Lympho-epithelial cross talk in the gut: implications for maintaining and restoring immune homeostasis

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Abstract

Intestinal immunological environment is shaped by a continuous cross-talk involving three major components: intestinal epithelium, immune system and local microbiota. This work intended to investigate the role of lympho-epithelial interactions in response to food components and microorganisms.

As part of this work the role of the immune-derived cytokine interleukin (IL)-12 in development of food allergy has been investigated. It was previously found that in peanut-sensitized mice there was decreased level of IL-12 in the intestine. We have observed that this is accompanied with increase in thymic stromal lymphopoietin (TSLP), a cytokine produced by the intestinal epithelium and indispensable for development of allergy. Oral delivery of recombinant *Lactococcus lactis* secreting bioactive IL-12 resulted in amelioration of the allergic symptoms and decrease in TSLP, thus suggesting a regulatory interaction between these two cytokines. Further investigation of the mechanism of this cross-regulation revealed that intestinal epithelial cells express incomplete but functional IL-12 receptor. However, mice deficient in IL-12 signalling displayed normal levels of TSLP implying involvement of other factors in the IL-12/TSLP axis.

In addition, the availability of mice defective in IL-12 associated pathways prompted us to test the hypothesis that alteration of the cytokine network in the gut may contribute to shape the intestinal microbiota. Thus, by using 16S pyrosequencing we have studied the composition of the gut microbiota in mice deficient for IL-12p40, IL-12Rβ2, and IFN-γ in comparison to WT mice. In parallel, we have monitored the metabolome of the microbiota and the host intestinal tissue.

Finally, we have described a novel pathogen-exclusion mechanism mediated by $CX3CR1^{+}$ cells that migrate into the intestinal lumen upon *Salmonella* infection. This event is orchestrated by a lympho-epithelial crosstalk involving MyD88-dependant epithelial signal. CX3CR1-dependant migration is vital for pathogen protection in the early stages of infection.

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Video S3 Tissue integrity was preserved during multiphoton procedure. 3D reconstruction and 360˚ rotation of the imaged tissue as described in Figure S5.

Preface

The work done for this PhD project has resulted in the following publications:

Scientific paper publications:

Man A. L.*, **Gicheva N.*,** Regoli M., Rowley G., Wellner N., Bertelli E., Nicoletti C. (2015) Migration of CX3CR1^{+} cells contribute to pathogen-exclusion during the initial stage of *Salmonella* infection. Under revision *Proceedings of the National Academy of Sciences of the United States of America (*equal contribution)*

Gicheva N., Dumont Fernandez A., Man A. L., Ivory K., Deen C., Bertelli E, Regoli M., Narbad, A., Nicoletti C. (2015) Interleukin-12 suppresses food allergy by indirectly down-regulating production of thymic stromal lymphopoietin by intestinal epithelial cells. Submitted at *Allergy*

Oral presentations:

Gicheva, N., Le Gall, G., Tett, A., Narbad, A., Nicoletti, C. Immunological signals shape microbiota and metabolic status of mouse gastrointestinal tract. *The 1st Norwich Students Microbiology Symposium, John Innes Conference Centre, Norwich, UK (1st December 2014)*

Gicheva, N., Le Gall, G., Tett, A., Narbad, A., Nicoletti, C. Immunological signals shape microbiota and metabolic status of mouse gastrointestinal tract. *Inaugural Annual Symposium, Institute of Food Research, John Innes Conference Centre, Norwich, UK (6th May 2015)*

Posters:

Gicheva, N., Tett, A., Narbad, A., Nicoletti, C. Host-derived immunological signals shape microbial community in mouse gastrointestinal tract. *National Institutes of Bioscience Conference, Roslin Institute, Edinburgh (18th-20th Jun 2013)*

Deen C., **Gicheva, N.,** Nicoletti, C. IL-12 responses at the epithelial surface – small talk or a deep conversation? *Inaugural Annual Symposium, Institute of Food Research, John Innes Conference Centre, Norwich, UK (6th May 2015)*

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administration schedule for the delivery of recombinant *Lactococcus lactis* and for being involved in the collection of some of the experimental results presented here. Dr. Dumont actively participated in the execution of some of the tasks described here mainly with regard to monitoring allergic symptoms following oral delivery of recombinant *L. lactis*. I would also like to thank Dr. Michael Comeau (Amgen) for the anti-TSLP antibody.

I am grateful to Dr. Adrian Tett and Dr. Udo Wegmann for their advice regarding the 16S microbiota composition analysis; to Dr. Arjan Narbad and Dr. Nathalie Juge for the advice on sample collection, sequencing and planning of future studies of the intestinal microbiota. Thanks to Dr. Gwen Le Gall for performing the NMR analysis.

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Chapter 1: General introduction

1. Gastrointestinal (GI) tract

The **gastrointestinal (GI) tract** with help from the salivary glands, liver, and pancreas fulfils ingestion, digestion, absorption and defecation.

The GI tract can be divided into upper and lower[1]. The upper GI tract consists of mouth, pharynx, oesophagus, stomach, and duodenum. The lower GI tract consists of jejunum, ileum, cecum, colon, rectum, and anal canal. This division delineates the embryonic origins of the different compartments as being foregut or midgut derived. The exact demarcation between the upper and lower tracts is the suspensory ligament of the duodenum (also known as the Ligament of Treitz) (Mazziotti et al., 1997, AMR, 1991) (**Figure 1**).

There is no specific anatomical landmark that divides jejunum from ileum. It is accepted that the first 40% of the length are jejunum and the remaining 60% are ileum (Seiden et al., 2013).

Together the duodenum, jejunum, and ileum constitute the **small intestine (SI)** whereas the cecum, colon, rectum, and anal canal[2] constitute the **large intestine (LI)** (**Figure 1**).

[1] Other classifications may refer as lower GI tract only to the colon, rectum, and anal canal.
[2] Often the anal canal is not included

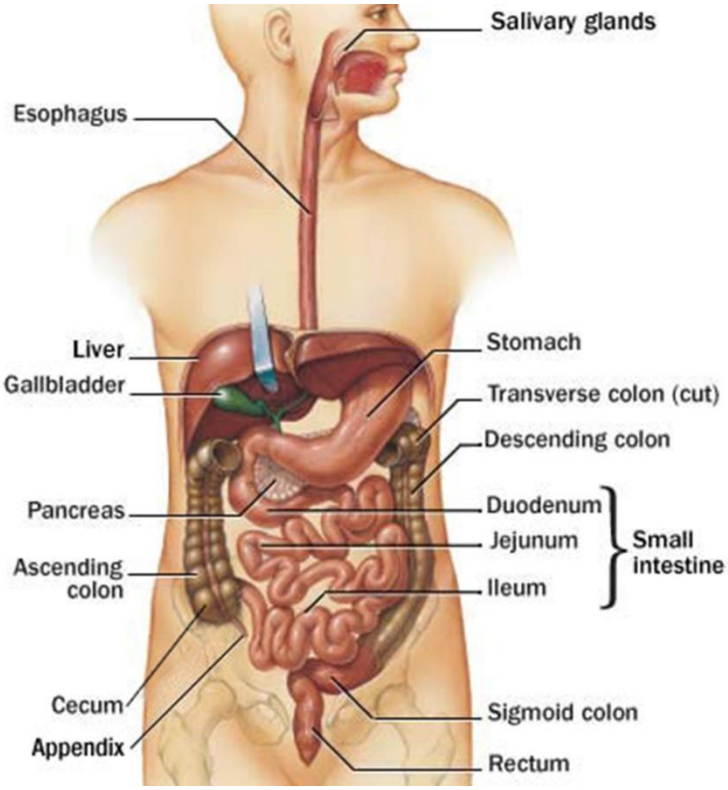


Figure 1 Anatomy of the human GI tract (Research, 2015).

Each segment of the GI tract has specific function and its anatomical features are suited accordingly but in most broad terms the GI tract is a multilayered tube. The series of tissue layers that build the tube are referred to as the GI wall. The cavity within the GI tract is called GI lumen. Four general layers (ordered from the lumen inward) could be distinguished, namely: mucosa, submucosa, muscular layer, and serosa/adventitia (**Figure 2**).

For the purpose of this work mucosa of the SI (and to a lesser extend LI) will be described in detail.

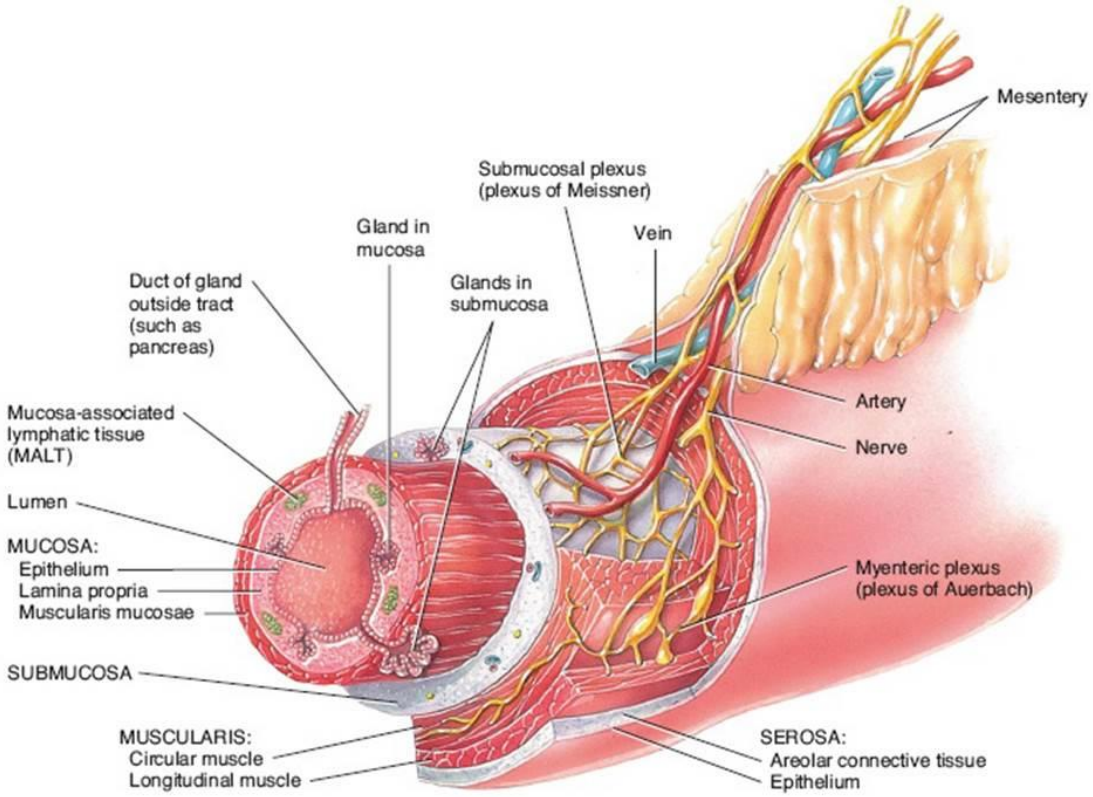


Figure 2 The four layers of the GI tract are the mucosa, submucosa, muscularis, and serosa (John Wiley & Sons, 2011).

2. Mucosal associated lymphoid tissue (MALT)

Organs of the immune system are distributed throughout the body such that defence can be provided regardless of pathogen entry route. The spleen is the main organ involved in generation of response against pathogens that have entered the tissues and spread into the blood stream. Equally important site of adaptive immune response generation is the mucosal immune system that is located near the environmentally exposed and highly vulnerable mucosal surfaces of the body. The mucosal surfaces are thin and permeable barriers due to their physiological activities such as gas exchange (lungs), nutrient absorption (intestine), secretory activities (eyes, nose, mouth), and reproduction (uterus and vagina). The mucosal associated lymphoid tissue (MALT) of the body include: the gut associated lymphoid tissue (GALT), broncho associated lymphoid tissue (BALT), nasal associated lymphoid tissue (NALT), genital associated lymphoid tissue (GENALT).

All mucosal surfaces are protected by an epithelial layer which is often reinforced by mucus and antimicrobial products as well as both innate and adaptive components of host defence (Rubtsov et al., 2008). In case of the gut and the lung this epithelium consists only of a single layer of cells whereas the vaginal and mouth epithelium is stratified. Underneath the epithelium usually there is a layer of connective tissue and a muscle layer. In addition, mucosal surfaces are heavily populated by immune cells (Chaudhry et al., 2011). Immune cells originating from each mucosal site could migrate to anatomically associated lymph nodes. For example, the tracheobronchial lymph nodes are involved in immune response generation towards antigen encountered in the lung mucosa, whereas the mesenteric lymph nodes are part of the GALT (Chaudhry et al., 2011).

3. Intestinal mucosa

Intestinal mucosa consists of **epithelial layer** and underlying **lamina propria (LP)** (**Figure 3**). Intestinal LP is a loose connective tissue, rich in vascular networks and lymphatic vessels. A myriad of cells reside within the LP, such as myofibroblasts, fibroblasts, mural cells (pericytes), bone marrow–derived stromal stem cells (Powell et al., 2011), neural endings (Furuya et al., 2005), and numerous lymphoid and myeloid immune cells.

Essential for the coexistence of commensal microbiota and mucosal immune cells is the ability to maintain the separation between host and microorganisms. The intestinal epithelium forms a physical and biochemical barrier to both commensal and pathogenic microorganisms present in the lumen, thus acting as a separator between the external environment and the immune system. Moreover, intestinal epithelial cells (IECs) can sense and respond to microbial stimuli and are involved in the immune response in both cases of tolerance and anti-pathogen immunity. The surface area of the GI mucosa is vast and the lumen is inhabited by billions of microorganisms which means that the GI mucosa is a crucial site of innate and adaptive immune regulation (**Figure 3**).

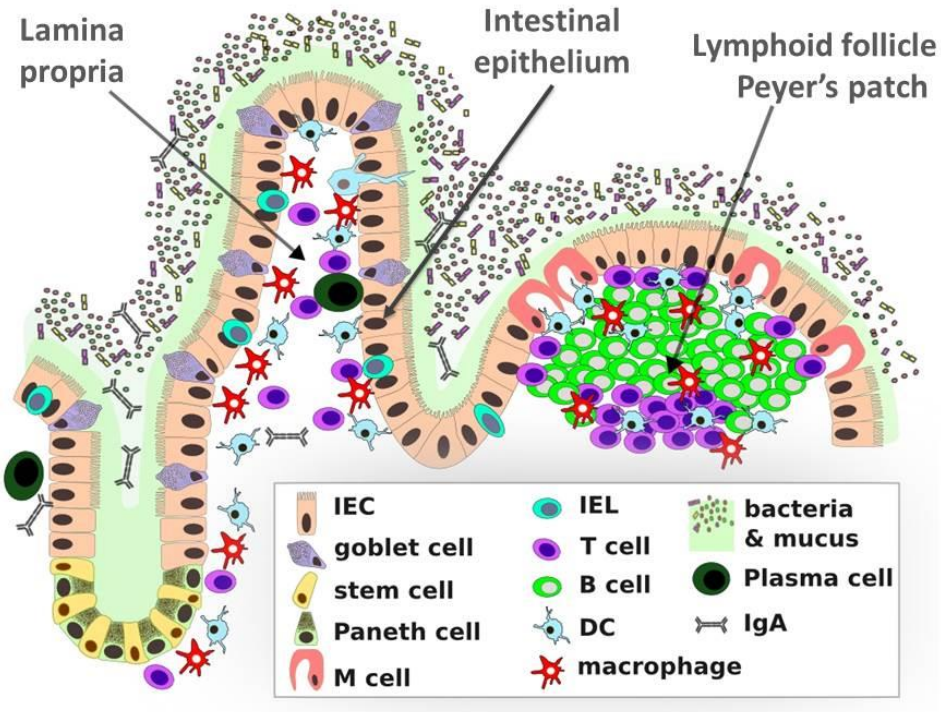


Figure 3 Schematic illustration of the intestinal mucosa. The microbes, intestinal epithelium and the underlying immune system interact closely to establish and maintain intestinal immune homeostasis. The presence of pathogen-associated molecular pattern receptors on IECs and antigen transport across follicle-associated epithelium (FAE) M cells allow the gut immune system to examine the luminal contents and to generate appropriate response in case of pathogen invasion. After the initial interaction within the Peyer's patches, the inductive sites of mucosal immunity, antigen-specific B and T cells recirculate to the effector site, i.e. the LP. A mucus layer produced by the goblet cells protects the epithelium; the mucus contains antimicrobial peptides and a large amount of secretory IgA the main role of which is to prevent microbes from crossing the epithelial barrier. IEC= intestinal epithelial cell; IEL= intra-epithelial lymphocyte; DC= dendritic cell; M cell= microfold cell; IgA= immunoglobulin A.

In mammalian gut the intestinal immunological microenvironment is shaped by this **continuous cross talk** involving the intestinal epithelium, the immune system and the local microbiota (**Figure 4**). In order to gain in depth

understanding of this entity, its components should be simultaneously investigated.

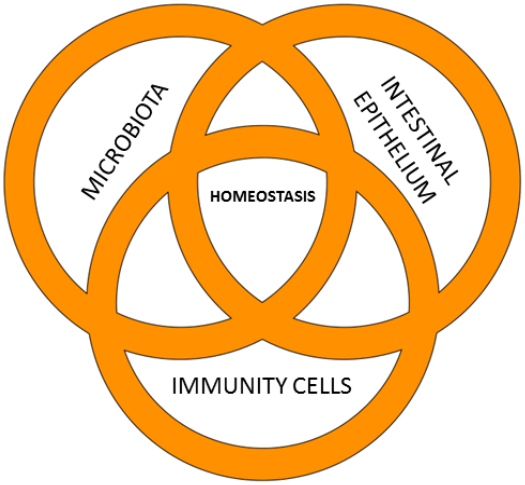


Figure 4 Three-way cross talk in the gut. Homeostasis in the mammalian gut is established and kept by an interaction between three components: the host immune system, the intestinal epithelium which separates the inner milieu from the environment, and the myriad of microorganisms inhabiting the gut.

3.1. Gut-associated lymphoid tissue (GALT)

The GALT consists of i) secondary lymphoid organs: Peyer's patches (PPs) and mesenteric lymph nodes (MLNs); ii) tertiary organized lymphoid tissues: cryptopatches and isolated lymphoid follicles; iii) dispersed cells: intraepithelial lymphocytes (IELs), LP immune cells.

3.1.1. Mesenteric lymph nodes (MLNs)

Lymph nodes are important immunological structures performing a series of vital functions: recruiting large numbers of naïve lymphocytes from the blood; collecting antigen and DCs from peripheral tissues; providing environment for antigen-specific tolerance; modulating the homing characteristics of effector or memory T cells.

MLNs are lymph nodes located within the layer of mesentery and are designated to receive antigen originating from the gut.

Two main regions can be distinguished histologically in the MLNs — the cortex and the medulla. The cortex itself is composed of paracortex (the T-cell area), and a more superficial B-cell area. The B-cell follicles are the main site of humoral responses, whereas the paracortex is the site where **DC-T cell interactions** occur. The medulla is a labyrinth of lymph-draining sinuses that are separated by medullary cords containing many plasma cells, some macrophages and memory T cells (von Andrian and Mempel, 2003).

In the MLNs, conventional DCs (cDCs) can be generally classified into lymphoid tissue resident cDCs, which are believed to originate from circulating precursors that enter directly from the blood, and migratory cDCs that arrive in organized lymphoid tissues via afferent lymph after acquiring antigen (Ag) in the intestine (Persson et al., 2013).

3.1.2. Peyer's patches (PPs)

Peyer's patches (PPs) are macroscopic aggregates of lymphoid follicles (**Figure 3**). They are present throughout the small intestines of humans and other mammals. Importantly, PPs are considered a gateway through which luminal dietary/microbial antigens are sampled by the mucosal immune system. Both complex and highly dynamic is the cellular composition of the PP, with at least four known major cell types identified as migratory in case of antigenic stimulation (Ahlawat et al., 2014) (**Figure 3**).

PPs consist of a number of large B-cell follicles intervened by T-cell areas. In the core of the follicle is the **germinal center (GC)** which is surrounded by **follicular mantle (FM)**. B-cell activation requires engagement of CD40 on conventional IgM^+ B cells by CD40 ligand (CD40L) on $CD4^+$ Th cells. Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion in the **dark zone** of the GC. During proliferation, the process of **somatic hypermutation (SHM)** introduces base-pair changes into the V(D)J region of the rearranged genes encoding the immunoglobulin variable region of the heavy chain and light chain. Centroblasts then differentiate into centrocytes and move to the **light zone**. With help from Th and DCs there the modified antigen receptor is selected for improved binding to antigen. A subset of centrocytes undergoes

immunoglobulin **class-switch recombination (CSR)**. In general, CSR diversifies the antibody effector functions by substituting the heavy-chain constant region of IgM and IgD with that of IgG, IgA, or IgE (with IgA being favored in the PPs). Cycling of centroblasts and centrocytes between dark and light zones seems to be mediated by a chemokine gradient, presumably established by the stromal cells. Centrocytes that produce an unfavorable antibody are eliminated by apoptosis. Antigen-selected centrocytes eventually differentiate into memory B cells or plasma cells (Klein and Dalla-Favera, 2008). FM comprises B-cells that have not passed through a GC (Isaacson and Du, 2004). The area between the follicle and the epithelium is the **subepithelial dome (SED)**. It is a diffuse area harboring numerous dendritic cells (DCs). In the SED there are also B cells which resemble the marginal zone (MZ) B cells of the spleen. These cells spontaneously release antibodies with low affinity and broad specificity (Cerutti et al., 2013).

A single layer of columnar epithelial cells, known as the **follicle-associated epithelium (FAE)** separates the lymphoid areas from the intestinal lumen. The FAE differs from the epithelium that covers the villus mucosa by means of less digestive enzymes and a less pronounced brush border. The most notable feature of the FAE is the presence of specialized enterocytes that have no microvilli. Due to their characteristic microscopic appearance those cells are called **microfold (M)**. M cells bind invasive pathogens, such as *Salmonella*, *Shigella*, *Yersinia* and reoviruses, and other particulate antigens which get transported across the epithelium and are then available for sampling by the immune system. Up until recently, M cells were considered to provide the main, if not the only, way in which complex antigens can gain access to the intestinal immune system. Other ways of antigen transport from the lumen are described in **Chapter 1 section 3.2.2.2 and Chapter 5.** The M cells do not process antigens themselves and they do not express MHC class II molecules. Instead, they provide a route of entry for intact antigen to be uptaken and processed by professional antigen presenting cells (APCs) in the dome region (Debard et al., 2001, Kerneis et al., 1997, Golovkina et al., 1999).

3.1.3. Cryptopatches (CP) and isolated lymphoid follicles (ILF)

In comparison to the PPs and the MLNs these lymphoid structures have been studied much less extensively. CP and ILF are relatively small lymphoid structures dispersed throughout the SI. They vary in size and cellular composition within the individual as well as significant variations is observed between species, ages and mouse strains (Pabst et al., 2005).

CP are numerous small and randomly distributed clusters of lymphoid cells in the basal LP of the murine intestine and are predominantly composed of lineage negative ($CD3^-CD4^-CD8^-B220^-$) cells expressing the stem cell factor c-kit (CD117) (Kanamori et al., 1996, Saito et al., 1998).

ILF have been described in human (Moghaddami et al., 1998), mouse (Hamada et al., 2002) and some other species (Rosner and Keren, 1984). There are approximately 100–150 ILF in the mouse gut. The cellular composition of ILF resembles that of PPs: predominantly filled with B cells. In addition ILF may possess germinal centers and a FAE containing M cells. Thus, ILF along with PPs are assumed inductive sites for intestinal immune reactions. They share common developmental traits as assembly of both structures requires IL-7Ra and LTa signaling (Pabst et al., 2005).

According to some authors CP and ILF serve non-overlapping functions and differ in localization, size and cellular composition. Therefore, they are usually regarded as two genuine and separate types of intestinal lymphoid aggregations (Hamada et al., 2002). On the other hand, there are publications suggesting that a clear-cut distinction between ILF and CP is not plausible (Pabst et al., 2005).

3.1.4. Lamina propria (LP) immune cells

In order to understand the immunological processes in the intestinal mucosa it is important not to underestimate its complexity and the numerous interactions occurring between the varieties of cell types present. This section does not attempt to provide a comprehensive description but is rather a brief account acknowledging this complexity. There are recent reviews for the individual cell types, discussing their function both in regards to the systemic and mucosal immunity.

LP Dendritic cells (DCs) and macrophages (MΦs)

Inducing tolerance to harmless antigens, initiating protective immunity against intestinal pathogens, and contributing to intestinal diseases including celiac disease and the inflammatory bowel diseases (IBD) are all suggested to be mediated by DCs (Persson et al., 2013).

The characterization of DCs in the intestinal LP has been the subject of intense investigation and controversy, mainly due to most of their surface markers (including CD11c, CD11b and MHC class II (MHCII)) being shared with the macrophages (MΦs) (Persson et al., 2013).

DCs can be distinguished (but not exclusively) from the MΦs by their lack of expression of the high-affinity IgG receptor FcγR1 (CD64) (Tamoutounour et al., 2012). In addition, MΦs do not express the integrin αE chain CD103. Substantial number of the $CD64^{+}$ appear to be $CX3CR1^{high}$ and there are also some $CX3CR1^{int}$ (Tamoutounour et al., 2012). By and large, $CD11c^{+}MHCII^{+}CD64^{-}$ cells are $F4/80^{-}$ and the majority of these cells express CD103. These $CD103^{+}$ DCs are considered the prototypic migratory cDCs in the LP and can be divided into two main populations differing in their localization, transcription factor requirements, and function: $CD103^{+}CD11b^{+}$ and $CD103^{+}CD11b^{-}$ cDCs (Persson et al., 2013). Further discussion of the intestinal DCs and MΦs is provided in a **Chapter 5**.

Neutrophils

Polymorphonuclear leukocytes (PMN), also called neutrophils are a vital element of the innate immune system and comprise a critical component of the army of first responses to sites of inflammation (Fournier and Parkos, 2012).

Neutrophils can secrete cytokines such as TNF-α, IL-1β, IL-1ra (Nikolaus et al., 1998), IL-17 (Ferretti et al., 2003, Zindl et al., 2013), IL-22 (Zindl et al., 2013).

Unfortunately, clear cut understanding of PMN function in inflammatory diseases such as IBD (which is characterized by intestinal infiltration of neutrophils) has not yet been achieved (Fournier and Parkos, 2012). A detailed description of other neutrophil features is provided elsewhere (Mantovani et al., 2011).

Basophils

Basophils were discovered by Paul Ehrlich in 1879 (Ehrlich, 1879) but still there are many unknowns in regards to their function. Basophils constitute less than 0.3% of leukocytes in the peripheral blood. They are functionally closely related to mast cells as both cell types express the high-affinity receptor for IgE and produce similar effector molecules, including histamine, lipid mediators (eg, leukotrienes and prostaglandins), serine proteases, and interleukins (eg, IL-4, IL-13, and IL-6) (Falcone et al., 2006).

Basophils are effector cells of the innate immune system that are associated with allergic inflammation and infections with helminth parasites (Ohnmacht and Voehringer, 2009). Upon infection with the helminth *Nippostrongylus brasiliensis* increased *de novo* production in the bone marrow leads to basophilia. In such cases basophils are found near the marginal zone in the red pulp of the spleen, in the LP of the small intestine, and in the lung parenchyma.

Basophils are also found in the lungs of patients with allergic asthma (Devouassoux et al., 1999). Increased number of basophils is found in the skin of contact dermatitis or atopic dermatitis patients during late-phase response (Dvorak and Mihm, 1972).

Eosinophils

The GI tract is a primary site for normal eosinophil residence. Significant progress has been made in elucidating that eosinophils are integral members of the GI mucosal immune system. In physiological states, small numbers of eosinophils are found throughout the GI tract except the esophagus (Zuo and Rothenberg, 2007).

Mast cells

Mast cells comprise 2–5% of mononuclear cells in the LP of the normal GI tract. Previous studies have documented an increased number of mast cells in gastrointestinal mucosa of patients with gastrointestinal diseases such as irritable bowel syndrome (Ramsay et al., 2010).

Mast cells are preferentially located next to nerve terminals in the LP, where they are activated by secreted neuropeptides such as substance P. When

stimulated by substance P, these mast cells release inflammatory mediators, such as serotonin and proteases, as well as proinflammatory cytokines. In addition release of histamine and prostaglandin D2 by intestinal mast cells is important for chloride and water secretion as well as control of intestinal motility (Ramsay et al., 2010).

Innate lymphoid cells (ILCs)

Now we had a look at the usual suspects, it is time to introduce some new stars of immunology – innate lymphoid cells (ILCs). According to the current classification (Spits et al., 2013) the **natural killer (NK)** cells and the **lymphoid tissue-inducer (LTi)** cells also belong to the ILCs. Indeed, both NK and LTi cells have been known for 40 years (Kiessling et al., 1975) but for all that time nobody suspected that there will be even more such cells out there.

Three main features define the ILCs: i) the absence of recombination activating gene (RAG)-dependent rearranged antigen receptors; ii) a lack of myeloid cell and iii) dendritic cell phenotypical markers; and their lymphoid morphology (Spits and Cupedo, 2012).

Similarly to NK cells and lymphoid tissue-inducer (LTi) cells ILCs depend on γc and ID2 for their development (Boos et al., 2007). In addition, ILCs also require signaling through interleukin-7 receptor subunit-α (IL-7Rα) for development and maintenance (Spits et al., 2013). Different ILC populations have distinct patterns of cytokine production mirroring the cytokine-secreting profiles of Th cell subsets (Spits and Di Santo, 2011).

The proposed classification of ILCs identifies three groups; all based on functional criteria. **Group 1 ILCs** are defined by their capability to produce interferon-γ (IFNγ) (NK cell belong to this group). **Group 2 ILCs** are able to produce T helper 2 (Th2) cell-associated cytokines, such as IL-5 and IL-13. **Group 3 ILCs** are capable of producing IL-17 and IL-22 (Spits et al., 2013). The prototypical Group 3 ILCs are LTi cells, which are crucial for the formation of secondary lymphoid organs during embryogenesis (Spits et al., 2013). These cells have also been reviewed recently (Spits et al., 2013).

ILCs have been appreciated as key orchestrators of immune defenses at mucosal surfaces. For example, ILC3s have been shown to mediate intestinal epithelial cell (IEC) glycosylation by stimulating Fut2 expression in the epithelium. Epithelial fucose is used as a dietary carbohydrate by many of the commensal bacteria thus mediating host-microbiota interaction. Fucosylation of IECs in response to pathogens such as *Salmonella enterica* Typhimurium is also mediated by ILC3 (Goto et al., 2014).

Example of the ILC2s (previously referred to as nuocytes) intestinal mucosal function is their expansion *in vivo* in response to the type-2-inducing cytokines IL-25 and IL-33. ILC2s represent the predominant early source of IL-13 during helminth infection with *Nippostrongylus brasiliensis* (Neill, 2010).

T-cells

The amount of information on systemic and mucosal T-cells is startling. Therefore this section is by no means comprehensive. Nevertheless, a short outline of the intestinal mucosa T-cells will be provided, just to underline the complexity and variety of the intestinal mucosal immune system. The intraepithelial lymphocytes (which are also T-cells) are discussed in this section despite being located in the intraepithelial region instead of the LP.

Homing of T-cells in the gut is believed to be mediated by CCL25 (Wurbel et al., 2007).

$CD8^+$ T-cells

LP resident $CX3CR1^+$ cells induce differentiation of precursor cells into $CD8^+$ T-cells expressing IL-10, IL-13, and IL-9 via antigen presentation. These $CD8^+$ T-cells are capable of migrating into adjacent compartments (such as the intraepithelial) and control the activation of $CD4^+$ T-cells in the small intestine (Chang et al., 2013).

$CD4^+$ T-cells

Among the intestinal lymphocytes, the $CD4^+$ T cells are key mediators of host protective and homeostatic responses. The greatest proportion of $CD4^+$ T cells in both small intestine and colon LP express $CD44^{hi}CD62L^-$ effector memory phenotype of antigen-experienced cells (Targan et al., 1995, De Maria et al.,

1993). Only a minority of LP $CD4^+$ T cells harbor surface markers associated with naïve T cells (Cose et al., 2006). What are the functions of those cells and whether they can undergo priming in the LP is not yet studied (Shale et al., 2013).

Presumably, priming of $CD4^+$ T cells that populate the LP occurs predominantly in the MLN by DCs originating from the intestinal mucosa (Shale et al., 2013, Coombes and Powrie, 2008). During the interaction between DCs and naïve $CD4^+$ T cells cytokine receptor ligation can modulate the activation of the T cells into particular types (also referred to as lineages or subsets). These $CD4^+$ T cell subsets can be identified by expression of certain transcription factors and effector molecules. There is evidence accumulating that these $CD4^+$ T cell subsets are not terminal and their differentiation exhibits a certain degree of plasticity (Brucklacher-Walderet et al., 2014; Hooper and Macpherson, 2010). The most widely recognized subsets are Th1, Th2, Th17, and Treg (Brucklacher-Waldert et al., 2014) (**Figure 5**).

DCs' production of IL-12 is essential for Th1 differentiation. IL-12 effects are mediated via STAT4 and along with T-bet and H2.0-like homeobox 1 are deemed essential for Th1 IFNγ production (Heufler et al., 1996, Lugo-Villarino et al., 2003). IL-18 is a cytokine that has been described to amplify the effects of IL-12 (Vossenkamper et al., 2004). Th1 responses are essential for the elimination of intracellular pathogens (Jong et al., 1998). Liu et al. (2014) have shown that TGFβ, IL-6, and IL-2 can drive Th1 to Th17 conversion in the intestine (Liu et al., 2015, Lee et al., 2009).

Development of Th2 requires IL-4 (Swain et al., 1990); in addition IL-13, which has partially overlapping functions with IL-4, can independently drive Th2 polarization (Punnonen et al., 1993), whereas IL-12 is a potent Th2 inhibitor (Gavett et al., 1995). The actions of IL-4 and IL-13 are mediated by the transcription factor STAT6 (Kaplan et al., 1996). Another transcription factor required for the Th2 effector properties, such as IL-3 and IL-13 secretion, is GATA-3 (Kitamura et al., 2005). It exerts STAT6 independent auto-activation creating a feedback mechanism that stabilizes the Th2 commitment (Ouyang et al., 2000). Th2 responses are essential for protective immunity against intestinal

helminths (Turner et al., 2003) whereas their inappropriate exacerbation has been associated with allergic hypersensitivity (Deu et al., 2010; Licona-Limon et al., 2014). Initially linked to Th2 phenotype, IL-9 production is now attributed to a separate class of $CD4^+$ T helper cells - Th9, which are also expressing the transcription factor PU.1 (Gessner et al., 1993, Veldhoen et al., 2008, Gerlach et al., 2014). TGF-β is essential for Th2 to Th9 reprograming (Veldhoen et al., 2008).

Cytokines implemented in the differentiation of Th17 include IL-23, IL-6 (Zhou et al., 2007) and to some extent TGF-β (Ghoreschi et al., 2010; McGeachy et al., 2007). IL-6 induces expression of IL-21 that amplifies an autocrine loop leading to enhanced expression of IL-21 and IL-23 receptors, where IL-21 and IL-23 stimulate IL-17 production. STAT3 is required for mediating IL-6 and IL-21 signaling and along with RORγt promote IL-17 upregulation (Zhou et al., 2007). It has been suggested that adenosine 5'-triphosphate derived from the gut microbiota activates $CD70^{high}$ $CD11c^{low}$ DCs to produce IL-6, IL-23p19 and TGF-β-activating integrin-αV and -β8 thus driving Th17 differentiation (Atarashi et al., 2008). Another microbiota-induced, intestine-specific factor involved in Th17 response is IL-1β (Show et al., 2012). IL-10 and IL-2 have been shown as negative Th17 regulators (Huber et al., 2011; Yang et al., 2011). MLNs rather than intestinal tissue are essential for generation of gut homing Th17 cells (Kawabe et al., 2013). Th17 cells are important for the defense against extracellular bacteria such *Citrobacter rodentium* and *Klebsiella pneumoniae*, as well as other pathogens of fungal (*Candida albicans*) and viral (influenza H1N1) origin (Conti et al., 2009; Huang et al., 2004; Ye et al., 2001; Crowe et al. 2009). A number of pathologies have been associated with aberrant Th17 response, one example being IBD (Fujino et al., 2003; Seiderer et al., 2008). Th17 are enriched at mucosal sites and produce IL-17A, IL-17F, and IL-22 (Littman and Rudensky, 2010). However, not all Th17 secrete IL-22; what is more, there are IL-17 negative $CD4^+$ helper T cells that secrete IL-22 which are referred to as Th22 (Duhen et al., 2009, Basu et al., 2012). *Citrobacter rodentium* infection induces IL-22 in ILCs and $CD4^+$ T cells. IL-22 production by ILCs is heavily

dependent on IL-23 whereas Th22 development occurs via IL-6 dependent mechanism. $CD4^+$ T cells, differentiated to Th22 (but not Th17) in the presence of IL-6 and absence of TGF-β, when transferred to infected IL-22-deficient mice are able to establish protection against *Citrobacter rodentium* (Basu et al., 2012).

Compared to the systemic immune system, the intestinal mucosa is enriched for Treg cells (Uhlig et al., 2006a). Characteristic for those is the expression of the transcription factor Foxp3 and secretion of IL-10. A number of small intestinal LP IL-10 producing T cells are Foxp3 negative and are classified as Tr1 (Maynard et al., 2007). LP DCs promote Treg differentiation by TGF-β and retinoic acid secretion (Sun et al., 2007). Treg cells, after their generation in the MLN, home to the gut where they undergo local expansion which is important for oral tolerance (Hadis et al., 2011a). Mice with Treg specific ablation of IL-10 show that IL-10 production by Treg cells is not required for the control of systemic autoimmunity but is critical for maintaining homeostasis at mucosal sites such as colon and lungs (Rubtsov et al., 2008). In addition, mice with Treg-specific deletion of IL10Ra and STAT3 also exhibit severe colitis (Chaudhry et al., 2011). The Treg-derived cytokine IL-35 has been recently identified as a contributor to their suppressor activity (Sawant et al., 2014).

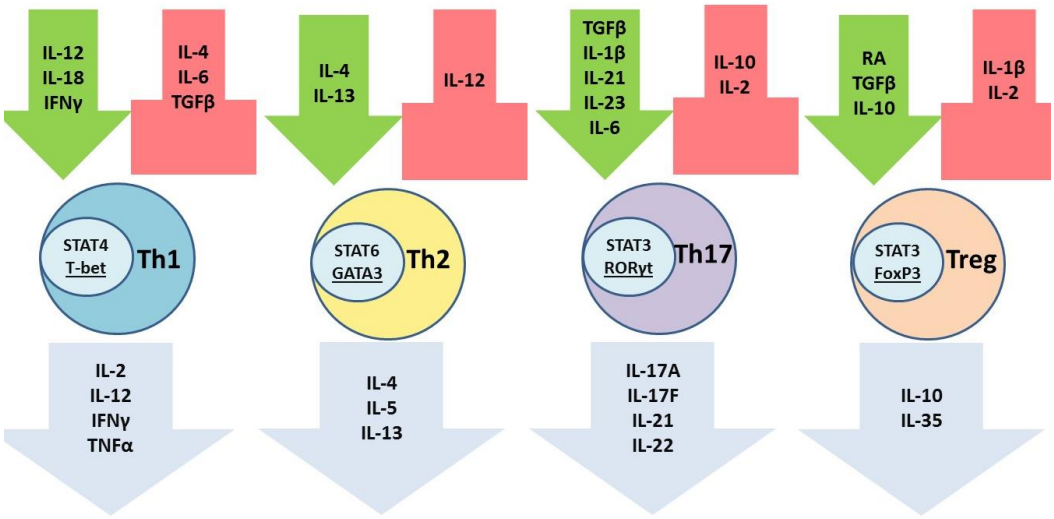


Figure 5 There are four widely recognized $CD4^+$ T cell subsets. They are characterized by expression of certain transcription factors and secretion of effector molecules. The cytokines positively regulating each subset differentiation are in green and the negative regulators are in red.

Intraepithelial lymphocytes (IELs)

IELs comprise antigen-experienced T cells belonging to both the T cell receptor-γδ $(TCR\gamma\delta)^+$ and $TCR\alpha\beta^+$ lineages. Particular IEL subsets are found in the epithelium of the small and large intestine (Cheroutre et al., 2011). The small intestinal IELs could be up to 60% $TCR\gamma\delta^+$ antigen-experienced cells, expressing activation markers, such as CD44 and CD69 (Cheroutre, 2004). These cells are tissue associated and are not recirculating. On average for every five IECs there is one intestinal IEL (Hershberg and Mayer, 2000). Effector cytokines expressed by IELs include IFNγ, IL-2, IL-4 or IL-17 (Offit and Dudzik, 1989, Guy-Grand, 1991, Guy-Grand et al., 1991, Roberts et al., 1993, Chardes et al., 1994, Muller et al., 2000, Shires et al., 2001, Tang, 2009).

IELs interact with IECs via the constitutively expressed CD103 (also known as the αE integrin). CD103 binds E-cadherin on IECs (Kilshaw and Murant, 1990, Cepek, 1994).

Activated small intestinal IELs tend to express CD8αα homodimers (Leishman, 2002, Denning, 2007). CD8 exists on the T-cell surface in two isoforms, CD8αα

and CD8αβ. CD8αβ is a much stronger co-receptor for MHC-I than CD8αα, the expression of CD8β enhances TCR sensitivity about 100-fold compared to CD8αα (Yachi et al., 2005). CD8αα might be a negative regulator of MHC-I T-cell activation (Cheroutre and Lambolez, 2008). The thymus leukaemia antigen (TLA) is a non-classical MHC class I molecule that is widely expressed by mouse small intestinal ECs and is ligand for the CD8αα. Some TCRαβ IELs along with CD8αα also express CD4 or CD8αβ, but very few of the IELs are $CD4^+$ compared to the LP lymphocytes (Cheroutre et al., 2011).

IELs can be divided in two subsets depending on how they have acquired their antigen-experienced phenotype. The first subset are the thymus activated (also called 'natural'/type b) IELs that have maturated during development in the thymus in the presence of self-antigens. They are either $CD8\alpha\alpha^+$ or $CD8\alpha\alpha^-$ T cells that express TCRγδ or TCRαβ but do not express CD4 neither CD8αβ (Cheroutre et al., 2011). Natural IELs are the first type of antigen-experienced T cells to populate the gut, even before birth.

The second subset (also known as 'induced'/type a) derive from conventional $CD4^+$ or $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ T cells, that have been activated post-thymically in response to peripheral antigens (Cheroutre et al., 2011). They typically express a 'memory-like phenotype' ($CD2^+$ $CD5^+$ $CD44^+$ $LFA1^+$ $THY1^+$), and are CD8αα positive. The conventional T cells in the periphery have polyclonal origin, whereas induced IELs are found oligoclonal based on their TCR diversity. At birth there are barely any induced IELs, but their population grows with age as the organism encounters more and more exogenous antigens. The small intestine contains at least ten times more IELs than the colon (Cheroutre et al., 2011).

B-cells

The traditional view about the intestinal immune system is that it includes an immunoglobulin (Ig)A-inductive site represented by the PPs and an IgA effector site represented by the LP (**Figure 3**). As described above, B cells undergo IgM to IgA class switch in the PPs but recently studies have shown the presence of active IgA class switching also in the LP (Cerutti, 2008).

3.2. Intestinal epithelial cells (IECs)

Intestinal epithelium is formed by a single layer of cells, held tightly together by specialized proteins. Four cell lineages: absorptive enterocytes, which make up most IECs, mucus-producing goblet cells, hormone-producing enteroendocrine cells, and Paneth cells, which produce antimicrobial peptides or lectins, could be found in the intestinal epithelial layer.

Intestinal epithelium is simultaneously a link and separator between the luminal content and the immune system. It is no longer just a nutrient transporter, now we know that it is also capable of fine-tuning the immune response and breach of this epithelial function might be cause of disease. Before looking into the immunological "vocabulary" of the IECs we will first have a glance at the intricate lifecycle of these cells and their constant renewal occurring with remarkable speed and precision.

3.2.1. Life and death of the IECs

Mammalian intestinal epithelium is renewed every 3 to 5 days (Creamer et al., 1961) with up to 10^{11} IECs (~200 g) being lost every day in humans (Barker, 2014). This process of renewal must be tightly regulated in order to ensure continuous integrity of the epithelial layer where the epithelial cells are tightly attached to each other without any gaps that might compromise the barrier.

In the small intestine each villus is "fed" with cells by at least six crypts of Lieberkühn, which house **dedicated populations of LGR5$^+$ (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) crypt base columnar (CBC) stem cells** that divide and produce all the cell types present in the epithelium. Stem cell activity is strictly regulated during homeostasis by a complex array of signals delivered by neighbouring cells, such as Paneth cells, which are found in close association with the stem cells at the crypt base and are an important source of various niche factors, including epidermal growth factor (EGF), WNT3A, and Notch ligand. In addition Paneth cells enable adequate response to change of nutrient availability. In case of calorie restriction Paneth cells reduce mTOR complex 1 (mTORC1) signalling, thus resulting in a rapid reduction of the LGR5$^+$ stem cell pool (Barker, 2014).

The colonic stem cell niche is less well defined. Since Paneth cells are generally absent from the colon it is believed that $CD24^+$ and KIT^+ goblet cells that are located in close proximity to $LGR5^+$ stem cells at the crypt base are instead involved in the steam cell niche (Rothenberg et al., 2012).

The small populations of adult stem cells regularly divide to produce highly proliferative progenitors known as transit-amplifying cells. After 2 – 3 divisions transit-amplifying cells gradually commit to terminal differentiation and migrate upwards towards the beginning of the villus (Rothenberg et al., 2012). Despite the outlined above findings there is still no consensus on the true identity (transit-amplifying versus $LGR5^+$) of adult intestinal epithelial stem cells. It is likely that the niche harbours a lot of plasticity and the dedicated active stem cells ($LGR5^+$) are responsible for daily epithelial homeostasis but in case of injury the more quiescent 'reserve' stem cells (the transit-amplifying cells) can be activated in order to facilitate repair.

With the recent advance of molecular biology several signalling pathways involving WNT, bone morphogenic protein (BMP) (He et al., 2004), and Notch (VanDussen et al., 2012) have been identified as involved in maintaining the stem cell niche and driving intestinal epithelial differentiation.

WNT signals are important to maintain a proliferative, undifferentiated environment for the LGR5 stem cells, as well as driving Paneth cell maturation.

The seven transmembrane segment receptor LGR5, which is used as a stem cell marker, is itself linked to stem cell homeostasis. Conditional epithelial deletion of LGR5 and its close homologue LGR4 results in the suppression of WNT signalling and stem cell death (de Lau et al., 2011). LGR5 at the plasma membrane recruits **roof plate-specific spondin (R-spondin)-**a WNT agonist, thus amplifying WNT signalling and ensuring stem cell homeostasis *in vivo* (Glinka et al., 2011) (Carmon et al., 2011).

Intestinal cell differentiation is additionally controlled by **Notch** which maintains the undifferentiated proliferative state of crypt progenitors and also controls the differentiation of the transit-amplifying cells into absorptive enterocytes or secretory cells (Fre et al., 2005). Notch inhibition induces the expansion of secretory cell populations, mainly goblet cells, at the expense of enterocytes.

Understanding of these interactions enabled in 2014 a homogenous niche-independent culture of $LGR5^+$ stem cells to be maintained (Yin et al., 2014).

Notch and WNT signalling just build a general scaffold of intestinal stem cell renewal and differentiation but it is likely that other pathways are also involved and allow for fine-tuning and adjustment to the environmental changes such as calorie restriction (as already described) or pathogenic challenge. It has been found that $Lgr5^+$ stem cells constitutively express the intracellular receptor Nod2. Nod2 stimulation by muramyl-dipeptide (MDP), a peptidoglycan motif common to all bacteria, prompts stem cell survival and protection against oxidative stress-mediated cell death (Nigro et al., 2014).

Whereas WNT signalling is characteristic of the crypt region, **BMP signalling** is active in the villi and antagonizes WNT-induced crypt formation and stem cell renewal, limiting crypt expansion. **Noggin** and **Chordin1** are BMP antagonists expressed in the crypt base and prevent BMP-induced WNT inhibition (He et al., 2004) (Kosinski et al., 2007).

Epithelial BMP signalling plays an important role in the terminal differentiation of the intestinal secretory cell lineage (Auclair et al., 2007). BMP receptors (BMPR) Ia, BMPR Ib, and BMPR II are all expressed in colonic epithelial cell lines (HT29, Caco-2, DLD-1, SW480, HCT116, and LS174-T). *In vitro* BMP2 promotes apoptosis, differentiation and supresses cell growth. BMP2, BMPRIa, BMPRIb, BMPRII, phosphorylated Smad1, and Smad4 are expressed predominantly in mature colonocytes at the epithelial surface in normal adult human and mouse colon (Hardwick et al., 2004).

Studies on IECs differentiation have identified 1113 genes differentially expressed between the crypt and villus (Mariadason et al., 2005). For example, villus cells have increased cell surface membrane protein glycosylation (Weiser, 1973). MHCI is found in villus and crypt IECs independent of the inflammatory state of the mucosa. In contrast, MHCII in the non-inflamed mucosa of healthy controls and CD patients in remission is restricted to the villi whereas during CD ileitis, both villus and crypt IECs show staining for MHCII (Hundorfean et al., 2007). Yet another example is IL-32 being largely expressed in the differentiated colon epithelial cells but not in the colonic crypts (Netea et al., 2005).

Once the IECs reach the villus tip they undergo **shedding**. Mathematical modeling suggests that in the mouse, 1400 mature enterocytes are likely to shed from a single villus tip daily (Williams et al., 2014). Regardless of the high rate of physiological IEC loss from individual villi, shedding events are observed relatively rarely in fixed specimens due to the very short time it takes for each cell to be extruded. Examination of hematoxylin and eosin (HE)–stained sections of human small intestine reveals shedding cell in less than 6% of villus sections (Bullen et al., 2006).

Pathological shedding has been observed in humans with inflammatory bowel disease (IBD) (Kiesslich et al., 2012), in mice following systemic tumor necrosis factor (TNF) administration (Watson and Hughes, 2012), and during acute murine endotoxic shock (Williams et al., 2013).

3.2.2. Immunological properties of the IECs

This section outlines some of the immunological properties of the IECs and aims to show that IECs are as important for the maintenance of immune homeostasis as are any of the hematopoietic derived immune cells.

Our research group has focused its attention on the cross talk taking place between gut epithelium and cells of the underlying immune system. Indeed, in the past few years it has emerged that lympho-epithelial cross talk plays a key role in the control of inflammation in the gut by shaping the appropriate immunological microenvironment (Swamy et al., 2010). It is well documented that IECs signal to the immune system via the secretion of regulatory cytokines. In the presence of non-invasive commensal bacteria, intestinal epithelial cells secrete molecules such as thymic stromal lymphopoietin (TSLP) (Taylor et al., 2009) and TGF-β (Barnard et al., 1989); in contrast, pathogens cause epithelial cells to release pro-inflammatory factors, such as IL-8 (CXCL-8), MCP-1 (CCL2) and MIP-3α (CCL20) (Jung et al., 1995) (Izadpanah et al., 2001).

3.2.2.1. IECs interaction with the commensal microbiota and pathogens

A layer of **mucus** protects the epithelium. In the LI the mucus layer is thick and at its closest to the epithelium is considered to be microbe free. In the SI the mucus is thinner and more loose which is suggested to provide more

opportunities for microbe-epithelium interaction. Nevertheless, antimicrobial peptides are produced by the Paneth cells of the SI which aim at reducing the microbial load reaching the IECs (Atuma et al., 2001, Bevins and Salzman, 2011). Despite these lines of protection microbes or microbial products do reach the epithelium. IECs possess a range of receptors evolved to recognise those microbes and their products. IECs are structurally and functionally polarized, with an apical surface facing the intestinal lumen and a basolateral surface facing the underlying basement membrane and LP. Some of the membrane bound **microbe associated molecular patters (MAMPs) recognition receptors** are expressed apically, others basolaterally, and some could be both. Additional clarification that should be made is that these receptors are often referred to as pathogen-associated molecular patterns recognising but in case of the gut - commensal microorganisms are also recognised therefore here the term MAMPs will be used (Abreu, 2010). Finally, MAMPs receptors are by no means restricted to the IECs; many other immune and nonimmune cells are capable of expressing MAMPs receptors.

Toll-like receptors (TLRs) are probably the most studied of all MAMPs receptors. In mice and humans combined there are 13 paralogs (TLR10 is only present in humans and TLR11–13 is only present in mice). TLRs are single-pass (Type I) transmembrane proteins characterized by multiple extracellular leucine-rich repeats (LRRs). TLRs possess a single intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domain (Moresco et al., 2011).

TLR2 in conjunction with either TLR1 or TLR6 recognises lipopeptides and other components of Gram-positive bacterial cells. TLR4 is the receptor for lipopolysaccharide (LPS). There are additional molecules involved in TLR4-LPS binding and signalling, namely CD14, MD-2 and sometimes β2 integrins (Fitzgerald et al., 2004).

TLR5 recognizes flagellin. TLR3 is implicated in the recognition of viral dsRNA (usually *in vitro* activated with the synthetic analogue poly I:C), whereas TLR7 and TLR8 are implicated in ssRNA recognition. Unmethylated DNA and CpG-oligodeoxynucleotides (CpG-DNA) are detected by TLR9 (Moresco et al., 2011). Up until 2014, TLR10 was orphan but now it is suggested that it is a modulatory

receptor with mainly inhibitory effects. The modulatory effects of TLR10 seem to be determined by competition for ligands or by the formation of heterodimer receptors with TLR2, and also PI3K/Akt-mediated induction of the anti-inflammatory cytokine IL-1Ra (Oosting et al., 2014).

TLR11 and TLR12 recognise the profilin from *Toxoplasma gondii* (Koblansky et al.). And finally, bacterial 23S rRNA is ligand for TLR13 (Oldenburg et al., 2012).

Apart from the TLRs there are other membrane bound MAMP receptors. For example, **C-type lectins**, such as DC-SIGN (CD209), Dectin-1 (CLEC7A), Dectin-2, Mincle (CLEC4E), Mannose receptor (CD206) have been shown to participate in microbe recognition and immune response (Cunha et al., 2012). Other examples include Galectin-3, CR3 (CD11b/CD18), CD36 (Cunha et al., 2012).

In terms of intracellular receptors, the nucleotide-binding oligomerization domain receptors, in short **NOD-like receptors (NLRs)**, also called the CATERPILLAR family, have been most widely studied. At present they comprise more than 20 different mammalian proteins which mostly contain three distinct domains: a carboxy terminal ligand recognition (LRR) domain, a centrally located nucleotide binding domain (NBD), and a structurally variable amino terminal effector binding domain (Cario, 2005). Research has mostly focused on two cytosolic receptors of this family, NOD1 (also designated CARD4) and NOD2 (CARD15), which sense the cytosolic presence of bacterial peptidoglycan fragments and their activation leads to phosphorylation of mitogen-activated protein kinase (MAPK) and the transcription factor NF-κB. A different set of NLRs induces caspase-1 activation through the assembly of large protein complexes named inflammasomes (Kanneganti et al., 2007).

NOD1 recognizes the dipeptide γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) (Chamaillard et al., 2003), which is produced by most Gram-negative and certain Gram-positive bacteria (Hasegawa et al., 2006). Muramyl dipeptide (MDP), which is peptidoglycan motif common to all bacteria, is the ligand for NOD2 (Girardin et al., 2003).

3.2.2.1. Cytokine signalling and the IECs

IECs are capable of producing a myriad of cytokines. For example, IECs regulate colonic $CD4^+$ T-cell function through production of **IL-18**. Under homeostatic conditions, IL-18R1 signalling limits colonic Th17 cell differentiation, whereas during inflammation, Foxp3 Treg cell expression of IL-18R1 is critical for prevention of experimental colitis (Harrison et al., 2015).

Another cytokine form the IECs' arsenal is **IL-15** which is closely relate to IL-2 sharing similar four-helix bundle structure. IL-15 is found to activate natural killer (NK) cells and induce B cell proliferation and differentiation *in vitro*. Both IL-2 and IL-15 require IL-2Rβ and γc for signalling which are found on IECs surface (Reinecker et al., 1996). In contrast to IL-2, which is produced by activated T cells, IL-15 seems to be expressed by a variety of cell types. It has been shown that both human and rodent IECs can express IL-15, particularly in the terminal ileum and the colon (Reinecker et al., 1996). Human colon intestinal epithelial cell lines (Caco-2, HT-29, and T84) constitutively express IL-15 (Reinecker et al., 1996). Within 4 h of stimulation with IFNγ Caco-2 cells up-regulate IL-15 mRNA expression which slightly drops after 12 h and is almost back to normal at 18 h (Reinecker et al., 1996). Interestingly IECs not only produce IL-15 but are also capable of responding to it. Upon stimulation with IL-15 within minutes STAT3 phosphorylation is observed (Reinecker et al., 1996). In addition IL-15 treatment induces cell proliferation in Caco-2 (Reinecker et al., 1996).

Other epithelial derived cytokines include **IL-25** and **IL-33** which are produced during helminth infection and facilitate ILC2s expansion and IL-13 production necessary for parasite expulsion (Neill, 2010). IL-33 has also been implicated in IBD where increased expression of full-length IL-33, representing the most bioactive form, is detected in UC epithelium, while elevated levels of cleaved IL-33 are present in IBD serum. Infliximab (anti-TNF) treatment of UC decreases circulating IL-33, whereas stimulation of HT-29 IEC confirms IL-33 and sST2 regulation by TNF (Pastorelli et al., 2010).

IECs also secrete **IL-7** (Watanabe et al., 1995), **IL-6** (McGee et al., 1993), **IL-8** (Steiner et al., 2000), **IL-10** (Hyun et al., 2015, Dvorak et al., 2003), **IL-26** (Dambacher et al., 2009), **IL-32** (Netea et al., 2005), **IL-37** (Imaeda et al., 2013)

Factors produced by the IECs such as **TSLP**, **TGFβ** and **retinoic acid** condition the nearby DCs towards more tolerogenic phenotype (Peterson and Artis, 2014) (Mosconi et al., 2013). The production of TSLP has been identified as critical to the maintenance of intestinal immune homeostasis. On one hand, the constitutive low level production by IECs of this cytokine ensures that the immunological environment of the gut is polarized towards a T helper-2 (Th2) anti-inflammatory response. On the other hand, over-expression of TSLP is linked to the genesis of allergic responses both in the respiratory (Li et al., 2011) and gastrointestinal (Blázquez et al., 2010) tract.

The work described in **Chapter 2 and 3,** shows that the mouse model of food allergy is characterized by reduced levels of IL-12 in the gut and overexpression of TSLP. Oral delivery of *Lactococcus lactis* engineered to secrete bioactive IL-12p70 resulted in a reduction of TSLP. **The mechanisms involved in this regulatory interplay between the epithelium-derived TSLP and the lymphoid-derived IL-12 have been one of the main objectives of this thesis.**

3.2.2.2. Further immunological roles of the IECs

IgA transport

Immunoglobulin (Ig)A constitutes the most abundant antibody in the gut, where it provides immune protection through both high- and low-affinity binding systems. While high-affinity IgA neutralizes toxins and pathogens, low-affinity IgA blocks the adhesion of commensal bacteria. Epithelial cells express a polymeric Ig receptor that shuttles IgA oligomers secreted by B cells from the LP into the lumen. Recent findings indicate that epithelial cells function not only as IgA transporters, but also as IgA inducers (Cerutti, 2008). He et al. (2007) have found that TLR stimulation prompts colonic epithelial cells to secrete BAFF and APRIL which have been proposed to drive local switching to IgA (Chu et al., 2013).

Antigen sampling/presentation by IECs

As mentioned previously (**section 3.1.2**), specialised type of epithelial cells (M cells) associated with the PPs transport particulate antigens originating in the lumen. It has also been suggested by Karlsson *et all.* that supra-molecular, exosome-like structures assembled in and released by the small intestinal epithelial cells are also involved in antigen transport. Those vesicles were named "**tolerosomes**" because when isolated from serum shortly after antigen feeding or from an *in vitro* pulsed intestinal epithelial cell line they were fully capable of inducing antigen specific tolerance in naïve recipient animals. These structures carry **MHC class II** with bound antigenic peptides sampled from the gut lumen (Karlsson et al., 2001). Yet another way of antigen transport via the small intestine **goblet cells** delivering low molecular weight soluble antigens from the intestinal lumen to the underlying $CD103^+$ DCs is also likely to take place (McDole et al., 2012). Mouse IECs likewise express **CD1d** and are capable of lipid antigen presentation. Reduction of the epithelial CD1d has been implicated in colitis (Olszak et al., 2014).

4. Immunological processes of the GI tract

Mammalian digestive tract is populated by numerous and complex microbial community. The number of microbial cells can reach 10^{10}-10^{12} cell/ml, which outcompetes any other known microbial ecosystem. Hundreds of species comprise these communities, the diversity and metabolic properties of which vary from host to host and are also dependant on the gut segment. At birth the digestive tract is sterile but gets rapidly colonized. Most of the gut associated microbes are not pathogenic and are even beneficial for the host. On the other hand, many could cause serious illness. The gastrointestinal part of the immune system has the intricate duty of creating tolerance towards the food antigens and the mutualistic bacteria whilst combating against invading pathogens. Here it will be discussed how the anatomical and cellular characteristics of the GI tract outlined in the previous sections interact in order to serve both functions. Processes of both steady state and disease will be conferred.

4.1. Defence against intestinal pathogens

Intestinal mucosa is exposed to billions of antigenic molecules. The majority of those belong to harmless commensal microorganisms or are food derived and therefore the immune system remains unresponsive to them and is even tolerised as discussed in **section 4.2**. On the other hand many pathogens make their invasive way via the oral root. Those include bacteria such as *Salmonella* species, certain *Escherichia coli* serotypes, certain *Vibrio* spp, certain *Clostridium* spp, *Campylobacter* spp, etc.; viruses, protozoans and multicellular parasites. Several layers of innate and adaptive immune defence are in place to protect the GI tract.

As mentioned previously, a layer of **mucus** protects the epithelium form mechanical, chemical and microbial stress. In the SI the mucus is thinner and more loosely attached. In the LI the mucus consists of two layers – upper loose and inner dense and microbe free. A range of **antimicrobial peptides** are secreted by the epithelium and released into the mucus layer (Tollin et al., 2003).

It has recently been shown that **extrusion of infected enterocytes** is a way of reducing pathogen load. This mechanism is advantageous in case of acute insults such as *Salmonella* Typhimurium that can invade and breach the mucous and epithelial barrier. The epithelium-invading *Salmonella* Typhimurium initially replicate within the infected IECs, expulsion of the infected enterocytes into the lumen limits this stage of the infection. Epithelium restricted deletion of inflammasome components (NAIP1-6, NLRC4, caspase-1/-11) results in more than 100-fold higher intraepithelial loads and faster lymph node colonization (Sellin et al., 2014) suggesting that these inflammasome mediators are important players in this pathogen-protection mechanism.

Another early infection defence mechanism first documented by our group is described in **Chapter 5.** It involves **migration of macrophages** into the lumen after *Salmonella* challenge.

Copious amounts of **immunoglobulin A (IgA)** are produced by B cells residing in the LP and then transported/released in the lumen by the IECs. IgA interferes with the earliest steps in the infection process by blocking toxins and pathogens

adherence to the intestinal epithelium (Mantis et al., 2011). Approximately 25% of intestinal IgA and IgG antibodies are polyreactive but the majority are antigen-specific. Specificity is not only directed against enteropathogenic but also against commensal microbes and self-antigens. Regardless of their reactivity, all intestinal antibodies are somatically mutated and are likely to be a result of antigen-mediated selection (Benckert et al., 2011).

Craig and Cebra were the first to show that PPs are a source of IgA precursor cells (Craig and Cebra, 1971). They demonstrated that cells derived from PPs were able to replenish lethally irradiated rabbits with IgA-producing cells. They clearly showed that the intestinal LP of recipient animals was repopulated with IgA-producing cells of donor origin after transfer of PPs, but not of popliteal LN cells. The cellular organisation of the PPs is described in **section 3.1.2**.

TGF-β cooperates with CD40 ligand (CD40L, also known as CD154), a tumour necrosis factor (TNF) family member expressed by $CD4^+$ T cells, to trigger IgA class switch recombination (CSR) and generate antigen-specific IgA^+ B cells, which represent nearly 70% of germinal center B cells in the PPs. B cell-specific TGF-β-receptor-deficient mice are severely impaired in their steady-state and antigen-induced IgA responses. In addition to TGF-β, IL-4, IL-6, and IL-10 also facilitate the expansion and differentiation of B cells to IgA-secreting plasma cells (Cerutti and Rescigno, 2008).

Although important, PPs and MLNs are not essential for intestinal IgA production. Severe IgA deficiency in lymphotoxin-deficient ($LT^{-/-}$) mice could be restored by reconstitution with LT-expressing bone marrow, despite the absence of both LNs and PPs. IgA deficiency in $LT^{-/-}$ mice is reversed by the transplantation of a segment of RAG-1 (recombination-activating gene I)-deficient intestine, which confirms the dispensability of the MLNs and PPs and the sufficiency of the LT-mediated gut microenvironment for IgA production (Kang et al., 2002).

Immunoglobulin E (IgE) has been implicated in the clearance of intestinal parasites. For example, rapid worm expulsion is impaired in IgE-deficient but not in IgG1-deficient mice. IgE mediates activation of basophils promotes the recruitment of other effector cells into the small intestine and induces

expression of antihelminthic proteins resistin-like molecule β and mucin 5ac. Selective deletion of IL-4/IL-13 in basophils results in impaired worm expulsion. Therefore IgE-mediated activation of basophils and the release of basophil-derived IL-4/IL-13 are critical steps in protective immunity against helminths (Schwartz et al., 2014).

IgE is believed to be one of the major mediators of immediate hypersensitivity reactions that underlie atopic conditions such as urticaria, allergic rhinitis, asthma and anaphylaxis.

Location and kinetics of class switching to IgE are still not fully understood. Further advances in understanding IgE^+ B cells have been hindered by their small numbers under physiological conditions. Relevant findings have been reviewed by (Akdis and Akdis, 2012).

4.2. Oral tolerance

The mammalian immune system has extraordinary potential to combat an array of pathogens with constantly evolving invasion strategies. But even more astonishing is its capacity of distinguishing between friends and foes. Non-responsiveness towards body's own antigens is maintained by the means of **systemic tolerance**, whereas non-responsiveness against foreign innocuous antigens, such as food and commensal microbiota, is perpetuated by **peripheral tolerance**.

Essential for systemic tolerance is the **negative selection** of the newly developed B and T cells occurring in the bone marrow and the thymus respectively. If the B cells are found to be highly reactive to self, three mechanisms can occur in order to preserve self-tolerance: receptor editing, clonal deletion or anergy (Sandel and Monroe, 1999). Auto-reactive T cells are either eliminated, become anergic or they start expressing the transcription factor forkhead box P3 (Foxp3) and become natural regulatory T cells (nTregs). Both deletion of self-reactive T cells and differentiation of nTreg require the interaction of the T-cell receptor with its cognate antigen in the thymus. The promiscuous expression of tissue-restricted self-antigens by medullary thymic epithelial cells ensures that all possible self-antigens are available during the

selection process (Kyewski and Klein, 2006). However, these mechanisms are not suitable for preventing responses against food and commensal microbiota antigens that have not been found at the sites of lymphocyte differentiation, therefore additional layers of peripheral tolerance must exist (Pabst and Mowat, 2012).

Oral tolerance represents a state of local and systemic immune unresponsiveness that is induced by oral administration of harmless antigen such as food components. It is disputed that similar but not identical mechanisms account for the toleration of the intestinal microbiota. Tolerance induction at the mucosal surface of the gut is believed to prevent intestinal disorders such as food allergy, celiac disease, and inflammatory bowel diseases (Pabst and Mowat, 2012). Systemic delayed-type hypersensitivity, T-cell proliferation, and cytokine production as a result of mucosally induced tolerance are common measures of this process. Serum antibody levels can also be suppressed (particularly IgE and Th1-dependent IgG2a) as well as the mucosal T-cell and IgA responses. Furthermore, oral tolerance has been shown to suppress immunopathology in experimental models of autoimmune encephalitis (Higgins and Weiner, 1988), collagen-induced arthritis (Nagler-Anderson et al., 1986), and type 1 diabetes (Zhang et al., 1991).

Oral tolerance varies with **age**. For example, B6D2F1 mice, are very susceptible to tolerance induction by a single gavage with 20 mg of ovalbumin (OVA) at age 8 weeks, but as they age the degree of tolerance induction declines and at age 70 weeks could no longer be achieved. However, 70-week-old mice may be rendered tolerant by repeated ingestion of OVA. Mice orally exposed to OVA at age 8 weeks remain tolerant at age 70 weeks which suggest that only the induction but not the maintenance of tolerance is impaired in ageing (de Faria et al., 1998). On the other hand, neonatal and new born immune system is also incapable of mounting oral tolerance (Strobel and Ferguson, 1984).

In order to understand the molecular and cellular basis of oral tolerance it is vital to identify which **anatomical locations**, apart from the intestinal mucosa, are involved in the process. Several studies indicate that the liver is a major organ involved in the induction of oral tolerance. Injection of antigen into the

portal vein, which drains blood from the intestine to the **liver**, is known to induce antigen specific tolerance (Goubier et al., 2008). In addition, generation of oral tolerance can be prevented by diverting the blood flow away from the liver by portocaval shunting (Callery et al., 1989). It has been also established that **MLNs** are required for oral tolerance whereas **PPs** are dispensable (Spahn et al., 2002) (Kraus et al., 2005), in particular, CCR7-dependant transport of antigen from the LP into the MLNs by $CD103^+$ DCs is the key event for inducing the systemic consequences of oral tolerance. Thus, genetic deficiency in CCR7 prevents T cells recognition of the fed antigen in the MLNs and impairs the induction of oral tolerance (Worbs et al., 2006). Limited number of papers have looked at the involvement of **thymus** in oral tolerance. According to (Song et al., 2004) (Song et al., 2006) thymectomised adult mice are not protected from experimental autoimmune encephalomyelitis (EAE) after myelin basic protein feeding. On the other hand, Mattingly and Waksman (1978) observe similar responses to orally fed sheep erythrocytes in normal and thymectomised adult rats (Mattingly and Waksman, 1978).

One may question whether any **food antigens** endure the acidic environment of the stomach and the enzymatic digestion in the small intestine in order to trigger mucosal immune response. It has been demonstrated that orally administered antigen within 30 min enters the intestinal epithelium. After 6 h it appears in the intestinal LP, and disappears at 24 h (Goubier et al., 2008). In healthy adult humans within 2-3 h after a test meal there are up to 10.5 ng ovalbumin (OVA)/ ml serum; the OVA being intact and unbound or present as part of the immune complexes (Husby et al., 1985, Husby et al., 1987). How the intact OVA makes its way into the blood stream remains largely unknown and the biological significance of this phenomenon is questionable.

Specialised methods of **antigen transport** take place at the mucosal surface and they are believed to functionally contribute in the establishment of oral tolerance. Material of low molecular weight, such as haptens and polypeptides, may pass directly across the epithelium by **paracellular diffusion** through pores in the epithelial cells tight junctions (Pabst and Mowat, 2012). The importance of **M cell** antigen transport for the induction of oral tolerance is unclear. First, M

cells are inefficient at uptake of soluble protein antigens. They are specialized to take up particulate antigens or those antigens for which these cells express receptors (eg, poliovirus) (Chehade and Mayer, 2005). Second, as already mentioned, PPs are not essential for oral tolerance; therefore the M cell antigen transport might contribute but is likely to be secondary to oral tolerance induction. Though, on the other hand, there are studies demonstrating that targeting protein antigen directly to the M cells facilitates tolerance induction (Suzuki et al., 2008), and one study observed that inhibition of PP development during gestation reduced oral tolerance to protein but not haptens in adult mice (Fujihashi et al., 2001). Other routes of antigen transport - "**tolerosomes**" and small intestine **goblet cells** have been described in **section 3.2.2.2.** Finally, most popular around the mucosal immunologists' community in the recent years way of antigen transport from the lumen to the LP is the so called **DC/macrophage "sampling"** (Rescigno et al., 2001). More detailed information regarding its mechanism and its significance is presented in **Chapter 5**. Mice that lack $CX3CR1^{+}$ macrophages and are incapable of antigen sampling but have fully functional $CD103^{+}$ DCs cannot mount oral tolerance against OVA (Hadis et al., 2011b).

Before proceeding further with the cellular mechanism of oral tolerance here we should spend a moment on the antigen's role in the process. Amongst the most widely used "model" oral tolerance **antigens** are: egg or egg derived proteins such as OVA and hen egg lysozyme; cow's milk and proteins derived from it such as β-lactoglobulin and α-casein. Some other antigens capable of inducing oral tolerance are: heat shock protein 65 (Harats et al., 2002); collagen (Trentham et al., 1993); keyhole limpet hemocyanin (used as novel antigen); myelin basic protein (Khoury et al., 1992). Example of proteins that cannot induce oral tolerance and indeed abrogate it are cholera toxin (CT) (Elson and Ealding, 1984) which will be further discussed in **Chapter 2 and 3,** and heat-labile enterotoxin (Clements et al., 1988). There is dearth in the literature regarding the antigens' capabilities and properties enabling oral tolerance induction. From the examples listed above one could see that generally soluble globular proteins tend to mount oral tolerance. Little is known about the role of

protein glycosylation in this case or whether non-proteinaceous antigens have the same effect. In addition, there is lack of evidence whether/how antigens originating from the commensal flora may induce oral tolerance.

Many different **doses and regimens** of single and multiple feeds induce oral tolerance successfully. There is limited evidence that single administration of high dose of antigen (> approximately 0.5 mg/g body weight in mice) induces clonal deletion or anergy (Garside et al., 1995) (Melamed and Friedman, 1994) whereas multiple low doses (< approximately 0.1 mg/g body weight in mice) are more likely to generate regulatory cells (Strobel and Mowat, 1998).

Intestinal **microbiota** is a constant participant in the maintenance of gastrointestinal immune homeostasis and is expected to affect most if not all associated regulatory pathways. Germ free (GF) mice fed with OVA seem to mount oral tolerance, but the difference between fed GF and non-fed GF animals is smaller than that observed between fed and non-fed conventional mice (Walton et al., 2006). Monocolonisation of germ free mice with certain probiotic strains can modulate oral tolerance. For example mice monoassociated with *Lactobacillus paracasei (NCC 2461)* but not *Lactobacillus johnsonii (NCC 533)* or *Bifidobacterium lactis Bb12 (NCC 362)* develop higher tolerance against β-lactoglobulin (Prioult et al., 2003).

A recent study implicates **mucus** as modulator of tolerance. Antigen sampling cells from the small intestine can uptake MUC2 (major component of gut mucus) which drives their anti-inflammatory properties. *Muc2*$^{-/-}$ mice gavaged with OVA cannot mount intestinal or systemic tolerance. The impairment of tolerance in *Muc2*$^{-/-}$ mice is not due to bacteria-induced inflammation. Germ free *Muc2*$^{-/-}$ do not attenuate OVA-specific delayed type hypersensitivity, IgG, and IgE responses after gavage with OVA. Interestingly, oral tolerance induction is restored in *Muc2*$^{-/-}$ if gavaged with a combination of OVA and MUC2 (Shan et al., 2013). Thus, mucus is much more than a nonspecific physical barrier and it is also capable of limiting the immunogenicity of gut antigens by delivering tolerogenic signals.

The evidence outlined above hopefully demonstrates the multitude of factors influencing oral tolerance. Since major focus of this dissertation is the **lympho-**

epithelial cross-talk regulating the immune homeostasis, it is ought to discuss the epithelial contribution to the phenomenon of oral tolerance. For example, supernatants from IECs from antigen fed animals exert a dramatic inhibitory effect on the proliferative reaction of antigen-primed T cells in response to the antigen presented by syngenic spleen cells. This inhibitory effect can be reversed by neutralising anti- **TGF_β** antibodies (Galliaerde et al., 1995). Serum TGF_β levels increase after oral administration of TGF_β. Balb/c mice treated orally with OVA and TGF_β show enhanced reduction of OVA-specific IgE and IgG1 antibodies, T-cell reactivity, and immediate-type skin reactions when compared with the mice treated orally with OVA alone (Ando et al., 2007). Epidemiologic studies suggest that TGF_β in breast milk provides protection against allergic disease during infancy. Cytokines in milk, such as IL-10, IL- 4 and TGF_β influence antigen priming. The importance of neonatal exposure to TGF_β in breast milk is highlighted by TGF_β knockout mice which do not survive after weaning due to an uncontrolled inflammatory state unless suckled on TGF_β producing mothers (Letterio et al., 1994). It is suggested that the anti-inflammatory action of TGF_β is mediated by modulation of IL-18, IL-6, IFN_γ, IL-1_β, and IL-1Rα mRNA expression and active protein secretion in the intestine (Penttila et al., 2003).

On the other hand, overexpression of certain cytokines by the epithelium has been shown to have negative effect on tolerance. For example, mice genetically engineered to overexpress IL-7 in the intestinal epithelium develop chronic colitis (Watanabe et al., 1998). Increased production of epithelia IL-18 has been associated with Crohn's disease (Pizarro et al., 1999) and exogenous application of this cytokine has been found to prevent induction of oral tolerance (Eaton et al., 2003).

4.3. Food allergy

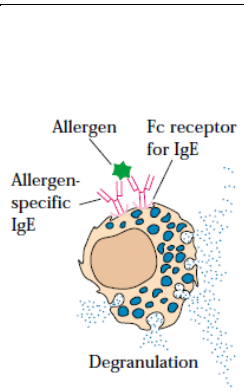
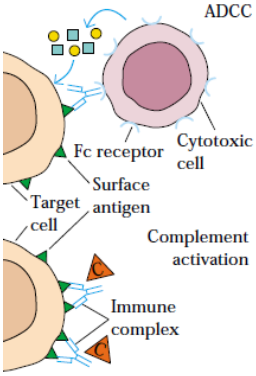
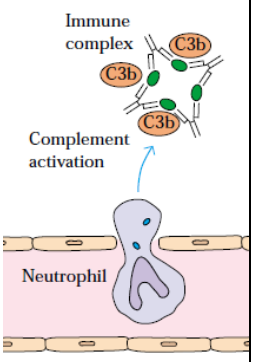
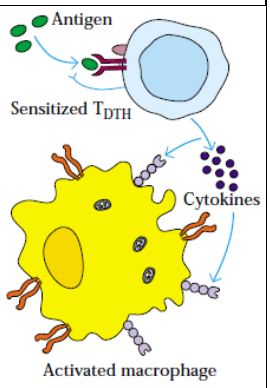
Under certain circumstances dysregulation of the immune response can result in significant tissue damage and even death. This inappropriate immune response is termed hypersensitivity and includes allergies and autoimmunity.

Depending on whether the symptoms of hypersensitivity develop instantly or after certain time post antigen exposure the hypersensitivity reactions could be

accordingly classified as “immediate” or “delayed”. In the first instance, different antibody isotypes drive the aberrant reaction, for example, IgE antibodies induce mast-cell degranulation and release of histamine. IgG and IgM antibodies induce hypersensitivity by activating complement (membrane-attack complex and split products such as C3a, C4a, and C5a). T cells secrete various cytokines acting as effector molecules in the delayed-type hypersensitivity reactions (**Table 1**) (Kindt et al., 2007).

Food allergy presumably occurs when oral tolerance fails to develop normally or breaks down and involves an abnormal response to otherwise harmless orally acquired antigen. Food hypersensitivity develops in genetically predisposed individuals (Sicherer et al., 2000). Depending on the effector molecules/cells involved, IgE-, cellular-, or mixed food hypersensitivity disorders have been described (**Table 2**).

Table 1 The four types[3] of hypersensitivity responses according to Coombs and Gell (Gell, 1963). Adapted from (Kindt et al., 2007).

IgE-Mediated Hypersensitivity	IgG-Mediated Cytotoxic Hypersensitivity	Immune Complex-Mediated Hypersensitivity	Cell-Mediated Hypersensitivity
			
Type I	Type II	Type III	Type IV
Ag induces crosslinking of IgE bound to mast cells and basophils with release of vasoactive mediators	Ab directed against cell surface antigens; meditates cell destruction via complement activation or antibody-dependent cell-mediated cytotoxicity (ADCC)	Ag-Ab complexes deposited in various tissues induce complement activation and an ensuing inflammatory response mediated by massive infiltration of neutrophils	Sensitized Th1 cells release cytokines that activate macrophages or Tc cells which mediate direct cellular damage
Typical manifestations include systemic anaphylaxis and localized anaphylaxis such as hay fever, asthma, hives, food allergies, and eczema	Typical manifestations include blood transfusion reactions, erythroblastosis fetalis, and autoimmune haemolytic anemia	Typical manifestations include blood transfusion reactions, erythroblastosis fetalis, and autoimmune haemolytic anemia	Typical manifestations include contact dermatitis, tubercular lesions and graft rejection

[3] Recently a fifth type has been added to this classification. It is also considered to be a subclass of Type II RAJAN, T. V. 2003. The Gell–Coombs classification of hypersensitivity reactions: a re-interpretation. *Trends in Immunology*, 24**,** 376-379.

Table 2 Food hypersensitivity disorders adapted from (Sampson, 2004)

IgE mediated	
Gastrointestinal	Oral allergy syndrome, gastrointestinal anaphylaxis
Cutaneous	Urticaria, angioedema, morbilliform rashes and flushing
Respiratory	Acute rhinoconjunctivitis, bronchospasm (wheezing)
Generalized	Anaphylactic shock
Mixed IgE and cell mediated	
Gastrointestinal	Allergic eosinophilic esophagitis, allergic eosinophilic gastroenteritis
Cutaneous	Atopic dermatitis
Respiratory	Asthma
Cell mediated	
Gastrointestinal	Food protein–induced enterocolitis, food protein–induced proctocolitis, food protein–induced enteropathy syndromes, celiac disease
Cutaneous	Contact dermatitis, dermatitis herpetiformis
Respiratory	Food-induced pulmonary hemosiderosis (Heiner syndrome)

According to recent estimates IgE-mediated food allergies affect 3.5% to 4% of Americans and most importantly this number has been increasing (Sampson, 2004). The prevalence of food hypersensitivity is higher in children; in some cases allergy could be outgrown (Hourihane et al., 1998). A variety of foods can cause IgE-mediated reactions but most studies have focused on cow's milk, hen's egg, peanut, tree nut, wheat, soya, fish and shellfish since they are the most common triggers (Gray et al., 2015). Allergies to ovalbumin and cow's milk are most common among children, whereas peanuts induce the most severe form of the food allergy (Lack, 2008). Comprehensive list of plant-derived allergens and discussion of their structural characteristics contributing to allergenic properties are summarised elsewhere (Breiteneder and Ebner, 2000).

Depending on the anatomical location of sensitization induction food allergies could be divided in two groups. In class 1 the sensitization occurs in the gastrointestinal tract and it is more likely to affect young children. On the other hand, class 2 is mainly observed in adults and sensitization takes place in the respiratory mucosa (Breiteneder and Ebner, 2000).

Interestingly, not all individuals that produce food-specific IgE will have symptomatic reaction when the food in question is consumed. Unfortunately,

the mechanisms underlying this difference between food allergic and food sensitized individuals are not completely understood (Dang et al., 2013, Sicherer and Sampson, 2007). Higher plasma levels of IL-4, IL-13, IL-12p70 and lower IL-10 are present in food-sensitized compared to nonsensitized infants. IL-10 and IL-6 are lower in allergic compared to sensitized infants. Interestingly, higher IL-13 and IL-12p70 were found in egg-allergic compared to peanut-allergic patients (Dang et al., 2013).

IL-4, IL-13 and IL-5 are some of the cytokines most commonly studied in allergy. The importance of IL-4 in allergy has been confirmed in a series of experiments involving wild type and IL-4 deficient Balb/c mice sensitized with OVA. Increased level of IL-4 was measured in spleen and MLN cells of WT mice after sensitization and continuous oral rechallenge. In addition, these animals had high anti-OVA IgE and IgG1 in the serum and lost body weight. In contrary, IL-4 deficient mice failed to develop symptoms (Dourado et al., 2010). In the blood of allergic patients increased IL-4 expression has been detected in CD25 (IL-2 receptor alpha)$^{+}$ population. Further studies indicate that within this population it is the basophils, NK, and NK-T cells rather that the CD4^{+} T cells that exhibited positive intracytoplasmic staining for IL-4. Allergic basophils express higher levels of MHCII and CD25 (Caubet et al., 2014). IL-4 is also essential for intestinal mast cell expansion in the case of food allergy (Burton et al., 2013). The role of IL-13 and IL-5 in allergy has been primarily studied in the context of airway hypersensitivity (Rael and Lockey, 2011, Kouro and Takatsu, 2009). Association studies have shown that certain IL-13 variants could possibly contribute to elevated IgE in atopic children and are strongly associated with sensitization to food allergens (Zitnik et al., 2009). In eosinophilic esophagitis IL-5 facilitates eosinophil trafficking, growth, activation and survival (Stein et al., 2006).

What factors contribute to the production of those pro-allergic cytokines is still not fully understood. Since the epithelial cells are located at the site of allergen entry it is likely for them to also be some of the primary responders and initiators of the allergic cascade. The role of the lympho-epithelial crosstalk in sensitization with food antigens will be discussed in more detail in **Chapter 2 and 3**.

5. Summary

Accumulating evidence suggests that lympho-epithelial interactions in the intestine help to promote mucosal homeostasis. In addition, the epithelium is an important player that also mediates cross-talk with the commensal microbiota as well as gastrointestinal pathogens. IECs contribute to the innate defense mechanisms by producing antimicrobial peptides and mucus (Dahan et al., 2007). What is more, the epithelium is also involved in establishment and modulation of adaptive immune responses. For example, factors produced by the epithelium such as TSLP, TGFβ, and retinoic acid can promote IL-10 production by DC hence contributing to maintenance of tollerogenic state (Man et al., 2008, Liu et al., 2007). IECs are capable of producing a number of other cytokines upon bacterial invasion, some examples being IL-8, monocyte chemotactic protein 1, and macrophage inflammatory protein 3α (Dahan et al., 2007). In addition to secretion of signaling molecules IECs have been implemented in cell-to-cell interactions with macrophages (Rescigno et al., 2001) and T cells (Gonnella and Wilmore, 1993).

On the other hand, immune cell derived mediators can affect the epithelium. One example is the macrophage migration inhibitory factor (MIF) which is produced rapidly after *in vivo* bacterial challenge by $CD11c^+$ cells. It has been shown to act on IECs and induce M-cell like phenotype thus facilitating antigen transport (Man et al., 2008). Another example is the induction of epithelial cyclooxygenase-2 by inflammatory mediators which is essential for epithelial repair (Subbaramaiah et al., 2004). Macrophages have been shown to interact with the epithelial stem cell progenitors in the colon and facilitate repair of the injured epithelium (Pull et al., 2005).

These are just a few examples of the myriad of interactions occurring at the lympho-epithelial interface in the gut. **Chapter 2 and 3** will discuss further how the epithelial-derived cytokine TSLP participates in sensitization to food components and its potential cross-regulation with IL-12, a cytokine produced by the lymphoid compartment. **Chapter 4** explores the intestinal microbiota and metabolic status of mice deficient in IL-12 signaling. Finally, **Chapter 5** will focus

on a defense mechanism occurring in the gut early during bacterial infection. Of great importance for this mechanism is the capability of the IECs to send appropriate MyD88- dependent signals to the $CX3CR1^{+}$ macrophages that mediate protection.

Chapter 2: Lympho-epithelial crosstalk in the gut plays a major role in food allergy, IL-12 and TSLP

1. Introduction

Aberrant response to food components is termed food allergy. Depending on the effector molecules/cells involved food hypersensitivity can be classified as IgE-, cellular-, or a combination of those two (examples are provided in **Chapter 1 section 4.3.**). Both IgE- and cytotoxic-dependant reactions can cause anaphylaxis though the term "anaphylaxis" usually applies for IgE-dependant events and the IgE-independent are classified as "anaphylactoid" but are otherwise clinically similar. Allergen-specific IgE could bind to FcεRI receptors on mast cells and basophils, subsequent interaction with the antigen is central to the initiation and propagation of the immediate hypersensitivity reactions. Degranulation of mast cells and basophils leading to release of biologically active mediators such as histamine, tryptase, chymase, heparin, prostaglanding D_2, leukotriene B_4, platelet-activating factor, and cysteinyl leukotrienes drives the pathologic symptoms of anaphylaxis (Kemp and Lockey, 2002). Activation of histamine receptor H_1 results in pruritis (itchy skin), rhinorrhea (nasal mucus overproduction), tachycardia (rapid heartrate), and bronchospasm. Engagement of both H_1 and H_2 receptors leads to headache, flushing, and hypotension (Kaliner et al., 1981). Activation of complement and blood clotting could also occur during anaphylaxis (Kemp and Lockey, 2002).

Normally serum IgE has short lifespan and its concentration is low (lowest among the five immunoglobulin subtypes). During childhood serum IgE levels increase up to 10-15 years of age and then gradually decline throughout adulthood. Environmental factors (such as pollen exposure), genetics, immune status can influence total IgE levels (Smith P, 2009). Class switching to IgE is tightly regulated and requires two signals the first being IL-4 and IL-13. The second signal is ligation of CD40 on B cells with T cell CD40L (Gould and Sutton,

2008, D., 2009, Stutz and Woisetschlager, 1999). Several additional mechanisms of IgE class switch recombination control have been described and it is likely that further are yet to be discovered (Geha et al., 2003). Prior to the B-T cell interaction the T cells acquire polarisation signals and are primed to the specific antigen by DCs (Kawabe et al., 2013). A large body of evidence, part of which collected in Nicoletti's lab suggested that dendritic cells with their ability to direct immune responses play an important role in the generation of adverse reaction to food.

1.1. Work leading to the project

In our laboratory the role of DCs in the early stage of allergic sensitization has been studied in detail and several allergy-related immunoregulatory features of DCs observed and reported. First, we described evidence showing that production of allergen-specific IgE and IgG can be provoked by DCs isolated from cow's milk (CM)-allergic mice when passively transferred into naïve syngeneic mice, even in the absence of antigen exposure (Chambers et al., 2004). Regardless of the allergic status and differences in potential to regulate antibody responses, the DC population analysed from both PPs and spleen from all groups of mice (allergic and non-allergic) was characterised with low expression of regulatory molecules, such as CD40, CD80, CD86 and CD8a, indicating a low degree of maturation of these $CD11c^{+/hi}$ $B220^{-}$ DCs. Moreover, the level of MHC II was not significantly different between PP- and splenic DCs and was not affected by the allergic sensitization. Furthermore, it was observed that both systemic and gut-derived DCs from allergic mice showed an increased resistance to T cell–mediated cell death compared with DCs from control donors (Man et al., 2004). Additional experiments have demonstrated that in both allergic and control mice, T cell– mediated DC apoptosis is dependant on the presence of the specific antigen, requires MHC II, and is only partially CD95-CD95 ligand dependent. This demonstrated that the reciprocal, finely balanced regulation between DCs and T cells, which plays a central role in controlling immune responses, is altered in allergy. The biological relevance of increased DC resistance to T-cell mediated apoptosis was ultimately confirmed by

experiments of adoptive transfer of “apoptosis-resistant” DC from OVA-sensitized into naïve mice (Arques et al., 2008). After isolation surviving apoptosis-resistant DCs were subsequently co-cultured with OVA-specific $CD62L^{hi}CD44^{low}$ naïve T cells or passively transferred into naïve syngenic recipients and the *in vitro* profile of DC and T cell lymphokine production, chemokine receptors expression and *in vivo*, post- DC transfer T helper (Th) and IgE responses were assessed. Naïve OVA-specific T cells, when co-cultured with apoptosis-resistant DCs from either OVA-sensitized or immunized mice, produced increased levels of IL-4 and reduced levels of IFNγ and showed increased expression of Th2 related CCR4 and CCR8 chemokine receptors. Finally, in the absence of antigen challenge, higher levels of OVA-specific IgE were elicited by adoptive transfer of apoptosis-resistant DC compared to freshly isolated DCs from both sensitized and immunized donors. These findings suggest that increased numbers of apoptosis-resistant T cell-activating DCs is associated with sensitization. The apoptosis-resistant DCs are likely to contribute to the generation/maintenance of IgE-mediated allergic reaction.

In another study of peanut food allergy it was found that $CD11c^{+}$ $B220^{-}$ cells isolated from PPs of peanut-allergic C3H/HeJ mice are unable to produce sufficient IL-12p70 upon stimulation with IL-4 compared to Balb/c (Temblay et al., 2007). *In vivo* data confirmed this insufficiency to be restricted to the gut immune system, and not to be associated with reduced expression of IL-4 receptor or the lack of functional TLR4. Instead, a pathway critically involved in IL-4–dependent production of IL-12p70 was dysregulated since IL-4 failed to inhibit IL-10 production by PP-DCs. Importantly, neutralization of IL-12 within PPs by oral delivery of microencapsulated anti-IL-12 antibody during antigen presentation significantly augmented Balb/c susceptibility to food allergy. Thus, reduced intestinal level of IL-12 is a hallmark of allergic sensitization to food components. IL-12 is a typical T helper type 1 cytokine and its role in the genesis and as potential therapeutic in the treatment of allergy has been suggested (Hogan et al., 1998). However, although the potential of IL-12 in suppressing allergic reactions has been reported (Lee et al., 2001, Hogan et al., 1998), how this is brought about and its mechanism of action remained to be determined.

1.2. Strategic approach

In collaboration with the laboratory of Dr. Arjan Narbad at IFR a strategy was devised for restoring physiological levels of IL-12p70 in mice undergoing allergic sensitization based on oral delivery of *Lactococcus lactis* engineered to produce biologically active IL-12p70 (Fernandez et al., 2009). This approach provided us with a reliable delivery tool to test the hypothesis that restoring levels of IL-12 in the gut immune system at the time of antigen presentation might prevent allergic sensitization to an orally delivered allergen and possibly to identify cytokine regulatory pathways affected.

1.3. The IL-12 cytokine family and its role in allergy

IL-12 was identified as a natural killer stimulating factor (NKSF) in 1989 (Kobayashi et al., 1989) and was the first known cytokine with heterodimeric structure where two independently transcribed and translated protein subunits are covalently linked by a disulphide bond (Palmer and Weaver, 2010). Since then other cytokines with analogical architecture have been discovered, namely IL-23, IL-27, and IL-35. These cytokines are classified as the IL-12 family and are schematically illustrated in **Figure 6**.

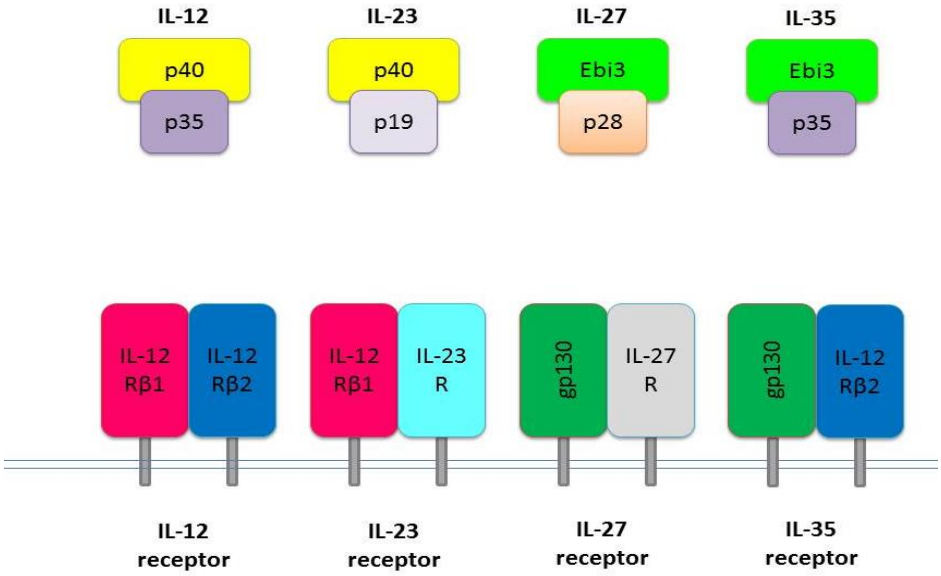


Figure 6 Schematic overview of IL-12 family of cytokines and their corresponding receptors.

According to the generally accepted view, IL-12 consist of p40 and p35 protein subunits (together making p70) and its receptor is formed by IL-12Rβ1 and IL-12Rβ2 (Palmer and Weaver, 2010). It is suggested that p40 is important for receptor binding, while p35 is responsible for the biological activity (Ling et al., 1995), however there is evidence that both subunits are needed for binding and intracellular responsiveness (Chua et al., 1995b).

Within the IL-12 family many structural components are shared. The IL-12Rβ1 subunit is part of both IL-12 and IL-23 receptors (Trinchieri et al.). Similarly, the IL-12Rβ2 subunit is part of IL-12 and IL-35 receptors (Collison et al., 2012b). Despite sharing structural features and intracellular signalling pathways the overall biological activity of these cytokines is very diverse (Vignali and Kuchroo, 2012). For example, IL-35 is produced by regulatory T-cells and is suppressive, while IL-12 is involved in initiation of Th1 differentiation (Collison et al., 2012b, Uhlig et al., 2006b).

The gp130 that is involved in IL-35 and IL-27 signalling provides a crosslink with other cytokines families as it is a component of receptors for IL-6, IL-11, leukocyte-inhibitory factor, oncostatin M, ciliary neurotrophic factor, cardiotrophin 1 and cardiotrophin-like cytokine (Silver and Hunter, 2010). In addition to their canonical function as heterodimers, some members of the IL-12 family can function autonomously as monomers or homodimers. For example, p28 alone, in the absence of its regular partner Ebi3, has been shown to bind to the receptor chain gp130 without provoking further signalling and thereby antagonizing IL-6 and IL-27, limiting the production of IL-17 and IL-10, respectively (Stumhofer et al., 2010). Similarly, p40 has been shown to form homodimers (p40)2 antagonising IL-12 driven responses (Gately et al., 1996). In contrast, there is no clear evidence suggesting that p19 or p35 can be secreted without a partner and although Ebi3 is autonomously secreted it is not confirmed to have biological function (Vignali and Kuchroo, 2012).

Some of the receptors in the IL-12 family were also found to function not only as heterodimers but as homodimers and/or monomers. For example, $CD4^{+}CD45RB^{hi}CD25^{-}$ naive T cells lacking just one chain of the putative IL-35R are partially resistant to IL-35, which suggested that signalling can occur with

the expression of only one chain of IL-35R. It should be noted that whereas some biological functions are partially affected others are completely abolished (Collison et al., 2012a). If expressed individually, human IL-12Rβ1 and IL-12Rβ2 bind to IL-12p70 with low affinity and when they are coexpressed both low and high affinity binding is observed accompanied with IL-12 responsiveness (Presky et al., 1996). In contrast, mouse IL-12Rβ1 is the primary binding component conferring both low and high affinity sites (Chua et al., 1995a) but IL-12Rβ2 is required for biological activity of IL-12p70 (Wu et al., 2000). Interestingly, (p40)2 signals via IL-12Rβ1 (Jana and Pahan, 2009). In a study by Collison et al. (2012) IL-12p70 triggered formation of IL-12Rβ2 homodimers even though at a very low level (Collison et al., 2012a).

Antigen presenting cells (APCs) are considered to be the main source of IL-12 (Liu et al., 2006). IL-12 drives Th1 polarisation, secretion of IL-2 and IFNγ (Collison et al., 2012b, Uhlig et al., 2006b), where IFNγ is part of synergistic loop (Snijders et al., 1998) upregulating expression of both IL-12 (Liu et al., 2003) and its receptor subunits (Kano et al., 2008). A potent stimulator of IL-12 expression is the interaction between CD40 on DCs with its ligand (CD40L) on the T cell surface (Cella et al., 1996). In addition to IFNγ another cytokine synergising with IL-12 is IL-18, also contributing to development of Th1 cells and capable of upregulating IL-12 receptor subunits (Sinigaglia et al., 1999).

An important function of IL-12 is linking the early innate responses with subsequent antigen specific immunity (Chehimi and Trinchieri, 1994). This cytokine is vital for combating intracellular infections of viral and bacterial origin such as influenza A (Hama et al., 2009), norovirus (Fang et al., 2013), *Staphylococcus aureus* (Hultgren et al., 2001), and *Mycobacterium spp*. (McNab et al., 2014). Evidence has accumulated that TLR engagement can play an important role in the balance between production of IL-12 and other members of the family. Simultaneous activation of more than one TLR has been shown to produce much stronger IL-12 activation (Gautier et al., 2005). Most importantly, selective activation of TLRs can discriminate between stimulation of different IL-12 family members. For example, TLR4 engagement can drive production of IL-12 and IL-23, whereas activation of TLR2 stimulates only IL-23 (Re and

Strominger, 2001). A number of microorganisms have been shown to modulate IL-12 production by DCs. For example, *Lactobacilllus acidophilus* NCFM is promoting, whereas *Lactobacillus reuteri* 5289 is supressing (Amar et al., 2015). Cytokines such as IL-10 (Aste-Amezaga et al., 1998) and TGFβ (Pardoux et al., 1997) have been demonstrated to downregulate IL-12. Complex regulatory interactions link IL-4 and IL-13 with IL-12. Interestingly, addition of IL-4 and IL-13 to peripheral blood mononuclear cells (PBMCs) inhibits IL-12 induced by LPS or *Staphylococcus aureus* when all the stimuli are applied simultaneously, however, if the PBMCs are pre-treated with IL-4 or IL-13 for more than 20 h, exactly the opposite effect is observed (D'Andrea et al., 1995). A detailed study of IL-12 p40, p70 and (p40)2 production of freshly isolated mouse and human DCs have revealed that IL-4 inhibits (p40)2 and increases p70. Dual application of IFNγ and IL-4 produces strong synergism and leads to optimal production of p70 with minimal (p40)2 (Hochrein et al., 2000).

While stimulating Th1 polarisation and secretion of IFNγ, IL-12 supresses Th2 responses (Davidson et al., 1998, Lee et al., 2001). For example, infection with *Leishmania major* initiates strong Th2 response which leads to eosinophilic inflammation, airway hyperresponsiveness and release of IgE. This exacerbated Th2 reaction can be reversed by intraperitoneal administration of IL-12 (Nabors et al., 1995). Allergic asthma, known to be characterised by exacerbated Th2 response, is also linked to reduced IL-12 levels (van der Pouw Kraan et al., 1997) which if restored by gene delivery lead to significant amelioration of the allergic symptoms (Hogan et al., 1998). Similarly to asthma, food hypersensitivity has been established as a Th2 associated condition. Lee et al. (2001) investigated the effects of oral IL-12 treatment in a murine model of peanut allergy and found that supplementation of IL-12 could both prevent and reverse hypersensitivity (Lee et al., 2001).

Intracellular pathways associated with IL-12 and IL-12R

Canonical IL-12 signalling activates the intracellular mediators Janus kinase (JAK2) and tyrosine kinase 2 (TYK2) ultimately leading to phosphorylation of a number of signal transducer and activator of transcription (STAT) proteins.

Highest activation is reported for STAT4 but other members such as STAT1, STAT3, and STAT5 are also phosphorylated (Watford et al., 2004). Another cytokine from the IL-12 family capable of eliciting phosphorylation of the same STAT proteins is IL-23 but major difference is that in the latter case it is STAT3 that achieves highest activation (Teng et al., 2015). Phosphorylation of STAT4 is not mediated by extracellular signal-regulated kinase (ERK) or c-Jun N-terminal kinase (JNK) in T cells and NK cells. Instead, it has been shown that IL-12 stimulation leads to mitogen activated protein kinase (MAPK)p38 activation via MAP kinase kinase MKK6 (Visconti et al., 2000). Interestingly, an alternative IL-12Rβ1- and STAT4- independent pathway of IL-12 signalling exists. T cells deficient in IL-12Rβ1 could still respond to IL-12 by increased IFNγ; this process requires MAPKp38 and MKK1/2 (Verhagen et al., 2000).

1.4. Mouse model of peanut allergy

For a first time mouse model of food allergy was described by Li et al. (1999). They have utilised intragastric administration of cow's milk (CM) along with cholera toxin (CT) to sensitize C3H/HeJ mice. This treatment required several boosts at weekly intervals that ultimately resulted in increased CM-specific IgE at 3 weeks which reached maximum at 6 weeks after the first sensitization dose. Systemic anaphylaxis was provoked after intragastric challenge with CM at week 6. Most importantly, this mouse model successfully recapitulated symptoms associated with food hypersensitivity in human. In addition to the production of allergen-specific IgE, the anaphylactic reaction elicited with the recall CM challenge at week 6 was associated with vascular leakage, elevated plasma histamine, and increased intestinal permeability to casein. It was also evident that the IgE production was likely to be a result of excessive Th2 polarisation since *in vitro* stimulation of splenocytes from allergized mice induced significant increase in IL-4 and IL-5 but not IFNγ (Li et al., 1999).

A year later Li et al. (2000) demonstrated successful sensitization of C3H/HeJ mice with crude peanut extract (CPE) (Li et al., 2000) (Error! Not a valid bookmark self-reference.). Again CT was applied as an adjuvant alongside the food antigen. In this case two doses were administered within seven day

interval and then three weeks after the first sensitization the animals were rechallenged with peanut protein alone. Similarly to the CM allergy model, this treatment resulted in increased antigen-specific IgE production and anaphylactic reaction after peanut protein challenge accompanied by increased mast cell degranulation and plasma histamine. The allergen-specific B- and T-cell responses provoked by the sensitization in this model were similar to those observed in patients with peanut hypersensitivity. Interestingly, in both CM and CPE sensitization the lower dose of allergen administered for sensitization resulted in higher IgE and more severe anaphylactic reaction (Error! Not a valid bookmark self-reference. **A**).

Some of the questions this work provokes are whether other mouse strains could be sensitized in a similar fashion and whether the C3H/HeJ malfunctioned TLR4 (Poltorak et al., 1998) plays a role.

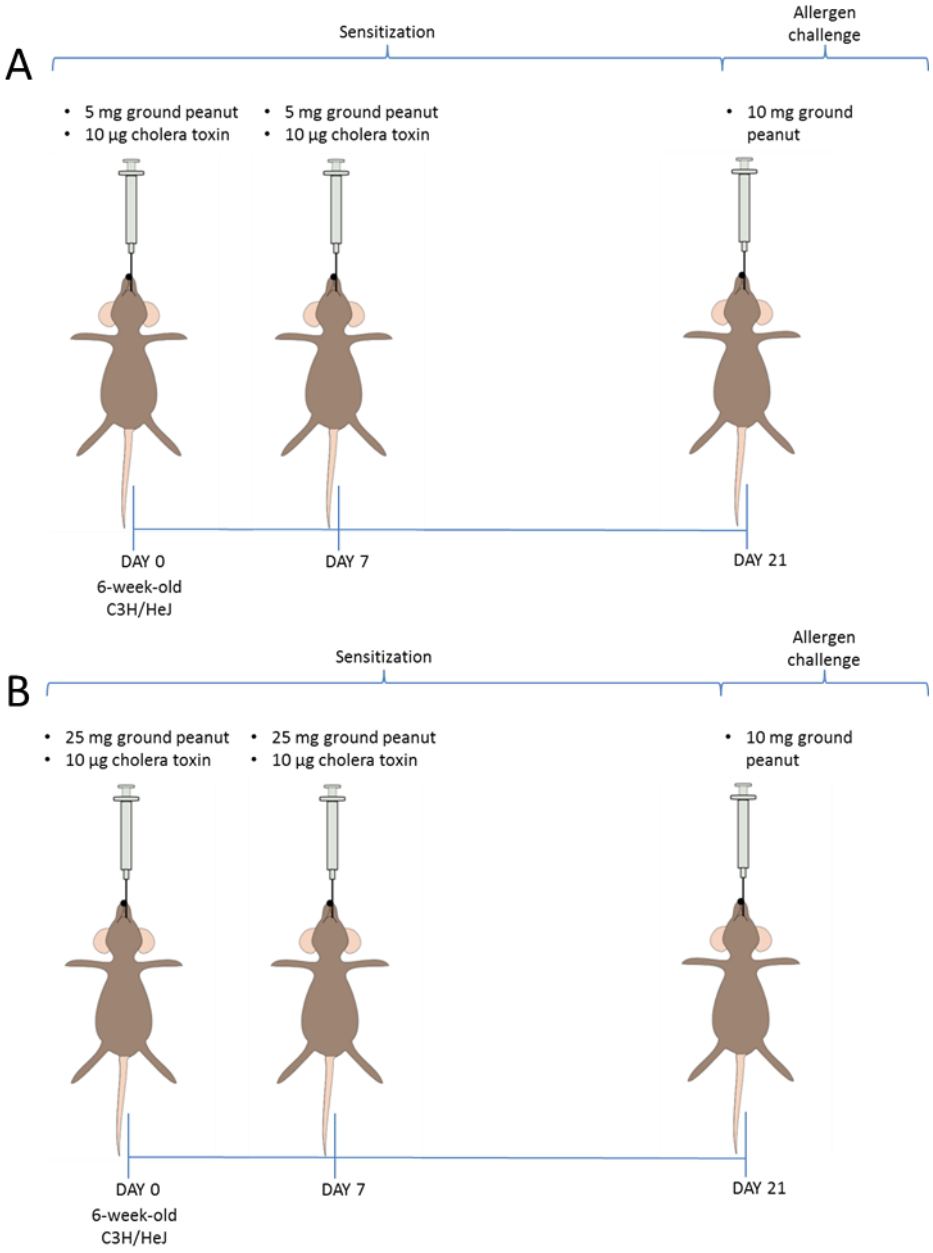


Figure 7 Original protocol for the mouse model of peanut allergy (Li et al., 2000). The model was described by Li et al. (2000) for low (A) and high (B) dose of allergen. This model has been used by many laboratories (including Nicoletti's) to study variety of aspects of food allergy. Indeed this model fatefully replicates the type I hypersensitivity reaction observed in human (see text for details).

Strain-specific susceptibility to food allergy

In another paper by the same group (Morafo et al., 2003) the authors mention that according to unpublished data the above described protocol (Error! Not a valid bookmark self-reference.) did not induce anaphylactic reactions in Balb/c mice but did induce peanut-specific IgE. Even when a more severe protocol for peanut sensitization involving increased CT dose and number of boosts alongside $NaHCO_3$ was used, no hypersensitivity reactions were detected in Balb/c mice, despite the presence of significant serum peanut-specific IgE levels (Morafo et al., 2003). Other studies argue that Balb/c mice are not only capable of producing peanut-specific IgE and IgG1 but also exhibit anaphylactic reaction, however with lesser severity (Adel-Patient et al., 2005, Jhaveri and Bonfield, 2015, Husain et al., 2011, Mondoulet et al., 2012). A study by Smit et al. (2011) compared sensitization in C3H/HeOuJ, Balb/c and C57BL/6 mice. They used relatively rigorous sensitization scheme involving CT and CPE intragastric administration at days 0, 1, 2, 7, 14, 21 and 28 followed by CPE rechallenge at day 35 (Smit et al., 2011). This protocol resulted in CPE-scpecific T cell responses, IgE, IgG1 and IgG2a and mast cell degranulation that differed in their levels between the three mouse strains. Most importantly, anaphylactic symptoms after systemic challenge were high in C3H/HeOuJ and C57BL/6 but were absent in Balb/c.

Studies that looked at the effects of the genetic make-up of the host in the development of food allergy also brought about the notion that specific genes, involved in microbial recognition may play significant role in the sensitization process. Indeed, although still a matter of debate it has been suggested that Toll-like receptor-4 (TLR4) might be involved in determining susceptibility to food allergy. Bashir et al. suggested that the lack of a functional TLR4 is what renders C3H/HeJ mice susceptible to Type I allergic reaction. What is more, they also show that removal of the intestinal flora with antibiotics in TLR4 sufficient animals can also lead to exacerbated anaphylactic reaction and increased splenocyte production of IL-5 and IL-13 upon stimulation with peanut (Bashir et al., 2004). Using the mouse model of cow's milk allergy it has been shown that germ free (GF) animals are more susceptible to allergy as indicated by the lower

rectal temperature after sensitization and have increased allergen-induced degranulation of mast cells (mMCP-1 release). Despite the fact that GF mice also score higher anaphylactic scores they are not significantly different from those of conventional mice (Rodriguez et al., 2011). In another study response towards peanut allergen similar to that of C3H/HeJ mice was seen in congenic $TLR4^{+}$ C3H/HeOuJ mice, suggesting that genetic susceptibility to food allergy does not depend solely on the presence of a functional LPS (TLR4) receptor (Temblay et al., 2007).

2. Hypothesis

Previous studies done in the Nicoletti lab prompted us to test the hypothesis that replenishing the levels of IL-12 via oral administration of genetically engineered *L. lactis* secreting the biologically active variant of the cytokine IL-12 (IL-12p70) could prevent allergic sensitization. Also, we hypothesized that the significant decline of IL-12 in allergic mice might lead to alteration of the intestinal immunological homeostasis by triggering the production of pro-allergic cytokine(s) by the intestinal epithelial cells.

3. Methods

Animals

C3H/HeJ mice were purchased from Harlan (Shardlow, UK). Balb/c mice were purchased from Disease Modelling Unit, UEA. Experiments were approved by the University of East Anglia Welfare and Ethical Review Body (AWERB; ref: 70/7582) and conducted under the guidelines of Animals Scientific Procedures Act (1986) of the United Kingdom. Also, experimental protocols were approved by the Animal Care and Use Committee of the University of Siena (no. G2007/11/2) under the “Guiding Principles for Research Involving Animals and Human Beings”.

Recombinant Bacterial strains

The heterologous *Lactococcus lactis* strains FI5876/pTG262 empty vector (FI10632) (LAB) and FI5876/pFI2596 secreting IL-12p70 (FI10611) (rLAB-IL12) were used. *L. lactis* strains were grown in M17 medium (Oxoid) supplemented

with 0.5% (wt/vol) glucose (GM17 medium) at 30° C without agitation. For experimentation *L. lactis* strains were grown to an OD600 of approximately 1.0. The recombinant bacterial strains were generated by Dr. A. Fernandez-Dumont in Dr. Narbad's laboratory at IFR. Description of the WT strain, generation of the recombinant strain, levels of IL-12p70 secreted and its biological activity have been described in detail elsewhere (Fernandez et al., 2009).

Mouse model of food allergy and administration of rLAB-IL12

Five weeks old female C3H/HeJ mice were sensitized according to an established protocol (Li et al., 2000). Briefly, mice received intragastric administration of 1 mg/mouse of ground crude peanut extract (CPE) together with 10 μg of cholera toxin (List Biological Laboratories) in PBS on days 0, 7, 14, 21, 28. On day 35 all mice were challenged by intragastric gavage with CPE (10 mg/mouse divided into 2 doses at 30 min intervals). To assess the role IL-12 in suppressing food allergy three groups of animals were intragastrically gavaged with PBS (control group), *L. lactis* FI10632 (LAB) and *L. lactis* FI10611 secreting mIL-12 (rLAB-IL12) (8-10 mice/group). Details of the administration regimen are shown in **Figure 8 A** below. Bacterial cell pellets were collected, washed with PBS and resuspended in 2% $NaHCO_3$. A suspension of 100 μl containing 10^9 colony-forming units/ml was administrated by oral gavage in a single dose to the mice 24 h and 2 h before administration of the sensitizing mixture. Parallel experiments assessed the role of TSLP in the development of food allergy in the same mouse model. Animals were gavaged with CPE+CT, CT alone, or PBS; where the mice receiving CPE+CT were either administered with rLAB-IL12 or PBS before each sensitization as already described. Intestinal tissue for gene expression analysis and microscopy was collected within an hour of CPE restimulation. Further experiments assessing the role of TSLP included groups of mice injected i.p. with two doses of 0.5 mg/mouse of anti-TSLP blocking antibody (clone M702, Amgen) 12 h and 3 h prior to the delivery of each sensitizing dose (**Figure 8 B**). In all these experiments the percentage of mice that developed type I hypersensitivity reaction was assessed using a scoring system previously described (Jong et al., 1998). Serum levels of CPE-specific IgE,

IgG1 and IgG2a antibody were determined by ELISA in all groups of sensitized and control mice (see below). Plasma levels of histamine were detected by using an enzyme immunoassay kit (Immunotech S.A.S.) whereas vascular leakage was monitored by injecting Evans blue dye solution (100 μl of 0.5% w/v) (Sigma) into the tail vein to three animals from each group and footpads were examined 40 minutes after the dye injection. Body temperature was monitored using a rectal probe (Kent Scient. Corp.). Finally, levels of the cytokines IL-4, IL-5 and IFN-γ produced by splenocytes *in vitro* following antigen (CPE 10 μg/ml) recall challenge were determined in culture supernatant by ELISA kits (Biolegend) following 72 h challenge. For isolation of splenocytes, spleens were treated with 400 U/ml collagenase D (Roche) in RPMI 1640 (Sigma) and incubated at 37° for 10 min and were afterwards gently dispersed with glass homogenizer. Cells were spun at 400 g for 5 min and red blood cells were removed by addition of 1 ml/spleen Red Blood Cell Lysing Buffer (Sigma) after a minute incubation the cells were washed with 20 volumes of medium. Cells were grown in RPMI 1640 (Sigma) with 10% FCS, penicillin (final conc. 100 U/ml) and streptomycin (final conc. 100 μg/ml) (Invitrogen).

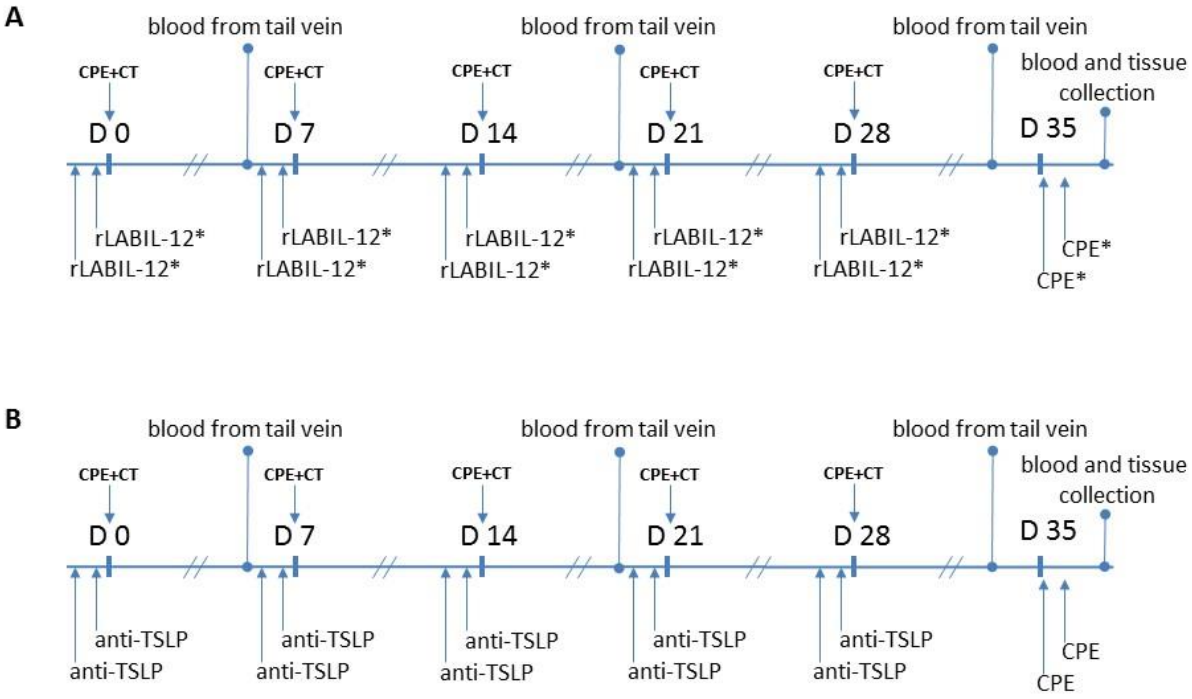


Figure 8 Peanut sensitization scheme. To assess the effect of IL-12 reconstitution on the development of allergy (A) five sensitization doses of CPE

and CT were intragastrically administered at seven day intervals. Twenty-four and two hours before each sensitization a group of animals received gavage with *L. lactis* FI10611 secreting bioactive mouse IL-12p70 (rLAB-IL12), alternatively* another group received *L. lactis* FI10632 (LAB) or PBS (control group). Blood was withdrawn from tail vein on days 7, 21 and 35 for evaluation of plasma CPE-specific antibodies. On day 35 the animals from all groups were recall challenged with CPE alone and within 1 h blood for measurement of histamine and spleens were collected. Alternatively* mice were not recall challenged with CPE and intestinal tissue was collected for evaluation of IFNγ. To assess the role of TSLP in the development of food allergy (B) again mice were sensitized with CPE and CT as before. In this instance anti-TSLP blocking antibody was administered twelve and three hours before each sensitization. Tissue and blood collection was performed as described in A).

Effect of CT gavage on IECs IL-12Rβ2 expression

Mice (4 animals per group) were intragastrically administered with 10 µg CT in 300 µl PBS or only 300 µl PBS as a control. After 48 h small intestine was collected for IECs isolation for flow cytometry. Faecal material and fat were removed and intestinal tissues were washed in 25 ml PBS with 0.1% (vol/vol) sodium azide, 2% (vol/vol) FCS and 4mM EDTA (PBSAFE) which contained 0.05% (vol/vol) of protease inhibitor cocktail (Sigma Aldrich) (per 6 cm intestine) and subsequently washed twice into 25 ml PBSAFE containing 0.8 mM DTT (Sigma Aldrich). IECs were detached by incubation in PBS containing 10% FCS, 1 mM EDTA at 37 °C for 20 min and vigorously shaken 20 times. This step was repeated until no more IECs were detaching and visual inspection showed bare villi. Cells were filtered through 40 µm ø strainers, spun (10 min at 300g) and washed once in PBSAFE, resuspended appropriately and counted. For flow-cytometry analysis $1x10^6$ cells were stained in 100 µl final volume with APC anti-mouse E-cadherin, Clone: 114420 (1:10; R&D Systems); APC rat IgG2a, Clone: 54447 (1:10; R&D Systems); FITC rat anti-mouse CD45, Clone: 30-F11 (1:20; Caltag laboratories); PE anti-human/mouse IL-12Rβ2, Clone: 305719 (1:10; R&D

systems); PE mouse IgG1, Clone: 11711 (1:10; R&D systems). After one wash with PBSAFE samples were fixed by adding equal volume 2% formalin and analysed with EC800 (Sony Technologies), software version 1.3.6 (Sony Technologies).

Effect of CT gavage on intestinal IL-12 and TSLP expression

Mice (5 animals per group) were intragastrically administered with 10 μg CT in 300 μl PBS or only 300 μl PBS as a control. Small intestinal tissues for protein and mRNA analysis were collected 6 h, 16 h, and 72 h post gavage.

ELISA assay for serum and fecal peanut-specific IgE, IgG1, and IgG2a

On days 7, 21 and 35 following initial sensitisation and before each sensitisation process, tail vein blood extraction was performed. Sera were collected and stored at –70° C. Levels of peanut-specific IgE antibody were determined by ELISA as described in detail previously (Chambers et al., 2004). Serum levels of peanut-specific IgG1 and IgG2a were measured as follows (Lee et al., 2001). Briefly, Nunc-Immuno 96 Maxisorp plates (Fisher scientific) were coated with 300 μl per well with CPE or dinitrophenyl-albumin (DNP-albumin) (Calbiochem) at a concentration of 1 μg/ml in coating buffer at pH 9.6. As previously illustrated by Lee et al., 2001 due to the fact that there is a lack of commercially available anti-peanut antibody the levels of IgE, IgG1 and IgG2a were determined by comparison with a reference curve created with DNP-albumin and its correspondent anti-DNP IgE (Sigma), IgG1 and IgG2a (Biocytex) mouse monoclonal antibodies. After two hours at room temperature the plates were washed six times with PBS with 0.05% Tween-20 (PBST). To generate the standard curve, 100 μl of anti-DNP IgE, IgG1 and IgG2a at dilutions of 10^6 pg/ml per well to 1 pg/ml per well were loaded in one half of the plates. Mouse sera (100 μl) were added at dilutions from 1:10 to 1:100 for IgE and from 1:1000 to 1:10000 for IgG1 and IgG2a to the other half of the plates. Plates were incubated for 90 min at room temperature, washed in PBST six times and then anti-mouse IgE-HRP (Serotec), anti-mouse IgG1 and anti-mouse IgG2a (Sigma) from goat was incorporated. In the case of IgG1 and IgG2a a second step was included by the incubation with anti-goat IgG-HRP (Sigma). Enhanced K-blue

TMB substrate (100 μl) (Skybio) was added and incubated for 10 min. Afterwards, the reaction was stopped with 50 μl of 2M sulphuric acid.

Protein isolation for cytokine measurement

Protein was extracted by running samples in tubes with acid washed 3 mm ø glass beads (VWR) on fast prep (6x 1 min with rest on ice inbetween, speed 4 m/s) in CelLytic MT (Sigma) containing 1% (vol/vol) protease inhibitor cocktail (Sigma). Samples were centrifuged (800 g for 5 min), transferred and centrifuged again (100 g for 10 min) and supernatants were collected and protein concentration determined with Nanodrop ND-1000 Spectrophotometer. Intestinal levels of TSLP (pg/ml) were measured by using ELISA MAX Deluxe set (Biolegend) according to the manufacturer's description.

Immunohistochemistry

Frozen sections (8 μm) were fixed with 10% buffered formalin for 30 minutes and endogenous peroxidases were quenched with 0.3% hydrogen peroxide for 1 hour and stained with biotinylated goat anti-mouse TSLP (R&D Systems) and then treated with Vectastain Elite ABC kit (Vector Laboratories). Fluorescein deposit was achieved on the site of the immunoreaction with the TSA Plus fluorescence system (Perkin-Elmer). Sections were counterstained with mouse anti-β-actin Mab (Sigma-Aldrich) followed by TRITC-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Lab-Starfish, Milan, Italy).

Q-PCR

RNA extraction was carried out using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions with additional RNase-free DNase set (Qiagen) treatment. Quantity and purity of each RNA sample extracted was determined with Nanodrop ND-1000 Spectrophotometer. The mRNA (300 ng/reaction) was reverse transcribed into cDNA with the QuantiTect Reverse transcription Kit (Qiagen). Q-PCR was performed with ABI 7500 or ABI 7300 sequence detection systems (Applied Biosystems) and mRNA expression of IL-12p40 and TSLP was measured using the following primers from QuantiTect primer Assay (Qiagen): Mm IL12b_1_SG; Mm Tslp_1_SG. Gapdh and 18S mRNA were used as - endogenous controls and quantified with QuantiTect primers (Qiagen) Mm

GAPDH_3_SG and Mm Rn18s_3_SG. SYBR GREEN Mastermix contained 10% (vol/vol) of 20 mg/ml BSA (Biolabs new England); 1% (vol/vol) 50 mg MgCl2 (Bioline); 2% (vol/vol) ROX reference Dye (Invitrogen); 10% (vol/vol) of Quantitect primer Assay; 0.6% (vol/vol) of 100x SYBR Green nucleic acid staining (Invitrogen); 1,4% (vol/vol) water; 50% (vol/vol) 2x Immomix (Bioline); 25% (vol/vol) cDNA. Real-time PCR reactions were performed, using a 10 min hot start at 95 °C, followed by 50 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 35 s. Reactions were carried out using three technical replicates for every biological replicate. All samples were normalized to 18S expression using the ΔΔCT method.

4. Results

4.1. Oral delivery of *L. lactis* secreting IL-12p70 suppresses food allergic reaction to peanuts.

L. lactis producing bioactive IL-12p70 (Fernandez et al., 2009) was delivered prior the administration of the sensitizing mixture and its effects on food allergic reaction were monitored. First, mice treated with rLAB-IL12 showed a significant decline of allergen-specific IgE compared to sensitized control mice or LAB-treated over the observation period (**Figure 9 A**). Also, in agreement with a previous report (Swain et al., 1990) reduced levels of IgE, although not statistically significant were observed in mice treated with the wild type (WT) bacterial vector *L. lactis*, suggesting that this bacterial strain has intrinsic anti-allergic properties. Levels of plasma histamine were significantly reduced by treatment with rLAB-IL12. In sensitized untreated mice levels reached 2023+307 ng/ml and were reduced to 1670+450 ($p<0.65$) and 515±120 ng/ml ($p<0.01$) in LAB and rLAB-IL12-treated mice respectively (**Figure 9 B**). Treatment with rLAB-IL12 also affected cytokine production following *in vitro* recall antigen challenge of splenocytes. Increased vascular permeability leakage is an additional feature of type I hypersensitivity reaction; indeed, this was reduced in mice treated with rLAB-IL12 compared to sensitized mice (**Figure 9 C**). In addition, levels of IgG1 and IgG2a were monitored at the end of the observation period (day 35 post initial sensitization) (**Figure 9 D**); levels of IgG1 ranged between 66.2±21 μg/ml

in sensitized mice and 51±12 μg/ml and 14.9±4.7 μg/ml in LAB and rLAB-IL12-treated mice respectively; also the visual recording of the overall anaphylaxis score showed that mice treated with rLAB-IL12 exhibited an overall reduction of allergy-related symptoms (**Figure 9 G**). Levels of both IL-4 and IL-5 in culture supernatants were significantly reduced ($p<0.01$) while the production of IFNγ was significantly increased ($p<0.01$) only in rLAB-IL12-treated mice (**Figure 9 E, F**). Furthermore, repeated delivery of IL-12 could affect the inflammatory status of the gut; however, quantification of the pro-inflammatory cytokine IFNγ the production of which is directly up-regulated by IL-12 (Liu et al., 2015), showed that repeated delivery of IL-12 via rLAB does not induce local long-lasting inflammation as determined six days after the termination of the treatment (**Figure 9 H**).

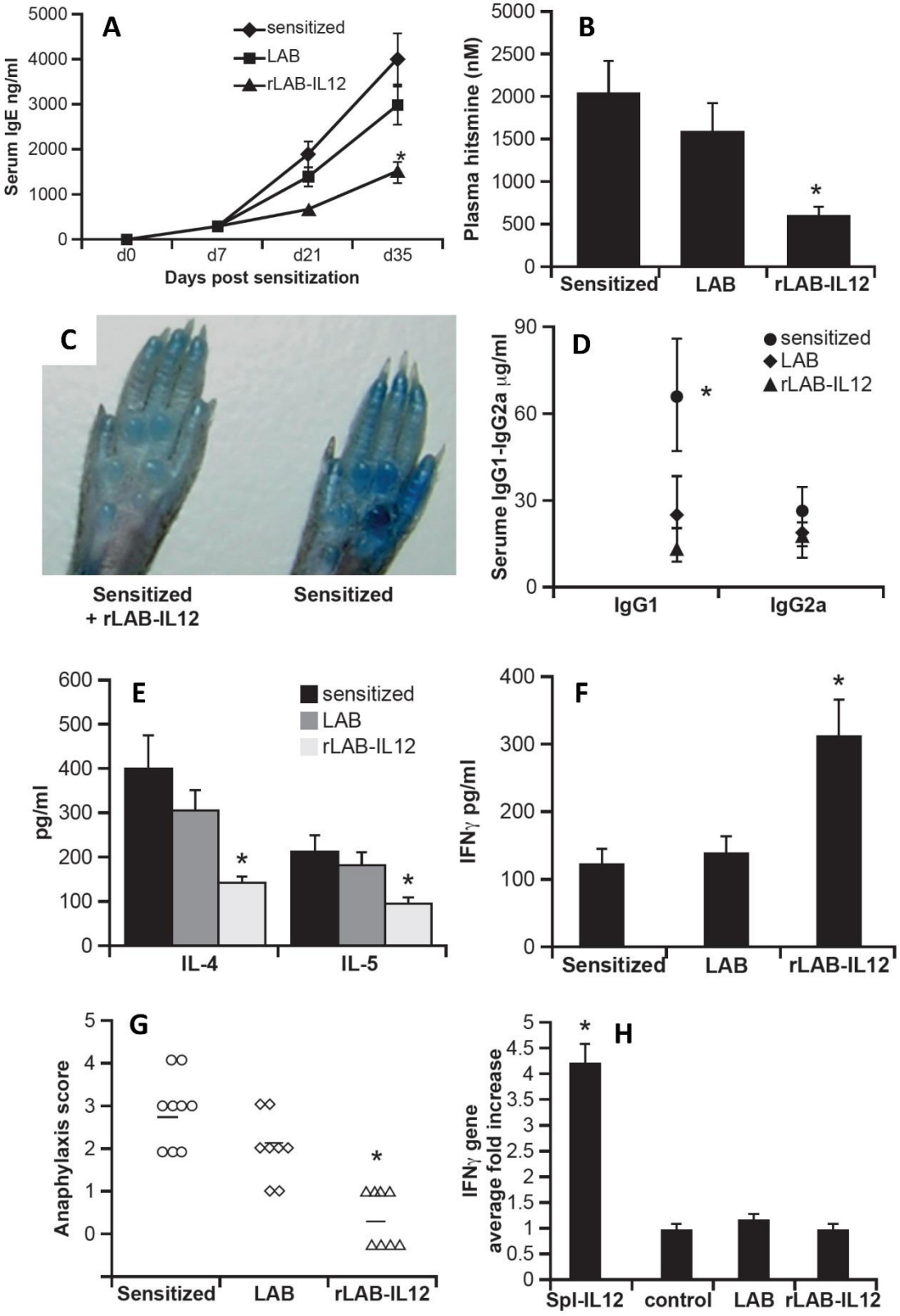

A
sensitized
LAB
rLAB-IL12
Serum IgE ng/ml
5000
4000
3000
2000
1000
0
d0
d7
d21
d35
Days post sensitization
*
B
Plasma hitsmine (nM)
5000
2500
2000
1500
1000
500
0
Sensitized
LAB
rLAB-IL12
*
C
Sensitized
+ rLAB-IL12
Sensitized
D
Serume IgG1-IgG2a μg/ml
90
60
30
0
sensitized
LAB
rLAB-IL12
*
IgG1
IgG2a
E
sensitized
LAB
rLAB-IL12
pg/ml
600
500
400
300
200
100
0
*
*
IL-4
IL-5
F
IFNγ pg/ml
400
300
200
100
0
*
Sensitized
LAB
rLAB-IL12
G
Anaphylaxis score
5
4
3
2
1
0
*
Sensitized
LAB
rLAB-IL12
H
IFNγ gene
average fold increase
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Spl-IL12
control
LAB
rLAB-IL12

Figure 9 Prior to sensitization mice were intragastrically delivered *L. lactis* FI10632 (LAB), *L. lactis* FI10611 secreting mIL-12 (rLAB-IL12) or PBS. The group receiving rLAB-IL12 showed significant reduction in serum IgE (A), plasma histamine (B), vascular leakage (C), IgG1 but not IgG2a (D), and exhibited ameliorated anaphylactic symptoms (Jong et al., 1998) (G). Splenocytes from mice receiving rLAB-IL12 produced significantly lower levels of IL-4 and IL-5 (E), and more IFNγ (F) after *in vitro* CPE restimulation. Finally rLAB-IL12 treatment did not induce long-term local inflammation since 6 days after the last application there was no significant increase in intestinal IFNγ (H). (Experiments performed in triplicate, n=8 to 9 mice/group; two-way ANOVA in A, one-way ANOVA in B, D, E, F, G, H; error bars represent standard error of the mean (SEM))

4.2. Oral delivery of IL-12 down-regulates allergy-associated increase of IEC-derived TSLP

Several reports have indicated TSLP as a key cytokine in allergic response (Han et al., 2012, Liu et al., 2007, Soumelis et al., 2002). Therefore the production of TSLP during sensitization in the presence or absence of oral administration of rLAB-IL12 was investigated. First, quantitative PCR analysis showed that TSLP gene expression was significantly up-regulated in sensitized mice as well as in mice treated with CT alone, compared to control non-sensitized (PBS-treated) mice (**Figure 10 A**). This suggested that CT is required to induce a strong TSLP-mediated Th2 polarized immune response. Next, we observed that administration of rLAB-IL12 prior to the delivery of the sensitizing dose significantly suppressed the expression of TSLP. The regulatory effect of IL-12p70 on IEC-derived TSLP was further confirmed by immunohistochemistry analysis (**Figure 10 B-E**). The latter approach did not detect TSLP neither in PBS nor rLAB-IL12 treated mice (**Figure 10 B, C**) but only in sensitized mice (**Figure 10 D, E**). These results demonstrated the existence of a regulatory effect exerted by IL-12p70 on the production of TSLP by IEC and, thus concurring with several reports (Han et al., 2012, Liu et al., 2007, Soumelis et al., 2002) suggesting an important role of TSLP in allergy.

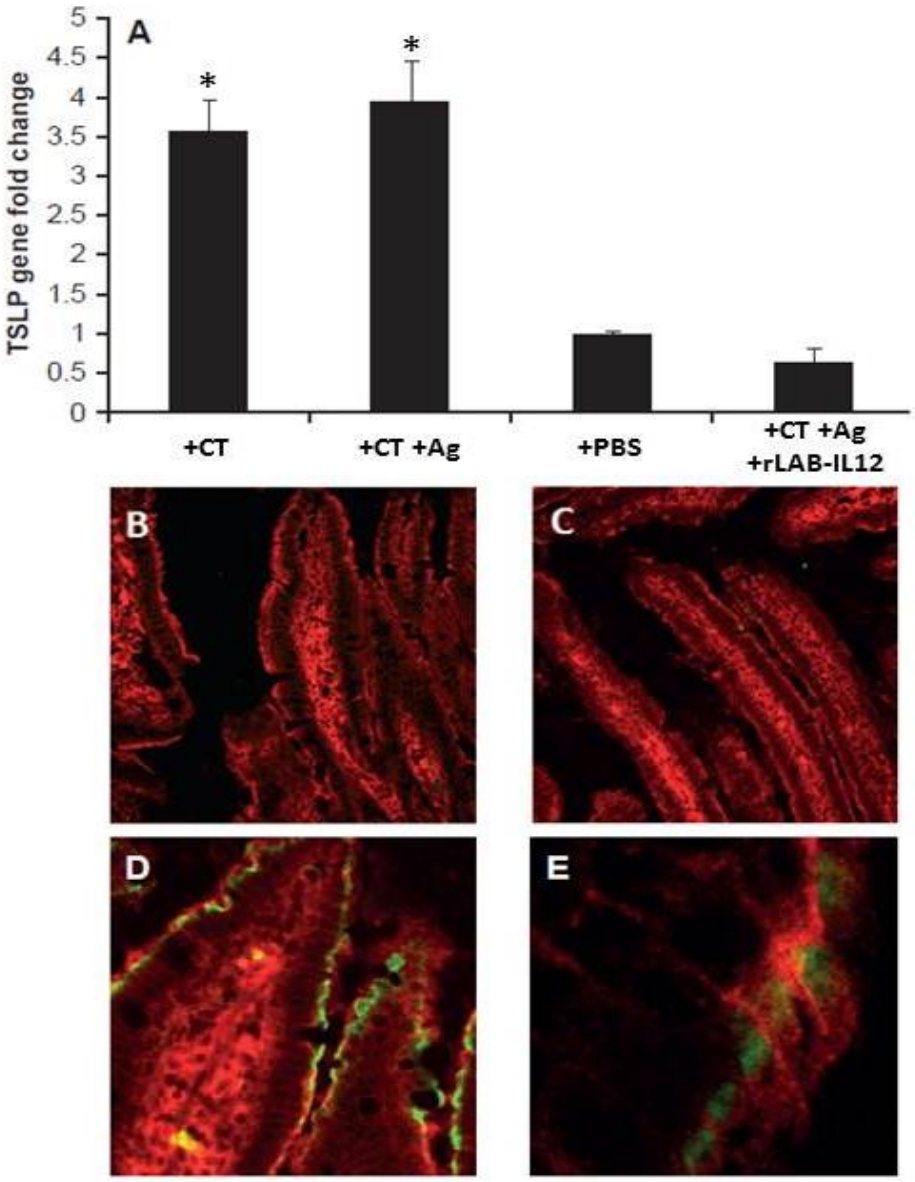


Figure 10 TSLP in the intestine is upregulated as a result of sensitization and treatment with rLAB-IL12 supresses TSLP overproduction. Application of CPE antigen (Ag) in combination with cholera toxin (CT) or CT on its own lead to significant increase in TSLP expression. When rLAB-IL12 was applied before each sensitizing dose there was no increase in TSLP (A). (Experiment performed in triplicate, n=7-9 mice/group); fold change difference >2 is considered significant, error bars represent SEM) Immunohistochemistry of small intestinal tissue sections form PBS (B) and rLAB-IL12 (C) groups showed no detectable TSLP whereas there was enhanced signal in the epithelium of sensitized mice (D) and higher magnification (E).

4.3. Requirement for TSLP in the sensitization process

It remained to be determined as to whether down-regulation of TSLP is directly linked to suppression of allergic reaction. To this end, mice were injected i.p. with two doses of anti-TSLP blocking antibody prior to the delivery of each sensitizing dose. In contrast to what has been observed by others (Chu et al., 2013) administration of anti-TSLP antibody prior sensitization in C3H/HeJ mice had a significant reduction effects on allergic reaction to peanuts. Levels of allergen specific IgE were significantly reduced in mice treated with anti-TSLP antibody (**Figure 11 A**) compared to sensitized control mice; this was accompanied by significant decrease in the production of both IL-4 and IL-5 during *in vitro* antigen recall challenge (**Figure 11 E**) as well as of plasma histamine (**Figure 11 B**). Of note is the observation that in contrast to rLAB-IL12 the administration of anti-TSLP antibody suppressed allergic reaction without affecting levels of IFNγ produced in an *in vitro* antigen recall challenge (**Figure 11 F**). This further highlighted the existence of multiple pathways that can trigger/regulate allergic immune responses. Furthermore administration of anti-TSLP antibody induced a significantly suppressed vascular leakage (**Figure 11 C**) and allergen-specific IgG1 antibody but did not affect antibody of IgG2a isotype (**Figure 11 D**). Finally, lack of decrease in body temperature (**Figure 11 G**) and lower anaphylaxis score (**Figure 11 H**) were observed in mice treated with anti-TSLP antibody**.**

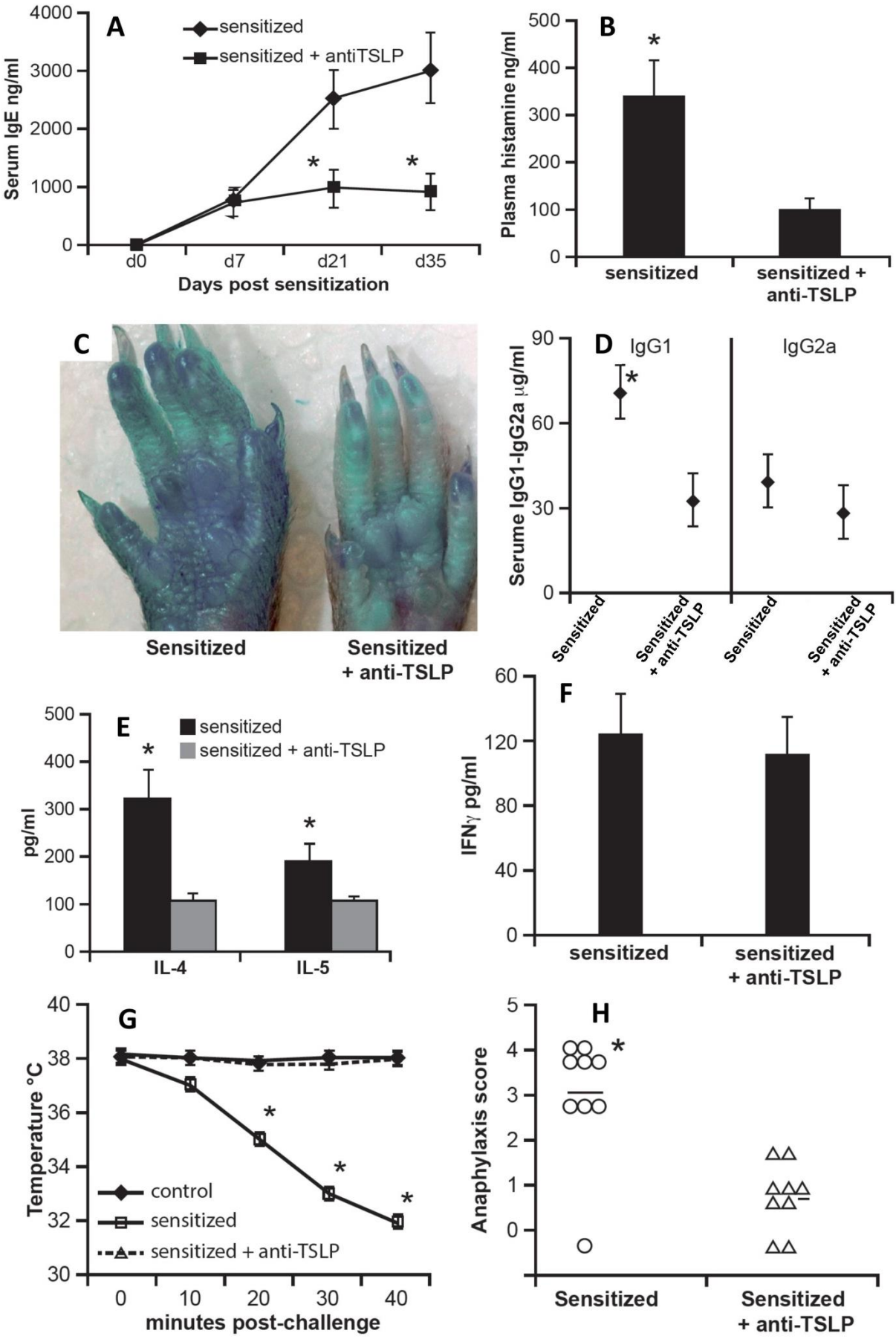

A
sensitized
sensitized + antiTSLP
Serum IgE ng/ml
4000
3000
2000
1000
0
d0
d7
d21
d35
Days post sensitization
B
Plasma histamine ng/ml
500
400
300
200
100
0
sensitized
sensitized + anti-TSLP
C
Sensitized
Sensitized + anti-TSLP
D
IgG1
IgG2a
Serume IgG1-IgG2a µg/ml
90
60
30
0
Sensitized
Sensitized + anti-TSLP
Sensitized
Sensitized + anti-TSLP
E
sensitized
sensitized + anti-TSLP
pg/ml
500
400
300
200
100
0
IL-4
IL-5
F
IFNγ pg/ml
60
120
80
40
0
sensitized
sensitized + anti-TSLP
G
Temperature °C
40
38
36
34
32
30
control
sensitized
sensitized + anti-TSLP
0
10
20
30
40
minutes post-challenge
H
Anaphylaxis score
5
4
3
2
1
0
Sensitized
Sensitized + anti-TSLP

Figure 11 Mice were injected i.p. with two doses of anti-TSLP blocking antibody prior to the delivery of each sensitizing dose. A number of parameters were measured including serum IgE (A), plasma histamine (B), vascular leakage (C), serum IgG1 and IgG2a (D), and rectal temperature (G). Anaphylactic score was assigned as previously described (Jong et al., 1998) (H). Splenocytes isolated from the sensitized animals were re-stimulated with allergen and IL-4, IL-5 (E) and IFNγ (F) were assessed by ELISA. (Experiments performed in triplicate, n=7-9 mice/group; t-test in B, D, E, F; two-way ANOVA in A and G; error bars represent SEM)

4.4. Kinetics of TSLP and IL-12 production in the gut following oral administration of CT

Oral administration of CT, either alone or in combination with antigen, induced a significant increase in TSLP gene expression (**Figure 10**). This observation, along with the notion that CT directly suppressed the production of IL-12 and its specific receptor (IL-12R) (Braun et al., 1999) prompted us to determine the levels of TSLP, IL-12 and IL-12R over time following a single oral delivery (10μg) of CT. Levels of TSLP were not affected during the first 16 h post oral administration of CT; however expression of TSLP significantly increased at 72 h after CT treatment. The pattern of the IL-12 (p40 subunit) expression showed different kinetics. Indeed, a significant decline of IL-12 expression in the gut tissue was detected already at 16 h post-treatment and remained lower at 72 h. Thus, oral delivery of CT initially suppressed the expression of IL-12 and subsequently up-regulated the production of TSLP by IEC (**Figure 12**). Interestingly we observed, for the first time that a substantial percentage of IECs in the small intestine expressed the IL-12Rβ2 chain; however, we also observed that its constitutive expression on IECs **(Figure 13 A)** was not significantly affected by the administration of CT **(Figure 13 B)**.

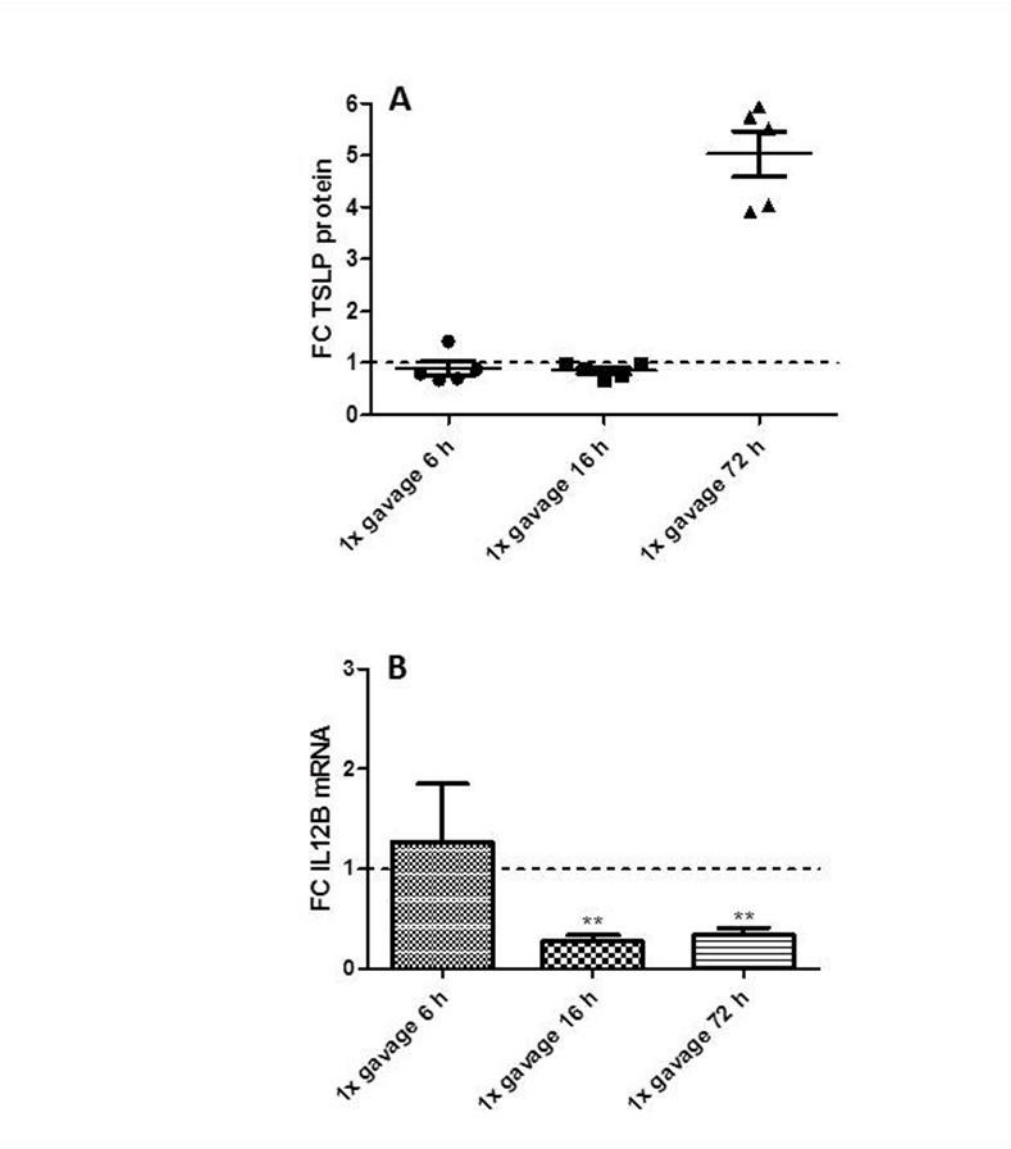


Figure 12 TSLP protein (A) and IL-12p40 (IL-12B) mRNA (B) were measured in mouse small intestine after intragastric administration of a single dose of CT. Reduction in IL-12p40 mRNA expression preceded overexpression of TSLP protein. Decrease in IL-12p40 mRNA was evident as early as 16 h post CT administration while at this early time points TSLP protein levels remained unchanged and increased only after 72 h. (n=5; one-way ANOVA, Bonferroni Multiple Comparison post-test, $p<0.01$=**; error bars represent SEM)

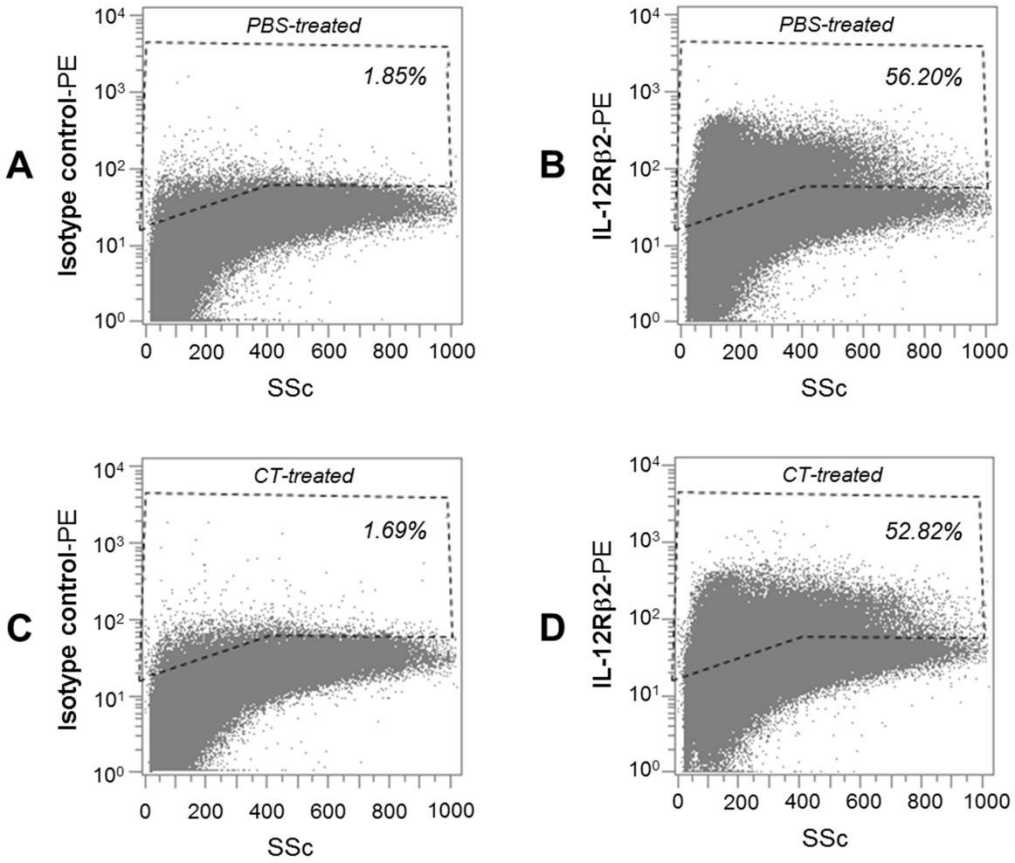


Figure 13 Effect of CT on IEC IL-12Rβ2 expression. Mice were gavaged with PBS (A, B) or CT (C, D) for 48 hours before sacrifice. Isolated IECs were stained with PE-coupled isotype control immunoglobulin (A, C) or anti-IL-12Rβ2-PE antibody (B, D). IL-12Rβ2 expression was compared between the treatments after data acquisition by flow cytometry. (This experiment was performed three times, each time n=4, t-test $p>0.5$)

5. Discussion

The main finding of this work is that oral delivery of recombinant *Lactococcus lactis* secreting the bioactive form of IL-12 (IL-12p70) prior the administration of allergen in a mouse model of food allergy prevented allergic sensitization to food components (peanuts) by possibly down-regulating the production of the pro-allergic cytokine TSLP by the intestinal epithelial cells. The importance of this finding is twofold. First, we have identified a novel regulatory loop that appeared to be important for the intestinal immune homeostasis based on the reciprocal control and regulation between the immune derived IL-12p70 and the epithelium-derived TSLP. Second, we have further shown the biological importance of a finely tuned interaction between the gut epithelium and the underlying immune system. TSLP is a critical cytokine in establishing and maintenance of the intestinal immune homeostasis; it has been shown that the production of (TSLP) plays a major role regulating the generation of non-inflammatory DCs and regulatory T cells (Ito et al., 2005, Rimoldi et al., 2005). However, overproduction of this cytokine is essential for development of food hypersensitivity.

This work further highlights the importance of lympho-epithelial cross talk at mucosal interface in the gut in maintaining health. However, although it is well known that epithelium-derived cytokines such as, TSLP, IL-25 and IL-33 have an impact on the function of cells of the immune system, much less is known about immune cell-derived cytokines that participate in the cross-talk between the gut epithelium and the immune cells and their regulatory function on IECs. It has been previously shown that following microbial challenge the cytokine Macrophage-Migration Inhibitory Factor (MIF) produced by $CD11c^{+}$ cell in the subepithelial area of the specialized follicle-associated epithelium of the Peyer's patch up-regulate microfold (M) cell transport of luminal antigen; thus playing an important role in initial steps of mucosal immune responses (Man et al., 2008). The current work provides an additional example of how an immune-derived factor controls critical features of IECs.

The IL-7-like cytokine TSLP is an epithelial-derived soluble factor with the ability to shape the immunological microenvironment of both respiratory and intestinal mucosae. TSLP is produced by epithelial cells, stromal cells, and basophils and acts on DCs, mast cells, T cells, and B cells (Takai, 2012). TSLP signals by binding to a heterodimeric receptor consisting of the IL-7 receptor α-chain and the low affinity TSLP receptor chain (TSLPR). The IL-7Rα is required for the generation of a high affinity binding site and for signalling (Pandey et al., 2000). Exposure of ECs to viral, bacterial, and parasitic pathogens, as well as to various cytokines can induce TSLP expression (Takai, 2012). It promotes Th2-type intestinal immunity against helminthic infections. This cytokine negatively regulates production of IL-12/23 p40 by DCs *in vivo* and *in vitro* and suppresses Th1 responses (Taylor et al., 2009). Interestingly, mRNA expression of TSLP is significantly reduced in patients with CD, whereas TSLP is overexpressed in patients with UC (Rimoldi et al., 2005, Tanaka et al., 2010). $TSLPR^{-/-}$ mice have pronounced Th1 and Th17 responses that lead to worsened DSS colitis and pathologic Th1-mediated inflammation in *Trichuris muris* infection (Taylor et al., 2009). Critically, TSLP has been identified as a mediator of allergic reactions including atopic dermatitis, asthma and gastrointestinal allergy (Zhou et al., 2005, Ziegler, 2012, Blázquez et al., 2010). In addition to this, here we describe that the production of TSLP by IEC is possibly regulated by IL-12p70 and that this regulatory pathway has a critical role in shaping intestinal immunity. It has been shown previously that TSLP supresses the production of IL-12 in DCs (Rimoldi et al., 2005); our study suggest that in turn IL-12 possibly controls the production of TSLP by IEC in a regulatory feedback loop that contributes to maintaining the intestinal immune homeostasis. In our opinion this observation shows interplay between these two cytokines produced by the intestinal epithelium and the immune system that is central in the control of intestinal immunity.

It has been previously shown that different mouse strains exhibit variable degree of susceptibility to sensitization. For example the C3H/HeJ mice are highly susceptible whereas Balb/c under the same sensitization regimen and although producing a significant amount of IgE antibody do not develop type I

hypersensitivity reaction. Using this model it has been demonstrated that reduced levels of IL-12 in the gut, but not systemic, immune system is critical for the development of food hypersensitivity (Temblay et al., 2007). The availability of this model provided us with the possibility to test the biological relevance of the TSLP/IL-12 cross regulation and its implication in health and disease. We observed that by restoring levels of IL-12 in the gut we could abolish the expression of TSLP by IEC and in so doing suppressing food allergy to peanut. This demonstrates that lack of IL-12 and the subsequent over-expression of TSLP play a major role in shaping the proper immune response to food components. Interestingly, it has been previously shown in the same mouse model of food allergy that oral delivery of a similar bacterial vector secreting IL-10 suppressed food allergy. The notion that cytokines with very different biological activity, such as the anti-inflammatory IL-10 and the pro-inflammatory IL-12 both have the ability to suppress IgE-mediated allergy illustrates the complexity of the cytokine network involved in the regulation of immune response to food components. In addition, although the possibility of using recombinant bacterial vector to deliver IL-12 to treat allergy will require further investigation it is worth noticing that, at least in mice repeated delivery of rLAB-IL12 did not compromise the inflammatory status of the gut and no side effects were detected. However, in the case of IL-12 it was still plausible that restoring the balance between TSLP and IL-12 did not have a direct effect on peanut allergy in our experimental model. To address this we administered anti-TSLP blocking antibody prior to each sensitization dose in mice. This step was necessary since it has been suggested that TSLP is not central to peanut allergy while IL-33 plays a more critical role (Vossenkamper et al., 2004). Although we have not addressed the role of IL-33 we have observed that in contrast to what was previously reported specific blocking antibody to TSLP suppressed allergic reaction as monitored by a variety of immunological and visual parameters. This discrepancy may reflect the different genetic make-up of the host and its effect on susceptibility/resistance to peanut allergy; however at this stage we are not in the position to reconcile these discordant observations. In addition, our findings are in agreement with a number of studies that prescribe TSLP an

essential role in allergy. For example, application of anti-TSLP antibody in asthma reduces allergen-induced bronchoconstriction (Gauvreau et al., 2014). Furthermore, skin-specific overexpression of TSLP has been reported to result in atopic dermatitis associated with pronounced increase in Th2 $CD4^+$ cells homing to the skin and elevated serum IgE (Yoo et al., 2005). Similarly, lung-specific TSLP overexpression is sufficient to elicit severe airway allergic inflammation and development of asthma-like phenotype, accompanied with Th2 $CD4^+$ infiltrates, eosinophilia and increased serum IgE (Zhou et al., 2005).

The regulatory effects of IL-12 on the production of TSLP were further supported by the observation that oral delivery of CT differentially affected the kinetics of the TSLP and IL-12 production in the gut. Indeed, CT-mediated down-regulation of IL-12 production was already evident at 16 h following the delivery of CT while expression of TSLP was unaffected at 16 h but showed a significant up-regulation at 72 h post-administration; thus further suggesting that reduced production of IL-12 plays an important role in TSLP overexpression in allergy.

Overall, these data showed that the production of TSLP by IECs is under the control, either directly or in-directly by IL-12 and that this regulatory mechanism plays an important role in maintaining the intestinal immune homeostasis.

Chapter 3: IL-12-mediated regulation of TSLP production by IECs - preliminary study on the mechanism

1. Introduction

The way we look at the intestinal epithelium has dramatically evolved in the past few years. Although the main task of the epithelium overlying mucosal surfaces of the GI tract is to provide an effective barrier to the vast majority of macromolecules and microorganisms present in the intestinal lumen; it has become evident that the signals originating from the variety of cells forming the intestinal epithelium contributes to shape the immunological environment of the gut. The role of this selective barrier is achieved by several means. Firstly, the epithelium is composed of cells joined by tight junctions allowing passage of water and ions but providing an effective mechanical barrier to macromolecules (Duffey et al., 1981). Secondly, local secretions of mucus, secretory IgA antibodies, and a thick glycocalix cover the mucosal surfaces (Maury et al., 1995, Frey et al., 1996, Kett et al., 1995, Johansson et al., 2011). In addition to this vital function, the intestinal epithelium plays a key role in shaping mucosal immune responses by creating the appropriate immunological microenvironment described in detailed in **Chapter 2**. Indeed, IECs are capable of producing a number of cytokines upon bacterial invasion, some examples being IL-8, monocyte chemotactic protein 1, and macrophage inflammatory protein 3α (Dahan et al., 2007). In addition to secretion of signaling molecules IECs have been implicated in cell-to-cell interactions with macrophages (Rescigno et al., 2001) and T cells (Gonnella and Wilmore, 1993). However, the cytokine traffic is not by all means unidirectional and although this part of the lympho-epithelial cross talk has received less attention by the scientific community immune-derived factors can have an impact on the IECs function.

One of the first studies that addressed the role of immune–derived signals in shaping regulatory features of the IEC was carried out by Haller et al. (2000).

Their work has first shown that the presence of lymphocytes is essential to impart IEC (human Caco2 cells) the ability to respond to bacterial challenge (Haller, 2000). It is to be noted that the IEC and PBMC were co-cultured using Transwell culture devices and were not in direct cell-to-cell contact (Parlesak et al., 2004). Although the soluble factors responsible and the overall mechanism underlying these events were not identified it became clear that cytokine-mediated cross talk between immune cells and IECs is an important part of regulatory network operating at the mucosal interface in the gut. Another important example of the impact of immune-derived factors that contribute to shape the function of the epithelial cells is represented by macrophage migration inhibitory factor (MIF). Indeed, MIF is produced by $CD11c^+$ cell present in the SED area of the PPs rapidly after *in vivo* bacterial challenge. It has been shown to act on IECs and induce M-cell like phenotype thus facilitating antigen transport (Man et al., 2008). To these examples we can now add our finding that IL-12 regulates the production of TSLP from IECs. However, despite the fact that the latter observations pertain to events that participate in important aspects of intestinal immunity the mechanism of action and the way immune-derived cytokines interact with IECs remained to be determined.

2. Hypothesis

In **Chapter 2** we have reported that a substantial percentage of IECs did express the β2 subunit of the IL-12R. This prompted us to investigate the role of IL-12Rβ2 on IECs. There were several possible scenarios for IL-12-mediated regulation of TSLP. One possibility was that IL-12, either on its own or in synergy with unknown partner, could regulate TSLP directly by binding the IL-12R. Alternatively, IL-12 action could take place indirectly by inducing the production of additional (intermediate) soluble factors that in turn can impact on TSLP expression.

3. Methods

Animals

Female mice from B6.12931-IL12b^{trn1Jm}/J (IL-12p40 knockout) and B6.12931-IL12Rβ2^{trn1Jm}/J (IL-12Rβ2 knockout) strains, along with the appropriate wild type C57BL/6J controls, were purchased from Jackson Laboratories. Balb/c and C57BL/6 mice were purchased from Disease Modelling Unit, UEA. All procedures were carried out in accordance with U.K. Home Office regulations, license conditions and University of East Anglia guidelines on animal welfare and the current Italian law for animal experimentation.

MODE-K cells cultures

Mode-K cells (Vidal et al., 1993) originally isolated from the same mouse strain used for the *in vivo* study on food allergy (C3H/HeJ) were cultured either alone or co-cultured in the presence of gut (Peyer's patch-derived) lymphocytes.

A-MODE-K cells were washed with Hank's Balanced Salt solution (HBSS Gibco) and detached with 1 ml TrypLE (Gibco) per 25 cm^2 and incubated for 10-15 minutes at 37° C. The cells were transferred into Falcon tubes containing 6 volumes of HBSS and spun down (300 g for 10 min). After counting the cells were seeded into 12-well plates, 25cm^2 or 75 cm^2 T flasks at 2.5 x 10^4 cells/cm^2 and incubated at 37° C, 5% CO_2. Growth media for MODE-K: high glucose DMEM medium (Invitrogen), 10% (vol/vol) heat-inactivated FCS, penicillin (final conc. 100 U/ml) and streptomycin (final conc. 100 µg/ml) (Invitrogen). For flow cytometry, cells were washed with and PBS with 0.1% (vol/vol) sodium azide, 2% (vol/vol) FCS and 4mM EDTA (PBSAFE). Subsequently, the cells were detached with a cell scraper (Griener Bio-one), resuspended by pipetting, passed through a needle (0.3 mm x 0.13 mm) and filtered with a cell strainer (ø 40 µm BD falcon).

B- MODE-K cells were seeded in the top compartment of 12 mm ø Transwells, pore size 3 µm ø (Costar) at 1x10^5/well. After four days of growth PPLs were added to the culture. First, media was exchanged with fresh DMEM (Invitrogen), 10% (vol/vol) heat-inactivated FCS, penicillin (final conc. 100 U/ml), streptomycin (final conc. 100 µg/ml) (Invitrogen) and gentamycin (final conc. 50

µg/ml). PPLs were isolated from PPs by mashing between frost edged sterile glass slides and filtered through strainer (ø 40 µm BD falcon). Washed with media once and resuspended to $1x10^6$/ml. $5x10^5$ PPLs were added in the top compartments of the Transwells and the MODE-K layer was scraped to ensure appropriate contact. For non-contact co-culture $1x10^6$ PPLs were added to the bottom compartments, MODE-K layer was scraped to ensure that the conditions the same among all treatments. After three days expression of IL-12Rβ2 was examined by flow cytometry.

Antibodies

The following specific antibodies and isotypes were used for flow cytometry: Alexafluor 488 goat anti-rat IgG2b, Clone: poly4054 (1:50; Biolegend); anti-mouse CD130 (GP130), Clone: 4H1B35 (1:50; Biolegend); Rat IgG2b, Clone: RTK4530 (1:50; Biolegend); APC anti-mouse E-cadherin, Clone: 114420 (1:10; R&D Systems); APC rat IgG2a, Clone: 54447 (1:10; R&D Systems); FITC rat anti-mouse CD45, Clone: 30-F11 (1:20; Caltag laboratories); PE anti-human/mouse IL-12Rβ2, Clone: 305719 (1:10; R&D systems); PE mouse IgG1, Clone: 11711 (1:10; R&D systems); PE anti-mouse IL-12Rβ2, Clone REA200 (1:20; Miltenyi); PE Isotype (A3) REA293 (1:20; Miltenyi); PE anti-mouse CD212 (IL-12Rβ1), Clone: 114 (1:20; BD Pharmigen); PE mouse IgG2a, Clone: G155-178 (1:20; BD Pharmigen)

Flow cytometry

For detection of extracellular markers $1x10^6$ cells were stained in 100 µl final volume. For intracellular detection of phosphoproteins cells were fixed with equal volume of 8% buffered formalin. After 10 minutes at 37° C, fixed cells were washed once with PBSAFE and allowed to cool on ice followed by permeabilisation on ice for at least 30 minutes with Perm Buffer III (BD Biosciences). Permeabilised cells were washed twice with PBSAFE and stained for 30 minutes at room temperature. For intracellular detection of cytokeratin cells were washed once with 1% Saponin Buffer (SB; 1% Saponin in PBSAFE), resuspended in SB and incubated with anti-cytokeratin FITC (Beckman Coulter) for 1 hour on ice followed by one wash in SB and a second wash in PBSAFE.

Samples were fixed by adding equal volume 2% formalin and analysed with EC800 (Sony Technologies), software version 1.3.6 (Sony Technologies).

Isolation of primary intestinal epithelial cells for flow cytometry

Faecal material and fat were removed and intestinal tissues were washed in 25 ml PBSAFE which contained 0.05% (vol/vol) of protease inhibitor cocktail (Sigma Aldrich) (per 6 cm intestine) and subsequently washed twice into 25 ml PBSAFE containing 0.8 mM DTT (Sigma Aldrich). IECs were detached by incubation in PBS containing 10% FCS, 1 mM EDTA at 37 °C for 20 min and vigorously shaken 20 times. This step was repeated until no more IECs were detaching and visual inspection showed bare villi. Cells were filtered through 40 µm ø strainers, spun (10 min at 300g) and washed once in PBSAFE, resuspended appropriately and counted.

Isolation of primary intestinal epithelial cells for functional studies

The following protocol was used to extract IECs from the small intestine of 2 mice, volumes and amounts were scaled according to number of mice used for experiments.

Mice were sacrificed by cervical dislocation; small intestines were removed, dissected longitudinally and washed with sterile PBS. Tissue was cut into 0.5 cm pieces and washed with sterile PBS. To remove mucus, the tissues were first washed in 20 ml PBS containing 10% FCS and 1 mM DTT. Subsequently IECs were detached by incubation in 20 ml PBS containing 10% FCS, 15 mM EDTA at 37 °C, 250 rpm for 15 and 20 minutes, respectively. Before and after each incubation, tissue-buffer mix was vortexed for 1 minute. Cells were filtered through a 70 µm ø cell strainer and the filtrate was centrifuged at 300 g, 7 minutes at 4° C. The resultant pellets from each wash were pooled, resuspended in PBS containing 10% FCS and stored on ice. $CD45^+$ cells were removed from crude IEC preparations using magnetic labelling with anti-CD45 Microbeads (Miltenyi Biotec) and magnetic separation with LD MidMACS depletion columns (Miltenyi Biotec).

Testing functional integrity of IL-12Rβ2 through MAPKp38 phosphorylation

Mouse IEC were washed twice in DPBS (Sigma-Aldrich) then placed in a 37° C water bath in the presence of 500 μM H_2O_2 or 2 μg/ml IL-12p70 (Biolegend) for various times. Control cells were left untreated. At the end of the incubation period and while the cells were still in the water bath, an equal volume of 8% buffered formalin was added. After 10 minutes fixed cells were washed once with PBSAFE and allowed to cool on ice followed by permeabilisation on ice for at least 30 minutes with Perm Buffer III (BD Biosciences, UK). Permeabilised cells were washed twice with PBSAFE and stained for 30 minutes at room temperature with rabbit anti-phospho-MAPKp38 (Cell Signaling, USA) or normal rabbit IgG (Santa Cruz Biotechnology, USA). After one wash with PBSAFE cells were incubated for further 30 minutes with donkey anti-rabbit IgG-FITC (Santa Cruz) and washed once with PBSAFE. Data were acquired by flow cytometry. For viability assessments cell were washed and resuspended in 1ml PBS. Dead cells were stained using Live/Dead Fixable Violet Dead Cell Stain Kit (Life Technologies, UK) according to the manufacturer's instructions. Surface staining was performed before and intracellular staining after treatment with viability dye.

Transmission electron microscopy

Small fragments of gut were fixed in 4% paraformaldehyde and, following dehydration, embedded in Epon 812. After cutting and mounting on Formvar-coated grids, unspecific binding sites on sections were quenched with 1% ovalbumin dissolved in PBS. Then, sections were incubated overnight at 4° C with rabbit anti-IL12Rβ2 (Bioss Inc.) followed by 15 nM gold-conjugated anti-rabbit IgG (BB International). After washing, sections were air-dried, stained with uranyl acetate, and later examined with a 201 Philips transmission electron microscope.

Organoid isolation and culture

Small intestine was flushed with DPBS (Sigma-Aldrich), cut longitudinally and the villi were scraped off with a glass coverslip. Mucus was washed off three times with cold DPBS 2mM EDTA. Tissues were incubated for 30 minutes at 4° C

in DPBS 2mM EDTA. The incubation step was repeated into fresh DPBS 2mM EDTA for another hour at 4°C. Remaining villi were filtered out with 70 µm ø cell strainers. Pelleted crypts were resuspended in Matrigel matrix (Corning) and 20 µl was seeded per well of a pre-heated (37° C) 48-well tissue culture plate (Breiner Bio One). The plate was incubated for 5 minutes (37° C) before adding 0.25 ml media. Organoids were grown at 37° C and 5% CO_2 and media was renewed daily. After 7 days organoids were passaged by dissociating from the matrix by pipetting with a 200 ml pipette, spun down (600 rcf for 5 min), and resuspended into fresh Matrigel (ratio 1:3). Media: advanced DMEM/F12 (Invitrogen), 1% (vol/vol) Glutamax 100x (Invitrogen), 5mM HEPES (Invitrogen) and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), 0.02% (vol/vol) B27 supplement (Invitrogen), 0.01% (vol/vol) N2 supplement (Invitrogen), 1.25 mM n-Acetylcysteine (Sigma-Aldrich), 50 ng/ml recombinant mouse epidermal growth factor (EGF) (Invitrogen), 4 ng/ml mouse Noggin (Peprotech), 500 ng/ml recombinant mouse R-spondin (R&D) and 10 µM ROCK kinase inhibitor (Sigma-Aldrich).

Q-PCR

RNA extraction was carried out using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions with additional RNase-free DNase set (Qiagen) treatment. Quantity and purity of each RNA sample extracted was determined with Nanodrop ND-1000 Spectrophotometer. The mRNA (300 ng/reaction) was reverse transcribed into cDNA with the QuantiTect Reverse transcription Kit (Qiagen). Q-PCR was performed with ABI 7500 or ABI 7300 sequence detection systems (Applied Biosystems) and mRNA expression of TSLP and IL-12Rβ2, was measured using the following primers from QuantiTect primer Assay (Qiagen): Mm Tslp_1_SG; Mm IL12Rb2_1_SG. Gapdh and 18S mRNA were used as endogenous controls and quantified with QuantiTect primers (Qiagen) Mm GAPDH_3_SG and Mm Rn18s_3_SG. SYBR GREEN Mastermix contained 10% (vol/vol) of 20 mg/ml BSA (Biolabs new England); 1% (vol/vol) 50 mg MgCl2 (Bioline); 2% (vol/vol) ROX reference Dye (Invitrogen); 10% (vol/vol) of Quantitect primer Assay; 0.6% (vol/vol) of 100x SYBR Green nucleic acid staining

(Invitrogen); 1,4% (vol/vol) water; 50% (vol/vol) 2x Immomix (Bioline); 25% (vol/vol) cDNA. Real-time PCR reactions were performed, using a 10 min hot start at 95° C, followed by 50 cycles of denaturing at 95° C for 30 s, annealing at 60° C for 30 s and extension at 72° C for 35 s. Reactions were carried out using three technical replicates for every biological replicate. All samples were normalized to 18S expression using the ΔΔCT method.

Protein isolation for cytokine measurement

Protein was extracted by running samples in tubes with acid washed 3 mm ø glass beads (VWR) on fast prep (6x 1 min with rest on ice inbetween, speed 4 m/s) in CelLytic MT (Sigma) containing 1% (vol/vol) protease inhibitor cocktail (Sigma). Samples were centrifuged (800 g for 5 min), transferred and centrifuged again (100 g for 10 min) and supernatants were collected and protein concentration determined with Nanodrop ND-1000 Spectrophotometer. Intestinal levels of TSLP (pg/ml) were measured by using ELISA MAX Deluxe set (Biolegend) according to the manufacturer's description.

4. Results

4.1. IL-12R expression and function on primary IECs

In the previous chapter we have shown that a substantial percentage of IECs constitutively expressed the β2 chain of the IL-12R (IL-12Rβ2). This prompted us to determine whether IL-12 directly controlled the production of TSLP by binding to the receptor or whether the action of IL-12 is mediated by additional immune mediator(s) produced by IECs following interaction with IL-12p70. First, we used flow cytometry analysis to characterize the IL-12R.

Expression of IL-12Rβ2 was observed on approximately 58% of intestinal (ileum) $CD45^-$ cells and in a small proportion (>1%) of $CD45^+$ cells (**Figure 14 A**); the epithelial nature of these cells was confirmed by monitoring the expression of IL-12Rβ2 on E-cadherin$^+$ cells (**Figure 14 E**). However, the expression of IL-12Rβ2 on these cells was not paralleled by the expression of the complementary (Vignali and Kuchroo, 2012) IL-12Rβ1 chain (**Figure 6**) (**Figure 14 C**).

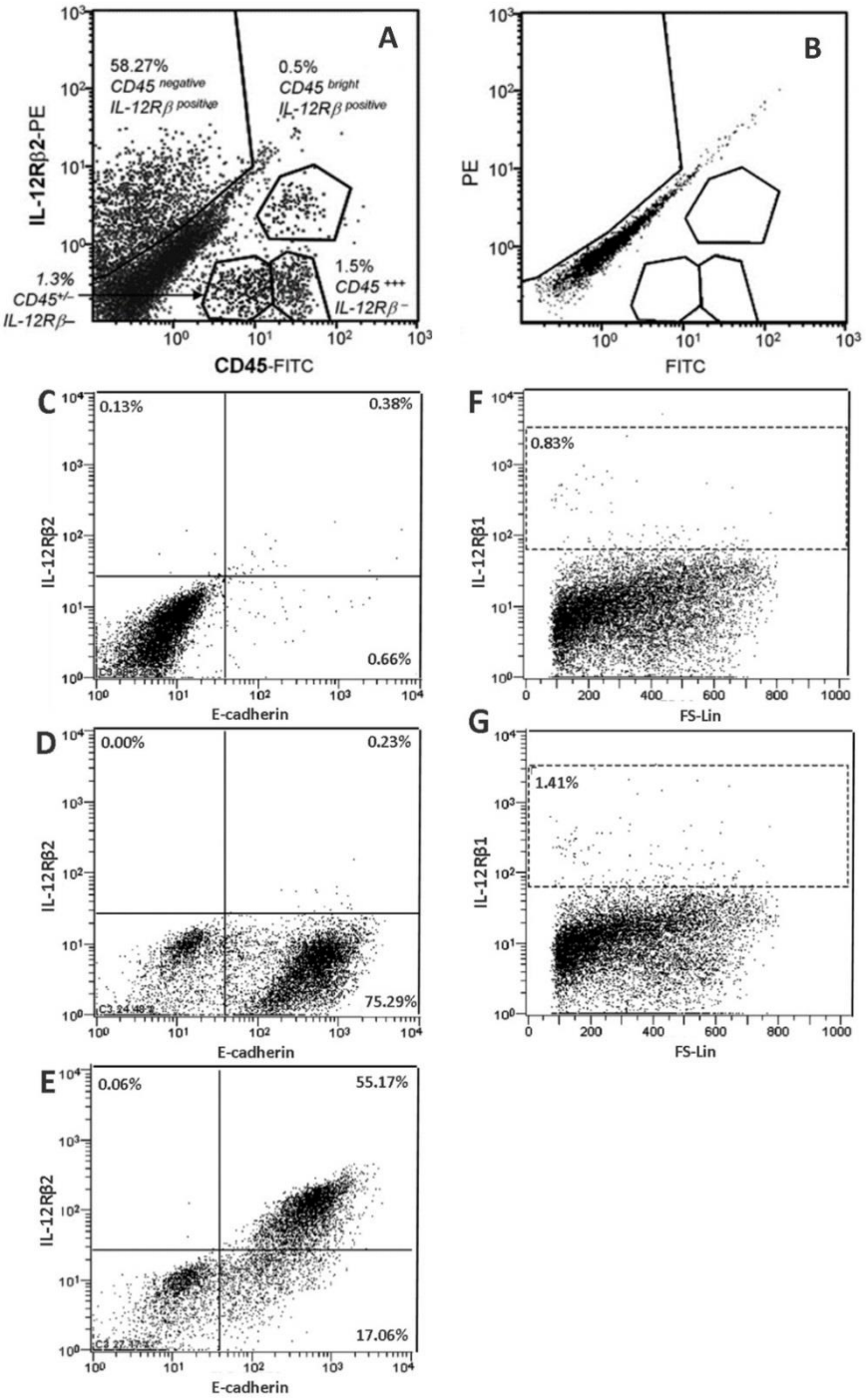


Figure 14 Intestinal epithelial expression of IL-12R. IL-12Rβ2 was detected on approximately 50% of intestinal (ileum) $CD45^-$ cells and in a small proportion (>1%) of $CD45^+$ cells (A), appropriate isotype control (B). The majority of the IL-12Rβ2$^+$ cells were also E-cadherin$^+$ (E), where appropriate isotype control is shown in C and single E-cadherin staining in D. However, despite the presence of IL-12Rβ2 on these cells no corresponding IL-12Rβ1 chain could be detected (G) with corresponding isotype control in G.

Expression of the IL-12Rβ2 chain was further confirmed in IECs following immuno-gold staining in the Golgi area (**Figure 15 A**), in the proximity of the basolateral membrane (**Figure 15 B**) and most important at the interface between IEC and lymphoid cells (**Figure 15 C**).

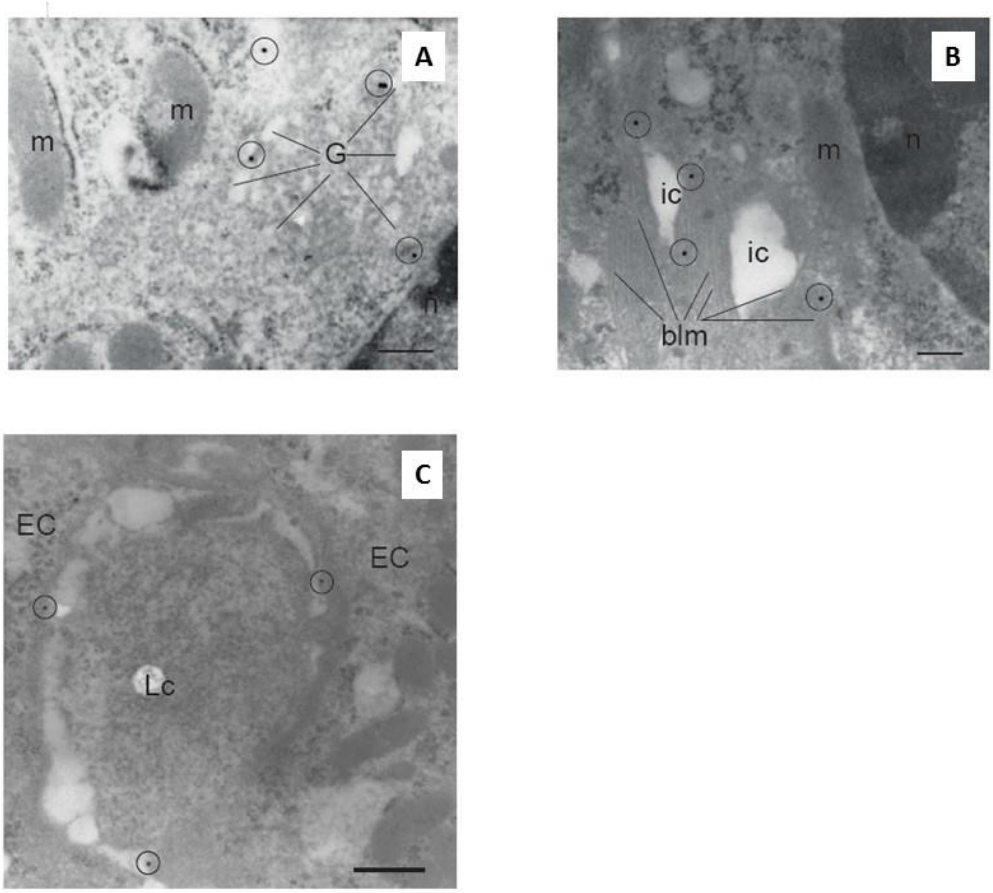


Figure 15 TEM of mouse small intestine showing that IL-12Rβ2 in IECs is found in the Golgi (A) as well as the basolateral membrane (B) and in the interface between IECs and lymphoid cells (C). EC=epithelial cell, LC=lymphoid cell, m=mitochondria, n=nucleus, ic=intracellular space, blm=basolateral membrane

IL-12Rβ2 is not only capable of associating with IL-12Rβ1 to form IL-12R but also it has been reported to interact with CD130 (gp130) as part of the IL-35 receptor (Collison et al., 2012a).Therefore, in order to better characterize the IL-12Rβ2 the presence of CD130 on IECs was also investigated. However, flow cytometry showed that gp130 was not expressed by freshly isolated IECs **(Figure 16).**

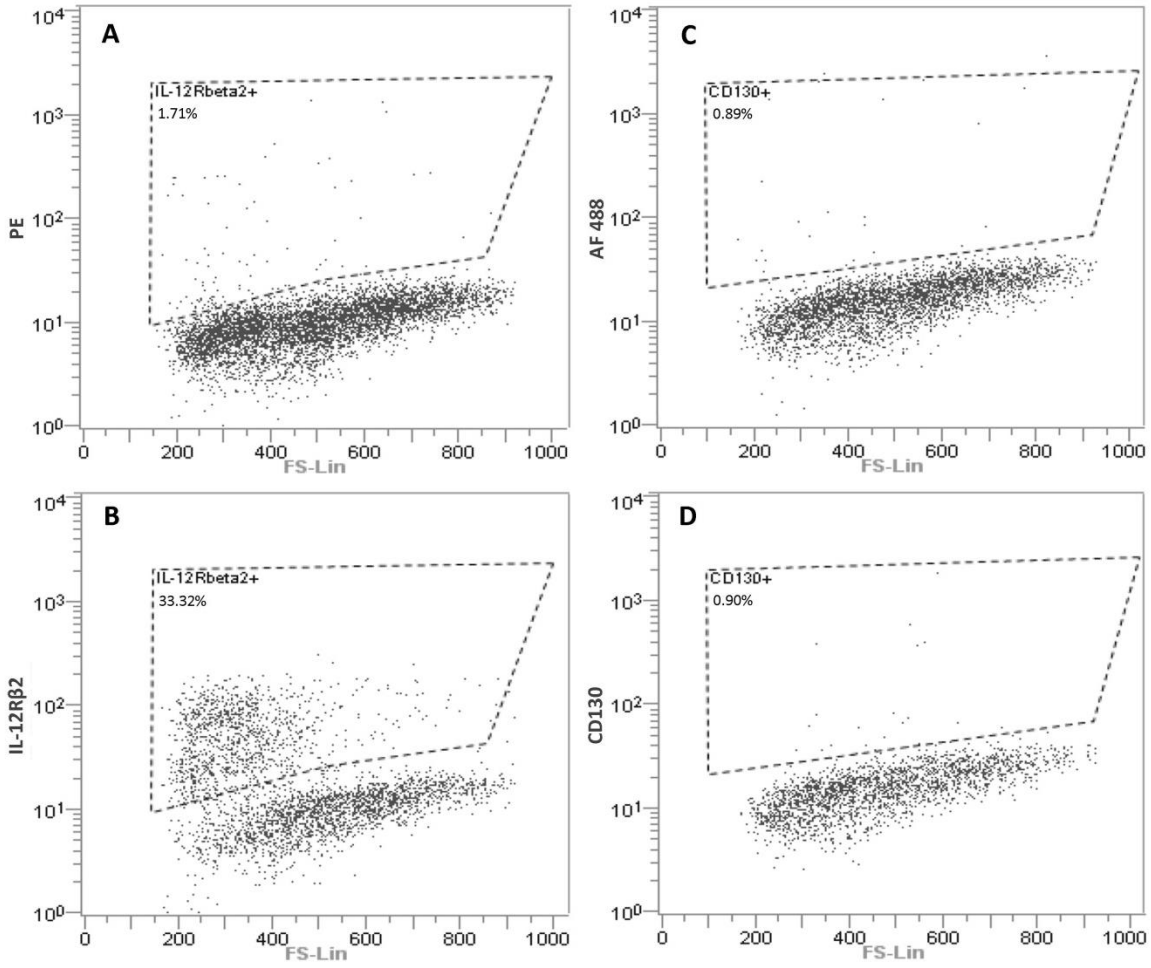


Figure 16 E-cadherin^{+} IECs from mouse jejunum were positive for IL-12Rβ2 (B) where the appropriate isotype control is displayed in (A). IECs did not express CD130 (D); the appropriate isotype control is displayed in (C). (This experiment was performed three times, n=6 mice, representative results are shown)

4.2. Investigating the functionality of IL-12Rβ2 on primary IECs

We then asked the question whether the IL-12Rβ2 chain expressed on freshly isolated primary IECs retained the ability to respond following the engagement with IL-12p70. MAPKp38 has been previously shown to participate in alternative IL-12Rβ1-independent IL-12 signalling cascade (Verhagen et al., 2000). Thus, to assess the function of IL-12Rβ2, freshly isolated IECs were cultured in the presence of IL-12p70 or hydrogen peroxide (H_2O_2) a non-specific activation stimulus. Rapidly, after the application of the stimuli, significant increase of phospho-MAPKp38 levels was observed following challenge of IECs (**Figure 17**).

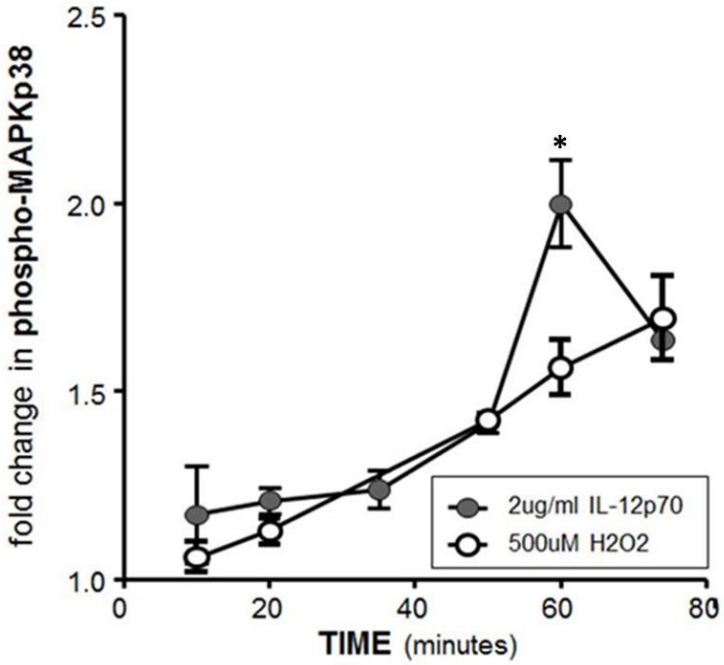


Figure 17 *Ex vivo* stimulation with IL-12p70 lead to increase in intracellular phosphorylation of MAPKp38 in primary small intestinal epithelial cells. Stimulation with H_2O_2 was used as a positive control that produced gradual increase in phospho-MAPKp38 with time. (Experiment performed three separate times, n=9 reactions/condition; two-way ANOVA; error bars represent SEM)

4.3. Levels of TSLP did not increase in IL-12Rβ2$^{-/-}$ mice

The expression of a functional, albeit incomplete form of the IL-12R raised a series of questions, the most notable being: does IL-12p70 suppress the production of TSLP by IECs directly by binding the IL-12Rβ2 chain on IECs or indirectly via the production of intermediate soluble factors? To start answering this complex question we determine the levels of TSLP in IL-12Rβ2$^{-/-}$ mice. Both levels of TSLP gene expression and tissue protein in the intestine were assessed by Q-PCR and ELISA. We observed that the production of TSLP was not significantly different when compared to WT controls (**Figure 18 A, B)**. Similarly, no change in TSLP mRNA was detected in knockout mice lacking IL-12p40 **(Figure 18 C)**. This result showed that IL-12 controlled the production of TSLP indirectly and strongly suggested that other immune mediator(s) that remain to be identified participate in the IL-12/TSLP regulatory loop.

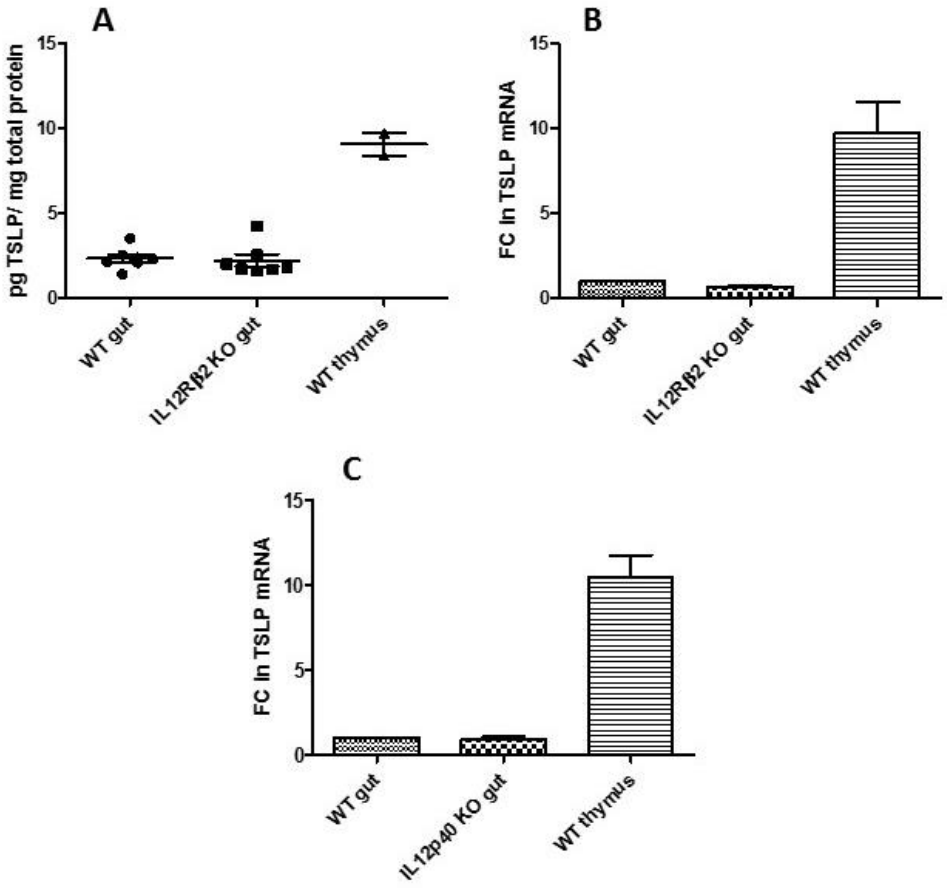


Figure 18 No change was observed in the intestinal TSLP protein (A) and mRNA (B) levels in IL-12Rβ2 knockout mice. In addition, mice deficient in IL-12 signalling due to gene knockout of IL-12p40 had intestinal TSLP mRNA levels

similar to those observed in WT (C). (n=6 to 7; comparison between WT and KO gut, thymus measurement used as positive control but not included in the analysis, fold change difference >2 is considered significant; error bars represent SEM)

4.4. Developing models to study the function and expression of IL-12Rβ2 on IECs:

4.4.1. Intestinal organoids

Parallel experiments were devised to investigate the expression and function of IL-12β2 on IECs. Fragile when separated from each other, the poor survivability of IECs *ex vivo/in vitro* prohibits investigation of the long term response to IL-12p70 stimulation. In order to overcome this problem we adopted organoid culture (**Figure 19 A**) as a method of keeping IECs *ex vivo/in vitro* for a period of time sufficient to observe change in mRNA and protein after stimulation with IL-12p70. First step in validating this model was to monitor whether the IECs retain their expression of IL-12Rβ2 (**Figure 19 B**). Flow cytometry analysis performed on mouse small intestine organoids cultured for 7 days showed that, although the organoids expressed E-cadherin, a canonical marker for mature IECs (**Figure 19 B**), these cells were characterized by the lack of expression of both IL-12Rβ2 and the accessory molecule GP130.

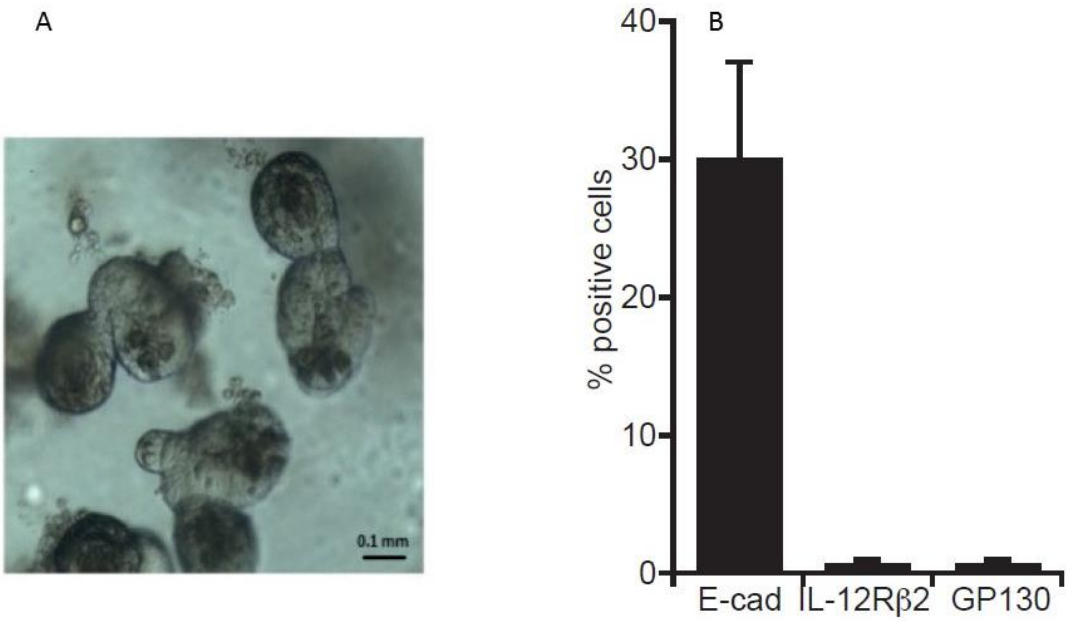


Figure 19 Mouse small intestinal organoids. A) Representative light microscopy image after five days of growth. B) Expression of specific surface markers on the organoids after seven days of growth. Data is pooled from two independent experiments. (This experiment was performed two times, each time n=4 wells of organoids)

4.4.2. MODE-K cell line as a model for studying IECs responses to IL-12

The lack of expression of the IL-12Rβ2 on organoid-associated IECs prompted us to investigate the expression of the receptor on mouse small intestine cell line MODE-K (Vidal et al., 1993). Incidentally, this cell line had the benefit of originating from the same mouse stain (C3H/HeJ) used for the work on food allergy described in **Chapter 2**. In addition to this, the cell lines are easy to culture for long period of time and significantly less expensive compared to the intestinal organoids. However, given that they have been immortalized by SV40 large T gene transfer through a murine ecotropic virus, these cells cannot be considered as fully "normal" IECs. First, we compared the expression of the IL-12Rβ2 on freshly isolated IECs and MODE-K cell line. We observed that the expression of the latter was negligible (**Figure 20**).Data collected from primary IECs, intestinal organoids and MODE-K raised an important question. Does the

expression of IL-12Rβ2 on IECs require immune-derived signals? To answer this question we adopted the strategy based on the use of well-established models of cell culture that allows the co-culture of IECs and gut-derived lymphocytes. MODE-K cells were grown either in contact with gut (PP)-derived lymphocytes or on Transwell polycarbonate membrane in the presence of lymphocytes seeded on the bottom chamber **(Figure 21 B)**. It appeared that both soluble and physical (cell-to-cell) mediated signals from immune cells play a role in the regulation of the expression of IL-12Rβ2. Indeed, the expression of the receptor was significantly higher in both co-culture models compared to control culture although its expression reached its peak in the presence of cell-to-cell signals (**Figure 21 A**). These results taken together demonstrated once again the biological importance of lympho-epithelial cross talk in the gut.

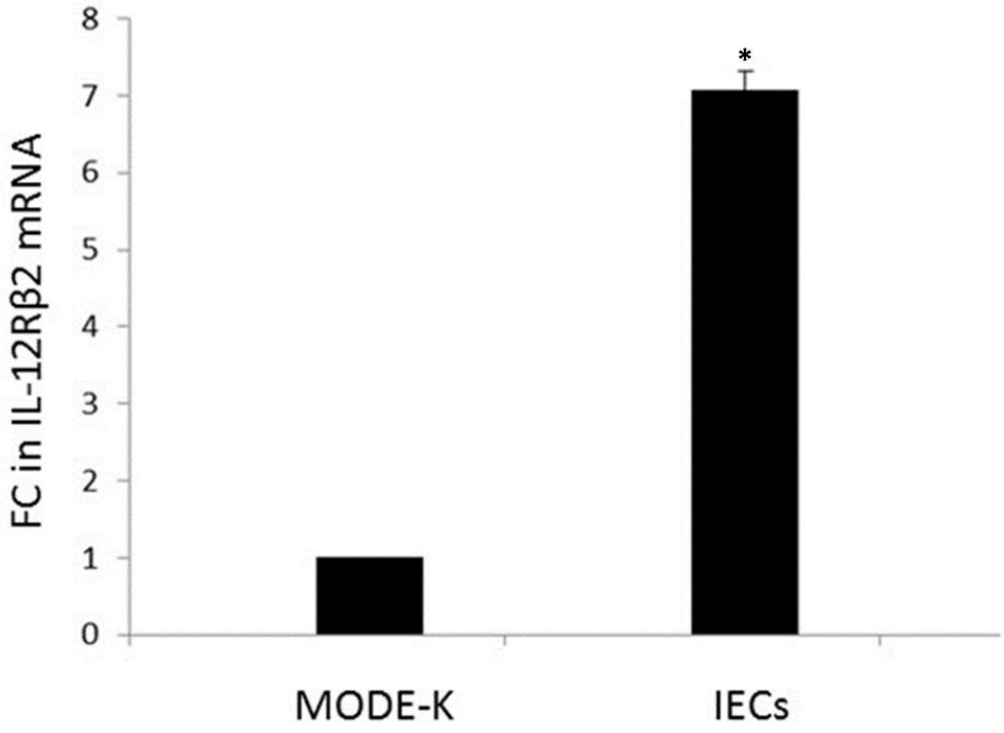


Figure 20 MODE-K expression of IL-12Rβ2. Quantitative PCR analysis showed that the expression of IL-12Rβ2 was significantly higher on freshly isolated primary IECs compared to three day old intestinal epithelial cell line MODE-K. (Experiment performed three times, each time n=2 mice donors of IECs; fold change difference >2 is considered significant; error bars represent SEM)

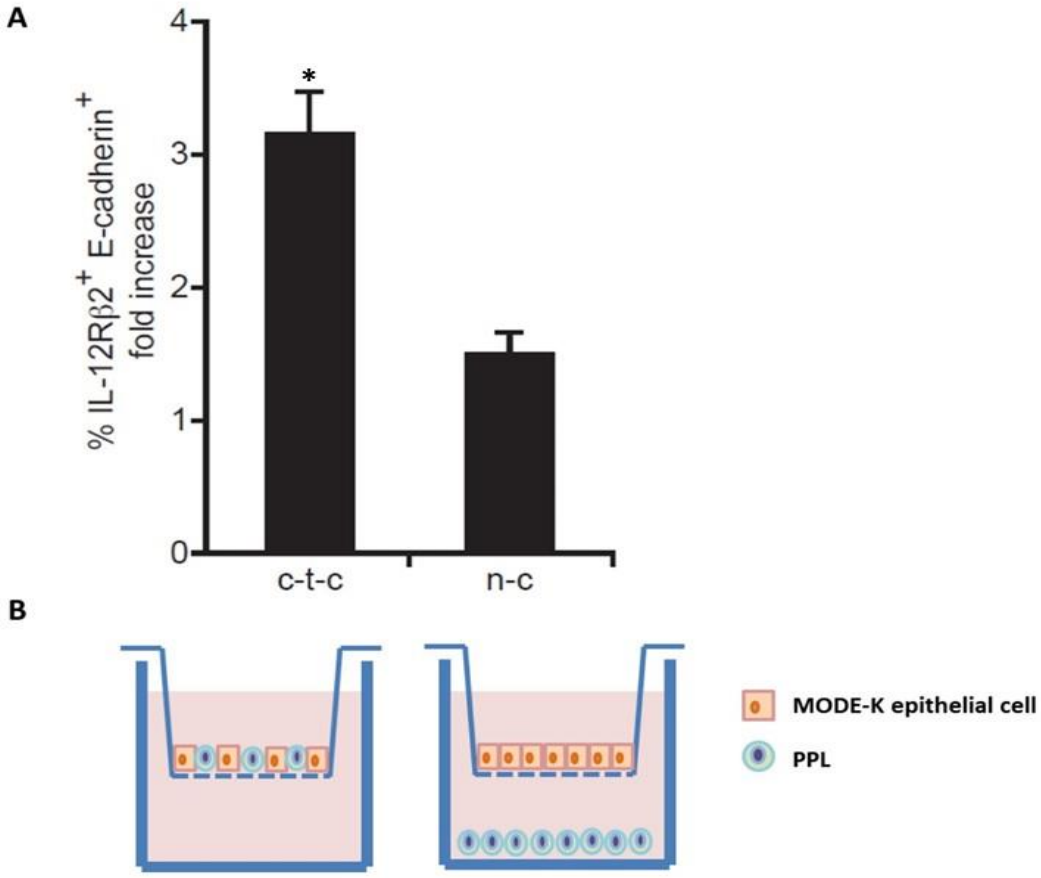


Figure 21 Co-culture with Peyer's patch derived lymphocytes (PPLs) led to an increased expression of the IL-12Rβ2 in MODE-K. (A) The levels of IL-12Rβ2 were assessed by flow cytometry. (B) Schematic representation of the culture conditions: in the left the MODE-K cells were in a cell-to-cell (c-t-c) contact with the PPLs, on the right the MODE-K cells were cultured in the top compartment of the Transwell and the PPLs at the bottom so that no contact (n-c) occurs between the cells and only soluble mediators participate in the cross-talk.

Higher expression of IL-12Rβ2 was seen when PP-lymphocytes were seeded in the bottom compartment of the Transwells culture system not in contact with MODE-K cells (1.5 fold) (n-c); however, a more pronounced increase (3.2 fold) was seen when PP-lymphocytes were allowed cell-to-cell interaction with the MODE-K cells (c-t-c)**.** (Experiment performed three times, each time n=3 wells/condition; fold change difference >2 is considered significant; error bars represent SEM)

5. Discussion

The main finding of this part of the work is that IECs do express a functional, although incomplete form of the IL-12R (IL-12Rβ2 chain only) that is likely to play an important role in the control and regulation of TSLP production in the intestinal epithelium by IL-12. Further experiments have shown however, that IL-12 does not directly regulate TSLP and that other factor(s) participate in the IL-12/TSLP axis. The pro-allergic (Th2) TSLP and anti-allergic (Th1) IL-12 seemed to be part of immune-regulatory feedback loop. It has been shown that TSLP directly suppressed the production of IL-12 by DCs and mice lacking TSLP receptor (TSLPR) showed very high level of IL-12 (Massacand et al., 2009). We have now demonstrated that IL-12 in turn keeps the production of TSLP by IECs under control and that deviation from this pattern is likely to lead to alteration of the intestinal immune homeostasis as in the case of allergic response to food components as reported in **Chapter 2**. The latter arm of the regulatory loop has been demonstrated by several converging pieces of evidence. First, as shown in **Chapter 2** allergic mice displayed a significant reduction of IL-12 and a parallel increase of TSLP; the supplementation of IL-12p70 by recombinant *L. lactis* restored physiological levels of TSLP and led to suppression of the allergic symptoms. Subsequent experiments also showed that following oral administration of CT, either alone or in combination with the allergen, levels of IL-12 in the gut declined before upregulation of the TSLP production by IECs could be detected. The latter data lead to two different conclusions. Firstly, CT does not directly induce the production of TSLP by IECs (also confirmed *in vitro* by challenging MODE-K epithelial cells with CT, data not shown) and secondly that CT-mediated up-regulation of TSLP possibly depends on its ability to suppress the production of IL-12.

Interestingly, mice deficient for either IL-12p40 subunit or IL-12Rβ2 showed normal levels of TSLP in the gut. This experiment brought about a series of important considerations. The use of p40-deficient mice in combination with β2 chain-deficient mice was required to ascertain whether IL-12 could down-regulate TSLP expression bypassing the need for a specific receptor. Indeed,

although the conventional mechanism of action of cytokine include its binding to specific receptor, in some case it has been observed that a cytokine can actually activate the target cell by entering the cells via micropinocytosis and initiate the intracellular signalling cascade by binding to transcription factors. A typical example is the intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1 (Kleemann et al., 2000). More importantly, the absence of increased expression of TSLP in IL-12Rβ2- and p40-deficient mice raised the fundamental question about the mechanism underlying IL-12-mediated suppression of TSLP.

One possible explanation of the discrepancy observed between the CT sensitization model and the knock-out mice is that in addition to the downregulation of IL-12 a second signal is required to drive the overproduction of TSLP. Although the nature of the second signals remains to be determined it is possible to hypothesize that CT treatment provides both signals thus leading to the up-regulation of TSLP production; in IL-12Rβ2 and p40-deficient mice only the signal provided by IL-12 pathway is missing while the remaining one is still in place to regulate TSLP production.

There are numerous examples of crucial immunological processes requiring secondary signal. For instance, T cell activation requires two events: TCR engagement with MHC accompanied with CD28 ligation to CD80. There are also cytokines whose expression requires dual input, one example being IL-13. Neill et al. (2010) have shown that ILC2 require both IL-33 and IL-7 stimulation in order to express IL-13. The fact that TSLP is a critical cytokine essential for the control of intestinal immune homeostasis suggests that a tight regulatory mechanism is in place to control its production thus making the hypothesis of the double signal-dependent regulation plausible. Experiments are now in progress that include the monitoring of global gene expression (microarrays) and proteomics analysis in peanut-sensitized and IL-12-treated mice. In order to identify the missing pieces of the IL-12/TSLP regulatory pathway the development of appropriate experimental models is necessary. One of the main technical difficulties encountered in this work is the fragility and short *ex-vivo* life spun of primary IECs. We have started to address the issue by using mouse

small intestine organoids and intestinal epithelial cells lines. The approaches undertaken showed, once more that a finely tuned dialogue between IECs and immune cells is required for the expression of molecules critical in immune regulation. We observed that both intestinal organoids and cell line originating from the intestinal epithelium lacked the expression of the IL-12Rβ2. This, however, was significantly up-regulated by the presence of both soluble and cell-to-cell immune-derived signals. This finding will provide an important tool to address the regulatory events in IECs that follow the engagement of IEC-associated IL-12Rβ2 and its ligand. Experiments are now in progress in the lab to study global gene expression in epithelial cells expressing IL-12Rβ2 following exposure to IL-12p70.

Chapter 4: Lympho-epithelial cross talk in the interaction with the gut microbiota

1. Introduction to the gut microbiota

Since the microbiome field's inception some 15 years ago thousands of research papers have been published on the subject with the microbiota craze peaking in 2012 (**Figure 22**).

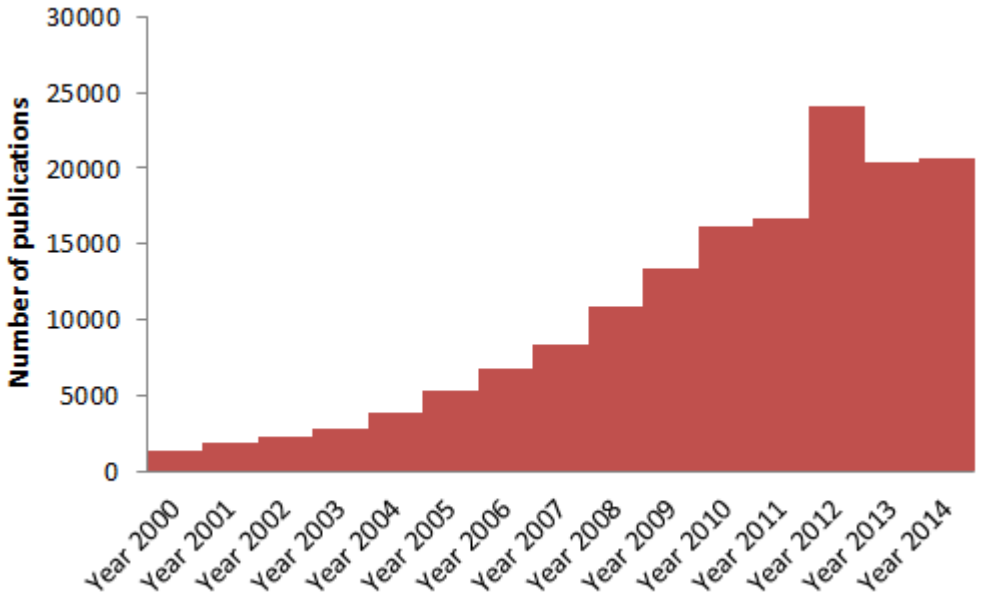


Figure 22 Number of publications as determined by search in Google Scholar for "intestinal microbiota".

It has been established that diet and host genetics as well as other host-associated factors can influence the microbial composition of the gut. On the other hand, change in the microbial community can have profound effects on the host. So far microbiota research has given a lot of evidence for this two-way interplay but little understanding of the mechanisms that drive it. Currently, more and more mechanistic studies are emerging which imply a vast number of pathways and regulatory mechanisms engaged in the host-microbiota interaction.

1.1. Composition of the gut microbiota

The microorganisms that live in coexistence with their host are referred to as the microbiota, microflora, or normal flora. They are found on most environmentally exposed surfaces (such as the skin, mouth, gut and vagina), but the majority of them live in the gut. Mammalian large intestine harbours around 10^{13} bacteria and is the most densely populated ecosystem (Peterson et al., 2014). Bacteria of the mammalian gut are known to provide some beneficial functions to the host: they supply essential nutrients, metabolize indigestible compounds, defend against colonization by pathogens and are also involved in contributing to the intestinal architecture (Round and Mazmanian, 2009). The composition and roles of the bacteria that are part of this community have been studied most. However, the roles of viruses, archaea, and unicellular eukaryotes that inhabit the mammalian digestive tract are less well known.

Currently (2014), there are around 80 bacterial phyla described in the Greengenes database (Youssef et al., 2014). Only two of which - the Bacteroidetes and the Firmicutes comprise the majority of human gut microbiota. Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria are also present but less abundant (Eckburg et al., 2005). As in humans, the two bacterial divisions with highest relative proportion in mouse gut are the Firmicutes (60-80% of sequences) and the Bacteroidetes (20-40%) (Ley et al., 2005). Of the Firmicutes more than 75% are in *Clostridium* cluster XIVa, which is a clade also present in human and includes butyrate producers. The majority (>88%) of the Bacteroidetes belong to Bacteroidetes 4b Proteobacteria and Actinobacteria (Ley et al., 2005).

Estimates of the number of bacterial species present in the human gut vary widely between different studies, but it has been generally accepted that it is somewhere between 500 to 1000 species (Xu and Gordon, 2003). Analysis involving multiple subjects has predicted that the collective human gut microbiota is composed of at least 1,800 genera and ≈15,000–36,000 species of bacteria, depending on whether species are conservatively (97% OTUs) or liberally (99% OTUs) classified (Frank et al., 2007). It is also suggested that the so far sampled sequences represent around 50% of the predicted species-level

diversity, thus promising an overwhelming microbial diversity yet to be characterized within the human gut (Frank et al., 2007).

Intestinal microbiota is not homogenous. For example, bacterial load, species richness and relative distribution in the small intestine are different from that in the large intestine (Sekirov et al., 2010). What is more, microorganisms in the intestinal lumen differ significantly from those attached and embedded in the mucus layer or those present in the immediate proximity of the epithelium. For example, *Bacteroides*, *Bifidobacterium*, *Streptococcus*, members of Enterobacteriacea, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus* are present in faeces, but only *Clostridium*, *Lactobacillus*, and *Enterococcus* are found in association with the mucus layer within crypts of the small intestine (Swidsinski et al., 2005).

Age, diet, host physiology can all affect the microbial composition of the gut at species level but it remains fairly stable at the phylum level. The major groups that dominate the human intestine are conserved between all individuals, although the proportions of these groups can vary.

Within phyla, the inter-individual variation of species composition is high. This may indicate that although there is a selective pressure for the maintenance of certain microbial groups (phyla), the functional redundancy within those groups allows for differences in the composition of the microbiota between individuals without compromising the maintenance of adequate function. However, this hypothesis remains to be further investigated (Sekirov et al., 2010).

1.1.1. Of mice and men

Unravelling the mechanisms by which gut microbiota contributes to human health and disease requires use of animal models. The work described in this chapter utilizes mice as experimental organisms and therefore this section reflects the essential similarities and differences that exist between the mouse and human digestive tract and their microbiota.

One of the reasons why mouse models are so widely used is that mouse and human share anatomical and physiological features. In particular, the gastrointestinal tract in both species is composed of anatomically similar

organs. The differences between murine and human GI tract are possibly evolutionary consequences of their distinct diets, body sizes and metabolic features.

The relative proportions in terms of length of the small and large intestines are similar in humans and mice (approximately 80% and 20%) (Casteleyn et al., 2010) whereas, the proportion of surface area of the small and large intestines is different. In humans, the surface area of the large intestine is approximately 400 times smaller than that of the small intestine but this is only 18 times in mice (Casteleyn et al., 2010). What is more, the mouse cecum is also relatively larger and has important role for fermentation of plant materials as well as for the production of vitamin K and B. Those nutrients are later reabsorbed through coprophagy. On the contrary, the human cecum is relatively small, anatomically similar to the colon and is of unknown function.

Generally, the gut microbiota of human and mice are dominated by two major phyla, Bacteroidetes and Firmicutes, however, on a deeper taxonomic level 85% of bacterial genera found in the mouse gut microbiota are not present in human (Ley et al., 2005). Use of different research techniques imposes difficulties in comparing murine and human microbiome. First, in human studies usually the analysis is performed on faecal samples whereas work done in mice most often assesses the cecal microbiota. It is only longitudinal studies in mice that sample faecal pellets. Looking at five murine faecal 16S rDNA studies and four public 16S rDNA healthy adult human datasets Nguyen et al. (2015) estimated that there are 79 genera occurring in both human and mouse intestinal microbiota (Nguyen et al., 2015). They have also found that genera highly abundant in human gut microbiota, as compared to mouse, include *Prevotella*, *Faecalibacterium* and *Ruminococcus*, whereas *Lactobacillus*, *Alistipes* and *Turicibacter* are more abundant in mouse gut microbiota. *Clostridium*, *Bacteroides* and *Blautia* are similarly abundant in both organisms (Nguyen et al., 2015). Though, much larger sample size is needed to validate those findings.

When assessing the effect of high fat diet it seems that both mouse and human microbiota behave similarly. For instance, mice fed on a high animal-fat diet show a decrease in Bacteroidetes to Firmicutes ratio (Murphy et al., 2010)

which is in compliance with observations of human dietary studies (Le Chatelier et al., 2013).

Up until recently it was believed that presence of community clusters characterized by differences in the abundance of signature taxa, referred to as enterotypes are found only in human and some primates populations but not in mice. A study by Wang et al. (2014) suggests that the wild domestic mouse population also experience enterotype-like clustering (Wang et al., 2014). Human enterotypes were first described in 2011 in a study investigating the phylogenetic composition of 39 faecal samples from 6 nationalities, where metagenomic reads were mapped, using DNA sequence homology, to 1,511 reference genomes. Multidimensional cluster analysis and principal component analysis (PCA) revealed that the samples formed three distinct clusters that were designate as enterotypes. These enterotypes could be identified by variation in signature genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (Arumugam et al., 2011). In contrary only two enterotypes were evident when cecal microbiota of wild mice was analysed (Wang et al., 2014). The signature taxa of the two mouse enterotypes are *Bacteroides* as the largest contributor (31.68%) to dissimilarity between groups and similarity within enterotype 1 (77.78%) and the second largest contributor (8.59%) to dissimilarity between groups is *Robinsoniella*, which contributes 14.04% to similarity within enterotype 2 and is also the most abundant genus in this group (average 13.6%) (Wang et al., 2014). In both mouse and man dietary history is suggested to play a role in the establishment of enterotypes. In contrary to this view are the findings by Hildenbrand et al. which suggest that strain-independent murine enterotypes are associated with the inflammation marker calprotectin (Gerlach et al., 2014).

Segmented filamentous bacteria (SFB) interact closely with mouse intestinal epithelium and modulate mucosal immunity (for more information see **Chapter 4 section 1.2.**). There are only sporadic reports (Klaasen et al., 1993, Jonsson, 2013) about presence of SFB in man. It is still not known whether SFB or any other members of the human intestinal microbiota are capable of such a close interaction with the epithelium and immune modulation.

1.1.2. Of time and space

Space/cage effect

At birth mammalian gut is sterile but it soon gets colonised by a simple microbial community which undergoes a succession with increasing complexity reaching climax in early childhood when the composition is thought to then remain relatively stable (Palmer et al., 2007).

The composition of the microbiota could be shaped by both environmental and stochastic factors. Family members are likely to share more similar microbiota than unrelated individuals (Turnbaugh et al., 2009). This could be due to either close genetic relatedness or common environment. Similarly, mice that share cage are likely to have related microbiota. Early studies on the intestinal microbiota done with denaturing gradient gel electrophoresis (DGGE) to profile PCR-derived amplicons from bacterial 16S rRNA genes have shown that genotype has a strong influence on the community structure, whereas environmental factors are contributing to a much lesser extent. Work done by Hufeldt et al. (2010) have shown that neither sex or individually ventilated cage effect are significant contributors to the composition of murine cecal microbiota (Littman and Rudensky, 2010). On the contrary, later studies by Campbell et al. (2012) show that along with the genotype, cohabitation and litter also have detectable, although reduced effect (Campbell et al., 2012). Instead of DGGE this later study utilizes pyrosequencing for analysis which might explain higher sensitivity.

A study by McCafferty *et al.* (2013) suggests that cage effects are driven by stochastic differences that occur over time within each cage, rather than the composition of the initial microbial community (McCafferty et al., 2013). It is also suggested that cage effects show higher variation explained (above 31%) at OTU level and the effect is smaller (22.6%) at the phylum level. It is suggested that genotype and cage conditions have independent effect on the microbiota composition (Gerlach et al., 2014). When Balb/c, C57B/6, FVB and non-obese diabetic (NOD) mice are cohoused for 3 weeks in terms of alpha diversity cage effect has no impact (Gerlach et al., 2014). In addition, cage effects in the Hildebrand et al. study are attributed to *Helicobacter* infections (Gerlach et al.,

2014). Removal of the *Helicobacter* OTUs from the dataset results in the difference between cages at OTU level to be no longer significant (Gerlach et al., 2014). It remains to be further confirmed whether *Helicobacter* is responsible for cage effects in experiments done in other mouse facilities.

Maternal influence on the microbiota composition

A study by Ley *et al.* (2005) suggests that kinship has effect over the gut microbiota. For the purpose of their work Ley at al. (2005) describe three C57BL/6J ob/+ matings. The microbiota of all offspring as well as that of the three mothers has been analysed. The new-born mice have been raised together with their mother and littermates until weaning and then housed individually in microisolator cages until 8 weeks of age, when they have been killed. The results reveal that mothers and their offspring share cecal microbiota with similar community membership at the level of genera (Ley et al., 2005). However, regardless of family membership, the obese (ob/ob) genotype is associated with a large shift in the relative abundance of specific taxa present (Ley et al., 2005), which indicates that even if the maternal influence has an effect on the microbiota it cannot mask stronger effects such as the ob/ob genotype in this case. What is more, the maternal effect observed could be simply a random drift in the microbiota. Mice are coprophagic and if they share a cage they are likely to acquire each-other's microbiota. In this case, the offspring is initially housed with their mother, which allows for them to acquire the same microbiota. Then the animals are housed individually and this prevents acquisition/exchange of further species. It is intriguing whether the same effect would be observed if after weaning all three litters were housed together for a sufficient period of time before testing. It should be noted that a maternal effect is not always evident. For example, a study by Kovacs et al. (2011) concluded that whilst microbiota is substantially different in different mouse genetic backgrounds, little, if any, maternal effect is observed (Kovacs et al., 2011).

1.2. Role of the gut microbiota in shaping the host

Experiments with reciprocal transplantation of mouse and zebrafish microbiota suggest that mouse host is affected in a similar manner by both mouse and zebrafish microbiota despite the different bacterial composition (Rawls et al., 2006). It has been found that similar number of mouse host genes change in response to the native mouse microbiota (500) and to the zebrafish microbiota (525) (Rawls et al., 2006). However, only half of the genes are responsive to both microbial communities; the majority of those genes (96.4%) are also regulated in the same direction. Shared enrichment of genes involved in (1) biosynthesis and metabolism of fatty acids; (2) metabolism of essential amino acids (valine, isoleucine, and lysine); (3) metabolism of amino acids that contain the trace element selenium; (4) metabolism of butyrate; and (5) biosynthesis of bile acids has been observed. Unfortunately, Rawls *et al.* (2006) do not discuss the other ≈250 genes that the mouse host expresses differently depending on whether colonised with mouse or zebrafish microbiota (Rawls et al., 2006).

Colonisation of the gut with bacteria has been shown to induce fucosylation of the epithelial surface proteins. Mono-colonisation with *Bacteroides thetaiotaomicron* is sufficient to maintain the fucosylation as long as the bacterium is capable of utilising L-fucose as a carbon source (Bry et al., 1996).

Transplanting the gut microbiota from normal mice into germ-free recipients increases their body fat without any increase in food consumption. It is proposed that this is achieved by two complementary mechanisms: 1) the composition of the microbial community in the gut affects the amount of energy extracted from the diet and 2) microbiota alters the metabolic status of the host (Backhed et al., 2004). In addition, colonization of WT germ-free mice with microbiota from obese (ob/ob) mice results in a significantly greater increase in total body fat than colonization with a normal (lean) microbiota (Turnbaugh et al., 2006).

As previously mentioned SFB are found in a very intimate interaction with the mouse epithelium and are “kept in check” by the secreted IgA. This raises the question whether they can be truly classified as commensals. The findings so far indicate that SFB colonization leads to the maturation of the gut mucosal

lymphoid tissue, induces a strong and broad IgA response, stimulates the T-cell compartment, and upregulates intestinal innate defence mediators (Ivanov et al., 2009, Gaboriau-Routhiau et al., 2009). In addition, SFB colonization exerts an adjuvant effect on systemic responses. SFB colonisation can aggravate pathologic responses in mouse models of encephalitis and arthritis. On the contrary, SFB have been found protective against the development of type I diabetes in genetically predisposed mice (Lee et al., 2011, Wu et al., 2010, Chappert et al., 2013, Kriegel et al., 2011, Yurkovetskiy et al., 2013).

Mice subjected to stress experience shift in the microbiota accompanied by increase in the circulating levels of IL-6 and MCP-1, which are significantly correlated with stressor-induced changes to three bacterial genera (i.e., *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea*). Interestingly, when the mice are treated with an antibiotic cocktail exposure to stress fails to increase IL-6 and MCP-1. Therefore stress exposure significantly affects bacterial populations in the intestine but also suggest that the microbiota are necessary for stressor-induced increases in circulating cytokines (Bailey et al., 2011).

1.3. Diet, microbiota, metabolism & immunity

Diet, microbiota, metabolism and immunity are clearly correlated but the pathways involved in their interplay are still unclear. It is likely that several determinants interact together to form certain community structure. For example, on one hand, diet is an important environmental variable and serves as both a source of colonizing bacteria and alters the gut nutritional environment, thus affecting the composition of the gut microbiota (Muegge et al., 2011, Wu et al., 2011). On the other hand, sex is a genetic trait, also known to influence the gut microbiota (Markle et al., 2013). Interestingly, it has been shown that diet has a sex-specific effect on host gut microbiota (Bolnick et al., 2014).

Interaction between the immune system and the microbiota has been implemented in metabolic control. Genetic deficiency in TLR5 causes hyperphagia and development of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity in mice

as well as changes in the composition of the gut microbiota. Wild-type germ-free recipients inoculated with gut microbiota from TLR5-deficient mice develop metabolic syndrome (Vijay-Kumar et al., 2010).

It is likely that multiple pathways are engaged in the continuous interaction between immunity, metabolism, diet and the microbiota. Trompette et al. (2014) have proposed that the ratio of Firmicutes to Bacteroides is dependent on the fermentable dietary fiber which is metabolized by the microbiota and consequently leads to increase in the concentration of circulating short-chain fatty acids (SCFAs). High-fibre diet is associated with increased circulating levels of SCFAs and is protective against allergic inflammation. Treatment of mice with the SCFA propionate leads to generation and tissue infiltration of DCs with high phagocytic capacity but an impaired ability to promote T helper type 2 (Th2) responses, an effect dependent on G protein–coupled receptor 41. These results show that dietary fermentable fibre and SCFAs can shape the immunological environment and modulate allergic inflammation (Trompette et al., 2014).

Propionate and butyrate have also been shown to activate intestinal gluconeogenesis which has beneficial effects on glucose and energy homeostasis. Butyrate activates intestinal gluconeogenesis gene expression through a cAMP-dependent mechanism, while propionate acts via a gut-brain neural circuit involving the fatty acid receptor FFAR3 (De Vadder et al., 2014).

There is evidence that the three-way interaction between the immune system, the intestinal epithelium and the microbiota regulates gut metabolic state. In the absence of B cells/IgA, and in the presence of the microbiota, the intestinal epithelium engages its own protective mechanisms at the expense of its metabolic function thus resulting in decreased body fat due to lipid malabsorption (Shulzhenko et al., 2011).

1.4. Role of the host in shaping its own gut microbiota

To test how factors specific to host gut habitat shape microbial community structure, Rawls *et al.* (2006) performed transplantations of mouse microbiota into germ-free zebrafish and zebrafish microbiota into germ-free mice. It was found that in regards to the lineages present the transplanted community bared similarity with its community of origin. However, the relative abundance of the lineages changes to resemble the normal gut microbiota of the recipient host. Rawls et al. (2006) suggest that the differences in community structure between zebrafish and mice arise in part from distinct selective pressures imposed within the gut habitat of each host (Rawls et al., 2006).

Another example of host effect on the microbiota are the ob/ob obese mice. Those genetically obese (ob/ob) mice have 50% fewer *Bacteroidetes*, and correspondingly more *Firmicutes*, than their lean wild type (WT, +/+) siblings (Ley et al., 2005).

In the intestines of mice with deficiency of activation-induced cytidine deaminase (AID), the absence of hypermutated IgA is partially compensated for by the presence of large amounts of non-mutated IgM and normal expression levels of defensins. Despite that, predominant and persistent expansion of SFB throughout the small intestine of $AID^{-/-}$ mice is observed. Reconstitution of lamina propria IgA production in $AID^{-/-}$ mice rescues the normal composition of gut flora (Suzuki et al., 2004). This opens the question to what extent are the SFB commensal, do they provide benefit to the host, is their overgrowth associated with aberrant effect, does other bacterial groups also change? Some of these questions are discussed in the **section 1.2.** of this chapter.

There is some evidence that ageing of the host leads to reduced diversity of the gut microbiota but unfortunately the study in question (Claesson et al., 2012) fails to control appropriately and no clear discrimination can be made between the effect of ageing and the effect of diet.

Pregnancy is another instance where dramatic alternation of the intestinal microbiota has been observed. As pregnancy advances, microbiota becomes more like that of people with metabolic syndrome. These changes seem not

damaging to maternal health and correspond with increase in blood glucose and fat deposition thought to help nourishing the fetus (Koren et al., 2012).

If mice are exposed to a social stressor called social disruption, circulating cytokines increase and prime the innate immune system for enhanced reactivity associated with change in community structure of the microbiota. Most notably, the relative abundance of bacteria in the genus *Bacteroides* decreases, while that of *Clostridium* increases (Bailey et al., 2011).

2. Hypothesis

Main goal of this pilot study was to test the hypothesis that alteration of the lympho-epithelial cross-talk in the gut would result in altered microbial community structure, metabolite profile of the microbiota and the host. Mice deficient in molecules related to IL-12 signalling were supplied as part of the work described in **Chapter 2** where we investigated the regulatory interplay between IL-12 and TSLP but this also provided an opportunity to look into the intestinal microbiota of these animals.

3. Methods

Animals

Female mice from B6.12937-Ifng^{trn1TS}/J (IFNγ knockout), B6.12931-IL12b^{trn1Jm}/J (IL-12p40 knockout), and B6.12931-IL12Rβ2^{trn1Jm}/J (IL-12Rβ2 knockout) strains, along with the appropriate wild type C57BL/6J controls, were purchased from Jackson Laboratories. All animals were shipped simultaneously and allowed for acclimatisation at the DMU facility for two weeks before experimentation. Mice from the same strain were housed in microisolator cages (up to 5 animals per cage), maintained under 12 h light–dark regime and fed *ad libitum* standard chow. At eight weeks of age mice were sacrificed by cervical dislocation at the same time of day (±3 h) and samples were collected. All procedures were carried out in accordance with U.K. Home Office regulations, license conditions and University of East Anglia guidelines on animal welfare.

Sample collection

Immediately after the mice were killed, the gut tube was cut open longitudinally and gastrointestinal content was scraped from the terminal ileum (along 10–15 mm of the ileum entering the cecum) or the colon (along 30–35 mm exiting the cecum). Those samples along with whole ceca were frozen in dry ice and stored at -80° C until used.

DNA extraction

From the scraped intestinal content 70(±30) mg were used for DNA extraction with FsatDNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's instructions.

16S PCR amplification and 454 sequencing

PCR amplification of the 16S using the universal V4-V5 primers U515F GTGYCAGCMGCCGCGGTA and U927R CCCGYCAATTCMTTTRAGT (Tang et al., 2014, Fabrice A, 2009) and consequent 454 sequencing was done by a crevice provider (Veterinary Laboratories Agency, UK).

Sequencing data analysis

Raw data from 454 sequencing (.sff format) was analysed with QIIME (Caporaso et al., 2010b). Briefly, de-multiplexing and initial filtering was done with the split_libraries.py script set to exclude reads outside 310-480 nucleotides length, window quality scores 50, with < 1 mismatched primers. Denoising with denoise_wrapper.py (Reeder and Knight, 2010) was performed on a high performance cluster. Picking of OTUs was accomplished using the QIIME workflow function pick_otus_through_otu_table.py with default settings including: clustering by uclust method (Edgar, 2010), cluster seed chosen as representative, PyNAST alignment (Caporaso et al., 2010a), conservative (97% similarity) rdp taxonomy assignment (Wang et al., 2007) according to the Greengenes database (McDonald et al., 2012, Werner et al., 2012), building phylogenetic tree with FastTree (Price et al., 2010). Chimeric sequences were removed with ChimeraSlayer (Haas et al., 2011). One sample from IL12p40 KO colon group, one from WT ileum, and one sample from WT colon group were removed from further analysis due to low sequence number. Before calculation

of diversity the OTU table was rarefied with upper limit of rarefaction depth seta at 2200. UniFrac was used for calculating beta-diversity (Lozupone and Knight, 2005).

Sample preparation for metabolomics

While still frozen ceca were cut longitudinally. Luminal content was gently separated from the host tissue. An average of 169 (±68) mg luminal content was mixed with 1 ml deuterated buffer (Le Gall et al., 2011) for 2 minutes. After centrifugation for 10 min at 800g supernatants were stored at -80° C until analysed the same week. Each sample of mouse tissue was washed with 10 ml PBS and transferred to tube with 10 glass beads 3 mm ø (VLR International). To an average of 60 (±15) mg of tissue was added 1 ml deuterated buffer and the samples were processed on FastPrep FR120 (BIO101 Thermo Savant) at 4 m/s three times 90 s bursts with 2 min rest on ice in-between. Samples were spun for 2 min at 6000 g. Supernatants transferred into fresh Eppendorf tubes and centrifuged for 15 min at 9000 g. Supernatants were stored at -80° C until analysed the same week.

4. Results

4.1. Analysis of microbiota composition

4.1.1. 16S metagenomics analysis

4.1.1.1. Demultiplexing and initial filtering

In the raw data the majority of the reads were 420 nucleotides long. After removal of the forward primer (18 nucleotides), the reverse primer (19 nucleotides), and the barcode (10 nucleotides) the highest number of reads was around 370 nucleotides long.

Table 3 Metagenomics raw data. Number of reads combined for all 52 samples in the raw data and number of reads used for further analysis after selecting for length, window quality score of 50 and no mismatched primers.

Selection length (in nucleotides)	***Number of raw reads***	***Number of reads passing the selection criteria***	***Calculated median read length across all filtered samples***	***370 nucleotides long reads from all filtered (from all raw)***
310-480	430853	382445	365	88% (78%)

The number of operational taxonomic units (OTUs) varied depending on whether the data were denoised or not and whether chimeric sequences were eliminated but this change had no effect on the way the samples clustered according to the multivariate analysis. If OTUs were assigned straight after the split_libraries.py there were 4359 OTUs. OTU number dropped to 1875 after denoising and was reduced even further to 633 if the chimeric sequences were removed. While the OTU number dropped by around 66% only 12.5% of the reads were removed as chimeric.

4.1.1.2. Rarefaction and alpha diversity

After rarefaction of the OTU table, rarefaction curves were constructed based on the calculated species richness. The number of species discovered is plotted as a function of the number of sequences. Steep slope indicates that a large fraction of the species diversity remains to be discovered. If the curve becomes flatter to the right, a reasonable number of individual samples have been taken meaning that more intensive sampling is likely to yield only few additional species. Amongst all groups higher species richness was observed in the colon compared to the ileum (**Figure 23**).

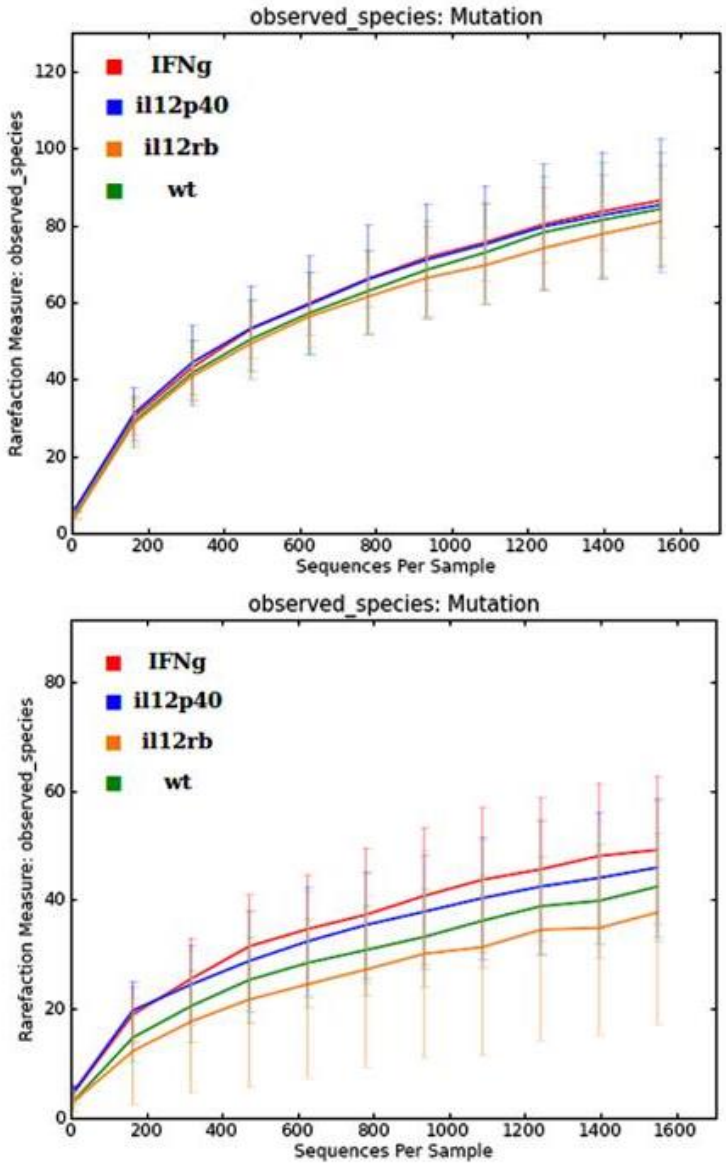


Figure 23 Rarefaction curves for colon (top) and ileum (bottom) for the four groups. Higher species diversity was observed in the colon compared to the ileum. Flattening of the lines and particularly the ones for ileum suggests that at the current sequencing depth the majority of the species present had been detected.

The highest number of observed species was evident in the IFNγ group whereas IL12Rβ2 group showed reduced species richness both in the colon and the ileum (**Figure 23**) but those differences did not reach statistical significance (p=0.635; n=10 to 12; ANOVA; Bonferroni Multiple Comparison post-test).

4.1.1.3. Taxa summary

Despite the interindividual variation, difference could be observed in the taxonomic distribution between the four groups (**Figure 24**).

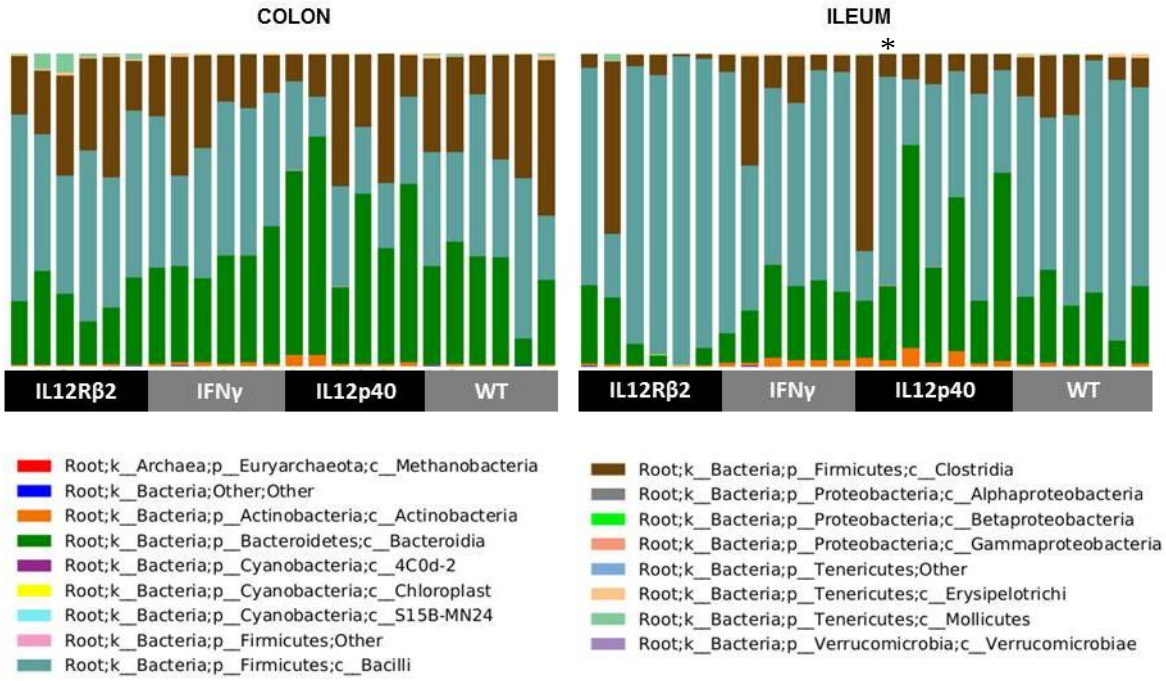


Figure 24 Relative taxonomic abundance at the level of class. The first bar in the colon group and the first bar in the ileum group were taken from the same mouse; the rest of the samples follow this order apart from the extra ileal sample marked with *.

When looking at the level of phyla similar pattern could be observed in both colon and ileum samples. Actinobacteria and Bacteroidetes were most abundant in the IL12p40 KO mice and least abundant in the IL12Rβ2 group with the difference being more pronounced in the ileum (**Figure 25**). In the ileum the abundance of Actinobacteria was higher (mean 1.5%) compared to the colon (mean 0.9%) ($p=0.0014$; $n=24$; Wilcoxon matched-pairs signed rank test). On the contrary, there were more Bacteroidetes in the colon (mean 33.79% vs. ileum mean 23.25%) ($p=0.0002$; $n=24$; Wilcoxon matched-pairs signed rank test).

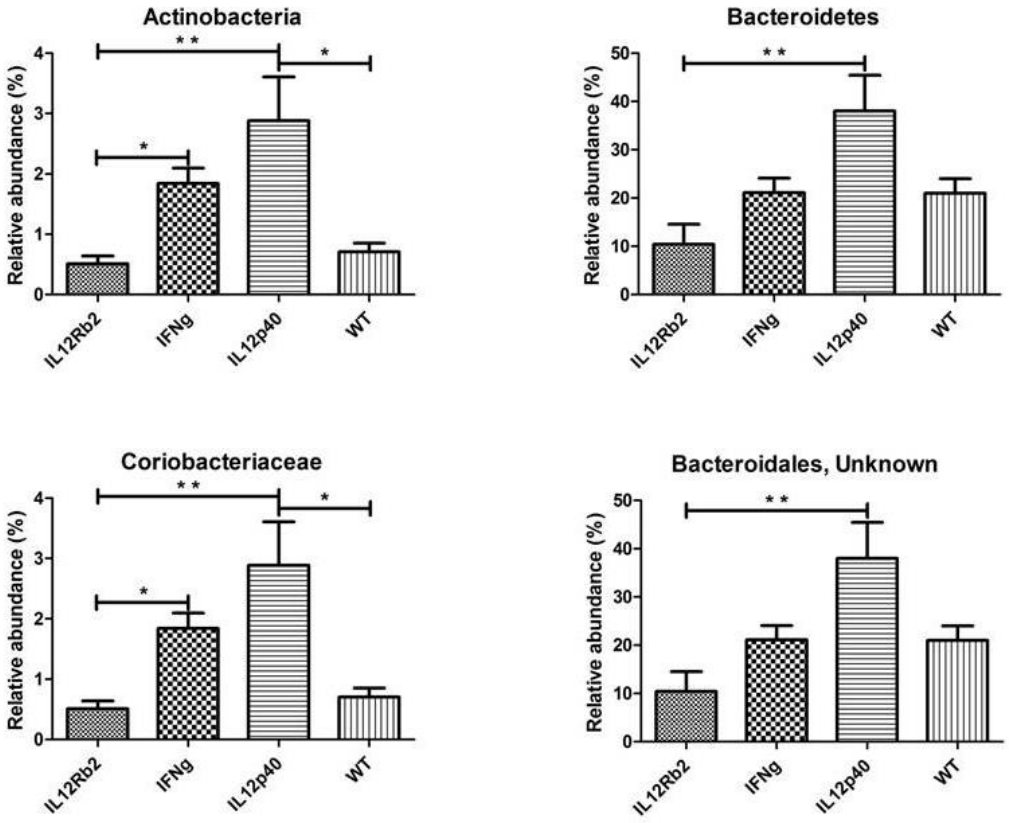


Figure 25 Actinobacteria and in particular representatives of the family *Coriobacteriaceae* were significantly more abundant in the ilea of IL12p40 KO mice compared to IL12Rβ2 KOs (p=0.0008; n=5 to 6; Kurskal-Wallis test; Dunn's Multiple Comparison post-test). An unknown family of order Bacteroidales was responsible for the difference evident at phylum level for the Bacteroidetes. Again, IL12p40 KO group had pronouncedly higher relative abundance compared to IL12Rβ2 KO (n=5 to 6; one-way ANOVA; Bonferroni Multiple Comparison post-test; p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****).

Bacteria from phylum Firmicutes were more abundant in the ileum (mean 74.72% vs. colon mean 64%) (p=0.0005; n=24; Wilcoxon matched-pairs signed rank test). For both colon and ileum, IL12p40 KO mice had the lowest abundance whereas the IL12Rβ2 KOs had the highest. This difference between the KOs was more pronounced in the colon (**Figure 26**).

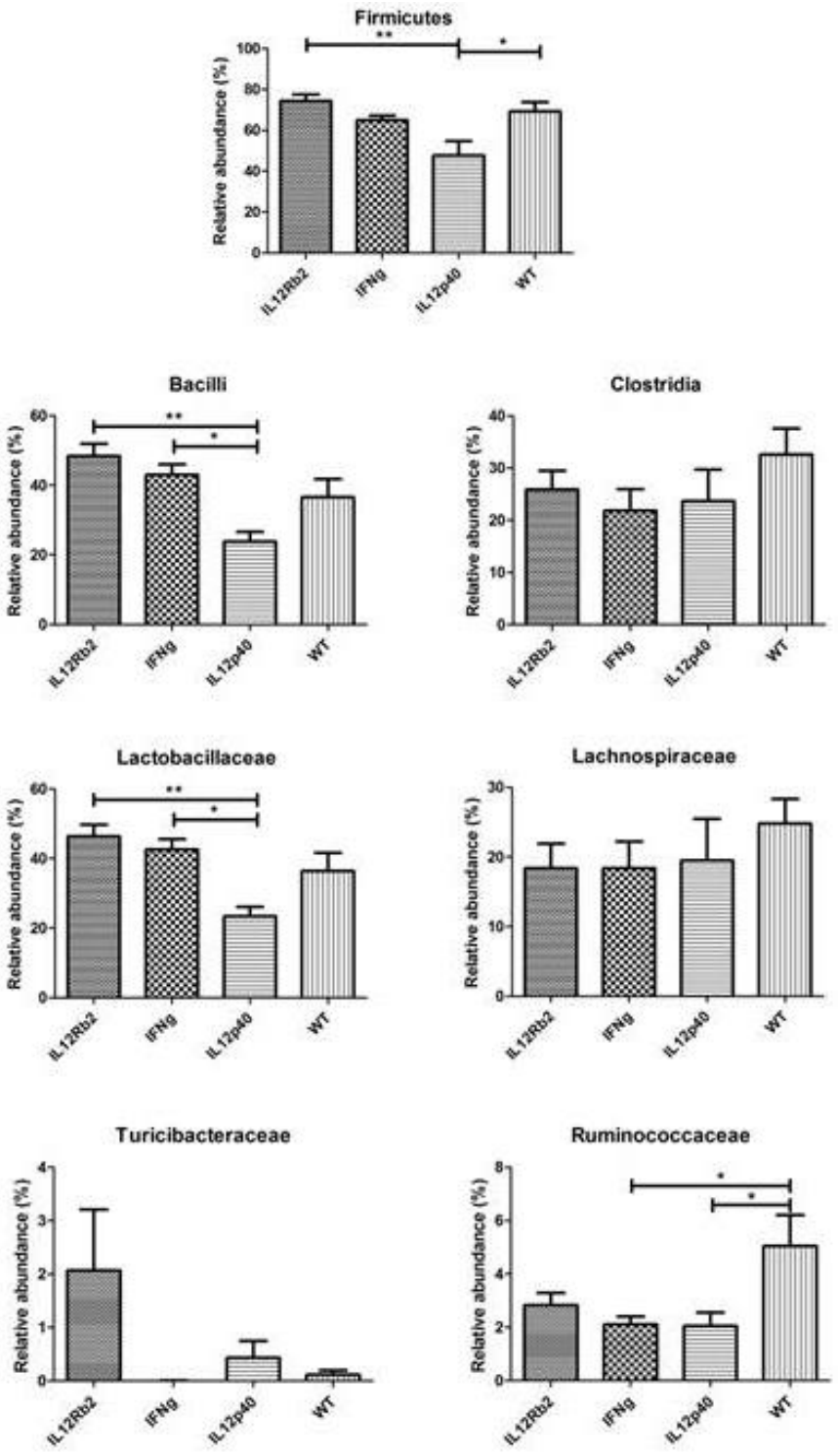


Figure 26 The overall difference observed in the colon microbial communities for phylum Firmicutes was driven by difference in class Bacilli, more particularly in order Lactobacillales, family *Lactobilacillaceae* (n=5 to 6; one-way ANOVA; Bonferroni Multiple Comparison post-test; p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****).

Finally, bacteria belonging to the phylum Tenericutes were also present but at a relatively low level (**Figure 26**). On average there were 1.23% in the colon and 0.46% in the ileum (p=0.0016; n=24; Wilcoxon matched-pairs signed rank test).

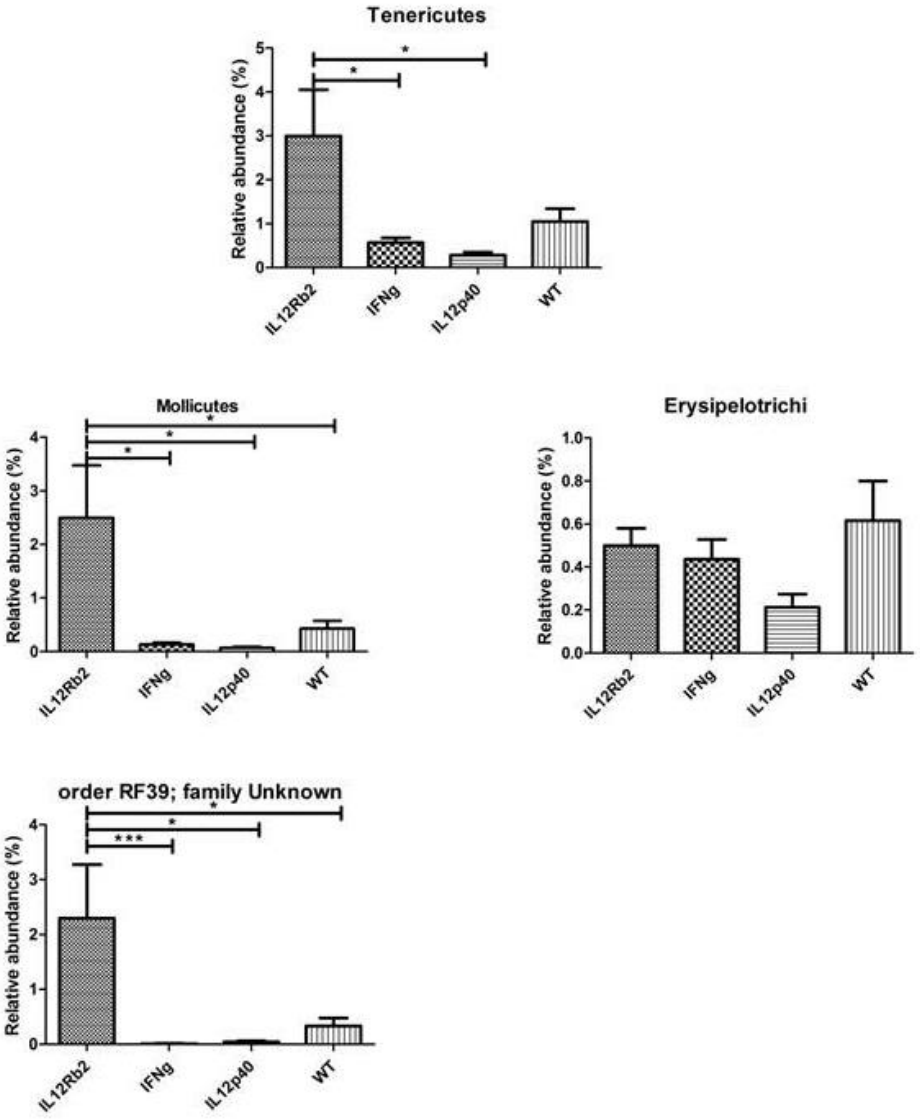


Figure 27 In the colon of IL12Rβ2 mice there were more bacteria from unknown family belonging to order RF39, class Mollinutes (n=5 to 6; Wilcoxon matched-pairs signed rank test; p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****).

4.1.1.4. Beta diversity

UniFrac (Lozupone and Knight, 2005) was used to calculate the distance measure between the microbial communities whilst incorporating phylogenetic information. Then Principal Coordinates Analysis (PCoA) was used to extract and visualize the most informative components of this multidimensional data (**Figure 28**). This transformation maps the samples present in the distance matrix to a new set of axes. The maximum amount of variation is explained by the first principal coordinate, the second largest amount of variation is explained by the second principal coordinate, etc.

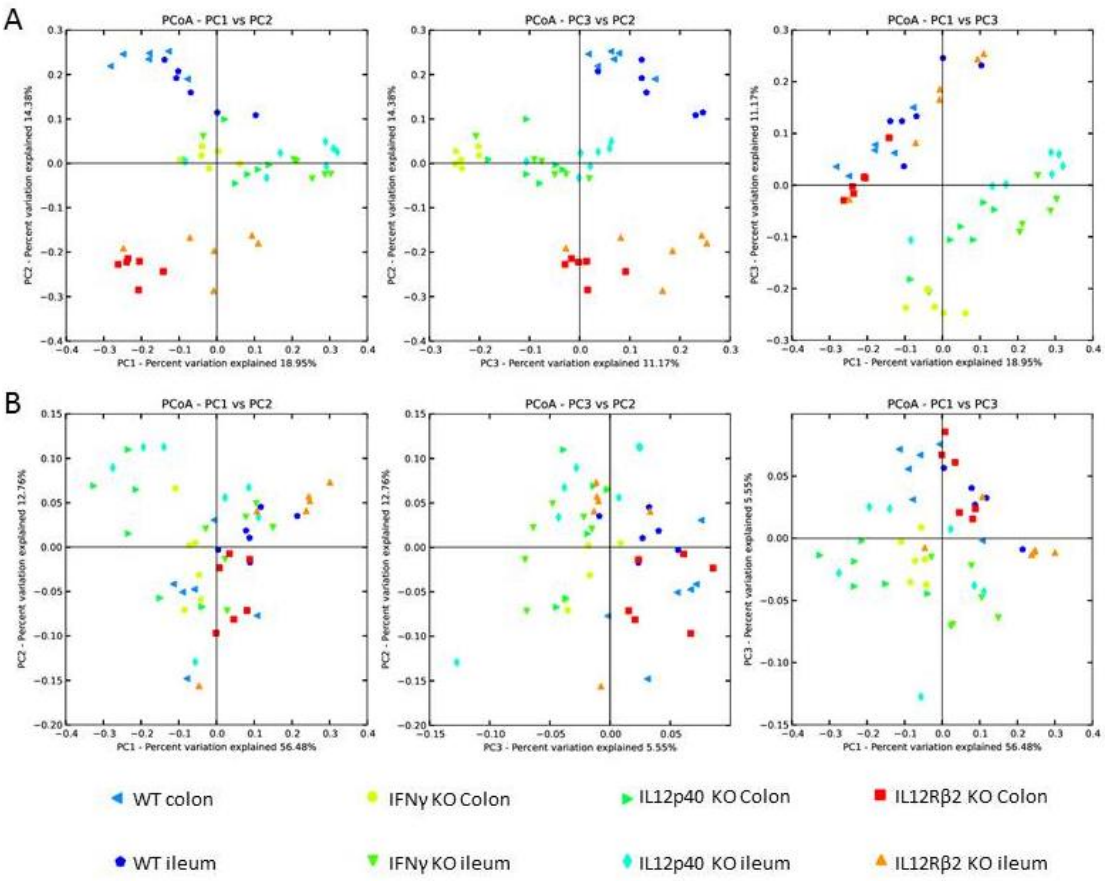


Figure 28 Principal Coordinate Analysis (PCoA) of the unweighted (A) and weignted (B) UniFrac distance for all samples (n=49); the first three principal coordinates are shown.

According to the unweignted UniFrac (**Figure 28 A**), gut microbial communities from IFNγ and IL12p40 KO were similar and the two groups clustered together when the first three coordinates were considered. When the microbial communities were analysed by weighted Unifrac (**Figure 28 B**) IFNγ and IL12p40 KO groups again clustered together and were different from WT and IL12Rβ2 groups.

4.2. Metabolomics

4.2.1. Metabolic profile of the luminal content

Across all luminal content samples more than thirty metabolites have been identified. The overall profiles of the IL12p40 and IFNγ KO mice showed similarities and were different from WT and IL12Rβ2 (**Figure 29**). A number of amino acids (**Figure 34**, **Figure 35**) were contributing to the separation of IL12Rβ2 samples form WT along the first principle component (**Figure 29, Figure 30**). Difference in some metabolites was predominant in only one group of mice (Figure 31, Figure 32, Figure 34), whereas other metabolites exhibited more complex pattern of abundance (**Figure 33**).

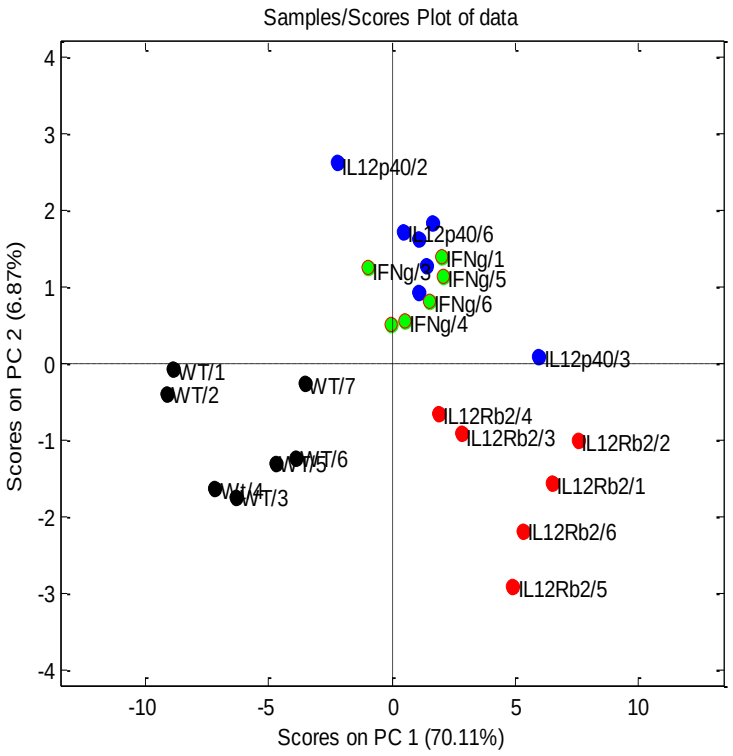


Figure 29 Principle Component Analysis (PCA) on 30 1H NMR spectra of metabolites present in the luminal content. IL12Rβ2 KO is the group that differs the most from WT. IL12p40 KOs and IFN-γ KOs have similar metabolic profiles.

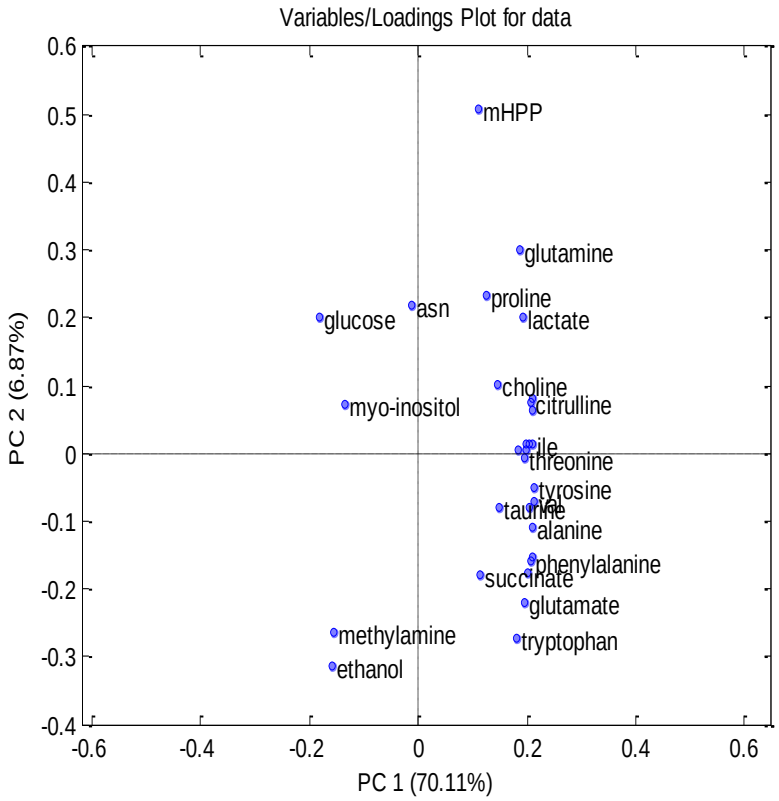


Figure 30 Separation of the luminal content metabolic profiles is based on the difference in levels of the 30 metabolites selected for the PCA. Glucose, myo-inositol, methylamine and ethanol levels are higher in WT while the levels of the other 26 metabolites are lower in WT.

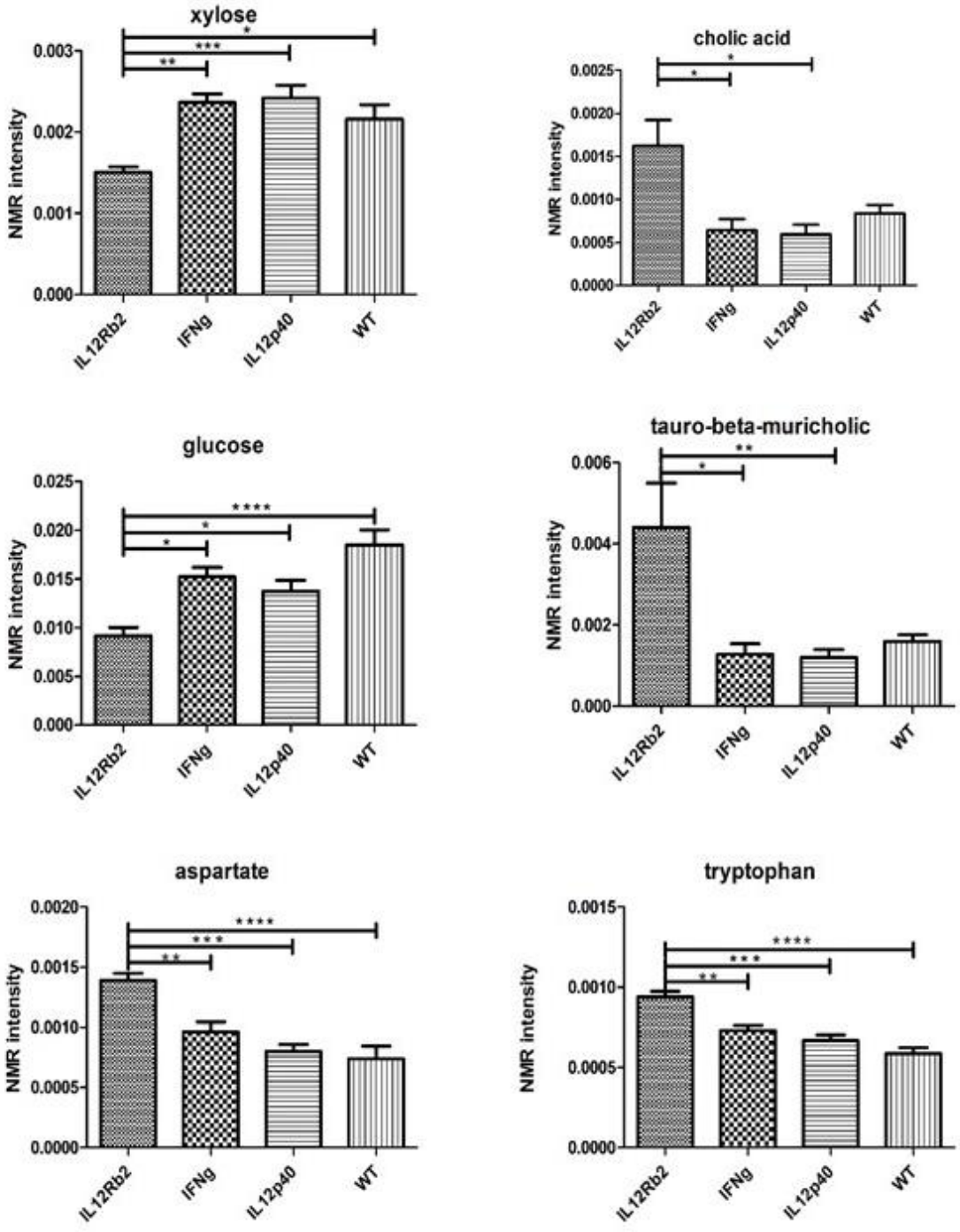


Figure 31 Metabolites identified as different in IL12Rβ2 group (ANOVA or Kurskal-Wallis p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****).

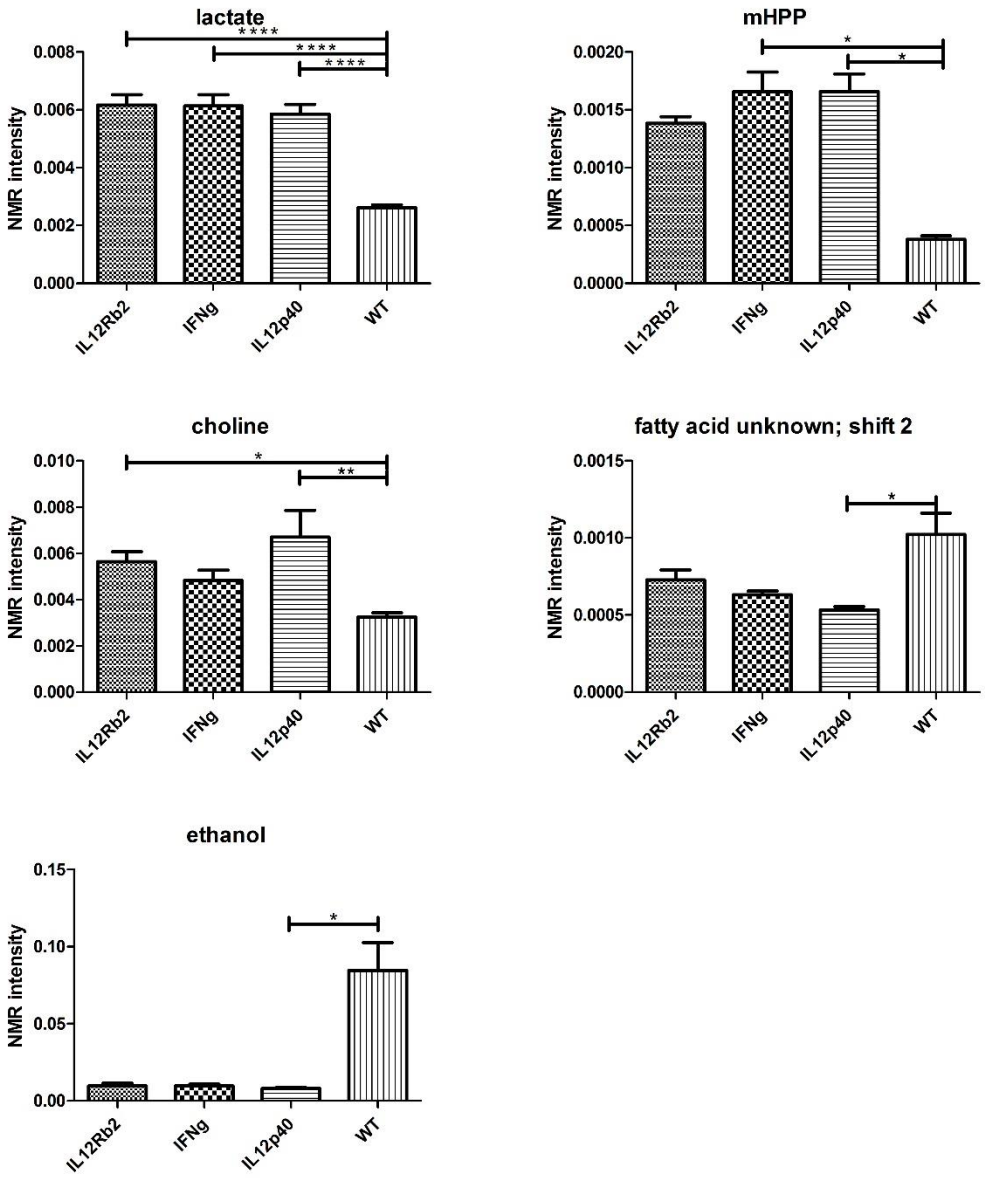


Figure 32 Metabolites identified as different in WT group (ANOVA/Kurskal-Wallis $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p<0.0001$=****).

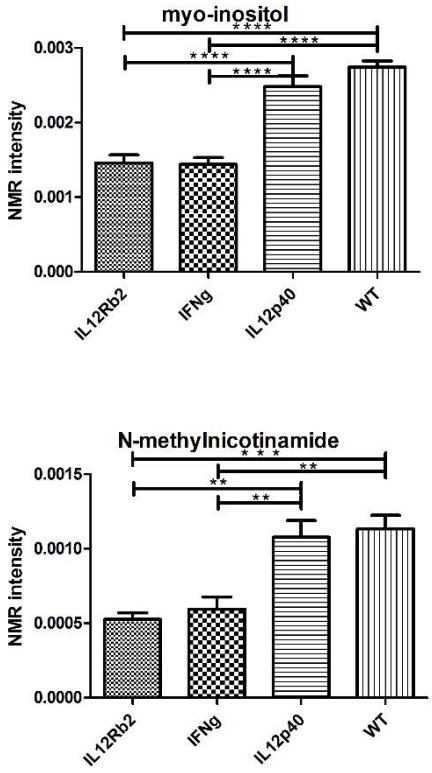


Figure 33 Metabolites that were low in two of the groups and high in the other two (ANOVA/Kurskal-Wallis $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p<0.0001$=****).

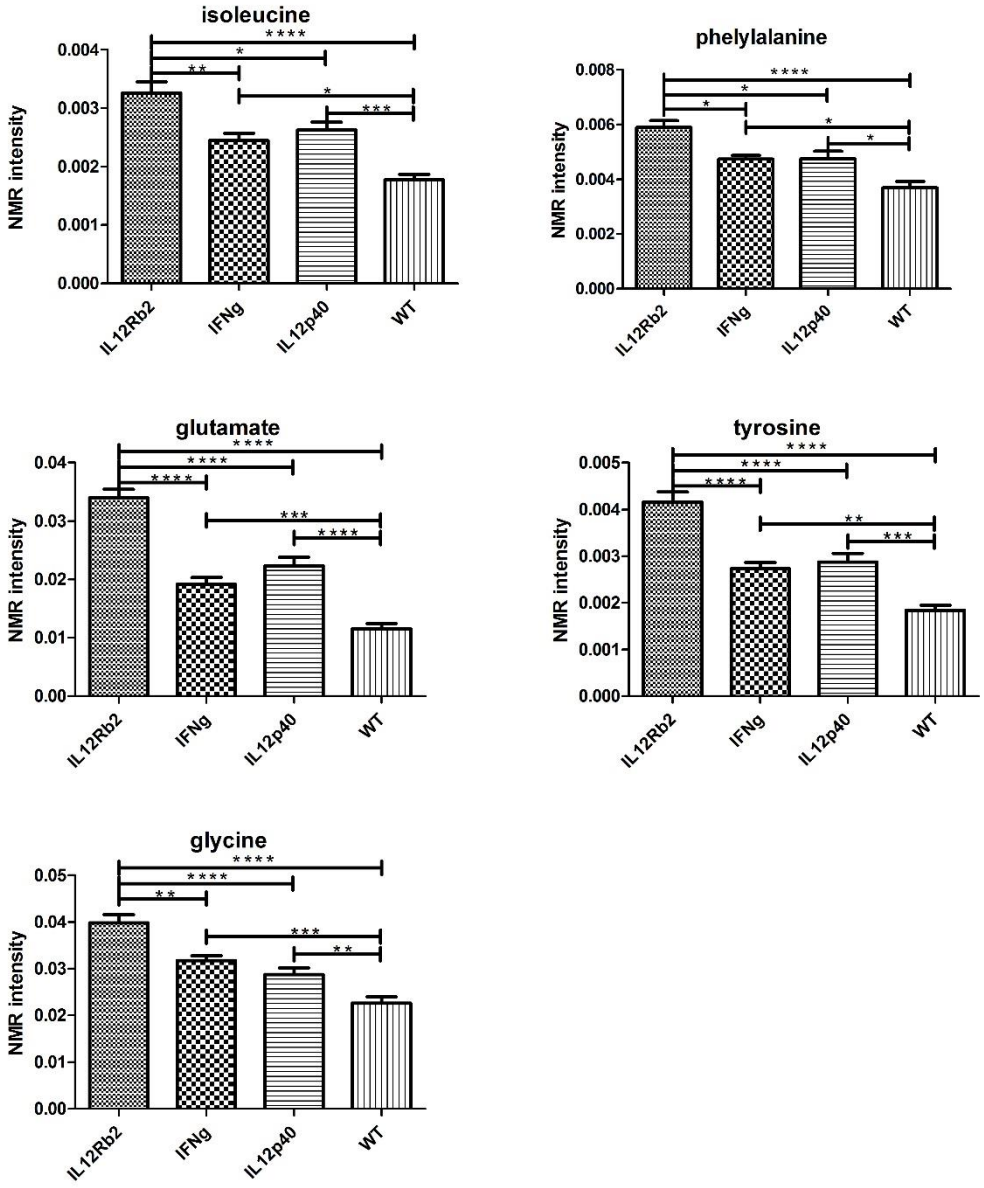


Figure 34 Five of the identified amino acids had highest NMR intensity in the IL12Rβ2 group; lower in the IFNγ and IL12p40 which were not significantly different between each other but were significantly different from WT group that had the lowest intensity (ANOVA/Kurskal-Wallis $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p<0.0001$=****).

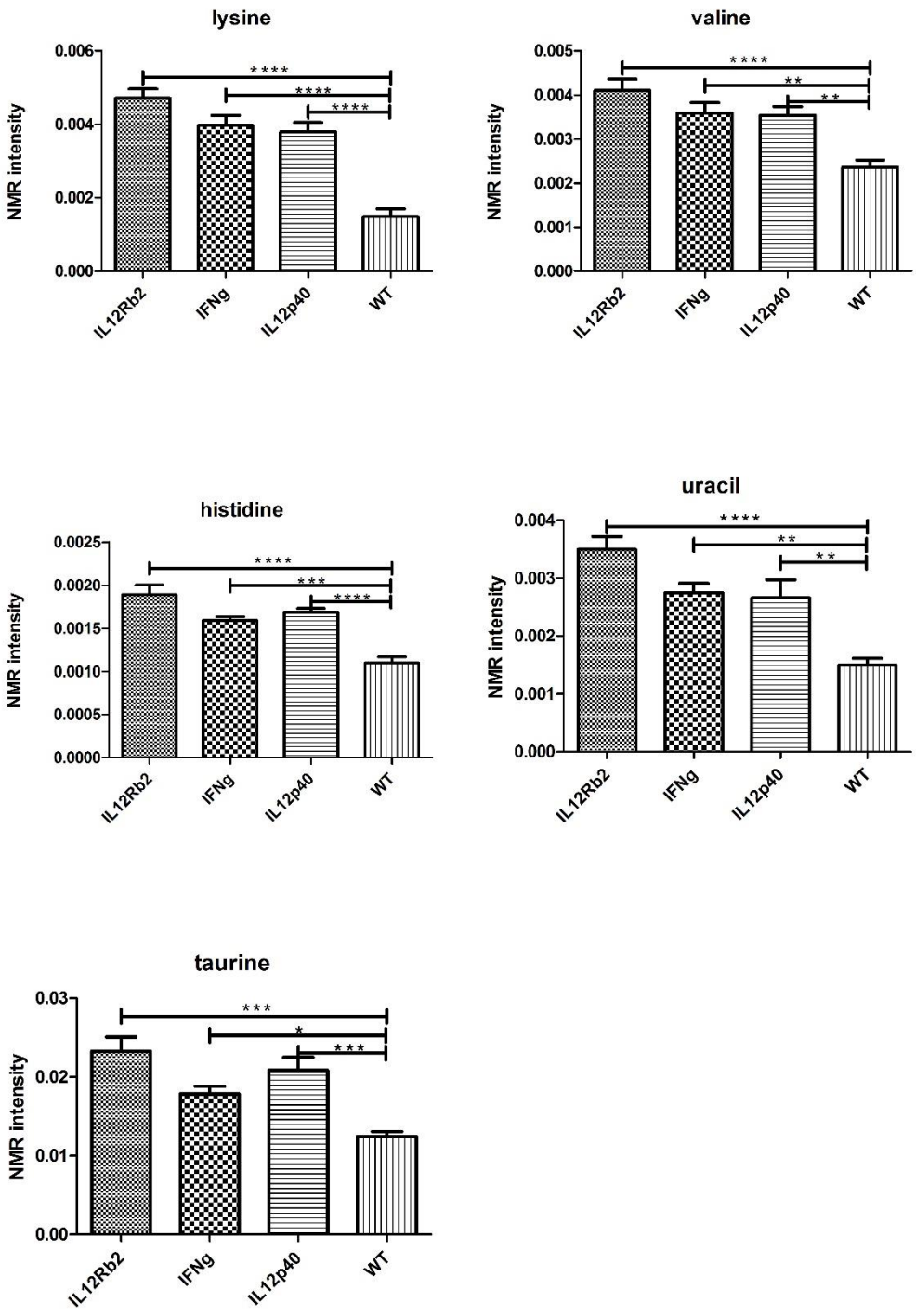


Figure 35 For another five of the identified amino acids and taurine, again the highest intensity was seen in the IL12Rβ2 group and the lowest in the WT but the difference was significant only for the comparison of WT against the other three groups (ANOVA/Kurskal-Wallis $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p<0.0001$=****).

4.2.2. Metabolic profile of the host intestinal tissue

We were interested to know whether the deficiency in IL-12 related genes will have an effect on metabolic profile of the gut and whether metabolic status of the luminal content will be reflected in the host intestinal tissue.

In contrast to the PCA of the luminal metabolites (**Figure 29**), the PCA of the mouse gut tissue (**Figure 36**) showed separation between IL12Rβ2 and IFNγ on one side against WT and IL12p40 on the other. The group that differed most was IL12p40 (**Figure 37, Figure 38**).

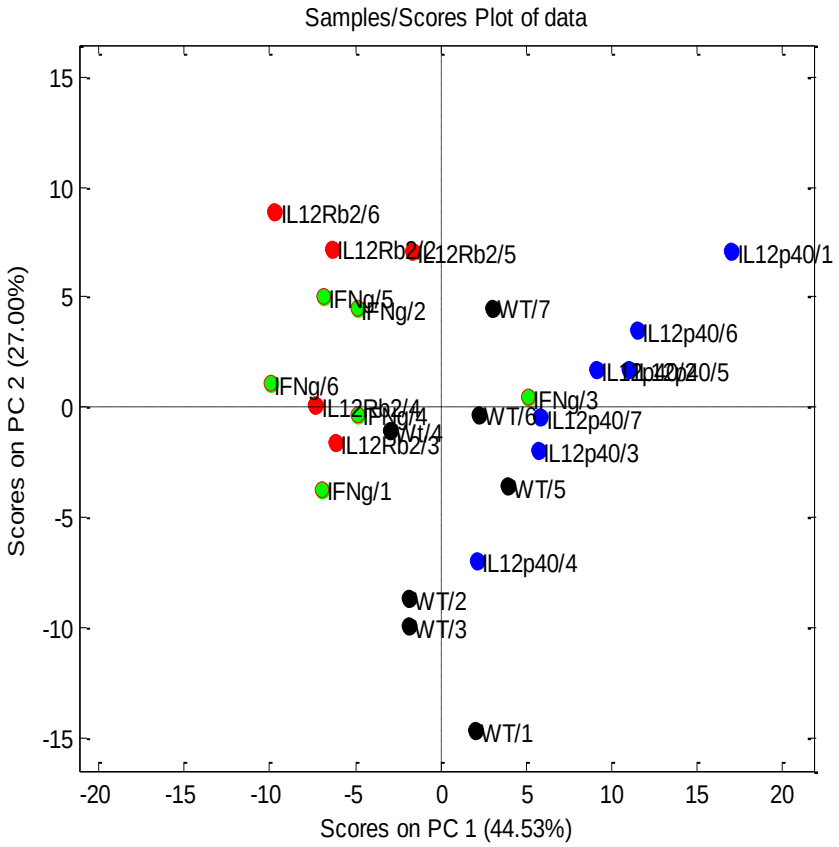


Figure 36 PCA on 1H NMR spectra of thirty metabolites present in host intestinal tissue.

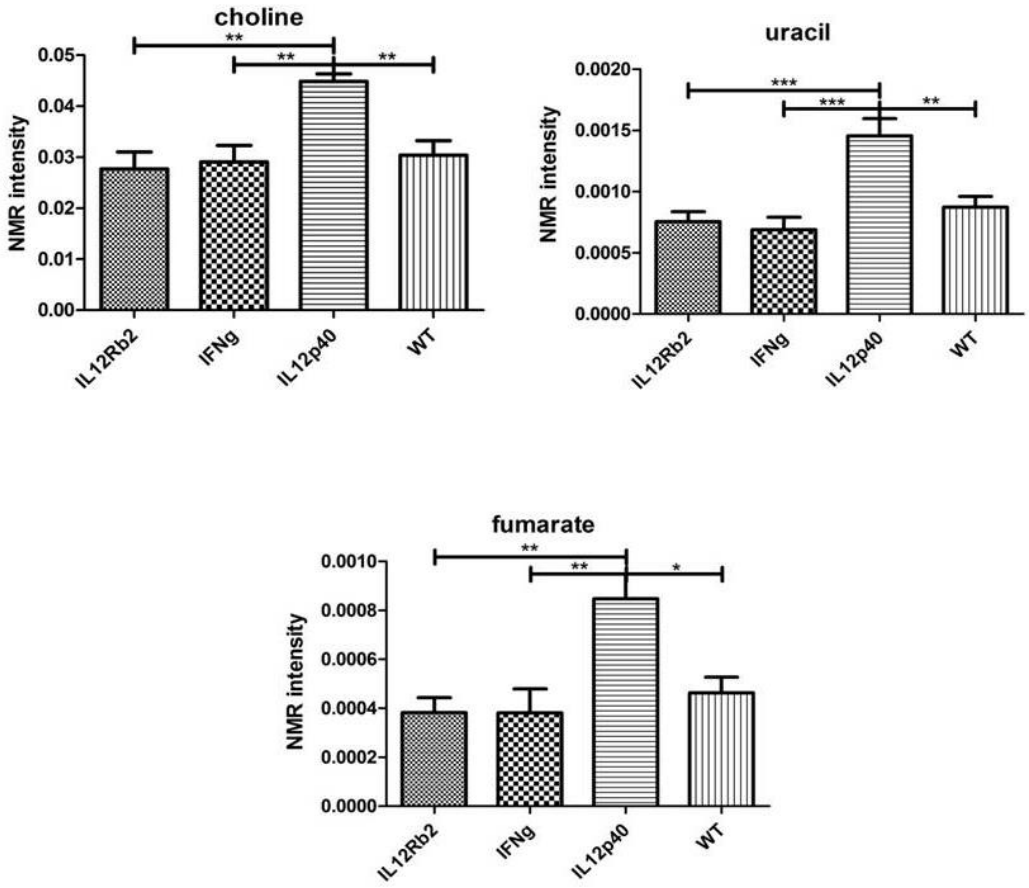


Figure 37 Metabolites differentially abundant in the gut tissue of IL12p40 KO mice (ANOVA/Kurskal-Wallis $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p<0.0001$=****).

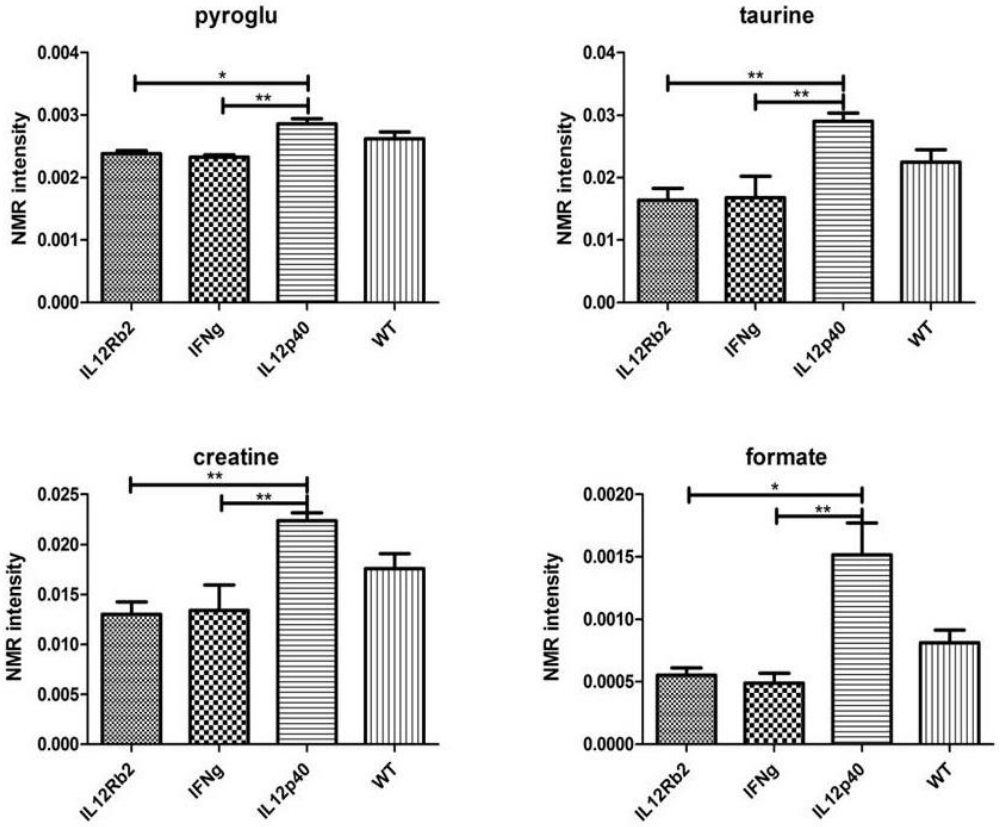


Figure 38 Examples of metabolites that were significantly different between IL12p40 and IFNγ/IL12Rβ2 KO mice (ANOVA/Kurskal-Wallis p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****).

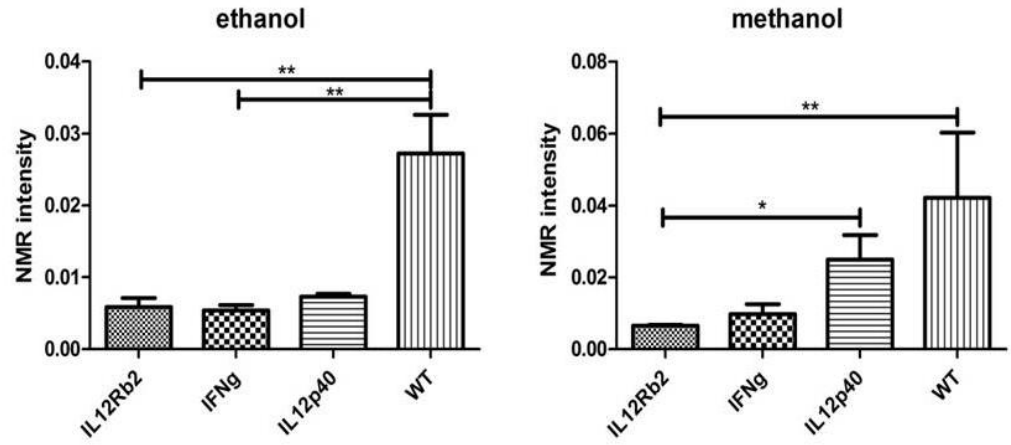


Figure 39 Similarly to the findings for the luminal content, ethanol as well as methanol were elevated in the WT group (ANOVA/Kurskal-Wallis p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****).

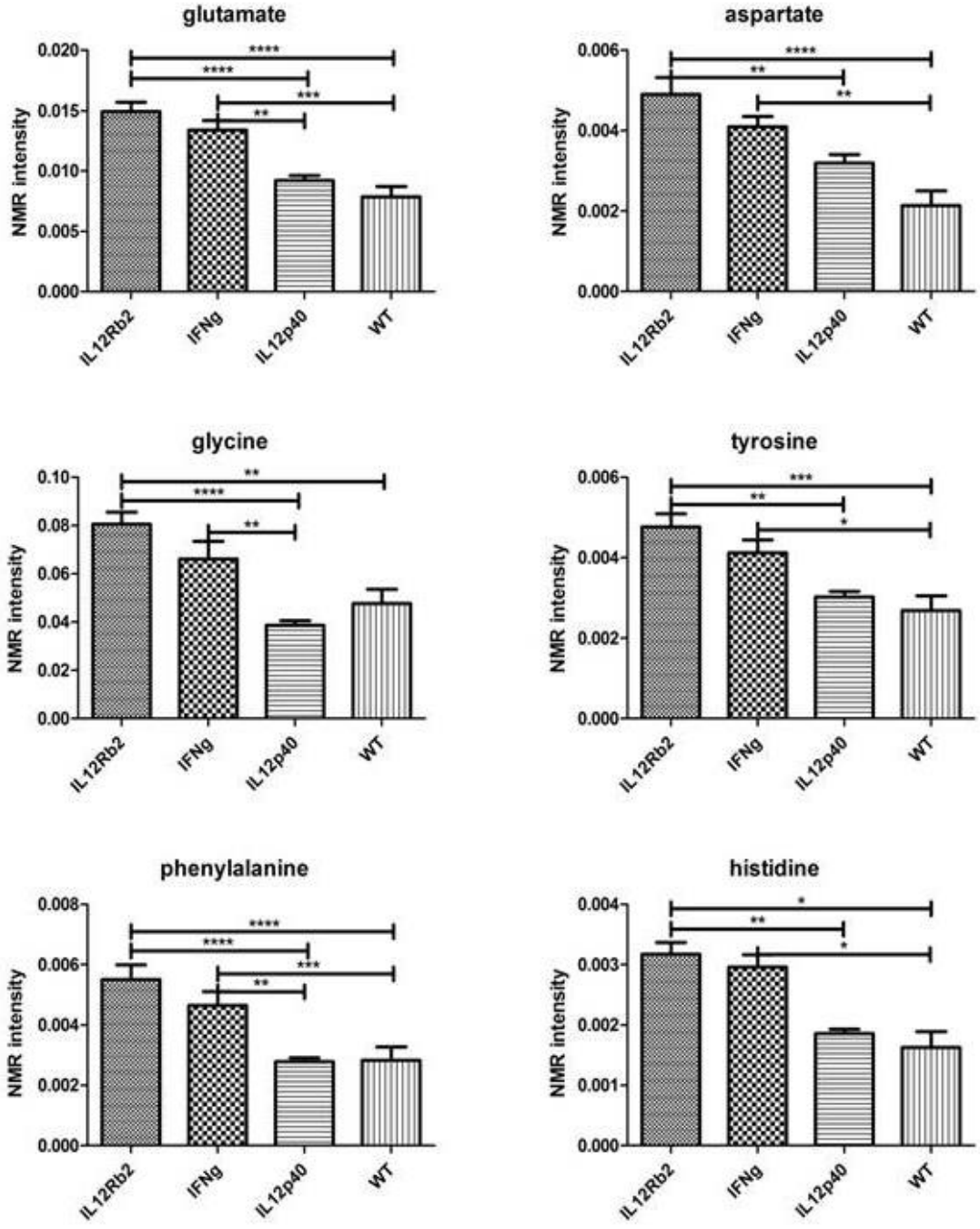


Figure 40 Most of the amino acids were similar between IFNγ and IL12Rβ2 KO groups whilst differing from IL12p40 and WT (ANOVA/Kurskal-Wallis $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p<0.0001$=****).

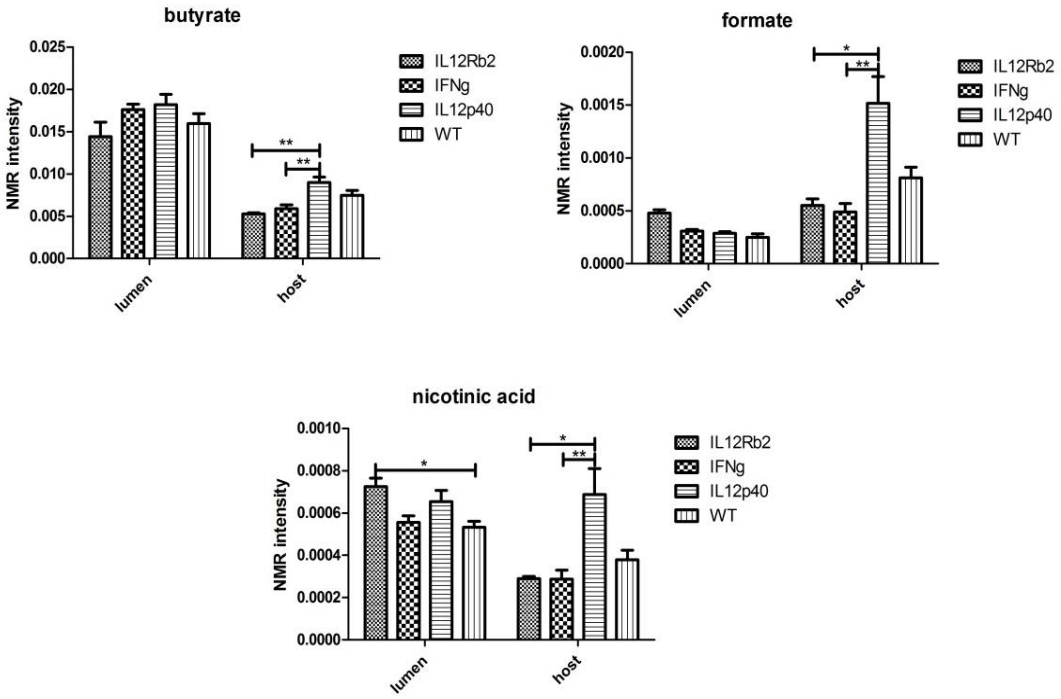


Figure 41 Examples of metabolites that have different patterns of abundance between the groups depending on whether the lumen or the host tissue have been analyzed (ANOVA/Kurskal-Wallis $p<0.05$=*, $p<0.01$=**, $p<0.001$=***, $p<0.0001$=****).

5. Discussion

In the past decade sequencing technology has become widely affordable allowing for bloom in the field of microbiota research (**Figure 22**). In addition, gut microbiota has been recognised as a contributor in both health and disease. Conditions such as IBD, obesity, and metabolic syndrome had been associated with intestinal dysbiosis. These factors lead to increased interest in how microbiota modulates the mammalian host. It is likely that the host-microbiota interaction is a reciprocal process and the host is also capable of driving changes in the bacterial communities resident in the gut. Therefore we hypothesised that alternation in the host's immune status leads to altered intestinal microbiota.

The choice of KO mouse strains in this study was opportunity driven. The IL12Rβ2$^{-/-}$, IL12p40$^{-/-}$, IFNγ$^{-/-}$ and WT mice were available as a part of the work described in **Chapter 3**.

The results so far suggest that IL12p40 and IFNγ KO mice harbour relatively similar microbial communities (**Figure 25, Figure 26, Figure 29**). At phylum level the differences between IL12p40 and IL12Rβ2 KO mice were with highest statistical significance (**Figure 25, Figure 26,)**. IL12Rβ2 and IL12p40 KO mice are both deficient in IL12p70 signalling but due to subunit sharing in the IL-12 cytokine family IL12Rβ2 KOs are also lacking IL-35 whereas IL12p40 KOs have no IL-23 signalling. IL-12 and IL-23 are mainly proinflammatory cytokines involved in the development of the Th1 and Th17 subsets of helper T cells, respectively (Vignali and Kuchroo, 2012). On the other hand, IL-35 is a potent inhibitory cytokine involved in tolerance possibly via suppression of Th17 (Shen et al., 2014). This establishes a functionally balanced dichotomy between IL-12/IL-23 and IL-35 and possibly underlines the differences observed between the microbiota and metabolic profiles of IL12p40 and IL12Rβ2 KO mice.

One of the limitations of the 16S metagenomics analysis is that while providing phylogenetic and abundance information about the bacteria present it does not give any indication of the function these microbes perform. There is possibility that remotely related (distant 16S) species occupy the same ecological niche. Or

alternatively, closely related species (similar 16S) may have very distinct roles. For that reason, the metabolic profile of the luminal content was also analysed. In this instance, again the IL12p40 and the IFNγ groups exhibited parallels in terms of their metabolic profiles (**Figure 29**).

Consistent trend in the abundance of the detected amino acids was evident where the NMR intensity was highest in the IL12Rβ2 group and lowest in the WT, with IL12p40 and IFNγ being at intermediate level (**Figure 26, Figure 35**). We were also interested to know whether the metabolic compounds present in the associated host gut tissue would reflect the metabolic profile observed in the lumen. Similarly to the metabolic analysis of the lumen, intestinal tissue from the IL12Rβ2 group displayed elevated abundance of amino acids (**Figure 40**). IL12Rβ2 are known to have increased protein in the urine and decreased protein in the blood (Airoldi et al., 2005). It is possible that microbiota capable of increasing the nutrient availability of amino acids is selected in those animals.

A number of compounds, including butyrate and nicotinic acid (niacin), showed different patterns of abundance depending on whether lumen or tissues were analysed (**Figure 41**). Butyrate was elevated in the tissue of IL12p40 mice while being no different in the lumen. This could possibly suggest that these microbiota derived compounds (Singh et al., 2014) are not simply diffusing into the host tissue. Indeed, evidence from the literature indicates that there are specialised butyrate (Hadjiagapiou et al., 2000, Lecona et al., 2008) and nicotinic acid (Takanaga et al., 1996, Nabokina et al., 2005) transporters. For example, monocarboxylate transporter 1 (MCT1) transports butyrate across the apical membrane of colonocytes (Thibault et al., 2007) and it has been shown that immunological signals can influence its expression (Thibault et al., 2007). Both butyrate and nicotinic acid can activate GPR109A (encoded by Niacr1) receptor (Singh et al., 2014). GPR109A signalling promotes anti-inflammatory polarisation of colonic macrophages and DCs. Moreover, it is essential for butyrate-mediated induction of IL-18 in colonic epithelium (Singh et al., 2014). Our finding that IL12p40 deficiency is related to higher concentration of butyrate and nicotinic acid in the gut alongside the knowledge built about

transport and immunological function of these compounds establishes an exciting platform for further research.

6. Concluding remarks

The work described here represents a pilot investigation aiming at identifying suitable targets for future studies. One main issue that requires addressing is discrimination between the cage effect and the effect of the host genotype on the microbiota.

With the advance in the microbiota field more and more evidence has accumulated in the literature in regards to the presence of cage effects. It has also been shown that transfer of disease-associated microbiota occurs when healthy WT mice are cohoused with colitic animals (Garrett et al., 2007). Coprophagy contributes to the exchange of microbiota between animals sharing the same cage. It has been suggested that stochastic differences in community assembly occur within each individual cage microenvironment. Attempts to eliminate cage effects by standardizing the initial microbial community within cages or with identical initial gavage to multiple animals are therefore likely to fail (McCafferty et al., 2013). Experiments where different mouse genotypes are compared require several micro-isolators, each housing a mixture of mice from the different genotypes. On the other hand, there are studies demonstrating that when cages containing only one mouse strain are compared with those containing two strains of mice, host genetics contributed to a greater extend to the differences in the microbiota. Campbell et al. have concluded that environmental effects are weaker than underlying host genetics in shaping cecum bacterial communities (Campbell et al., 2012) supporting the notion that the differences reported here possibly reflect the effect of IL-12 signalling deficiency on the intestinal microbiota.

Chapter 5: Extending the study of lympho-epithelial cross talk - a new role for CX3CR1^{+} cells in the gut: pathogen exclusion

1. Introduction

One of the main tasks of the epithelium overlying the mucosal surfaces of the gut is to provide an efficient barrier to microorganisms present in the intestinal lumen. Firstly, this is achieved by the presence of tight junctions that allow passage of water and ions but provide an effective mechanical barrier to macromolecules and microbes (Turner, 2009). Secondly, a combination of mucus and secretory IgA provide an efficient gel that sequestrates harmful microorganisms and prevent them from crossing the epithelial barrier in a process known as immune-exclusion (Mantis et al., 2011, Johansson et al., 2011). Furthermore, it has been recently shown that a few hours after infection the epithelium-intrinsic NAIP/NLRC4 inflammasone drives the expulsion of infected epithelial cells to restrict *Salmonella* replication in the mucosa (Sellin et al., 2014). Ultimately, the aim of these protective mechanisms is to prevent pathogens from traversing/colonizing the intestinal mucosa. We have previously reported that intestinal challenge with *Salmonella* Typhimurium induced, very shortly after infection, the migration of *Salmonella*-capturing CX3CR1^{+} cells into the intestinal lumen (Arques et al., 2009). Interestingly, in the occurrence of infection with *Salmonella*, CX3CR1^{+} cells display dual behaviour and these cells can also directly sample bacteria by using cellular extensions that protrude between epithelial cells and shuttle bacteria across the epithelium to initiate immune responses (Rescigno et al., 2001, Niess et al., 2005). Most importantly, presence of the CX3CR1 receptor is essential for both processes. To further investigate its role we have utilised a mouse model which was first described by Jung et al. in year 2000 (Jung et al., 2000a). They have replaced the *CX3CR1* gene with *green fluorescent protein (GFP)* reporter. This approach enabled them to assign CX3CR1 expression to monocytes, subsets of NK and dendritic cells,

and the brain microglia of mice. CX3CR1 is a seven-transmembrane receptor for fractalkine (FKN, CX3CR1L, neurotactin).

FKN is produced in two isoforms: membrane bound and secreted. It allows for both adhesion and chemottraction (Jung et al., 2000a). Soluble FKN induces intracellular calcium fluxes but does not activate integrins (Haskell et al., 1999). FKN mediated cell adhesion in the presence of pertussis toxin indicates that CX3CR1 acts primarily as an adhesion molecule, rather than as a signalling molecule but mechanism of signalling independent of G-protein is possible (Haskell et al., 1999).

FKN is expressed by activated endothelial cells in the vasculature (Haskell et al., 1999), steady state neurons in the central nervous system (Harrison et al., 1998), iris and retina of the eye (Silverman et al., 2003), steady state basal keratinocytes of the epidermis (Lucas et al., 2001), IFN-γ stimulated bronchial epithelial cells (Fujimoto et al., 2001), and steady state intestinal epithelial cells (Muehlhoefer et al., 2000). Expression of the FKN in the gut is at its highest level in IECs isolated from the terminal ileum (Niess et al., 2005). In both $CX3CR1^{+/gfp}$ and $CX3CR1^{gfp/gfp}$ the fluorescent cells form a net like structure under the IECs in the villi of the small intestine. The interaction of epithelial FKN with CX3CR1 on immune cells is a classic example of how the lympho-epithelial crosstalk could be mediated at a molecular level.

The lamina propria (LP) $CX3CR1^{+/gfp}$ cells are interconnected and in a steady state situation there are 3.1 ± 0.8 connections per cell (Niess and Adler, 2010). These cells are also present in the PP and MLNs. Lamina propria $CX3CR1^{+}$ cells from $CX3CR1^{+/gfp}$ and $CX3CR1^{gfp/gfp}$ mice also express major histocompatibility complex (MHC) class II, CD80, and CD86 at levels that are comparable to $CX3CR1^{+/+}$.

Germ free (GF) $CX3CR1^{+/gfp}$ mice have reduced number of CX3CR1 positive cells in the LP and the MLN, hence presence of commensal flora is required for the local accumulation of these cells (Niess and Adler, 2010) underlining the importance of the three-way cross-talk in the gut involving not only the IECs and the LP immune cells but also the resident microbiota.

There is a lot of controversy in regards to the role of CX3CR1^{+} cells in the process of antigen sampling and their interaction with the nearby CD103^{+} cells in the LP. Schulz et al. have reported that CD103 and CX3CR1 phenotypically and functionally characterize distinct subsets in the LP. CX3CR1^{+} cells represent a slow turnover, non-migratory gut-resident population, whereas the CD103^{+} DCs migrate into the MLNs. CX3CR1^{+} cells display poor T cell stimulatory capacity *in vitro* and *in vivo* and are less efficient at generating retinoic acid compared to the CD103^{+} DC. These findings indicate that CD103^{+} DCs serve as classic DCs, whereas CX3CR1^{+} populations are macrophage-like and might modulate immune responses directly in the mucosa and serve as first line barrier against invading enteropathogens.

We have previously reported that intestinal challenge with *S. enterica* Typhimurium induced the migration of *Salmonella*-capturing CX3CR1^{+} cells into the intestinal lumen (Arques et al., 2009). The migration of CX3CR1^{+} cells was restricted to the small intestine in a flagellin/MyD88-dependent manner and did not affect the integrity of the epithelial barrier. Interestingly, in the occurrence of infection with *Salmonella*, CX3CR1^{+} cells display dual behaviour and these cells can also directly sample bacteria by using cellular extensions called trans-epithelial dendrites (TEDs) that protrude between epithelial cells and shuttle bacteria across the epithelium to initiate immune responses (Rescigno et al., 2001, Chieppa et al., 2006). Most important, the presence of the FKN receptor CX3CR1 appeared to be essential for both events (Niess et al., 2005, Nicoletti et al., 2010). We sought to investigate the biological relevance of these two CX3CR1-mediated events by using *in vivo* real time imaging and a combination of mouse strains that differed in their ability to undergo CX3CR1- mediated direct sampling and intraluminal migration during *Salmonella* infection

Interestingly, while luminal sampling is a strain specific event observable only in mice on C57BL/6 but not Balb/c background (Vallon-Eberhard et al., 2006), luminal migration is a universal mechanism (Arques et al., 2009, Chieppa et al., 2006). Formation of TEDs in first generation hybrid CX3CR1$^{+/gfp}$ mice with wild-type CX3CR1 allele from Balb/c parent and CX3CR1^{GFP} allele from the C57BL/6 parent still occurs. Thus, the Balb/c allele encodes an active CX3CR1 receptor

and the absence of TEDs in Balb/c mice is a recessive phenotype not attributed to the CX3CR1 locus (Vallon-Eberhard et al., 2006).

The availability of wild type C57BL/6 sampling-competent/migration-competent, Balb/c sampling-deficient/migration-competent, and CX3CR1$^{gfp/gfp}$ sampling-deficient/migration-deficient mice provided an opportunity to investigate the biological relevance of the CX3CR1-mediated events.

2. Hypothesis

The observations described above prompted us to test the hypothesis that interaction between *Salmonella* and IECs drives pathogen-capturing CX3CR1^{+} cells to migrate rapidly into the intestinal lumen to limit the number of pathogens crossing the epithelial barrier. Main objective of this work was documenting the CX3CR1-mediated luminal migration by using *in vivo* real time imaging and to assess the differential contribution of sampling and migration to pathogen protection by comparing C57BL/6 sampling-competent/migration-competent, Balb/c sampling-deficient/migration-competent, and CX3CR1$^{gfp/gfp}$ sampling-deficient/migration-deficient mice.

3. Methods

Mice

CX3CR1 GFP knock-in mice (Jung et al., 2000b) on Balb/c background were kindly provided by Prof Oliver Pabst. Six-week-old female CX3CR1$^{+/gfp}$ mice were used for experimentation. The genotype of the animals was confirmed with PCR analysis (**Appendix 2**). CX3CR1$^{-/-}$ (C57BL/6 background) were purchased from Taconic and 6-8 week old WT Balb/c and C57BL/6 were purchase from Charles River. Villin-Cre MyD88 (MyD88$^{\Delta IEC}$) (C57BL/6 background) were kindly provided by K. Hughes and originated from the Welcome Trust Sanger Institute, Hinxton, UK. Mice were maintained and bred under specific pathogen-free conditions. Experiments were approved by the University of East Anglia Welfare and Ethical Review Body (AWERB; ref: 70/7582) and conducted under the guidelines of Animals Scientific Procedures Act (1986) of the United Kingdom. Also, experimental protocols were approved by the Animal Care and Use Committee

of the University of Siena (no. G2007/11/2) under the "Guiding Principles for Research Involving Animals and Human Beings".

Bacteria

InvA⁻AroA⁻, *InvA⁺AroA⁻ and InvA⁺AroA⁺ Salmonella* Typhimurium SL1344 bacterial strains were provided by P. Mastroeni and D. Pickard (Hoiseth and Stocker, 1981, Raffatellu et al., 2005).

Bacterial challenge

Isolated loops were injected with $1x10^7$ non-invasive/non-replicating *InvA⁻AroA⁻* for intravital imaging experiment or invasive/non-replicating *InvA⁺AroA⁻ Salmonella* for collecting intraluminal CX3CR1$^{+/gfp}$ cells for phenotypic and quantitative analysis. Oral challenges were performed by gavages that were delivered five to ten minutes after administration of a solution of $NaHCO_3$ (10% wt/vol/200µl). In order to monitor intraluminal migration of CX3CR1$^+$ cell or bacterial load in the gut within 5 h after infection mice received a single oral dose of $1x10^7$ of either *InvA⁺AroA⁻, InvA⁻AroA⁻* or *InvA⁻AroA⁺ Salmonella*; to determine long-term (5 days post-infection) bacterial load mice received a single dose of $1x10^7$ of *InvA⁻Aro⁺* strain. To determine strain specific susceptibility to *Salmonella* infection mice received a single dose of $1x10^8$ wt (*InvA⁺AroA⁺*) *Salmonella*; finally to investigate both humoral and cellular immunity to non-invasive Salmonella mice received three doses of $1x10^8$ *InvA⁻ AroA⁻ Salmonella* at three day interval.

In order to monitor intraluminal migration of CX3CR1$^+$ cells and faecal bacterial load mice received a single oral dose of $1x10^7$ of *InvA⁺Aro⁻ Salmonella*. To determine translocation of non-invasive *InvA⁻ Salmonella* two approaches were undertaken. For short term experiments mice (n=8-10mice/group) were orally administrated with a single dose of *InvA⁻AroA⁻ Salmonella* and sacrificed at 30, 60, 180 and 270 minutes post-infection. For long term experiments mice received the same dose of *InvA⁻Aro⁺* and mice were sacrificed 5 days post-infection. Tissues (small intestine and PPs for short term experiments; PP, MLN and spleen for long term experiments) were harvested weighed and treated with gentamicin (1 h at 37° C). After repeated washings in PBS tissues were

homogenized. Serial dilutions of the homogenates were plated on LB agar and incubated overnight at 37° C.

Surgical procedure

Animals were starved for 6 h prior anaesthesia with isoflurane, opening of the abdominal cavity and ligation of the ileum. The lumen of the intestinal loop was injected with *Salmonella* as described before (Arques et al., 2009). An hour later a 15 mm longitudinal incision was made such that the intestinal tube was cut open without damaging the blood supplying vessels or the mucosa chosen for imaging. The tissue was washed gently with warm PBS to remove residual luminal content. On the glass window of the microscope stage a drop of *Salmonella* in PBS was used to immerse the exposed luminal side. The tissue was held in place by covering it with a 13 mm in diameter round glass coverslip clamped to the stage. Importantly the pressure used to hold the specimen in place was enough to prevent displacement of the view field by weak peristaltic movement but also allowing for efficient blood supply and preserving the structure and functional integrity of the gut. To prevent drying of the exposed tissue it was covered with a cotton pad soaked in PBS which was regularly re-moisturised. During incubation and imaging the temperature of the animal was maintained by the enclosed microscope temperature control system (Life Imaging Services, Basel, Switzerland) part of the multiphoton setup.

Multiphoton imaging

The Multiphoton microscope System from LaVision BioTeC (Bielefeld, Germany) used consisted of TriM Scope II single beam 2-photon microscope system including Nikon Eclipse Ti optical inverted microscope; Chameleon Ultra II Ti: Sapphire laser and Chameleon Compact Optical Parametric Oscillator (OPO) (Coherent Inc., USA); holder (Life Imaging Services, Basel, Switzerland); 2-axis galvanometric XY-scanner; Multi-PMT adapter for up to 4 PMTs, containing dichroic beam splitters at 495, 560 and 625 nm. The system was controlled by Inspektor Pro 4.0 software.

With the excitation laser output of 3380 mW split 5%:95% between the laser scanner and the OPO, the Ti:Sa laser power was 169 mW, and the OPO output

was ~300 mW at 1000 nm. The attenuators setting for the Ti:Sa power was 20.25%, and the OPO power 10.75%, giving effective power values on the sample of 35 mW at 860 nm, and ~30 mW at 1000 nm.

Imaging was done with the galvanometric scanner covering an area of 200x200 microns with 1000*1000 pixels (nominal 0.2 micron) image resolution, using a Nikon CFI-Plan Apochromat "VC" 20X air lens; corrected for cover slips. 3-5 line averages were used to improve the signal/noise ratio. Z-stacks were acquired with 1 micron steps. Selective imaging of the GFP^+ cells was done with 860 nm excitation.

Data analysis

Image analysis was done with the Fiji/ImageJ package (Schindelin et al., 2012)

The imaging data were saved as four individual ome.tif files from the 4 PMTs.

For display, the intensities of the rgb channels were individually adjusted (low, high). To get a better signal/noise ratio a median filter was applied to the images. 3D displays and video clips of the data sets were made with the ImageJ 3D viewer plugin.

The TrackMate_ (Fiji) plugin (Authors: Nick Perry, Jean-Yves Tinevez, Johannes Schindelin) was used to follow the movement of individual cells. Because of the irregular shapes of the $CX3CR1^+$ the tracking points were placed manually on the centres of cells. To account for tissue movement/distortions during the imaging, the movement of single cells were determined relative to 'fixed' (i.e. not moving in the tissue) neighbouring cells. This gave more adequate results for local movements than global registering of tissue stacks.

Flow cytometry and isolation of $CX3CR1^+$ cells

Following bacterial challenge, luminal contents were carefully recovered by gently flushing the intestine with PBS. Intraluminal $CX3CR1^{+/gfp}$ cells were isolated and characterized by flow cytometry as described in details elsewhere (Arques et al., 2009). Samples were analysed by BD FACSAria II (BD Biosciences). The following antibodies were used: CD11c (HL3) (BD Biosciences), CD103 (M290) (BD Biosciences), CD103 (2E7) (eBioscience), F4/80 (BM8) (eBioscience), MHC II (M5/114.15.2) (eBioscience), SiglecF (E50-2440) (BD Biosciences). For

the isolation of CX3CR1^{+} cells intestinal tissue from CX3CR1$^{+/gfp}$ mice were collected and tissues repeatedly treated with HBSS containing EDTA (2mM). After each treatment tissues were shaken and supernatant discarded. After each wash an aliquot from the supernatant was analysed by microscopy to detect the presence of IEC; EDTA treatment was stopped (usually after 3-4 treatments) when epithelial cells were not present in the supernatant. Tissues were then treated for 50 minutes in RPMI 1640 with 10% FCS, 0.24mg/ml collagenase VIII (Sigma) and 40 U/ml DNase I (Roche) (Schulz et al., 2009). After shaking cells suspensions were filtered and then purified by gradient separation as described before (Schulz et al., 2009). Cells were sorted (>95% purity), suspended in PBS and injected into the intestinal lumen for pathogen exclusion assay.

Immunity to non-invasive *Salmonella*

Salmonella specific IgG and IgA were detected in serum and faeces. Briefly, serum was obtained from blood after 1 h incubation at 37° C and collected after centrifugation. Faecal samples were weighed and resuspended in PBS in presence of proteases inhibitors (Sigma-Aldrich); debris-free supernatants were then collected after centrifugation. ELISA plates (Costar) were coated with lysate from wt *Salmonella* obtained as described before (Beal et al., 2004). Plates were blocked and then incubated with dilutions of both serum and faecal solution. After repeated washings plates were incubated with anti IgA- and anti IgG biotinylated antibody (Abcam); this was followed by incubation with Streptavidin Peroxidase (Abcam). Also, numbers of single IgA-antibody forming cells (AFC) were detected using a modified ELISPOT assay developed in our lab (Nicoletti et al., 2010). Briefly, 96-well membrane ELISPOT plates (Whatman) were coated with lysate from wt *Salmonella*. After blocking (2% FCS and 2% milk powder) PP-lymphocytes cells (10^6) were added in 5% FCS RPMI and incubated overnight at 37° C and 5%CO_2. Plates were washed with PBS 10mM EDTA, rinsed with cold PBS and then incubated with goat HRP-labelled anti-IgA (Life Technology) antibody overnight at 4° C. This was followed with incubation with HRP colour development reagent (BioRad). ELISPOT assay was performed in

duplicate and spots enumerated by diaphanoscope. *In vitro Salmonella*-specific T cell proliferation was measured following co-culture of T cells from *InvA*$^{-}$*AroA*$^{-}$ infected CX3CR1$^{+/gfp}$ and CX3CR1$^{gfp/gfp}$ mice with T cell-depleted splenocytes from naïve wt syngeneic mice in presence or absence of *Salmonella* lysate and treated with mytomicin C. T cells and T-cell-depleted splenocytes were prepared using MACS Cytokine Secretion Assay (Miltenyi Biotec) following instructions provided by the manufacturer. T cell proliferation was measured after 72 hours by flow cytometry. Parallel recall challenge cultures were set to determine levels for IFNγ in the supernatant by ELISA (Biolegend).

4. Results

4.1. *Salmonella*-induced migration of CX3CR1^{+} macrophages occurred through paracellular channels in the epithelium.

We used a genetically modified mouse strain (Abreu, 2010) in which CX3CR1^{+} cells are expressing GFP (**Figure 42**). With the help of intravital two-photon microscopy we imaged the transepithelial migration of the CX3CR1^{+} cells in these CX3CR1$^{+/gfp}$ mice shortly (2 h) after the introduction of *InvA*$^{-}$*AroA*$^{-}$ *S. enterica* Typhimurium into isolated ileal loops. First, GFP-expressing CX3CR1^{+} cell protruded into the intestinal lumen from the surface of the intestinal epithelium (**Figure 43 A**) (**Appendix 3 Figure S3 A**) and progressed further into the lumen (**Figure 43 B, C**) (**Appendix 3 Figure S3 B, C**) before moving away from the entry site (**Figure 43 D, E**) (**Appendix 3 Figure S3 D, E**). It also appeared that the imaged cell was immediately followed by another CX3CR1$^{+/gfp}$ cell migrating via the same opening in the epithelium (**Figure 43 D, E**) (**Appendix 3 Figure S3 D, E**). The integrity of the tissue remained intact throughout the experiment (**Figure 44**) (**Appendix 4 Figure S4, Figure 44**). This migratory pattern was also seen by TEM (**Figure 45**). The cells migrating into the lumen, one of which was in close contact with *Salmonella* appeared to move through paracellular channels. The migration of CX3CR1^{+} cell is unidirectional; after collecting intraluminal GFP-CX3CR1 cells and reintroducing them into freshly isolated intestinal ileal loop no GFP-CX3CR1^{+} cells traversing the epithelial barrier to migrate back into the intestinal tissue. The majority of intraluminal CX3CR1^{+} cells (**Figure 46**) at 5 h

post-infection displayed the phenotype of gut resident macrophages MHCII^{+} F4/80^{+} CD11c^{+} CD103^{-} SiglecF^{-} that in steady-state situations do not migrate to mesenteric lymph-node, display poor T cell stimulatory capability and possess high phagocytic activity both *in vitro* and *in vivo* (Schulz et al., 2009, Bain et al., 2013).

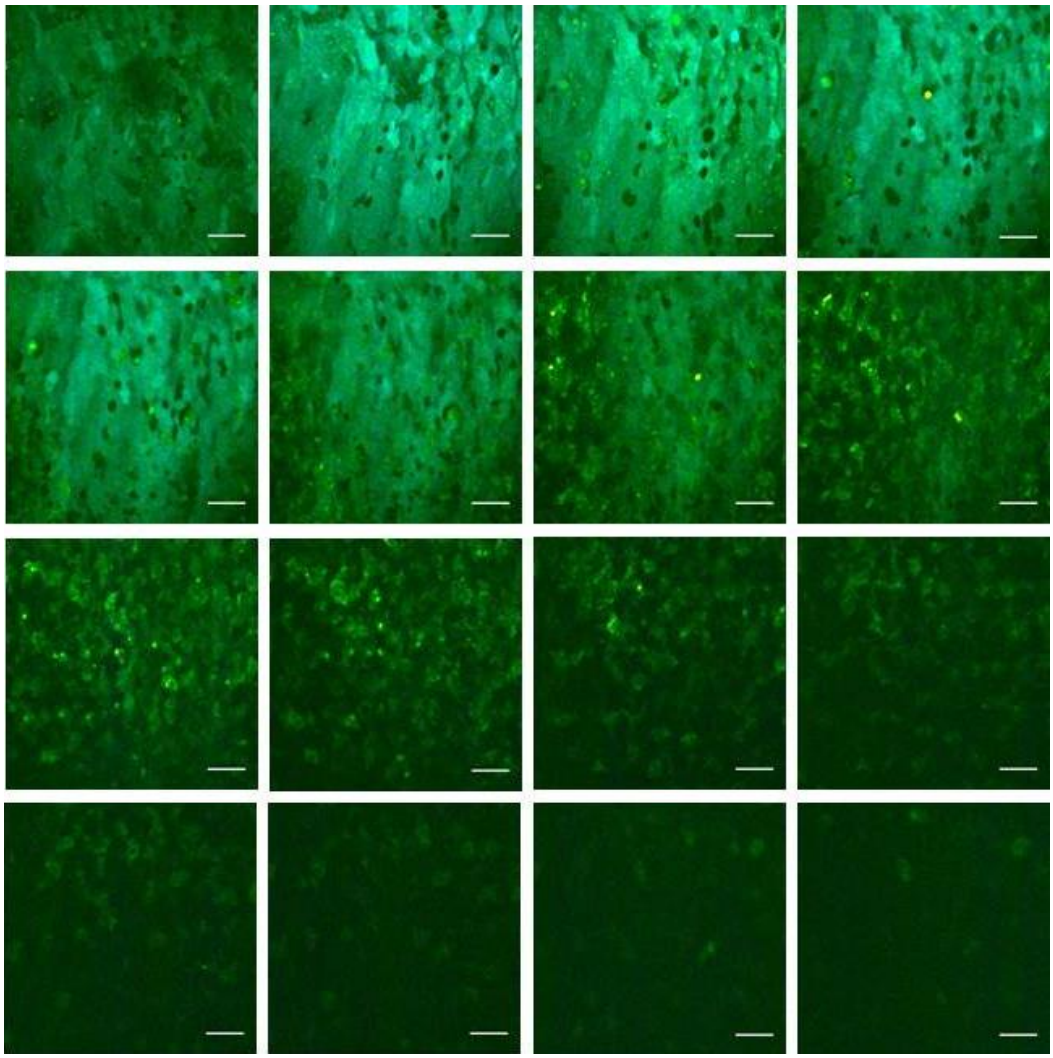


Figure 42 Using *in vivo* multiphoton microscopy series of consecutive optical sections of CX3CR1$^{+/gfp}$ unchallenged mouse intestine were obtained, starting at the luminal surface of the epithelium (turquoise). Underneath the epithelium, at about 15 µm depth are situated a number of interconnected CX3CR1$^{+/gfp}$ cells (green). Scale bar is equivalent to 30 µm.

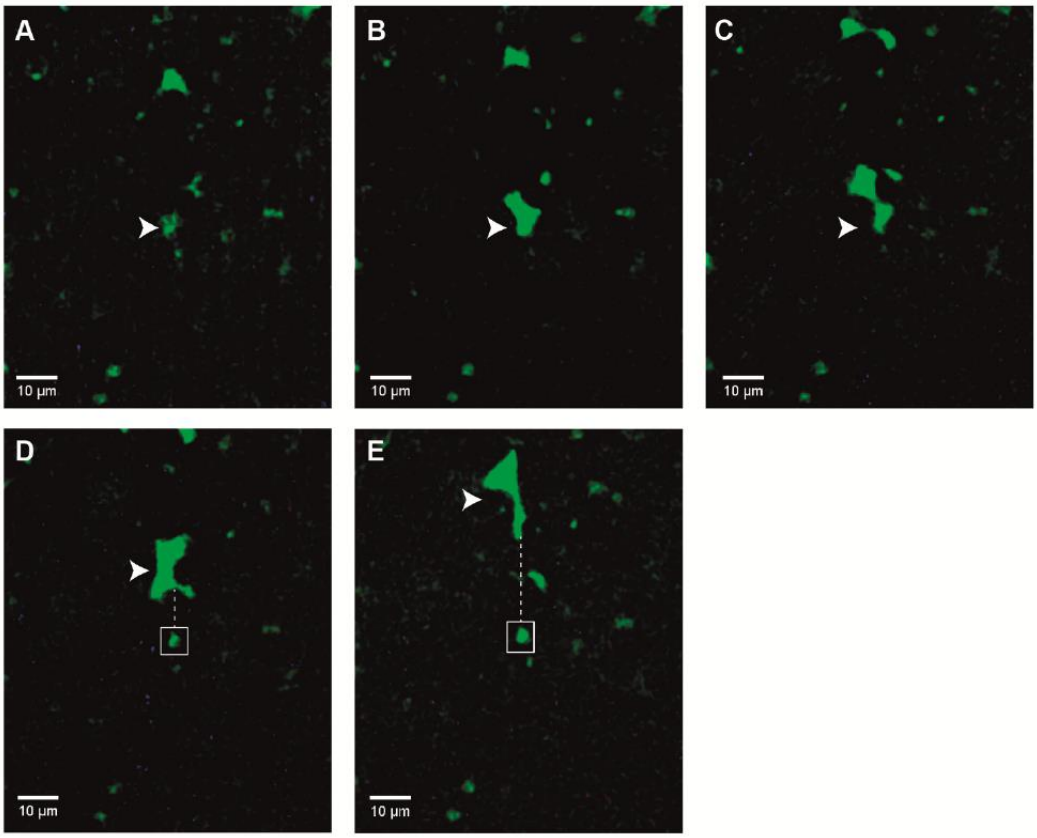


Figure 43 Still images from real time imaging analysis of CX3CR1$^{+/gfp}$ cells migrating into the intestinal lumen. Images were obtained after short term (2 h) challenge with non-invasive *InvA*$^{-}$*AroA*$^{-}$ *Salmonella* Typhimurium in CX3CR1$^{+/gfp}$ mice. CX3CR1$^{+/gfp}$ cell (arrow head) in progress of protruding into the intestinal lumen from the surface (XY plan) of the intestinal epithelium (A) and then continuing to move out into the lumen through an opening of the intestinal epithelium (B-C). The migrating cell did not act as antigen sampling cell and did not retract the sampling devise within the tissue; instead the cell moved entirely within the lumen and away (dotted line) from the entry site (white box) (D-E). This cell is immediately followed by another CX3CR1 $^{+/gfp}$ cell protruding into the lumen from the same opening (D-E) (white box). Orthogonal 3D sections are displayed in (**Appendix 3**).

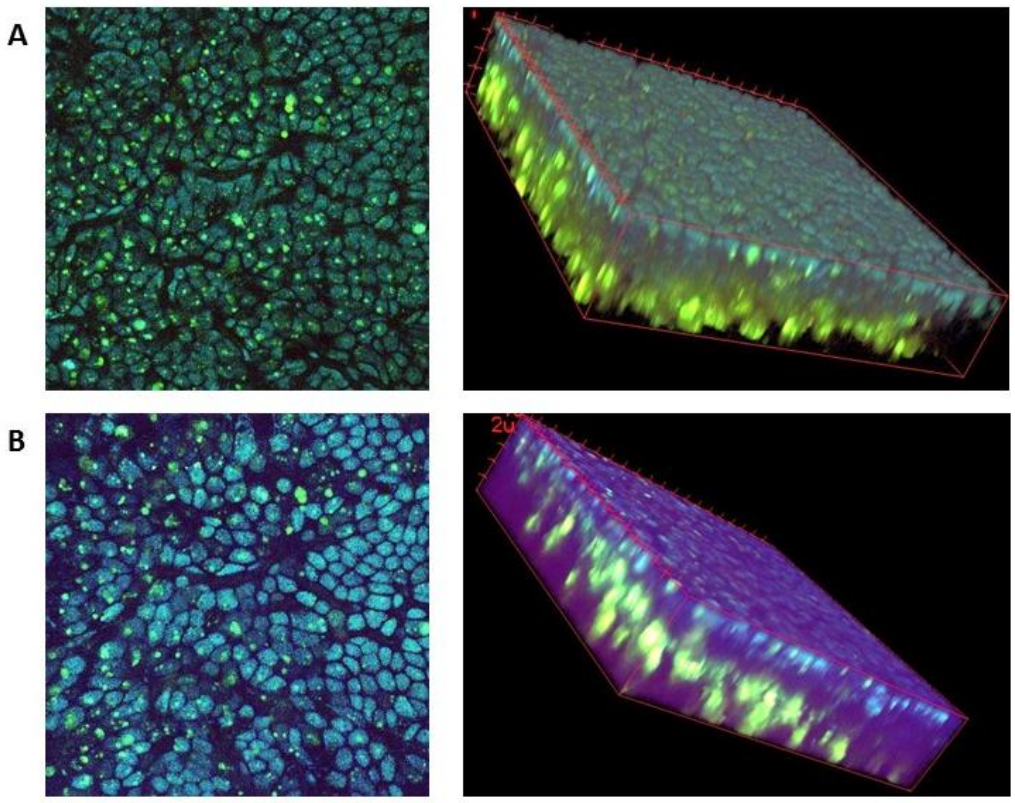


Figure 44 Throughout the *in vivo* multiphoton image acquisition the tissue remained intact. Epithelial layer integrity and three dimensional structure at the beginning of the experiment (A) are comparable to the ones observed at the end (B) of data acquisition.

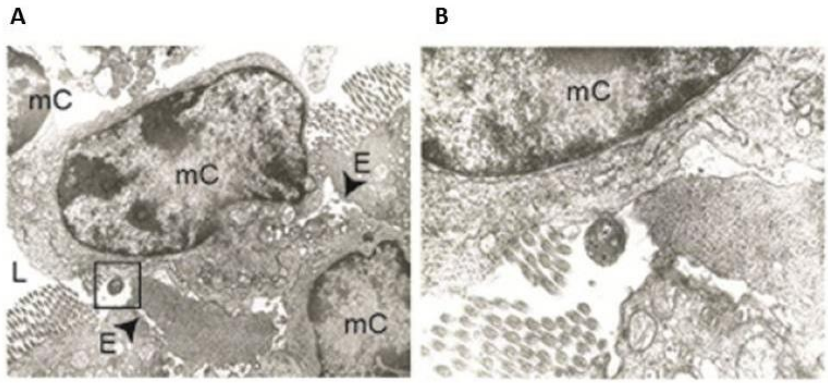


Figure 45 Migration pattern of CX3CR1^{+} cells was further investigated by TEM. Migrating cells (mC) move into the lumen (L) via paracellular space (arrow heads) between adjacent enterocytes (E). Also in (A) one migrating cell is in close contact with *Salmonella* (box) (detail in B).

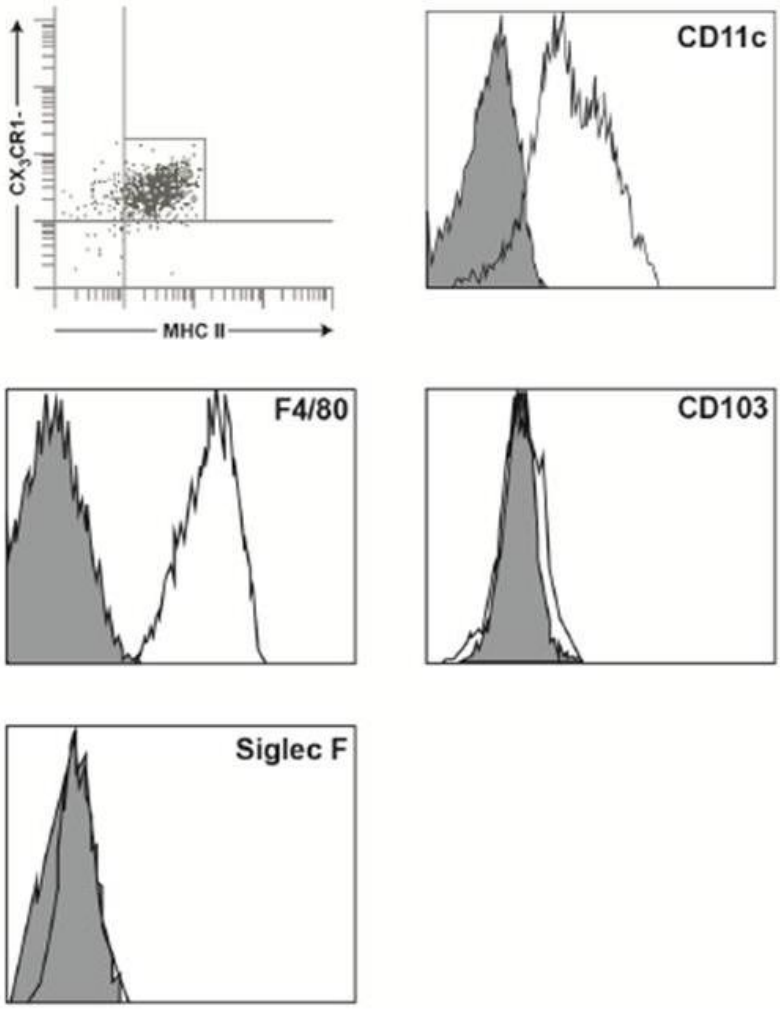


Figure 46 Phenotypic analysis of intraluminal CX3CR1^{+} cell population of CX3CR1$^{+/gfp}$ (Balb/c background) 5 hours following intestinal challenge with 1x10^{7} *InvA*$^{+}$*AroA*$^{-}$ *Salmonella.* The vast majority of the cell population rapidly recruited into the intestinal lumen showed the phenotype of resident (stationary) macrophage with poor T cell stimulatory activity and high phagocytic activity. These cells were MHCII^{+} F4/80^{+} CD11c^{+} but did not express the canonical marker for gut-derived dendritic cells, CD103; also, these cells lacked the neutrophil marker SiglecF. Grey areas correspond to isotype control and white areas represent specific staining. (Experiment performed in triplicate, n=3 mice, representative results are shown)

4.2. Intraluminal cell migration is absent in CX3CR1$^{gfp/gfp}$ and MyD88$^{\Delta IEC}$ mice and is higher in response to invasive *Salmonella*.

Salmonella-induced migration of CX3CR1^{+} cells was further investigated in CX3CR1$^{gfp/gfp}$, CX3CR1$^{+/gfp}$ and villin-Cre MyD88$^{\Delta IEC}$ mice carrying targeted deletion of MyD88 in the IECs (Frantz et al., 2012). After the introduction of *InvA*$^{-}$*AroA*$^{-}$ *Salmonella* Typhimurium into isolated intestinal loops of CX3CR1$^{+/gfp}$ mice the number of CX3CR1^{+} cells appearing in the gut lumen 30 minutes post infection (**Figure 47 A**) reached $0.71x10^{4} \pm 1.2x10^{3}$ which was approximately >30 fold higher than levels seen in CX3CR1$^{gfp/gfp}$ mice ($1.3x10^{2} \pm 1x10^{2}$). The numbers of intraluminal CX3CR1^{+} cells increased steadily between 90 and 270 minutes reaching $8.9x10^{4} \pm 5x10^{3}$. In contrast, no increase in the number of intraluminal CX3CR1^{+} cells was observed in CX3CR1$^{gfp/gfp}$ mice at any time point after infection; thus, showing that, the migration is absent, and not simply delayed in mice lacking a functional fractalkine receptor. Furthermore, we show that lympho-epithelial cross talk is essential for this event. Indeed, IEC-derived signals are instrumental in triggering the migration of CX3CR1^{+} cells in response to *InvA*$^{-}$*AroA*$^{-}$ *Salmonella* Typhimurium. The migration was completely suppressed in MyD88$^{\Delta IEC}$ mice compared to WT counterparts ($1.5x10^{2} \pm 1x10^{2}$ and $6.5x10^{4} \pm 1.1x10^{4}$ respectively at 5 h post infection) (**Figure 47 B**).

We then evaluated the CX3CR1^{+} cells migration in response to oral delivery of *Salmonella* strains that differed in their capacity to invade the host. Intraluminal migration in CX3CR1$^{+/gfp}$ mice (**Figure 47 C**), was significantly higher after infection with the invasive *Salmonella* strain ($4.3.x10^{2} \pm 1.2x10^{2}$) compared to non-invasive strain ($2.1.x10^{2} \pm 1x10^{2}$) already within 30 minutes post-infection. The number of intraluminal cells steadily increased with time and, after 5 h it reached $4.4x10^{4} \pm 9x10^{3}$ and $1.7x10^{4} \pm 1.2x10^{3}$ cells for invasive and non-invasive *Salmonella*, respectively. Migration was significantly reduced in response to both *Salmonella* variants at 12 h post-infection consistent with intraluminal migration being restricted to the initial stage of infection. Cell migration was also significantly higher in *InvA*$^{+}$-treated mice ($1.1x10^{3} \pm 0.4x10^{3}$ and $4.1x10^{2} \pm 1.3x10^{2}$ cells for *InvA*$^{+}$ and *InvA*$^{-}$ respectively after 270 min). CX3CR1^{+} cells were the only intraluminal cell population harbouring intracellular

Salmonella shortly after infection (Arques et al., 2009) suggesting a possible role in pathogen-exclusion. In agreement with this interpretation we observed that at 5 h after infection migration-competent CX3CR1$^{+/gfp}$ mice had a significantly higher faecal bacteria load compared to migration-deficient CX3CR1$^{gfp/gfp}$ mice (**Figure 47 D**).

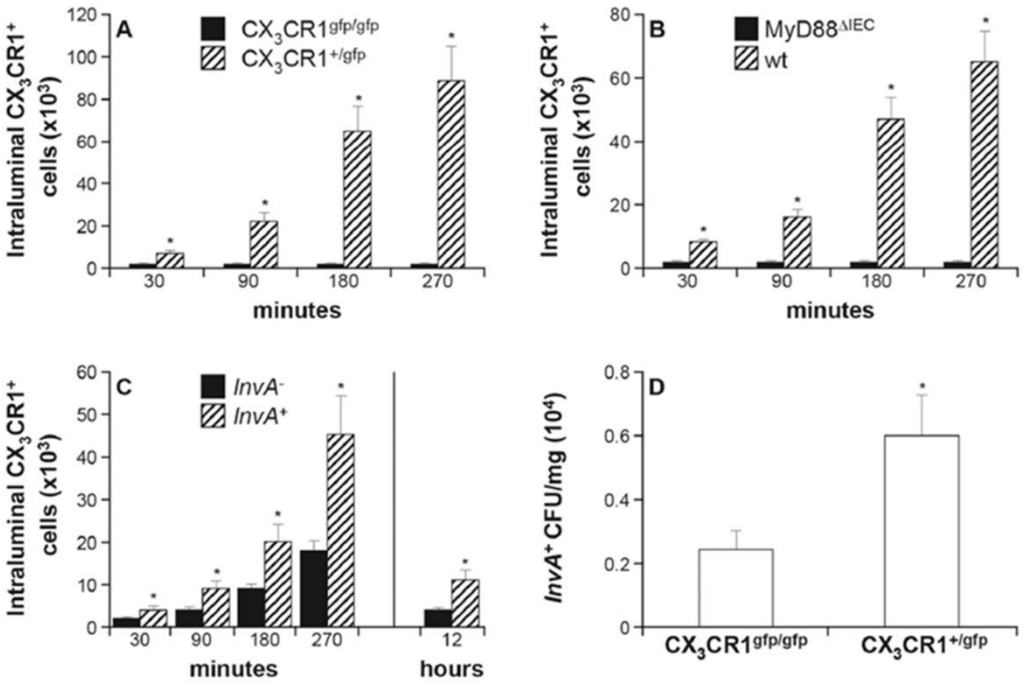


Figure 47 The role of CX3CR1 in *Salmonella*-induced migration was assessed in mice with a functional (CX3CR1$^{+/gfp}$) or non-functional (CX3CR1$^{gfp/gfp}$) receptor. Intraluminal migration of CX3CR1^{+} cells was absent in CX3CR1-deficient mice (A) (7-8 mice/group) that had been challenged with 1x10^{7} *InvA*$^{-}$*AroA*$^{-}$ *Salmonella* Typhimurium. The lack of the fraktalkine receptor completely abolished, and not simply delayed the pathogen-induced migration. *Salmonella*-dependent intraluminal recruitment of CX3CR1^{+} cells was also absent in mice with a target deletion of MyD88 in the IECs (MyD88$^{\Delta IEC}$) (B) (5-6 mice/group). In (C) it is shown that intraluminal migration is significantly more pronounced in response to oral challenge (1x10^{7}) with invasive (*InvA*$^{+}$) *Salmonella* variant (7-8 mice/group). Migration appeared to be restricted at the initial stage of infection and it declined significantly 12 h after infection for both invasive and non-invasive strains. The presence of intraluminal CX3CR1^{+} cells led to a significant

increase in faecal bacterial load (D) (7-8 mice/group) compared to CX3CR1$^{gfp/gfp}$ mice 5 h after oral delivery of invasive *Salmonella* as in (C). (Experiments performed in triplicate; two-way ANOVA in A, B and C, Bonferroni Multiple Comparison post-test, where significance ($p<0.05$=*) for the comparisons at each time point is indicated; t-test in D ($p<0.05$=*); error bars represent standard error of the mean (SEM))

4.3. Bacteria translocation is increased in CX3CR1-deficient mice

Bacteria translocation is increased in CX3CR1-deficient mice. It has been suggested that non-invasive *InvA*$^{-}$ *Salmonella* traverse the epithelium exclusively via CX3CR1-direct sampling also known as indirect route (Martinoli et al., 2007); thus we asked whether translocation of *InvA*$^{-}$ *Salmonella* differed in mouse strains either able (C57BL/6) (Chieppa et al., 2006) or unable (Balb/c mice and CX3CR1$^{-/-}$) (Niess et al., 2005, Vallon-Eberhard et al., 2006) to sample luminal bacteria via the indirect route. Following oral delivery of *InvA*$^{-}$ *Salmonella* antigen sampling-competent/migration-competent C57BL/6 and sampling-deficient/ migration-competent Balb/c had similar numbers of *Salmonella* penetrating both the conventional and the specialized follicle-associated (FAE) epithelia of Peyer's patches (PPs) (**Figure 48 A, B**) at any time point during the initial stages of the infection. By contrast, sampling-deficient/migration-deficient CX3CR1$^{-/-}$ mice showed significantly higher numbers of bacteria after 30 and 60 minutes post infection within the PPs and the small intestinal lamina propria that remained significantly higher throughout the experiment (**Figure 48 A, B**). Increased bacterial translocation across the gut epithelium in CX3CR1-deficient mice (both CX3CR1$^{-/-}$ and CX3CR1$^{gfp/gfp}$) was not the result of increased permeability of the epithelial barrier as shown by using soluble tracer and microparticles (**Appendix 5**). Indeed, serum levels of orally delivered fluorescent FITC-dextran and numbers of orally delivered FITC-labelled latex microparticles were similar to wt mice. In addition, the number of replicating *InvA*$^{-}$ *AroA*$^{+}$ *Salmonella* recovered from PP, mesenteric LN and spleen 5 days after oral delivery was higher in CX3CR1$^{-/-}$ mice compared to wt counterparts (**Figure 48**

C). Increased bacterial transport in the gut of CX3CR1$^{gfp/gfp}$ mice was also seen following infection with *InvA*$^{+}$ *AroA*$^{+}$ *Salmonella*. CX3CR1$^{gfp/gfp}$ mice succumbed 7 days after oral delivery of lethal dose of invasive *Salmonella* at a significantly faster rate compared to their CX3CR1$^{+/gfp}$ counterparts (**Figure 49 A**) and showed higher bacterial load in their organs (**Figure 49 B**). These results taken together would suggest that the lack of *Salmonella*-induced CX3CR1^{+} cells intraluminal migration favours bacterial translocation.

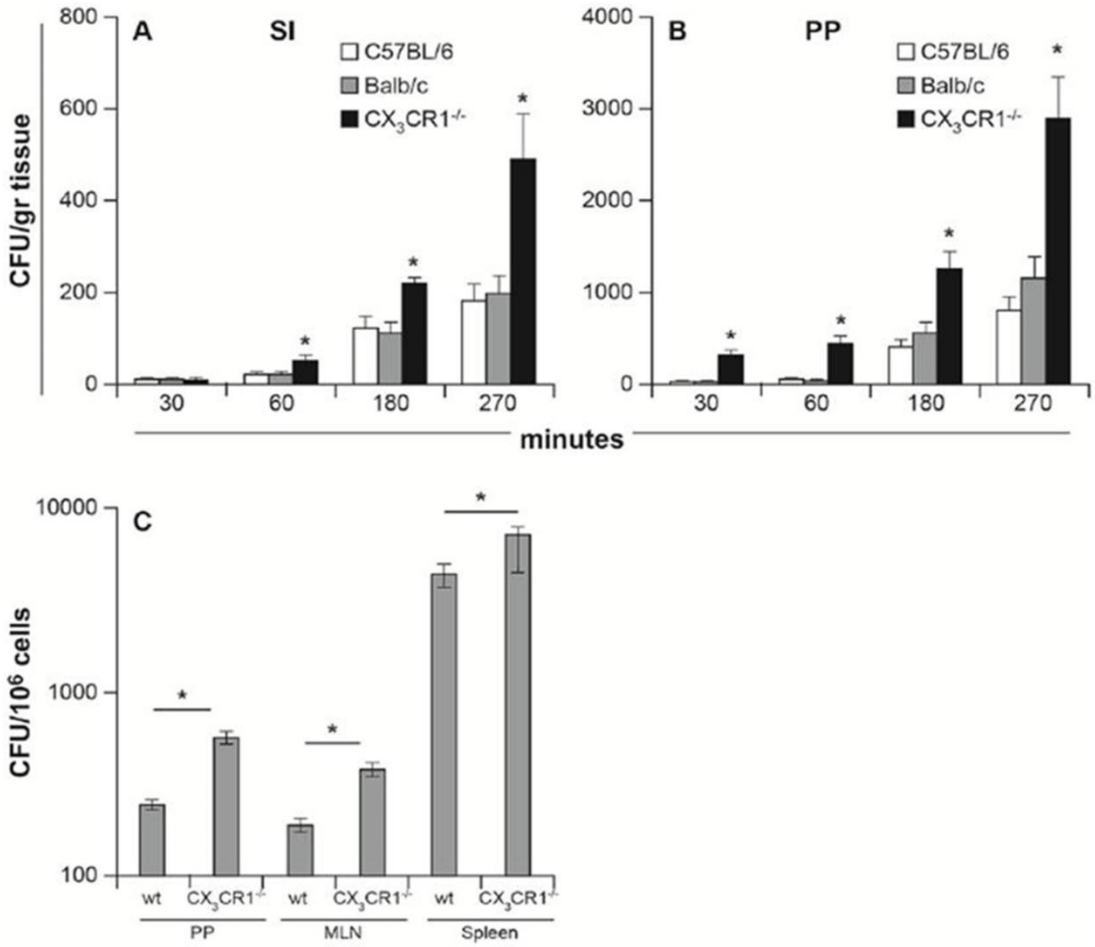


Figure 48 Role of CX3CR1-mediated sampling in the uptake of non-invasive *InvA*$^{-}$ *Salmonella*. Numbers of *Salmonella* traversing the conventional (A) (small intestine, SI) and specialized (B) (Peyer's patches, PP) epithelia did not differ in mouse strains that have been shown to be either sampling-competent/migration-competent (C57BL/6) or sampling-deficient/migration-competent (Balb/c). In contrast, *Salmonella* uptake was significantly higher in CX3CR1$^{-/-}$ mice that were both sampling-deficient and migration-deficient (8

mice/group). Similarly higher numbers of *Salmonella* were found in the GALT (PP and MLN) and spleen of CX3CR1$^{-/-}$ mice compared to wt mice (10 mice/group), 6 days after a single oral delivery of non-invasive-replicating *InvA*$^{-}$ *AroA*$^{+}$ *Salmonella* (C). Asterisk (*) indicates significant statistical difference. (Experiments performed in triplicate; two-way ANOVA in A and B, Bonferroni Multiple Comparison post-test, where significance ($p<0.05$=*) for the comparisons at each time point is indicated; t-test in D, where each anatomical location was considered as an independent data set; error bars represent standard error of the mean (SEM))

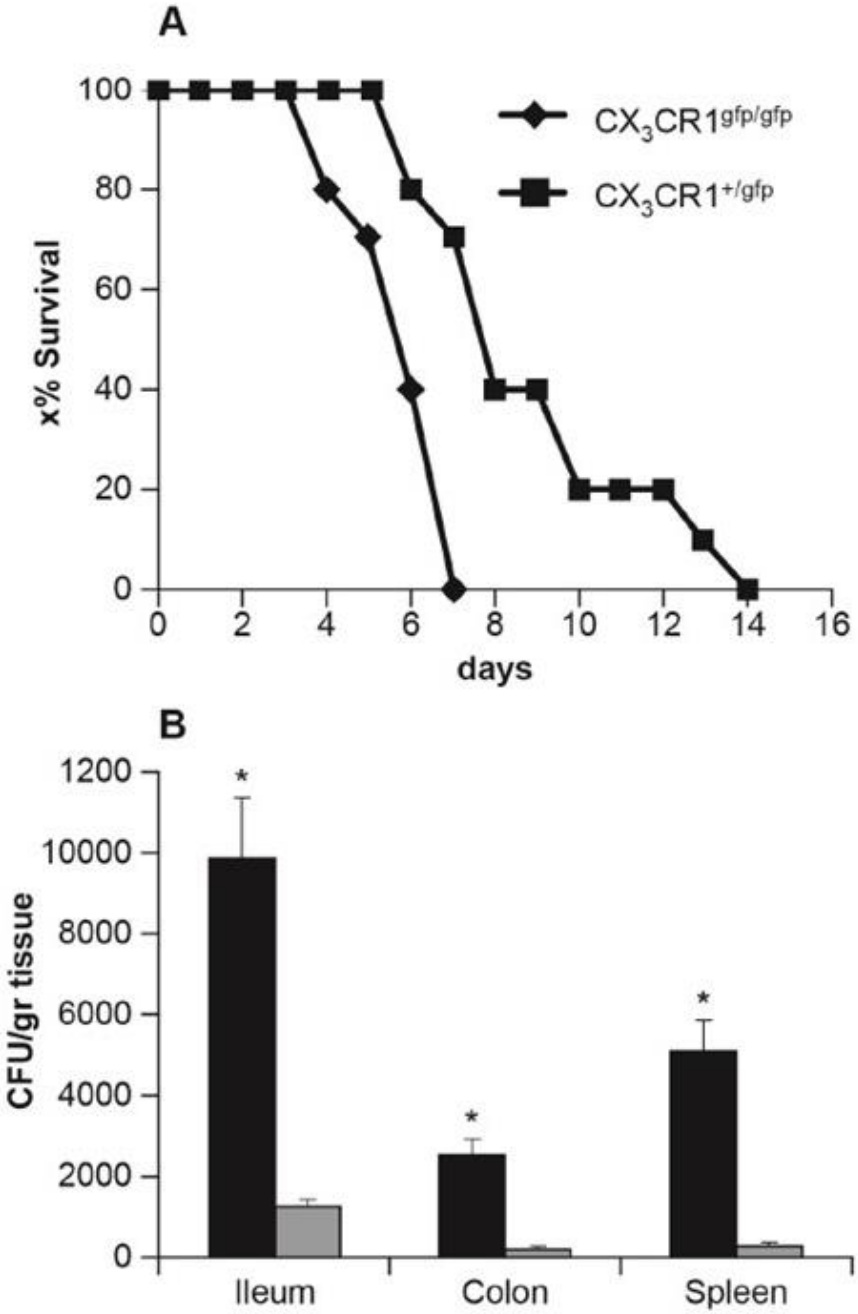


Figure 49 Survival of mice with functional (CX3CR1$^{+/gfp}$) and non-functional (CX3CR1$^{gfp/gfp}$) fraktalkine receptor after oral administration of 1x10^{8} *InvA^{+}AroA^{+} Salmonella*. CX3CR1-deficient mice succumbed within 7 days from infection at a faster rate compared to CX3CR1$^{+/gfp}$ mice (A) (12 mice/group). Also, in spite of lacking the ability to sample *Salmonella* via the indirect route CX3CR1$^{gfp/gfp}$ mice (black bars) showed significantly higher bacterial load in the gut and other organs compared to CX3CR1$^{+/gfp}$ (grey bars) (B). Furthermore, increased susceptibility of these mice to infection could not be attributed to reduced

immunity to *Salmonella* as shown in **Figure 50**, **Figure 51**. (In B experiments performed in triplicate, n=2 to 3 (in total n=8-10 mice/group); t-test, where each anatomical location was considered as an independent data set; error bars represent standard error of the mean (SEM))

4.4. Lack of CX3CR1-mediated sampling did not affect immunity to *Salmonella*.

We next addressed the possibility that the lack of CX3CR1-mediated sampling impaired immunity to *InvA*$^{-}$*AroA*$^{-}$ *Salmonella*. Levels of serum IgG was consistently higher in CX3CR1$^{gfp/gfp}$ mice compared to CX3CR1$^{+/gfp}$ mice starting at week 2 post infection (**Figure 50 A**) although it did not reach statistical significance. Instead, IgA production in CX3CR1$^{gfp/gfp}$ mice, albeit significantly lower than the one induced by invasive/non-replicating *InvA*$^{+}$*AroA*$^{-}$ *Salmonella* (data not shown) was significantly higher compared to wt mice (**Figure 50 B**) starting from week 3 post-infection. The higher *Salmonella*-specific IgA response in CX3CR1$^{-/-}$ mice was confirmed by assessing the numbers of antibody forming cells (AFC) in the PPs (**Figure 50 C**). Also, we observed that *Salmonella*-specific T cell blastogenesis was increased in CX3CR1$^{gfp/gfp}$ mice compare to CX3CR1$^{+/gfp}$ (**Figure 51 A-C**) and secreted higher of levels of IFNγ (**Figure 51 F**). Thus, immunity to non-invasive *Salmonella* that specifically targets CX3CR1-mediated entry route was not impaired in CX3CR1-deficient mice.

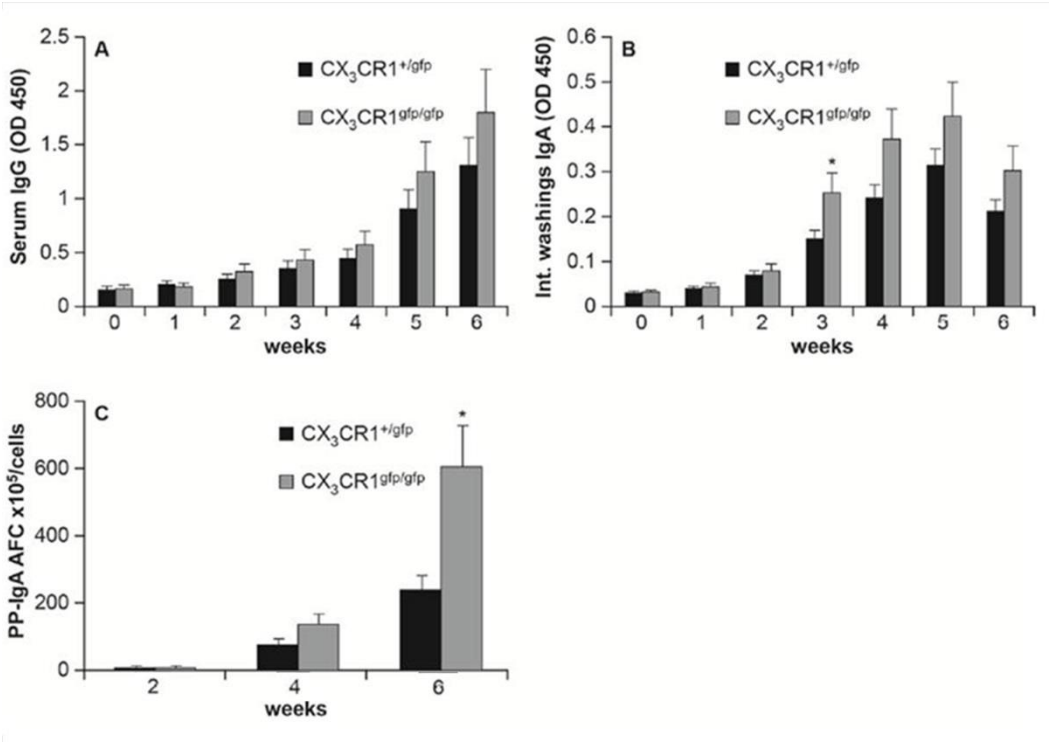


Figure 50 Humoral immunity to non-invasive *Salmonella* in CX3CR1-deficient mice. Mice (9-10 mice/group) received 3 consecutive doses of $1x10^7$ of non-invasive/nonreplicating *InvA*$^-$*AroA*$^-$ at 3 day interval. Levels of serum IgG (A) and intestinal IgA (B) *Salmonella*-specific antibodies were determined by ELISA. Both responses appeared to be higher in CX3CR1$^{gfp/gfp}$ compared to CX3CR1$^{+/gfp}$ mice although only intestinal levels of IgA were significantly different starting from week 3 after infection (*). Higher mucosal IgA immunity in CX3CR1$^{gfp/gfp}$ mice was further confirmed by monitoring numbers of AFC in PPs (C). Asterisk (*) indicates significant statistical difference. (Experiments performed three times, each time n=3 to 4 mice/group; two-way ANOVA, Bonferroni Multiple Comparison post-test, where significance ($p<0.05$=*) for the comparisons at each time point is indicated; error bars represent standard error of the mean (SEM))

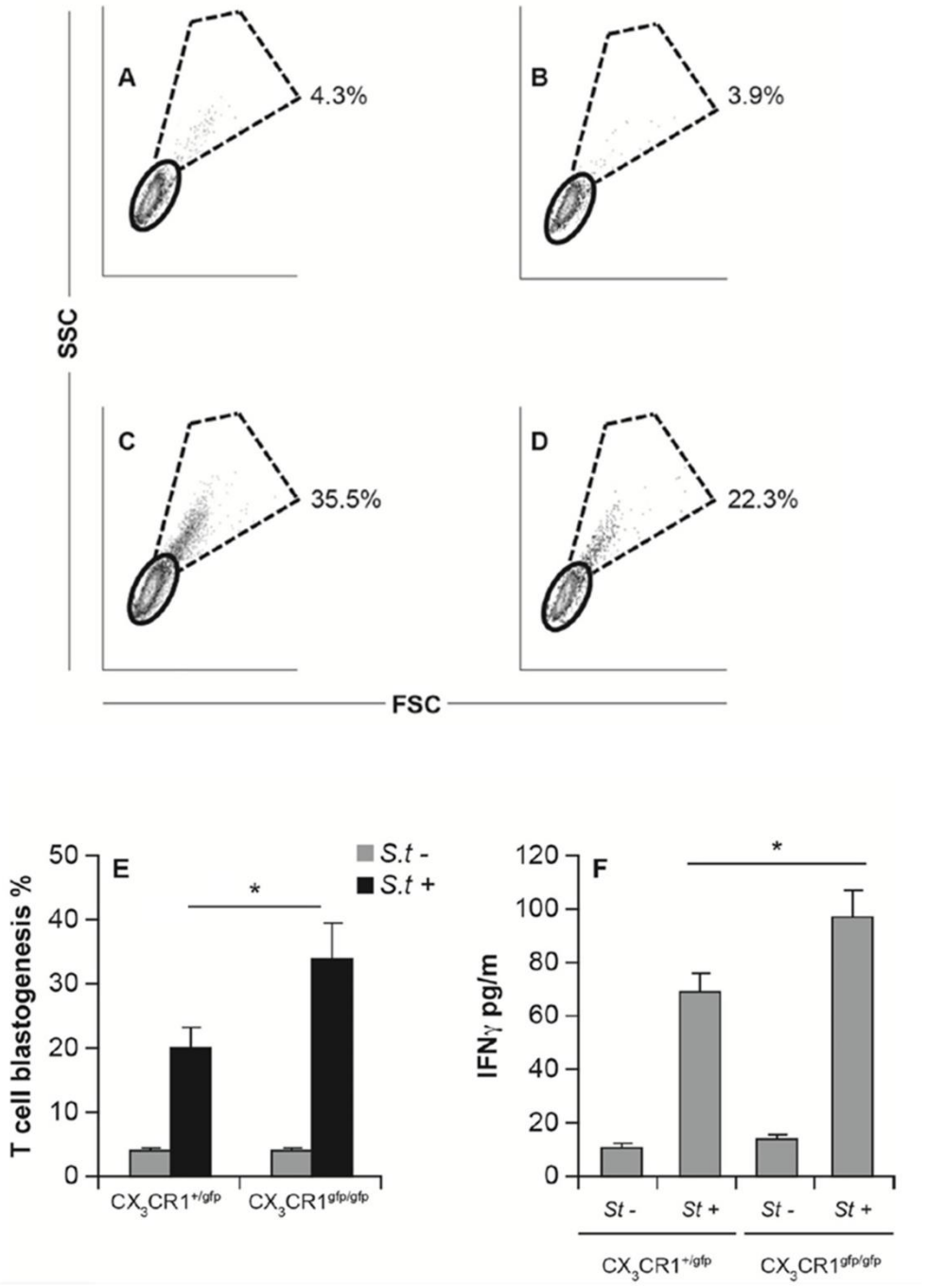


Figure 51 Cellular immunity to non-invasive *Salmonella* in CX3CR1-deficient mice. T cell blastogenesis was measured following *in vitro* recall challenge of T cells from CX3CR1$^{+/gfp}$ and CX3CR1$^{gfp/gfp}$ mice (9-10 mice/group) following oral delivery of *Salmonella* as for **Figure 50**. Lymphoblasts were recognized by flow cytometry based on size (side-scatter, SSC) and granularity (forward-scatter, FSC) (A-D). Blastogenesis in culture increased in presence of *Salmonella* lysate (C, D) compared to mock infected cultures (A, B) and it was significantly higher

in CX3CR1$^{gfp/gfp}$ mice (C) compared to CX3CR1$^{+/gfp}$ counterparts (D). Quantitative analysis is shown in (E). Also, parallel *in vitro* recall challenge cultures showed that CX3CR1$^{gfp/gfp}$ mice secreted higher levels of IFNγ compared to CX3CR1$^{+/gfp}$ (F). Asterisk (*) indicates significant statistical difference. (Experiments performed three times, each time n=3 to 4 mice/group; two-way ANOVA in E and F, Bonferroni Multiple Comparison post-test, where significance ($p<0.05$=*) is indicated; error bars represent standard error of the mean (SEM))

4.5. The presence of intraluminal CX3CR1^{+} cells reduced the number of *Salmonella* invading intestinal tissue.

Finally, we tested the hypothesis that intraluminal CX3CR1^{+} cells limit *Salmonella* penetration of the epithelial barrier. At various intervals, shortly following oral delivery of 1x10^{7} *InvA^{+}AroA^{-} Salmonella*, CX3CR1^{+} cells isolated from CX3CR1$^{+/gfp}$ donors were injected into the intestinal lumen of migration-deficient CX3CR1$^{-/-}$ mice (**Appendix 6 Figure S6**). Four groups of mice were used and the number of adoptively transferred cells at any given time point was based on the time course experiment (**Figure 47 C**). The adoptive transfer of intraluminal CX3CR1^{+} cells in CX3CR1$^{-/-}$ mice significantly reduced the number of *InvA^{+} Salmonella* traversing the epithelial barrier (**Figure 52**). In the presence of 0.5x10^{3} CX3CR1^{+} cells the number of tissue CFU of *InvA^{+}AroA^{-} Salmonella* declined (from 1.2x10^{3} ± 3.3x10^{2} to 2.1x10^{2} ± 1.4x10^{2}) at 30 minutes post-infection (group I). Similarly, significant reductions in bacterial load in the intestinal tissues were observed in group II and III. Finally, group IV was passively transferred, fifteen minutes after *Salmonella* administration with a number of CX3CR1^{+} cells (1x10^{4}) that far exceeded the number of CX3CR1^{+} cells found in the small intestine at the beginning of the infection. In this case, the presence of intraluminal CX3CR1^{+} cells almost completely abolished *Salmonella* invasion of intestinal tissue (CFU tissue <40). These results suggest that CX3CR1^{+} cells migrate rapidly into the lumen to prevent *Salmonella* from penetrating the intestinal epithelial barrier.

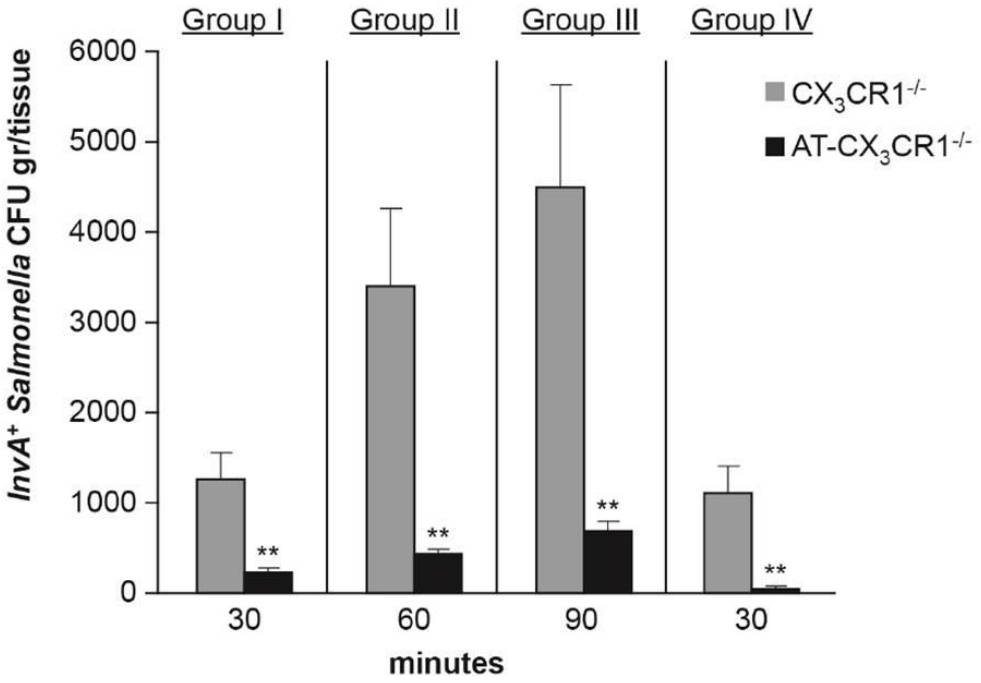


Figure 52 Role of intraluminal CX3CR1^{+} cells in pathogen-exclusion. CX3CR1$^{gfp/gfp}$ mice (6 mice/group) were infected with a single oral dose (1x10^{7}) of invasive/non replicating *InvA*$^{+}$*Aro*$^{-}$ *Salmonella*. *Salmonella* infecting the intestinal tissue were determined in the absence (CX3CR1$^{-/-}$, grey bars) or presence (AT-CX3CR1$^{-/-}$, black bars) of CX3CR1^{+} cells that were adoptively transferred (AT) directly into the intestinal lumen. A significant decline of *Salmonella* CFU g/tissue was observed in group I 30 minutes after infection following adoptive transfer of 0.5x10^{3} CX3CR1^{+} cells. Significant reduction in the number of pathogens invading the host was also seen in group II and III that received increasing numbers of CX3CR1^{+} that were determined according to the time course study shown in (**Figure 47 C**). In group IV the introduction in the lumen of larger, non-physiologically high number of intraluminal CX3CR1^{+} cells (1x10^{4}) (20 fold increase compared to the number of cells usually found in the gut 15 minutes after infection) nearly completely abolished *Salmonella* infection. (Experiment performed three times, each time n=2 mice/group; two-way ANOVA, Bonferroni Multiple Comparison post-test, where significance ($p<0.01$=**) is indicated; error bars represent standard error of the mean (SEM))

5. Discussion

Preventing pathogen invasion is, together with absorption of nutrients the main task of the intestinal epithelium. Indeed, the presence of a thick layer of mucus and secretion of IgA ensures that harmful microorganisms do not breach the intestinal epithelium. We report that when this barrier is breached by invasive pathogenic bacteria the host rapidly responds by sending into the intestinal lumen pathogen (*Salmonella*)-capturing CX3CR1^{+} cells to limit the number of bacteria penetrating the epithelium. The rapid migration is triggered by the initial interaction of pathogens with IEC since this migration is absent in mice lacking the adaptor molecules MyD88 solely within the epithelium. Interaction of *Salmonella* and the IEC-associated TLR may lead to two different events. CX3CR1^{+} cells can directly sample bacteria and shuttle them across the epithelium (Rescigno et al., 2001); alternatively these cells could also move through the epithelium and migrate into the lumen where they capture *Salmonella* (Arques et al., 2009). However, while it was suggested that CX3CR1-sampling was relevant to mounting pathogen-specific response the role and biological relevance of the rapid migration of *Salmonella*-capturing CX3CR1^{+} cells in the lumen remained to be determined. CX3CR1-sampling and -migration showed some differences the most notable being that, while CX3CR1-migration in response to *Salmonella* takes place in different mouse strains, such as C57BL/6 and Balb/c (Arques et al., 2009) the ability to sample luminal bacteria via the indirect route is strain-specific (Chieppa et al., 2006, Vallon-Eberhard et al., 2006). We exploited this feature to investigate the biological relevance of these two CX3CR1-mediated events during the initial stage of *Salmonella* infection. We observed that, in spite of lacking the ability to sample bacteria via the indirect route, Balb/c mice showed very similar uptake of non-invasive *Salmonella* in the gut compared to sampling-competent wt C57BL/6. This observation is of particular relevance when examined in the context of *Salmonella* uptake in CX3CR1-deficient mice that lacked both indirect sampling (Niess et al., 2005) and intraluminal migration (Nicoletti et al., 2010). We observed that CX3CR1-deficient mice showed a significantly higher bacterial

load both at mucosal level and systemically compared to both C57BL/6 and Balb/c. Increased pathogen uptake in CX3CR1-deficient mice did not depend on intrinsic alteration of the integrity of the epithelial barrier in these mice (both CX3CR1$^{-/-}$ and CX3CR1$^{gfp/gfp}$) since permeability to either soluble or particulate tracers was similar to wt counterparts. Overall these results would suggest that sampling via the indirect route does not play a significant role in pathogen uptake through the intestinal epithelium. Instead they point to the lack of rapid intraluminal migration of CX3CR1^{+} cells as the critical event favouring bacterial translocation. This hypothesis is further strengthened by the observation that intraluminal migration of CX3CR1^{+} cells in CX3CR1$^{+/gfp}$ mice was associated with increased faecal bacterial counts compared to CX3CR1$^{gfp/gfp}$ mice. Furthermore, CX3CR1-deficient mice are more susceptible to *Salmonella* infection, as seen by us and others (Niess et al., 2005); however, the reason for this remained to be determined. One possibility was that the lack of CX3CR1-mediated sampling led to an impaired immunity to *Salmonella*. Surprisingly, the absence of CX3CR1-mediated sampling does not appear to be critical for adaptive anti-*Salmonella* response. In these mice, oral delivery of non-invasive/non-replicating *InvA*$^{-}$*AroA*$^{-}$ *Salmonella* mutant that specifically target the indirect route induced similar levels of anti-*Salmonella*-specific IgG and higher spleen T cell proliferation. Also, CX3CR1-deficient mice developed a significantly higher mucosal (IgA) response to non-invasive *Salmonella* that is characterized by poor mucosal antigenic properties (Martinoli et al., 2007). It is likely that this could be attributed to higher numbers of *Salmonella* reaching the PPs, the inductive sites of IgA-mediated immunity, in CX3CR1-deficient mice. Taken together these results strongly suggest that increased susceptibility to *Salmonella* infection in CX3CR1-deficient mice (Niess et al., 2005) could be due, partly to the lack of protection afforded by CX3CR1- mediated pathogen exclusion early in infection. As a parallel observation, these data also confirmed previous reports suggesting that *Salmonella* type III secretion system (*InvA*) facilitates up-take by FAE-microfold (M) cells but its absence does not totally compromise the ability of *Salmonella* to target this entry route (Galán and Curtiss, 1989, Jepson and Clark, 2001, Martinez-Argudo and Jepson, 2008). Intraluminal migration of CX3CR1^{+} cells is

unidirectional and once into the lumen they do not traverse back the intestinal epithelium. Determining the fate of intraluminal *Salmonella*-capturing CX3CR1^{+} cells was prompted by the important finding that in mice *T. gondii*-infected neutrophils that had migrated into the intestinal lumen at a later stage (one week post-infection) during the infection can move back into the intestinal tissue (Coombes et al., 2013). The opportunity to utilize GFP-CX3CR1^{+} cells (Jung et al., 2000a) provided us with the tool to address this issue by using a more reliable system compared to the one we previously used that involved membrane-labelling with DiI dye (Arques et al., 2009). The observation that CX3CR1$^{+/gfp}$ in contrast to infected neutrophils undertake a one way journey suggested that, neutrophils may be intrinsically different from CX3CR1^{+} cells in their ability to traverse back the epithelium. Alternatively, in contrast to *Salmonella*, *T. gondii* may trigger the secretion/expression of cytokine/surface molecules in infected cells that favour this event. The unidirectional migration of CX3CR1^{+} cells into the lumen very early during *Salmonella* infection contributes to pathogen-exclusion. Direct introduction of these cells into the lumen of migration-deficient CX3CR1$^{-/-}$ mice shortly after oral delivery of invasive *Salmonella* significantly reduced the number of pathogens traversing the epithelial barrier. Furthermore, although at this time we cannot rule out the possibility that other cell types may participate to this protective response at a later stage in infection the previous observation that CX3CR1^{+} cells was the only intraluminal cell population harbouring intracellular *Salmonella* (Arques et al., 2009) strongly suggested that these cells are the main player in the very early stages of infection. We would like to propose that the CX3CR1-mediated pathogen-exclusion is part of a defensive strategy that includes multiple players. In addition to the already known protection barriers present at the intestinal mucosa such as the mucus layer, antimicrobial peptides, and sIgA information is emerging about new defence mechanism. For example, recently it has been shown that intraepithelial *Salmonella* Typhimurium population expands through a combination of *de novo* invasion and intraepithelial replication. The latter is counteracted by epithelium-intrinsic NAIP(s) and the NLRC4 inflammasome promoted removal of infected epithelial cells (Sellin et al., 2014). The work

presented in this chapter describes another mechanism of defence against intestinal pathogens (**Figure 53**). If the other lines of defence are breached IEC-derived signals readily trigger the intraluminal migration of CX3CR1^{+} cells that are present in large number in the subepithelial area where they form an intricate cell network.

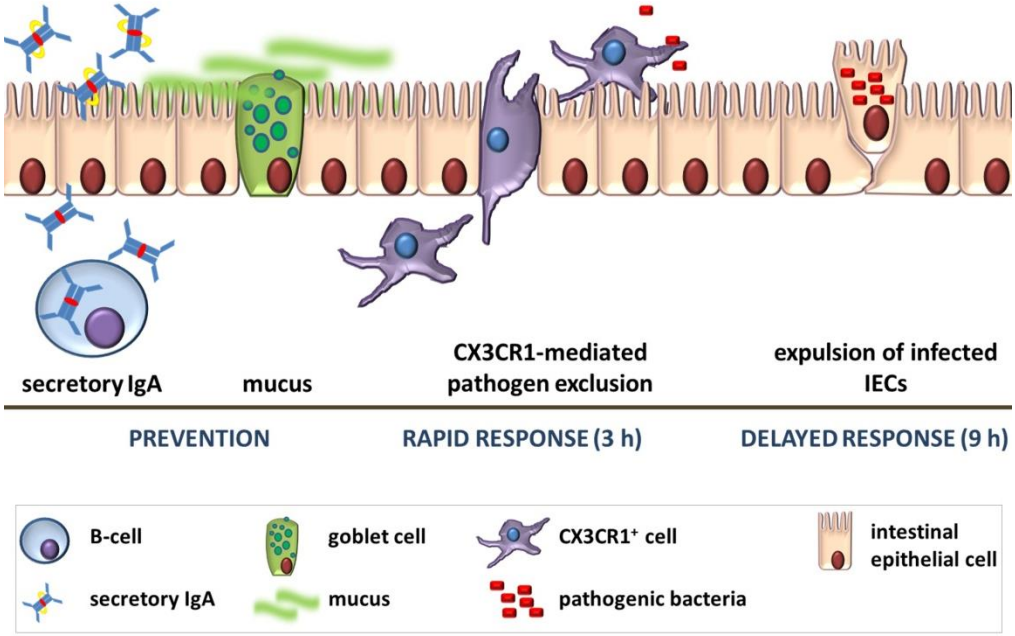


Figure 53 Several defence mechanisms are in place to prevent pathogen invasion of the intestinal mucosa. Mucus layer containing IgA and antimicrobial peptides covers the intestinal epithelium and protects it against potential pathogens. If this defence is breached, at the early stages of infection, CX3CR1-positive cells migrate into the lumen where they capture pathogens. If epithelial cells get infected they are eliminated by expulsion in order to minimise pathogen proliferation and spread. Elements of this figure are based on (Strugnell and Wijburg, 2010, Johansson et al., 2013, Sellin et al., 2014)

Appendix 1

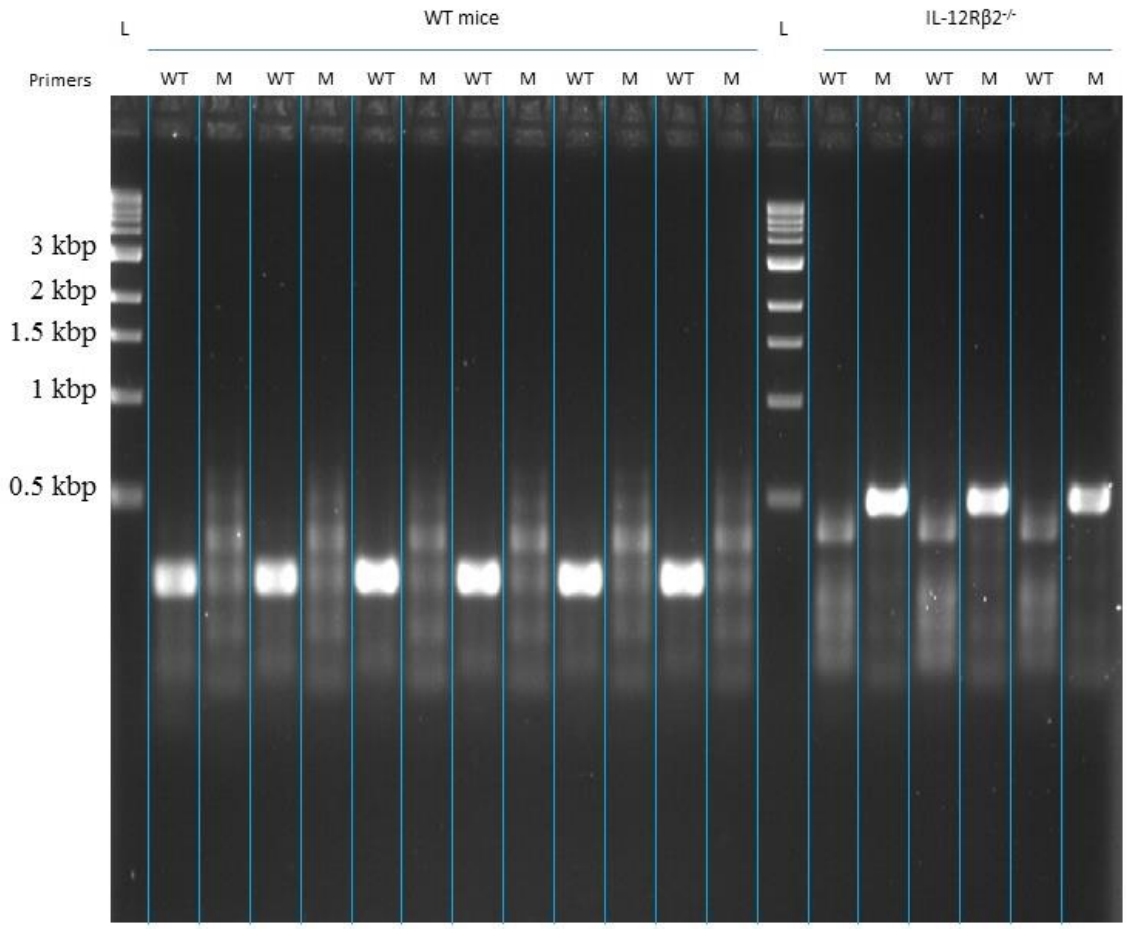
Confirming the genotype of IL-12Rβ2$^{-/-}$ mice

Primers used for confirming the genotype of IL-12Rβ2$^{-/-}$ mice (Wu et al., 2000):
Mutant (M):
5'-GAAGCGGGAAGGGACTGGCTGCTA-3' (PGK-1 neo/ Neo-specific forward primer);
5'-CGGGAGCGGCGATACCGTAAAGC-3' (PGK-1 neo/ Neo-specific reverse primer);
5'-GTGTGCAAGCTTGGCACTGTGACCGTCCAG-3' (exon 3/ IL-12R β2 exon 3 forward primer);
Wild type (WT):
5'-GTTTAGCTTGCAGACAAACAAGGTCATACC-3' (exon 3/ IL-12R β2 exon 3 reverse primer).

Wild type (WT) product: 265 bp
Transgenic/mutant (M) product: 500 bp

PCR programme (Techne Flexigene):

Heated lid: 105°C
Loo1: 1 cycle
Seg: 95°C/2 min
Loop2: 35 cycles
Seg: 95°C/35 sec
Seg: 51°C/30 sec
Seg: 72°C/35 sec
Final extension: 72°C/5min


L
WT mice
L
IL-12Rβ2-/-
Primers
WT M WT M WT M WT M WT M WT M
WT M WT M WT M
3 kbp
2 kbp
1.5 kbp
1 kbp
0.5 kbp

Appendix 2

Confirming the genotype of CX3CR1$^{gfp/gfp}$ and CX3CR1$^{gfp/+}$ mice

Primers used for genotyping of the CX3CR1 colony (NYCSM):

Wild type: 5'-AGCCGGAAGCCCAAGAGCATC-3'

Common: 5'-CCGCCCAGACGCCCAGACTA-3'

Mutant: 5'-TGCTGCTGCCCGACAACCAC-3'

Wild type (WT) product: 291 bp

Transgenic/mutant (M) product: 650 bp

PCR parameters:

Hotstart: 10 min at 95° C

35 cycles:

Denaturating: 30 sec at 94° C

Annealing: 30 sec at 60° C

Extension: 45 sec at 72° C

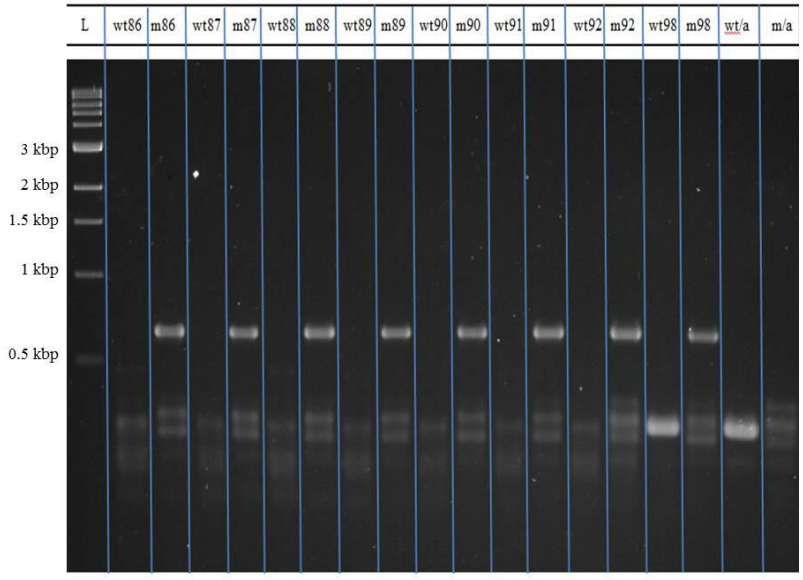


Figure S1 Example of PCR products from genotyping mice №86 to №98 along with wild type control "a". For each mouse there are two lanes - one with combination of primers detecting the wild type version of the CX3CR1 allele (product of 291 bp) and one detecting the transgene (product of 650 bp).

Appendix 3

Intraluminal migration of CX3CR1$^{gfp/+}$ cell following challenge with *Salmonella*

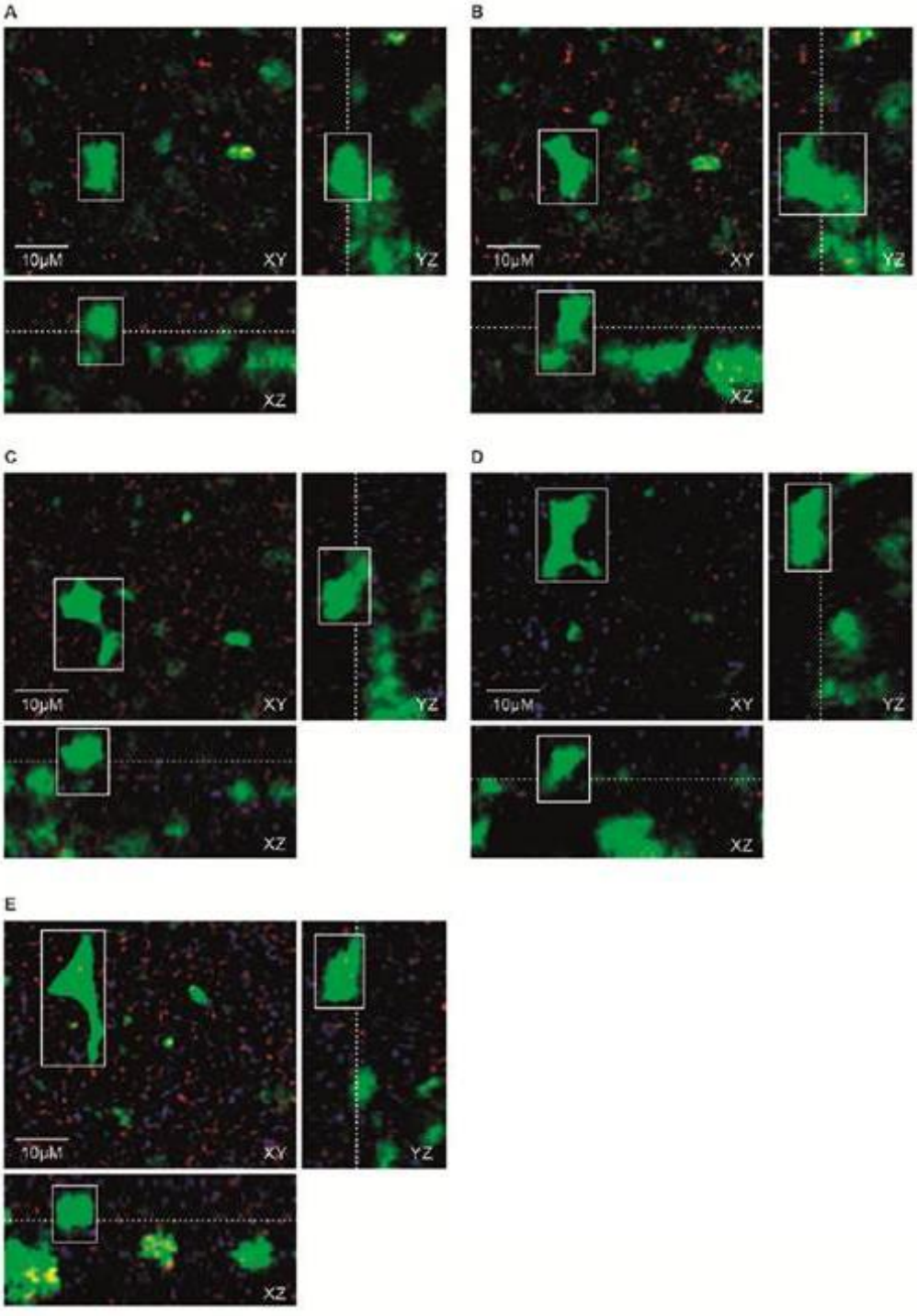


Figure S2 Intraluminal migration of CX3CR1$^{gfp/+}$ cell following challenge with *Salmonella*: orthogonal cross-sections through the 3D stacks in successive time-

frames. In (A) CX3CR1$^{gfp/+}$ cell is slightly protruding from the epithelial surface (dotted line); the outward movement being more pronounced in (B) and (C). The cells then moves away from the exit site (D) and (E). Scale bar = 10 μm. (see Supplementary Video S1 and S2)

Video S1 Intraluminal migration of CX3CR1$^{gfp/+}$ cell following challenge with *Salmonella.* The video footage on which **Figure 47** and **Figure S3** were based.

Video S2 Intraluminal migration of CX3CR1$^{gfp/+}$ cell following challenge with *Salmonella.* The cells are marked with yellow tracking points. There are four reference cells that are inside the tissue and remain relatively stationary (they move along with the whole tissue due to peristaltic movement but are otherwise not participating in intraluminal migration). The migrating CX3CR1$^{gfp/+}$ cell in the first two frames moves outward into the lumen and then away from the exit site (as in **Figure S3**)

Appendix 4

Intestinal permeability to dextran: *in vivo* multiphoton imaging

Staining with fluorescent dextran was also used to validate the tissue integrity during the multiphoton procedures. Before mounting on the imaging glass slide the tissue was immersed in 200 µl of 10 mg/ml Dextran-Texas Red 70 kDa (Life Technologies).

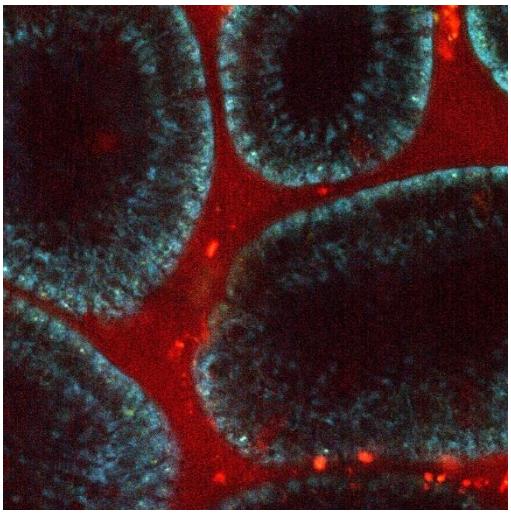


Figure S3 Tissue integrity was preserved during multiphoton procedure. Fluorescent Dextran (red) could be seen in the space between individual villi. The epithelium (blue) was intact and therefore impermeable to the Dextran none of which was observed inside the villi. A three dimensional reconstruction of the tissue in this figure is provided in (**Supplementary Video S3**)

Video S3 Tissue integrity was preserved during multiphoton procedure. 3D reconstruction and 360˚ rotation of the imaged tissue as described in **Figure S5**.

Appendix 5

Intestinal permeability to dextran: oral admiration

Intestinal permeability to dextran was measured in 6-8 week old CX3CR1$^{gfp/gfp}$, CX3CR1$^{gfp/+}$ and syngeneic WT mice (4 mice/group). FITC-labelled dextran (Sigma-Aldrich) was dissolved in PBS at a concentration of 100 mg/ml and administered to each mouse (44 mg/100 g body weight) by oral gavage. Blood samples were collected after 6 hours and the plasma analysed using fluorescence spectrometer at excitation wavelength of 490 nm and emission wavelength of 520 nm. Standard curves to calculate fluorescent dextran concentration in the samples were prepared from dilutions in PBS. This work was performed by collaborators at the University of Siena.

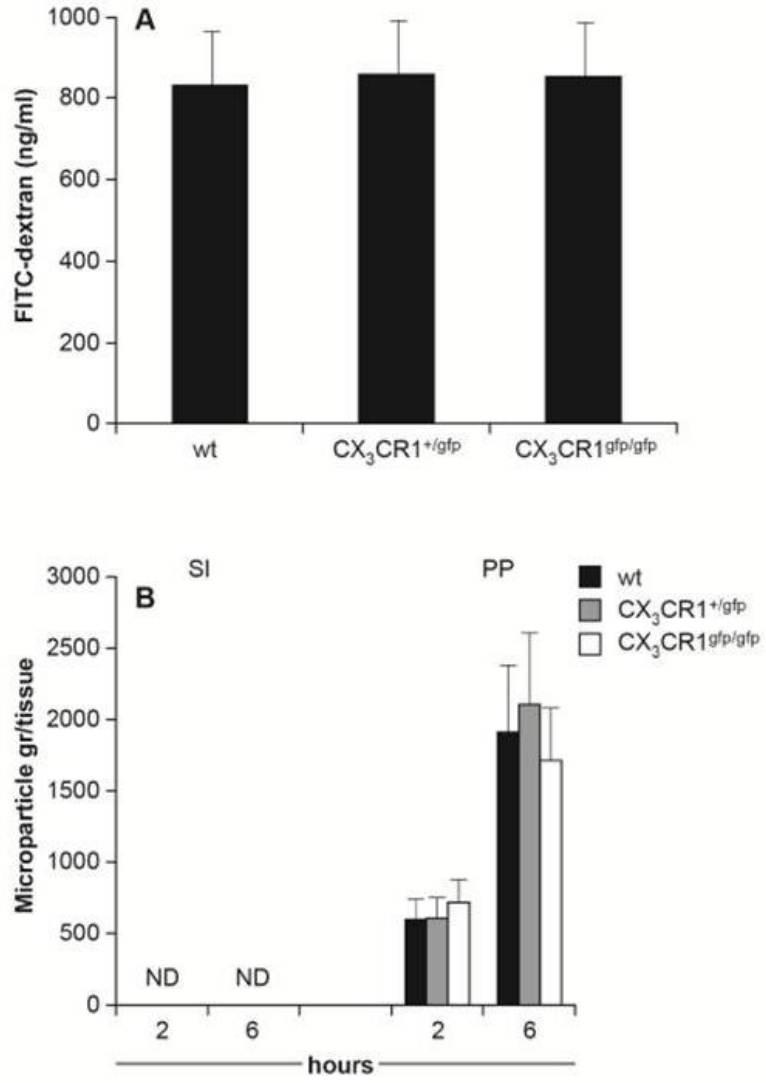


Figure S4 Intestinal permeability in CX3CR1-deficient mice. Oral delivery of a single dose of either FITC-dextran (A) or yellow-green fluorescent polystyrene microparticles (B) showed that intestinal permeability to both soluble and particulates tracers was not affect by the lack of functional fraktalkine receptor (4 mice/group). This shows that the higher bacterial load in the intestine as shown in **Figure 48** could not be attributed to an intrinsic "leaky" gut in CX_3CR1-deficient mice. Asterisk (*) indicates significant statistical difference

Appendix 6

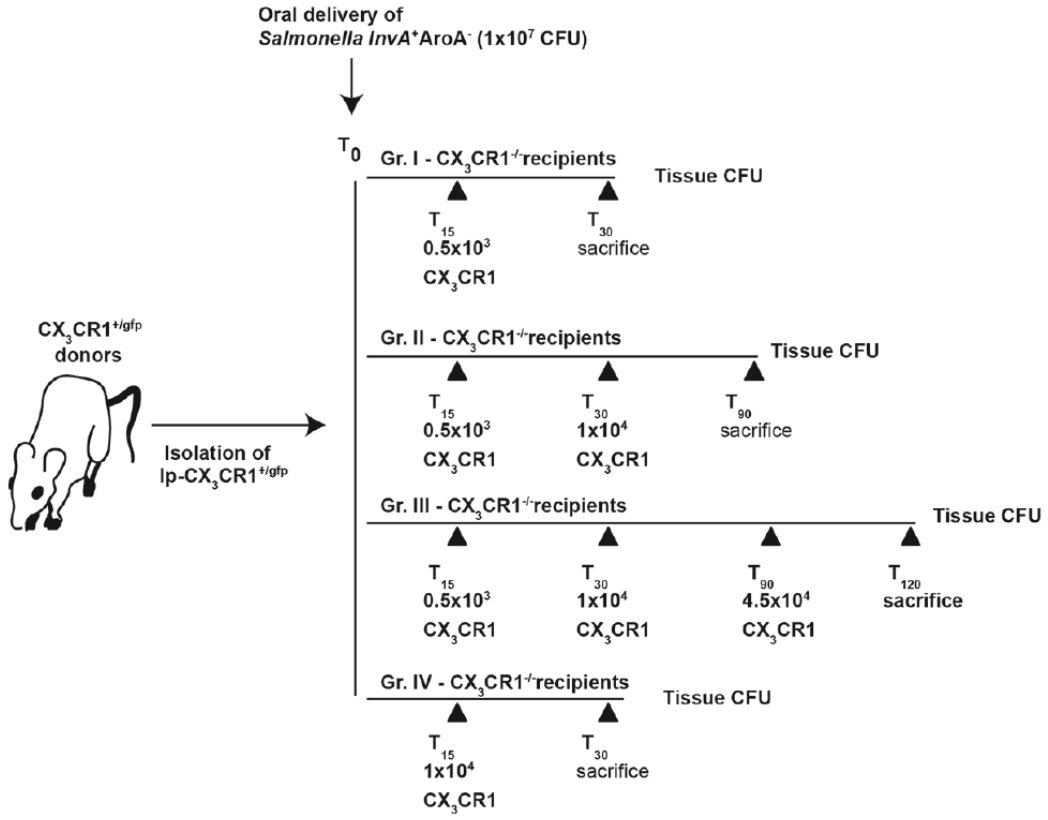


Figure S5 Schematic summary of pathogen-exclusion assay in CX3CR1$^{-/-}$ mice. Four groups of mice (6 mice/group) received a single oral delivery of 1x10^7 invasive/nonreplicating *InvA*$^+$*AroA*$^-$ at T0. The number of CX3CR1$^+$ cells for the adoptive transfer of intraluminal cells in Gr I, II and III at any given time (T15, T30 and T90) was established according to the results of the time course experiment illustrated in **Figure 47**. Group IV received a number of CX3CR1$^+$ cells (1x10^4) that far exceeded the number of cells observed in the lumen 15 minutes (T15) after infection. At the end of the experiments mice were sacrificed and CFU g/tissue determined.

Glossary

Ag	antigen
AFC	IgA-antibody forming cells
AID	activation-induced cytidine deaminase
APC	allophycocyanin
APCs	antigen presenting cells
BALT	broncho associated lymphoid tissue
BMP	bone morphogenic protein
CD	cluster of differentiation
CP	cryptopatches
CPE	ground crude peanut extract
CSR	class-switch recombination
CT	cholera toxin
CT*	cycle threshold (not to be confused with cholera toxin*)
DC	dendritic cell
DGGE	denaturing gradient gel electrophoresis
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FAE	follicle-associated epithelium
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FKN	fractalkine (CX3CR1L, neurotactin)
FM	follicular mantle
Foxp3	factor forkhead box P3
GALT	gut associate lymphoid tissue
GC	germinal center
GENALT	genital associated lymphoid tissue
GF	germ free
GFP	green fluorescent protein
GI	gastro intestinal
HBSS	Hank's Balanced Salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IEL	intra-epithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	Innate lymphoid cells
ILF	isolated lymphoid follicles

Lactococcus lactis	LAB
LI	large intestine
LP	lamina propria
LT	lymphotoxin
LTi	lymphoid tissue-inducer
M cell	microfold cell
MALT	mucosal associated lymphoid tissue
MAMPs	microbe associated molecular patters
MAPK	mitogen-activated protein kinase
MDP	muramyl dipeptide
MHC	major histocompatibility class
mHPP	m-hydroxyphenylpropionic acid
MLN	mesenteric lymph nodes
МΦ	macrophage
NALT	nasal associated lymphoid tissue
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NMR	nuclear magnetic resonance
OTU	operational taxonomic unit
OVA	ovalbumin
PBS	phosphate-buffered saline
PBSAFE	Phosphate-buffered saline, azide, foetal calf serum, ethylenediaminetetraacetic acid
PCA	Principle Component Analysis
PCoA	Principal Coordinates Analysis
PE	phycoerythrin
PMN	polymorphonuclear leukocytes
PP	Peyer's patch
PPL	Peyer's patch lymphocytes
RPMI	Roswell Park Memorial Institute medium
SB	saponin buffer
SCFAs	short-chain fatty acids
SED	subepithelial dome
SFB	segmented filamentous bacteria
SHM	somatic hypermutation
SI	small intestine
TCR	T cell receptor
TEDs	thransepithelial dendrites
TEM	transmission electron microscopy
TGF	transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRITC	tetramethylrhodamine
TSLP	thymic stromal lymphopoietin
WT	wild type

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