Mucosal immunity: the thin line between hypo- and hyperresponse to gut associated bacteria

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Contents

1. Introduction

The human gastrointestinal tract consists of the small intestine, caecum and the colon. From the jejenum, the second part of the small intestine onwards, the gut harbours an increasing number of bacteria [1, 2]. It is estimated that the human intestine is home to trillions of bacteria belonging to 15000 to 36000 individual species [3]. The largest number of these bacteria lives in a symbiotic relationship with their host. Their original role is thought to be enhancement of the digestive efficiency by degrading polysaccharides [4-6], but thousands of years of co-evolution [7] have led to commensal bacteria fulfilling other roles as well. They provide for example signals for intestinal epithelial cell maturation [9,10] and angiogenesis [11]. They also fulfil an important role in immune protection by competing with pathogenic microbes [12] and by priming the immune system. Together with daily uptake of food, this confronts the gut with the largest number of antigens in the body. So the gut associated lymphoid tissue (GALT), which is made up of the Peyer's Patches (PP), the lamina proper and the mesenteric lymph nodes, has to be able to mount a fast and strong response to prevent opportunistic invasion of microbes. At the same time the GALT has to prevent a strong reaction to commensal bacteria to prevent a constant inflammatory reaction in the gut. An overreaction of this sort not only compromises the host by removing the beneficial effects of commensal bacteria but also damages the host tissue. Such overreactions to commensal bacteria can lead to coeliac disease or inflammatory bowel disease (IBD) if the inflammation is prolonged [13, 14]. Both are chronic inflammations of the gut, in which an overreaction to commensal bacteria leads to significant tissue damage. In the case of IBD, flare-ups of the inflammation can often be observed in patients, which hints at an underlying deregulation of the immune system.

The most important task of the GALT is to maintain immune homeostasis, in which only invading or pathogenic bacteria elicit an immune response while commensal bacteria are unharmed by the immune system, while also keeping the gut tissue intact [15]. To be able to do so, the cells in the GALT sometimes react differently to antigens when compared to immune cells elsewhere in the body. The immune system of the gut also contains several barriers directed at minimizing the contact of microbes with immune cells to prevent hyperreactions and tissue damage.

But commensal bacteria not only pose a challenge for the immune system; they actually appear to be necessary for its maturation [16-18]. This applies not only to the intestinal immunity, but to the systemic immunity as well since alteration of intestinal microbiota can have negative effects on inflammatory diseases in other parts of the body [19-21, 152]. Priming of the immunity to commensal bacteria seems to be an important mechanism to ensure prolonged health of the host [19-21]. A role of commensal microbes has also been described in autoimmunity [22]. The interaction between microbes and the immune system is not one sided however, as the composition of the microbes of the gut (microflora) can easily be influenced by immune mechanisms [23]. This is necessary to prevent opportunistic infections by otherwise benign microbes [24,25]. Commensal bacteria can also dampen the inflammatory response thus protecting the host against some pathogens [26]

Unfortunately not all microbes ingested in food and water are benign or even neutrally towards the human host. Some bacteria have developed intricate mechanisms to subvert host immune defenses and can cause severe disease [27,28]. Bacteria of the species S*almonella* even go as far as using host immune mechanisms against commensal bacteria to ensure their survival in the gut [29].

The intestinal immune system and its interaction with resident and pathogenic bacteria has been a field of intense research in the past years. The purpose of this thesis is to give an overview of the different parts of the intestinal mucosal immunity and to discuss how they interact with microbes to confer protection against pathogenic species as well as preventing unnecessary reactions to commensal bacteria. Inflammatory bowel disease as an example of overreaction to intestinal microbes will also be discussed as well as induction and prevention of autoimmunity and allergy through intestinal bacteria. The last part will discuss *Shigella*, a pathogen which makes its home in the intestine of the host and how it can escape the immune defenses and even use them for its advantage. Since many questions still remain to be answered in this field of research, this thesis can only give an overview of the current hypothesis and models of immune action.

2. The gut associated immune system

The gut associated immune system is diverse and harbours many cellular and humoral components to ward off defenders and at the same time prevent inflammation due to commensal bacteria (unless they try invade the host). The purpose of this section is to describe these components and their role in the immune response.

2.1 Humoral components

The main purpose of the houmoral components of the gut associated immune system is to prevent microbes from coming into contact with the epithelium. There are 3 humoral agents at work in the gut: mucus, antimicrobial peptides and Immuneglobuline A (IgA)[30]. Together these form a barrier which most antigens of commensal bacteria do not penetrate (fig 1).

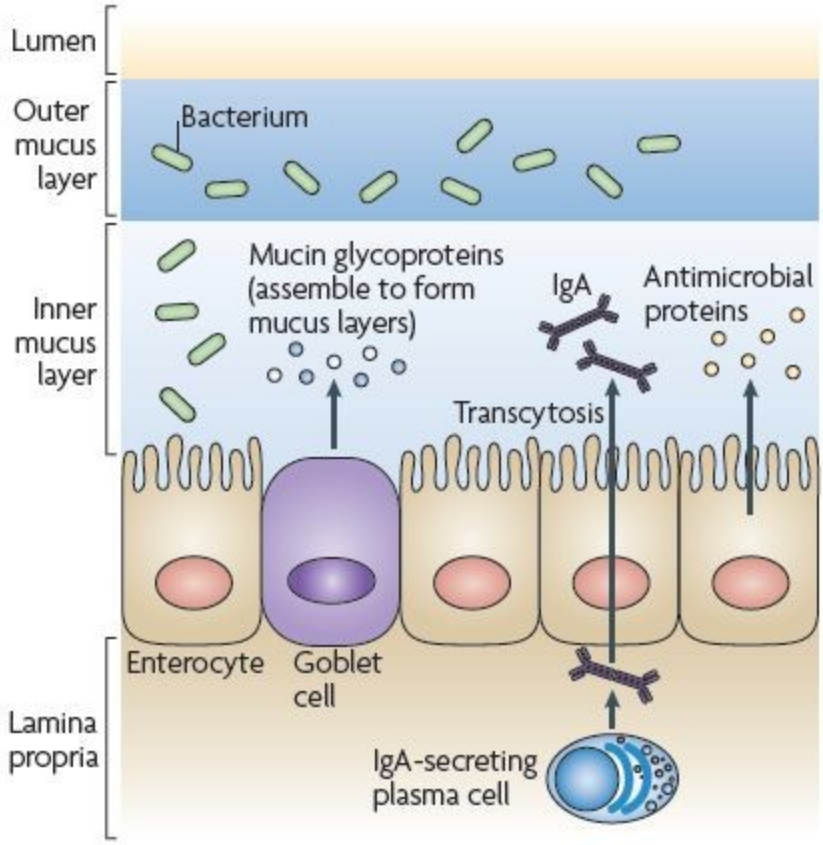

Figure 1 | **Immune mechanisms that limit bacteria–epithelial cell interactions.** Several immune mechanisms work in concert to limit contact between the dense luminal microbial community and the intestinal epithelial cell surface. Goblet cells secrete mucin glycoproteins that assemble into a thick, stratified mucus layer. Bacteria are abundant in the outer mucus layer, whereas the inner layer is resistant to bacterial penetration. Epithelial cells (such as enterocytes, Paneth cells and goblet cells) secrete antimicrobial proteins that further help to eliminate bacteria that penetrate the mucus layer. Plasma cells secrete IgA that is transcytosed across the epithelial cell layer and secreted from the apical surface of epithelial cells, limiting numbers of mucosa-associated bacteria and preventing bacterial penetration of host tissues

Figure 1: Taken from Hooper LV, Macpherson AJ. Immune adaptions that maintain homeostasis with the intestinal microbiota. Nature Rev Immunology 2010 10:159-169

The mucus layer is composed of mucin glycoproteins which are produced by specialized goblet cells in the intestine. Mucins assemble into a viscous layer composed of 2 distinctive strata about 150 ?m from the intestinal epithelial cells (IEC) [31]. The inner stratum is almost devoid of bacteria whereas the outer one harbours many microbes [31]. The inner layer is thus preventing bacteria from penetrating further and as such important for immunity. This has been shown by using mice engineered to lack the mucin MUC-2, which are prone to spontaneous inflammation of the gut [32].

Not surprisingly a number of pathogens have devised strategies to traverse the mucus layer in the colon, for example by lowering the viscosity of the mucus [33] or by propelling through it using flagella [34]. It should be noted however that even commensal bacteria sometimes penetrate the mucus layer without causing any problems for their host [35].

Antimicrobial proteins are secreted by almost all IECs (fig. 1) [36] and include defensins, cathelicidins and C type lectins. Most of these directly disrupt the bacterial cell wall or membrane to destroy them [30], but a small subset including lipocalin 2 also deprives bacteria of essential heavy metals [37]. Antimicrobial proteins are virtually absent from the lumen and heavily concentrated at the inner mucus layer [38], which probably increases their concentration and thus effectiveness which would be poor in the much larger sized lumen. The most prominent antimicrobial proteins are α-defensins produced by Paneth cells which are effective against a range of pathogenic bacteria [39]. Most of these α-defensins are constantly produced in the lumen along with several other antimicrobial proteins [40]. Many other antimicrobial proteins are only secreted upon stimulation of pattern recognition receptors (PRRs), for example toll like receptors (TLRs) [41]or nucleotide-binding and oligomerization domain(NOD) containing proteins [42]. These PRRs are stimulated by pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), which is present on the cell walls of gram negative bacteria. If production of these antimicrobial proteins is impaired, for example by removing the Paneth cells of the gut in mice, microbes in the gut penetrate the mucosa significantly more often than in wild type mice [43].

IgA is produced by plasma cells in the lamina proper and then transported across the epithelium by transcytosis [30]. IgA prevents association of bacteria with the epithelium[44] and restricts bacterial penetration of the gut [45], probably by binding to surface antigens thus blocking their binding sites. The most efficient differentiation of B-cells, which produce IgM, to IgA plasma cells takes place in the Peyer's Patches (PP) of the GALT. Microfold-cells and dendritic cells (DCs) sample antigens near the patches and present them to resident T- and B-cells in so called germinal centres within the patches (fig.3) [46]. Recognition of an antigen by B-cells under proper stimulation of T-cells induces their proliferation and induces a activation-induced cytidine deaminase (AID) dependent class switch from IgM to IgA [47, 48]. Research has shown that production of IgA can also be induced independent of T-cells or the PP [45].
Once IgA B-cells and plasma cells have been created, they are thought to migrate into mucosal lymph nodes and from the lymph to the thoracic duct. There they enter the blood stream and home back to the lamina proper of the gut (fig.2) [49]. The high endothelial venules of the lamina proper express MadCAM-1 adressin, which binds to the α4β7 integrin on the IgA producing cells [50]. IgA producing cells, but not other Ig cells, express CCR9. The chemokine TECK/CCL25 produced in the small intestine is the ligand of CCR9 and thus allows homing of IgA cells [51,52]. The peritoneal cavity harbours another population of B cells which can take part in IgA production: B1 cells (fig.2) [53]. These cells normally produce low-affinity IgM antibodies [54], but can switch to IgA by antigenic stimulation [55] independent of T-cells [56]. In case of an inflammation of the gut or the presence of bacterial antigens B1 cells also migrate into the lamina proper of the gut [57]. DCs and IECs have been described to be able to induce class switching of nave B-cells to IgA cells without the aid of T-cells in the lamina proper[58]. This process is thought to be dependent on B-cell activating factor (BAFF) secreted by DCs and a proliferation inducing ligand (APRIL) secreted by DCs and IECs, which can activate the B cell maturation antigen (BCMA), the BAFF receptor (BAFFR) and cytophilin ligand interactor (TACI) receptors on B-cells [59].
IgA plays an important role in mediating the composition of the microflora, preventing it to turn opportunistic [58, 60]. IgA is also important because it is an immune mechanism which can combat bacterial infection without using an inflammatory response and so without causing any damage to the gut tissue. IgA even acts anti-inflammatory and can thus prevent inflammatory diseases in the gut [61].

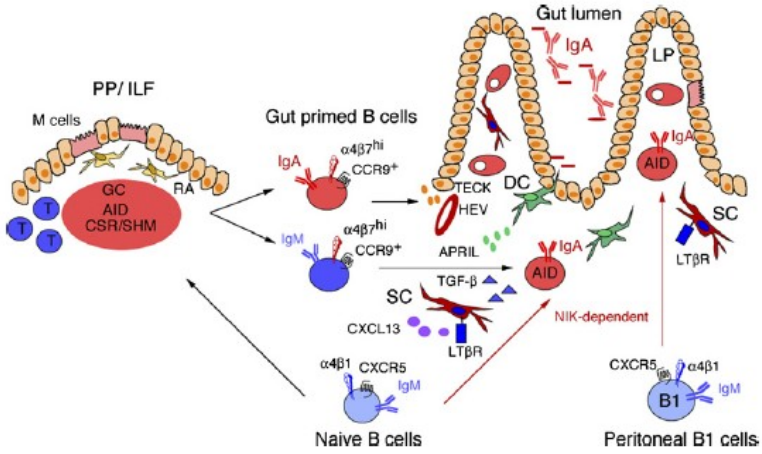

Fig. 1. Multiple pathways for migration of B cells and generation of IgA in gut. Schematic representation of gut, with organized follicular structures, such as Peyer's patches (PP) and isolated lymphoid follicles (ILF) and diffuse tissues of lamina propria (LP). PPs are composed of a subepithelial dome rich in dendritic cells (DCs), T cells and B cell-follicles that contain germinal centers (GCs). In these structures, B cells undergo efficient class switch recombination (CSR) to IgA and somatic hypermutation (SHM) and acquire gut-seeking properties through enhanced expression of integrin α4β7 and CCR9 by the retinoic acid (RA) produced by DCs. Migration of both IgA and IgM B cells from the PPs into the intestinal villi takes place through the postcapillary venules (HEV) expressing MadCAM-1, requires CCR9–TECK interactions and is independent of NF-κB activation through LTβ-NIK of LP stromal cells (SCs). LTβR signalling through NIK on LP SCs is essential for their synthesis of CXCL13 and VCAM-1, which is most likely required for the recruitment of CXCR5 and integrin α4β1 expressing naïve B cells and peritoneal B1 cells into the gut. Once in the LP, after local activation and up-regulation of AID expression, these IgM B cells will switch and differentiate into IgA plasma cells in situ with the help of TGFβ and APRIL produced by SCs and DCs respectively. IgAs secreted into the intestinal lumen regulates both composition and appropriate segmental distribution of gut microbiota.

Figure 2 taken from 58: Tsuji M, Suzuki K, Kinoshita K, Fagarasan S. Dynamic interactions between bacteria and immune cells leading to intestinal IgA synthesis. Sem Immunol 2008; 20: 59-6

2.2 Intestinal Epithelial cells

The intestinal epithelium is composed of only one thin layer of epithelial cells. Some of these, like the aforementioned goblet and Paneth cells, fulfil a special function (fig.3) [30]. The majority however primarily acts as a barrier between the lumen of the gut and the lamina proper. These cells were once thought to just act as a physical barrier, but in the past years have been described to be able to aid in distinguishing between commensal and pathogenic bacteria and modify the immune response accordingly [62].

Apart from having to keep bacteria from breaching the cell membranes of IECs, the cells also have to limit paracellular traffic in the gaps between them. This is done by tight junctions and adjacent adherens junctions between the cells [63]. Adherens junctions are formed by cadherins and are necessary for the assembly of tight junctions, which are mainly built of claudins and occludins [63]. Paracellular transport across the IEC can take place via 2 pathways, which appear to be regulated separately [64]. The leak pathway allows passage of large molecules including proteins and LPS [64]. The second path is through small pores thought to be defined by claudins of the tight junction and also shows charge specificity [65]. Molecules larger than 4 A are excluded from this path [64]. Immune cytokines such as tumor necrosis factors (TNF) can influence the tight junctions by activating myosine light chain kinase (MLCK) [66]. Although it is not completely clear how MLCK achieves this, the paracellular flux is increased through the leakage pathway [67]. Endocytic removal of occludin from the tight junctions has been described as part of the process [66]. These regulations are rather quick and also easily reversible [63] in contrast to the change in expression of claudins which occurs in intestinal epithelial cells [68]. Claudin 2 expression for example is increased in patients suffering from IBD [68], leading to an increased passage through the small pore pathway [69]. It should be noted however, that an increased leakage is not enough to induce disease of the gut [63].

The IECs express various PRRs allowing them to respond to bacterial factors such as LPS, flagellin

or unmethylated CpG-containing DNA [70]. As mentioned above, recognition of PAMPs can induce APRIL expression by IECs and lead to IgA expression [59]. Upon stimulation by commensal bacteria IECs produce anti-inflammatory cytokines and are important in immune regulation, as will be described in chapter 3.2 [62], but can also support inflammation depending on context [121]. Stimulation of TLR2 can maintain the integrity of tight junctions during inflammatory stress by activating protein kinase C αβ [71]. TLR2 and TLR5 can induce protection against apoptosis in IECs during inflammation by phosphorylating Akt 1 and its downstream components through MyD88 , since the phosphoinositide 3-kinase/Akt 1 pathway is involved in stress induced apoptosis and inflammatory response [72].

MyD88 also is an important molecule in it's own right, since it is involved in signalling through all TLR receptors except TLR3 [73]. The MyD88 pathway involves a lot of signalling and adaptor molecules and hence can be inhibited or influenced at various points by regulatory molecules or even by microbes. TLRs recruit MyD88 after recognition of PAMPs. MyD88 then interacts with Il-1 receptor-associated kinase-4 (IRAK-4) at their death domains. IRAK-4 then associates with IRAK-1, which leads to its phosphorylation and activation. Both IRAK-1 and IRAK-4 migrate from away from the complex and interact with the tumor necrosis factor receptor associated factor-6 (TRAF-6). TRAF-6 then forms a complex with transforming growth factor-β-activated kinase-1 binding protein-1 (TAB-1) and TAB-2. TAB-1 and 2 then phosphorylate transforming growth factor-β-activated kinase-1 (TAK-1). TAK-1 then interacts with kinases upstream of the inhibitory κB kinase (IKK) complex, which leads to activation if NF-κB [73]. NF-κB usually activates the immune system and induces clearence of the microbes which were recognized via PAMPs by the TLRs, but it can laos have an inhibitory role as described later in this thesis. It is also impüortant to note that although most TLRs function mainly via MyD88, research suggests MyD88 independent pathways as well for example in TLR4 signalling [73].

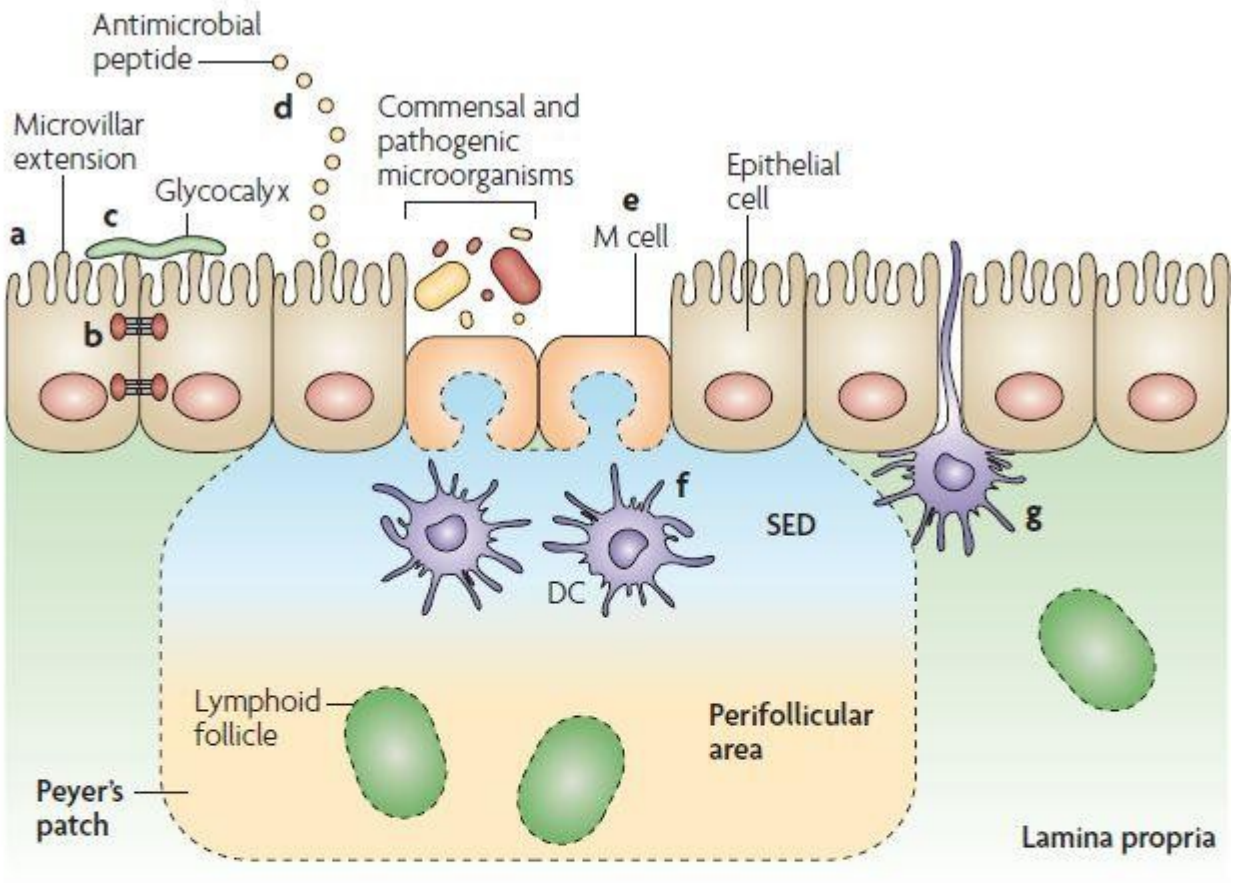

Figure 3 | **The intestinal epithelial-cell barrier.** Simple columnar epithelial cells exhibit physical and biochemical adaptations to maintain barrier integrity including actin-rich microvillar extensions (**a**), epithelial-cell tight junctions (**b**), apically attached and secreted mucins that form a glycocalyx (**c**) and the production of various antimicrobial peptides (**d**). Specialized intestinal epithelial cells known as M (microfold) cells overlie Peyer's patches and lymphoid follicles to facilitate luminal sampling. M cells exhibit reduced mucin secretion and have modified apical and basolateral surfaces (**e**) to promote uptake and transport of luminal contents to professional antigen-presenting cells that inhabit the subepithelial dome (SED) of the Peyer's patches and lymphoid follicles (**f**). Specialized dendritic cell (DC) subsets can also extend dendrites between the tight junctions of intestinal epithelial cells to sample luminal contents (**g**).

Figure 3 taken from 62: Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nature Rev Immunol 2008; 8:411-420

2.3 Professional antigen presenting cells: M-cells, dendritic cells and antigen sampling

Antigen presenting cells enable recognition of antigens by lymphatic cells. Through co-stimulation they can also strongly influence the reaction which these antigens will cause [30, 75]
Microfold or M-cells are specialized cells that are located above the PP and lymphoid follicles in the gut [30,74]. Their primary role is transport of antigens from the lumen into the lymphatic tissue below by transcytosis [30]. They secrete less mucus than other IECs and have short irregular microvili at their apical membrane to promote binding and uptake of antigens [62, 75]. M-cells basically just channel the antigens into the lymphoid tissue without processing them very much

[75].

Dendritic cells (DCs) are cells specialized in presenting antigens to B and T-cells [30]. They load the antigens into MHC II molecules and present them in a context appropriate to the antigen, which means that theoretically they induce tolerance to harmless antigens while inducing an immune response in B- and T-cells to pathogenic antigens [30]. DCs induce class switching to IgA in B-cells as mentioned before and help homing of lymphocytes to the gut by inducing expression of CCR9 [30, 51, 52]. It should be noted that DCs do not migrate further than the mesenteric lymph nodes due to their limited life span. This prevents spread of sampled live bacteria into the periphery [30]. DCs have 4 ways to acquire antigens which they then present, the best known being uptake of antigens which were transported into the lymphoid structures of the gut by M-cells [76]. IECs can also take up antigens and expose them to DCs, especially in areas where there are not many M-cells present [77]. The third mechanism is facilitated by the fetal Fc receptor on IECs which can bind antigens bound to IgG. The complexes are then shuttled through to the underlying DCs [78]. The last mechanism by which DCs can come in contact with luminal antigens is by extending processes called sampling dendrites through the epithelium and establishing direct contact with the lumen(fig. 3 g) [62, 79]. Generation of these dendrites seems to be dependent on TLR signalling of IECs [80,81].
The DCs of the lamina proper can be divided into two major groups. The first group is named CX3 CR1+ and tends to express high amounts of costimulatory molecules as well as TNF-α [80,82]. These DCs induce the Th1 or Th17 effector response (discussed below) and serve to initiate an immune response [82]. CX3CR1- DCs on the other hand respond to commensal bacteria and oral antigens [83]. They express CD103 which is a ligand of E-cadherin on IECs and closely associate with the epithelial layer [80]. They also induce maturation of naive T-cells to Treg cells and imprint lymphocytes with gut homing markers such as CCR9 [51, 84].

2.4 Other cells of the innate immune system in the gut

Apart from the cells mentioned in 2.3 a number of other cells of the innate immune system are active in the immunity of the gut [30].

Macrophages in the gut are located in the lamina proper and the PP [85]. Macrophages are numerous in the gut but differ greatly from macrophages elsewhere in the body [30]. In the rest of the body phagocytic killing of cells by macrophages is accompanied by strong inflammatory responses mediated by presentation of antigens to Th cells [85]. In the gut however macrophages are highly effective at phagocytosis even without prior stimulation without causing an inflammatory response [86, 87]. They show a markedly lower expression of co-stimulatory molecules such as CD40, CD80 and CD86 [85]. Intestinal macrophages also show no secretion of inflammatory cytokines such as IL1, IL.6 or TNF-α in reaction to PAMPs, but instead react to whole bacteria by emitting the anti-inflammatory cytokine IL-10 [87]. This response is attributed to the low or even absent expression of TLRs by intestinal macrophages [88] and other receptors for an inflammatory response such as Fcα R which initiates as respiratory burst after IgA mediated uptake of microbes in macrophages [89, 90].
It is not known how this different phenotype of macrophages in the intestine is achieved, but production of TGFβ, IL-10, thymic stromal lymphopoietin (TSLP) and retinoic acid (RA) by IECs is thought to be involved. Indeed experiments have shown that a hyporesponsive phenotype of macrophages can be achieved by culturing them with IECs [91]. Endotoxin resistance was also observed in intestinal macrophages, since subsequent exposure to LPS limited the TLR4 response and downregulated TLR4 production [92,93]. IL-10, which can be produced by several parts of mucosal immune system has also been suggested to be involved in the process since IL-10 deletion mice show an increased inflammatory response in macrophages upon stimulation of TLRs [87]. Some or even all of these effects could induce the priming of macrophages in the gut.

Another function of intestinal macrophages is support of the Th2 response against helminths, by changing to the phenotype of alternatively activated macrophages (AA-macrophages) found after stimulation with IL-4 or IL-13 [94]. AA-macrophages produce chemokines such as CCL22 to attract Th2 cells [95] and have also been implicated in tissue restoration after the Th2 response [85].

Basophils which make up less than 1% of the circulating leukocytes are also a player in the intestinal immunity [96]. Being a close relative to mast cells, they are also involved in the Th2 response against helminths and in the development of allergies [96]. By virtue of their high-affinity IgE receptor FcεRI they can rapidly release Th2 associated cytokines when an IgE-antigen complex approaches them [97]. They have also been described as an important part of the memory Th2 response by producing IL-4 rapidly on secondary exposure [98]. Basophils are part of the response against intestinal parasites through the Th2 response which is characterized by IgE production and inflammation [96].

Another population of circulating leukocytes which are involved in the mucosal immunity of the gut are neutrophils. Neutrophils fulfil basically the same role in the intestine as elsewhere in the body. They can sense tissue damage and infection and upon doing so attract DCs and monocytes [99, 100]. They can activate DCs by cell-cell contact, drive maturation and proliferation of B-cells by B-lymphocyte stimulator (BlyS) and activate macrophages and drive differentiation of T-cells with interferon-γ (IFNγ) [101]. They support an inflammatory response to microbes this way. Neutrophils themselves mediate small scale tissue destruction and pus formation in sites of infection [101]. They so by emptying granules which contain proteases, defensins and/or reactive oxygen species into the inflammatory site if they cannot find a microbe to unleash them on although sensing an infection [101]. This non-specific destruction of macromolecules usually helps a lot in clearing out infections [101]. Neutrophils can also mediate their own response: in the early phase of inflammation they recruit and activate more neutrophils while killing tissue and bacteria [102], but label themselves for phagocytosis by macrophages and undergo apoptosis once the infection is under control [103].

Induction of apoptosis usually is one of the responsibilities of NK-cells. In the gut however there is a peculiar population of NK-cells in the gut which only has weak conventional NK-cell functions (I.e. killing of infected cells), but promotes epithelial cell homeostasis and secretion of antimicrobial factors through IL-22 [104]. There are also normal NK-cells in the gut which act in defense against intracellular bacteria which have downregulated MHC1 [30].

The last population of the innate immunity with a well-described funtion in the gut are mast cells whose functions in the intestinal immunity are diverse. They can mediate inflammation by IL-1,-6,-8,-16-18 and TNFα, support wound healing by TGFβ or use IL-3,-5 and IL-13 to support the Th2 response [105]. To do so they possess a wide array of TLRs [105, 106]. Mast cells react to bacteria by producing cytokines needed for innate and adaptive immune response [107]. Mast cells are also heavily involved in allergies and react with degranulation to allergens [105]. Mast cells can modulate the intestinal barrier, the inflammatory response and T-cell maturation by secretion of histamine [105]. The most prominent function of mast cells in the intestine however is the defense against helminths in cooperation with eosinophils and Th2-cells [108].

2.5Cells of the adaptive immunity

B and T-cells are the primary players of adaptive immunity, each group being made up of several subsets. The only groups of B-cells showing a strong connection to the gut are IgA producing B-cells, which have been described in under 2.1 [30, 46] above and IgE cells, which take part in the response against intestinal parasites as described under 2.4 [97]. The rest of the B-cells are primarily

involved in systemic immunity and outside the scope of this work.

$CD4^+$ T-cells in the gut can be divided into 4 distinctive groups which all have their own set of receptors and effector mechanisms: TH1 cells, TH2 cells, Th17 cells and Treg cells [109].

Th1 cells are involved primarily in cellular defense against intracellular bacteria and virus [109] in the gut. They produce IFNγ to improve the phagocytic killing of infested cells by macrophages. Macrophages and DCs which have sampled microbial products secrete IL-12 in turn, which has a positive feedback to Th1 cells [30]. This response is important to prevent opportunistic infections in the intestine.

The second group of $CD4^+$ cells in the gut are Th2 cells which manage defense against helminths in a response which is antagonistic to the Th1 mediated response. Th2-cells promote secretion of humoral IgE to mark and block the helminths and attract and activate mast cells and eosinophils to kill them (see 2.4) [97, 109]. Eosinophils and mast cells react by degranulation to helminths and their granules contain factors, like the major basic protein, which can harm helminths which are resistant to most secreted humoral factors and to big to be killed by phagocytosis. Th2-cells are hardly found at all in a healthy intestine however [30] and overall do not play a major role in the immune system of the gut.

The Th17-cells which are the most recently discovered of the family mediate the immune response against extracellular bacteria and are therefore of utmost importance in the gut, since commensal and pathogenic bacteria both fall under this category (at least before entering the tissue) [30, 109]. The TH17 response is antagonistic to both Th1 and Th2, although it has been suggested that Th1 cells are involved in the later stages of the Th17 response [109]. Initiation of Th17 maturation requires stimulation of naive $CD4^+$ cells by a high dose of antigen and co-stimulation by CD40 [110]. In the case of a severe infection this stimulation favors Th17 over Th1 [111]. Production of IL-6 by DCs seems to be crucial for the Th17 development as well [109]. After activation Th17 cells secrete their effector cytokines IL-17 and IL-22, which induce the production of antimicrobial peptides such as defensins [112]. IL-17 also prolongs the life of macrophages and attracts neutrophils and increases their cytotoxic and phagocytic abilities (fig. 4) [109]. The main role of Th17 is thus the attraction and activation of innate immune cells and support of production of humoral components of the innate immune system to achieve clearance of extracellular bacteria.

TH17 cells have also been implicated to play a part in defense against fungal infections. Fungal antigens are recognised not by TLRs, but other PRRs like dectin-1 or the mannose receptor on the T-cells [109]. There probably are yet indiscovered PAMP receptors on the Th17 cells which can work without involvement of TLRs and the discovery of these receptors may allow more understanding on the diverse actions of Th17 cells. The exact nature of the antifungal Th17 response however is unknown, but it has been shown that IL-17 deficiencies increase the severity of several fungal infections [113, 114]. A role for Th17 has also been reported for clearance of intracellular bacteria, but there are no unambiguous reports on the effects of Th17 yet [109].

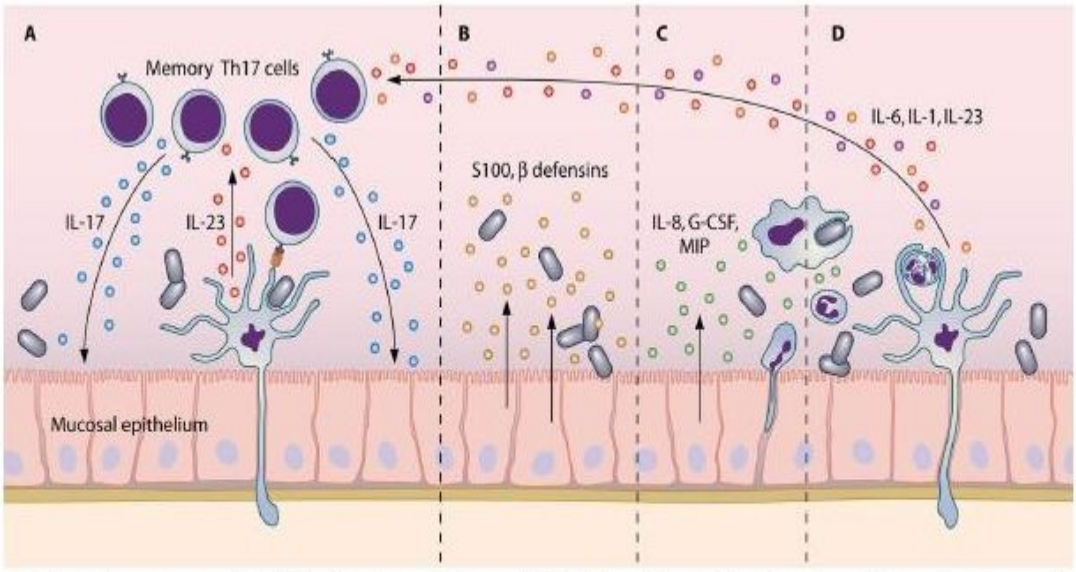

FIG. 4 Mucosal infection engages the Th17 axis of host defense. (A) Peripheral dendritic cells activated by pathogens produce IL-2 upregulates IL-17 and IL-22 production from resident memory Th17 cells. (B) Synergy between IL-17 and IL-22 induces secretion of antim peptides from epithelial cells. (C) IL-17 also drives epithelial expression of granulopoeitic and chemotactic factors. (D) As innate immu accumulate at the mucosal surface, dendritic cells that have phagocytosed infected, apoptotic neutrophils respond by secreting proinflan cytokines that support Th17 differentiation from uncommitted infiltrating $CD4^+$ T cells.

Figure 4 taken from 109:Peck A, Mellins ED. Precarious balance: Th17 cells in host defense. Infect and Immun 2010; 78:32-38

Regulatory T-cells (Treg) are necessary to prevent hyperreactions of the immune system in the body. They are defined by the transcription of FOXP3 They produce various regulatory factors such as IL-10 or TGFβ [115] and are mainly involved in toning down or even terminating the Th1 or Th17 response [30]. Due to the fact that the intestine harbours millions of bacteria which could potentially lead to an immune response while actually being benign towards the host, the actions of Treg are of special importance there [30]. Spontaneous inflammation occurs in mice which are deficient of IL-10 or TGFβ [116, 117] or factors necessary for Treg cell maturation [30].

There is small set of CD8+ T cells in the gut, called γδT lymphocytes. They possess receptors very much alike to the ones found on natural killer cells (NKR), but the exact ligands for these receptors as well as the function of γδTL remain elusive. The γδTL in the gut differ from the ones found in the rest of the body and are thought to act like gut specific cytotoxic T lymphocytes [118]. This means they kill of infected host cells after recognition but they do not use MHC I signalling like CTLs but rather NK receptors like NK cells. γδTL also seem to recognize stress signals present on infected cells and kill them [119]. They produce kerantinocyte growth factor and are involved in intestinal epithelial repair [120], a process which seems to be depended on the presence of intestinal microflora [30]. NK-cells can also induce tissue repair via this pathway, which means that it is probably mediated receptors related or even identical to NKRs[118].

3. Commensal bacteria in the gut

As said before the gut is home to numerous bacteria. This section will focus on the effect of commensal bacteria on the immune system of the gut in a healthy state and how the immune system is able to maintain a stable state in the face of this huge number of antigens.

3.1 The effect of commensal bacteria in the gut

The intestinal microbiota can influence the development, distribution, differentiation, activation and inflammatory behaviour of macrophages, NK cells, B cells and both kinds of T cells within the intestine and even in peripheral sites of the body [121, 122]. Without a microflora immunological development is severely hampered as was tested in sterile mice [8, 121]. For example T- and B-cell development in the peripheral lymph nodes is impaired in germ-free mice [123]. Administration of specific antibiotics to mice lowers the number of Th17 cells in the intestine, which shows dependence of T helper development on the microflora [124]. The PP and B-cells in the lamina proper of the gut are also altered in germ-free animals [8].

Commensal bacteria are also directly involved in the defense against various pathogens. Alterations in the microflora are observed in enteric infections and often precede them, which leads to the hypothesis that such alterations are not a consequence of the infection, but actually increase the risk of infection [121, 125]. This has been tested with antibiotic induced alterations of the microflora [125]. Further alteration of the microflora by pathogens then lead to a more favourable environment for the pathogen [125]. This vicious circle can often only broken once the adaptive immune response against the pathogens takes action or through therapy.
Commensal bacteria have also found to be involved in termination of the inflammatory reaction after clearance of pathogens, at least during salmonella infections [126]. This anti-inflammatory action of commensal bacteria can for example be mediated by their ability to interfere with immune signalling, for example via the NF-κB pathway. Pathogenic bacteria activate this pathway by interacting with TLRs and Nod like receptors, leading to inflammation [62]. NF-κB is usually kept in the cytoplasm of cells by its interaction with IκB. TLR stimulation leads to phosphorylation of IκB which targets the molecule for ubiquitylation and subsequent degradation. Without IκB the NF-κB nuclear localization sequence unmasked and allows it to relocate into the nucleus and initiate transcription of cytokines mainly involved in inflammation [127]. Commensal bacteria can interfere with this pathway by preventing the degradation of IκB [128].Some species of *Bacteriodes,* which grow as commensal bacteria in the gut, can induce the expression of peroxisome-proliferation-activated receptor γ, which associates with NF-κB in the nucleus after which the whole complex is transported out of the nucleus [129]. The commensal bacterium *Lactobacillus acidphilus* can produce a specific surface layer A protein which allows it to bind to DCs, thus limiting production of the inflammatory cytokine IL-12 while inducing production of IL-10 [130]. Commensal bacteria possess the ability to actively support immune homeostasis by these mechanisms.

3.2 Immune homeostasis: Induction of tolerance to commensal bacteria

It should be obvious by now that the immune system of the gut possesses potent mechanisms to deal with microbial invaders no matter if their nature is pathogenic or opportunistic. The problem is keeping these potent killing systems from acting against commensal bacteria and to achieve a state of immune homeostasis, in which the inflammatory response to the foreign bacteria is sufficiently dampened as not to harm them or the host tissue. There are multiple signalling pathways in the immune system of the gut to enable such a state.

First of all antigens or bacteria sampled by DCs are transported to the mesenteric lymph nodes where they can interact with resident lymphocytes and initiate Treg maturation. As said before, commensal bacteria hardly ever penetrate further into the body since the DCs survival is limited and they are efficiently destroyed by macrophages in the mesenteric lymph nodes. Immune cells recirculate through the blood to the whole area of the intestinal mucosa. All this allows the initiation of a mucosal immune response without priming a systemic adaptive response [131]. Disruption in the barriers of the gut or impairment of the innate immunity this compartmentalization of the immune response is lost, as can be seen in IgA deficient mice [45]. The net effect of this compartmentalization is that of keeping the lymphocytes in the gut restricted to the gut. This allows

them to develop a local tolerance of commensal bacteria while the same bacteria would initiate a response of the immune system if they penetrate into the systemic circulation. It also allows them to take local effects on bacteria without initiating a systemic immune response, which mimimizes tissue damage.

The seeming unresponsiveness to antigens taken up in food or water is called oral tolerance and can be used for therapeutic means. Oral tolerance is often dependent on CD4+ cells and requires the antigen to be presented in the MHC II of DCs in absence of costimulatory molecules such as CD80 or CD40 or together with inhibitory co-stimulatory molecules such as CTLA-4 [75]. This induces differentiation of Treg cells.

Treg downregulate immune reactions in the gut just like in other parts of the body. The production of IL-10 by various mechanisms mentioned above supports Treg differentiation, which then dampen the immune response [30]. The importance of this is shown in murine models lacking IL-10, which are prone to colitis induced by *Heliobacter Hepaticus*, a common part of the microflora [132].

As mentioned above, IECs can produce various anti-inflammatory cytokines such as IL-10, TGF-β, IL-25 and TSLP thus modulating the innate and adaptive immune response by virtue of their PRRs [133, 121]. Low activation of TLRs on IECs activates the usually pro-inflammatory NF-κB pathway, which under these circumstances allows production of the anti-inflammatory cytokines IL-10 and IL-8 [72]. TSLP secreted by IECs upon stimulation by commensal bacteria induces secretion of IL-10 and IL-4 by resident DCs [133]. TGF-β and retinoic acid from epithelial cells has also been shown to upregulate $CX3^{+}CR1^{-}$ DCs, which subsequently induce differentiation of Treg cells by production of the same two factors in the lymph nodes and PP [134]. Experimental blocking IL-10 and TGF-β from IECs also induces IEC apoptosis disrupts the epithelial barrier function [135]. DCs have been found to be able to prevent tissue damage by attenuation of the TLR-2 response and subsequent downregulation of the inflammatory axis of the NF-κB path upon stimulation via NOD2 by microbes [136]. This basically makes the inflammation in response to helminths less severe and shorter, leading to less damage to the intestinal epithelium.

Intestinal macrophages have a very simple answer to the question of how to promote tolerance and prevent inflammation: they simply lack expression of many inflammatory receptors. As mentioned above they have a very low or even absent expression of many TLRs and the Fcα R and do not react with inflammation even if they recognize bacteria [88, 89, 90]. Another function of macrophages in the gut is presentation of antigens in MHC II to T-cells without expressing any co-stimulatory molecules which induces the differentiation of Treg cells [137].
Like macrophages and DCs, mast cells from the intestine also show an attenuated reaction to TLR ligands such as LPS in comparison to mast cells harvested from sterile sites of the tissue (for example blood) [105]. This decreased reaction of innate immune cells to antigens in the gut is a passive mechanism supporting immune homeostasis.

TLRs are generally associated with recognition of PAMPs followed by an immune response. They belong to the innate branch of the immune system and are prominent in immune cells of the mucosa. Stimulation of these TLRs however does not necessarily lead to an inflammatory immune reaction as has been noted before in this thesis. The conditions of the TLR stimulation dictate their action on the immune system [80].
TLR2 receptors can be linked to TLR1 or TLR6. Signalling via TLR2 and TLR1 is characterized by high amounts IL-12 and low amounts of IL-10, basically supporting an immune response, while signalling via TLR2 and TLR6 results is characterized by high amounts of IL-10 but low IL-12 which has the opposite effect on immune regulation [138].
TLR4 is usually associated with an inflammatory response when stimulated with LPS. Chronic

stimulation in mucosal tissue may lead to non-responsiveness to LPS as well as other ligands. This is achieved by multiple factors blocking TLR4 and its signalling pathway, for example IRAK-M [139].

There are probably more mechanisms which maintain homeostasis of the immune system in the gut and if the intense research of the last few years will continue more molecular pathways will be uncovered. All in all our knowledge of the whole system is far from complete and needs further investigation.

4. Immune dysbalance

Although commensal bacteria and the immune system usually live in a state of truce in which neither harms the other for mutual benefit, even slight variations in the behaviour of either of them can lead to serious consequences for the host. This part of the thesis will discuss 4 medical conditions resulting from a dysbalance of the intestinal immune homeostasis.

4.1 Inflammatory Bowel disease and Irritable bowel syndrome

An example of dysbalance of immune homeostasis is inflammatory bowel disease (IBD), which is characterized by a chronic inflammation in parts of or even the whole intestine. It can be divided into ulcerative colitis (UC) and Crohn's disease (CD), which usually is more severe [140]. First of all it should be noted that in mouse models it was not possible to initiate IBD without the presence of bacteria, so the process really is an overreaction to intestinal microbes [141]. Next it should be noted that observations concerning the microflora or tissue in the gut are ambiguous in IBD since it is often hard to discern whether they are the cause of the inflammatory response or only altered as a consequence of it [140]. IBD has also been identified as a polygenic disorder, so development of IBD differs per patient [142]. This also explains while downregulation of specific cytokines is seen in some patients while it is normal in others.

Genetic variations of NOD2 show a strong connection with development of CD, especially the region of NOD2 responsible for interacting with the bacterial cell wall peptidoglycan component muramyl dipeptide [MDP). MDP stimulation in mice with a mutation in NOD2 leads to lower production of defensins by Paneth cells and increased inflammatory signalling via NF-κB [143]. This effect could become pathological in CD.
Reduced expression of IL-8 due to an NOD2 mutation has been suggested to result in the recruitment of an insufficient number of neutrophils, thus leaving the work of phagocytosis to macrophages which are less suitable for the job [144].This leads to an increase in the inflammatory response since the bacteria cannot be cleared away efficiently any more [140].

A decrease in defensin production due to a mutation in the wingless type gen has also been implied in CD, as well as a decrease in the anti-microbial effect of defensins and other anti-microbial peptides [145].
A mutation in the autophagy 16 related-like 1 gene has also been associated with an impairment of antimicrobial protein release by inhibiting exocytosis of secretory granules in Paneth cells [146].
Loss of the transcription factor X-box-binding-protein 1, which is necessary for normal Paneth and goblet cell development, has been shown to trigger spontaneous intestinal inflammation in mice and was also linked to IBD in humans [147].
Reduced actions of antimicrobial peptides would increase adhesion of microbes to IEC and might support damage to mucosa.

Defects of the mucosal barrier in IBD can be found before the onset of inflammation in identical

twins of patients [148] and are therefore thought to be involved in the pathogenesis. Increased permeability results in an increase of the breaching by microbes, which in turn increases the inflammatory response [140].
Use of rodent models has shown that the paracellular permeability of the mucosal epithelium can be increased by mast cell derived proteases during stress [105]. How far this relates to IBD is not known.
Flagellin, a surface protein involved in mobility which is expressed by many bacteria in the gut, is linked to CD, as antigens to flagellin have been found in many CD patients [149]. Leaks of the barrier which allow flagellin to pass could thus contribute significantly to the development of CD.
Furthermore damage to the intestinal mucosa has been shown to recruit a large number of macrophages to the site of inflammation. These seem not to belong to the intestinal type of macrophages but are recruited from the blood stream and thus do not show hyporesponsiveness to the microflora [150]. These cells will now produce IL-8 to recruit even more macrophages and granulocytes to the gut. Upon activation by microbes the macrophages will secrete IL-12 which induces expression of IFNγ by T cells. IFNγ then further increases epithelial permeability and gives a positive activation feedback to the macrophages. Stimulated by this macrophages will secrete IL-1 and TNFα which drive apoptosis in IEC, vascular damage and necrosis. Both factors also induce fibroblasts to degrade the extracellular matrix by production of matrix metalloproteinases [85]. Macrophages thus significantly deteriorate the mucosal barrier and contribute greatly to the damage done in IBD.

A reduced diversity of the microflora has been observed in IBD, which implicates a role in pathogenesis [148]. Indeed although the diversity and number of bacteria is lowered during CD, a subtype of E.coli which can adhere to the epithelium and even replicate in macrophages has been found in unusually high numbers [151], implicating its involvement in pathogenesis of CD.
To sum it up IBD is a multifactorial disease, but most mechanisms which can lead to its development are involved in the maintenance of the immune homeostasis.

Another medical condition associated with dysfunction of the gut is the irritable bowel syndrome (IBS), which is associated with pain in the visceral area, deviations in gut motility and psychological problems in the patients [152]. Although IBS is considered a psychosomatic disorder with only secondary influence of the gut via the brain-gut axis, research has shown that at about 30% of the patients develop IBS after an acute gastroenteritis [152]. Using the inflammatory cytokine IL-1β as a marker, it was found that these patients do not properly downregulate the inflammatory response after infection [153]. This persistent low grade inflammation of the gut is hypothesized to be responsible for IBS in this sub-group of patients [154]. The effect of the immune system on the neural system of the gut has been investigated more closely following this hypothesis. IBS patients, even those without a history of gastroenteritis, were found to harbour an increased number of inflammatory cells in the colon or terminal ileum [155, 156]. Supernatant of mucosal biopsies from IBS patients has also been shown to have a an effect on sensory neurons in the gut as well as on the intestinal epithelial barrier function [157, 158]. These results strongly suggest a connection between dysregulation of the immune system of the gut and the neurological defects of the enteric nervous system observed in IBS.
Analysis of the microflora of patients suffering from IBS have also been found to differ from healthy individuals [152]. This led to the speculation that the microflora might be involved in the pathogenesis of IBS as well. Indeed *E.Coli nissle 1917*, a probiotic bacteria, was shown to be able to enhance gut contractility [159], whereas *Lactobacillus rhamnosus*, a commensal bacteria also found in yoghurt, decreases gut contractility [160]. The visceral pain response can also be altered by the microbiota, as treating mice with oral antibiotics increases their susceptibility to visceral pain [161]. The alteration of the microflora due to the antibiotics treatment also caused a small increase in inflammatory cells and inflammatory state of the gut similar to the low-grade inflammation seen in

IBS patients [152]. This effect can only be tied to the microbiota, as treatment with probiotics can re-establish the state before antibiotic treatment in mice [161]. Recent research also suggests a connection between behaviour and the microbiota [162]. This means that most symptoms connected to IBS can be influenced by the microflora of the gut, which strongly hints at a connection between perturbations in the microflora and the development of IBS. This link is only increased by the fact that many risk factors of IBS, such as acute infection, dietary changes or use of oral antibiotics, also cause changes in the microflora of the gut [163-165]. It can be hypothesized that IBS and perturbations in the microflora form a vicious circle in the pathogenesis of IBS: changes in the microflora support development of IBS while IBS creates an unfavourable environment for the commensal microbes in the gut. This further changes the gut microflora, which in turn increases the symptoms of IBS [152].
There seems to be an intimate connection between the immune system and the microflora of the gut and the development of IBS. Although probably not all cases of IBS can be traced to dysregulations of the immune response or the microflora in the gut, both pose a valid target for treatment of IBS. The research in this subject has picked up during the pat few years but is far from complete, since although many connections have been found the molecular mechanisms underlying them have not yet been described in detail.

4.2 The role of microbes in the development of autoimmunity and allergy

Allergy has been described as a hyperreaction of the immune system against harmless antigens, which have for example been taken up in food, water or even air. In contrast to this, autoimmunity is a reaction of the immune system against antigens present in the own body, for example against surface molecules present on β-cells, which leads to type 1 diabetes (T1D). The microflora of the gut and pathogens associated with the gut have been described to be able to invoke as well as suppress both of these inappropriate responses of the immune system [166, 167]. One thing that has to be noted is that both allergies and autoimmunities are strongly influenced by the genetic background of afflicted individuals, so microbes are not the only factor determining the pathogenesis. There are also some autoimmune diseases which are solely based on the genetic background of the patient and are not influenced by microbes at all [166].
Although allergies and autoimmune diseases differ greatly from one another, an underlying general mechanism for their development in relation to microbes has been proposed. Autoimmune diseases are often depended on interleukin-2 (IL-2) and interferon γ (IFN-γ) produced by Th1 cell while allergies depend on IL-4 and IL-5 originating from Th2 cells [167]. Th1 and Th2 cytokines usually downregulate one another, which suggests that an overreaction of one of the responses as seen in autoimmunity or allergy can be connected to dysregulation of the regulation and that microbes could be involved in preventing this dysregulation [167]. There also seems to be a correlation between allergies and autoimmune diseases in individual patients, which suggests a common dysregulation in both forms of immune hyperreaction [168, 169]. This dysregulation can apparently be prevented by priming if the immune system by microbes [166, 167].

There are numerous autoimmune diseases, which can be divided into three groups depending on their relation to microbes. The first group is not influenced by microbes at all and solely depended on genetic factors, for example the X-linked syndrome [166]. The second group is induced by microbes, as can be seen in rheumatic fever, which is depended on antigens to streptococcal polysaccharides [170]. The microbes in this group are, to my knowledge, exclusively pathogens. The third group also depends on genetic predisposition, but the pathogenesis can be influenced by commensal microbes. This third group is the only one which involves commensal microbes and therefore will be the focus in this thesis. It should also be noted that not all autoimmune diseases can easily be divided into these groups, since the mechanisms pathogenesis are often not completely understood yet [166].

Probably the best described autoimmune disease belonging to group 3 is type 1 diabetes [171]. In most recent experiments about the interactions of microbes and T1D non-obsese diabetic (NOD) mice were used with and without a knock-out of the MyD88 gene, which has strong relations to the development of T1D. These have then been raised under normal, germ-free (without commensal or pathogenic bacteria) or specific pathogen-free (SPF) conditions, the latter meaning that they do have commensal bacteria but will not meet pathogenic ones. MyD88 knock-out (KO) mice did not develop T1D during a period of 30 days when housed under normal or SPF conditions, which shows the dependence of T1D on TLR signalling (since most TLRs signal via MyD88) [166]. They do develop T1D when housed in germ-free conditions however [171] which means that NOD MyD88 KO mice are protected from T1D by their commensal bacteria (which are present in SPF conditions but not during germ-free housing). Furthermore, colonization of germ-free NOD MyD88 KO mice by a mix of commensal bacteria lowered the incidence of T1D from >80 to 34% in males [171]. In studies using the same model it was also found that the anti-diabetogenic effect of the MyD88 KO in mice was restricted to the pancreatic lymphoid nodes, which receive antigens from the gut and pancreas, further hinting at the importance of antigens derived from the gut [171].
Another example of involvement of commensal microbes in autoimmune disease would be the mouse model of experimental autoimmune encephalomyelitis. In this model mice under SPF conditions produced less autoimmune T-cells than mice housed under germ-free conditions [166]. A negative example of the involvement of microbiota would be the mouse model of spontaneous ankylosing enthesopathy, an autoimmune joint disease. In contrast to T1D mice housed under germ-free conditions do not develop a disease phenotype while mice housed under SPF conditions which harbour commensal bacteria do develop the disease [172]. The same was true in a mouse model for lymphocyte-driven arthritis [173].
There are two models which can explain why microbes support the development of autoimmunity. The first one is molecular mimicry, in which an antigen on the microbe closely resembles an antigen of the host and if an immune reaction against the microbe should become necessary, for example in the case of an opportunistic infection by commensal bacteria, the immune system will also attack host cells which express the resembling antigen [166]. This model also explains while some specific microbes are associated with specific autoimmune diseases, for example the association of streptococci with rheumatic fever; the microbes simply express antigens much alike to those of the afflicted host cells. The other model is bystander activation, in which antigen presenting cells which have been activated by a microbe present self-antigens to lymphocytes while also expressing activating cytokines due to their activation by the microbe [166]. Both models require self-reactive T-cells to escape selection in the thymus in the first place however and can thus not induce autoimmunity if the immune system does not do its part in the development as well [166].
The reason for commensal bacteria protecting against certain autoimmune diseases is less well understood. It could however been related to the afore mentioned ability of commensal microbes to induce tolerance via TLRs, as it has been found that mutant mice with a duplication of the TLR7 gene have an increased susceptibility to autoimmune diseases. More copies even increase this effect in a dosage dependent manner[174]. Taking into account the example of MyD88 in T1D, it could be hypothesized that excessive TLR signalling can be a cause of autoimmunity. This also means that the induction of tolerance discussed under 3.2 also protects from autoimmunity by attenuating the immune response. The hypothesis that priming of the immune system of the gut can induce protection against autoimmunity is also backed up by the mechanism of oral tolerance. It was observed that taking up antigens in the food apparently induces tolerance of the immune system to these antigens. Collagen induced arthritis for example can be suppressed by feeding with collagen [175]. T1D can also be delayed by feeding with insulin. In both cases the number of IL-10 producing cells in the Peyer's Patches and mesenteric lymph nodes was increased [175]. This also supports the claim that attenuation of the immune response in the gut can induce protection against autoimmune diseases.

A similar protection by induction of tolerance has been suggested for allergies as well in the hygene hypothesis. The hypothesis basically states that encountering more microbes and antigens early in life lowers the chance of development of allergies. This hypothesis was formulated after comparing the data of incidences of allergies in developed, more hygienic to data from less developed countries [105]. This has been tested by administering lactobacillus to pregnant women suffering from atopic dermatitis, a skin allergy, and their offspring after birth, which significantly decreased the development of atopic dermatitis in the children [176]. Including probiotics in the diet of children suffering from atopic dermatitis also improved their symptoms [177]. This underlying mechanisms for this effect have not been made completely clear yet however. IL-10 has been proposed as a protective agent in allergies and IL-10 production is increased to downregulate the immune response after resolving an infection [178]. Stimulation of mast cells by microbes leads to induction of a Th1 response, which inhibits an allergic Th2 response [105].
On the other hand microbial infections have been observed to aggravate the allergic response in individuals who have already developed an allergy [179], supposedly by activating PRRs on mast cells which already have been primed to stimulate an allergic response [105].

The mechanisms involved in protection from autoimmunity and allergies by microbes seems to be related and just like in IBD and IBS tightly connected to a healthy microflora. The hygiene hypothesis also further emphasizes the role of commensal bacteria in the gut in the development of the immune system.

5. Intestinal Pathogens and immunity. The example of *Shigella* infections

Although the mucosal immune system of the gut possesses many diverse and effective mechanisms to deal with invading pathogens, many pathogens ate able to escape immunity, at least long enough to multiply within the host and assure spread to other hosts. There are many diverse strategies involved in the escape from immunity and discussing them all would be outside of the scope of this thesis. Shigella will be discussed as an example of a pathogen which cannot only escape, but actually mediate immunity to serve its own means to a certain extent.

Shigella, a Gram negative enteroinvasive bacterium can cause shigellosis in humans and exclusively in them. *Shigella* is transmitted via the feco-oral route from an infected patient or indirectly through water and food [180]. Because of this, shigellosis is primarily a burden in developing countries and has been speculated to cause as much as 1 million deaths in the world per year, primarily in children under the age of 5 [180]. The symptoms of shigellosis are diverse and range from mild diarrhea to a severe form accompanied by blood in the stool, high fever and abdominal cramps. The more severe symptoms reflect an extensive destruction of the mucosal tissue of the gut due to an inflammatory reaction [180].
Shigella crosses the mucosal barrier in M-cells and then escapes into the lymphoid follicles where it can interact with IECs, macrophages and DCs [181]. This invasive phenotype of shigella is governed by a 213-kb virulence plasmid [182]. The plasmid contains genes encoding a type 3 secretion apparatus (TTSA) and substrates secreted by it [183]. The TTSA structure resembles a needle and can be used by *Shigella* to inject effector proteins into the cytoplasm of host cells [184]. The Ipa surface protein is also encoded by this plasmid [180] and this protein allows *Shigella* to escape from the vacuole in macrophages by lysis of the vacuole [185]. IpaB then induces activation of the macrophages own caspase-1, which induces apoptosis in the macrophages and allow *Shigella* to escape into the tissue [185]. The activation of caspase-1 one followed by cell death has recently been described as pyroptosis instead of apoptosis in infection by *Salmonella* [186]. The mechanisms described in pyroptosis closely resemble what is described in the caspase-1 mediated apoptosis in *Shigella* [180, 186], which is why I suggest that *Shigella* actually induces pyroptosis as well. The main differences between apoptosis and pyroptosis is the lysis of the cell membrane in pyroptosis

[186] and the cleavage and activation of inactive precursors of the inflammatory cytokines IL-1γβ and IL-18 by caspase-1 [187]. These cytokines are then released into the surrounding tissue after lysis of the macrophages and induce a strong inflammatory response [188]. The initiation of pyroptosis in macrophages is dependent on the TTSA and flaggelin in *Salmonella* and probably *Shigella* as well [186] and has also been described for DCs in addition to macrophages [186, 189]. The induction of pyroptosis is rather rapid, but can artificially delayed by stationary phase substrates of the TTSA [189]. This allows the bacteria to multiply inside of the cytosol of the host cells before lysis and induction of an inflammatory response occur.
The question that comes to mind is why *Shigella* induces pyroptosis and the release of inflammatory mediators when inflammation usually aids in the clearance of pathogens from the host. The answer to that is that cells who have undergone apoptosis possess an intact cell membrane and are cleared away by apoptosis. This would trap Shigella in the dead cell and force it to yet again make an escape from being digested [180]. In addition the inflammation actually aids *Shigella*, since the resulting diarrhea insures the spread to other hosts [186]. The inflammation of the gut also attacks the commensal bacteria and hamper their ability to protect the host by competing with *Shigella* in the gut [186]. Furthermore the destruction of host tissue by the innate immune system (especially mast cells) increases the permeability of the mucosal and epithelial barrier and promotes infiltration of *Shigella* [186]. Another way of Shigella to induce this beneficial inflammation is by alter the transcription of the IECs to attract polymorphonuclear neutrophils (PMN) by injecting the epithelial cells with yet undetermined factors through the TTSA [190]. The main cytokine involved in this response seems to be IL-8 which mediates the early inflammatory response in shigellosis [190]. IL-8 is also produced by the IECs when stimulated with basolateral or intracellular LPS. This means that they only react to LPS which already has breached the intestinal barrier and so to the LPS on *Shigella* which have escaped from innate immune cells [191, 192]. It seems the main population of cells involved in the inflammation induced by *Shigella* is PMN [180].
In addition to mechanisms to induce inflammation, *Shigella* also possesses strategies which can inhibit inflammation in the gut. The protein ShiA, a protein encoded on the pathogenicity island of *Shigella*, has been reported to downregulate inflammation, which can be seen in ShiA mutants which elicit a stronger inflammatory response [193]. The mechanisms by which the downregulation is achieved is elusive until now. OspG, a protein secreted by the TTSA, has also been reported to inhibit inflammation. OspG can can prevent the degradation of the phosphorylated inhibitor of NF-κB type α and thus prevent TNF-α mediated activation of the pro-inflammatory NF-κB pathway [196].
The ability of *Shigella* to both up- and downregulate inflammation would suggest a certain optimal level of inflammation which ensures diarrhea and spread of *Shigella* while preventing a sufficiently strong inflammation to kill the pathogen. An early response also has to be prevented, since *Shigella* needs time to adapt to the conditions in the host before efficient growth of the population takes place [180].

There are other immune mechanisms apart from inflammation which *Shigella* has to escape to colonize the host successfully. One of the important effectors in mucosal immunity are antimicrobial proteins. *Shigella* has been found to be able to downregulate production of some of these antimicrobial proteins, for example β-defensin-1 [197]. Shigella can also escape autophagic destruction in infected macrophages and DCs by secreting IcsB through the TTSA [198]. IcsB can bind to the *Shigella* protein VirG, which is required for intracellular motility. VirG is usually recognized by the host and induces autophagy, but can be masked by binding to IcaB which prevents the induction of autophagy [198]. Another potential threat to *Shigella* is the cytokine IFN-γ, which is produced by NK-cells when they recognize a macrophage infected by *Shigella.* IFN-γ induces digestion of *Shigella* by the macrophages before the bacteria can escape and is crucial for the protection in primary infection [199]. A downregulation of IFN-γ during *Shigella* infection was observed and research showed that through a yet unidentified bacterial effector secreted by the

TTSA Shigella can inhibit IFN-γ production. This is achieved by blocking IL-12, a potent inducer of IFN-γ production [180].

All of the immune mechanisms discussed so far belong to the innate immunity. But since the activation of the adaptive immune system relies heavily on innate immune recognition, Shigella can prevent an effective adaptive response as well. Since Shigella is a intracellular pathogen, a Th1 response would be appropriate to deal with it. By inhibition of both IL-12 production and the NF-κB pathway an effective development of a TH1 response is inhibited [180, 196]. The production of anti-inflammatory cytokines like IL-10 during the early stages of Shigella infection also impair T-cell differentiation [200]. The heavy inflammation in the lamina proper of the gut during later stages of infection also leads to induction of apoptosis in resident cells. Samples from patients have shown that about 40% of the resident T-cells underwent apoptosis, not counting those that died due to necrosis [180, 201]. The inhibition of Th1 differentiation coupled with the high number of dead T-cells in the gut during shigellosis can explain why the adaptive immune response developed against *Shigella* is usually not efficient in preventing re-infection [202]. This emphasizes the role of the innate immune system in the protection of the gut mucosa.

Despite all these elaborate strategies to prevent attacks by the immune system most Shigella infections are eventually cleared from the gut [180]. This clearance is achieved by neutrophils, the very cells that favour the infection during early stages of shigellosis by disruption of the epithelial barrier [180]. Neutrophils can prevent the escape of Shigella from phagocytic vacuoles by virtue of the protein human neutrophil elastase (NE). NE has been described to be able to degrade virulence factors of *Shigella* efficiently enough to prevent escape of the pathogen [203]. This allows killing of *Shigella* inside the vacuole by proteolytic enzymes, antimicrobial proteins and reactive oxygen species [180]. Neutrophils also secrete neutrophil extracellular traps (NET) into the surrounding tissue. NET are able to trap and kill bacteria [204] and also prevent diffusion of granule proteins released from neutrophils and prevent unnecessary damage to the surrounding host tissue [180]. Apparently *Shigella* has found a way to neutralize this threat by inducing necrosis in neutrophils after initially recruiting them to induce inflammation through IpaB and IpaC secreted by the TTSA [205]. Clearance is probably achieved by neutrophils in later stages of infection due to *Shigella* being unable to defend against the large number of invading neutrophils [180].

Most strategies used by *Shigella* are dependent on the type three secretion apparatus. *Shigella* is not the only pathogens possessing the plasmid with the genes encoding this apparatus however. Salmonella for example uses the same TTSA [206]. *Salmonella* uses the induced inflammation to out-compete commensal resident bacteria in the colonization of the gut and it has even been hypothesized that the invasive *Salmonella* sacrifice themselves to induce the inflammation and aid their relatives in the lumen of the gut [206], since they are cleared away rather rapidly after breaching the mucosal barrier, but not before inducing pyroptosis [186]. *Salmonella* are also eventually cleared by neutrophils [206].

This example hows that although the immune system of the gut employs many defensive mechanisms, pathogens still pose a threat to humans particularly since they can often easily access the gut via food and water. It also emphasizes the importance of commensal bacteria in the defense, since both *Salmonella* and *Shigella* use the immune system to fight of these competitors.

6. Conclusion

The mucosal immune system has to face the herculean task of tolerating commensal bacteria while terminating pathogenic or opportunistic microbes. To be able to fulfil this task, the system possesses a dazzling array of cellular and humoral factors. A recurring factor to induce tolerance to commensal

bacteria seems to be a chronic low stimulation of immune cells via PRRs. In contrast to immune cells in the rest of the body, the cells in the gut respond to this stimulation with anti-inflammatory signalling molecules. Other parts of the mucosal immune system, like IgA and macrophages, are usually indifferent to such stimulation and can prevent bacterial intrusion without any response at all, in the case of IgA even without harming the bacteria. These more passive mechanisms aim to keep the commensal bacteria in the lumen where they do not harm the host and but still benefit from the favourable conditions in the gut and have a beneficial effect for the host. It is also clear that disturbance in even one of the many mechanisms of inducing tolerance can lead to severe inflammation and tissue damage.

It should also be clear that although the response of the immune system often induces tissue damage, such a harsh reaction to a breach in the mucosal barrier is necessary to eliminate pathogenic bacteria, which often have developed evasive mechanisms to escape the immune mechanisms present in a healthy gut environment like defensins and mucosal macrophages.

That being said the immune system of the intestinal mucosa is far from fully described until now and discovery of new molecular mechanisms can only help in therapy to maintain tolerance (and prevent IBD for example) and terminate pathogens which subvert the immune system.

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