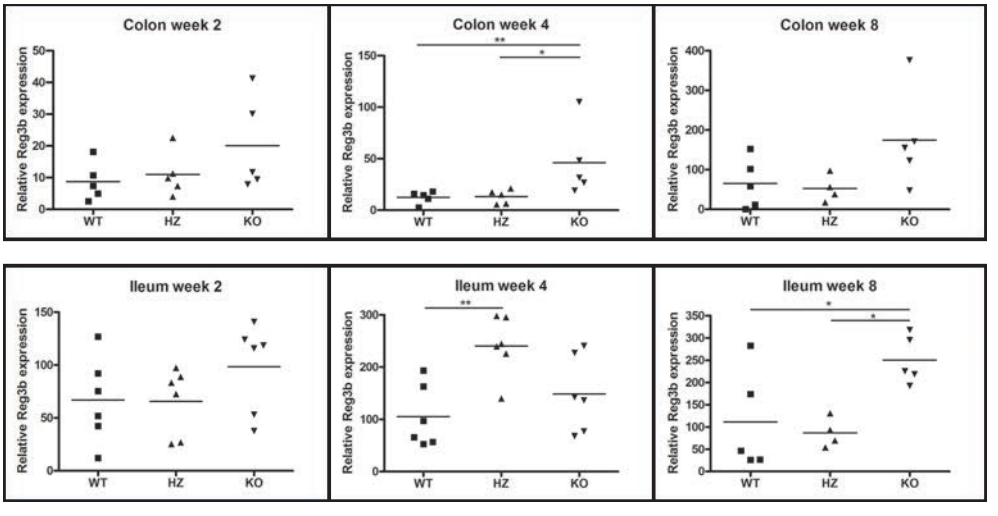


**Figure 3.9.** QPCR measurement of RegIIIβ expression in proximal colon and ileum of wild-type (WT), heterozygote (HZ) and knock-out (KO) mice at week 2, 4 and 8. * p< 0.05, ** p<0.01.

The *Fut2* gene encodes α-1,2-fucosyltransferase, which is responsible for expression of α1,2-linked fucose-containing glycans on the epithelial cell surface to which specific bacteria can bind.[30] Using qPCR, expression of *Fut2* was shown to significantly increase in the ileum of Muc2$^{-/-}$ mice at weeks 4 and 8 compared to WT mice (Fig. 3.11).

**RegIIIβ binds to fucosylated glycans**

As fucosylated glycans on the gut epithelial surface can serve as receptors for pathogenic viruses and bacteria for Norovirus,[31] human immunodeficiency virus (HIV),[32] *Helicobacter pylori*[33] and *Campylobacter jejuni*[34] we hypothesized that RegIII proteins, which are induced during infection, might bind to these targets. RegIIIβ was produced as a native secreted protein using a baculovirus expression system in insect cells and purified. The protein was labelled with 555 Alexa Fluor, re-purified and hybridized to a glycan

array. As expected, RegIIIβ showed significant binding to several fucosylated glycans (Fig. 3.12).

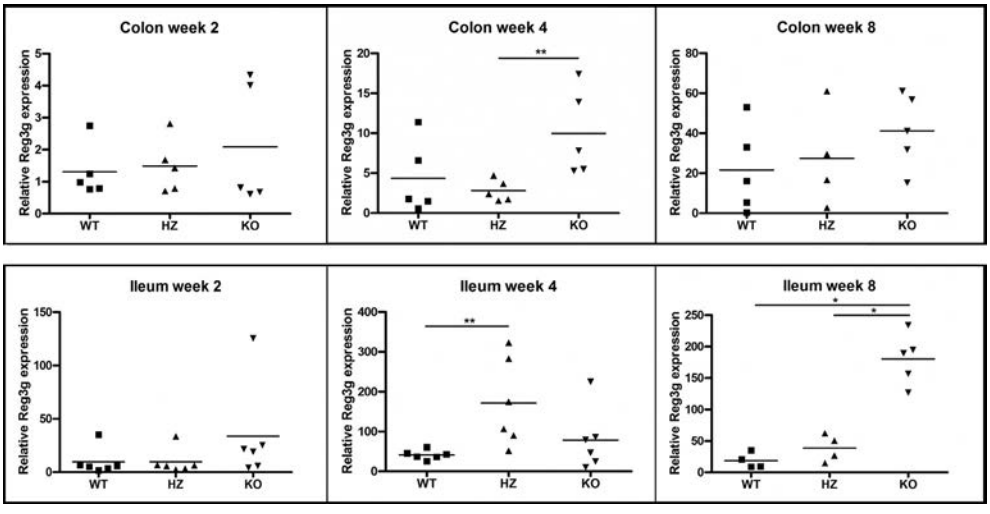


**Figure 3.10.** QPCR measurement of RegIIIγ expression in proximal colon and ileum of wild-type (WT), heterozygote (HZ) and knock-out (KO) mice at week 2, 4 and 8. * p< 0.05, ** p<0.01.

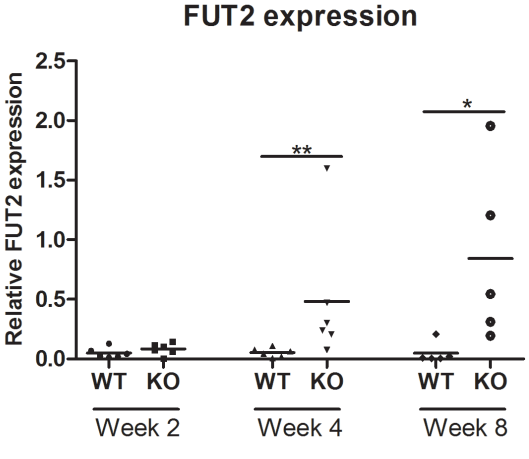


**Figure 3.11.** FUT2 expression in ileum at 2-week-old, 4-week-old and 8-week-old, in wild-type (WT) and Muc2 knock-out (KO) mice. * p<0.05, ** p<0.01.

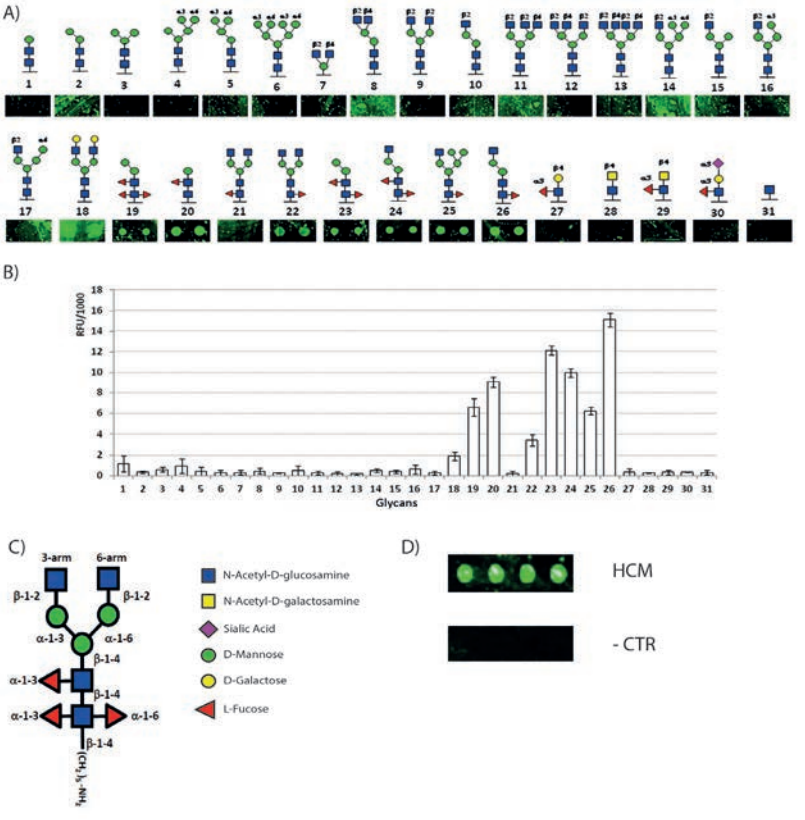


**Figure 3.12.** A) N-Glycan structures included in this study. The panels underneath the structures indicate binding of labelled RegIIIβ to the glycan structures. B) Fluorescence intensities for binding of RegIIIβ to the glycan structures. C) Exemplary bisfucosylated oligosaccharide, pictogram representation according to Consortium of Functional Glycomics guidelines, and a legend of used figures. D) Binding of labelled RegIIIβ to human colonic mucin (HCM) printed on the array. - CTR is negative control.

## DISCUSSION

Muc2 is the major secreted intestinal mucin and its absence in Muc2$^{-/-}$ mice leads to colitis, which starts in the distal colon.[13, 14] Previously, microarray analysis and qPCR of innate signalling receptors and cytokines were employed to reveal distinct phases in colitis development pre- and post-weaning, presumably as a consequence of changes in microbiota diversity and density.[14] The effect of Muc2 deficiency has not been previously investigated in the small intestine, and it is not known if and how adaptive responses compensate for the lack of a mucus layer. To gain more insights into the role of mucus in the ileum we compared age-dependent changes in morphology and temporal gene

expression patterns in the ileum and compared them to the proximal colon.

As previously reported Muc2$^{-/-}$ mice develop colitis in the proximal colon after about 4 weeks as evidenced by the increased thickness of the mucosa, an abnormal morphology, flattening of the epithelial cells and ulceration accompanied by blood in the faeces and weight loss.[13, 14] The histological changes are characteristic of the murine models for IBD and the clinical symptoms of inflammatory bowel disease in humans. Microarray analysis of the proximal colon of WT and Muc2$^{-/-}$ mice revealed induction of pathways involved in TLR signalling, production of cytokines and chemokines including *Tnf-α*, *Interferon* (*Ifn)-γ, Ltb, IL-1β*, NF-κb signalling activity and immune pathways related to B and T cell activity and antibody production. Additionally, there was increased transcription of the T cell markers *Cd4* and the T cell maturation marker *Cd3e* in Muc2$^{-/-}$ mice, consistent with the mild influx of immune cells that we observed by histology. We compared the genes that were differentially expressed in the colon of Muc2$^{-/-}$ mice (compared to WT) at week 4 and week 8 with 32 genes known to be up or down regulated in IBD.[10] Similar genes and numbers of IBD genes were affected in the Muc2$^{-/-}$ mice (weeks 4 and 8) as in the mouse DSS model of colitis where altered mucus permeability allows increased contact of the epithelium with microbiota.[5, 10] The most strongly down-regulated gene was *Abcb1a*, a gene expressing an epithelial cell surface-located transporter that is proposed to export toxins from the mucosa into the lumen. *Abcd1a* is also down-regulated in UC, which most likely impacts on the capacity of the epithelium to detoxify compounds in the mucosa. The most strongly up-regulated genes include genes encoding MMPs, which are involved in the tissue remodelling and leukocyte infiltration in UC and CD.

Neurotransmitter and GPCR signalling pathways were down-regulated in Muc2$^{-/-}$ mice, which may reflect damage to or disappearance of interstitial cells of Cajal and cells of the myenteric nerve system; damage to these cells is observed in inflamed colon and may be at the basis of the reduced gastrointestinal motility in IBD.[35]

Hz mice do not develop colitis, although qPCR data showed that Muc2 expression is lower in Hz mice than WT mice at week 2 before the onset of colitis. There is also less intense staining of mucus in the colon Hz mice compared to WT. Although the Hz mice do not develop colitis, several immunity genes and 4 IBD-related genes were differentially expressed in the colon at week 8, compared to WT mice. The differentially expressed genes included *Ltb*, that encodes a protein produced by Th1 cells to promote diapedesis, and suggests that Hz mice have increased mucosal inflammation compared to WT mice, even though tissue damage was not apparent by histological examination. The up-regulation of *Mmp3* and *Mmp7* in Hz mice suggests increased epithelial cell turnover, as *Mmp3* is involved in tissue remodelling, perhaps due to the reduced production of mucus and increased damage to the epithelium. Lack of secreted Muc2 was not compensated by increased expression of two other secreted mucins Muc5AC and Muc6 in

either colon or ileum. In contrast to the colon, lack of Muc2 did not induce histological signs of tissue damage in the ileum; instead we observed an increased villus length at week 4 and in week 8, suggesting increased cell proliferation. We did not observe increased cell sizes; increased cell proliferation was in agreement with induction of pathways involved in cell cycle and mitosis, protein translation and the induced expression of genes involved in cell division and differentiation, including the S100a family of genes and *Timp1,* that can promote cell proliferation. In the ileum of Muc2$^{-/-}$ mice immune signalling pathways including B and T cell receptor signalling were repressed compared to WT, whereas such pathways were upregulated in the colon at week 4 and 8. This may be related to an increased proliferation and differentiation status of the epithelium, influencing expression of TLR and innate response pathway genes. One of the strongly down-regulated genes in the ileum was *Tlr5*, which induces NF-κb activation upon binding of bacterial flagellin. Indeed, down-regulation of *Tlr5* in colitis has been previously observed.[36] Furthermore *Nfap*, a transcriptional activator of NF-κb, was down-regulated whereas *Iκ*β, a NF-κb inhibitor that binds to cytosolic NF-κb to prevent nuclear translocation, was among the most strongly upregulated genes. This apparent repression of innate inflammatory signalling via NF-κb appeared to be an important mechanism preventing immune-mediated pathology in the ileal mucosa of Muc2$^{-/-}$ mice.

Another factor that may have contributed to the protection of the ileum in the absence of a protective mucus layer was the increased expression of RegIIIγ and RegIIIβ. At week 8 both genes were significantly upregulated in the ileum of Muc2$^{-/-}$ mice and in the ileum of Hz mice at week 4. This correlates with decreased expression of secreted *IL-22ra2*, a soluble inhibitor of IL-22, which is an inducer of *RegIII* expression. RegIIIγ has been reported to be bactericidal against Gram-positive bacteria, whereas *in vitro* killing activity against Gram-negative bacteria has been shown for RegIIIβ.[37, 38] *In vivo,* RegIIIβ has also been shown to protect against infection with Gram-negative *Salmonella enteritidis*, but not Gram-positive *Listeria monocytogenes.*[39] Expression of RegIII proteins is induced by infection or inflammation, which is in agreement with the increased expression of immunity genes in Muc2$^{-/-}$ mice. *Defb37*, a beta-defensin, was also upregulated in Muc2$^{-/-}$ mice, further emphasising important roles of RegIII proteins and other defensins in protection of the gut barrier function. Expression of the RegIII proteins was also increased in the colon, but the overall amount of transcription in colon was much lower due to the absence of Paneth cells, which express relatively high amounts of these proteins. This lower expression of RegIII proteins in mouse colon than in ileum was also shown by Burger-van Paassen *et al.*[16] Defensins were not upregulated in the colon of Muc2$^{-/-}$ mice, presumably due to the effect of inflammatory cytokines and nitric oxide on protein folding, which causes the accumulation of unfolded proteins inside the endoplasmic reticulum, which affects the function and localization of several proteins, including defensins.[40]

Apart from their reported bactericidal effects the RegIII proteins may have anti-inflam-

matory properties. In IBD patients increased hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP, similar to murine RegIIIγ and RegIIIβ) transcription and serum levels of the protein are correlated with disease severity, and secretion of pro-inflammatory cytokines by colonic tissue of patients with active CD was inhibited by addition of purified native HIP/PAP.[41] Furthermore PAP has been shown to attenuate NF-κb signalling in human monocytes and epithelial cells.[41] Additional evidence for an anti-inflammatory function of RegIII proteins comes from studies in rats where administration of anti-PAP antibodies increased inflammation in an experimental model of taurocholate-induced acute pancreatitis.[42] In endothelial cells, purified human PAP decreased expression of surface receptors involved in leukocyte recruitment suggesting that PAP might dampen inflammatory responses by inhibiting leukocyte recruitment into the intestine.[41] It is currently not known whether murine RegIIIγ or RegIIIβ have similar anti-inflammatory effects but if so, induction of high amounts of RegIII proteins in the ileum of Hz and Muc2$^{-/-}$ might be a major mechanism preventing inflammation-mediation tissue damage, or promoting tissue repair.



Although we did not observe tissue damage in the ileum at all weeks, in agreement with the repression of inflammatory pathways, mouse orthologues of human IBD-related genes were differentially expressed in the ileum of Muc2$^{-/-}$ mice compared to WT mice. These genes included members of the S100a family of genes involved in cell cycle progression and differentiation, *Mmp3* and *Timp1*, which regulates MMP activity and promotes cell proliferation (see also above). Other IBD-related genes involved in chemotaxis and activity of granulocytes such as *Mip-1α (Ccl3), Tnf-α* and *Ccl17* were also up-regulated in the ileum of Muc2$^{-/-}$ mice suggesting a heightened inflammatory status.

*Fut2,* encoding an α-1,2-fucosyltransferase, responsible for expression of α1,2-linked fucose containing glycans on the cell surface of the intestinal mucosa,[30] was significantly upregulated in the ileum of Muc2$^{-/-}$ mice at week 8. Human genetic variants of FUT2 that have a non-secretor phenotype have been reported to be to be more resistant to gastro-intestinal infection with Norovirus but more susceptible to CD and certain bacterial infections due to bacterial binding to fucosylated glycans.[31] As expression of RegIII proteins is known to be strongly induced during mouse intestinal infection or inflammation[16, 37] we hypothesised that RegIII proteins might bind to fucosylated glycans, for instance to inhibit bacterial colonization. Using a glycan array, we indeed found that RegIIIβ was able to strongly bind to several glycan structures containing one or more fucosyl groups. Both *Fut2* and *Muc2* are upregulated during colonization of germ-free mice and have been proposed to promote growth and attachment of beneficial symbionts such as *Bacteroides thetaiotaomicron* which metabolize fucose containing glycans.[43] It might seem paradoxical that RegIII proteins bind to fucosylated mucin as they would compete with symbionts. Possible explanations are that RegIII proteins regulate availability of fucosyl binding sites through competition for binding, or that the mucus is also a matrix for retaining RegIII proteins throughout the mucus and on the mucosal

epithelial surface. Support for the latter hypothesis comes from recent molecular docking experiments showing that RegIII proteins have at least two different glycan binding sites, of which one can bind fucosylated carbohydrates (unpublished, Chapter 4).

Muc2 deficiency causes colitis, which is most severe in the distal colon where RegIII gene expression is lowest[16] but also involves the proximal colon. In contrast, no tissue damage is observed in the ileum, where RegIII protein expression is substantially higher than in the colon, suggesting the involvement of RegIII proteins in protection of the ileum against tissue damage. Thus the reported antimicrobial and anti-inflammatory properties of RegIII proteins may be involved in protection against tissue damage in the ileum. RegIIIβ was shown to bind to glycan structures containing N-acetyl glucosamine sugars linked to fucose, which may be a novel mechanism to prevent pathogen infection or colonisation. Further studies are in progress to investigate the protective role of RegIII proteins in the $Muc2^{-/-}$ model. Additionally, microbiota profiling is being used to reveal how mucus and inflammatory responses modify microbial ecology before and after the onset of colitis, which may enable us to correlate the gene expression changes to specific components of microbiota.

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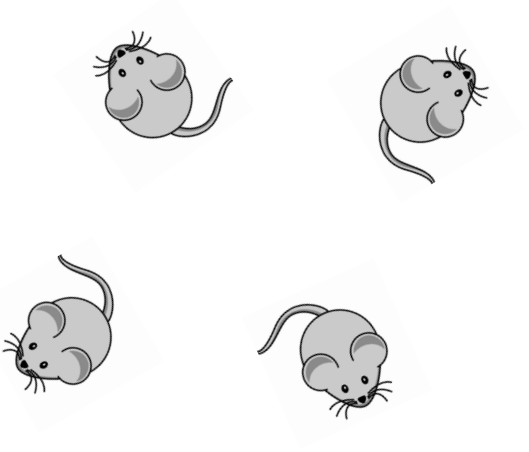

# Chapter 4

# Purification and characterization of recombinant mouse RegIII proteins

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**ABSTRACT**

Mammalian RegIII proteins, including mouse RegIIIβ and RegIIIγ and human HIP/PAP, are expressed predominantly in the small intestine. RegIII proteins have been suggested to have a role in intestinal defences against bacteria as these proteins are known to be upregulated by bacterial colonization and inflammatory stimuli driven by mucosal inflammation and damage. Recombinant RegIIIβ has been proposed to kill Gram-negative bacteria and a number of Gram-positive bacteria, whereas recombinant RegIIIγ and human HIP/PAP have been reported to be bactericidal for Gram-positive bacteria through binding to peptidoglycan. In this study recombinant RegIII proteins were produced either in insect cells using the baculovirus expression system and purified from the supernatant, or in *E. coli* and isolated as inclusion bodies and refolded. Assays were performed with recombinant RegIIIβ and RegIIIγ to determine their possible bactericidal or bacteriostatic effects, and their binding characteristics to different bacteria. Additionally, we generated structural models of RegIII proteins for *in silico* docking experiments with known ligands.

Our results showed binding of both RegIIIβ and RegIIIγ to *L. monocytogenes, E.coli* and *S. enteritidis*. Cleavage of the N-terminus enhanced the binding, but this processed RegIIIβ was not found to be bactericidal or bacteriostatic for *L. monocytogenes* and *S. enteritidis* based on CFU plate counts. Thus, the *in vitro* bactericidal effects RegIIIβ reported in the literature appear to be dependent on the exact preparation of protein, bacteria and methodology. Furthermore, molecular docking experiments suggest the presence of two ligand binding sites on RegIIIβ, which may allow for binding to lipid A and/or peptidoglycan and mucus simultaneously. Thus RegIIIβ may be able to immobilize bacteria in the intestinal mucus layer.

## INTRODUCTION

The consortium of intestinal microbes colonizing the mammalian intestine provides benefits to the host through the antagonism of pathogens and by degrading complex carbohydrates to produce sugars and metabolites that can be utilized by the host for energy. Several adaptations of the host mucosa have evolved to maintain a beneficial relationship with the microbiota and to prevent damage from pathogens. One of the mechanisms involves sequestering of bacteria in the luminal compartment to avoid colonisation of the epithelial cell surface and invasion by opportunistic pathogens. Here the secreted mucus layer overlaying the epithelium plays an important role. In humans the mucus layer is most dense in the colon where microbial numbers are highest and can reach $10^{12}$ colony forming units (CFU) per gram of luminal content.[1] There are relatively small numbers of bacteria in the duodenum and jejunum but in the ileum densities reach around $10^{7}$ to $10^{8}$ CFU bacteria per gram of luminal content. Here, the mucus barrier is thinner but IgA and the secretion of several Paneth and epithelial cell-produced antimicrobial factors enhance the mucus barrier.[2]

A key human antimicrobial factor secreted in the intestinal lumen is HIP/PAP (Hepatocarcinoma-intestine-pancreas/pancreatic associated protein), which was originally identified in rats as an abundant secretory protein expressed during pancreatitis.[3] Further studies revealed expression in mice, where it was called RegIII,[4] in humans[5, 6] and in rats.[7, 8] The family of RegIII proteins is divided into 4 subclasses, RegIIIα, RegIIIβ, RegIIIγ and RegIIIδ,[4, 9]based on the primary structures of the proteins.



Mammalian Reg proteins consist of a carbohydrate recognition domain (CRD) and an N-terminal secretion signal. Several RegIII family members are expressed predominantly in the small intestine, including mouse RegIIIβ and RegIIIγ[8, 10, 11] and human HIP/PAP.[12, 13] The known environmental triggers for inducing expression of RegIII proteins are intestinal colonization by bacteria,[11] infection[14] or inflammatory stimuli driven by mucosal inflammation and damage[15] implicating roles for RegIII proteins in mucosal defence and immune homeostasis.

Recombinant and refolded RegIIIβ has been proposed to kill Gram-negative bacteria and certain species of Gram-positive bacteria,[16, 17] although the reasons for specificity are unknown. The bactericidal effect was reported to be optimal in early logarithmic phase of growth and in the case of Gram-negative bacteria, involves RegIIIβ binding to the lipid A anchor of lipopolysaccharide,[17] although the precise mechanisms leading to bacterial death are not known. Apart from binding to lipid A and peptidoglycan (PGN), we showed previously that RegIIIβ can also bind to mucus and fucosylated glycans (Chapter 3 this thesis, unpublished data).

Another RegIII family member expressed in the mouse intestine is RegIIIγ. Recombinant and refolded RegIIIγ protein and human HIP/PAP have been reported to be bactericid-

al to Gram-positive bacteria *in vitro*, through binding to PGN.[11] RegIIIγ possesses an N-terminal pro-segment sequence that is proposed to interact with a charged surface on RegIIIγ and HIP/PAP to prevent antimicrobial activity.[18, 19] Proteolytic cleavage of this pro-segment in RegIIIγ and HIP/PAP by trypsin was shown to enhance bactericidal effects and generate an active polypeptide.[18, 19] Similar regulation of RegIIIβ antimicrobial activity by trypsin processing of a flexible N-terminal pro-segment has also been proposed, but not been conclusively demonstrated. The *in vitro* bactericidal activity of RegIIIγ or HIP/PAP is inconsistent with other publications where recombinant HIP/PAP from humans and rats was reported to lack bactericidal activity but induced bacterial aggregation.[19, 20]

Both HIP/PAP and RegIIIγ proteins have been reported to have anti-inflammatory effects in pancreatitis and colitis.[15, 21, 22] Administration of recombinant RegIIIγ reduced mortality and weight loss due to colitis caused by *Citrobactor rodentium* infection in mice.[22] Protection was attributed to anti-inflammatory effects *in vivo* as the recombinant protein lacked bactericidal activity *in vitro*.

The aim of this study was to use recombinant RegIIIβ and RegIIIγ from inclusion bodies produced by *Escherichia coli* and RegIIIβ secreted by insect cells via baculovirus transfection and characterize the bactericidal, bacteriostatic and binding properties using flow cytometry and CFU counts. Additionally we sought to investigate their potential to attenuate inflammatory signalling *in vitro*. Structural models of RegIII proteins were generated for docking experiments with known ligands, including fucosylated glycans in order to gain further insights into the binding sites and residues conferring ligand specificity.

## MATERIAL AND METHODS

### Production of recombinant RegIII proteins

Recombinant RegIII proteins were produced and refolded by GenScript (GenScript Corp., New Jersey, USA). Both RegIIIβ and RegIIIγ, possessing an N-terminal histidine-containing peptide tag (HIS-tag), were over-expressed in *E. coli* and refolded from purified inclusion bodies. Additionally, soluble RegIIIβ was secreted from insect cells using a baculovirus vector with a C-teminal HIS-tag to facilitate affinity purification.

### *In vitro* processing of RegIII proteins

Trypsin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1M HCl (pH 3) according to the manufacturers protocol to a concentration of 1 mg/ml. Trypsin was added to RegIII protein solutions at a concentration of 1/20 (w/w) and incubated at 37˚C for 1 h.

**SDS-PAGE and Western blot**

Protein was denatured by heating it at 95˚C for 5 min in 1x Laemmli buffer (4x buffer containing 160 mM Tris (pH6.8), 8% SDS, 40% glycerol, 0.05% (w/v) bromophenol blue) with 0.1M DTT and loaded onto a 12% SDS-PAGE gel (Bio-rad, Hercules, CA, USA). The gel was either stained with Coomassie blue (Instant blue, Westburg, Leusden, the Netherlands) or used for Western blotting by transferring the proteins to a PVDF membrane (Immobilon, Millipore, MA, USA). Western blots were first incubated for 1 h with blocking buffer (Li-cor, Lincoln, NE, USA) and then with custom made polyclonal antibodies (Eurogentec, Belgium)[10, 23] diluted 1:20 000 in PBS-T 1:1 diluted with blocking buffer for 1 h. Antibodies to RegIIIβ and RegIIIγ-specific peptides were generated in rabbits by conjugation to keyhole limpet hemocyanin (KLH) and then affinity purified by chromatography using the immobilized peptides. For RegIIIγ antibodies were raised against the N-terminus of the protein (EVAKKDAPSSRSSC) or a peptide sequence near the middle of the protein (MIKSSGNSGQYVC). RegIIIβ was detected using polyclonal antibodies recognizing the N-terminus (GEDSLKNIPSARISC) or a peptide sequence near the middle of the protein (STALDRAFCGSLS). The latter antibodies did not detect RegIIIβ effectively therefore we used a commercially available antibody recognising an epitope located in the middle of the protein (1:2000, R&D systems, Minneapolis, MN, USA). The primary antibodies were detected using donkey anti-rabbit IRDye800 or goat anti-rat IRDye680 (both diluted 1:20 000, Li-cor) and an Odyssey scanner (Li-cor).

**RegIII binding to bacteria**

Binding of RegIII proteins to bacteria was carried out with both processed (trypsin cleaved as described above) and unprocessed (uncleaved) RegIII proteins. Bacteria were grown to stationary or mid-exponential phase from a diluted overnight culture, washed once in PBS by centrifugation (5 min at 3300g) and re-suspension of the bacterial pellet. Approximately $10^7$ CFU bacteria were resuspended in a mixture of 20 µg RegIIIβ produced in insect cells or RegIIIγ produced in *E. coli,* in a total volume of 200 µl PBS containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich). This mixture was incubated at for 1 h in a slowly shaking heating block at 25˚C, after which the bacteria were washed once in PBS as described above. Primary antibody (anti-RegIIIβ, R&D systems, 1:1000, or anti-RegIIIγ, custom made, 1:10 000) was then added to the bacteria and incubated for 30 min at room temperature (RT). Subsequently, bacteria were washed and incubated with the secondary antibody (anti-rat or anti-rabbit with APC label, 1:50, Jackson ImmunoResearch) for 30 min at room temperature in the dark. Finally, bacteria were washed once more and measured by flow cytometry (FACS Canto, Becton Dickinson (BD), NJ, USA).

**Bactericidal assays**

Bacteria were grown to stationary or mid-exponential phase from a diluted overnight culture and washed once in MES buffer (25 mM MES, 25 mM NaCl, pH 6) by centrifugation for 5 min at 3300g. Approximately $10^7$ CFU bacteria (*Salmonella enterica* subspe-

cies *enteritidis*, *Listeria monocytogenes*, *E. coli* Nissle, *E. coli* LF82) were pelleted and resuspended in MES buffer containing 20 µg processed RegIIIβ produced in insect cells or refolded RegIIIγ in a final volume of 200 µl. The solution was incubated for 1 h at 37˚C in a slowly shaking heating block and then the bacteria were recovered by centrifugation for 5 min at 3300g and 150 µl of supernatant was removed. The bacterial pellet was resuspended in the remaining 50 µl from which serial dilutions were made. To determine CFU, 100 µl of each dilution was spread on agar plates as follows: *L. monocytogenes* on Bacto™ Brain Heart Infusion agar (BHI, BD), *S. enteritidis*, *E. coli* LF82 and *E. coli* Nissle on Luria Bertani (LB) agar (Merck KGaA, Darmstadt, Germany) and incubated overnight at 37˚C. Colonies were counted the next day.

Bactericidal activity of RegIII proteins was also investigated using live/dead staining kit (Invitrogen, molecular probes) according to the manufacturer's protocol. Briefly: bacteria (*E. coli* VE7108 and *E. coli* Nissle) were resuspended in a 0.85% NaCl buffer, stained with SYTO 9 and propidium iodide for 15 minutes in the dark, and analysed by flow cytometry (FACS Canto, BD).

**Bacterial growth curves**

Processed refolded RegIIIβ or RegIIIγ (20 µg) were added to an overnight culture of bacteria diluted 1:200 in a total volume of 200 µl in a flat bottom 96 well plate (Costar, Corning Incorporated, NY, USA). Due to the lower concentration of baculovirus produced RegIIIβ we used 10 µg of protein and 100 µl of bacteria from a 1:200 dilution of an overnight culture, in a total volume of 200 µl per well. To control for dilution of culture medium 100 µl of PBS was added to 100 µl of bacteria. Growth was measured very 10 min by optical density $(OD)_{(600\ nm)}$ in a Spectramax M5 (Molecular devices, CA, USA) during an 8 hour incubation. Plates were shaken briefly in the machine prior to measurement.

**Molecular Docking and Evolutionary Conservation Estimation**

Homology models of RegIIIβ and RegIIIγ were obtained from the Database of Structure & FUNction Predictions of Proteins from Representative Organisms (SUNPRO) 1. The 'GAEPNGG' and 'LKWNDMT' motifs of RegIIIβ and 'GYEPNRG' and 'LKWRENYC' of RegIIIγ were treated as flexible regions of the protein, while the remaining parts were treated as 'rigid body' during the docking simulations. Molecular docking was performed using AutoDock VINA[24] and the graphical front-end AutoDockTools (ADT).[25] Glycan ligand structures (obtained in Chapter 3, this thesis) were converted into the SMILE format using Marvin Sketch and optimized with eLBOW.[26] ADT were employed to protonate the ligands and identify rotatable bonds in the ligands. Grid maps of 32x32x32 Å, were defined to include sugar recognition sites and $Ca^{2+}$-coordination motifs (i.e. loop1 and loop2) conserved in different C-type lectins. The default settings in AutoDock VINA were used for other variable parameters. The contacts between the lowest energy pose of each glycan and RegIIIβ and/or RegIIIγ were calculated using Contact CCP4.[27] The limits

for contact calculation were set at 4.1 Å. The molecular models were visualized using PyMOL or Chimera. ConSurf was employed to estimate the evolutionary conservation of amino acid positions in RegIIIβ based on the phylogenetic relationship between homologous sequences.[28]

## RESULTS

### Structural models of RegIIIβ and RegIIIγ

Like other members of the mammalian RegIII protein family, the protein sequences of RegIIIβ and RegIIIγ possess secretion signal peptides, which target the protein to the endoplasmic reticulum membrane for secretion. The overall amino acid identity between the secreted pro-proteins of human PAP and mouse RegIIIβ is 70% and between PAP and RegIIIγ is 67%. Given the high sequence identity we generated predictive structural models for the secreted proteins based on the crystal structure of human PAP. The three dimensional structure of human HIP/PAP exhibits a C-type lectin fold with its characteristic 'long loop' structure,[29] comprising two distinct sub-domains, commonly designated as 'loop 1' (residues 107-121) and 'loop 2' (residues 131-145)[30] which are also apparent in the predicted structures of RegIIIβ and RegIIIγ. The N-terminal region of secreted RegIIIβ has sequence similarities to the N-terminal region of RegIIIγ and human HIP/PAP (Fig. 4.1A), which are processed by trypsin to induce a conformational switch and subsequent bactericidal activity.[18] Indeed, the predicted cleavage site of RegIIIβ, 37R-38I is identical to human PAP 37R-38I.



Antibodies generated against variable peptide sequences found in the N-terminus and exposed regions of the RegIII proteins (loop) are shown in figure 4.1B. The amino acid sequence identity between RegIIIβ and RegIIIγ is 71% and several areas of variable sequence reside in within the exposed loops 1 and 2 of the folded protein (Fig. 4.1C and D). The 'EPN' motif in loop 1 is involved in $Ca^{2+}$-independent recognition of PGN by HIP/PAP[30] and is conserved in RegIIIβ and RegIIIγ (Fig. 4.2). Moreover, RegIIIβ and RegIIIγ also possess a 'DPT' motif in loop 1 which is identical to human PAP (Fig. 4.2), similar to the 'DPQ' motif in the loop 1 region of Reg4, another human Reg family lectin involved in $Ca^{2+}$-independent mannan binding[31] (Fig.4.2).

In the carbohydrate binding domain of the mannose-specific $Ca^{2+}$-dependent C-type lectins such as DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) and MBP-A (mannose binding protein) A, the 'EPN' motif in loop 1 and the 'WND' motif in the β4 strand are conserved (Fig. 4.1B, 4.2). These motifs coordinate the principal $Ca^{2+}$ contacts with the sugar hydroxyls involved in sugar binding.[29, 30, 32, 33] RegIIIβ and RegIIIγ lack the 'WND' motif in the β4 strand, which is consistent with their $Ca^{2+}$-independent binding to sugars.[11, 17] The 'ERN' motif in loop 2 of RegIIIβ, which is involved carbohydrate recognition and Lipid A binding, is conserved in human PAP,

but variable among other members of the C-type lectin family.

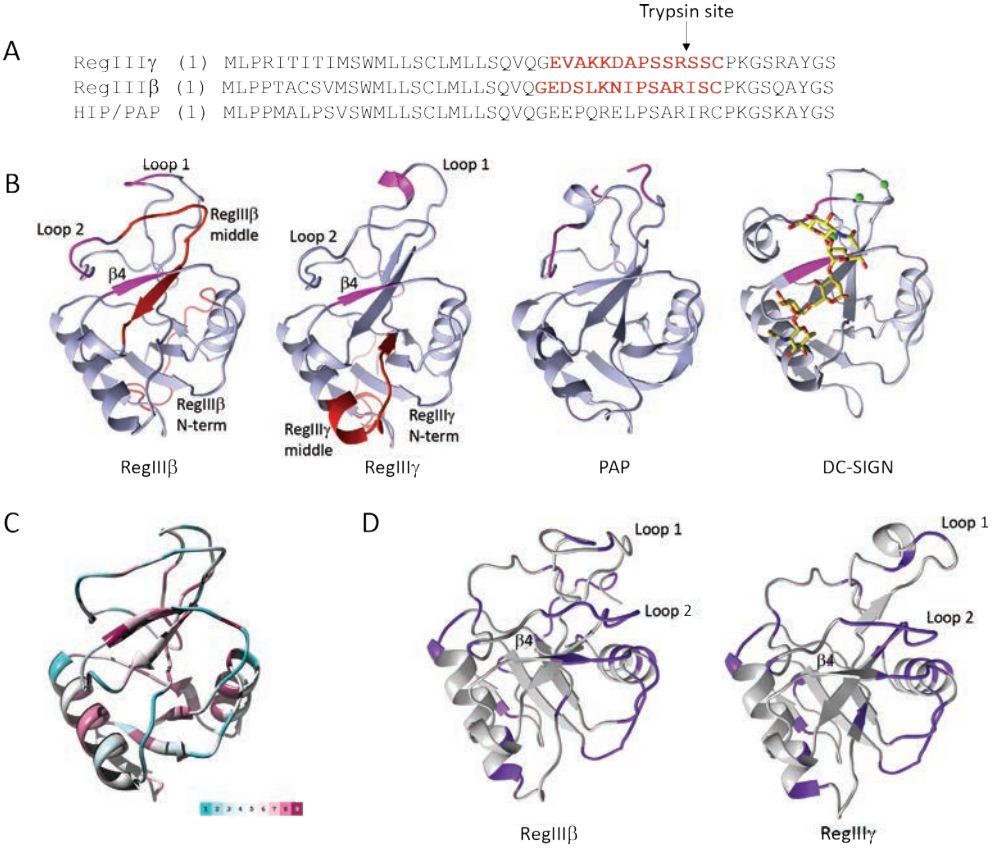


**Figure 4.1.** A) Amino acid sequence of the N-terminus of RegIIIγ and RegIIIβ, with the trypsin cleavage site (arrowed) and peptides used to generate antibodies recognizing the N-terminus (indicated in red). B) Cartoon structures of calcium-independent C-type lectins RegIIIβ, RegIIIγ and human PAP and the calcium-dependent DC-SIGN. Regions involved in carbohydrate recognition are shown in magenta. The ligand-binding loops of RegIIIβ and RegIIIγ are indicated, and the antibodies recognition regions are shown in red. C) Cartoon showing the conserved regions in around 70 different C-type lectins. Blue is variable, white is average, and pink is conserved. D) Cartoons showing the regions of the proteins that are variable between RegIIIβ and RegIIIγ in purple.


**Figure 4.2.** Sequence alignment of mouse RegIIIγ, mouse RegIIIβ, human PAP I, human Reg IV, human Langerin, DC-SIGN and MBP-A. Site I mannan-binding site residues are labelled in light blue and site II mannan-binding site residues are in light green. The ¨EPN¨ motif of PAP I and ¨DPQ¨ motif of Reg IV are highlighted in magenta frames. The ¨EPN¨ and ¨WND¨ motifs responsible for $Ca^{2+}$ coordination are highlighted in red frames. The "ERN" motif of RegIIIβ and PAP I are highlighted in a purple frame. Residues predicted to be involved in contacts with glycans binding to RegIIIβ and RegIIIγ are in Bold and labelled according to the number of glycans interacting with a particular residue following the presented colour scheme (modified from Ho *et al*.[31]).

## Production and refolding of recombinant RegIII proteins

SDS-PAGE of soluble and insoluble fractions revealed that both RegIII proteins were absent from the soluble fraction of *E. coli* lysates and were found in inclusion bodies. The inclusion bodies were partially purified by centrifugation and further purified by HIS tag affinity chromatography under denaturing conditions and subsequently refolded by step-wise dilution and dialysis. Both proteins were estimated to be at least 75% pure by SDS-PAGE gel with a yield of about 3 mg/L LB culture and a final concentration of 0.47 mg/ml for RegIIIβ and 0.43 mg/ml for RegIIIγ.

RegIIIβ, containing its native signal secretion leader and a C-terminal HIS affinity tag, was also expressed in insect cells transfected with a baculovirus expression vector. Soluble secreted RegIIIβ protein was recovered from the supernatant by affinity chromatography to more than 90% purity as judged by Coomassie stained SDS-PAGE. The protein was stored in aliquots at -80°C at a concentration of 0.12 mg/ml.

Soluble RegIIIβ produced in the baculovirus system and *E.coli* were centrifuged at 10 000 g for 10 min and the supernatant and pellet were analysed by SDS-PAGE and Coomassie staining with or without DTT in the loading buffer. Most of the protein was present in the supernatant but a proportion was recovered from the pellet suggesting precipitation and/or the formation of aggregates after purification. A proportion of the soluble protein appeared to form multimers (Fig. 4.3A) in the absence of DTT. RegIIIβ produced in either *E. coli* or insect cells was of the expected sizes. The RegIIIβ protein produced in *E.coli* was larger (18.8 kDa) than the RegIIIβ from insect cells (17.4 kDa) due to the presence of an enterokinase cleavage site between the HIS-tag and RegIIIβ protein.

**RegIIIβ and RegIIIγ are proteolytically processed by trypsin *in vitro***

The bactericidal activity of mouse RegIIIγ and human HIP/PAP was previously shown to be regulated by an N-terminal pro-segment that is removed by trypsin.[18, 19] High conservation of amino acids in the cleavage site of the pro-segment suggested that RegIIIβ would also be proteolytically activated by trypsin (Fig. 4.1A). To confirm this prediction we generated two different peptide-specific antibodies for each RegIII protein, one recognizing the N-terminal pro-segment and the second recognizing an internal domain expected to be surface exposed (Fig 4.1B).

After treatment of RegIIIγ protein with trypsin the protein is not recognized by the antibody binding to the small cleaved N-terminal pro-segment (Fig. 4.3B, left panel), indicating the expected cleavage. Similarly, RegIIIβ proteins from both sources (insect cells and *E. coli* refolded) were also reduced in size by treatment with trypsin (Fig. 4.3B, right panel) and could not be detected by the antibody recognizing the N-terminal peptide. The soluble RegIIIβ protein from insect cells shows the predicted small reduction in molecular weight after cleavage with trypsin and is present as a single protein band. Strikingly, trypsin treatment of the refolded RegIIIβ protein generated multiple protein bands (Fig. 4.Bb, right panel) suggesting increased susceptibility to trypsin proteolysis. Detection of refolded RegIIIβ produced in *E. coli* with an antibody that recognizes the N-terminal pro-segment showed that processing was incomplete (Fig. 4.3B, right panel) and a small portion of the protein remained uncleaved.

**N-terminal processing enhances binding of RegIIIβ and RegIIIγ to bacteria**

Soluble RegIIIβ produced using the baculovirus system and refolded RegIIIγ were tested for binding to different bacteria using a flow cytometric assay. Different concentrations of RegIII proteins were incubated for 1 h with bacteria from stationary or exponential growth phases in PBS, containing BSA as a non-specific binding inhibitor. Detection of

RegIIIβ binding was gated using bacteria incubated with RegIII antibodies without RegIII protein as a control (Fig. 4.4A) and binding events (e.g. 'stained' bacteria, thus bound to RegIII) were calculated as a percentage of the total bacteria. Trypsin cleavage of the N-terminal pro-segment of RegIIIβ was required for efficient binding to *S. enteritidis* and *L. monocytogenes* (Fig. 4.4B and C) with approximately 20-fold less binding detected using unprocessed RegIIIβ. Similarly, binding of RegIIIγ to bacteria was also greatly enhanced by processing of RegIIIγ with trypsin. Binding of RegIIIβ to both *S. enteritidis* and *L. monocytogenes* was dose-dependent and not appreciably affected by bacterial growth phase or the inclusion of reducing agents (data not shown). Similarly, RegIIIγ bound to the tested bacteria, although the percentage of bound bacteria was lower (around 20%) and more variable than for RegIIIβ.

**Bactericidal assays with RegIII proteins**

The growth phase of bacteria can influence their susceptibility to antimicrobials, therefore bacteria at the mid-exponential phase and the stationary phase were used in our killing assays with N-terminally processed RegIII proteins. Addition of 6 μM RegIIIβ or RegIIIγ to *S. enteritidis* harvested in exponential growth phase did not reduce the CFU after 1 h incubation at 37°C (Fig. 4.5A). Although RegIIIβ did not kill *L. monocytogenes*, RegIIIγ did reduce CFU of *L. monocytogenes* in two out of three experiments. Bactericidal assays with stationary phase bacteria showed reduction of *E.coli* Nissle CFU by RegIIIγ, but no effect on viability of adherent invasive *E. coli* strain LF82. Stationary phase *S. enteritidis* and *L. monocytogenes* were not killed by incubation with either protein (Fig. 4.5B).



We also investigated the bactericidal effects of RegIIIβ on exponentially growing *E.coli* VE7108 and *E.coli* Nissle using a live/dead stain and flow cytometry to quantify the different populations. In absence of RegIIIβ protein, the percentage of dead exponential phase bacteria was around 4% to 15%, depending on the strain. Addition of RegIIIβ for 1 h increased this percentage of dead *E.coli* VE7108 from 15% to 42%, whereas the percentage of dead *E.coli* Nissle increased from 4% to 10%. Similar results were obtained in a second, separate experiment. Taken together, these results show that the bactericidal effect of the recombinant RegIII proteins are at best modest under these conditions and are dependent on bacterial growth phase as well as on the strain and species of the bacteria investigated.

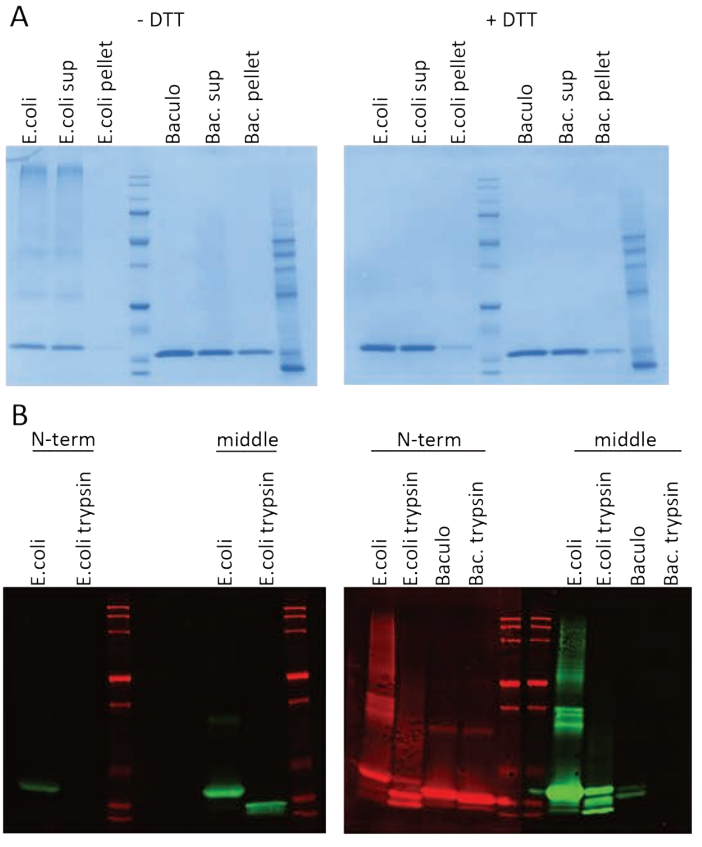


**Figure 4.3.** A) Coomassie-blue stained SDS-PAGE gel showing recombinant RegIIIβ produced using baculovirus (bac) or *E. coli*. Supernatant (sup) or pellet after centrifugation of protein solution, with or without DTT in the buffer. B) Western blot showing N-terminal cleavage of RegIIIγ (left panel) or recombinant RegIIIβ proteins (right panel). RegIII proteins were detected using antibodies recognising the N terminus (N-term) or other parts of the protein predicted to be surface exposed (middle).

**Effect of RegIII protein on bacterial growth *in vitro***

As an additional assay for the antimicrobial activities of recombinant RegIII proteins we measured the effect of RegIIIγ or RegIIIβ on bacterial growth. *S. enteritidis* or *L. monocytogenes* were grown for 8 hours in 96-wells plates at 37°C, with or without processed RegIIIβ or RegIIIγ. Neither of the refolded RegIII proteins (Fig. 4.6A and B), or soluble RegIIIβ produced in insect cells (Fig. 4.6C and D) inhibited the growth of bacteria compared to the control. In fact, the bacteria incubated with refolded RegIIIβ or RegIIIγ

reached higher numbers than untreated samples in the lag phase and early log phase. Based on these results there appears to be no inhibitory effect of RegIII proteins on the growth of *S. enteritidis* and *L. monocytogenes*. However, in *L. monocytogenes* cultured with RegIIIγ the final OD was lower than that of the control without RegIIIγ, indicative of a lower final number of bacteria.

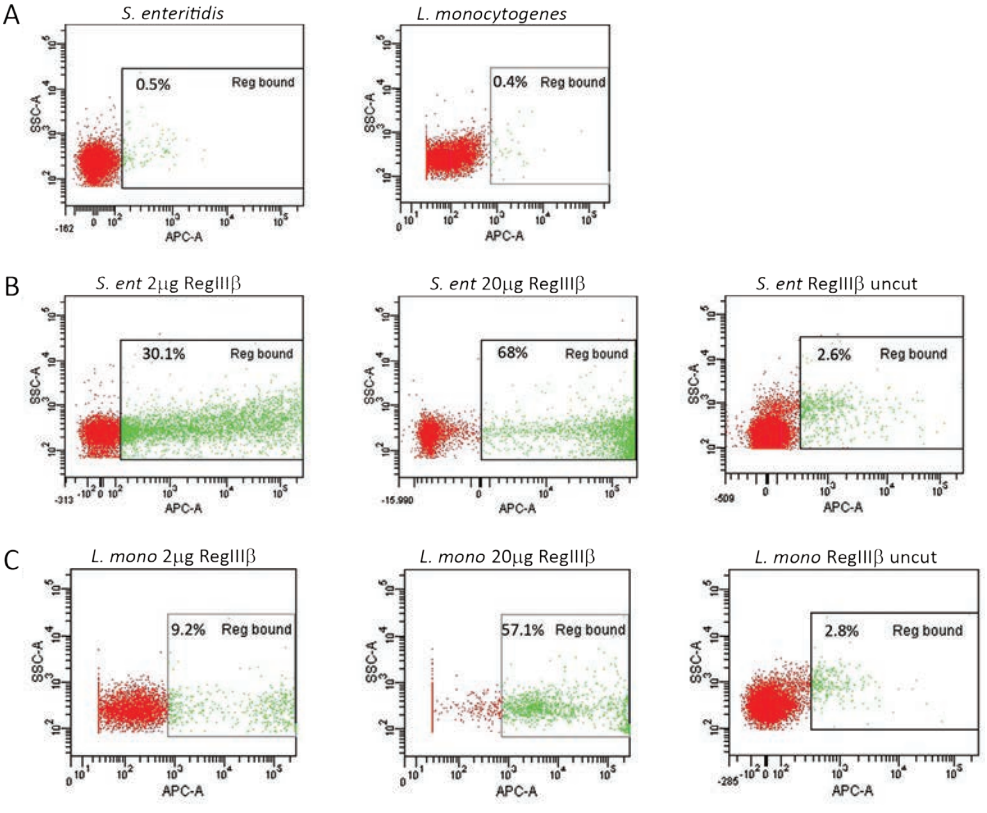


**Figure 4.4.** Flow cytometrical analysis of A) Negative control: *S. enteritidis* and *L. monocytogenes* incubated without RegIII protein. B) *S. enteritidis* incubated with different concentrations of RegIIIβ. C) *L. monocytogenes* incubated with different concentrations of RegIIIβ. Percentage of total bacteria detected using fluorescent anti-RegIIIβ antibody are indicated in the gated area.

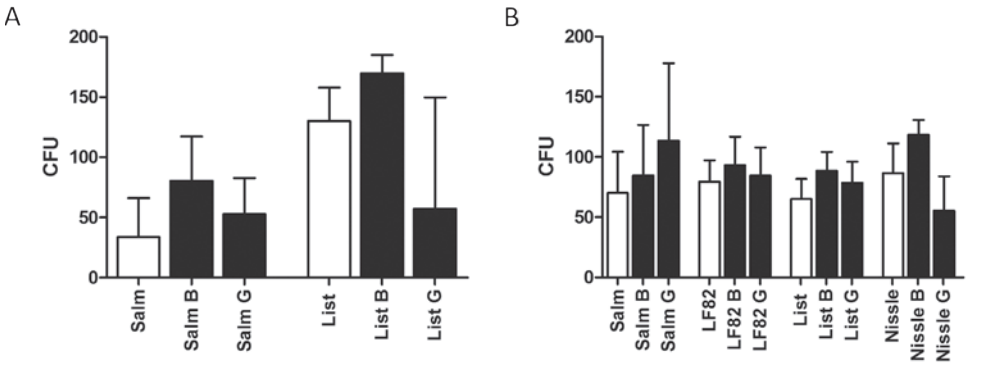


**Figure 4.5:** A) CFU of mid-log phase cultures of *S. enteritidis* (salm) and *L. monocytogenes* (list) incubated with and without RegIIIβ (B) and RegIIIγ (G). B) CFU counts with stationary phase cultures of *S. enteritidis*, *L. monocytogenes*, *E. coli* LF82 or *E. coli* Nissle incubated with and without RegIIIβ (B) and RegIIIγ (G).

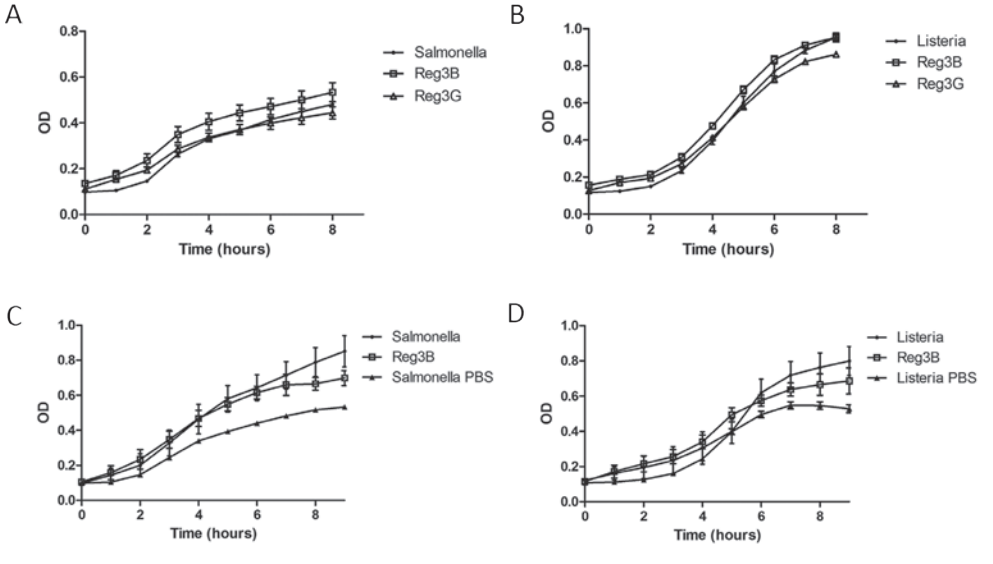


**Figure 4.6.** Growth curves of *S. enteritidis* (A) and *L. monocytogenes* (B) for 8 hours with or without processed RegIIIγ or RegIIIβ. The two lower panels show incubation of *S. enteritidis* (C) and *L. monocytogenes* (D) with the secreted RegIIIβ produced using the baculovirus system.

**Molecular docking**

The binding modes of the previously identified fucose-containing glycans 20, 23, 24, 26 (Chapter 3), the carbohydrate part of Lipid A and the carbohydrate repeat unit of PGN (β-(1,4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid) to RegIIIβ were modelled by molecular docking (Table 4.1). The lowest energy predicted binding poses of all carbohydrates dock into one of two binding sites on RegIIIβ, designated as binding site 1 (BS1) and binding site 2 (BS2).

Interestingly, the lowest energy binding poses of PGN and the fucose-containing glycans 20 and 24 docked into BS1 whereas lipid A, and the glycans 23 and 26 docked into BS2. The only known ligand of RegIIIγ, PGN, was shown to dock into a region in loop 1 which is largely conserved in RegIIIβ (BS1, Table 4.1). A space-filling model of RegIIIβ shows the binding of glycan 20 to BS1 and lipid A to BS2, and also shows that BS2 is not conserved in RegIIIγ (Fig. 4.7).

| Glycan | Monosaccharides | Links | Predicted Binding Site in RegIIIβ |
|---|---|---|---|
| **20** | GlcNAc, Man, Fuc | Manβ1-4(Fucα1-3)GlcNAcβ1-4GlcNAcβ-$O(CH_2)_5NH_2$ | BS 1* |
| **23** | GlcNAc, Man, Fuc | Manα1-3Manβ1-4(Fucα1-3)GlcNAcβ1-4(Fucα1-6)GlcNAcβ-$O(CH_2)_5NH_2$ | BS 2** |
| **24** | GlcNAc, Man, Fuc | GlcNAcβ1-2Manα1-3Manβ1-4(Fucα1-3)GlcNAcβ1-4(Fucα1-6)GlcNAcβ-$O(CH_2)_5NH_2$ | BS 1* |
| **26** | GlcNAc, Man, Fuc | GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-$O(CH_2)_5NH_2$ | BS 2** |
| **Lipid A** | GlcNAc | GlcNAcβ1-6GlcNAc | BS 2** |
| **peptidoglycan** | MurNAc, GlcNAc | MurNAcβ1-4GlcNAc | BS 1 * |

**Table 4.1** Glycans and their predicted binding sites in RegIIIβ. * BS1 involves interactions in and around the 'EPN' and the DPT' motives in loop 1. ** BS2 involves interactions in and around the ERN motif in loop 2

## DISCUSSION

The goal of this study was to characterize the binding properties and bactericidal or bacteriostatic effects of recombinant RegIIIβ and RegIIIγ. Additionally we compared the predicted structure and ligand binding sites using molecular docking tools *in silico.*

High-level expression of RegIIIβ and RegIIIγ in *E. coli* leads to formation of inclusion bodies, which could be isolated, denatured, affinity purified and refolded to generate soluble RegIII proteins. In contrast, secretion of soluble recombinant RegIIIβ could be achieved in insect cells using the baculovirus expression system. The size of RegIIIβ produced in insect cells and of RegIIIβ produced in *E. coli* was as predicted, suggesting that the proteins were not post-transcriptionally modified by glycosylation. This is in agreement with the lack of a consensus sequence for N and O-glycosylation (Asn-Xaa-Ser/Thr) in RegIIIβ and with the reported data on RegIIIγ.[34] In the absence of reducing agents, the recombinant RegIII proteins form multimers, which were evident in SDS-PAGE. Indeed, RegIIIβ was previously reported to form aggregates after N-terminal processing.[35-36] Aggregation of bacteria by PAP has been reported previously and was proposed as an antimicrobial function.[19, 20] However, we did not observe aggregation of bacteria in our bactericidal assays. Previously, Hassanain *et al.*[37] demonstrated that aggregates of PAP had a different function than non-aggregated PAP.

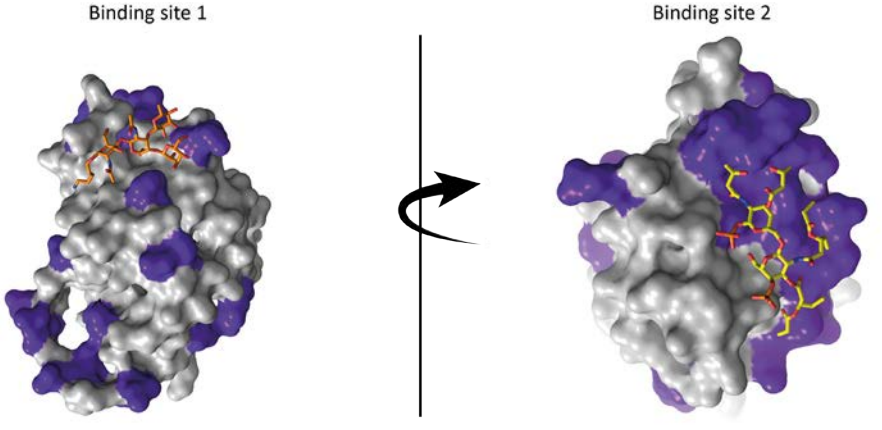


**Figure 4.7.** Space-filling model of RegIIIβ. Residues which are different from RegIIIγ are shown in purple. Ligands (glycan number 20 in BS1 and Lipid A in BS2) are shown as sticks. Rotation of RegIIIβ by 180° is indicated by an arrow.

A structural model of RegIIIβ showed the N-terminal pro-segment sequence that is proposed to interact with a charged surface on in RegIIIγ and HIP/PAP to prevent antimi-

crobial activity. Cleavage of this pro-segment by trypsin was shown to enhance bactericidal activity of RegIIIγ.[18, 19] This may be a mechanism to allow the host to restrict the binding activity of RegIII proteins to the lumen or to avoid detrimental effects inside host cells during storage or secretion. We demonstrated in this study that the predicted pro-segment was also cleaved from RegIIIβ by trypsin. Immunoblotting showed that trypsin treatment of soluble RegIIIβ produced in insect cells generated a single major protein whereas the refolded RegIIIβ produced in *E. coli* appeared to be cleaved into at least 3 polypeptides of different sizes. Analysis of the potential trypsin cleavage sites within the RegIIIβ primary protein sequence indicated several possible cleavage sites (not shown), which may have become accessible to trypsin in alternative protein conformations. These results point to the fact that refolding of RegIIIβ may have increased its susceptibility to proteolysis by trypsin. In contrast to RegIIIβ, recombinant RegIIIγ yielded a single protein band upon cleavage with trypsin as previously described.[18] One explanation for the difference in trypsin digestion patterns of RegIIIβ and RegIIIγ might be the fewer number of potential trypsin cleavage sites in RegIIIγ. For this reason the RegIIIβ protein produced in insect cells was used in the bactericidal and binding assays although refolded RegIIIβ was included in some assays for comparison.

We showed that an important consequence of N-terminal processing is enhanced binding of both RegIIIβ and RegIIIγ to bacteria, further supporting the idea that cleavage of the N-terminal peptide indeed activates the protein. Unexpectedly, we observed binding of RegIIIγ to Gram-negative bacteria although the only known bacterial ligand is PGN[11] which is not exposed in Gram-negative bacteria. This suggests that RegIIIγ may bind to another glycan, possibly in the carbohydrate O-antigen of LPS.

RegIIIβ bound to *L. monocytogenes, E. coli* and *S. enteritidis* as did RegIIIγ, despite having only a reported bactericidal activity on Gram-positive *L. monocytogenes*.[11] Although processed RegIIIβ bound to all bacteria tested, it was not bactericidal for *L. monocytogenes* and *S. enteritidis* based on CFU plate counts. Using flow cytometry and live/dead staining on logarithmically growing *E. coli* strains we did measure moderate killing of *E. coli* strain VE7108 and small effects on *E. coli* Nissle in the absence of any apparent bacterial aggregation. The conditions for binding and bactericidal assays were the same as those described previously for RegIIIγ[11] and RegIIIβ.[16] Addition of reducing agent DTT had no effect on binding activity. Recently, recombinant RegIIIβ was shown to reduce CFU of *E. coli* harvested in late log phase to about 20% of the starting value[16] after 30 min incubation. *Clostridium butyricum* was also killed by RegIIIβ, but not the more aerotolerant Gram-positive bacteria *Lactobacillus murinus* and *Enterococcus feacalis,* suggesting bactericidal activity against some, but not all, Gram-positive bacterial species. In a follow up paper by the same group the bactericidal effect of RegIIIβ on *Salmonella typhimurium* was reported to be optimal in early logarithmic growth phase,[17] for reasons that are not yet fully understood. However, the bacterial growth phase appeared to have no effect on RegIIIβ binding or bactericidal activity against *S. enteritidis*, *E. coli*

or *L. monocytogenes* in our experiments. In recent studies RegIIIβ was shown to bind lipid A[17] and PGN.[17, 30] This would explain binding of this protein to both Gram-positive and Gram-negative bacteria although RegIIIβ would need to penetrate the hydrophobic outer carbohydrate part of the LPS to reach the lipid A component near the outer membrane.

To investigate potential bacteriostatic effects of RegIII proteins on bacterial growth we added N-terminally processed and unprocessed proteins to low density bacterial cultures. This had no influence on bacterial growth, as judged by the growth curve. With *L. monocytogenes*, the final OD is lower after addition of RegIIIγ compared to the control, even though the growth rate was not influenced. A disadvantage of this method is that we cannot rule out aggregation of the bacteria, which could interfere with the OD measurement. The added RegIII proteins could still be detected in the supernatant after completion of the growth curve measurements as was shown by western blot (data not shown). However, it might be that the RegIII proteins are not active under these conditions.

Degradation of our RegIII proteins during different incubation steps was not observed, but it is possible that they lose activity over time. Although our Reg3 proteins showed binding to bacteria, it is possible that the refolded Reg3 proteins produced in E. coli were incorrectly folded explaining the inconsistencies with published results on their bactericidal activity. The latter is difficult to check, as crystallization and X-ray crystallography was not possible. Inconsistencies in the bactericidal role of human PAP have also been reported by others.[19, 20] The different results found by Medveczky,[19] Mukherjee[18] and us could be related to different bacterial strains used, different PGN preparations, possible interference of bacterial aggregation with viability assays and proteolytic instability of the PAP pro-segment.[19] In particular, measurement of reduction in bacterial CFU as a method for assessing bactericidal activity, does not exclude the possibility of bacterial aggregation unless this is carefully checked by other methods.

Overall, these results support a role for binding of RegIII proteins to bacteria but the *in vitro* bactericidal effects described in the literature appear difficult to reproduce, even using the same assay conditions and bacterial strains. The bactericidal effects reported to date were in any case relatively modest suggesting that the assay conditions were not optimal or that refolded recombinant RegIII proteins were not fully active *in vitro*. Therefore, future efforts to optimize the expression of soluble RegIII proteins in mammalian cells, seems warranted.

Structural models of RegIIIβ and RegIIIγ were generated based on the crystal structure of human PAP.[38] The carbohydrate binding domains of the C-type lectin family involves the characteristic long loop (comprising loop 1 and loop 2), which is surface exposed and these loops are also present in RegIIIβ and RegIIIγ. To investigate the ligand binding sites in RegIIIβ and RegIIIγ the ligands known to bind (unpublished

data, Chapter 3) were docked to the predicted structures. In RegIIIβ two binding sites were identified, the results suggest that BS2 binds to lipid A and two other glycans and the BS1 binding to PGN and 3 different fucose-containing glycans previously identified. PGN was predicted to dock in BS1 containing an EPN motif that was shown to be necessary for binding of HIP/PAP to PGN. Indeed, both RegIIIβ and RegIIIγ show this EPN motif. However, mutation of this motif in RegIIIβ was shown previously not to affect binding to PGN.[17] The so-called BS2 interacts with residues within and around the ERN motif in loop 2, as previously shown.[17] These interacting resides are not conserved in RegIIIγ. The variability of the ERN motif and the surrounding residues in loop 2 might therefore determine the differences between carbohydrate recognition and bactericidal activity between RegIIIβ and other C-type lectins, including RegIIIγ, which is in agreement with the findings of Miki *et al.*[17]

RegIIIβ and RegIIIγ were found to contain a BS1 involving different interacting residues including the EPN and DPT motifs in loop1 that are conserved in both proteins, which would explain the finding that RegIIIβ and RegIIIγ can both bind PGN.[11, 17, 30] The other important implication of these results is that RegIIIβ has two ligands binding sites. As the BS1 was shown to dock to fucose-containing glycans, we hypothesize that RegIIIβ could bind to both mucus and bacterial lipid A, thereby trapping bacteria in the mucus. The binding of RegIIIβ to mucus might also have an effect on mucus structure and or penetrability of bacteria. Further work is in progress to test these hypotheses and identify other possible ligands of RegIIIγ.

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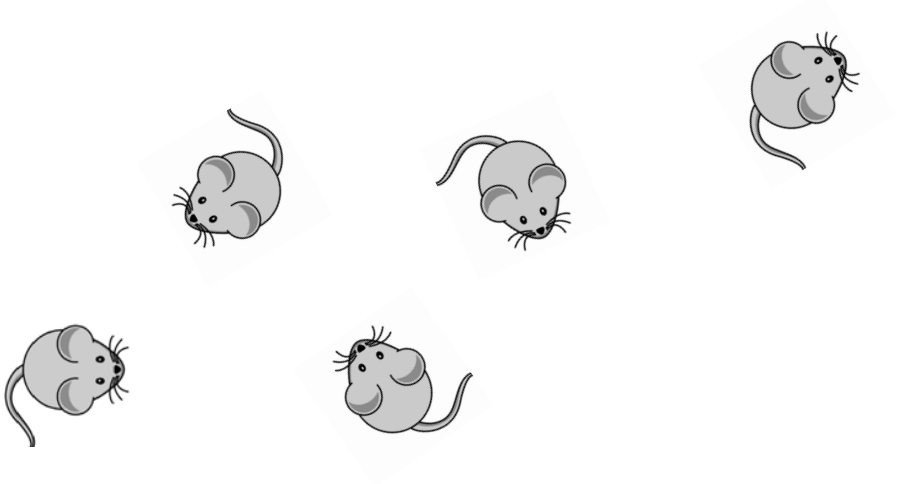

# Chapter 5

# Intestinally secreted C-type lectin RegIIIβ attenuates Salmonellosis but not Listeriosis in mice

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**ABSTRACT**

**Background:** The RegIII protein family, including the human member designated pancreatitis-associated protein, are secreted proteins that contain a C-type lectin domain involved in carbohydrate binding. They are expressed by intestinal epithelial cells. Colonization of germ-free mice and intestinal infection with pathogens increases the expression of RegIIIγ and RegIIIβ in the murine ileum. RegIIIγ is directly bactericidal for Gram-positive bacteria but the exact role of RegIIIβ in bacterial infections is unknown.

**Methods:** To investigate the possible protective role of RegIIIβ in intestinal infection RegIIIβ-knockout($^{-/-}$) mice and wild-type (WT) mice were orally infected with the Gram-negative *Salmonella enteritidis* or the Gram-positive *Listeria monocytogenes*. At day 2 (*Listeria* infection) and at day 4 (*Salmonella* infection) after oral infection mice were sacrificed to collect intestinal and other tissues for pathogen quantification. Protein expression of RegIIIβ and RegIIIγ was determined in intestinal mucosal scrapings of infected and non-infected mice. In addition, ex-vivo binding of ileal mucosal RegIIIβ to *Listeria* and *Salmonella* was investigated.

**Results:** Whereas recovery of *Salmonella* or *Listeria* from feces of RegIIIβ$^{-/-}$ and WT mice was not different, significantly higher numbers of viable *Salmonella*, but not *Listeria*, were recovered from the colon, mesenteric lymph nodes, spleen and liver of the RegIIIβ$^{-/-}$ mice than from those of WT mice. Mucosal RegIIIβ binds to both bacterial pathogens and may interfere with their mode of action.

**Conclusion:** RegIIIβ plays a protective role against intestinal translocation of the Gram-negative bacterium *S. enteritidis* in mice, but not against the Gram-positive bacterium *L. monocytogenes.*

## INTRODUCTION

In many countries in the industrialized world, foodborne intestinal infections are continuing to increase.[1] For example, outbreaks of salmonellosis and listeriosis have been reported for decades, but within the past 25 years their incidence has increased on many continents.[2] To inhibit the colonization (adhesion to the intestinal epithelium) and translocation (invasion of host tissues) of foodborne pathogens and commensals the intestinal mucosal surfaces are armed with an array of physical and chemical defence mechanisms. The low pH of the gastric compartment, and bile acids secreted in the proximal intestine, reduce the number of bacteria that will survive within the gut.[3, 4] Other important defence mechanisms against pathogens include competition for nutrients and adhesion sites from commensal bacteria, a thick mucus layer on the luminal side of the epithelium and the rapid innate response of the intestinal epithelium.[5, 6]

Studies in rodents indicate that innate recognition of bacteria or bacterial components triggers epithelial expression of secreted C-type lectins RegIIIγ and RegIIIβ.[7-10] In rodents mucosal and faecal levels of RegIII protein are associated with the severity of infection.[9] The human homologue of this protein, i.e. they are on the amino acid level the most identical genes between two species (bidirectional best hit), is pancreatitis-associated protein (PAP). PAP is also detectable in faeces and its use as biomarker to monitor or discriminate human intestinal disease is the subject of ongoing studies.

Currently, the role and function of the Reg3 family members is of much interest. The murine RegIIIγ and human PAP have been shown to have antimicrobial activity against Gram-positive bacteria but not the Gram-negative *Escherichia coli*.[8] PAP and RegIIIγ were shown to bind peptidoglycan carbohydrate, which is critical for bacterial killing.[11] Expression of RegIIIγ is greatly reduced in mice deficient in MyD88, an intracellular adaptor protein involved in most Toll-like receptor (TLR)-mediated signalling. MyD88-deficient mice have been shown to be more susceptible to *L. monocytogenes* infection compared with wild-type (WT) mice.[12] Injection of recombinant RegIIIγ into the lumen of MyD88-deficient ligated ileal loops before inoculation with *L. monocytogenes* was shown to reduce *L. monocytogenes* survival in the gut. Other *in vivo* studies showed that genetic ablation of RegIIIβ, which is another RegIII murine isoform, resulted in impaired clearance of the bacterial load in ileal Peyer's patches during *Yersinia pseudotuberculosis* infection without affecting luminal bacterial levels.[13] It remains unclear, however, how RegIIIβ protects against bacterial translocation and whether this is specific for Gram-negative pathogens.

In this study we used RegIIIβ-knockout($^{-/-}$) mice and their WT littermates to investigate the protective role of RegIIIβ following oral challenge with Gram-negative *Salmonella enteritidis* or Gram-positive *L. monocytogenes.* Additional *ex-vivo* experiments were performed to gain insights into the mechanisms involved in the protective function of

RegIIIβ.

## MATERIALS AND METHODS

### Animals and diet

The experimental protocol was approved by the animal welfare committee of Wageningen University (Wageningen, the Netherlands). RegIIIβ$^{-/-}$ mice with a C57Bl/6 x 129O1a background were generated as described.[14] Knock-out mice were obtained by breeding heterozygote mice. Genotyping was performed as described previously[14] and $^{-/-}$ mice were used as knockout and $^{+/+}$ littermates were used as WT control. The mice were selected on genotype and age and were fed a semi-purified AIN93-G[15] diet (Abdiets, Woerden, The Netherlands). At 7-9 weeks of age mice with an average body weight of 22 g (range 16–31 g) were housed individually. They received a purified diet containing per kg: 200 g acid casein, 326 g cornstarch, 174 g glucose, 160 g palm oil, 40 g corn oil, 50 g cellulose, and vitamin and mineral mix (without calcium) according to AIN93.[15] Diets were supplemented with $CaHPO_4.2H_2O$ (Merck, Darmstadt, Germany) to a final concentration of 30 mmol/kg. To mimic the composition of a Western human diet, the prepared diets were relatively low in calcium and high in fat compared to standard rodent diets.[15] Food and demineralized drinking water were supplied ad libitum. Mice were separated into groups and age and gender were randomized over all groups as much as possible. The power analysis used to calculate the minimal number of animals needed per group was based on previously performed infection studies in this model (unpublished results). In total 3 RegIIIβ$^{-/-}$ and 3 WT groups were formed. From each mouse strain one group was orally infected with *Salmonella* ($^{-/-}$: n=10, WT: n=12), a second group of each strain was orally infected with *Listeria* ($^{-/-}$: n=7, WT: n=8) and a final group was sham treated ($^{-/-}$: n=5, WT: n=6). Body weight was measured every two days before infection and daily after infection.

### Nramp1 genotype of Salmonella infected mice

To monitor equal susceptibility to *Salmonella* infection the natural resistance-associated macrophage protein 1 (Nramp1) genotype was determined in the *Salmonella* infected RegIIIβ$^{+/+}$ and RegIIIβ$^{-/-}$ mice. A PCR product was created from the genomic DNA by using primers 5'GGAATGAATGTCAAGCAGCCAG 3' and 5'ATCCCACCTCATAGCCGAAG 3' in order to sequence the area of the gene that is known to vary between different mouse strains (G or A at position 596) in the Nramp1 gene.[16] The PCR cycle was 3 min at 95˚C, 35 cycles of 30 s at 95˚C, 30 s at 58˚C and 1 min at 72˚C, ending with 5 minutes at 72˚C. The PCR product was subsequently sequenced by Baseclear (Leiden, the Netherlands), using the following primers 5' CACCCCATTTCCAGTAGGG 3' and 5' CCTGTGACACCTGGATGTTC 3', to determine the Nramp1 genotype of the Salmonella infected mice.

**Infection**

After adaptation to individual housing and diet for 8 days, mice were orally infected by gavage with 0.2 mL saline containing $5*10^8$ CFU of *S. enteritidis* (clinical isolate, phage type 4; strain NIZO1241, NIZO food research, Ede, the Netherlands) or *L. monocytogenes* (animal isolate, EGD-e, serotype 1/2a, NIZO food research). The virulence of each strain was sustained by routine oral passage in RegIIIβ$^{+/-}$ mice and subsequent isolation of the pathogen from extra-intestinal organs. *S. enteritidis* was grown on BGAM agar plus Sulphamandalate supplement (Oxoid, Basingstoke, United Kingdom) and quantified as described.[17] *L. monocytogenes* was grown and determined by similar culturing methods but by using PALCAM agar[18] plus PALCAM selective supplement (Oxoid).

**Collection of biological samples and bacterial quantification**

A pilot study indicated that determination of the number of *L. monocytogenes* and *S. enteritidis* in organs of mice was optimal, which means that *Listeria* and *Salmonella* CFU could be detected in organs, at 2 and 4 days after oral infection, respectively (unpublished results). Non-infected mice were sacrificed 3 days after oral sham treatment. Mice were anaesthetized by isoflurane and blood was collected via an orbital puncture to isolate heparin plasma. After cervical dislocation the colon and the distal 1/3 of the small intestine, representing the ileum, were excised. Approximately 1 cm was cut from the middle of each intestinal segment to quantify the number of viable *Salmonella* or *Listeria* present in colonic and ileal tissue. This piece was cut open longitudinally, briefly flushed in sterile saline, homogenized in 250 µl of saline and 10-fold dilutions were plated for bacterial growth on selective agar for *Salmonella* or *Listeria* (as described above). The remaining parts of the ileum and colon were cut open longitudinally, flushed with saline and the mucosa was isolated by scraping with a spatula. Mucosal samples were immediately frozen in liquid nitrogen for protein analysis. Furthermore, the mesenteric lymph nodes (MLN), spleen and liver were removed, and after homogenization in sterile saline, directly used for *Salmonella* or *Listeria* quantification as described above. Bacterial counts were expressed as the total $\log_{10}$ CFU per gram tissue.

**RegIIIβ and RegIIIγ protein analysis in the ileal mucosa**

RegIIIγ and RegIIIβ expression were determined in the ileum because this is the area where expression is upregulated during infection of rats[9, 10] and upon microbial colonization of germ-free mice.[8] Frozen mucosal scrapings of the ileum were pulverized under liquid nitrogen. Approximately 2/3 of the pulverized tissue was suspended in a 0.2 mol/l sucrose buffer of pH 7.4 containing 20 mmol/l trishydroxymethylaminomethane (Tris) and a protease inhibitor cocktail (Complete, Roche Diagnostics). To homogenize the samples they were sonicated on ice for 30 s at level 4 (Sonicator XL2020, Heat Systems, Farmingdale, NY, USA) and protein concentrations were determined using the BC Assay (Omnilabo, Breda, the Netherlands) according to the manufacturer's protocol. Samples from each group were pooled per group and 100 µg protein was denatured at 95°C for 10 min in sample buffer (4x buffer: 160 mM Tris, pH 6.8, 8% SDS, 40% glycerol,

0.05% w/v bromophenol blue), subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (4.8% stacking-gel, pH 6.8, 12.5% separation-gel, pH 8.8) and transferred to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA, USA). After blocking, the membranes were incubated with polyclonal anti-RegIIIβ (1:50 000) or anti-RegIIIγ (1:10 000) antibodies (custom made by Eurogentec, Seraing, Belgium). These antibodies were generated in rabbits against synthetically produced peptides, using the peptide sequences GEDSLKNIPSARISC (RegIIIβ) and EVAKKDAPSSRSSC (RegIIIγ). The chosen peptide sequences correspond to unique sequences within the RegIII protein and allow differentiation between the RegIIIβ and RegIIIγ proteins. Serum from immunized rabbits was affinity purified using the same peptides. The signal of the secondary HRP-conjugated antibody (Goat-anti-Rabbit HRP, 1:100 000, Jackson ImmunoResearch, Suffolk, UK) was detected by using the ECL Plus chemiluminescent detection kit (GE Healthcare, Den Bosch, the Netherlands).

**Myeloperoxidase analysis in ileal mucosa and serum amyloid A detection in plasma**
A mouse myeloperoxidase (MPO) ELISA test kit (Hycult biotechnology, Uden, the Netherlands), was used according to the manufacturer's guidelines to determine the concentration of MPO in ileal mucosal scrapings.

Serum amyloid A (SAA) 2, an acute phase apolipoprotein present in plasma,[19] was determined to investigate systemic inflammation. A mouse SAA2 ELISA kit (Life Diagnostics, Inc. West Chester, Pennsylvania, USA) was used according to the guidelines of the manufacturer.

**Pull-down assay**
To investigate the possible mode of action it was evaluated whether RegIIIβ present in the ileal mucosa binds to *S. enteritidis* or *L. monocytogenes* in a pull-down assay. *S. enteritidis* and *L. monocytogenes*, identical to the strains used in the infection study, were grown overnight (37°C in a horizontal shaker at 250 rpm) in LB and BHI broth, respectively.

Overnight grown bacteria were spun down (3 min at 2350 g) and $1*10^8$ bacteria were resuspended in 20 µl ileal mucosal scraping extracts from *Salmonella* infected RegIIIβ$^{-/-}$ or WT mice, which were diluted 1:5 in the above described sucrose buffer. The bacteria were incubated for 30 min at 37°C. After spinning down the bacteria (5 min at 1500 g) presence of RegIIIβ was investigated in the supernatant (15 µl) by SDS-PAGE as described above. Proteins were transferred to an Immobilon-FL membrane (Millipore) and after blocking exposed to rat anti-mouse RegIIIβ antibodies (MAB5110; R&D systems, Minneapolis, USA, 1:2000). The secondary antibody used was Goat-anti-Rat IRDye® 680CW (Li-Cor, Nebraska, USA, 1:20 000) and the blots were scanned by using the Odyssey scanner (Li-Cor). Experiments were performed in triplicate.

**Statistics**

All data are expressed as mean ± SEM and statistical analysis was performed by using Prism 5.0 Software (GraphPad, San Diego, California, USA). Our aim was to investigate the role of RegIIIβ in infection. Therefore the pre-defined comparisons were RegIIIβ$^{-/-}$ versus WT. Except for in vitro investigations to evaluate binding of RegIIIβ to *Salmonella* and *Listeria* when binding to these pathogens were investigated. Data were tested for normality by Kolmogorov-Smirnov normality test and Shapiro-Wilk normality test. If normally distributed, differences were tested for significance using Student's *t* test (two-tailed). Data with unequal variances were tested by using the Mann-Whitney U test. *Salmonella* output in feces was determined at multiple time points and therefore these data were analyzed by repeated-measures two-way ANOVA (mixed-model). Differences were considered statistically significant when $P<0.05$.

## RESULTS

**Body weight**

To monitor the general condition of the mice, body weight was determined during the experiment. At the start of the experiment average body weight was 22 ± 0.7 g. During the study there was no significant difference between the body weight of RegIIIβ$^{-/-}$ and WT mice (data not shown). Average body weight before infection (average weight during 2 weeks prior to infection) was 22.8 ± 1.4 g and 23.1 ± 1.4 g for WT and RegIIIβ$^{-/-}$ groups, respectively. Post-infection body weight of WT and RegIIIβ$^{-/-}$ groups was 23.2 ± 1.4g and 23.2 ± 1.5 g (average of the post infection period).

**Bacterial infection increases RegIIIβ and RegIIIγ in the ileum mucosa**

To evaluate presence of RegIIIβ and RegIIIγ protein in the ileal mucosa of WT and RegIIIβ$^{-/-}$ mice, and subsequent levels upon oral infection, mucosal scrapings were evaluated by immunoblotting using antibodies specific for RegIIIβ and RegIIIγ. As expected, RegIIIβ was not detected in the ileal mucosa of RegIIIβ$^{-/-}$ animals (Fig 5.1). After *Salmonella* and *Listeria* infection the protein level was higher in the mucosal samples of WT mice than before infection (Fig 5.1). The isoform RegIIIγ was not detectable in mucosal scrapings of non-infected RegIIIβ$^{-/-}$ mice (Fig 5.1). In contrast, the WT mice had detectable levels of this Reg3 isoform and levels increased during *Salmonella* and *Listeria* infection in WT and RegIIIβ$^{-/-}$ mice.

**RegIIIβ$^{-/-}$ mice are more susceptible to Salmonellosis but not to Listeriosis than WT mice**

In order to ensure similar Nramp1 genotypes in all *Salmonella* infected animals these mice were sequenced for a previously reported point mutation in this gene, which could affect infection susceptibility.[16] All mice were shown to be homozygous for the Nramp1 susceptibility allele (data not shown). To investigate the role of RegIIIβ in intestinal colo-

nization, the numbers of viable *Salmonella* were investigated in fresh faecal samples of infected WT and RegIIIβ$^{-/-}$ mice. Colonization of *Salmonella* in RegIIIβ$^{-/-}$ mice was not different from that of WT mice (Fig 5.2). In the colonic tissue, which was first flushed with saline to remove intestinal content, higher numbers of *Salmonella* were recovered in RegIIIβ$^{-/-}$ mice than in WT mice (Fig 5.3A; $P<0.05$). In the ileal tissue *Salmonella* levels were identical in RegIIIβ$^{-/-}$ and WT mice. Furthermore, in MLN, spleen and liver these levels were higher in RegIIIβ$^{-/-}$ mice than in their WT counterparts (Fig 5.3B; $P<0.05$).

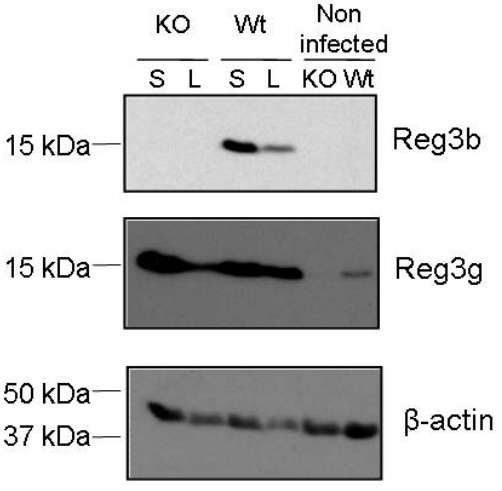


**Figure 5.1**. Western blot analysis of RegIIIβ and RegIIIγ in pooled ileal mucosa samples of RegIIIβ$^{-/-}$ mice (KO) or WT mice (WT)**.** RegIIIβ$^{-/-}$ mice (n=10) and WT mice (n=12) were orally infected with *S. enteritidis* (S) or RegIIIβ$^{-/-}$ mice (n=7) and WT mice (n=8) were orally infected with *L. monocytogenes* (L). Non-infected mice (n=5) received saline as control treatment.

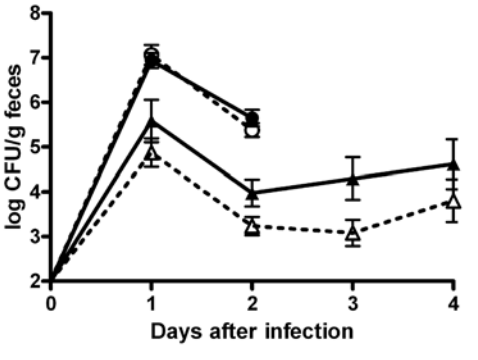


**Figure 5.2**. Salmonella (▲, Δ) and Listeria (●, ○) excretion in faeces. Individually housed mice were orally infected at day 0. RegIIIβ$^{-/-}$ mice (closed symbols) were infected with $5*10^8$ colony-forming units (CFU) of S. enteritidis (n=10) or L. monocytogenes (n=7) and WT littermates (open symbols) with identical CFU of S. enteritidis (n=12) or L. monocytogenes (n=8). Values are means ± SEM. Mucosal and

systemic inflammation.

Intestinal colonization of *Listeria* was identical in RegIIIβ$^{-/-}$ and WT mice as found for *Salmonella* (Fig 5.2). To further investigate effects of RegIIIβ on *Listeria* translocation the CFU in ileal and colonic tissue and extra-intestinal organs were determined. The number of *Listeria* recovered from WT did not differ from RegIIIβ$^{-/-}$ mice in ileal and colonic tissues, (Fig 5.3C) and in MLN, spleen, and liver (Fig 5.3D).

The role of RegIIIβ in inflammation was investigated by measuring the levels of the inflammation marker myeloperoxidase (MPO) in the ileum mucosa at 2 and 4 days after oral infection of *Listeria* and *Salmonella* groups, respectively. MPO levels of Salmonella (0.49± 0.05 ng/mg protein) and Listeria (0.39 ± 0.06 ng/mg protein) infected groups did not differ significantly from the non-infected groups (0.35 ± 0.05 ng/mg protein; data not shown). To study systemic inflammation SAA levels in plasma were determined at the same time points. The levels were elevated from 0.1 ± 0.001 µg/ml (non-infected mice) to 1164 ± 186 and 502 ± 174 µg/ml upon *Salmonella* and *Listeria* infection, respectively ($P$<0.05; data not shown). However, there were no significant differences between WT and RegIIIβ$^{-/-}$ mice.

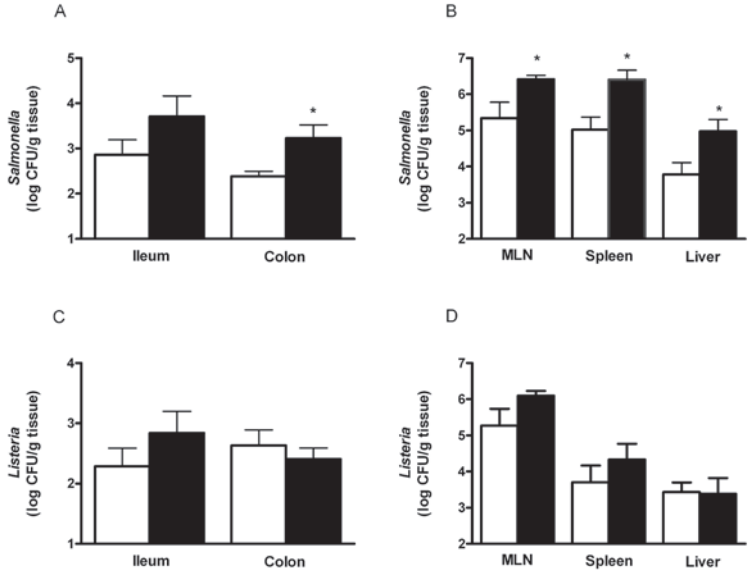


**Figure 5.3**. Salmonella (A, B) and Listeria (C, D) in intestinal and extra-intestinal tissues at 4 and 2 days after oral infection, respectively. CFU of pathogens in the ileal and colonic mucosa (A, C) and in mesenteric lymph nodes (MLN), spleen and liver (B, D) of RegIIIβ$^{-/-}$ mice (n=10; black bars) or WT mice (n=12; white bars). Values are means + SEM. *Different from WT, $P$<0.05.

**RegIIIβ binds to *Salmonella* and *Listeria***

To investigate the possible mode of action it was evaluated whether RegIIIβ present in the ileal mucosa binds to *S. enteritidis* and *L. monocytogenes* in a pull-down assay. Upon ex-vivo incubation of mucosal samples from infected mice with these bacteria it was shown that RegIIIβ protein levels decreased in the supernatant (Fig 5.4). It was mainly the lower-molecular-weight form of the proteolitically processed protein that decreased after incubation with bacteria. This indicated that it is the cleaved form of RegIIIβ that binds to the pathogens and not the full length polypeptide. We have shown previously that the RegIII protein is not precipitated even when centrifuged at 15000 *g*.[9] Therefore, the results presented here indicate that ileal mucosal RegIIIβ is able to directly bind both *Salmonella* and *Listeria*.

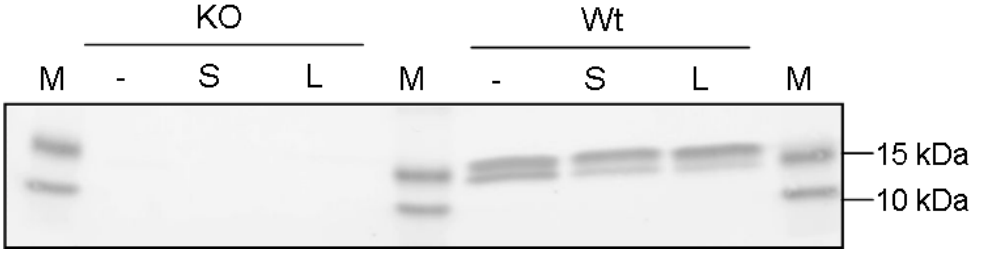


**Figure 5.4**. *Listeria* and *Salmonella* pull-down assays. Presence of RegIIIβ, detected by Western blot analysis, in supernatant fractions of ileal mucosa samples from *S. enteritidis* infected WT (WT) or RegIIIβ$^{-/-}$ (KO) mice incubated with *Salmonella* or *Listeria.* Mucosal samples incubated without bacteria served as negative control (-). The pre-stained protein marker is indicated by M.

## DISCUSSION

Both microbial infections of conventional mice and intestinal colonization of germ-free mice by commensals increases ileal expression of the secreted C-type lectins RegIIIγ and RegIIIβ.[7-10] While RegIIIγ has been shown to have a bactericidal activity against Gram-positive bacteria,[8] the physiological function of RegIIIβ remains unknown. This study demonstrates a role for RegIIIβ in protection against infection with the Gram-negative *S. enteritidis*. Despite the fact that similar levels of viable *Salmonella* were recovered from faeces higher numbers were present in MLN, spleen and liver of infected RegIIIβ$^{-/-}$ mice compared to WT mice, indicating that the translocation and dissemination of *Salmonella* in host tissues is increased in the absence of RegIIIβ. As the mice were bred on a mixed background (C57Bl/6 and 129O1a) and these strains differ in their susceptibility to *Salmonella* infection due to a mutation (G → A) in the Nramp1 gene, all mice were genotyped individually.[16] All the *Salmonella* infected animals were shown to be ho-

mozygous for the Nramp1 susceptibility allele, ruling out the possibility that the Nramp status would affect the results. In colonic tissue *Salmonella* levels were also increased in RegIIIβ$^{-/-}$ mice compared with WT mice. The MLN drain the small intestine, which is a main site of *Salmonella* invasion.[20] Therefore reduced levels of *Salmonella* in the MLN of WT mice suggest protective effects of RegIIIβ on *Salmonella* translocation in the ileum. Although we did not observe differences in *Salmonella* counts in the ileal tissues of WT and RegIIIβ$^{-/-}$ mice, this may be due to lack of statistical power in this assay, as SEM levels are higher in ileal tissue than those in colonic tissue. Moreover the investigated ileal tissue did not contain Peyer's patches and M cells, which are considered, in addition to absorptive enterocytes, to be important sites of *Salmonella* translocation.[21, 22] Together the experimental infection data strongly suggest that RegIIIβ inhibits *Salmonella* translocation from the gut lumen into intestinal tissues and further extra-intestinal tissues but does not kill *Salmonella* in the gut lumen. These results are supported by recent findings of Dessein *et al*., who showed that genetic ablation of RegIIIβ did not affect the bacterial load of the Gram-negative pathogen *Yersinia pseudotuberculosis* in the intestinal lumen[13] but significantly increased bacterial burden in the Peyer's patches, which are the main route of entry for this pathogen. In contrast, RegIIIβ did not to influence resistance to infection with the Gram-positive *Listeria monocytogenes*, as identical numbers of this pathogen were recovered from intestinal and extra-intestinal tissues in RegIIIβ$^{-/-}$ and WT mice as found in our study.

As murine RegIIIγ and human PAP are directly bactericidal for Gram-positive bacteria *in vitro*[8, 23] it seemed possible that RegIIIβ would have similar antimicrobial activity. However, in this study there was no effect of RegIIIβ on the colonization and translocation of *L. monocytogenes*. The strain of *L. monocytogenes* used in our infection study was identical to that used previously for *in vitro* antibacterial assays with RegIIIγ.[8] Previous investigations with rat PAP1, which is the homologue of the murine RegIIIβ, also showed no direct bactericidal effect against *L. monocytogenes in vitro.*[9] Others also report a lack of bactericidal effect of this protein.[13] The co-existence of the two closely related RegIII proteins in the mouse strongly suggests a different function for each of these proteins. Here we provide evidence that RegIIIβ has a protective role against Gram-negative *Salmonella* translocation but not *Listeria* infection. The exact mechanism by which RegIIIβ inhibits *Salmonella* translocation into host tissues is unclear.

The observed difference of RegIIIβ functionality between Listeriosis and Salmonellosis might be due to differences in physical appearance and host invasion mechanisms of the two pathogens but might also be related to differences in host responses induced by these pathogens. We can only speculate about the latter as we did not monitor the exact (immune) response of the mice. Previous studies by Brandl *et al*.[12] indicated that the innate immune defence against *L. monocytogenes*, which requires Toll-like receptor (TLR)-mediated signals, plays a crucial role in RegIIIγ related protection against this pathogen. As *S. enteritidis* and *L. monocytogenes* may trigger different (TLR-mediated)

innate immune signals,[24] this might be a cause of differential regulation of RegIIIβ expression and therefore the subsequent protective effects of this protein. On the other hand, it might be that RegIIIβ modulates the immune response directed against *Salmonella* but not the response against *Listeria*. Although measured at a single time point in the current study, RegIIIβ did not affect the systemic inflammatory response as measured by SAA levels in serum. The local inflammatory response in our infection models was relatively low, as MPO levels did not increase upon infection in the ileal mucosa. This was likely due to the relatively short period that mice were exposed to the orally administered pathogens because our focus was on pathogen colonization and translocation which normally precedes inflammation. Thus based on our study design, we cannot fully exclude absence of effects of RegIIIβ on the inflammatory response. However, previous investigations in our lab with a similar *Salmonella* infection model in rats indicated that mucosal inflammation in this infection model is relatively low at day 4 post infection.[25] Further analysis of the collected intestinal tissues from the current study might give insight on the role of the innate (immune) response in RegIIIβ related protective effects. For example, micro-array analysis of intestinal tissues may identify whether and which host defence pathways are associated with protective effects of RegIIIβ. Performing additional *Listeria* and *Salmonella* infection experiments in mice deficient in specific TLRs might reveal the involvement of specific innate immune signalling in RegIIIβ mediated protection against intestinal infection.

Besides the proposed association of the host response with RegIIIβ related protection of intestinal infection, this lectin might also directly and differentially affect *Listeria* and *Salmonella.* The outer membrane of these pathogens is different, for example *Salmonella* contains immuno-reactive lipopolysaccharides (LPS) which are absent in Gram-positive bacteria such as *Listeria*. This influences host signalling pathways, e.g. TLR recognition. Our ex-vivo analysis revealed that ileal mucosal RegIIIβ showed binding affinity to both *Salmonella* and *Listeria*. Bactericidal activities of RegIIIγ depend on binding to cell wall peptidoglycan, a molecule exposed on the Gram-positive bacterial surface.[4] This peptidoglycan recognition is determined by the so called Loop1 EPN tripeptide motif, which is also present in RegIIIβ.[11] This motif is involved in peptidoglycan binding, and binding affinity for carbohydrate ligands depends on carbohydrate chain length.[11] Moreover RegIIIβ binds to the Gram-positive *Bacillus subtilis* peptidoglycan, mannose polysaccharides and chitin, which is a long-chain polymer of *N*-acetylglucosamine.[11] Our results suggest that RegIIIβ possibly recognizes carbohydrate structures on the bacterial surface of microorganisms which enter the intestinal mucosa. Currently we do not know what the specific target of RegIIIβ is on the outer membrane of bacteria. It may be related to LPS, which are large molecules consisting of a lipid and a polymeric carbohydrate structure on most gram negative bacteria.[26] This may be a binding target for the EPN motif in RegIIIβ. This motif is likely, as shown for RegIIIγ, also involved in binding of RegIIIβ to Gram-positive bacteria. The molecular mechanism by which RegIIIβ may recognize its binding target remains to be determined. Studies with purified RegIIIβ are

needed to determine what ligands specifically bind to this protein. To address this question it will be necessary to have purified biologically active RegIIIβ protein, and efforts are underway to produce and purify this protein in active form. Although binding may be crucial for its function it does not necessarily result in a protective effect *in vivo*, as possible binding of RegIIIβ to *Listeria* in the intestinal mucosa does not result in reduced *Listeria* levels in extra-intestinal organs. This might be caused by another important difference between the pathogens, which is that *Listeria* and *Salmonella* use complex and very different mechanisms of cellular attachment and invasion.[20, 27] *Listeria* can invade the human intestinal epithelium via the epithelial receptor E-cadherin. A single amino acid change in the murine E-cadherin, however, makes mice relatively resistant to intestinal infection.[28] As also shown in the present study, relatively high doses of *Listeria*, do achieve a significant invasive infection in rodents.[28] This probably involves a second but less well characterized surface protein of *L. monocytogenes* Internalin B, which enables *Listeria* to enter and survive within epithelial cells via interaction with three different epithelial membrane receptors.[20, 27] In the case of *Salmonella* the bacteria are not highly adherent, but their invasion machinery is particularly efficient.[20] *Salmonella* has another mechanism to invade cells which involves binding to epithelial cells by its type III secretory system (TTSS).[22, 29] The TTSS allows direct activation of components of the host-cell cytoskeleton by intracellular delivery of dedicated bacterial effectors, resulting in membrane ruffling and endocytosis. By this event *Salmonella* invade and reside in an atypical acidic compartment called the SCV (Salmonella containing vacuole) and survive inside the cell.[27] It might be possible that RegIIIβ interferes with the expression or function of *Salmonella* pathogenicity island 1 (SPI-1) TTSS invasion machinery.

In conclusion, RegIIIβ inhibits intestinal bacterial translocation upon oral infection with the Gram-negative *Salmonella*. In contrast, this protein does not have protective effects against intestinal infection with the Gram-positive *Listeria*. Inhibitory effects of RegIIIβ on bacterial infection may be linked to its observed binding to pathogens. The protective mechanism of RegIIIβ in Salmonellosis is not associated with direct bactericidal affects but may be related to interference with *Salmonella* virulence mechanisms or host responses to this pathogen.



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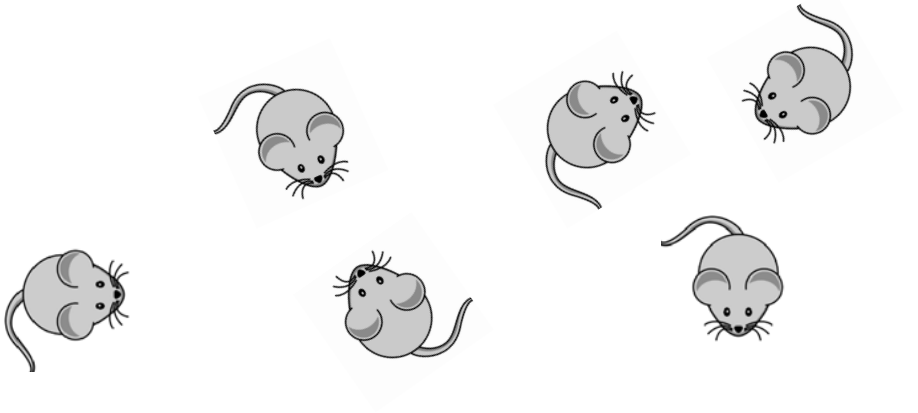

# Chapter 6

# Intestinal RegIIIγ plays a protective role against intestinal infection with both Gram-positive and Gram-negative pathogens

Linda M.P. Loonen, Ellen H. Stolte, Marcel T.J. Jaklofsky, Marjolein Meijerink, Jan Dekker, Peter van Baarlen and Jerry M. Wells

Submitted

**ABSTRACT**

RegIIIγ is considered to have a protective role against infection with Gram-positive bacteria due to its bactericidal activity, but evidence from *in vivo* studies is lacking. We generated a RegIIIγ$^{-/-}$ mouse, and investigated the effect of lack of RegIIIγ on intestinal mucus distribution, the spatial compartmentalization of bacteria and expression of innate immunity genes. Infection studies were also performed with Gram-positive and Gram-negative pathogens to investigate the antimicrobial role of RegIIIγ. RegIIIγ$^{-/-}$ mice display altered ileal mucus distribution, increased bacterial contact with the epithelium and elevated inflammatory markers in the ileal mucosa without histological evidence of pathology. Infection response pathway genes were differentially expressed in both *L. monocytogenes* and *S. enteritidis* infected RegIIIγ$^{-/-}$ and wt mice. Higher amounts of MPO were present in the ileal mucosa of RegIIIγ$^{-/-}$ than wt mice, but translocation to the organs was unaffected. We concluded that RegIIIγ has a protective role against mucosal infection with pathogenic *Listeria* and *Salmonella in vivo*. RegIIIγ is equally distributed throughout the mucus and its absence results in increased epithelial contact with the microbiota resulting in low-grade inflammation. RegIIIγ can bind to both Gram-negative and Gram-positive bacteria and influence mucus distribution in the ileum, properties which may contribute to mucosal protection.

## INTRODUCTION

The mammalian intestine harbours one of the richest and densely populated microbial communities on earth and several adaptations of the host mucosa have evolved to maintain this overall beneficial relationship. One of the main mechanisms involves sequestering of bacteria in the luminal compartment, to avoid colonisation of the epithelial cell surface and invasion by opportunistic pathogens. This is in part achieved through the secretion of secretory (s)IgA, which protects the epithelium by the immune exclusion of microbes[1] but also by the continuous production and removal of secreted mucin. In the small intestine, the mucus barrier is fortified by the accumulation of several Paneth and epithelial cell-produced antimicrobial peptides and proteins (AMP).[2] One key human antimicrobial protein is hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP) (RegIIIα, GeneID 5068) that has a murine homologue designated regenerating islet-derived 3 beta (RegIIIβ).

Mammalian Reg proteins consist of a carbohydrate recognition domain (CRD) and a N-terminal secretion signal. Some RegIII family members implicated in host defence are expressed predominantly in the small intestine, including mouse RegIIIβ and RegIIIγ[3-6] and human HIP/PAP.[7, 8] The main environmental triggers for expression of RegIII proteins are intestinal colonization by bacteria,[5] infection[9, 10] or inflammatory stimuli driven by mucosal inflammation and damage[4, 11] implicating broad roles of RegIII proteins in mucosal defence and immune homeostasis.

Recombinant and refolded RegIIIγ and the human orthologue HIP/PAP have been reported to be bactericidal to Gram-positive bacteria through binding to peptidoglycan.[5] The bactericidal activity of HIP/PAP is controversial as other authors report that HIP/PAP from humans and rats is not bactericidal, but can induce bacterial aggregation.[12, 13]

Loss of the antimicrobial RegIIIγ function was given as an explanation for the decreased spatial separation of commensals from the small intestinal epithelium in RegIIIγ knockout mice.[14] The only caveat being that this spatial separation was not based on visualisation of the mucus but inferred by fluorescent detection of the bacteria and epithelial nuclei. The firm mucus layer can easily become detached from the epithelial surface during the histological procedure, something which is only apparent by direct staining. Therefore it is important to visualise the mucus layer in order to accurately assess changes in bacterial exclusion and the extent to which bacteria penetrate the mucus.

The aim of this study was to determine the *in vivo* contribution of RegIIIγ to protection of the mouse mucosa from infection with Gram-positive *Listeria monocytogenes* and Gram-negative *Salmonella enteritidis*. We first generated and characterised a RegIIIγ$^{-/-}$ mouse, including histological and immuno-histochemical investigations of the mucus and spatial compartmentalization of bacteria in the intestine. Additionally, we investigated the effects of RegIIIγ deficiency in uninfected and pathogen infected mice using

microarray gene expression, qPCR and measurements of bacterial translocation and serum biomarkers.

## RESULTS

### RegIIIγ deficiency increases innate inflammatory markers in the ileal mucosa

In RegIIIγ$^{-/-}$ mice expression of Cre-recombinase leads to deletion of the RegIIIγ cDNA cloned between the loxP sites in the targeting vector, thereby bringing the enhanced GFP (eGFP) coding sequence under control of the canonical RegIIIγ promoter (supplement and supplementary Fig. S6.1). RegIIIγ$^{-/-}$ mice had a similar appearance to wt mice, showing no developmental abnormalities or signs of disease, and similar body weights. As expected, RegIIIγ was not expressed in the small intestine of RegIIIγ$^{-/-}$ mice (Fig. 6.1A). EGFP was expressed in the small intestine (Fig. 6.1B) and pancreas, but not in the liver, spleen or lungs of RegIIIγ$^{-/-}$ mice (data not shown). Intestinal expression of RegIIIγ was not altered in RegIIIγ$^{-/-}$ mice (Fig. 6.1C). Interleukin (IL)-22, an inflammatory response marker, was significantly increased in the ileum of RegIIIγ$^{-/-}$ mice (Fig. 6.1D). Furthermore, increased amounts of myeloperoxidase (MPO), a quantitative marker of polymorphonuclear granulocytes, were measured in the ileum of uninfected RegIIIγ$^{-/-}$ mice compared to uninfected wild type (wt) mice (Fig. 6.1E). To further investigate the immune status, a comprehensive panel of 58 serum biomarkers was measured. Macrophage inflammatory protein-2 (MIP-2) (P=0.0562), monocyte chemotactic protein-1 (MCP-1) (P=0.0117), and macrophage colony stimulating factor (M-CSF)-1 (P=0.0465), all of which are associated with the chemotaxis and development of neutrophils and monocytes, were upregulated in RegIIIγ$^{-/-}$ compared to wt mice (supplementary table S6.3). Fifteen cytokines measured in the serum of RegIIIγ$^{-/-}$ and wt mice were either not significantly different or below detection limit. To determine whether RegIIIγ deficiency had an effect on immune responses in mucosal lymphoid tissue, cytokine production was measured in *ex vivo* stimulated immune cells from MLNs. There were no significant differences in the production of IL-10, tumour necrosis factor (TNF)α, interferon (IFN)-γ, IL-4 and IL-6 between RegIIIγ$^{-/-}$ and wt mice (supplementary Fig. S6.2). Therefore, it appears that RegIIIγ$^{-/-}$ mice exhibit an increased innate inflammatory activity in the mucosa without evoking systemic inflammation.

### RegIIIγ affects the ileal mucus distribution and spatial separation of microbiota

The relatively higher amounts of IL-22 and MPO in the ileum of RegIIIγ$^{-/-}$ mice could have been caused by increased microbial translocation in absence of RegIIIγ. This prompted us to investigate the intestinal morphology and search for deviations from wt mucosal morphology and the location of microbiota. Crossmon-stained tissue of wt and RegIIIγ$^{-/-}$ mice showed indistinguishable villus-crypt architecture with similar crypt and villus lengths (data not shown). Interestingly, PAS/AB (Fig. 6.2A, C) and specific staining of

mucin with an anti-MUC2 antibody (Fig. 6.2B, D) showed a different mucus distribution in the ileum (Fig. 6.2), but not in the colon (data not shown). In all wt mice an intensely stained band of mucus was consistently observed, lying as a 'blanket' above the tips of the villi, surrounding the ileal content; however, this band was absent in each of the RegIIIγ$^{-/-}$ mice. QPCR on ileal tissue showed that this differential mucus distribution was not due to differences in expression of *Muc2* (data not shown). Moreover, distribution of goblet cells appears to be the same in wt and RegIIIγ$^{-/-}$ mice. No other secretory and membrane-bound mucins were differentially expressed in the intestinal tissues.

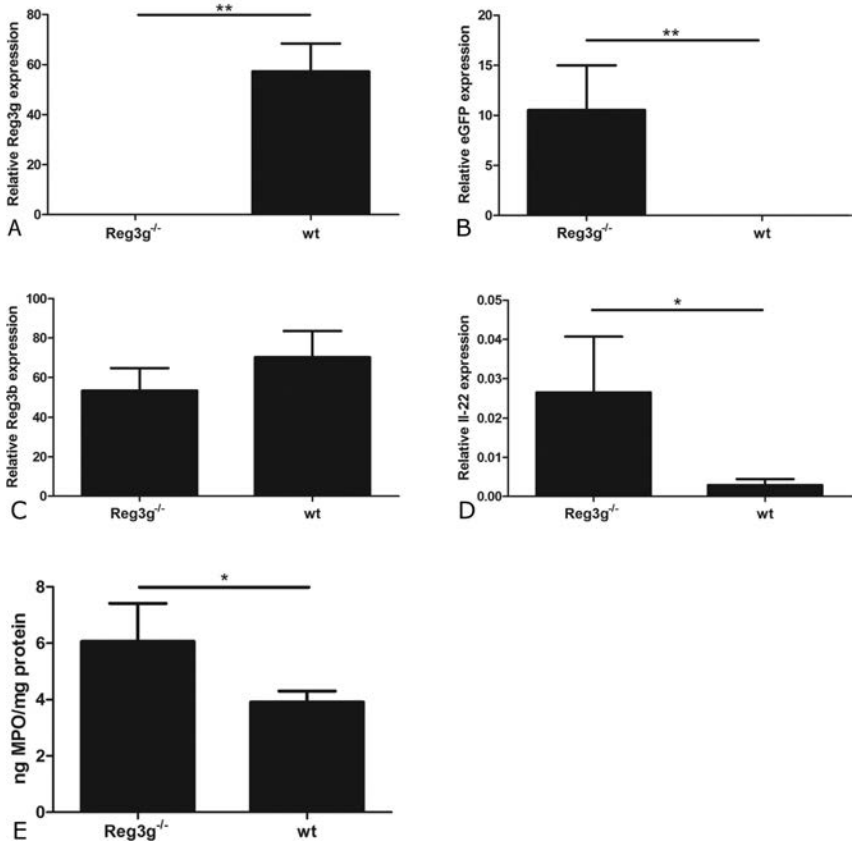


**Figure 6.1**. Ileal expression of RegIIIγ, RegIIIβ, eGFP, IL-22 and MPO in RegIIIγ$^{-/-}$ and wt mice. Relative mRNA expression determined by qPCR (A-D). ELISA of MPO protein in ileal extracts of control (uninfected) mice used in the infection study (E). * = P<0.05; ** = P<0.01.

In the small intestine, immunofluorescence staining of the mucus and bacteria showed that small numbers of bacteria were observed in contact with the epithelium in RegIIIγ$^{-/-}$ mice, but not in wt mice (Fig. 6.3A, B); no differences were observed in the colon (Fig. 6.3C, D). To investigate whether RegIIIγ was present only in the crypts or throughout the mucus we used immunofluorescent staining techniques to visualize RegIIIγ in Carnoy fixed sections of the ileum (Fig. 6.4). RegIIIγ was detected throughout the mucus in wt mice (Fig. 6.4A).

The findings described above show that in ileum but not in colon, lack of RegIIIγ protein leads to increased contact of microbiota with host with concomitant absence of a specific, darkly stained mucus band near the villus tips. Lack of RegIIIγ did not appear to lead to changes in intestinal crypt depth and villus lengths. Since lack of RegIIIγ led to increased contact between ileal microbiota and epithelia, we were interested to see if lack of RegIIIγ would promote host invasion by an intracellular Gram-positive and Gram-negative pathogen.

**Mucosal inflammation, but not pathogen translocation to the organs, is increased in infected RegIIIγ$^{-/-}$ mice**

During the course of the infection with *S. enteritidis* and *L. monocytogenes* body weight decreased, but not significantly compared to non-infected controls (supplementary table S6.4). The colony forming units (CFU) of *L. monocytogenes* in faeces were not significantly different for RegIIIγ$^{-/-}$ and wt mice. However, significantly higher CFU of *S. enteritidis* were present in the faeces of RegIIIγ$^{-/-}$ mice compared to wt (Fig. 6.5). No differences were found in translocation of *S. enteritidis* or *L. monocytogenes* to the spleen, liver or MLNs (Fig. 6.6A, B). This result was also obtained using a murinized *L. monocytogenes* strain (supplementary Fig. S6.3). As a systemic marker of bacterial translocation and inflammation we measured SAA2 levels in serum. In uninfected mice the levels of serum SAA2 were below the detection limit of the assay (<7.8 ng/ml) but levels increased substantially (>$10^5$ ng/ml) after infection with either pathogen. There were no significant differences between SAA2 in RegIIIγ$^{-/-}$ and wt infected mice (supplementary table S6.5), supporting similar bacterial translocation levels in all infected groups of mice. MPO in ileal tissue was significantly elevated in all infected compared to uninfected mice (Fig. 6.7). Furthermore, in infected mice, MPO levels were increased more in RegIIIγ$^{-/-}$ than in wt mice.

**Gene expression profiles of infected mice**

A transcriptomics approach was used to gain more insight into the potential pathways or mechanisms that might be modulated in the ileum as a consequence of the absence of RegIIIγ during microbial infection. Infection resulted in the differential ($p<0.02$) expression of 999 genes by *S. enteritidis* and 744 genes by *L. monocytogenes*. Gene ontology (GO) enrichment calculations by ErmineJ and gene set enrichment analysis (GSEA) showed that infection with either pathogen appeared to induce mucosal pathways in-

volved in DNA metabolism and cell cycle, interferon signalling and antigen processing and presentation, whereas diverse metabolic pathways including pathways involved in xenobiotic and fatty acid metabolism, PPARα signalling and peroxisome function appeared to have been suppressed. For detailed network and regulatory pathway analysis, array data were analysed using Ingenuity Pathway Analysis (IPA).

***S. enteritidis* induces an interferon-driven immune response via IL-1β-MyD88-STAT1/3-IRF axis**

IPA analysis showed that during infection with *S. enteritidis*, antigen presentation and diverse metabolic and disease pathways were significantly modulated, in addition to pathways involved in immune cell maturation and differentiation. IPA was also used to perform an upstream regulator analysis in order to identify the cascade of upstream transcriptional regulators that could explain the observed gene expression changes in the *S. enteritidis* infection dataset. The regulators that were most strongly induced during infection were *Ifn*-γ, *Signal Transducer and Activator of Transcription* (*Stat)1*, *IL-1β*, *Stat3*, *Suppressor of cytokine signalling* (*Socs)1*, *Myd88* and *Interferon regulatory factor* (*Irf)1* suggesting that an interferon-driven immune response via an IL-1β-Myd88-Stat1/3-Irf axis was a major determinant of the mouse response to infection. The major cellular responses were involved in activation of haematological system development, immune cell trafficking, cell-cell signalling and interactions, cell fate and an inflammatory response, and a repression of processes involved in infectious disease progression including replication of intracellular pathogens.

***L. monocytogenes* induces an interferon-driven immune response via TLR3-MyD88-STAT1/3-IRF axis**

IPA analysis showed that during infection with *L. monocytogenes*, metabolic and antigen presentation pathways and interferon signalling were significantly modulated, in addition to pathways involved in and driven by Janus kinase (JAK)-STAT signalling, the signalling pathway that strongly drives interferon response. Upstream regulator analysis showed that the regulators that were most strongly induced during infection were *Stat1*, *Irf1*, *Stat3*, *Toll like receptor* (*Tlr)3* and *Myd88* suggesting that an interferon-driven immune response via a Tlr3-Myd88-Stat1/3-Irf axis was a major determinant of the mouse response to infection. The major cellular responses were involved in (immune) cell fate, an inflammatory response to intracellular pathogens, and a repression of processes involved in small molecule biochemistry, lipid metabolism, and progression of infectious disease. RNA expression of a few infection-related genes was quantified by qPCR, transcription of *Ifn*-γ, *Mip-2* and *RegIIIβ* were all increased in infected mice compared to uninfected mice (Fig. 6.8A-D). In *L. monocytogenes* infected mice transcription of *Ifn-γ* was significantly higher in RegIIIγ$^{-/-}$ than in wt mice (Fig. 6.8D).

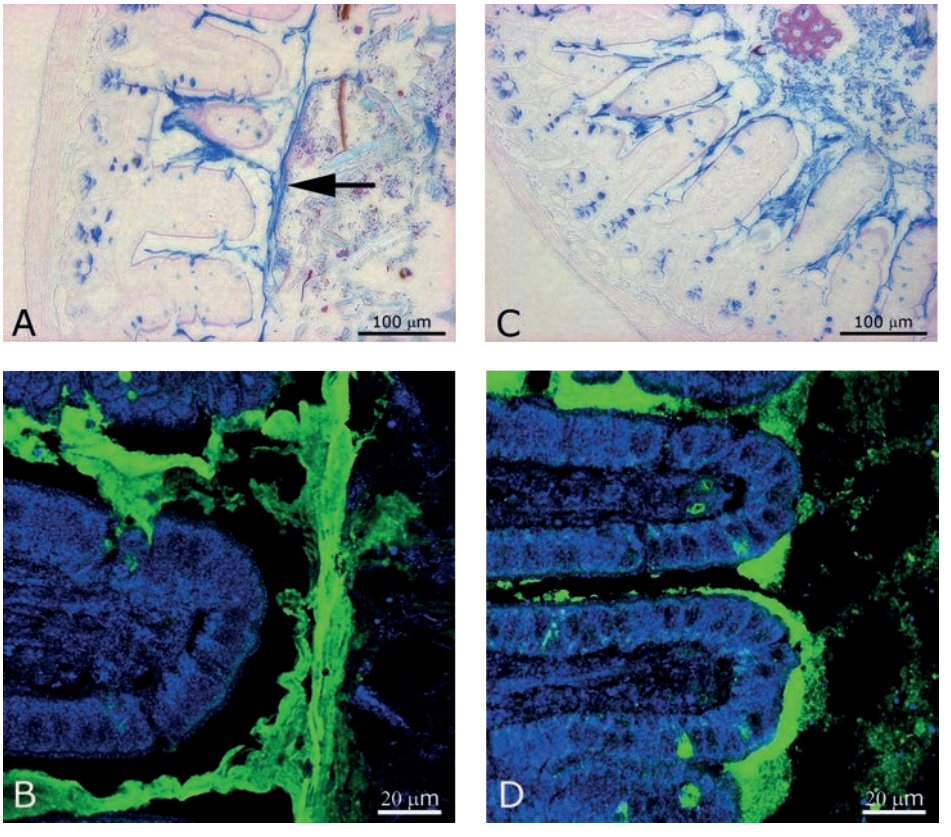


**Figure 6.2**. Mucus staining in Carnoy's-fixed ileal sections of wt (A, B) and RegIIIγ$^{-/-}$ (C, D) mice. PAS/alcian blue staining of ileum (A, C). Immunofluorescent detection of MUC2 (B, D) using an anti-MUC2 antibody and goat-anti-rabbit Alexa 488. Arrow in A indicates the continues layer of mucus overlaying the villus tips. Representative images are shown in each panel.

**Figure 6.3 (next page)**. Fluorescent microscopy of mucus and microbiota in Carnoy's-fixed sections of ileum and colon from RegIIIγ$^{-/-}$ and wt mice. Representative images of ileal tissue of wt (A) and RegIIIγ$^{-/-}$ mice (B); and of colon tissue of wt (C) and RegIIIγ$^{-/-}$ mice (D). MUC2 was detected by immunofluorescence using anti-MUC2 and goat-anti-rabbit Alexa Cy3 antibodies (red). Nuclei were visualised using DRAQ5 (blue). Bacteria were identified using FISH and the universal Euprobe 388 (green). Arrows in B indicate bacteria in contact with the epithelium.

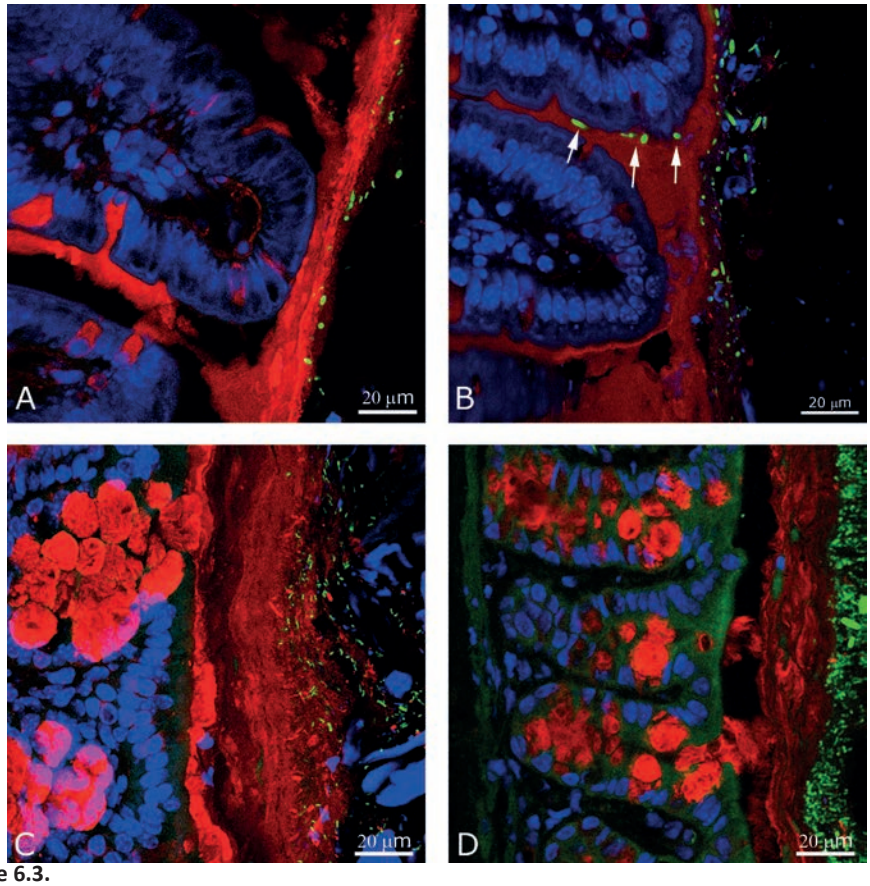


**Figure 6.3.**

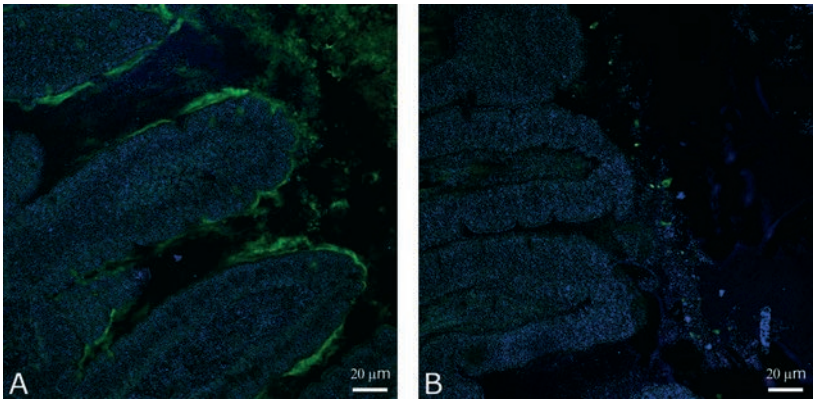


**Figure 6.4**. RegIIIγ staining in Carnoy's-fixed sections of ileum from wt (A) and RegIIIγ$^{-/-}$ mice (B), showing distribution of RegIIIγ throughout the mucus layer in wt animals. A RegIIIγ-specific antibody was detected using a goat-anti-rabbit Alexa 488 secondary antibody.

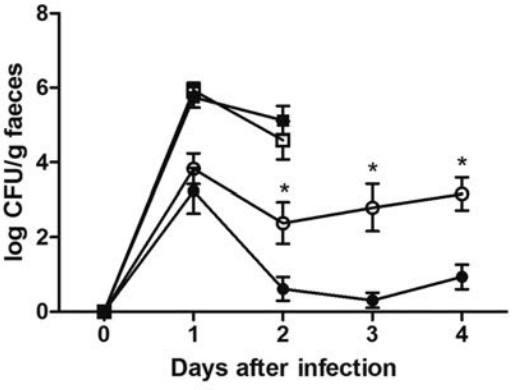


**Figure 6.5**. Pathogens excreted (CFU) in the faeces at different time points after infection. One *S. enteritidis* infected wt mouse was not included in this figure because it did not produce faeces. *L. monocytogenes* CFU are shown as squares and *S. enteritidis* CFU as circles. Open symbols represent CFU of RegIIIγ$^{-/-}$ mice and filled symbols CFU of wt mice. Error bars indicate SEM; * = P<0.05, comparing *S. enteritidis* infected RegIIIγ$^{-/-}$ versus wt mice.

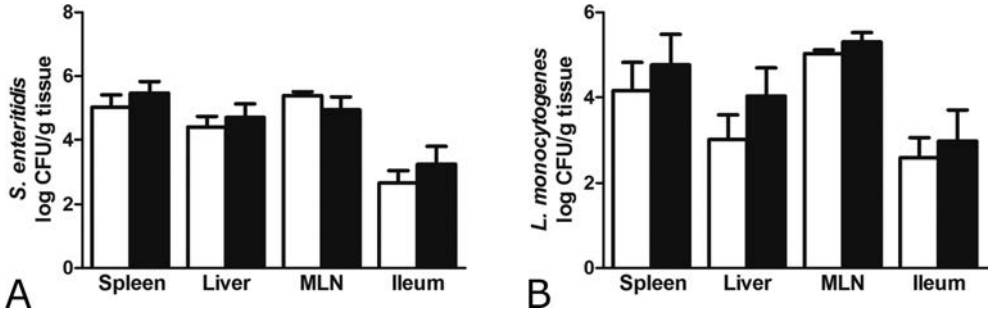


**Figure 6.6**. Pathogens (CFU) in ileum and organs of mice infected with *S. enteritidis* (A) and *L. monocytogenes* (B). White bars indicate log CFU/g of tissue from RegIIIγ$^{-/-}$ mice and black bars indicate log CFU/g of tissue from wt mice. Error bars indicate SEM.

**In RegIIIγ$^{-/-}$ mice the infection response is altered compared to wt mice**

To investigate if the transcriptome data revealed relevant differences between infected wt and RegIIIγ$^{-/-}$ mice, a protein-protein interaction network was generated in IPA for genes with GO terms involved in infectious and bacterial disease. These categories were most significantly enriched in the IPA analyses of mice infected with *L. monocytogenes* or *S. enteritidis*. This network was overlaid with gene expression data of infected wt and RegIIIγ$^{-/-}$ mice. The bacterial disease network included the nodes *Myd88*, *Stat1/3*,

*Irf1/8* and *Socs1/3* that were regulated both during infection by *S. enteritidis* and by *L. monocytogenes*.

In *S. enteritidis* infected mice the relative expression of 15 of the 80 differentially expressed network genes (19%) including the genes *Ifn*-γ, *IL-1*β and *Saa* was lower in RegIIIγ$^{-/-}$ mice than in wt mice, whereas 2 (3%) of the network genes (i.e. interferon gamma induced GTPase and ubiquitin D) were expressed at relatively higher levels in RegIIIγ$^{-/-}$ compared to wt mice (supplementary Fig. S6.4). In mice infected with *L. monocytogenes*, the same comparison showed that 2 of the 39 (5%) differentially expressed network genes (*Saa* and lipocalin 2) were expressed at relatively lower levels during infection of RegIIIγ$^{-/-}$ mice, whereas 12 genes (31%) including *Ifn*-γ, *IL-1*β, *Icam1* and C*hemokine (C-X-C motif) ligand* (*Cxcl)9/10*, were expressed at relatively higher levels (supplementary Fig. S6.5).

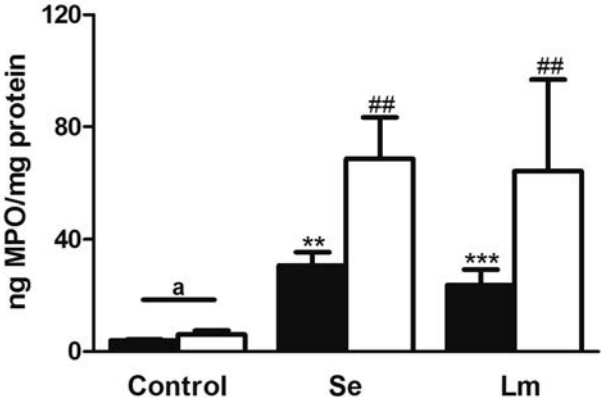


**Figure 6.7**. Amount of MPO in ileal protein extracts of wt (black bars) and RegIIIγ$^{-/-}$ mice (white bars) measured by ELISA. Significant difference between wt infected and uninfected mice, ** P=<0.01, *** P=<0.005; Significant difference between infected and uninfected RegIIIγ$^{-/-}$ mice, $^{\#\#}$ P=<0.01. MPO was also significantly different between uninfected RegIIIγ$^{-/-}$ and wt mice ($^{a}$ P=<0.05). Se is *S. enteritidis*, Lm is *L. monocytogenes*.



## DISCUSSION

To investigate the role and function of RegIIIγ *in vivo* we generated a RegIIIγ$^{-/-}$ mouse model that expresses an eGFP reporter under control of the RegIIIγ promoter. Furthermore, we investigated the hypothesis that absence of RegIIIγ would result in higher

susceptibility to infection with Gram-positive *L. monocytogenes* than to infection with Gram-negative *S. enteritidis,* due to its proposed bactericidal activity towards Gram-positive bacteria.

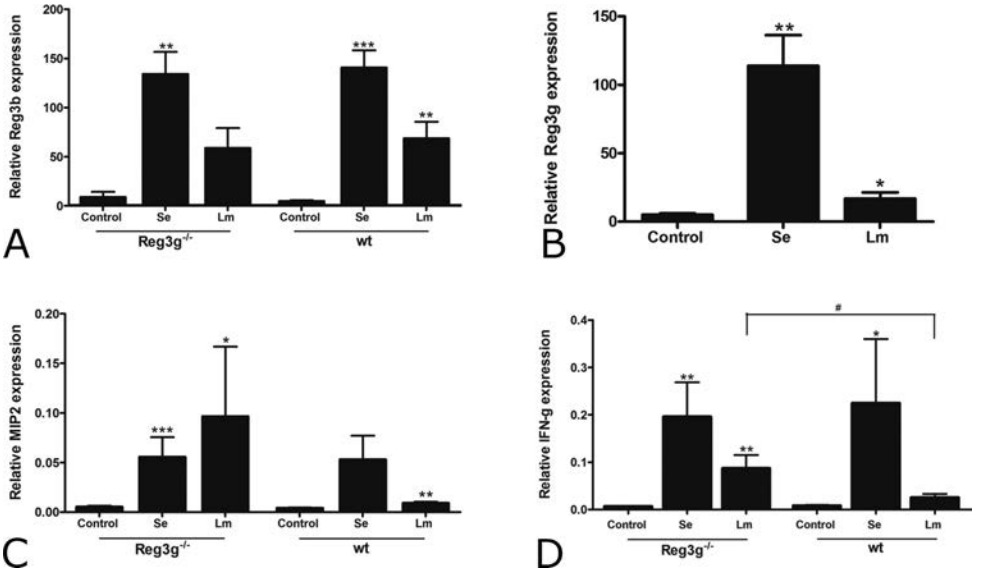


**Figure 6.8**. Relative mRNA expression of immune-related genes in uninfected and infected mice determined by qPCR. RegIIIβ (a); RegIIIγ (b); MIP2 (c) and IFN-γ (d). Error bars indicate SEM. Significant difference between infected and uninfected RegIIIγ$^{-/-}$ or wt mice, * P=<0.05; ** significance P=<0.01 *** P=<0.005. Se is *S. enteritidis*, Lm is *L. monocytogenes*.

Initial phenotypic histological analysis revealed no morphological differences between RegIIIγ$^{-/-}$ and wt mice, corroborating another RegIIIγ$^{-/-}$ mouse study.[14] However, we observed a significant increase in the relative amounts of IL-22 transcript and MPO protein levels in tissue samples from the ileum of RegIIIγ$^{-/-}$ mice relative to wt mice, suggesting elevated innate responses and chemotaxis of neutrophils. Moreover, significantly higher amounts of the chemokines MCP-1 and M-CSF-1, both of which are involved in monocyte and neutrophil recruitment, activation and differentiation, were found in serum. Interestingly, pro-inflammatory cytokines such as IL-1β, IL-12p70, TNF-α and IFN-γ, were not different between RegIIIγ$^{-/-}$ and wt mice, or were below detection limit. Therefore, it appears that RegIIIγ$^{-/-}$ mice exhibit an increased innate immune cell activity in the mucosa that successfully avoids systemic inflammation.

As MUC2, the major intestinal mucin, is highly glycosylated and RegIIIγ, a C-type lectin, is able to bind glycans, we hypothesised that loss of RegIIIγ might make the mucus more

accessible for bacteria and would allow increased contact between microbiota and epithelia. Indeed, sections of ileal tissue from RegIIIγ$^{-/-}$ mice, but not wt mice, consistently showed the presence of small numbers of bacteria at the epithelium, in agreement with the study of Vaishnava *et al*.[14] In the colon, increased co-localisation of bacteria and epithelial surfaces was not observed, presumably due to the thicker mucus layer[15] and the lower levels of RegIIIγ expression in the large intestine.[4] We next investigated the distribution and location of the mucus in wt and RegIIIγ$^{-/-}$ mice. Strikingly, mucus visualization revealed an intensely stained band of mucus consistently located above the villi and surrounding the ileal content in wt mice; this band was absent in RegIIIγ$^{-/-}$ mice. This was not reported by Vaishnava *et al*.[14] as the mucus was not visualized in that study. The changes in mucus were not due to lower expression of MUC2 mRNA in the ileum of RegIIIγ$^{-/-}$ mice, suggesting that RegIIIγ may directly affect the structure or the distribution of mucus in the ileum. As RegIIIγ was shown to be present throughout the mucus layer of wt mice, its absence may provide an explanation for the altered distribution of mucus in these RegIIIγ$^{-/-}$ mice. Alternatively, RegIIIγ itself may self-assemble into large multimeric complexes as previously described for PAP and other Reg family members[13] and stabilize the mucin structure by crosslinking the mucins in the mucus network. In wt mice there was a significant decrease in the faecal counts of Gram-negative *S. enteritidis* compared to RegIIIγ$^{-/-}$ mice, which may be due to increased entrapment of *S. enteritidis* in the mucus by RegIIIγ and subsequent killing by antimicrobial proteins including RegIIIβ, which is highly increased in expression after *S. enteritidis* infection. It is also possible that changes in the microbiota of RegIIIγ$^{-/-}$ mice led to less antagonism towards luminal *S. enteritidis.*[16]

As binding of RegIIIγ to bacteria might be a mechanism to trap bacteria in the mucus we investigated whether recombinant RegIIIγ could bind both pathogens *in vitro.* Indeed RegIIIγ could bind to both pathogens and binding was enhanced by proteolytic cleavage of the N-terminal propeptide, but no bactericidal effects were measured (data not shown). The *L. monocytogenes* strain used in our study was previously reported to be killed by RegIIIγ and HIP/PAP,[5] but inconsistencies in the bactericidal effect of RegIIIγ and HIP/PAP on Gram-positive bacteria have been described by others.[12, 13] This discrepancy may be due to the use of different bacteria, possible interference of bacterial aggregation during viability assays or conformational differences in the refolded proteins leading to differential proteolytic susceptibility or activity. Lack of RegIIIγ and concomitant decreased bacterial entrapment could thereby explain the increased mucosal inflammatory responses observed following infection of RegIIIγ$^{-/-}$ mice with either pathogen.

Infection of RegIIIγ$^{-/-}$ mice with either of the pathogens did not result in increased translocation to the spleen, liver or MLNs compared to wt infected mice. This result was fully reproduced using a 'murinized' *L. monocytogenes*, engineered to increase infectivity in murine models.[17] Although there was no increased translocation of pathogens in the organs of RegIIIγ$^{-/-}$ mice, we did measure increased amounts of MPO in ileal tissue of

infected RegIIIγ$^{-/-}$ mice compared to wt infected mice. MPO is produced mostly by neutrophil granulocytes, which are attracted to sites of infection suggesting that increased numbers of both pathogens translocated into the mucosa of RegIIIγ$^{-/-}$ mice compared to wt mice. Taken together these results suggest that the heightened mucosal inflammatory response observed in infected RegIIIγ$^{-/-}$ mice was sufficient to inhibit spread of the pathogens to the bloodstream and organs. In agreement with this, loss of RegIIIγ did not affect systemic inflammatory responses as no significant differences between wt and RegIIIγ$^{-/-}$ in serum SAA2 levels were measured. In line with this notion, it was observed that several genes in the infection response network were differentially expressed in RegIIIγ$^{-/-}$ compared to wt mice. For example, in *L. monocytogenes* infected mice, *Ifn*-γ, *Il-1*β, *Icam1, Cxcl9* were expressed at relatively higher levels in RegIIIγ$^{-/-}$ mice than in wt mice.

Overall these results suggest that the mucosal inflammatory response, involving monocyte and neutrophil recruitment and activity to pathogen infection was increased in RegIIIγ$^{-/-}$ mice. Reduced trapping of the bacteria in the mucus or increased movement of bacteria through the mucus might explain the increased mucosal inflammatory responses to both pathogens in RegIIIγ$^{-/-}$ mice. Our results showing presence of RegIIIγ throughout the mucus, altered mucus distribution in the ileum of RegIIIγ$^{-/-}$ mice and RegIIIγ binding to both pathogens support this hypothesis.

By generating a new transgenic RegIIIγ$^{-/-}$ mouse model we were able to demonstrate that RegIIIγ deficiency leads to altered mucus distribution, increased bacterial-epithelial contact and significant increases in the expression of several innate immunity genes in the ileum. During infection, RegIIIγ deficiency had no effect on translocation of pathogenic *L. monocytogenes* or *S. enteritidis* to the organs, but resulted in a heightened innate response in the mucosa. The increased amount of MPO measured in the mucosa of RegIIIγ$^{-/-}$ mice suggests the presence of higher numbers of polymorphonuclear granulocytes which are important in immunity to both *L. monocytogenes* and *S. enteritidis*. Additionally, our results suggest that RegIIIγ may have protective effects that go beyond direct bactericidal activity, including effects on mucus properties and trapping of bacteria in the mucus network.

## METHODS

### Animals

RegIIIγ$^{-/-}$ mice were generated using a Cre-Lox procedure and the genotype was verified by PCR (see supplementary materials and methods and table S6.1 for primer sequences). Specified pathogen free RegIIIγ$^{-/-}$ and wt mice on a C57Bl/6 background were reared on a semi-synthetic standard AIN-93G diet[18] (Research Diet Services, Wijk bij Duurstede, the Netherlands) and provided with sterilized drinking water *ad libitum*, unless stated otherwise. The animal welfare committee of Wageningen University (Wageningen, the Netherlands) approved the experimental protocol.

### Collection of biological samples for phenotyping

Mice (7-9 weeks old) were anaesthetized by isoflurane, blood was collected after orbital removal using BD Microtainer tubes (SST™ tubes, Becton Dickinson (BD), Franklin Lakes, NJ, USA) and mice were killed by cervical dislocation. Serum was analysed by Myriad-RBM (Rules Based medicine; Austin, Tx, USA) using the RodentMAP v2.0 platform. Different parts (approximately 1 cm) of the ileum and colon were either fixed in Carnoy's fixative or in 4% (w/v) paraformaldehyde (PFA) and embedded in paraffin. PFA fixed tissue sections were stained according to Crossmon, Carnoy's fixed tissue sections were stained with PAS/Alcian Blue (PAS/AB), anti-Muc2 antibody[15] or fluorescence *in situ* hybridisation (FISH) to detect bacteria using the universal bacterial probe EUB338[19] (for histological protocols see supplement). Unfixed tissue was snap frozen and stored at -80˚C prior to RNA isolation. Mesenteric lymph nodes (MLNs) were collected, the cells cultured and stimulated *ex vivo* for cytokine analysis (supplementary methods).

### Infection studies

Mice (7-9 weeks old) used in the infection study were housed individually from one week before the start of the infection and fed a modified diet, to mimic a Western-style human diet, by including 200 g/kg fat and reducing the calcium to 30 mmol/kg. The amount of calcium in a standard rodent diet is typically very high (124.75 mmol/kg feed[18]) but we lowered this because it has been shown to be protective against infection in the intestine of rats[20-23], and humans.[24, 25] This 'humanized' diet was fed to the mice from 7 weeks of age. Moreover this diet has been also used in a previous infection study using RegIIIγ$^{-/-}$ mice.[26, 27]

After adaptation, mice were orally infected by gavage with 0.2 ml saline containing $10^8$ colony forming units (CFU) of *Salmonella Enterica serovar enteritidis (S. enteritidis,* n=12 for RegIIIγ$^{-/-}$ and wt) or *Listeria monocytogenes* (*L. monocytogenes*, EGD-e, n=8 for RegIIIγ$^{-/-}$ and wt). The bacterial strains were passaged in mice and enumerated on selective medium as previously described.[27] Two groups of mice were sham treated (0.2 ml saline, RegIIIγ$^{-/-}$ and wt; both n=6). An additional infection study was performed with non-passaged murinized *L. monocytogenes*[17] (see supplement). Body weight was measured every two days before infection and daily after infection. Fresh faeces were

collected every day after infection.

**Collection of biological samples and bacterial quantification of infected mice**
Mice were sacrificed 2 (*L. monocytogenes*) or 4 days (*S. enteritidis*) after infection, the time points at which the number of translocated bacteria were highest in the organs.[27] Non-infected control mice were sacrificed 3 days after oral sham treatment. Blood was collected as above and after cervical dislocation the distal one third of the small intestine was excised for determining CFU of pathogens (shown as $\log_{10}$ CFU per gram wet weight of tissue). Approximately 1 cm from the middle of the ileum was cut open longitudinally, briefly flushed in saline, homogenized in sterile saline and plated. Other parts of the ileum were treated in the same way and frozen immediately in liquid nitrogen for protein and/or RNA isolation (see supplementary for protocols and table S6.2 for primer sequences for qPCR). Furthermore, MLNs, spleen and liver were removed and after homogenization in sterile saline, directly used for pathogen quantification.

**Myeloperoxidase quantification in ileal mucosa and serum amyloid A2 detection in serum**
To determine the ileal inflammation status, myeloperoxidase (MPO) was measured in ileal protein extracts using the manufacturer's MPO ELISA protocol (Hycult Biotechnology, Uden, the Netherlands). Serum Amyloid A (SAA) 2, was measured to investigate systemic inflammation according to the manufacturer's protocol (Life Diagnostics Inc., West Chester, PA, USA).

**Microarray and statistical analysis**
Total RNA was extracted from the ileum as described above, pooled (3 animals per group, randomly selected) and hybridised to the Mouse Gene 1.1 ST array (Affymetrix, Santa Clara, CA, USA). The labelling and hybridization methods, quality control procedures on RNA and datasets generated are described in supplementary methods.

**Biological interpretation of expression datasets**
Biological interaction networks among regulated genes activated in response to infection were identified using Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA) using all differentially regulated genes ($p$ value $<0.01$ and intensity $\geq 20$) as input. The output was used to prioritise differentially regulated pathways of interest for bacterial infections, to identify cascades of upstream transcriptional regulators that could explain the observed gene expression changes, and to reconstruct protein-protein networks that could be used to overlay gene expression data. For details, see supplementary methods.

**Statistics**
All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). Data shown are the means and the standard errors of the means (SEM). Data were tested for normality with the D'Agostino and Pearson normality test. Statis-

tical analysis of normally distributed data was performed with the two-tailed Student's t-test. Data that did not show normal distribution were analysed with the non-parametric Mann-Whitney test. The excretion of pathogens in faeces was measured at multiple time points, and thus analysed by a repeated-measures two-way analysis of variance (ANOVA) (mixed model). Differences were considered statistically significant when $P<0.05$.

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## SUPPLEMENTARY METHODS

### Generation of RegIIIγ transgenic mice

LoxP-flanked RegIIIγ mice were generated in collaboration with Ozgene (Bentley DC, Australia) as shown in supplementary figure S6.1. Initially a 246 bp fragment containing a poly-adenylation site (pA) was amplified from an Ozgene vector and cloned between the EcoR1 sites in pBAD (Invitrogen, Life technologies Ltd, Paisley, UK). A DNA fragment containing PGK-neo cassette containing a 3' loxP site was excised from FL Sniper vector (Ozgene) using PacI digestion and ligated into the PacI site downstream of pA in pBAD to generate pBAD-Neo. A 726 bp DNA fragment flanked by NheI restriction sites and a 5' loxP site containing the promoter and exons of RegIIIγ was then generated by spliced overlap extension PCR using C57BL/6 genomic DNA and cloned RegIIIγ cDNA as templates. The complete RegIIIγ promoter/cDNA fragment was then cloned into NheI digested pBAD to generate pBAD-R3. A DNA fragment containing pA and the downstream PGK-Neo selection cassette was then removed from pBAD-neo by digestion with EcoRI and ligated into the EcoRI site downstream of the RegIIIγ cDNA to generate pBAD-R3/Neo. A DNA fragment containing the enhanced green-fluorescent protein (eGFP) coding sequence and an unique pA site was cloned into pBAD using a similar strategy to that described for the RegIIIγ cDNA. The whole RegIIIγ cDNA PGK-neo cassette was then removed from pBAD-R3/Neo by digestion with NheI and ligated upstream of eFGP to generate pBAD-R3/Neo/eGFP. The final targeting vector contained DNA fragments (approx 6 Kb) with homology to the chromosomal integration site (homology arms) flanking the floxed selection cassette in pBAD-R3/Neo/eGFP. The targeting vector was constructed using PCR amplified genomic DNA and a similar cloning strategy to that described for the construction of pBAD-R3/Neo/eGFP. All DNA fragments cloned into the final targeting vector including the homology arms were verified by DNA sequencing at each stage.

The targeting vector was linearized by digestion with PvuI and electroporated into C57Bl/6 ES cells and grown under selection with G148. Resistant clones were picked into 96 well plates for freezing and genomic characterization. Recombinant ES clones were identified by Southern Blotting by digestion with BamHI and hybridization to the following genomic sequence probes (from C57BL/6 genomic DNA) (i) 5' homology arm nt 15952 to 16401 (supplementary table S1, P1062, (ii) 3' homology arm nt 30827 to 31822 (iii) enP nt 22001-22888 (for primers see supplementary table S6.1).

Correctly targeted ES clones were injected into C57Bl/6 blastocysts, which were transferred into pseudo-pregnant foster mothers, resulting in chimera identified by coat colour. These mice were crossed to C57Bl/6 mice to obtain heterozygous floxed RegIIIγ$^{+/-}$mice. Heterozygous floxed RegIIIγ$^{+/-}$ mice were crossed to generate floxed RegIIIγ$^{-/-}$ homozygotes on a C57Bl/6 background. In the presence of Cre-recombinase the sequence between the loxP sites in the targeting vector including the RegIIIγ cDNA and neo cassette are removed (supplementary figure S6.1) bringing EGFP under control

of the RegIIIγ promoter.

The genotype of floxed RegIIIγ$^{-/-}$ mice was confirmed by PCR using one primer set specific for a sequence in the targeting vector (508 bp). The second set of primers was specific for a fragment spanning the chromosomal homology arm (768 bp) (supplementary figure S6.1).

**Cre-induced RegIIIγ$^{-/-}$ mice**
To generate RegIIIγ$^{-/-}$ mice, floxed RegIIIγ$^{-/-}$ homozygotes were crossed with B6.C-Tg(CMV-cre)1Cgn/J mice (The Jackson laboratory, Bar Harbor, ME, USA) expressing Cre recombinase in the germ line. To verify the genotype of these mice PCR based genotyping was performed on the offspring using Cre-specific primers (The Jackson Laboratory) and primers specific for the RegIIIγ targeting vector as described above.

**Mesenteric lymph node cell culture and cytokine analysis**
Mouse mesenteric lymph nodes (MLNs) were gently disrupted in PBS containing 0.5% (w/v) bovine serum albumin (BSA) and 2 mM EDTA to obtain single cell suspensions for *ex vivo* cytokine analysis. 5x10$^6$ viable cells/ml were cultured in RPMI-1640 medium (Gibco, Life technologies Ltd, Paisley, UK) with Glutamax™, 25 mM HEPES, 10% heat inactivated FCS (PAA, Pasching, Austria) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The MLN cells were stimulated with the Concanavalin A (ConA) (5 µg/ml, Sigma-Aldrich) for 24 h at 37˚C and 5% $CO_2$. Culture supernatants were stored at -80˚C until cytokines were measured by multiplex analysis (Mouse Th1/Th2/Th17 CBA kit, Becton Dickinson, BD, Franklin Lakes, NJ, USA) according to the manufacturer's protocol on a FACS Canto II (BD) and analysed using BD FCAP software (BD).

| | | |
|---|---|---|
| Cloning primers | pA-F | C**GAATTC***TGTGCCTTCTAGTTGCCAGCC*<br>EcoRI |
| | pA-R | C**GAATTC**TTAATTAA*CCATAGAGCCCACCGCATCC*<br>EcoRI PacI |
| | R3P-F | *TGCTTATTCACTTTGATGCCCC* |
| | R3P-R | GTGATGGTTATACGGGGAAGCAT*CTTGTCTGTAAGGAAGGAAAAACCAG* |
| | R3CDS-F | CTGGTTTTTCCTTCCTTACAGACAAG*ATGCTTCCCCGTATAACCATCAC* |
| | R3CDS-R | *GGCCTTGAATTTGCAGACATAGG* |
| | R3cDNA-F | C**GCTAGC**ATAACTTCGTATAGCATACATTATACGAAGTTAT*TGTATTCTTCT-*<br>NheI loxP *CATTCCAGAATCAAAGAA* |
| | R3cDNA-R | C**GCTAGC**GAATTC*GGCCTTGAATTTGCAGACATAGG*<br>NheI EcoRI |
| | EGFPP-F | *TGCTTATTCACTTTGATGCCCC* |
| | EGFPP-R | CCTCGCCCTTGCTCACCAT*CTTGTCTGTAAGGAAGGAAAAACCAG* |
| | EGFPCDS-F | CTGGTTTTTCCTTCTTTACAGACAAG*ATGGTGAGCAAGGGCGAGG* |
| | EGFPCDS-R | *GCAGTGAAAAAAATGCTTTATTGGTGA* |
| | EGFPCD-NA-F | C**ATCGAT**GCTAGC*TGTATTCTTCTCATTCCAGAATCAAAGAA*<br>ClaI NheI |
| | EGFPCD-NA-R | C**ATCGAT**GGATCC*GCAGTGAAAAAAATGCTTTATTTGTGA*<br>ClaI BamHI |
| | H3-F | CA**GGCGCGCC**ATCGAT*ACAAAACCATCATCCTGCCACC*<br>AscI ClaI |
| | H3-R | CA**GGCGCGCC***TCCCCTGAATTACAATGCCAGG*<br>AscI |
| | H5-F | C**TACGTA***CCACACCTGGCTTTTTGTAGCTTG*<br>SnaBI |
| | H3-R | C**TACGTA**GGCGCGCC**GCATGC***TTTTGTTCCCTTCCCCTGGAAAC*<br>SnaBI AscI SphI |
| Southern blot | 5' F | TCAAAAAGTTGACCATGAGGGC |
| | 5'R | CCTGGGAAATGGATTTGTTGTTCC |
| | 3'F | GCTCTTCTTCAGCAACTCCTCAAC |
| | 3'R | ATGACAGGGTAGCAAAATCTCCAG |
| | enP F | AACGTGAACTCCTTTCTGCCTG |
| | enP R | ATGCTCATGCAAGTCAGGGAGG |
| Genotype | KO F | TTGCCAGCCATCTGTTGT |
| | KO R | GACGTGCTACTTCCATTTGTC |
| | WT F | GGGAGAATACCTATCCTCACA |
| | WT R | TCCAGAACAGATAGCGTC |

**Table S6.1.** Primer sequences for generating RegIIIγ$^{-/-}$ mice. Restriction sites used for cloning are indicated in bolt. Italic letters indicate homology with the backbone. The grey part indicates the loxP site.

**Histology**
Intestinal tissues were fixed in either Carnoy's fixative or in 4% (w/v) para-formaldehyde (PFA) and embedded in paraffin through a graded series of ethanol (50-100%) and xylene.

**Crossmon staining according to Masson**
Paraffin sections of ileum and colon (fixed in PFA) were cut at 4 µm and attached to slides coated with polylysine (Thermo scientific, Gerhard Menzel GmbH Braunschweig, Germany). After overnight incubation at 37˚C, slides were deparaffinised using a series going from xylene to distilled water with decreasing ethanol steps. The sections were submerged in Mayer's haemalum (BDH GurrCertistain, BDH laboratory supplies, Poole, UK) for 5 min followed by 10 min rinsing in running tap water. Sections were submerged for 3 s in acid fuchsin (Rubin S) (Merck KGaA, Darmstadt, Germany) with Orange G (Merck KGaA), containing 5 ml acetic acid (glacial) 100% anhydrous (Merck KGaA), 0.33 g Thymolcryst extra pure (Merck KGaA) and 500 ml of distilled water, followed by a washing step for 2 min in distilled water. After this washing step sections were incubated for 4 min in tungstophosphoric acid (Merck KGaA) and washed for 2 min in distilled water. Sections were submerged in a 1% light green SF solution (BDH GurrCertistain) and incubated for 10 min, washed 2 times for 1 min in distilled water and placed in 100% ethanol 3 times for 2 min. After this step the sections were submerged 2 times for 5 min in xylene and were finally mounted in DPX mounting reagent (BDH GurrCertistain) and air dried overnight at 37˚C. Measurements of the villi and crypt length were calculated from digital images using FIJI (ImageJ) and size calibration bars. Twelve to 15 villi or crypts were measured per animal.

**Pas/Alcian Blue staining**
Paraffin sections of ileum and colon (fixed in Carnoy's to preserve the mucus layer[1]) were cut at 4 µm and attached to slides coated with polylysine (Thermo scientific, Germany). After overnight incubation at 37 ˚C**,** slides were deparaffinised using a series going from xylene to distilled water with decreasing ethanol steps. Sections were stained in alcian blue (8GX Acros Organics, New Jersey, USA) for 35 min followed by a rinse in running tap water for 2 min and a rinse in distilled water. The sections were submerged in periodic acid 0.5% for 10 min and rinsed for 1 min in distilled water. After this washing step the sections were incubated in Shiffs reagents (PAS) (Merck, Germany) for 45 min and washed in freshly made $SO_2$ water (10 ml of 10% $K_2S_2O_5$ (Merck, Germany), 10 ml of HCl (1 mol/L) and 180 ml of distilled water) for 3 times 2 min, followed by a washing step in tap water for 5 min. After this step the sections were submerged 2 times for 3 min in 100% ethanol followed by a 3 time submersion in Xylene for 5 min and were finally mounted in DPX mounting reagent (BDH Gurr Certistain, England) and air-dried overnight at 37 ˚C.

**MUC2 staining**

Paraffin sections of both ileum and colon (fixed in Carnoy's) were cut at 4 µm and attached to slides coated with polylysine (Thermo scientific). After overnight incubation at 37˚C, slides were deparaffinised as above. An antigen retrieval step was performed by heating the sections for 20 min in 0.01 M sodium citrate (pH 6.0) at 95˚C. Sections were washed for 3 h in 3 replacements of PBS. A blocking step to reduce non-specific binding was included using 5% goat serum (Invitrogen, Life technologies Ltd, Paisley, UK) in PBS with 0.1% Triton X-100 for 30 min at room temperature. MUC2 expression was detected by incubating the sections with custom designed anti-MUC2 antibody[1] 1:500 in PBS containing 1% goat serum and incubated overnight at 4˚C. After primary incubation sections were washed 3 times in PBS for 10 min, followed by a secondary incubation using goat-anti-rabbit Alexa488 conjugated antibodies (1:1000) (Molecular Probes, Life technologies Ltd, Paisley, UK) for 1 hour at room temperature. Sections were washed 2 times in PBS for 10 min in the dark and incubated with DRAQ5 (Invitrogen) (1:1000) for 1 h at 4˚C to counterstain nuclei. Finally, sections were washed 2 times in PBS for 10 min, mounted in fluoromount G (SouthernBiotec, Alabama, USA) and stored at 4˚C.

**Fluorescence *in situ* hybridisation (FISH) to detect bacteria**

Paraffin sections of proximal colon and ileum including faecal pellets fixed in Carnoy's were cut at 4 µm and attached to slides coated with polylysine (Thermo scientific). After overnight incubation at 37˚C the slides were deparaffinised using series going from xylene to 100% ethanol. The tissue sections were incubated with the universal bacterial probe EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') (Isogen Bioscience BV, De Meern, the Netherlands) conjugated to Alexa Fluor488. A non-sense probe (5' CGACGGAGGG-CATCCTCA-3') conjugated to Cy3, was used as a negative control.[2] Tissue sections were incubated with 0.5 µg of probe in 50 µl of hybridization solution (20 mM Tris-HCl (pH 7.4), 0.9 M NaCl, 0.1% SDS) at 50˚C overnight in a humid environment using a coverslip to prevent drying of the sample. The sections were washed in wash buffer (20 mM Tris-HCl (pH 7.4), 0.9 M NaCl) at 50˚C for 20 min and co-staining was performed using the above mentioned protocol for immunostaining of MUC2 with the elimination of the antibody retrieval step and the 3 h washing step. Sections were finally mounted using fluoromount G (SouthernBiotec) and stored at 4˚C.

**RegIIIγ staining**

RegIIIγ immune staining was performed similar to the MUC2 staining (see above) including the antigen retrieval (1 h) and blocking step. RegIIIγ expression was detected by incubating the sections with custom designed anti- RegIIIγ antibody[3] 1:2000 in PBS containing 1% goat serum and incubated overnight at 4˚C. After incubation with the primary antibody sections were washed 3 times in PBS for 10 min, followed by a secondary incubation using goat-anti-rabbit Alexa488 conjugated antibodies (1:1000) (Molecular Probes) for 1 h at room temperature. Finally the sections were incubated with DRAQ5

and mounted as described above.

**RNA isolation, cDNA synthesis and qPCR**
Total RNA was isolated using the RNeasy kit (Qiagen, Venlo, the Netherlands) with a DNase digestion step according to the manufacturer's protocol. One µg of RNA was reverse transcribed using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's protocol. QPCR was performed on a Rotorgene 2000 real-time cycler (Qiagen) (see supplementary materials and methods and table S2 for primer sequences).

For qPCR 5 µl cDNA (1:20 diluted from cDNA synthesis mixture) was used, together with 300 nM forward and reverse primer (for qPCR primer sequences, see supplementary table S2), 6.25 µl 2x Rotor-Gene SYBR Green PCR kit (Qiagen, Venlo, the Netherlands), and demineralized water up to a total volume of 12.5 µl. QPCR was performed (2 min 95˚C, then 40 cycles of 15 s at 95˚C, 1 min at 60˚C, and a final step of 2 min at 60˚C) on a Rotorgene 2000 real-time cycler (Qiagen).

| | Forward primer | Reverse primer |
|---|---|---|
| GAPDH | GGTGAAGGTCGGTGTGAACT | CTCGCTCCTGGAAGATGGTG |
| HPRT | GTTAAGCAGTACAGCCCCAAA | AGGGCATATCCAACAACAAACTT |
| RegIIIγ | TTCCTGTCCTCCATGATCAAAA | CATCCACCTCTGTTGGGTTCA |
| RegIIIβ | ATGCTGCTCTCCTGCCTGATG | CTAATGCGTGCGGAGGGTATATTC |
| eGFP | CAGAAGAACGGCATCAAGGT | CGGTCACGAACTCCAGCA |
| IL-22 | AGACAGGTTCCAGCCCTACA | CAGGTCCAGTTCCCCAATC |
| MUC2 | ACCTGGGGTGACTTCCACT | CCTTGGTGTAGGCATCGTTC |
| IFN-γ | TCTTGGCTTTGCAGCTCTTC | TGTTGCTGATGGCCTGATTG |
| MIP-2 | CACCAACCACCAGGCTACA | GCTTCAGGGTCAAGGCAAAC |

**Table S6.2.** QPCR primer sequences

The raw data was analysed using the Rotorgene Analysis Software V5.0. Changes in transcript levels were calculated relative to the housekeeping genes according to the following equation:

$$\text{Ratio} = (E_{\text{reference}})^{\wedge} Ct_{\text{reference}} / (E_{\text{target}})^{\wedge} Ct_{\text{target}}$$

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT) were incorporated in all qPCR experiments as internal reference genes; results were similar following normalization to either gene. Both GAPDH and HPRT were not affected by the treatment compared to the sham mice. Non-RT and non-template controls were included in all experiments; no amplification above back-

ground levels was observed for these controls. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The PCR product of each template was checked by sequencing.

**Microarray**

Quality control of the hybridisations to the Mouse Gene 1.1 ST array and primary data analysis were performed according to strict criteria to ensure that the array data were of the highest possible quality. RNA quantity and quality was assessed on the total RNA obtained from ileum spectrophotometrically (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and with 6000 Nano chips (Bioanalyzer 2100; Agilent, Santa Clara, CA, USA), respectively. RNA was judged as being suitable for array hybridisation only if samples showed intact bands corresponding to the 18S and 28S ribosomal RNA subunits, displayed no chromosomal peaks or RNA degradation products, and had a RIN (RNA integrity number) above 8.0.

The Ambion WT Expression kit (Life Technologies) in conjunction with the Affymetrix GeneChip WT Terminal Labelling kit (Affymetrix, Santa Clara, CA) was used for the preparation of labelled cDNA from 100ng of total RNA without rRNA reduction. Labelled samples were hybridised on Affymetrix GeneChip Mouse Gene 1.1 ST arrays, provided in plate format. Hybridisation, washing and scanning of the array plates was performed on an Affymetrix GeneTitan Instrument, according to the manufacturer's recommendations. Detailed protocols can be found in the Affymetrix WT Terminal Labelling and Hybridisation User Manual, and are also available upon request.

Quality control (QC) of the datasets obtained from the scanned Affymetrix arrays was performed using Bioconductor[4] packages integrated in an on-line pipeline.[5] Various advanced quality metrics, diagnostic plots, pseudo-images and classification methods were applied to ascertain that only arrays that passed the most rigorous quality controls were used in the subsequent analyses.[6] An extensive description of the applied criteria is available upon request. The more than 825.000 probes on the Mouse Gene 1.1 ST array were redefined according to Dai[7] utilising current genome information. In this study, probes were reorganised based on the Entrez Gene database, build 37, version 1 (remapped CDF v13). Normalised expression estimates were obtained from the raw intensity values using the Robust Multiarray Analysis (RMA) pre-processing algorithm available in the Bioconductor library affyPLM using default settings.[8]

Differentially expressed probe sets were identified using linear models, applying moderated t-statistics that implemented empirical Bayes regularization of standard errors[9] using Bioconductor's limma package. The moderated t-test statistic has the same interpretation as an ordinary t-test statistic, except that the standard errors have been moderated across genes, i.e. shrunk to a common value, using a Bayesian model. To adjust for both the degree of independence of variances relative to the degree of identity and the relationship between variance and signal intensity, the moderated t-statistic was ex-

tended by a Bayesian hierarchical model to define an intensity-based moderated T-statistic (IBMT).[4] IBMT improves the efficiency of the empirical Bayes moderated t-statistics and thereby achieves greater power while correctly estimating the true proportion of false positives. Probe sets with raw expression intensity >20 and p values <0.01 were considered to be significantly regulated. The p<0.02 cut-off was chosen as this resulted in a set of genes with Gene Ontology (GO) annotations matching the global outcome of ErmineJ and gene set enrichment analysis (GSEA) in Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA) (see below).

**Biological interpretation of expression datasets**

Biological interaction networks among regulated genes activated in response to conventionalisation were identified using IPA. The IPA output includes modulated signaling pathways with statistical assessment of the significance of their modulation being based on a Fisher's Exact Test. IPA analyses included comparison of differentially regulated genes in the ileum following bacterial infection of wt or RegIIIγ$^{-/-}$ mice, in each case relative to expression observed in the sham-inoculated mice. The input was all differentially regulated genes (p value <0.01 and intensity ≥20). The output was used among others to prioritise differentially regulated pathways of interest for bacterial infections, and to reconstruct protein-protein networks that could be used to overlay gene expression data, either fold-change based on the limma statistics[5] or as ratio data of expression in RegIIIγ$^{-/-}$ versus wt mice. For IPA analysis, gene expression ratios *x* between 0 and 1 were transformed to negative fold-changes using the formula fc =-1/(ratio *x*). We also performed an IPA upstream regulator analysis to identify the cascade of upstream transcriptional regulators that can explain the observed gene expression changes in a user's dataset, which can provide insights into the biological activities occurring in the tissues or cells being studied.

## SUPPLEMENTARY FIGURES

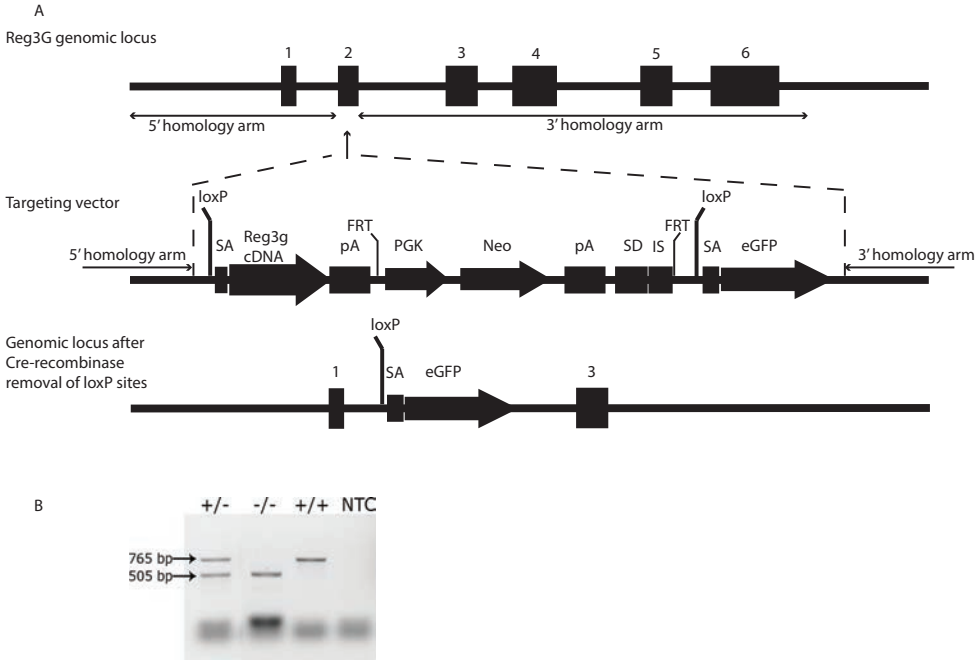


**Figure S6.1.** A) Schematic representation of the targeting vector used to generate RegIIIγ$^{-/-}$ mice.

Upper scheme: The six exons of mouse RegIIIγ and homology arms cloned into the targeting vector to promote recombination in the genome; exon 1 is not translated.

Middle scheme: The targeting vector showing loxP sites for Cre-mediated recombination, the cDNA for RegIIIγ, the selection cassette (PGK Neo), enhanced green fluorescent protein cDNA (eGFP), poly-adenylation (pA), splice acceptor (SA), splice donor (SD) and instability signal (IS) and homology arms that recombine with homologous genomic DNA to replace exon 2 with the vector sequences (between dotted lines).

Lower scheme: Schematic representation of the RegIIIγ locus after Cre-mediated excision of the DNA between the loxP sites showing absence of exon2.

B) Genotyping of transgenic mice. Genotyping of the mice was done by PCR, generating a band of 508 bp in case of a RegIIIγ$^{-/-}$, 768 bp for a wild type and both bands in case of a heterozygote. NTC means non-template control, where demineralised $H_2O$ was used instead of DNA.

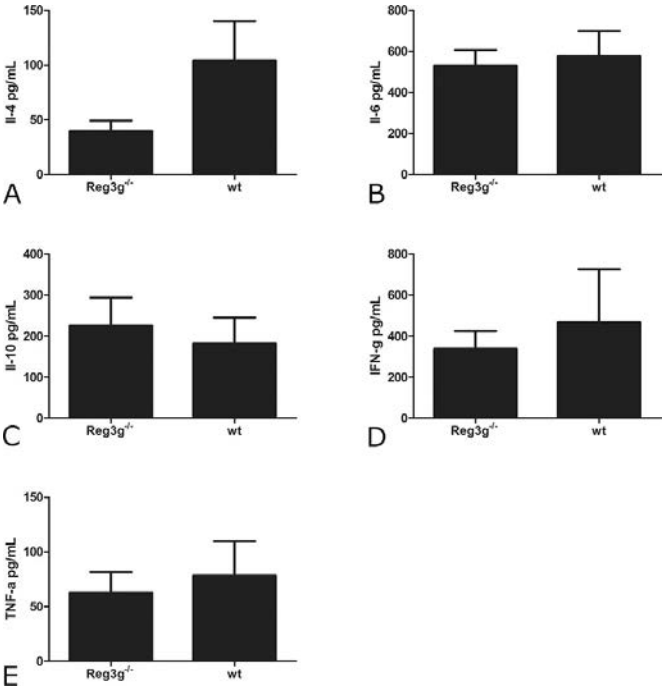


**Figure S6.2**. Cytokines produced by ConA stimulation of MLN cells isolated from RegIIIγ$^{-/-}$ and wt mice. Cells isolated from MLNs were stimulated with ConA and after 24 hour incubation the supernatant was removed after which levels of secreted IL-4 (A), IL-6 (B), IL-10 (C), IFN-γ (D) and TNF-α (E) were measured. Error bars indicate SEM.

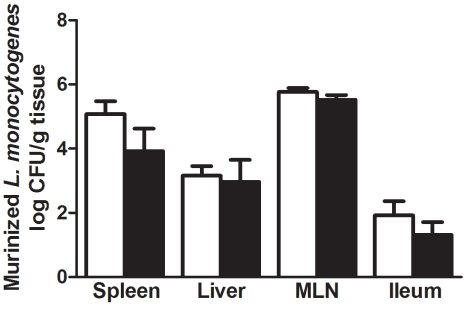


**Figure S6.3**. Pathogen colony forming units (CFU) in ileum and organs of mice infected with 'murinized' *L. monocytogenes*.[6] White bars indicate log CFU/g from RegIIIγ$^{-/-}$ (n=6) mice and black bars indicate log CFU/g from wt mice (n=8). Error bars indicate SEM. This murinized strain possesses a mutated form of Internalin A, which can bind to mouse E-cadherin at the tips of the villus where cell extrusion takes place[7] or at the goblet cells where E-cadherin is exposed during mucus secretion,[8] thereby increasing infectivity in murine models.

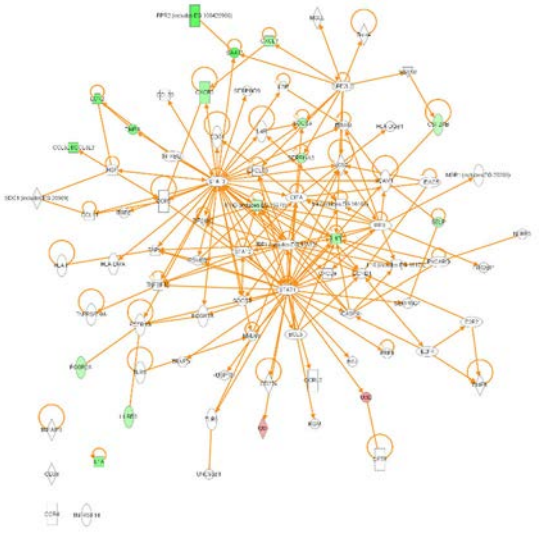

**Figure S6.4**. Network representation of interacting proteins encoded by differentially expressed genes (P <0.02) that are involved in responses to infectious disease and bacterial infection. The colour overlay represents genes that were expressed at higher (intenser shades of red) or lower (intenser shades of green) ratios for the comparison "*S. enteritidis* infection of RegIIIγ$^{-/-}$ mice vs *S. enteritidis* infection of wt mice". Coloured figures can be found in the pdf version of this thesis.

**Figure S6.5**. Network representation of interacting proteins encoded by differentially expressed genes (P <0.02) that are involved in responses to infectious disease and bacterial infection. The colour overlay represents genes that were expressed at higher (intenser shades of red) or lower (intenser shades

of green) ratios for the comparison "*L. monocytogenes* infection of RegIIIγ$^{-/-}$ mice vs *L. monocytogenes* infection of wt mice". Coloured figures can be found in the pdf version of this thesis.

| | Biomarker description | Mean KO | Mean WT | p value |
|---|---|---|---|---|
| **Eotaxin** | Enhanced in allergic inflammation | 349.80 pg/ml | 511.00 pg/ml | 0.0079 |
| **C-reactive protein (CRP Mouse)** | Acute phase reactant, used as biomarker for inflammatory diseases, infections, and neoplastic diseases | 3.68 µg/ml | 5.00 µg/ml | 0.0114 |
| **Monocyte Chemotactic Protein 1 (MCP-1)** | Involved in recruitment of monocytes to site of injury and infection | 58.60 pg/ml | 33.20 pg/ml | 0.0117 |
| **Serum Amyloid P-component** | Precursor of amyloid component P. Acute phase reactant | 19.60 µg/ml | 26.20 µg/ml | 0.0273 |
| **Macrophage colony stimulating factor-1** | Cytokines that act in hematopoiesis by controlling the production, differentiation, and function of granulocytes and the monocytes-macrophages | 6.28 ng/ml | 5.46 ng/ml | 0.0465 |
| **Macrophage Inflammatory Protein-2 (MIP-2)** | Potent neutrophil attractant and activator | 12.12 pg/ml | 8.88 pg/ml | 0.0562 |

**Table S6.3**. Measurement of serum biomarkers reveals few changes between RegIIIγ$^{-/-}$ and wt mice. 58 serum biomarkers were measured by RBM (see methods). The only five significantly changed markers are indicated and MIP-2 which was approaching significance at P=0.05. Cytokines measured in the serum were not different between RegIIIγ$^{-/-}$ and wt mice (IL-1a, IL-5 and IL-18) or below detection limits (IL-1b, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-11, IL-12p70, IL-17a, IFN-γ and TNF-α).

| | Wild type | RegIIIγ$^{-/-}$ |
|---|---|---|
| Pre-infection | 21.93 (+/- 3.00) | 21.65 (+/- 2.70) |
| *S. enteritidis* infection | 20.73 (+/- 3.26) | 22.05 (+/- 2.52) |
| *L. monocytogenes* infection | 20.10 (+/- 3.84) | 20.61 (+/- 2.62) |
| Uninfected controls | 22.53 (+/- 2.19) | 22.35 (+/- 2.04) |

**Table S6.4**. Average body weight of infected and control groups of RegIIIγ$^{-/-}$ and wt mice in grams. +/- indicates SD. No significant difference was observed between the mean body weights of the different groups.

| | Wild type | RegIIIγ$^{-/-}$ |
|---|---|---|
| *S. enteritidis* infection | 1.33 (+/- 0.30) | 1.46 (+/- 0.41) |
| *L. monocytogenes* infection | 2.31 (+/- 0.75) | 1.99 (+/- 1.03) |

**Table S6.5**. Serum Amyloid A2 levels in serum of infected wt and RegIIIγ$^{-/-}$ mice. In uninfected animals the levels were below detection limit. +/- indicates SD. Levels are shown in mg/ml.

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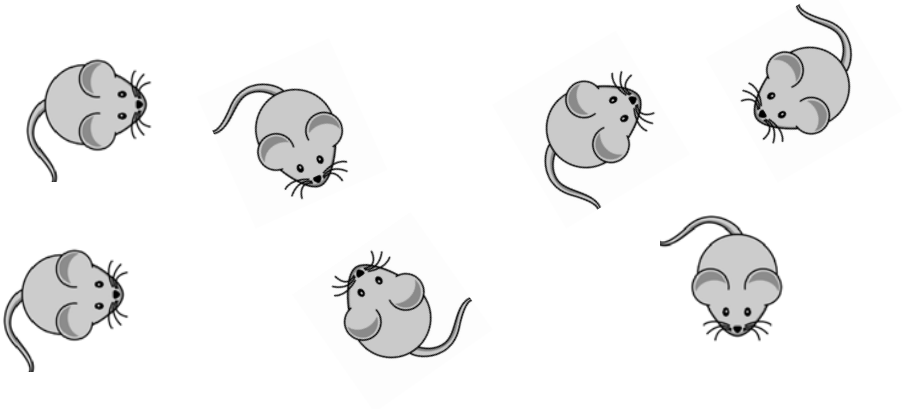

# Chapter 7

## General discussion

Linda M.P. Loonen

## BACKGROUND

Interest into the biology of the mammalian family of RegIII proteins increased substantially when it was shown that during active pancreatitis, human RegIIIα, commonly known as HIP/PAP (hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein), was one of the most abundant proteins in pancreatic secretions.[1] Nearly two decades later, it was found that in inflammatory bowel disease (IBD) patients, increased HIP/PAP transcription and serum levels of the protein were correlated with disease severity.[2] About the same time, using experimental mouse models, it was shown that RegIII proteins are expressed in the intestine and induced after conventionalization of germ free mice[3, 4] and in the colon of severe combined immune-deficient (SCID) mice after bacterial colonization.[5] In mice there are two intestinally expressed RegIII proteins, RegIIIγ and RegIIIβ that are both induced after weaning, in colitis and by intestinal infection.[4, 6]

Expression of RegIIIγ during infection was shown to be dependent on Myeloid differentiation primary response gene 88 (MyD88), a Toll like receptor (TLR) signalling adapter protein, and clearance of *Listeria monocytogenes* from the intestine of MyD88 knock out ($^{-/-}$) mice is impaired compared to corresponding wild-type (WT) mice.[6] More recently, MyD88$^{-/-}$ mice were also shown to be more susceptible to *Salmonella typhimurium* infection than WT mice, but transgenic expression of MyD88 in the Paneth cells of MyD88$^{-/-}$ mice restored RegIIIγ expression, and reduced *S. typhimurium* counts in the mesenteric lymph nodes (MLNs).[7] Antibiotic treatment of mice with vancomycin decreases RegIIIγ gene expression, presumably due to a reduction in MyD88 dependent signalling by the resident microbiota, favouring growth of vancomycin-resistant enterococci.[8] *RegIIIγ* and *RegIIIβ* gene expression is completely abolished in mice lacking interleukin (IL)-22, which is a T cell-derived cytokine that plays a role during the acute phase response. When *ex vivo* colon cultures of IL-22$^{-/-}$ mice were incubated with IL-22, expression of RegIII genes was restored, demonstrating the requirement for IL-22 in the regulation of RegIII expression. IL-22 is produced by Th17 cells and is among others required for protection of mice against *Citrobacter rodentium*. In IL-22$^{-/-}$ mice, recombinant RegIIIγ reduced mortality and weight loss after infection of mice by *C. rodentium*, demonstrating the specific role of this protein in protection from colitis caused by this pathogen, possibly by exerting anti-inflammatory effects.[9]

As for mice, the rat PAP orthologues are also thought to have anti-inflammatory activity, as administration of anti-PAP antibodies in an experimental model of taurocholate-induced acute pancreatitis in rats was associated with increased inflammation.[10] In human inflammatory bowel diseases (IBD) patients, secretion of proinflammatory cytokines in colonic tissue of patients with active Crohn's Disease (CD) was inhibited by addition of purified native human HIP/PAP.[2] *In vitro* experiments with human monocytes, epithelial cells and endothelial cells revealed its anti-inflammatory effects to be

due to attenuation of nuclear factor-kappa light chain enhancer of activated B cells (NF-κb) activation by tumour necrosis factor-alpha (TNF-α). Furthermore, human PAP decreased expression of surface receptors involved in leukocyte recruitment in endothelial cells suggesting that PAP could dampen inflammatory responses by limiting leukocyte recruitment into the intestine.[2] However, intravenous injections of PAP induced inflammation in the lung, concurrent with increased TNF-α expression in the liver[11] but prevented leukocyte-mediated pulmonary vasoconstriction and oedema formation,[12] suggesting that the biological effect of PAP might differ between organs. Clearly, the biological function(s) of RegIII proteins in maintaining and promoting tissue homeostasis are broad and depend on the organ.

The overarching goals of this thesis were:

- To investigate the protective role of RegIIIβ and RegIIIγ in the intestine
- To explore their role in colitis and in infection with enteropathogens
- To investigate whether RegIIIβ and RegIIIγ might be potential biomarkers of intestinal health

The approaches included both *in vitro* experiments using purified, recombinant RegIII proteins and *in vivo* studies using mouse models of colitis and experimental infections with bacterial pathogens in knockout mice. The insights gained from this research are discussed in the following sections.

**Expression of RegIII proteins in the intestine and their role in colitis**

*RegIII*β and *RegIII*γ are both upregulated within a few days after conventionalization or infection of mice and in mouse colitis, and *PAP* is upregulated in human CD patients.[2, 4] These findings suggest that the upregulation of RegIII or PAP could have been induced by increased proximity of bacteria to epithelial cells, possibly as a consequence of lower integrity of the mucus barrier. To gain more information on the location and expression of mouse RegIII proteins and the possible involvement of mucus barrier, we investigated expression of RegIIIγ and RegIIIβ in the colon and small intestine of WT mice and Muc2$^{-/-}$ mice, using a combination of microarray gene expression profiling, *in situ* hybridization and histological techniques, at 2, 4 and 8 weeks (**Chapter 2 and 3**). After 4 weeks, Muc2$^{-/-}$ mice showed histological changes characteristic of other murine colitis models and the clinical symptoms of human IBD. As Muc2 is the major secreted mucin produced in the intestine, its absence leads to increased contact of the microbiota with the epithelium.[13] In Muc2$^{-/-}$ and WT mice, we observed that expression of RegIII genes and proteins was substantially higher in the ileum than in the colon, confirming previous work in humans and mice.[2, 3, 4, 14] Highest production of RegIII genes and proteins was observed in Paneth cells; these cells are absent in the colon, explaining the low expression of RegIII in the distal intestine. Expression of RegIII proteins was also observed

in enterocytes, mainly around the crypt-villus junction (**Chapter 2**). Interestingly, in 2 week-old mice staining of RegIII proteins was evident in goblet cells but this decreased after weaning, perhaps due to a developmental switch in gene regulation. Nevertheless, this showed for the first time a new function of goblet cells in innate immunity, at least in young developing animals.

The Muc2$^{-/-}$ mouse model was used to explore expression of RegIIIγ and RegIIIβ prior to and after the onset of colitis. Muc2$^{-/-}$ mice develop colitis in the colon after 4 weeks as evidenced by the increased thickness of the mucosa, an abnormal mucosal morphology, flattening and ulceration of the epithelial cells accompanied by blood in the faeces and weight loss.[13] Microarray analysis showed that expression of *RegIIIγ* and *RegIIIβ* was increased in the proximal colon and ileum at week 2, 4, and 8, even prior to the onset of colitis (**Chapter 3**). Highest expression of both RegIII genes was in the ileum and was significantly induced compared to WT mice. Furthermore, the pathways involved in TLR signalling, cytokine and chemokine production (e.g. TNF-α, IFNγ (interferon gamma), Ltb (lymphotoxin B), IL-1β), NF-κb activity and other immune pathways related to B and T cell activity and antibody production were induced in the colon of Muc2$^{-/-}$ mice compared to WT mice. Additionally, there was increased expression of the T cell markers CD4 and CD3e in the colon of Muc2$^{-/-}$ mice, which is consistent with the mild influx of immune cells that we observed by histology. Furthermore, 14 out of a panel of 32 IBD-related genes were differentially expressed in the proximal colon of Muc2$^{-/-}$ mice at week 8. Fifteen of these IBD-related genes were also differentially expressed in the same way in the mouse DSS-colitis model due to increased contact of the epithelium with microbiota.[15, 16] Defensin genes were not upregulated in the colon of Muc2$^{-/-}$ mice. This could have been due to the effect of inflammatory cytokines and nitric oxide on protein folding, which causes the accumulation of unfolded proteins inside the endoplasmic reticulum and concomitant induction of the unfolded protein response, which affects the function and localization of several proteins including defensins.[17]

In contrast to what we observed in colon, the ileum of Muc2$^{-/-}$ mice showed no histological signs of damage. This was not due to differential expression of three other secreted mucins, Muc5AC, Muc5B and Muc6. However, an increase in villus length was observed in the ileum suggesting hyperplasia. This was in agreement with increased expression of pathways involved in cell cycle and mitosis, protein translation and the altered expression of the S100a family of genes that are involved in cell division and differentiation (**Chapter 3**). Strikingly, TLR and (innate) immune signalling pathways including B and T cell receptor signalling were down-regulated compared to WT mice. During the inferred down-regulation of immune signalling pathways, expression of *RegIIIβ* and *RegIIIγ* was significantly increased in the ileum of Muc2$^{-/-}$ mice. This is compatible with the increased expression of the gene encoding LtB, which is required for production of IL-22, which induces epithelial RegIII expression in enterocytes.[18]

Given the demonstrated anti-inflammatory properties of human PAP it is tempting to speculate that the mouse RegIII proteins may have played a role in the suppression of inflammatory pathways in the ileum where their expression is highest. Apart from suppression of inflammation, the lack of damage in the ileum may also have been associated with lower bacterial burden as numbers of bacteria are $10^2$-$10^4$ times lower in ileum compared to colon. Increased expression of RegIII proteins might further lower the number of bacteria in the ileum.[4, 19] However, the *in vitro* bactericidal properties of RegIII proteins are not consistently reported in the literature,[9] suggesting that the activity is dependent on the preparation of the protein and the assay conditions.

**Lessons from *in vitro* and *in silico* studies on recombinant RegIII proteins**

RegIIIγ has been proposed to kill Gram-positive bacteria upon binding to peptidoglycan (PGN) via unknown mechanisms. One possibility is that binding of RegIIIγ to PGN interferes with PGN biosynthesis, by binding to precursors causing osmotic cell lysis. Alternatively, RegIII proteins may kill bacteria by activating bacterial two-component stress response defence systems as described for other innate PGN binding proteins.[20] However, not all the groups investigating these proteins, have found bacterial killing by RegIII proteins or their human orthologue HIP/PAP. To further investigate anti-inflammatory and possible antimicrobial activities of RegIIIβ and RegIIIγ proteins, we expressed and purified recombinant forms of these proteins and tested their activities *in vitro* (**Chapter 4**). Expression of RegIIIβ and RegIIIγ in *E. coli* leads to formation of inclusion bodies as described previously for RegIIIγ.[21] This facilitates initial purification but requires denaturation and refolding of the proteins to obtain soluble protein, with the risk of losing or reducing the activity of native protein. Nevertheless, refolded RegIII proteins have shown bactericidal activities.[4, 22] We tried to crystalize refolded RegIII proteins produced in *E. coli,* but our attempts were unsuccessful, presumably due to the heterogeneity of the protein conformations in the final preparations. To overcome some of these problems expression in *Pichia pastoris* (yeast) and insect cells using the baculovirus system were explored. Production in *P. pastoris* did not yield purified, soluble RegIIIβ, but insect cells did secrete soluble RegIIIβ protein albeit in low yields compared to *E. coli*. As expected, the size of RegIIIβ produced in insect cells and in *E. coli* showed that RegIIIβ had not been post-transcriptionally modified by glycosylation, as shown before.[21] If no reducing dithiothreitol (DTT) was used, all of the purified RegIII proteins formed multimers as previously reported.[23-26]

Human PAP and mouse RegIIIβ and RegIIIγ proteins contain an N-terminal pro-segment that is cleaved by trypsin, resulting in enhanced PGN binding and bactericidal activity of RegIIIγ and PAP towards *Listeria monocytogenes.*[27] This may be a mechanism to allow the host to restrict the binding activity of RegIII proteins to the lumen and to avoid detrimental effects inside host cells during storage or secretion. We demonstrated that the predicted pro-segment was also cleaved from recombinant RegIIIβ by trypsin as previously described for RegIIIγ.[27] Immunoblotting showed that trypsin treatment of soluble

baculovirus produced RegIIIβ generated a single major protein whereas refolded RegIIIβ produced in *E. coli* appeared to be cleaved into at least 3 polypeptides of different sizes (**Chapter 4**). Analysis of the potential trypsin cleavage sites within the RegIIIβ primary protein sequence indicated several possible cleavage sites, which may have become accessible to trypsin in alternative protein conformations. These results suggest that refolding of RegIIIβ may have increased its susceptibility to proteolysis by trypsin. In contrast to RegIIIβ, refolded RegIIIγ protein yielded a single protein band upon cleavage with trypsin as previously described.[27] One explanation for the difference in trypsin digestion patterns of RegIIIβ and RegIIIγ might be the fewer number of potential trypsin cleavage sites in RegIIIγ. For this reason the soluble RegIIIβ protein, secreted by insect cells, was used in the bactericidal and binding assays, although refolded RegIIIβ was included in some assays for comparison (**Chapter 4**).

The published work on the bactericidal effects of RegIIIβ report around 80% reduction in colony forming unit (CFU) after incubation with most of the sensitive bacteria, which is a relatively weak bactericidal effect, compared to antibiotics and defensins.[22] Trypsin processing of baculovirus produced, soluble RegIIIβ led to enhanced binding to *L. monocytogenes* and *S. enteritidis* as determined by flow cytometry. However, in contrast to earlier reports,[19] RegIIIβ produced in our lab was not bactericidal for *S. enteritidis* during logarithmic or stationary phase, although similar buffers, protein concentrations and methods were used as reported by others (**Chapter 4**).[19, 21, 22] Immunoblotting showed that the lack of bactericidal activity was not due to degradation of our recombinant RegIII proteins during storage, but it is possible that the proteins loose activity over time. Differences in the bacterial strain or unreported details in the procedures for handling or purification may also have led to different results. Inconsistencies in the bactericidal activity of RegIII proteins have been reported by others as well,[28] suggesting that these proteins may be unstable or that their activity may be highly dependent on unknown assay conditions.

RegIII proteins may protect mice against bacteria by pathways that do not depend on bactericidal activity. For instance, RegIIIγ was not bactericidal for Gram-negative *Citrobacter rodentium in vitro* but when administered to IL-22$^{-/-}$ mice, that do not produce RegIII proteins, although it reduced mortality and severity of colitis.[9] Purified native rat PAP aggregated *E. coli,* but was not bactericidal[29] and recombinant human PAP purified and refolded from *E. coli* lacked bactericidal activity against *B. subtilis.*[28] Recombinant PAP from rat was also reported to lack bactericidal activity against *Salmonella enteritidis* and *Listeria monocytogenes.*[30] We were also unable to demonstrate a bactericidal effect of refolded RegIIIγ on the Gram-positive *L. monocytogenes*, in contrast to what had been reported by Cash *et al*.,[4] using the same methods and bacterial isolate. The N-terminal processed RegIIIγ did however show binding of RegIIIγ to *L. monocytogenes*

and, somewhat unexpectedly, even more strongly to Gram-negative *S. enteritidis* and *E*. *coli*. This suggested that RegIIIγ can also bind to lipopolysaccharide (LPS), a property common to many animal PGN binding proteins involved in innate immunity.[31-33]

One factor that may account for the inconsistent results obtained with recombinant RegIIIβ and RegIIIγ could be the use of CFU counts to measure bactericidal activity, as this method does not take into account bacterial aggregation by RegIII proteins. Aggregation of bacteria by RegIII proteins may depend on correct folding, something that is likely to be influenced by the refolding method and might even vary from one protein preparation to another. For this reason we performed some bacterial killing assays using flow cytometry and fluorescent live/dead staining techniques and microscopy. We observed weak bactericidal effects of RegIIIβ on logarithmically growing *E. coli VE7108* (25% killed) and on *E. coli Nissle* (6%) in the absence of any histological evidence for bacterial aggregation.

In addition to the bactericidal assays we tested whether RegIIIβ and RegIIIγ were bacteriostatic by adding N-terminally processed and unprocessed RegIII proteins to low density bacterial cultures of *L. monocytogenes* and *S. enteritidis*. This had no effect on bacterial growth over 8 hours. The added RegIII proteins could still be detected in the supernatant after completion of the growth curve measurements, ruling out their degradation. However we cannot exclude the possibility that the RegIII proteins are not active in bacterial culture medium.

To gain more insight into the binding sites of RegIII proteins and their known ligands, we unsuccessfully attempted to crystallize the mouse RegIII proteins. However, it was possible to generate models of the tertiary structure using the protein sequence of human PAP for which a protein crystal structure was available.[34] The structural models of RegIIIβ, RegIIIγ and PAP closely resemble each other in terms of the overall protein fold, and the amino acid sequences have between 67% and 71% identity. The structural models show the external N-terminal pro-peptide sequence that is proteolytically processed in the lumen to increase the binding activity of the proteins.[27, 28] Furthermore, docking experiments revealed a model that predicts RegIIIβ and RegIIIγ binding to bacterial PGN, as was shown in binding assays with insoluble PGN[4, 19] (this thesis, data not shown). This would explain why RegIIIβ can bind *L. monocytogenes* in our binding assays and had a bactericidal effect on *Clostridium butyricum,* although the other Gram-positive species tested, namely *Lactobacillus murinus* and *Enterococcus faecalis,* were resistant to killing.[22] The reasons for differential killing of Gram-positive bacteria are not clear but might involve PGN modifications or increased D-alanine-substitution on wall teichoic acids, which might inhibit binding of RegIIIβ.[35, 36]

RegIIIβ can bind LPS via the lipid A component[19] and docking experiments with this

ligand suggested that binding occurs at a different site on the protein than binding to PGN (**Chapter 4**). The interacting residues responsible for this lipid A binding were in an area with low conservation in RegIIIγ, suggesting that RegIIIβ and RegIIIγ have different specificities or affinities for ligands (Fig. 7.1). The interacting residues in the PGN-binding site were conserved in RegIIIβ and RegIIIγ, which is compatible with reports describing binding of both proteins to this ligand.

As PAP was previously shown to attenuate NF-κb signalling in human monocytes and epithelial cells,[2] we tested RegIIIβ for potential anti-inflammatory effects. Incubation of mouse BMDC (bone marrow derived DCs) with and without RegIIIβ and with or without a stimulus (lipopolysaccharide (LPS) or $Pam_3CysSerLys_4$ ($Pam_3CSK_4$), a TLR2 ligand) showed no anti-inflammatory effect of RegIIIβ on surface activation markers or cytokine secretion (not shown). Considering the binding of RegIIIβ to LPS, it is possible that RegIIIβ proteins sequester LPS, thereby reducing LPS concentrations and avoiding LPS entering cells and contributing to maintaining homeostasis.[37] In addition, to investigating potential anti-inflammatory effects of RegIIIβ, we also tested whether RegIIIβ had an effect on the trans-epithelial resistance (TER) in a Caco2 (human colonic cell line) cell culture. Pre-incubation of cells or bacteria with RegIIIβ did not have an effect on the decrease of the TER when *S. enteritidis* or pathogenic *E. coli* LF82 were added to the Caco2 cells, but RegIIIβ alone significantly increased the TER of the cells (not shown) in 3 independent experiments. Messenger RNA of several tight junction proteins was not upregulated as was shown by qPCR. Exactly how the TER is increased by RegIIIβ is not known yet, but it might involve modification of TJ complexes by binding of RegIIIβ to receptors or other extracellular molecules located on the apical membrane.

**Infection studies in RegIIIβ and RegIIIγ knockout mice**

At the beginning of this research project, we set out to study the phenotype of RegIIIβ and RegIIIγ knockout ($RegIII\beta^{-/-}$ and $RegIII\gamma^{-/-}$) mice and their sensitivity to infection with Gram-positive and Gram-negative pathogens. To obtain and breed specific pathogen free (SPF) $RegIII\beta^{-/-}$ mice took several months and the generation of our own $RegIII\gamma^{-/-}$ mouse took more than two years. Nevertheless, the results obtained with these models have been very enlightening and generated new ideas about the function of these proteins *in vivo*, as discussed below.

The mice used for the RegIIIβ infection experiment were bred on a mixed background (C57Bl/6 and 129O1a). These strains differ in their susceptibility to *Salmonella* infection due to a mutation in the natural resistance-associated macrophage protein (Nramp) 1 gene encoding the Slc11a1 transporter. Mice bearing specific alleles of Nramp1 are more resistant to *S. enterica* serovar typhimurium infection since production of specific transporters impairs growth of *S. enterica* in macrophages via stimulation of expression of lipocalin-2, an antimicrobial iron scavenger.[38] All control and *S. enteritidis* infected mice were genotyped and were shown to be homozygous for the Nramp1 susceptibility

allele, ruling out the possibility that the Nramp status would influence the results. Unchallenged RegIIIβ$^{-/-}$ mice have a normal appearance and body weight. However, when challenged with virulent *S. enteritidis*, higher numbers of *S. enteritidis* were present in colon, mesenteric lymph nodes (MLN), spleen and liver of infected RegIIIβ$^{-/-}$ mice compared to WT mice, indicating that the adherence, and translocation and dissemination of *S. enteritidis* is increased in the absence of RegIIIβ (**Chapter 5**). The MLN drain the small intestine, which is a main site of *S. enteritidis* invasion.[39] Therefore reduced levels of *S. enteritidis* in the MLNs of WT mice suggest protective effects of RegIIIβ on *S. enteritidis* translocation via the ileum. We did not observe differences in *S. enteritidis* counts in the ileal tissues of RegIIIβ$^{-/-}$ and WT mice. However, the investigated ileal tissue did not contain Peyer's patches (PP) and M cells, which are considered, in addition to absorptive enterocytes, to be important sites of *S. enteritidis* translocation.[40, 41] Together, the experimental infection data strongly suggest that RegIIIβ inhibits *S. enteritidis* translocation from the gut lumen into extra-intestinal tissues but does not kill *S. enteritidis* bacteria at-a-distance in the gut lumen. These results are supported by recent findings of Dessein et al., who showed that genetic ablation of RegIIIβ did not affect the bacterial load of the Gram-negative pathogen *Yersinia pseudotuberculosis* in the intestinal lumen[14] but significantly increased bacterial burden in the PP, which are the main route of entry for this pathogen.

In contrast, lack of RegIIIβ did not appear to influence resistance to infection with the Gram-positive *Listeria monocytogenes*, as identical numbers of this pathogen were recovered from intestinal and extra-intestinal tissues in RegIIIβ$^{-/-}$ and WT mice in our infection study (**Chapter 5**). This is compatible with our *in vitro* data showing no bactericidal effect of RegIIIβ on *L. monocytogenes,* although recombinant RegIIIβ appeared to bind both *S. enteritidis* and *L. monocytogenes in vitro,* suggesting that RegIIIβ might have a different function, such as trapping bacteria in the mucus instead of direct killing.

After infection with both *S. enteritidis* and *L. monocytogenes,* myeloperoxidase (MPO) protein levels in ileal scrapings were not different between RegIIIβ$^{-/-}$ and WT mice, indicating a low local inflammatory response. In absence of a differential inflammatory response, we cannot infer if RegIIIβ could have anti-inflammatory effects, as reported for human PAP. Further analysis of the collected intestinal tissues from this study might give insight on the role of the innate (immune) response in RegIIIβ related protective effects. For example, micro-array analysis of intestinal tissues may identify whether specific host defence pathways are associated with protective effects of RegIIIβ. In the future *L. monocytogenes* and *S. enteritidis* infection experiments in mice deficient in specific TLRs could be used to investigate their potential involvement in RegIIIβ-mediated protection against intestinal infection.

**Contributions of RegIIIγ to mouse innate immunity**

To investigate the *in vivo* role and function of RegIIIγ we generated a RegIIIγ$^{-/-}$ mouse

model that expresses an enhanced green fluorescent protein (eGFP) reporter under control of the RegIIIγ promoter (**Chapter 6**). Furthermore, based on its proposed bactericidal activity towards Gram-positive bacteria,[4] we investigated the hypothesis that absence of RegIIIγ would result in higher susceptibility to infection with Gram-positive *L. monocytogenes* than to infection with Gram-negative *S. enteritidis* using infection studies in our RegIIIγ$^{-/-}$ mice.

We first compared the phenotypes of uninfected RegIIIγ$^{-/-}$ and WT mice. No morphological differences between RegIIIγ$^{-/-}$ and WT mice were found by phenotypic histological analysis. However, we observed a significant increase in the relative amounts of IL-22 transcript and MPO protein levels in ileum samples of RegIIIγ$^{-/-}$ mice relative to WT mice (**Chapter 6**), suggesting elevated innate responses and chemotaxis of neutrophils. Moreover, significantly higher amounts of the chemokines monocyte chemotactic protein (MCP)-1 and macrophage colony stimulating factor (M-CSF)-1, both of which are involved in monocyte and neutrophil recruitment, activation and differentiation, were found in serum of RegIIIγ$^{-/-}$ mice. Interestingly, pro-inflammatory cytokines such as IL-1β, IL-12p70, TNF-α and IFN-γ were not different between RegIIIγ$^{-/-}$ and WT mice, or were below detection limit in serum. Therefore, it appears that RegIIIγ$^{-/-}$ mice exhibit an increased innate immune cell activity in the mucosa that successfully avoids systemic inflammation. Theoretically, the heightened inflammatory status of the mucosa might have been due to increased contact between the luminal bacteria and the epithelium, or because of an anti-inflammatory effect of RegIIIγ as previously proposed by Vaishnava *et al.*[42] and Zheng *et al*.[9] Therefore, to assess the distribution of bacteria in the mucus layer, we performed FISH staining on ileum and colon sections. Sections of ileal tissue from RegIIIγ$^{-/-}$ mice, but not WT mice, consistently showed the presence of small numbers of bacteria on the epithelium, in agreement with the study of Vaishnava,[42] who independently generated a RegIIIγ$^{-/-}$ mouse. In the colon, increased co-localisation of bacteria and epithelial surfaces was not observed. This supports the possibility that the increased IL-22 and MPO levels in the ileal mucosa of RegIIIγ$^{-/-}$ mice were due to decreased spatial segregation of bacteria resulting in increased innate inflammatory signalling. These results do not rule out possible anti-inflammatory effects of steady state levels of RegIIIγ *in vivo*.

Infection of RegIIIγ$^{-/-}$ mice with either of the pathogens did not result in increased translocation to the spleen, liver or MLNs compared to WT infected mice (**Chapter 6**). This result was fully reproduced using a 'murinized' *L. monocytogenes* strain, engineered to increase the epithelial route of infection in murine models.[43] Although there was no increased translocation of pathogens in the organs of RegIIIγ$^{-/-}$ mice, we did measure increased amounts of MPO in ileal tissue of infected RegIIIγ$^{-/-}$ mice compared to WT infected mice. MPO is produced mostly by neutrophil granulocytes, which are attracted to sites of infection suggesting that increased numbers of both pathogens had translocated into the mucosa of RegIIIγ$^{-/-}$ mice compared to WT mice. These results suggest

that the heightened mucosal inflammatory response observed in infected RegIIIγ$^{-/-}$ mice was sufficient to inhibit spread of the pathogens to the bloodstream and organs. In agreement with this, loss of RegIIIγ did not affect systemic inflammatory responses as no significant differences in serum SAA2 levels were measured between RegIIIγ$^{-/-}$ and WT mice.

Overall, the results suggest that the mucosal inflammatory response, involving monocyte and neutrophil recruitment and activity to pathogen infection was increased in RegIIIγ$^{-/-}$ mice. As suggested above, trapping of bacteria in the mucus or increased movement of bacteria through the mucus might explain the increased mucosal inflammatory responses to both pathogens in RegIIIγ$^{-/-}$ mice.

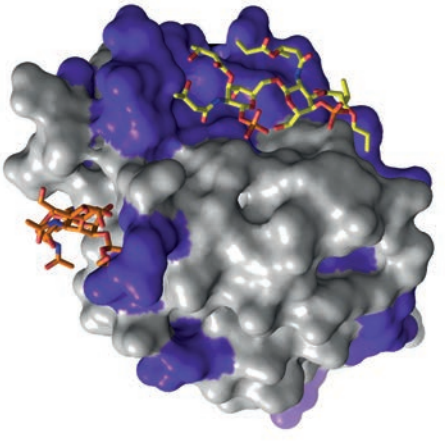

**Figure 7.1.** Space filling model of RegIIIβ showing the two different predicted binding sites for lipid A and PGN. Regions of RegIIIβ protein conserved in RegIIIγ are shown in grey and the variable regions are shown in purple.

**RegIIIβ is predicted to have two ligand-binding sites and can bind to glycans on human mucin**

We observed that an intensely stained band of mucus consistently located above the villi and surrounding the ileal content in WT mice was absent in RegIIIγ$^{-/-}$ mice, while the expression of Muc2 mRNA was not altered. We also showed that RegIIIγ was present throughout the mucus layer of WT mice (**Chapter 6**). If binding of RegIIIγ in the mucus layer can alter the structure or the distribution of mucus in the ileum it would provide an explanation for the altered distribution of mucus in RegIIIγ$^{-/-}$ mice. To investigate possible interactions of RegIII proteins with glycans we performed *in vitro* glycan binding arrays, initially with recombinant RegIIIβ produced by baculovirus, as

this protein was secreted in soluble form. Purified RegIIIβ was fluorescently labelled and hybridized to a glycan array of purified mucin and host glycans. Strikingly, RegIIIβ significantly bound several different fucosylated glycans, as well as purified human colonic mucin (**Chapter 3**). Docking approaches were used to predict the RegIIIβ binding sites of these ligands using models of the tertiary structure described above (**Chapter 4**). In RegIIIβ two binding sites were identified; binding site 1 (BS1) binds to PGN and 2 different fucose-containing glycans while binding site 2 (BS2) binds to lipid A, a part of the LPS layer in Gram-negative bacteria, and to two other glycans ligands on the array, suggesting that RegIIIβ may be able to bind to both mucins and PGN. (Fig. 7.2), From this model, it follows that RegIIIβ could be distributed throughout the mucus and could bind to mucins while at the same time, maintaining the ability to bind PGN exposed on Gram-positive bacteria. As RegIIIβ and RegIIIγ can self-assemble into large multimeric complexes as previously described for PAP and other Reg family members[28]innate immunity, lectin-like protein, N-terminal proteolytic processing, pancreatitis-associated protein (PAP it may crosslink mucins thereby stabilizing mucin structure or altering penetrability by bacteria. Interestingly, fucosylated glycans on the epithelial surface can serve as receptors for pathogenic viruses and bacteria.[44] Thus the induction of RegIIIβ and RegIIIγ expression during infection might help to prevent colonisation of the gut epithelium by pathogens, by reducing availability of receptor binding sites that could be bound by these pathogens.

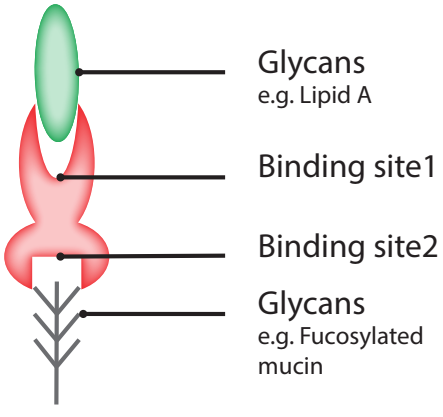


**Figure 7.2**. Hypothetical model of the two different binding sites on RegIIIβ, based on docking results obtained in this study.

**Current hypotheses about the role of RegIIIβ and RegIIIγ**

Our phenotyping study in RegIIIγ$^{-/-}$ mice strongly supports a protective role for RegIIIγ in compartmentalizing commensal bacteria in the lumen. Loss of this protein increased expression of mucosal innate inflammatory markers but not of systemic inflammatory

markers. The increased inflammatory response was modest, presumably due to the redundancy of innate defence factors including RegIIIβ, and the absence of pathogens in our SPF mice. Vaishnava et al.,[42] concluded that the spatial segregation of bacteria was due to bactericidal effects of RegIIIγ on Gram-positive bacteria. However, infection studies in our RegIIIγ$^{-/-}$ mice showed that mucosal inflammation was significantly increased when mice were infected with both Gram-positive *L. monocytogenes* and Gram-negative *S. enteritidis*. This does not exclude a bactericidal effect on *L. monocytogenes,* but it suggests that RegIIIγ has protective effects that go beyond direct specific bactericidal activity towards Gram-positive bacteria, including having an effect on mucus properties or trapping of Gram-positive as well as Gram-negative bacteria in the mucus network. Similar *in vivo* studies on the possible effects of RegIIIβ deficiency on the spatial segregation of bacteria are currently on going.

The expression of two closely related RegIII proteins in the mouse intestine suggests a (partially) different function for each of these proteins. One could speculate about two proteins, each having a specific bacterial target (i.e. Gram-negative or Gram-positive), in case of purely anti-microbial properties. However, when looking at the results obtained in this thesis (**Chapters 4, 5 and 6**) and work on the rat and human proteins published by others[2, 28, 29] this appears not to be the case. There are inconsistencies on bactericidal effects of RegIII proteins in the different published studies as well as our own data that suggest that RegIII proteins may bind to bacteria without inducing killing. The infection studies show that the two mouse RegIII proteins studied in this thesis have different functions to one another. RegIIIβ protects against *S. enteritidis* infection and translocation to the organs but not against *L. monocytogenes* infection. However, RegIIIγ seems to confer protection against both pathogens as infection of RegIIIγ$^{-/-}$ mice with either *S. enteritidis* or *L. monocytogenes* leads to heightened mucosal inflammation. Even in unchallenged RegIIIγ$^{-/-}$ mice the MPO levels were elevated in the ileal mucosa, which could be due to increased contact of bacteria with the epithelium (**Chapter 6**). An increase in MPO levels was not observed in uninfected RegIIIβ$^{-/-}$ mice compared to WT. Therefore, we might not find the same altered spatial compartmentalization of bacteria observed in the RegIIIγ$^{-/-}$ mice. The different results obtained in the infection studies with RegIIIβ$^{-/-}$ and RegIIIγ$^{-/-}$ mice might be partially caused by differences in the microbiota composition and their different antagonistic effects on the pathogens (i.e. colonization resistance).[45, 46] Thus, determining the microbial composition in RegIIIβ$^{-/-}$ and RegIIIγ$^{-/-}$ mice and abundance of specific bacterial species might give us more information on the possible targets of both RegIIIβ and RegIIIγ. Analogously, the microbiota composition in the Muc2$^{-/-}$ model could give us more information on the possible impact of presence or absence of specific bacterial groups on colitis. Attempts are underway to analyse the microbial composition in both the colon and the ileum of these models. One promising future tool is an inducible RegIIIγ$^{-/-}$ mouse model. In this model, RegIIIγ gene deletion is mediated through intra-peritoneal administration of tamoxifen, which induces cre-mediated deletion via recombination of loxP sites flanking RegIIIγ, bringing eGFP under

control of the cognate promoter. This model was recently generated and preliminary results look promising (Fig. 7.3). Administration of tamoxifen in different concentrations, increased relative expression of eGFP and significantly decreased relative expression of RegIIIγ.

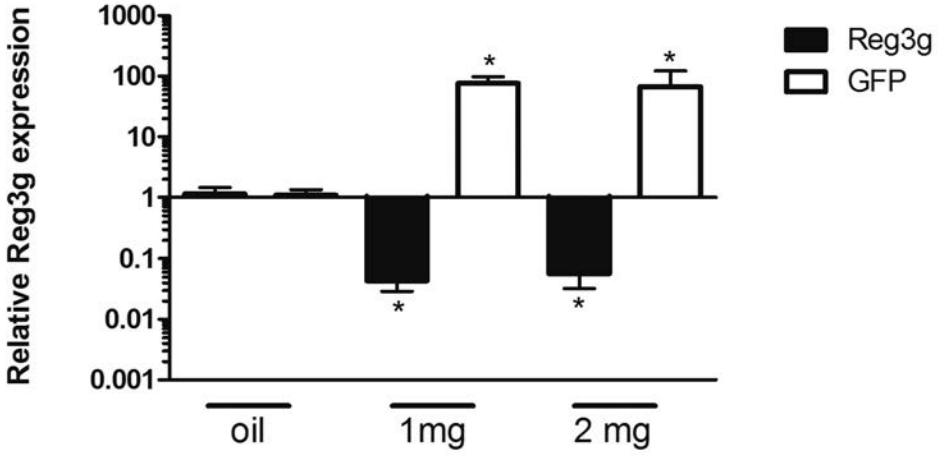


**Figure 7.3.** RegIIIγ and eGFP mRNA expression levels in the distal ileum of tamoxifen induced animals. mRNA expression data of 4 mice per group are shown relative to the housekeeping gene HPRT. Expression ratios are shown as log values. * indicates P<0.05, compared to the OIL control group, and significant for both reference genes.

More research needs to be done on this model before it can be used for studying the effect of RegIIIγ on the microbiota. To closely monitor potential changes in microbiota compositions due to the deletion of RegIIIγ, littermates should be used and gene deletion could be induced at different time points.

Although RegIIIβ and RegIIIγ appear to have protective effects, their absence does not cause disease in mice up to 8 weeks of age as the knock out animals appear normal and healthy. Based on *in vivo studies* to date (**Chapter 5** and Dessein *et al.*[14]), RegIIIβ seems to specifically protect against infection with Gram-negative bacteria, but a broader panel of pathogens including multiple Gram-negatives and Gram-positives should be tested to confirm this hypothesis. RegIIIγ seems to reduce mucosal inflammation caused by infection with either Gram-positive or Gram-negative pathogens, suggesting it has a more general anti-microbial or protective activity. In addition to this protection to bacteria, RegIIIγ may possess an anti-inflammatory activity, as higher amounts of inflammatory markers were measured in uninfected RegIIIγ$^{-/-}$ mice than WT mice. The antimicrobial effect of RegIIIγ might be due to binding to pathogens as shown *in vitro* (**Chapter 4**) or

a bactericidal activity although this is somewhat controversial as discussed above. The demonstration that RegIII proteins bind to mucus and are predicted to have more than one ligand binding site adds weight to the notion that trapping of bacteria in the mucus layer could be an important protective property. However, one should be cautious about drawing firm conclusions from *in vitro* data as the *in vivo* activity of RegIII proteins may require mucus or other secretory factors/conditions that are not reproduced *in vitro*.

The ability to produce secreted RegIII proteins in mammalian cells would be a significant advantage for further studies, as the function of refolded proteins may be different to the function of native proteins. The importance of the conformational structure of these proteins was shown previously by Hassanain *et al.*[47] who demonstrated that aggregated proteins could have a different function than non-aggregated proteins. Aggregation could take place after enzymatic processing of the N-terminus, possibly giving processed and unprocessed RegIII proteins multiple functionalities. To date, no one has succeeded in producing secreted RegIII protein in mammalian cells; our own attempts were also unsuccessful. Several groups produced RegIII in pancreas cells after inducing pancreatitis in those cells *in vitro,*[48-50] but we were not able to show any RegIII production in LPS-stimulated colonic and small intestinal epithelial cell lines. Also CHO and HEK293 cells, transfected with a construct coding for RegIIIβ or RegIIIγ under the control of a CMV promoter, did not secrete either of the two RegIII proteins (data not shown). One could speculate that cofactors such as chaperones or microRNAs need to be present, e.g. to stabilize RegIII mRNA. What might be worth to try in the future is to stimulate intestinal epithelial cells with IL-22, a natural inducer of RegIIIγ production and secretion.[9]

The possible mechanisms of RegIIIβ and RegIIIγ are summarized in figure 7.4, including the untested hypothesis that RegIII opsonizes bacteria thereby enhancing phagocytosis through binding to glycans on the antigen-presenting cells (APCs; panel A). In panel B we show the effect of RegIII proteins on TJs and the anti-inflammatory effect. An increase in the TER of Caco2 cell monolayers was reproducibly measured in presence of RegIIIβ (data not shown), but it is not known whether this is a direct effect of RegIIIβ on TJs, or due to cell signalling and effects on TJ composition. Nothing has been published on host receptors for RegIII proteins so we can only speculate about internalization of RegIII proteins and the resulting signalling pathways, but anti-inflammatory effects have been shown by several groups. The reported anti-inflammatory effect involves the stimulation of SOCS3 and the inhibition of NF-κb.[2, 10, 51, 52]

We have shown RegIII binding to bacteria (**Chapter 4**), to mucus and glycan structures (**Chapter 3 and 6**), and formation of RegIII multimers (**Chapter 4**) suggesting protein-protein interactions. These possible activities are summarised in panel C, where binding of RegIII to bacteria and glycans on mucins immobilizes bacteria in the mucus

layer (indicated by 1). Immobilisation of bacteria in the mucus would protect the host by preventing bacterial contact with epithelial cells. For RegIIIβ this is plausible as the structural models (**Chapter 4**) show two different ligand binding sites. Alternatively, 2) indicates a network of multimeric RegIII proteins and the mucus layer, forming a net-like structure preventing bacteria from establishing contact with host epithelia; this network-like structure including RegIII multimers and mucus thus exerts a protective effect. This hypothetical model is based on the observations that RegIII proteins can occur as multimers and can be bound to mucus. A precedent for this idea comes a recent publication showing that human beta defensin 6 forms a net-like structure.[53] Another possibility is indicated in 3) which is the killing of bacteria by RegIII proteins. Even though we did not measure substantial killing *in vitro*, and our *in vivo* results do not support biologically relevant instances of bacterial killing by RegIIIγ and RegIIIβ, we cannot ignore the literature about this topic. Panel D shows binding of RegIIIβ to fucosylated glycans that can be present on the membrane bound mucins of the glycocalyx, thereby hindering adherence of pathogens that utilize these carbohydrates for colonization.

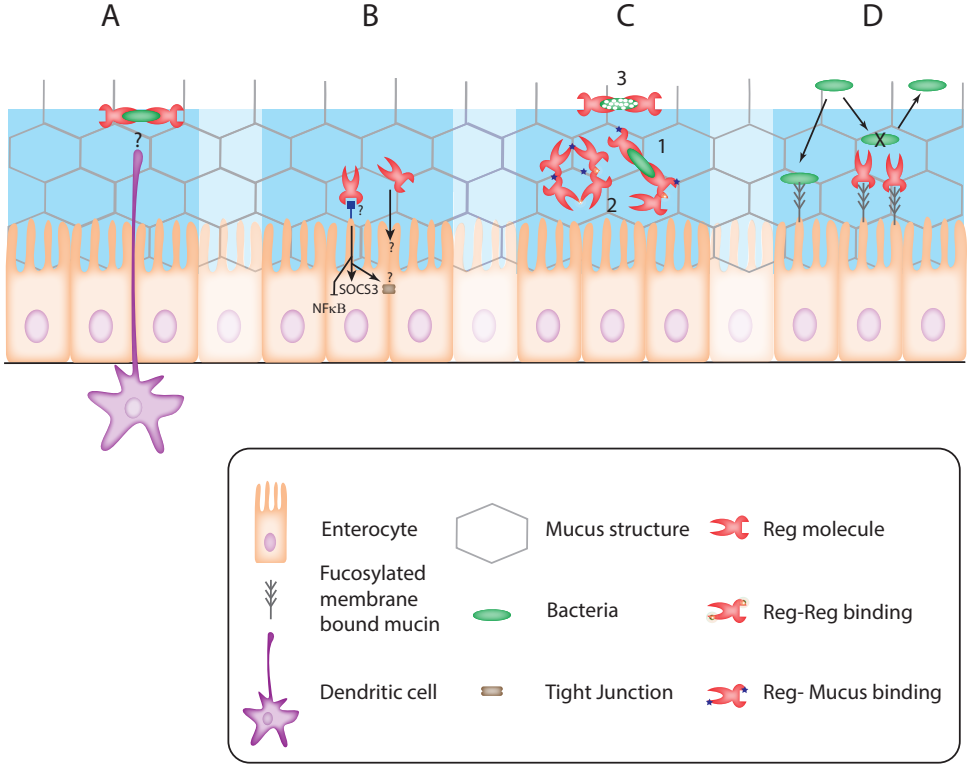


**Figure 7.4.** An overview of the possible protective mechanisms of RegIIIβ and RegIIIγ.

**Future perspectives**

The novel insights described in this thesis show that RegIII proteins are induced in the intestine under conditions of stress (i.e. infection or colitis) and have a protective function. They may have functions beyond the bactericidal effects described in the literature, including the ability to bind mucus and bacteria, which together with defensins and other factors appears to have a role in keeping microbes at a distance from the epithelium. Future research on the effect of the RegIII proteins on the microbiota, for example using the inducible knockout described above, would provide insights into the potential effects of RegIII proteins on microbiota composition and distribution. More effort is needed to produce RegIII proteins in mammalian expression systems and characterise their ligand binding and specificity *in vitro*. For example, competitive binding studies using different ligands could be used to identify binding sites for each of the RegIII proteins and could be used to confirm the potential of RegIIIβ and RegIIIγ to bind both mucus and bacteria. More research is also warranted on identifying host receptors; this is a prerequisite for unravelling the mechanisms via which anti-inflammatory effects and effects on TJs are modulated by RegIIIβ and/or RegIIIγ. Note that even for the well-studied human PAP protein, host receptors are unknown. As a first step to identify host receptors, it may be possible to demonstrate binding of dye-coupled RegIII proteins to epithelial or immune cells in *ex vivo* tissue, using the methods that were successfully used in the glycan array experiments (**Chapter 3**). Furthermore tissue explants can be used to study proposed anti-inflammatory effects of RegIII proteins, e.g. by measuring cytokine expression in Ussing chambers. Candidate receptors binding to RegIII might be identified by analysis of signalling pathways using cell reporters or transcriptomic studies and investigated further using blocking antibodies or binding of labelled RegIII proteins to protein fractions separated by SDS-PAGE.

The role of RegIII proteins in protecting against intestinal inflammation also warrants further study as they may ultimately have therapeutic potential. In the $Muc2^{-/-}$ mouse model, colitis was most severe in the distal colon where concentrations of RegIII are relatively low, in contrast to ileum where tissue damage was not observed and where RegIII concentrations are highest. We hypothesised that RegIII proteins play a key role in preventing inflammation-mediated tissue damage in the ileum. This could be tested by breeding a RegIII knock out on the $Muc2^{-/-}$ background. As RegIII production is highest in the ileum, it would be very interesting to see whether the lack of RegIIIβ or RegIIIγ would give a more severe pathology in ileum. External supplementation of RegIIIβ or RegIIIγ should also be investigated as a possible way to reverse inflammatory tissue damage. It could also be tested if protection against tissue damage is also dependent by killing of specific bacterial taxa; the other possible mechanisms outlined in figure 7.4 could also be evaluated using a $RegIII^{-/-}$ $Muc2^{-/-}$ mouse model.

As RegIIIβ and RegIIIγ are upregulated in mice suffering from gut barrier defects, for instance in the ileum of $Muc2^{-/-}$ mice, and during other intestinal inflammatory or stress

conditions, RegIII proteins have potential as biomarkers of intestinal health. Further research could be aimed at developing methods to accurately measure RegIII protein in faecal and blood samples and to evaluate usefulness of these measurements as biomarkers, first in mouse models and later in humans.

More research is warranted on the effects of RegIII proteins on different types of host cells including proliferating effects. Ultimately, these proteins may have therapeutic potential for diverse applications, for example as oral biologics or via strategies to stimulate their production including probiotics. They may even have possible systemic applications in the treatment of severe inflammatory conditions such as sepsis, e.g. as scavengers of toxic LPS molecules or immunogenic peptidoglycan-lipoprotein fragments. Clearly there is still a lot to be discovered about the functions of these fascinating proteins and in the future this may lead to novel applications in medicine.

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# Appendices

Summary

Samenvatting

Acknowledgements

Biography

List of publications

Education certificate

## Summary

Mammalian RegIII proteins are expressed in the intestine and in the pancreas in response to inflammation or infection. In the mouse intestine, expression of RegIIIβ and RegIIIγ is increased by microbial colonization, inflammation and infection. At the outset of this thesis human PAP and mouse RegIIIγ were reported to be bactericidal for Gram-positive bacteria. Additionally, human PAP had been shown to attenuate NF-κb signalling in human monocytes and epithelial cells and administration of anti-PAP antibodies increased inflammation in an experimental rat model of acute pancreatitis. The overarching goals of this thesis were to find out more about the protective role of mouse RegIIIβ and RegIIIγ in the intestine and explore their protective role in colitis and bacterial infection.

In **Chapter 2** we investigated expression of RegIIIβ and RegIIIγ in intestine of Muc2 knockout ($^{-/-}$) mice, which develop colitis after about 4 weeks, due to the absence of a secreted mucus layer in the small intestine or colon. RegIII proteins were expressed in Paneth cells, enterocytes and goblet cells pointing to a new function for goblet cells in innate immunity. Ang4 expression was confined to Paneth cells and goblet cells. Absence of Muc2 increased expression levels of RegIIIβ, RegIIIγ, and Ang4 and colitis appeared first in the distal colon where the RegIII expression is lowest.

In **Chapter 3** we investigated the distinct phases of colitis development in Muc2$^{-/-}$ mice from before weaning to 4 and 8 weeks of age, also taking into account the effect that mucin deficiency has in the ileum. Gene set enrichment approaches showed increased expression of innate and adaptive immune pathways associated with colitis over time, whereas in the ileum many immune signalling pathways were down-regulated. Nevertheless, RegIIIβ and RegIIIγ were significantly upregulated, suggesting their proposed antimicrobial and/or anti-inflammatory activities might be related to the suppression of immune pathways and avoidance of immune-mediated damage. Furthermore, we showed that RegIIIβ could specifically bind to mucin and fucosylated glycans *in vitro,* which may serve to inhibit bacterial binding to membrane bound mucins on the epithelium, and also enable RegIIIβ to be retained in the secreted mucin.

An *in vitro* approach was used in **Chapter 4,** where we investigated the activities of RegIIIγ and RegIIIβ by expressing and purifying recombinant proteins. Both proteins were insoluble when expressed in *E. coli* but RegIIIβ could be expressed and secreted in baculovirus as a soluble protein. As previous work reported that RegIII proteins were bactericidal even when produced as inclusion bodies in *E. coli* and refolded, we followed similar procedures to obtain soluble RegIII proteins. In our hands both the *E. coli* and baculovirus produced proteins bound strongly to both Gram-positive and Gram-negative bacteria after processing of an N-terminal pro-peptide by trypsin, but lacked any appreciable bactericidal activity. Furthermore these proteins did not influence the

growth of *Salmonella enteritidis* and *Listeria monocytogenes*. Attempts to crystallize the proteins were unsuccessful but structural models of the protein were generated based on the crystal structure of human PAP. These models were used to dock known ligands of RegIIIγ or RegIIIβ. Only one ligand is known for RegIIIγ, which is peptidoglycan, but for RegIIIβ the ligands include peptidoglycan, lipid A and the fucose-containing glycans identified in chapter 3. RegIIIβ was predicted to have two different binding sites which would allow it to bind to mucins and bacteria simultaneously, thereby preventing penetrating of the mucus.

In **Chapter 5** a RegIIIβ$^{-/-}$ mouse was used to study the role of the protein during infection with Gram-negative *Salmonella enteritidis* or Gram-positive *Listeria monocytogenes.* Whereas recovery of *S. enteritidis* or *L. monocytogenes* from faeces was similar in RegIIIβ$^{-/-}$ and wild type (WT) mice, significantly higher numbers of viable *S. enteritidis*, but not *L. monocytogenes*, were recovered from the colon, mesenteric lymph nodes, spleen, and liver of the RegIIIβ$^{-/-}$ than the WT mice. The results suggest that mouse RegIIIβ plays a protective role against intestinal translocation of the Gram-negative bacterium *S. enteritidis* but not against the Gram-positive bacterium *L. monocytogenes*.

In **Chapter 6,** the generation of a RegIIIγ$^{-/-}$ mouse is described. One of the main phenotypic differences between the RegIIIγ$^{-/-}$ and WT was an altered distribution of the ileal mucus and increased bacterial contact with the epithelium. Additionally, measurement of innate immune markers in the mucosa suggested heightened inflammation in the RegIIIγ$^{-/-}$ mice. Compared to WT mice, RegIIIγ$^{-/-}$ mice infected with *S. enteritidis* and *L. monocytogenes* showed an increase of mucosal inflammatory markers indicating protective, anti-microbial roles of RegIIIγ in defense against both Gram-positive and Gram-negative bacteria.

**Chapter 7** summarizes and discusses the key results of the thesis in the context of the wider literature and possible directions for future research.

## Samenvatting

RegIII eiwitten komen in de darm en de alvleesklier van zoogdieren tot expressie als reactie op een ontsteking of infectie. In de darm van muizen neemt de expressie van RegIIIβ en RegIIIγ toe na microbiële kolonisatie, ontsteking of infectie. Aan het begin van het in dit proefschrift beschreven onderzoek was het bekend dat het humane eiwit PAP en een muizen variant hiervan, RegIIIγ, antimicrobieel was voor Gram-positieve bacteriën. Daarnaast was het bekend dat humaan PAP de NF-κb signalering in (humane) monocyten en epitheel cellen kan verzwakken en dat de toevoeging van antistoffen tegen PAP alvleesklier-ontsteking deed toenemen in ratten. Het uiteindelijke doel van dit proefschrift was om meer over de beschermende rol van muizen RegIIIβ en RegIIIγ in de darm te weten te komen, en om de beschermende rol van deze eiwitten tijdens colitis (ontsteking van de dikke darm) en bacteriële infectie te onderzoeken.

In **hoofdstuk 2** hebben we naar de expressie van RegIIIβ en RegIIIγ in de darm van Muc2 knock out muizen gekeken. Deze muizen hebben geen beschermende mucus laag in de darm, en ontwikkelen als gevolg daarvan colitis op een leeftijd vanaf ongeveer 4 weken. In dit hoofdstuk hebben we gevonden dat RegIII eiwitten worden geproduceerd door zowel Paneth cellen, enterocyten als slijmbeker cellen, wat mogelijk duidt op een nieuwe rol voor slijmbekercellen in de aangeboren immuniteit. De expressie van Ang4, een ander antimicrobieel eiwit, vond plaats in Paneth cellen en slijmbekercellen. De afwezigheid van Muc2 induceerde een toename van RegIIIβ, RegIIIγ en Ang4, en daar waar de expressie van de RegIII eiwitten het laagst is, namelijk in laatste deel van de dikke darm, ontwikkelt de colitis het eerst.

In **hoofdstuk 3** hebben we gekeken naar de verschillende fases van de colitis ontwikkeling in de Muc2 knock out muis, waarbij we ook gekeken hebben naar het effect van de afwezigheid van Muc2 in de dunne darm. Transcriptoom analyse liet toegenomen expressie van genclusters betrokken bij aangeboren en adaptieve immuniteit zien, terwijl in de dunne darm veel clusters van immuun genen juist een afgenomen expressie lieten zien. Desondanks waren RegIIIβ en RegIIIγ significant toegenomen, wat kan duiden op een mogelijke onderdrukking van ontstekingen door deze eiwitten. Daarnaast hebben we *in vitro* laten zien dat RegIIIβ specifiek kan binden aan mucine en gefucolyseerde suikers. Dit zou de binding van bacteriën aan de suikers op het cel membraan kunnen remmen, en er daarmee voor zorgen dat RegIIIβ aanwezig blijft in de mucus laag.

We hebben een *in vitro* methode gebruikt in **hoofdstuk 4**, waar we de activiteiten van RegIIIβ en RegIIIγ onderzocht hebben door recombinant eiwit te produceren en te zuiveren. Beide eiwitten werden onoplosbaar geproduceerd door E. coli maar RegIIIβ werd als een oplosbaar, uitgescheiden eiwit geproduceerd door baculo virus. RegIII eiwitten lieten in eerdere studies antimicrobiële activiteit zien, zelfs wanneer ze als onoplosbaar geproduceerd waren in E. coli en hervouwen werden, dus hebben we vergelijkbare pro-

tocollen gebruikt om oplosbaar eiwit te verkrijgen. In onze experimenten bonden zowel het hervouwen als het oplosbare eiwit sterk aan Gram-positieve en Gram-negatieve bacteriën, maar was er geen antibacteriële activiteit. Daarnaast lieten de eiwitten geen effect zien op de groei van *Salmonella enteritidis* en *Listeria monocytogenes*. Pogingen om de eiwitten te kristalliseren zijn niet gelukt maar er zijn wel modellen gemaakt gebaseerd op de kristal structuur van humaan PAP. Deze modellen zijn gebruikt om te voorspellen waar liganden van RegIIIβ en RegIIIγ zouden kunnen binden. Er is tot nu toe maar één ligand bekend voor RegIIIγ, te weten peptidoglycan, maar voor RegIIIβ zijn de liganden peptidoglycan, lipid A en enkele fucose bevattende suikers bekend. Het model voorspelde dat RegIIIβ twee verschillende bindingsplaatsen zou hebben, wat zou kunnen leiden tot binding aan mucines en bacteriën tegelijkertijd. Hiermee zou voorkomen kunnen worden dat de bacteriën de mucus laag penetreren.

In **hoofdstuk 5** hebben we een RegIIIβ knock out ($^{-/-}$) muis gebruikt om de rol van dit eiwit tijdens een infectie met de Gram-negatieve *Salmonella enteritidis* en de Gram-positieve *Listeria monocytogenes* te bestuderen. De aantallen *S. enteritidis* en *L. monocytogenes* die werden teruggevonden in de feces van de muizen was vergelijkbaar tussen de RegIIIβ$^{-/-}$ muizen en de wild type (normale) muizen. Daarentegen zijn significant hogere *S. enteritidis* aantallen aangetroffen in de dikke darm, de lymfe klieren, milt en lever van de RegIIIβ$^{-/-}$ muis vergeleken met de wild type muis. Deze resultaten wijzen op een beschermende rol van RegIIIβ in de translocatie van de Gram-negatieve *S. enteritidis* in de muis, maar niet in de translocatie van de Gram-positieve *L. monocytogenes*.

In **hoofdstuk 6** is de ontwikkeling van een RegIIIγ knock out muis beschreven. Een van de grootste fenotypische verschillen van dit model vergeleken met de wild type muis is een andere distributie van de mucus laag in de dunne darm en een verhoogd contact van bacteriën met het darm epitheel. Daarnaast zijn een aantal markers voor het aangeboren immuun systeem verhoogd in de darm, wat duidt op een verhoogde mate van ontsteking in de RegIIIγ$^{-/-}$ muis. Vergeleken met de wild type muis laat de RegIIIγ$^{-/-}$ muis ook een verhoogde mate van mucosale ontstekingsmarkers zien na infectie met *S. enteritidis* en *L. monocytogenes*, wat wijst op een beschermende antimicrobiële rol van RegIIIγ in een infectie met deze bacteriën.

Tot slot worden in **hoofdstuk 7** de belangrijkste bevindingen van dit proefschrift beschreven en bediscussieerd en in een bredere context geplaatst, met aanbevelingen voor toekomstig onderzoek.

## Acknowledgements

'Nobody said it was easy,

No one ever said it would be this hard'

Coldplay, the Scientist

Persistence. That's mainly what it took me to be able to reach this point: the acknowledgements. The final part, now it is really finished! The song of Coldplay, one of my favorite bands, fits perfectly with how I felt during the past years. Of course it was not only hard, I've also really enjoyed my time at HMI. You can never do a PhD alone, so I would like to thank everybody who helped me in the past years to make it to this point, and some people in particular.

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And then there are of course the people outside of science that make the non-working life better. Ewa, Jasiu and Bas, thanks for our nice dinners and yes, there was some science talk, but the good food was more important! Same for Ansa and Hakan, thanks for the nice dinners with, but also without science talks!

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'Look at the stars,

Look how they shine for you'

Coldplay, Yellow

*Linda*

**Curriculum vitae**

Linda Maria Pietronella Loonen was born on May 20, 1983 in Oosterhout and grew up in a small village called Dorst. In 2001 she graduated from high school (Nassau scholengemeenschap) in Breda. She continued her education at the Avans hogeschool in Etten-Leur where she studied Biology and medical laboratory research (HLO). For her first internships she went to the University of Vermont in Burlington, USA, department of Microbiology and Molecular Genetics, under the supervision of Dr. Markus Thali, where she worked on developing tools to analyse trafficking of Feline Immunodeficiency Virus (FIV) during assembly and release. After that she went to the Erasmus medical centre in Rotterdam to determine the role of TRPV6 in human osteoblast mineralisation using RNA interference at the department of Internal Medicine, under the supervision of Dr. Bram van der Eerden. She obtained her BAS (Bachelor of Applied Science) degree in 2004, and decided to continue her master education Cell Biology at the Wageningen University. Here she did her major thesis at the Cell biology and Immunity group under the supervision of Dr. Yvonne Vissers and Prof. Dr. Ir. Huub Savelkoul. Her research focussed on the immunological mechanism of Sweet itch in horses. After her major thesis she went to the USA again for her MSc internship, which she performed at the Massachusetts General Hospital/Harvard Medical School, Boston, USA, at the department of Pediatric gastroenterology and nutrition, under the supervision of Dr. D. Newburg. Here she looked at the role of adiponectin on the fat and glucose metabolism in 2 day old mouse pups. She obtained her MSc in 2008 after which she started her PhD at the host-microbe interactomics group of Prof. Dr. Jerry Wells. The findings of her PhD research can be found in this thesis, entitled 'RegIII proteins as gatekeepers of the intestinal epithelium' and were part of the Top Institute Food and Nutrition project Nutrition and Health.

Since april 2013 Linda is working as a postdoc in the host-microbe interactomics group.

## List of publications

Wells JM, Loonen LMP, Karczewski JM. The role of innate signalling in the homeostasis of tolerance and immunity in the intestine. Int J Med Microbiol. 2010 Jan; 300(1):41-8

Van Ampting MT[#], Loonen LMP[#], Schonewille AJ, Konings I, Vink C, Iovanna J, Chamaillard M, Dekker J, van der Meer R, Wells JM, Bovee-Oudenhoven IM. Intestinally secreted C-type lectin Reg3b attenuates Salmonellosis but not listeriosis in mice. Infec Immun. 2012 Mar; 80(3): 1115-20 [#]equally contributed

Burger-van Paassen N, Loonen LMP, Witte-Bouma J, Korteland-van Male AM, de Bruijn AC., van der Sluis M, Lu P, van Goudoever JB, Wells JM, Dekker J, van Seuningen I, Renes IB. Mucin Muc2 deficiency and weaning influences the expression of the innate defense genes Reg3β, Reg3γ and angiogenin-4. PLoS One, 2012; 7(6):e38798

Loonen LMP, Stolte EH, Jaklofsky MTJ, Meijerink M, Dekker J, van Baarlen P, Wells JM. Intestinal Reg3γ plays a protective role against intestinal infection with both Gram-positive and Gram-negative pathogens. Submitted

Loonen LMP, Sovran B, Lu P, Stolte EH, Serna S, Burger-van Paassen N, de Vos P, Reichardt N, van Baarlen P, Dekker J, Renes IB, Wells JM. Homeostatic mechanisms preventing inflammation-mediated mucosal damage in the ileum of Muc2-deficient mice. In preparation

Loonen LMP, Taverne N, Velikova N, Stolte EH, Marina A, Dekker J, Wells JM. Purification and characterization of recombinant mouse RegIII proteins. In preparation

## Overview of completed training activities

### Discipline specific activities

*Courses*

| | |
|---|---|
| Systems biology course: Statistics of ~omics data analysis, VLAG and EPS | 2008 |
| Advanced visualisation, integration and biological interpretation of ~omics data, VLAG and WIAS | 2009 |
| The light in the intestinal tract tunnel, VLAG and ABS (Helsinki, Finland) | 2009 |
| Genetics and physiology of food-associated microorganisms, VLAG | 2010 |
| Proteomics, VLAG | 2011 |

*Meetings*

| | |
|---|---|
| European Mucosal Immunology Group (EMIG) meeting, Milan, Italy | 2008 |
| Host-Microbe Interactions Workshop, Sardinia, Italy | 2008 |
| The 3rd symposium & master classes on mucosal immunology, Rotterdam | 2008 |
| 14th international congress of mucosal immunology (ICMI), Boston, USA | 2009 |
| Voorjaarsvergadering NVGE, Veldhoven | 2009 |
| 5th International Yakult symposium, Amsterdam | 2009 |
| 11th Gut day, Vlaardingen | 2009 |
| Digestive Disease Week, New Orleans, USA (poster presentation) | 2010 |
| 12th Gut day, Gent, Belgium | 2010 |
| 13th Gut day, Wageningen (poster presentation) | 2011 |
| 4th Cross-talk network meeting, Wageningen (oral presentation) | 2011 |
| 2nd Network and straining Stars meeting, Siena, Italy (oral presentation) | 2011 |
| Keystone meeting: Innate Immunity: sensing the microbes and damage signals & The Microbiome, Keystone, Colorado, USA (poster presentation) | 2012 |
| 3rd TNO beneficial microbes conference, Noordwijkerhout (oral presentation) | 2012 |
| Cross-talk meeting Final symposium, Paris, France (oral presentation) | 2012 |
| 6th Seeon Conference Microbiota, Probiota and Host, Seeon, Germany (oral presentation) | 2013 |

### General courses

| | |
|---|---|
| VLAG PhD week, VLAG | 2008 |
| PhD afternoon: Effective Publishing Strategies & Networking workshop, TIFN | 2008 |
| Philosophy and Ethics of Food Science and Technology, VLAG | 2009 |
| PhD Competence Assessment, WGS | 2009 |
| Techniques for writing and presenting a scientific paper, WGS | 2010 |
| Effective behaviour in your professional surroundings, WGS | 2011 |
| Advanced course guide to scientific artwork, Wageningen UR library | 2012 |
| Adobe InDesign, Wageningen UR library | 2012 |

**Optional activities**

| | |
|---|---|
| Preparing PhD proposal | 2008 |
| HMI lab meetings | 2008-2013 |
| HMI journal club | 2008-2013 |
| TIFN meetings A-1001, Wageningen | 2008-2012 |
| TIFN program A days, Utrecht/Wageningen | 2008-2012 |

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