# Mucosal homeostasis and inflammatory bowel disease

## PhD thesis

**Dag Henrik Reikvam**

**2011**

UiO : University of Oslo

Laboratory for Immunohistochemistry and Immunopathology,

Centre for Immune Regulation,

Department of Pathology,

Institute of Clinical Medicine,

Faculty of Medicine,

University of Oslo, Norway



*Series of dissertations submitted to the*
*Faculty of Medicine, University of Oslo*
*No. 1168*

ISBN 978-82-8264-169-2



Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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## ACKNOWLEDGEMENTS

This thesis emanates from Laboratory for Immunohistochemistry and Immunepathology (LIIPAT) at Department of Pathology, Institute of Clinical Medicine at the University of Oslo. The first 14 months of this project were provided by LIIPATs recruitment fund, while the last three years were financed by the Norwegian Cancer Society. I am grateful for both of these fundings.

My first contact with LIIPAT started with a bold e-mail to Per Brandtzaeg, at the time the head of the group. I am thankful for that he, as one of his last deeds before retiring as group leader, was willing to recruit a physician with no scientific merits. I have also found his poignant and constructive criticism and vast knowledge of mucosal immunology inspiring and educating.

Finn-Eirik Johansen has been my head supervisor throughout this PhD project. I greatly appreciate his analytic mind, technical skills, optimism and availability, as well as the confidence he has had in my work.

Hege S. Carlsen has been the co-supervisor. With great enthusiasm and methodological skills in immunohistochemistry she confidently supervised the project resulting in the first two papers of this thesis.

The first two papers had never been if it was not for the persistent inclusion of patients, careful organization of background data, and executing skills of Gøri Perminow. I would also like to thank Lars Gustav Lyckander and Morten Vatn for their contributions to the IBSEN II project and Jon Michael Gran for statistical expertise.

Alexander Erofeev generously included me as collaborator on the first laboratory animal colitis experiments and gave me a flying start on these studies. I further thank my other co-authors at LIIPAT, Anders Sandvik, Vedrana Grcic, Rejoanoul Islam, Christina Bang, Espen Bækkevold, and Frode Jahnsen for all experimental collaborations, intellectual input, and for the (at times heated) discussions in our project group meetings. I also highly acknowledge Krzysztof Grzyb for his service as an expert histopathologist. A great salute goes to the rest of the scientific staff at LIIPAT for generating a positive and stimulating atmosphere and in particular to Mariann Friis-Ottesen whom I shared office with.

A number of collaborators outside LIIPAT have made important contributions to this research work. Peter Gaustad, Institute of Microbiology, UiO has supervised all conventional bacteriological work. Leonardo Meza-Zepeda, Norwegian Microarray Consortium, UiO provided facilities for and analyses of epithelial gene expression profiling. Kathy McCoy and Andrew Macpherson, University of Bern, Switzerland supplied germ free mice and valuable input on gnotobiotic animal research. Muriel Derrien and Hauke Smidt, Wageningen University, the Netherlands have been in charge of phylogenetic microarray data. They are all worthy of my sincere acknowledgements.

LIIPAT has a staff of highly skilled, positive, and committed technicians who all have made significant contributions to my experimental work. I am thankful for the help and support of Aaste Aursjø, Linda Manley, Linda Solfjell, Hogne Røed Nilsen, Kjersti Thorvaldsen, Vigdis Wendel, and Kathrine Hagelsteen. Anne Kilsund Axelssen at the Institute of Microbiology also provided excellent help with conventional bacteriological assays. I also thank the staff at Department for Comparative Medicine for helpful assistance

and careful tending to my laboratory animals. LIIPATs secretarial staff of Hege Eliassen and Gry Bruland Larsen has also been to great help.

Finally, I thank my queen, Stine Marit, and my two princesses, Tiril Helene and Helle Marie, who have made me rush home from work every day with a big smile on my face.

Oslo. April 2011

Dag Henrik Reikvam

## ABBREVIATIONS

| | |
|---|---|
| **AID** | Activation-induced cytidine deaminase |
| **AMP** | Anti-microbial peptides |
| **ANOVA** | Analysis of variances |
| **APC** | Antigen presenting cell |
| **BCR** | B cell receptor |
| **CD** | Crohn's disease |
| **CD*n*** | Cluster of differentiation *n* |
| **CSR** | Class switch recombination |
| **CTLA-4** | Cytotoxic T lymphocyte antigen 4 |
| **DC** | Dendritic cell |
| **dIgA** | Dimeric IgA |
| **DKO** | pIgR/$J_H$ double KO |
| **DSS** | Dextran sulfate sodium |
| **D*n*** | Domain *n* |
| **Foxp3** | Forkhead box P3 |
| **GALT** | Gut-associated lymphoid tissue |
| **HRP** | Horseradish peroxidase |
| **IBD** | Inflammatory bowel disease |
| **IBSEN** | Inflammatory Bowel Disease SouthEast Norway |
| **IEC** | Intestinal epithelial cell |
| **Ig** | Immunoglobulin |
| **Ig*X*** | Immunoglobulin, class *X* |
| **IHC** | Immunohistochemistry |
| **IL** | Interleukin |
| **ILF** | Isolated lymphoid follicle |
| **IFN-γ** | Interferon γ |
| **IPEX** | Syndrome of *I*mmune dysregulation, *P*olyendocrinopathy, *E*nteropathy, *X*-linked |
| **$J_H$** | Joining segment of Ig variable region heavy chain gene |
| **KO** | Knock-out |
| **LPS** | Lipopolysaccharide |
| **MAMP** | Microbe-associated molecular pattern |
| **MHC** | Major histocompatibility complex |
| **MLN** | Mesenteric lymph node |
| **NOD** | Nucleotide-binding oligomerization domain |
| **OR** | Odds ratio |
| **PBS** | Phosphate-buffered saline |
| **pIgR** | Polymeric immunoglobulin receptor |
| **PP** | Peyer's patch |
| **PRR** | Pattern recognition receptor |
| **qPCR** | Quantitative polymerase chain reaction |
| **RA** | Retinoic acid |
| **RNA** | Ribonucleic acid |
| **SC** | Secretory component |
| **SFB** | Segmented filamentous bacteria |
| **SIgA** | Secretory immunoglobulin A |
| **TCR** | T cell receptor |
| **TGF-β** | Transforming growth factor β |
| **Th** | T helper cell |
| **TLR** | Toll-like receptor |
| **TNBS** | Trinitrobenzene sulfonic acid |
| **TNF-α** | Tumor necrosis factor α |
| **Treg** | Regulatory T cell |
| **TSLP** | Thymic stromal lymphopoietin |
| **UC** | Ulcerative colitis |
| **WT** | Wild type |

# PAPERS INCLUDED

**Paper I** **Increased number and activation of colonic macrophages in pediatric patients with untreated Crohn's disease.**

G. Perminow, D. H. Reikvam, L.G. Lyckander, P. Brandtzaeg, M. Vatn, and H. S. Carlsen.

*Inflamm Bowel Dis*. 2009;15:1368-1378.

**Paper II** **Increase of regulatory T cells in ileal mucosa of untreated pediatric Crohn's disease patients.**

D. H. Reikvam, G. Perminow, L. G. Lyckander, J. M. Gran, P. Brandtzaeg, M. Vatn, and H. S. Carlsen.

*Scand J Gastroenterol*. 2011;46 (5):550-60. Epub 2011 Feb 1.

**Paper III** **Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression.**

D. H.Reikvam*, A. Erofeev*, A. Sandvik, V. Grcic, F. L. Jahnsen, P. Gaustad, K. D. McCoy, A. J. Macpherson, L. A. Meza-Zepeda, and F-E. Johansen.

*PLoS One.* 2011 Mar 21;6 (3):e17996.

**Paper IV** **Altered mucosal homeostasis in mice lacking secretory antibodies**

D. H. Reikvam, A. Erofeev, R. Islam, M. Derrien, V. Grcic, A. Sandvik, P. Gaustad, L. A. Meza-Zepeda, F.L. Jahnsen, H. Smidt, and F-E. Johansen.

*Submittet*. 2011

**Paper V** **Absence of the polymeric immunoglobulin receptor protects B cell-deficient mice from colitis.**

D. H.Reikvam, A. Erofeev, A. Sandvik, C. Bang, E. Bækkevold, F. L. Jahnsen, and F-E. Johansen.

*Manuscript.* 2011

* These authors contributed equally to this pape

# 1 PREFACE

Inflammatory bowel disease (IBD) includes the two disease entities ulcerative colitis (UC) and Crohn's disease (CD). These two chronic relapsing diseases are closely related and partly overlapping in terms of clinical picture, management and putative etiology. The diseases inflict disabling symptoms like diarrhea, abdominal pain, and fever and require advanced medication and often major surgery (Baumgart and Sandborn, 2007). Untreated the diseases confer increased risk of colonic cancer (Baumgart and Sandborn, 2007).

Historically IBD has been a disease of industrialized parts of the world with reported incidents rates in Europe and North America for both entities ranging from 3 to 15 ($10\ 000^{-1}$) (Loftus, Jr., 2004). After steady increase throughout the last century the adult incident rates have now stabilized in high incident areas while rates in previously low incident areas like Africa, Asia, and Latin America are increasing (Loftus, Jr., 2004). As chronic disorders the IBD burden is better described by the prevalence, which indicates that 1.4 million persons in the United States and 2.2 million persons in Europe suffers from IBD (Loftus, Jr., 2004). The direct costs per patient per year in the United States is estimated to be nearly 10 000 USD and higher for children than for adults (Kappelman et al., 2008). In addition come the hard-to-analyze indirect costs in terms of reduced employment and productivity (Park and Bass, 2010).

IBD has long been considered to occur as a response to environmental factors in genetically susceptible persons (Baumgart and Carding, 2007). The last decade has identified susceptibility genes for IBD, many of which have in common that they play a role in the human organism's handling of intestinal microbes. The "hygiene hypothesis" suggests that increased IBD incidence rates in areas adapting a western world standard of living, included improved hygiene, is caused by alteration in the composition of the intestinal microbiota (Baumgart and Carding, 2007; Koloski et al., 2008). However, despite intense research and great progress the pathogenic mechanisms underlying IBD remain enigmatic.

If able to identify and solve only one tiny piece in the giant puzzle of IBD pathogenesis, one could potentially move closer to prevention of the disease or to the invention of new therapies, which would reduce the disease burden both for individuals and society.

# 2 INTRODUCTION

## 2.1 IBD – clinical aspects

UC's is a relapsing chronic inflammation. The gut inflammation is restricted to the colon, where it starts distally and spreads continuously in the proximal direction, causing superficial mucosal ulcerations. The affected area at diagnosis generally corresponds to disease severity. Patients typically experience bloody diarrhea, which may include mucus and pus, often present at night and followed by abdominal cramps. Severe cases may present with systemic toxicity such as fever, tachycardia, anemia and colonic dilation. After medical induction therapy the prevalence of patients in remission is approximately 50%, but 9 out of 10 patients experience relapses. Total colectomy is curative and 10 years after diagnosis 1 out of 4 patients are colectomized.

CD is also a chronic and relapsing disease but distinguishes itself from UC in some aspects. CD causes transmural inflammation that may affect any part of the gastrointestinal tract. Lesions are generally discontinuous ("skipped lesions") and can be numerous. Half the patients present with lesions in the terminal ileum while approximately 1 out of 4 patients present with colonic lesions and 1 out of 4 with ileocolonic lesions. The transmural inflammation facilitates strictures and fistulas and the clinical presentation relates to the anatomical localization and type of complicating pathology and may change over time. The typical presentation is diarrhea, abdominal pain, fever, and signs of bowel obstruction. The first year after medical induction therapy more than half the patients are in remission, but only 1 out of 10 stays in remission for several years while the rest have a chronic active or chronic intermittent course. Twenty years after diagnosis most CD patients will have undergone surgery due to strictures or fistulas.

In CD, but not in UC, the life expectancy is slightly reduced compared with the general population. Both UC and CD confer increased risks for colonic cancer, most frequently adenocarcinoma. The risk of cancer seems to be correlated to the length of the affected gut, and is less increased in patients that adhere and respond to anti-inflammatory therapy.

After exclusion of other causes of enteritis the diagnosis of both UC and CD are clinical, based on history and physical examination, supplemented by laboratory tests and confirmed by histological assessments. Over the last decades endoscopy has become the key

examination, both for close-up visual evaluation of mucosal appearance and disease distribution and to obtain tissue specimens for histopathological evaluation. In addition, radiological imaging is important for assessment of the extra-colonic distribution of CD.

An overview of the clinical aspects of UC and CD is presented by Baumgart & Sandborn (Baumgart and Sandborn, 2007).

### 2.1.1 Pediatric vs. adult onset IBD

IBD may debut at any age. Worldwide, 10-25 % of all IBD patients are diagnosed before 18 years of age (Griffiths, 2004; Nieuwenhuis and Escher, 2008). Even though the total incidence rates of IBD has stabilized in industrialized countries (Loftus, Jr., 2004), there are several recent reports from industrialized countries pointing to an increased incidence of pediatric onset IBD over the last 20 years (Perminow et al., 2009; Benchimol et al., 2009; Turunen et al., 2006; Orel et al., 2009; Malaty et al., 2010).

Though the nature of the clinical presentation of pediatric IBD is the same as in adult-onset disease some differences exist (Sauer and Kugathasan, 2009; Griffiths, 2004): While UC and CD occur in similar rates in adults, there is a preponderance of CD in pediatric patients. Pediatric CD also occurs more frequently in boys than in girls while a gender difference is not observed in adults. Moreover, pediatric IBD differs from adult disease with respect to disease location. The most frequent disease location in adult CD is the terminal ileum. Isolated ileitis is less frequent in pediatric patients as adolescent patients more often present with a pan-enteric disease and the young children more often present with isolated colonic CD. Pediatric-onset UC more often presents with pan-colitis and a more severe disease phenotype at diagnosis. Ten years after diagnosis twice as many pediatric UC children have undergone surgery compared with adult-onset UC patients.

Pediatric IBD patients naturally have a shorter exposure time to environmental pathogenic factors than adults, and thus it has been proposed that genetics play a more important role in the disease development in children. Researchers argue that pediatric-onset disease should be regarded as a separate entity within IBD, and further research on pediatric IBD has been warranted (Kugathasan and Cohen, 2008).

## 2.2 The intestinal microbiota

The lumen of the mammalian gastrointestinal tract harbors a vast amount of microbes. It is estimated that the microbial concentration in the colon is $10^{11}$ cells/ml, which implies that the total number of microbial cells in the human gut is in the range of $10^{14}$ (Ley et al., 2006). This microbial community, often termed commensal or mutualistic, outnumbers the cells of the human organism by an order of magnitude. New DNA sequencing technology has enabled scientists to estimate the intestinal microbiota's collective genome (a.k.a. metagenome or microbiome) to contain more than $3x10^6$ different genes, which is 150 times the number of human genes (Qin et al., 2010). Considering this metagenome's proximity to the human genome a modern view is to look at man as a super-organism whose genetic information is provided both by the human cells and by the microbial metagenome (Gill et al., 2006; Peterson et al., 2008). Assessments of the intestinal microbiota were for many years based on bacterial cultivation. However, only a fraction of the intestinal microbiota can be cultured *ex vivo*. Sequencing and bioinformatic studies of the highly conserved 16S rRNA gene (See also section 5.5.2) present in all bacteria have tremendously improved our insight into the composition and the evolution of the intestinal microbiota (Peterson et al., 2008). Very recently, deep sequencing technology of the metagenome has provided additional information to metagenomic studies (Qin et al., 2010; Nelson et al., 2010).

The intestinal microbiota consists of all three domains of life: Eukarya (i.e. fungi, parasites), Archaea and Bacteria. 99% of the gut metagenome is of bacterial origin, which consequently is the most studied (Qin et al., 2010). Of 55 divisions in the kingdom of Bacteria only seven are represented in the gut, a low number compared to other ecological habitats. Two phyla, the Firmicutes and the Bacteriodetes, represent 98% of the 16S rRNA gene sequences in the healthy human gut (Ley et al., 2006). Within these phyla there are around $10^2$-$10^3$ different species, depending on the metagenomic definition of a species, and $10^4$ phylotypes on strain level. This pattern with a shallow widespread fan-like phylogenetic tree is typical for an environment with extreme selection pressure (Ley et al., 2006). To be established as gut residents bacteria have to overcome lack of light, low oxygen tension, limitations in substrates for energy production, rapid turnover of epithelial cells, the mechanical propulsion of luminal contents, and the potential combat with the host immune system (Ley et al., 2006; Sonnenburg et al., 2004). Also, from an evolutionary point of view, bacteria that provide benefits for the host will be selected. The microbiota provides an enzymatic machinery for digestion of plant saccharides, which complements the limited

glycosylhydrolase repertoire of the mammalian genome and allows increased energy utilization from a plant rich diet (Ley et al., 2008; Hooper et al., 2002). Microbial enzymes are also essential for synthesizing vitamin K and certain B vitamins (Hooper et al., 2002). The commensal microbiota occupy an ecological niche that could otherwise be exploited by pathogenic bacteria so they offer a colonization resistance to potential pathogens (Stecher and Hardt, 2008). The importance of the microbiota to stimulate the host immune system is presented in later sections.

Cultivation based studies have shown that newborns acquire their initial gut microbiota from the feces and vagina of their mothers at birth (Ley et al., 2006). The composition of the microbiota is chaotic the first year of life, but eventually stabilizes and shows little temporal variation in healthy adults (Rajilic-Stojanovic et al., 2009; Ley et al., 2006; Peterson et al., 2008). Potential modifications of the microbiota are derived from environmental factors such as diet and antibiotic use (Maslowski and Mackay, 2011; Willing et al., 2011). Preliminary studies of intra-individual spatial variations in the composition of the microbiota along the healthy gut have revealed small differences (Peterson et al., 2008; Eckburg et al., 2005). However, there is a gradient in the magnitude of the microbial load along the gut with bacterial densities of $10^2$ cells/ml in the proximal small intestines to $10^{11}$ cells/ml in the colon (Sartor, 2008). In summary, a healthy person's microbial composition is fairly stable and determined by the microbes one is exposed to after birth as well as to yet unidentified genetic factors (Spor et al., 2011).

Even though analyses of the microbial composition show intra-individual stability there are great inter-individual diversities (Peterson et al., 2008; Eckburg et al., 2005). Phylogenetic microarray and metagenomic catalogue studies have reported that less than 20% of the phylotypes at the species levels are uniformly present in unrelated persons (Rajilic-Stojanovic et al., 2009; Qin et al., 2010). It is believed that humans carry a core microbiota of essential species but that there is a vast inter-individual species-specific diversity outside of this core microbiota.

Recently, alterations in the composition of the intestinal microbiota have been shown to be associated with obesity, a finding that demonstrate the significance of the microbial community for human health (Turnbaugh et al., 2009). Furthermore, experimental animal models for several autoimmune diseases like type 1 diabetes mellitus, multiple sclerosis and ankylosing enthosopathy are dependent on microbial stimuli (Wen et al., 2008; Ochoa-

Reparaz et al., 2010; Chervonsky, 2010). Perturbations of the intestinal microbiota associated with IBD are discussed in section 2.4.

## 2.3 The intestinal mucosal immune system

All organisms have developed defense systems to protect themselves from foreign matter like microbes and viruses. Mammals have evolved a highly complex immune system; which, for didactic reasons is divided into two systems: the innate and the adaptive immune systems. The two systems overlap and interrelate extensively. An extensive and general overview of the innate and adaptive immune systems can be found in text books (Janeway, 2005). A minute introduction of the two systems' key characteristics exemplified by the defense against microbes in the gut is presented in this chapter. It further presents an overview of mucosal immunity and is followed by sections on elements of mucosal immunity related to the aims of this thesis.

The innate immune system refers to the antigen non-specific defense against a foreign threat. On front line, the defense system involves mechanical barriers like stratified epithelium of the esophagus, mucus lining the epithelial cells, which again are sealed together by tight junctions and constantly shed and renewed, and the propulsion of the luminal contents by peristalsis. Mammals also have chemical barriers in the gastrointestinal tract consisting of a low pH in gastric juices, non-specific enzymatic protein digestion, and secretion of anti-microbial peptides (AMP) from epithelial cells. Resident commensal bacteria occupying the ecological niche in the gut may also be regarded as part of the super-organism's innate defense system as they provide a colonization resistance to pathogenic microbes (Stecher and Hardt, 2008).

Leukocytes of hematopoietic origin are the central cell types (Figure 1) to combat microbes that overcome the physical and chemical barriers. Macrophages and neutrophils reside in or are rapidly recruited to the lamina propria, respectively. They have the capability to neutralize intruders by phagocytosis and subsequent intracellular destruction, or by degranulation of preformed granules containing toxic substances. Notably, the toxicity of degranulation works unspecifically and also causes harm to the host tissue (Mosser and Edwards, 2008). Dendritic cells (DC) are also phagocytic and are important for processing of

microbial molecules and presenting them for lymphocytes. Activated DCs migrate to lymphoid tissue and bridge the innate and adaptive immune system.

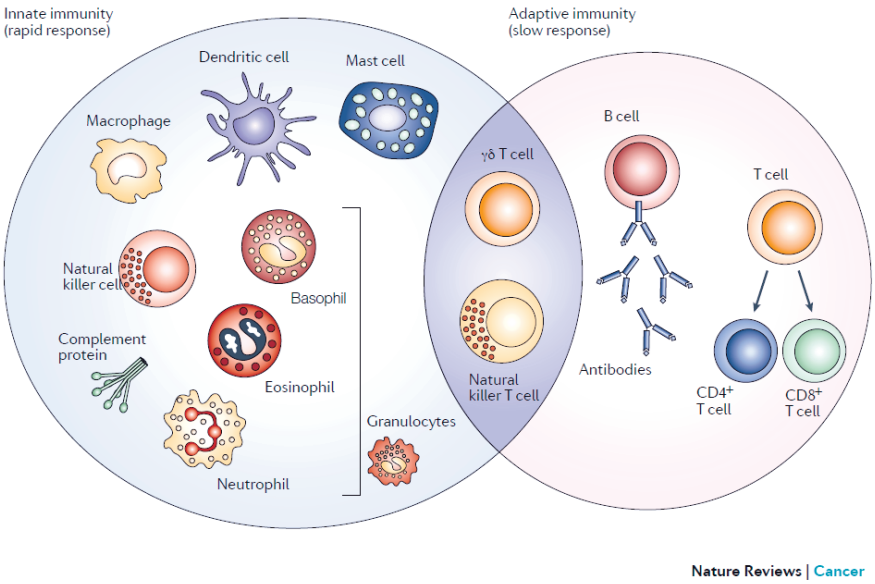


**Figure 1. Overview of innate and adaptive hematopoietic immune cells.** The innate immune response functions as the first line of defense against infection. In addition to tissue-specific cells (e.g. epithelial cells), the innate immune cells consist of hematopoietic cells including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells, and natural killer cells. The adaptive immune response is slower to develop, but is antigen specific and has memory. It consists of B cells and $CD4^+$ and $CD8^+$ T lymphocytes. Natural killer T cells and $\gamma\delta$T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity.



The ability to discriminate foreign material from the organism's own is essential to the immune system. For innate immune cells this is provided by a set of pattern recognition receptors (PRRs). PRRs are germ-line encoded and highly conserved. They are expressed by leukocytes and also by non-hematopoietic cells like epithelial cells. The transmembrane Toll-like receptors (TLR) and the cytosolic nucleotide-binding oligomerization domains (NOD) are two of the most studied classes of PRRs. The ligands for PRRs are conserved peptides

(e.g. flagellin), glycoproteins (e.g. peptidoglycan), lipopolysaccharide (LPS), or nucleic acid structures (e.g. unmethylated CpG, double-stranded RNA) that are common to a wide range of microbes, but not expressed by the mammalian cells. These ligands are collectively termed microbe-associated molecular patterns (MAMP). Engagements of PRRs by MAMPs induce signaling cascades facilitating transcription of pro-inflammatory molecules. In summary, the innate immune system discriminates between self and non-self and relies on germ line-encoded molecules that are partially preformed and rapidly mobilized. The innate immune response is an immediate and unspecific reaction that occurs in minutes and hours after an intruder has been recognized.

The adaptive immune response starts if an infection is not cleared by the innate immune response within hours. The adaptive response is mediated by B and T lymphocytes. B cells have the ability to differentiate into plasma cells which produce immunoglobulins (Ig) and are the main effectors of humoral immunity. T cells mediate cellular immunity through killing of infected cells by the subset cytotoxic T cells ($CD8^+$), or through $CD4^+$ T cell subsets' activation or modulation of B cells and cytotoxic T cells.

Essential in adaptive immunity are the antigen-specific response and the concepts of clonality and immunological memory. Each B or T lymphocyte has a single type of receptor with unique specificity, i.e. B cell receptor (BCR) or T cell receptor (TCR). High affinity interaction between a lymphocyte receptor and an antigen recognized as foreign leads to activation of the actual lymphocyte. The vast numbers of T and B cells present in the body collectively provide a near infinite number of BCR and TCR specificities. All cells derived from an activated lymphocyte will bear the identical receptor specificity as this lymphocyte. Igs have specificity identical with the BCR and are accordingly also of clonal origin. The antigen, most often of protein nature, has to be processed and presented as fragmented peptides to the TCR on major histocompatibility complex (MHC) II molecules, which are present on antigen-presenting cells (APC) like DCs, macrophages and B cells, or on MHC I molecules present on all nucleated cells. A lymphocyte that is activated by specific high-affinity binding to an antigen-MHC II complex will proliferate. This clonal expansion is important for the power of the immune response. The primary adaptive immune response is a slow reaction and takes hours and days to be raised. After the primary immune response has resolved most of the antigen-specific activated lymphocytes undergo apoptosis. However, a significant number of cells persist in a dormant state. These memory cells ensures a more rapid and effective response the second time the immune system encounter the same antigen.

Innate immune responses take place in all tissues. Gut mucosal adaptive immune responses are induced in specialized lymphoid tissue called gut-associated lymphoid tissue (GALT). GALT consists of Peyer's patches (PP) in the small intestines and isolated lymphoid follicles (ILFs) located throughout the gut mucosa. Antigens are sampled at these inductive sites and presented to lymphocytes, which subsequently drain to the mesenteric lymph nodes (MLN) and then to blood via the thoracic duct. Educated lymphocytes home to the lamina propria, the effector site of mucosal adaptive immunity, where they exert their effects.

### 2.3.1 Overview of mucosal immune homeostasis

In the gut, the cumulative area of the mucosal membrane is several hundred square meters (Brandtzaeg, 2009). A single layer of columnar epithelial cells divides the interior from the luminal exterior, which is occupied by $10^{14}$ microbes and a vast load of foreign dietary materials. Consequently the mucosal membrane is a vulnerable surface, and the ability to maintain homeostasis with tolerance to commensal microbes and concurrent responsiveness to pathogens and intruders is of paramount importance. This ability can be viewed as a hierarchy of three immunological barriers (Hooper and Macpherson, 2010). The first layer limits the physical contact between the intestinal microbes and the epithelial cells. The second layer provides rapid detection and elimination of microbes that manage to penetrate the epithelial barrier. Figure 2 gives an anatomical overview of the first two layers of the intestinal immunological barriers. The third layer consists of immune responses that minimize the exposure of microbes to the systemic immune system.

Three components contribute to prevent direct contact between microbes and the epithelium: mucus, AMPs, and secretory immunoglobulins (SIg). Specialized intestinal epithelial cells called Goblet cells localized in the mucosal crypts constitutively secrete mucin, which is a highly glycosylated protein (Johansson et al., 2008). The mucin forms a protective mucus layer up to 150 μm thick on the apical side of the epithelial cells. The mucus layer is divided into two layers were the inner and more densely stratified layer is devoid of bacteria (Johansson et al., 2008). Experimental deletion of the mucin production in mice leads to bacterial penetration and spontaneous colitis (Johansson et al., 2008; Van der et al., 2006).

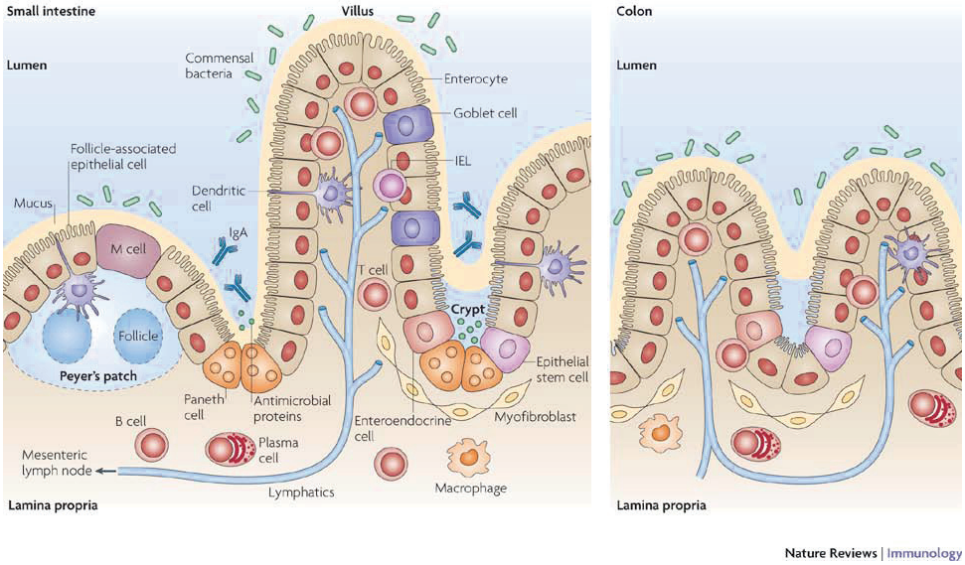


**Figure 2**. **Microanatomy of the intestinal immune system**. A single layer of intestinal epithelial cells (IECs) separates the intestinal lumen from the underlying lamina propria. The IECs lining the lumen are bathed in nutrients, commensal bacteria, IgA and goblet cell-produced mucus. Epithelial stem cells proliferate and give rise to daughter cells with the potential to proliferate. These IECs then differentiate into villous or crypt enterocytes, which absorb nutrients (small intestine) and water (colon). In addition to differentiated enterocytes and goblet cells, progenitor IECs differentiate into Paneth cells at the base of the small intestinal crypts. Beneath the IECs, the lamina propria is made up of stromal cells (myofibroblasts), B cells (especially IgA-producing plasma cells), T cells, macrophages and dendritic cells. Certain subsets of T cells and dendritic cells localize between the IECs. The small intestine has regions of specialized epithelium termed follicle-associated epithelium and microfold (M) cells that overlie the Peyer's patches and sample antigens from the intestinal lumen. IEL, intraepithelial lymphocyte.

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AMPs are small peptides of several families including α/β defensins, cathelicidins, RELMβ, lectins/REGIIIβ/γ, and Ang4. They exert strong antimicrobial activity, most frequently by enzymatic disruption of the bacterial cell wall or cell membrane (Hooper and Macpherson, 2010; Dann and Eckmann, 2007). Some of the AMPs are secreted constitutively from a wide range of cells types (Dann and Eckmann, 2007), while others are selectively expressed by specialized epithelial cells like Paneth cells in the small intestine and are induced by microbes (Brandl et al., 2008; Vaishnava et al., 2008; Nair et al., 2008) or

MAMPs (Cash et al., 2006) in a PRR-dependent manner (Vaishnava et al., 2008; Brandl et al., 2007; Kobayashi et al., 2005). AMPs are not found in the gut lumen but retained in the apical mucus layer, thus setting up an AMP diffusion gradient which microbes have to travel against to reach the epithelial surface (Meyer-Hoffert et al., 2008). Disrupted production of certain classes of AMPs is associated with Crohn's disease (Wehkamp et al., 2008).

SIgs, as the third contributing component to keep microbes off the epithelial surface are thoroughly discussed in the 2.3.3.*n* sections.

The second hierarchical layer providing mucosal homesteostasis is the rapid detection and elimination of microbes that have penetrated the epithelial barrier (Hooper and Macpherson, 2010). This is foremost facilitated by resident macrophages, which are discussed in section 2.3.4. Adaptive immunity also comes into play at this level. Microbial antigens are engulfed and digested by DCs and presented to $CD4^+$ T cells. DCs reside beneath the intestinal epithelium and are especially abundant beneath the follicle-associated epithelium of PPs. The follicle-associated epithelium lacks coverage of protective mucus and harbors scattered microfold (M) cells, which efficiently transcytose luminal products across the epithelium (Brandtzaeg, 2009). Mucosal DCs are of different functional subsets. Some DC subsets migrate constitutively to lymphoid tissue (i.e. PP, MLN) where they present their antigens to naïve $CD4^+$ T cells and where also naïve B cells are present (Coombes and Powrie, 2008). These lymphocytes are then activated and imprinted with the gut-specific homing molecules integrin $\alpha_4\beta_7$ and chemokine receptor CCR9, which make them - after circulation via lymph and blood - return to the gut lamina propria as antigen-specific activated lymphocytes (Brandtzaeg, 2009). Further details on the mucosal B cell activation is presented in section 2.3.3.1. Other DC subsets are non-migratory and present antigens to $CD4^+$ T cells in the lamina propria (Coombes and Powrie, 2008). The outcome of the DCs' antigen presentation and activation of T cells is dependent on conditioning of the DCs. In a homeostatic situation, the cytokine environment in the lamina propria is dominated by thymic stromal lymphopoietin (TSLP), transforming growth factor β (TGF-β), retinoic acid (RA), IL-10, and prostaglandins produced by epithelial cells, stromal cells, macrophages and lymphocytes (Rescigno and Di, 2009; Coombes and Powrie, 2008). These cytokines condition DCs to favor induction of the transcription factor Forkhead box P3 (Foxp3) in naïve $CD4^+$ cells, which then become regulatory T cells (Tregs) and maintain specific tolerance to the commensal microbes (See section 2.3.5 for further details). On the other hand, if a microbial product is perceived as a pathogen, the cytokine environment turns pro-inflammatory and

DCs will activate pro-inflammatory transcriptional programs in $CD4^+$ T cells, which become pro-inflammatory Th1 or Th17 cells (Coombes and Powrie, 2008).

An additional mechanism that contributes to this second hierarchical layer is the antigen excretion provided by secretory immunoglobulins (SIgs) and the polymeric immunoglobulin receptor (pIgR) (Strugnell and Wijburg, 2010). This mechanism is presented in detail in sections 2.3.3.2-3.

The third hierarchical layer of protection is a firewall between the mucosal and the systemic immunological compartments (Hooper and Macpherson, 2010). In healthy mice, commensal microbes can be cultured from MLN of immune competent mice but not from blood or systemic organs (Macpherson and Uhr, 2004). Live commensal microbes travel with migratory DCs from the lamina propria to MLNs and enhance activation of commensal-specific B and T lymphocytes, which are subsequently put into circulation and disseminated throughout the gut by homing mechanisms described above. The efficacy of the firewall depends on the competence of the first and second layer of protection. For instance, defective protection by IgA results in increased commensal specific serum IgG indicating systemic exposure to commensal microbes (Macpherson et al., 2000; Johansen et al., 1999; Sait et al., 2007).

### 2.3.2 Intestinal epithelial cells as immune cells

As mentioned in previous sections, a single layer of columnar epithelial cells provides the barrier between the organism's interior and exterior. The former is striving for sterility while the latter is comprised of a vast microbial load. The epithelial cell membrane is impermeable to hydrophilic molecules that lack specific transporters while tight junction protein complexes seal off the paracellular route (Turner, 2009). For long, the epithelium was only acknowledged for its simple mechanical barrier function in addition to exchange of nutrients and water. In recent years the importance of the intestinal epithelial cells' (IEC) contribution to the mucosal immune homeostasis has been recognized (Artis, 2008).

IECs are constantly renewed and originate from intestinal stem cells located near the crypt base (Scoville et al., 2008) (Figure 2). The IECs proliferate and differentiate in the crypts, migrate up, and eventually undergo apoptosis on the surface epithelium in an approximately five day long cycle (Abreu, 2010). Differentiated IEC to be mentioned for their

immune functions are: a) AMP-releasing Paneth cells in the lower parts of small intestine crypts; b) mucin-producing Goblet cells throughout the crypts; and c) M cells located in the subepithelial dome of Peyer's patches and ILFs, which facilitate transepithelial transport of luminal antigens for education of mucosal adaptive immune cells. In addition, the generally absorptive enterocytes have important innate immune functions. In steady state situation, IECs are important producers of the cytokines TSLP, TGF-β, prostaglandin E-2, and retinoic acid, which all are important for a tolerogenic and anti-inflammatory cytokine milieu in the lamina propria (Rescigno and Di, 2009; Shale and Ghosh, 2009). IEC also produce the two cytokines BAFF and APRIL which are considered to be important for immunoglobulin class switching to IgA (Cerutti and Rescigno, 2008). In contrast to these homeostatic features, IECs may contribute to mucosal inflammation by release of pro-inflammatory cytokines, chemokines and co-stimulatory factors for effector T cells (Baumgart and Carding, 2007).

IECs may express a wide array of PRRs and still remain tolerant to the commensal microbes in their immediate vicinity. Several models have been proposed to explain this paradox (Duerkop et al., 2009). In a homeostatic situation, IEC express negligent amount of TLR2, TLR4 and the LPS receptor CD14 (Abreu et al., 2001; Melmed et al., 2003; Lotz et al., 2006). TLR5 is only located at the basolateral side of the cells, which limits its engagement to flagellin on bacteria that have penetrated to the lamina propria (Gewirtz et al., 2001). Other TLRs are located only on intracellular membranes, which together with the cytosolic NODs make them oblivious to extracellular microbes (Abreu, 2010).

The effects of PRR activation in IECs are numerous: it increases the proliferative rate of the epithelium (Abreu, 2010) and stimulates secretion of some AMPs (Cash et al., 2006; Vaishnava et al., 2008; Brandl et al., 2007) as well as factors essential for mucus production (Abreu, 2010). TLR2 activation preserves tight junction-associated barrier function (Cario et al., 2007). Engagement of IEC PRRs also affects lamina propria adaptive immune responses by facilitating secretion of BAFF and APRIL (Cerutti and Rescigno, 2008) and by allowing DCs to project between IECs to sample luminal antigens (Chieppa et al., 2006). As previously mentioned, PRR activation generally induces a pro-inflammatory transcriptional program. However, in a landmark article by Rakoff-Nahoum *et al.* (Rakoff-Nahoum et al., 2004), it was recognized that constant low grade IEC PRR activation is beneficial to the mucosa. As summarized in figure 3, abrogated PRR activation reduces epithelial responsiveness and leaves the mucosa more susceptible to pathogens and injury whereas excessive PRR

activation promotes inflammation with subsequent tissue damage (reviewed in (Asquith and Powrie, 2010))

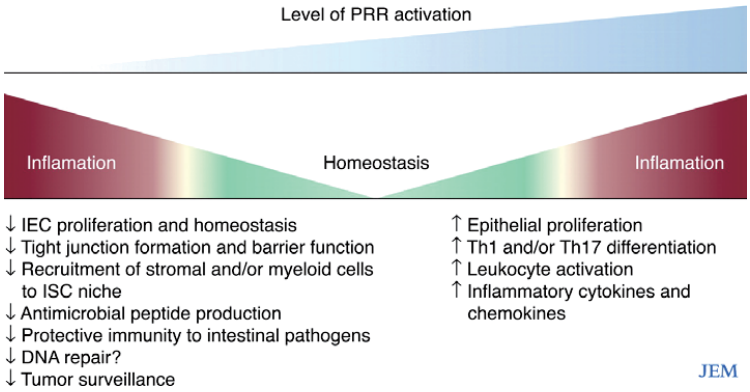



**Figure 3. Diminished or enhanced intestinal pattern recognition receptor (PRR) signals may promote intestinal inflammation and tumorigenesis**. PRR signals are maintained at a critical threshold to maintain intestinal homeostasis. PRR signals may be required to restore barrier function after epithelial insult and for protective immunity against pathogens; impairment of these processes caused by insufficient PRR signaling may result in pathogen outgrowth and, indirectly, excessive subsequent inflammation (left). Excessive PRR-driven repair or inflammatory responses (right) may also threaten homeostasis, e.g., through dysregulated epithelial proliferation leading to tumorigenesis and overexuberant pathogenic inflammatory responses to the intestinal microbiota.



### 2.3.3 Sectory immunoglobulins

IgA is the most prominent class of immunoglobulins in mammals. 80% of the antibody produced in the body originate from IgA-producing plasma cells (Brandtzaeg, 2009). Most of this IgA is produced in the gut as dimeric IgA (dIgA) covalently bound together by the J chain and transported from the stromal to the luminal side of the epithelium by the pIgR. It is estimated that in adult humans, 3 grams of dIgA is translocated in this fashion every day (Brandtzaeg, 2009). In addition, some polymeric IgM is transcytosed by the pIgR (Brandtzaeg, 2009), and IgG may also be secreted with the help of the neonatal Fc receptor (Baker et al., 2009). IgA composes a minor fraction of serum Igs, but as it is by far the most abundant Ig class in mucosal secretions the following sections are devoted to an overview of mucosal IgA biology.

#### *2.3.3.1 IgA*

The classical activation of B cells to become IgA-producing plasma cells takes place in GALT, primarily PPs but also in ILFs, and to some extent in MLNs, and is detailed in several reviews (Macpherson et al., 2008; Cerutti and Rescigno, 2008; Brandtzaeg, 2007) and summarized in the following paragraphs. A basic observation of IgA is that its production is dependent on the presence of microbial stimuli. IgA-producing cells are absent in neonates before colonization by commensal microbes, and they are reduced by 1-2 orders of magnitude in germ-free mice (Macpherson et al., 2008). The production of IgA requires the presence of intestinal microbes but most (>85%) of the produced IgA is polyspecific and recognizes antigens from a wide range of microbes. The rest of the IgA displays single antigen specificities (Jiang, 2004; Strugnell and Wijburg, 2010).

The CCR7-binding chemokines CCL19 and CCL21 are expressed in PP and mediate extravasation of naïve B cells from the blood stream through high endothelial venules. In the PP germinal centre the B cell encounters antigens which it engulfs with its BCR and subsequently digests and presents on its MHC II molecule. $CD4^+$ T cells - already activated by DCs that present the same antigen as the B cell - recognize the specific MHC II-antigen complex on the B cell and subsequently allows CD40 on the B cell to ligate to CD40 ligand (L) (CD154) on the T cell. In the crucial presence of TGF-β the CD40-CD40L interaction induces the enzyme activation-induced cytidine deaminase (AID), which facilitates both class switch recombination (CSR) in the B cell from IgM or IgD to IgA and somatic hypermutation with subsequent enhanced affinity for the target antigen. Other substances like RA, inducible nitric oxide synthase, the innate switch factors APRIL and BAFF, and the cytokines IL-2, IL-4, IL5, IL6, and IL-10 released in the germinal center also contribute to the activation and differentiation of intestinal B cells. It should be noted that in the PP, T cell-dependent activation of B cells may take place without the specific antigen recognition of the naïve B cells if the B cell is activated by PRRs (Suzuki and Fagarasan, 2009). This feature is not observed in peripheral lymph nodes and may contribute to the polyspecificity of IgA.

In parallel with CSR, RA promotes the expression of the homing molecules $\alpha_4\beta_7$ and CCR9 or CCR10. B cells leave the PP through the efferent lymphatics as IgA plasmablasts, re-enter systemic circulation and are then directed to the intestinal lamina propria by interaction of $\alpha_4\beta_7$ with MadCAM-1 and CCL25 with CCR9 (small intestine) or CCL28 with CCR10 (colon) on intestinal endothelial cells. In the lamina propria the plasmablasts undergo final differentiation to IgA-producing plasma cells.

CSR may also take place in a T cell-independent fashion. Still in the presence of TGF-β, BAFF and APRIL released by DCs in ILFs may enable class switch and activation of B cells without CD40-CD40L interactions. As BAFF and APRIL may be secreted by IECs of the colon in a PRR-dependent fashion it is proposed that T cell-independent CSR may take place in the lamina propria (He et al., 2007) but this remains debated (Brandtzaeg, 2009).

In humans there are two gene loci which encode the IgA properties on the Ig heavy chain domain resulting in two subclasses of IgA, named IgA1 and IgA2. IgA2 is more resistant to bacterial proteases and is the subclass dominating as secretory IgA (SIgA) in the colon. IgA1 is the subclass dominating in serum and in all other mucosal secretions (Brandtzaeg and Johansen, 2005). Mice only make one class of IgA, which structurally is equivalent to human IgA2 (Mestas and Hughes, 2004).

IgA (and IgM)-producing plasma cells in the gut also produce the J chain, which polymerizes IgA and IgM into dimers and pentamers, respectively. The transcriptional regulation of the J chain is poorly understood (Johansen and Brandtzaeg, 2004). The J chain is covalently linked by cysteine disufide bridges to two IgA heavy chain tail-pieces of opposing IgA monomers, which subsequently allows direct disulfide bonding between the penultimate cysteine residues of the two remaining heavy chain tail-pieces (Johansen et al., 2000). Polymers of both IgA and IgM may form without the J chain but these are, along with the monomers, not substrates for pIgR-mediated transport (Johansen et al., 2000).

There are other IgA receptors than pIgR. Best characterized is the FcαRI (CD89) which is present on circulating myeloid cells like monocytes and neutrophils but not on gut mucosa resident APCs. This suggests that IgA may have a role in opsonization of antigens for phagocytosis in the systemic compartment (Otten and van, 2004). Other IgA receptors are the Fcα/μR expressed on most B cells and macrophages but not on T cells or neutrophils, the transferrin receptor (CD71) expressed on human mesiangial kidney cells, and the asialoglycoprotein receptor expressed on hepatocytes. The significance of these receptors remains to be explored (2007).

The full biological role of systemic IgA is yet to be discovered. As opposed to IgM and IgG IgA does not activate compliment and is believed to play a homeostatic role (2007). However, the main functions for IgA are believed to be related to the interaction with pIgR on mucosal membranes and are described in section 2.3.3.3.

#### *2.3.3.2 The polymeric immunoglobulin receptor*

The pIgR is a ~100 kD protein produced by epithelial cells in literally all mucosal membranes but is particularly highly expressed in gut epithelium (Johansen and Brandtzaeg, 2004). It consists of five N-terminal extracellular domains (D) where D1 hosts the ligand-binding site. It further has a single membrane-spanning α helix and an intra-cellular C-terminal tail which provides the routing information for the receptor (Macpherson et al., 2008) The *PIGR* gene is constitutively expressed, but also subject to regulation by cytokines, hormones, and microbes and MAMPs (Johansen and Brandtzaeg, 2004). Interestingly, both Th1 and Th2 cytokines, namely interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IL-4 up-regulate pIgR expression in human intestinal epithelial cell lines. Activation of TLR3 and TLR4 also up-regulated pIgR expression *in vitro*, suggesting a role for viruses and commensal microbes in the transcriptional regulation of pIgR (Schneeman et al., 2005). Accordingly, colonization of germ-free mice up-regulated pIgR expression in the small intestine (Hooper et al., 2001).

After synthesis in the endoplasmatic reticulum, the pIgR is delivered to the basolateral side of the epithelial cell where D1 binds non-covalently to the dIgA/J chain complex (Johansen and Brandtzaeg, 2004). This binding promotes disulfide bonds between pIgR D5 and cysteins residues on the Cα2 domain of the IgA (Johansen and Brandtzaeg, 2004). The pIgR/dIgA complex is then subjected to clathrin-mediated endocytosis, fused with endosomes, routed to the apical cell membrane, and finally cleaved by endoproteases on the cell membrane. A sketch of this transcytosis is shown in figure 4. D1-5 of the pIgR, now termed secretory component (SC), together with the dIgA then forms SIgA (Macpherson et al., 2008). The SC portion has a biologically important function on the SIgA complex as it makes the dIgA less susceptible to protelytic enzymes (Phalipon and Corthesy, 2003). The fact that mice with deleted pIgR gene have absent IgA in mucosal secretions demonstrate that the pIgR is solely responsible for the epithelial translocation of IgA (Johansen et al., 1999; Shimada et al., 1999). Further knowledge gained from studies in pIgR knock out (KO) mice is presented in section 5.2.1.

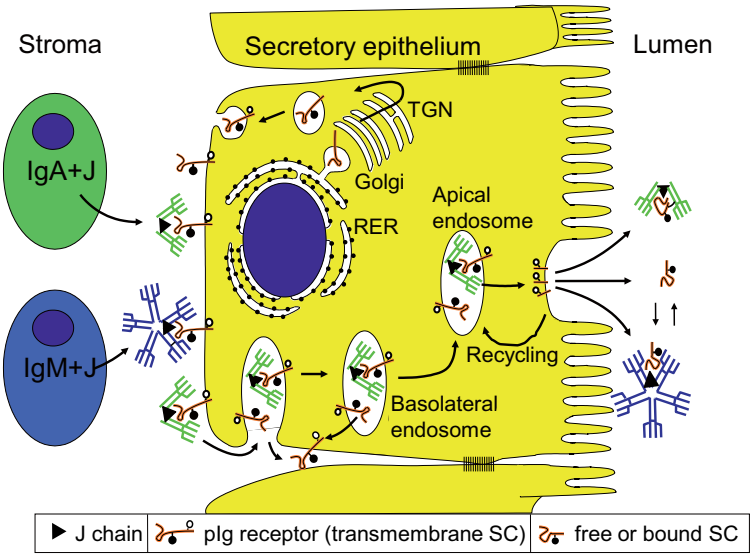



**Figure 4. Outline of pIgR-mediated transport of polymeric Igs**. Dimeric (d)IgA or pentameric (p)IgM, which is produced by plasma cells on the stromal side of mucosal membranesand held together by the J chain, is picked up by the polymeric immunolglobulin recetor (pIgR) on the basolateral side of epithelial cells. The dIgA- or pIgM-pIgR complex is then endocytosed, fused with endosomes, and transported to the luminal side where the extracellular domains of pIgR, a.k.a secretory component (SC), is cleaved off by endoproteases and the complex released in the lumen as secretory Ig (SIg). In addition, pIgR without an Ig cargo may travel the same route and be secreted as free SC. (Adapted from P. Brandtzaeg and F-E. Johansen)

In addition, pIgR that is not bound to dIgA may travel this same transcellular route and be cleaved off at the apical cell membrane and be released as free SC (Phalipon and Corthesy, 2003) (Figure 4). SC is extensively glycosylated which facilitates retention in the glycosylated mucus layer. *In vitro* experiments have demonstrated that free SC binds and neutralizes *Clostridium difficile* toxin A (Dallas and Rolfe, 1998) and *E.coli* strains (de, I et al., 2001). It has also been shown *in vitro* that SC inactivates the neutrophil attractant IL-8 (Marshall et al., 2001). However, whether the free SC survives microbial proteolysis in the gut and plays a physiological role in mucosal homeostasis has remained enigmatic (Phalipon and Corthesy, 2003) Further research on free SC is warranted (Strugnell and Wijburg, 2010).

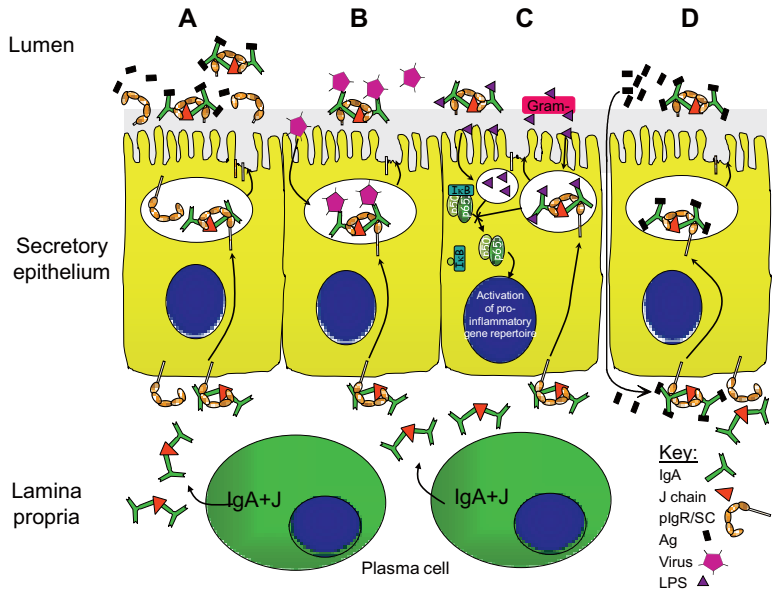



**Figure 5. Principles of pIgR/SIgA-mediated protection at mucosal surfaces**. SIgA mediate mucosal homeostasis by (A) trapping luminal antigens in the apical mucus layer preventing the antigens to reach the epithelial cell membrane, by (B) re-routing intracellular antigens to the luminal side, by (C) neutralizing intracellular antigens preventing them from activating the epithelial cell, and by (D) transporting antigens from the stromal to the luminal side. (Adapted from P. Brandtzaeg and F-E. Johansen)

### *2.3.3.3 The biological effect of SIgA*

Conventionally there are three principle non-exclusive models for how SIgA exerts its effects: luminal immune exclusion, intracellular neutralization, and antigen excretion (Strugnell and Wijburg, 2010) (Figure 5). To assess the separate contributions of each of the three mechanisms *in vivo* is notoriously difficult.

Immune exclusion refers to the concept that SIg is retained in apical mucus layer where it traps and agglutinates microbes ("flypaper") and prevents them from reaching the epithelial surface (Figure 5a). This mechanism assumes that the IgA is polyspecific and that pre-formed IgA cross-reacts with different species because antigen-specific IgA takes weeks to be induced (Strugnell and Wijburg, 2010) Genetically modified mice deficient in SIgA have been employed in various vaccine and infectious models with enteropathogens

(reviewed in (Strugnell and Wijburg, 2010). Although the results vary among different models and studies are hampered by co-contribution of SIgM and IgG, the overall conclusion is that lack of SIgA renders animals less protected by vaccine and more susceptible to infections by enteropathogens. In addition, an elegant study demonstrated that mice deficient of SIg were more likely than mice with intact SIg to transmit *S. typhimurium* to wild-type littermates This suggests that SIgA may be important for herd immunity as shed bacteria are covered with a SIgA coat (Wijburg et al., 2006)

*In vitro* and *in vivo* studies have showed that dIgA-pIgR complexes can bind to intracellular viruses and antigens like LPS and reroute them back into the lumen and thereby prevent infection and mucosal inflammation (Brandtzaeg, 2007) (Figure 5b,c). Finally, *in vitro* and *in vivo* experiments have demonstrated that IgA may bind antigens, including whole viruses, in the lamina propria and excrete them to the luminal side by dIgA-pIgR-mediated transcytosis (Strugnell and Wijburg, 2010; Robinson et al., 2001) (Figure 5d).

In addition to eliminating microbes from the mucosal membrane, it is proposed that SIgA carrying an antigen cargo may be trancytosed from the lumen to the lamina propria through M cells. Reports from Corthesy and colleagues indicate that SIgA-antigen complexes are then internalized in DCs, and this favors a tolerogenic DC respons (Corthesy, 2007). SIgA may thereby contribute to mucosal tolerance to commensal microbiota by supplying luminal antigens to DC without eliciting an inflammatory response. However, this model awaits for a SIgA receptor on the M cells to be identified (Brandtzaeg, 2007).

### 2.3.4 Intestinal macrophages

Macrophages are leukocytes. Distinct from lymphocytes, they originate from the granulocyte/monocyte colony-forming unit, which differentiates to monocytes in the bone marrow, and the monocytes are then released into the blood. The monocytes may extravasate and differentiate in tissues to macrophages or certain DC subsets (Geissmann et al., 2010; Gordon and Taylor, 2005). Monocytes can be divided into subsets on the basis of surface markers. However, one has not been able to identify if certain monocytes subsets are committed to become macrophages and DCs, respectively (Gordon and Taylor, 2005; Geissmann et al., 2010). The local tissue factors responsible for the differentiation of moncytes to macrophages have not been identified (Gordon and Taylor, 2005; Geissmann et al., 2010). In the gut there is also a great overlap in functional characteristic and surface

markers between macrophages and DCs (Kelsall, 2008). Still, it is considered that gut lamina propria resident macrophages functionally distinguish themselves from DCs in the same location by higher phagocytic capacity, increased intracellular killing, absent cytokine production, reduced antigen presentation, and inability to migrate to lymphoid tissue (Smith et al., 2010). Gut resident macrophages are non-proliferating and long-lived; probably they live for months after their final differentiation (Smith et al., 2010). Historically human macrophages have been identified by the cytoplasmic molecule CD68 (Pulford et al., 1990), but lower expression of this marker may be shared with DCs and other cells types. Resident gut macrophages, but not inflammatory macrophages (see below), are rather characterized by their lack of common monocyte-associated markers as reviewed by Smith et al. (Smith et al., 2010)

Resident gut macrophages express an array of TLRs, generally at a higher level than circulating monocytes, which enable them to distinguish self from microbes (Smith et al., 2010). It has been shown that engagement of TLRs on resident macrophages may facilitate the chemotaxis to and phagocytosis of various bacteria (Smith et al., 2010). However, in contrast to most monocyte-derived leukocytes TLR engagement in gut resident macrophages does not result in release of pro-inflammatory cytokines (Smith et al., 2010). This dampening of the inflammatory response is in particular mediated by TGF-β, which is produced by epithelial cells, stromal cells, mast cells, and apoptotic T cells and is abundantly retained in the lamina propria extracellular matrix (Smith et al., 2010). Resident macrophages are crucial for clearing microbes and debris from the lamina propria without eliciting a full inflammatory response, which would cause unnecessary tissue damage. They are also important for production of extracellular matrix and for tissue repair (Smith et al., 2010; Mosser and Edwards, 2008).

In mucosal inflammation such as IBD, new monocytes are recruited to the intestinal lamina propria by chemotaxis. Chemokines produced by leukocytes, stromal cells and endothelial cells at the inflammatory site mediate recruitment of inflammatory macrophages, which do not undergo suppressive differentiation like the resident macrophages but differentiate like classically activated macrophages (Smith et al., 2005; Mosser and Edwards, 2008). Hence, they generate a typical pro-inflammatory response with cytokines like TNF-α and IFN-γ. TLR engagement on these activated macrophages further increases the inflammatory response (Mosser and Edwards, 2008). Immunologically they express activation

molecules like CD40 and CD80/86 and Fc receptors for IgA and IgG (see (Smith et al., 2010) for full list), which are all absent on resident macrophages in a homeostatic situation.

### 2.3.5 Regulatory T cells in the intestinal mucosa

Together with gut resident macrophages and IgA-producing plasma cells, $CD4^+$ T cells constitute the majority of immune cells in the homeostatic gut mucosa. $CD4^+$ T cells are commonly divided into the functionally distinct subsets Th1, Th2, and Th17, which all are regarded as pro-inflammatory, and Tregs that have immunosuppressive functions.

Tregs originate from the thymus or from naïve $CD4^+$ T cells in the periphery. Tregs of thymic origin, so-called natural (n)Tregs, require high affinity interaction between TCR and MHC II on thymic stromal cells and co-stimulatory signals from CD40, CD28, IL-2 and IL-7 which together induce low levels of the Foxp3 that controls the suppressive transcriptional program of Tregs (Sakaguchi et al., 2010). nTregs are found circulating in peripheral blood and in tissues. The TCR repertoire of nTregs is thought to have specificity to self as they are considered crucial for protection against autoimmunity (Curotto de Lafaille and Lafaille, 2009; Sakaguchi et al., 2010).

In the gut lamina propria and lymphoid tissue, naïve $Foxp3^-CD4^+$ T cells may differentiate into $Foxp3^+$ induced (i)Tregs. This induction requires TCR-MHC II interactions between naïve $CD4^+$ T cells and conditioned DCs and a cytokine environment with sufficient IL-2, TGF-β, RA and low levels of IL-6 (Curotto de Lafaille and Lafaille, 2009) (Figure 6). Engagement of the co-stimulatory molecule cytotoxic T lymphocyte antigen 4 (CTLA-4) on the T cell is essential for iTreg formation (Curotto de Lafaille and Lafaille, 2009). As previously discussed (see section 2.3.1) and sketched in figure 6, the DC subset and conditioning is important for the T cell differentiation. Pro-inflammatory DC conditioning and cytokine environment would differentiate the naïve T cells into pro-inflammatory Th17 or Th1 while other DC subsets and conditioning induce iTreg differentiation (Figure 6). Recent data indicate that $Foxp3^+$ Tregs may lapse and become pro-inflammatory (Zhou et al., 2009; Murai et al., 2010). Gut iTregs have TCR specificities towards commensal microbes and dietary antigens (Curotto de Lafaille and Lafaille, 2009). In human gut mucosa nTregs and iTregs are indistinguishable and for the rest of this thesis they are collectively termed Tregs. In addition to $Foxp3^+$ Tregs naïve $CD4^+$ T cells may differentiate into $Foxp3^-$ T cells that have immunosuppressive capacities (i.e. Tr1, Th3) (Akbar et al., 2007).

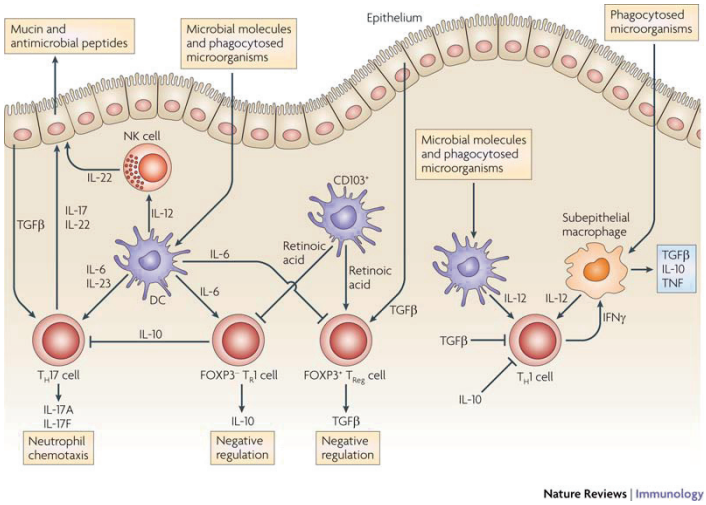



**Figure 6. Regulatory network for intestinal CD4$^+$ T cells**. T helper 17 ($T_H17$) cells are induced by transforming growth factor-β (TGFβ) and interleukin-6 (IL-6) and matured by IL-23 following the activation of intestinal dendritic cells (DCs) by phagocytosed microorganisms or stimulatory microbial molecules that have crossed the surface epithelial cell barrier and/or activated epithelial cells. The signature cytokines of $T_H17$ cells, IL-17A and IL-17F, have pro-inflammatory effects and mediate neutrophil chemotaxis. $T_H17$ cells also express IL-22, which contributes to epithelial homeostasis and stimulates the secretion of antimicrobial molecules. Acute inflammation is normally avoided through the induction of two classes of CD4$^+$ regulatory T ($T_{Reg}$) cells that can be differentiated on the basis of their expression of the transcription factor forkhead box P3 (Foxp3). Foxp3$^+$ $T_{Reg}$ cells are induced by retinoic acid produced by CD103$^+$ DCs in the presence of TGFβ. Conversely Foxp3$^-$ $T_R1$ cells are induced by IL-6 but inhibited by retinoic acid. $T_{Reg}$ cells secrete IL-10 and/or TGFβ, which have negative regulatory effects on effector T cells. Commensal bacteria and their associated molecules also stimulate DCs to secrete IL-12, which activates interferon-γ (IFNγ) secretion by $T_H1$ cells, which in turn activates phagocytic activity of subepithelial macrophages. NK, natural killer; TNF, tumour necrosis factor.



*In vivo* experiments in mice have demonstrated that specific bacteria (Gaboriau-Routhiau et al., 2009; Atarashi et al., 2011) or microbial products (Mazmanian et al., 2008; Donaldson et al., 2011) may induce Tregs. The TCR repertoire of Tregs in MLNs is distinct from that in peripheral lymph nodes indicating that the intestinal antigens shape the intestinal Treg pool (Barnes and Powrie, 2009). How PRR engagement affects the induction of Tregs is not univocal. Mice that lack TLR2 seem to have reduced Treg numbers while TLR9-deficient mice demonstrate an alteration of the Treg/ Th17 balance in favor of an increase of Tregs

(Curotto de Lafaille and Lafaille, 2009). It is also demonstrated that key bacterial species may alter the Treg/Th17 balance. Mice colonized with the *ex vivo* uncultivable anaerobes Segmented Filamentous Bacteria (SFB) have a reduced Treg/Th17 fraction in the small intestines (Ivanov et al., 2008; Gaboriau-Routhiau et al., 2009).

The identification of Tregs in humans has been heavily debated as no single cell marker exists to specifically detect all Tregs. In humans, the constitutive expression of CD25 (the high-affinity IL-2 receptor α chain) on $CD4^+$ cells with concomitant expression of Foxp3 is most often compatible with Tregs (Fontenot et al., 2003; Hori et al., 2003). However, both CD25 and Foxp3 may be transiently expressed on activated T cells *in vitro* (Buckner and Ziegler, 2008), and CD25 may even be expressed by activated macrophages (Smith et al., 2005). As mentioned above, $Foxp3^-CD4^+$ T cells with suppressive capacities may be present. Foxp3 is nevertheless considered the most relevant phenotypic marker for human Tregs. The crucial importance of this transcription factor in human gut homeostasis is demonstrated clinically when the *FOXP3* gene is mutated. The ensuing syndrome of *I*mmune dysregulation, *P*olyendocrinopathy, *E*nteropathy, *X*-linked (IPEX) displays an IBD-like enteropathy at infancy (Wildin et al., 2002). In addition to Foxp3 and CD25, other markers such as high expression of CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), combined with low expression of CD45RB and CD127, are all associated with $CD4^+$ T cells exhibiting suppressive capacity *in vitro*. However, none of these markers alone shows better selectivity for Tregs than Foxp3 (Akbar et al., 2007; Josefowicz and Rudensky, 2009).

The mechanisms for Treg-mediated suppression *in vivo* have been poorly assessed, but *in vitro* experiments have provided some clues (Shevach, 2009). Tregs require specific stimulation of their TCRs and co-stimulation by IL-2, but once they are activated suppression is not restricted to MHC II engagement. Tregs mediate bystander suppression to other immune cells and suppress effector T cells by releasing immunosuppressive cytokines (e.g. IL-10, TGF-β, IL-35), by consuming IL-2 (through CD25), and by cell-to-cell contact mechanisms. They also inhibit immune activation by suppressing DC function through CTLA-4-CD80/86 and LAG-3-MHC II interactions. Treg-mediated suppression may take place both in the mucosa and in GALT and MLNs. Experimental colitis studies indicate that Treg suppression in lymphoid tissue is more important than suppression in the mucosa (Barnes and Powrie, 2009).

### 2.3.6 Host influence on gut microbes

The previous sections have provided examples from recent years' intense research on how the commensal microbiota influences central elements of the host's intestinal mucosal immune system such as epithelial cells, SIgA, macrophages, and regulatory T cells. There is now increasing interest in how elements of the host immune systems reciprocally shape the intestinal microbiota.

It has been shown that monoassociating germ free mice with one microbial species elicits a specific IgA response against this microbe (Talham et al., 1999; Hapfelmeier et al., 2010) In a gnotobiotic model, Peterson et al. demonstrated that specific IgA inhibited the proliferation and fitness of a mutant *Bacteroides thetaiotaomicron* strain by modulating bacterial gene expression (Peterson et al., 2007). SFB induces a strong specific IgA respons (Talham et al., 1999). Mice deficient in AID, and consequently lacking IgA, have an increased load of SFB, which can be reversed by reconstitution of IgA (Suzuki et al., 2004). AID-deficient mice also have a general overgrowth of cultivable anaerobes (Fagarasan et al., 2002). Peterson et al. have proposed a model where one of the main tasks of IgA and adaptive mucosal immunity is to recognize new epitopes expressed in the intestinal microbial community and thereby enforce a selective pressure on emerging strains and ensure diversity of the intestinal microbiota (Peterson et al., 2008). Thus, in addition to immune exclusion and other functions of SIgA presented in section 2.3.3.3, SIgA shapes the composition of the intestinal microbiota and prevents dysbiosis.

Reports have also pointed out that elements of the innate immune system affect the composition of the intestinal microbiota. Lack of the PRR NOD1 or the AMP α-defensin in mice alters the composition of the microbiota without affecting the total number of gut bacteria (Bouskra et al., 2008; Salzman et al., 2010). Interestingly, in a mouse model it was reported that Gram negative bacteria could induce AMP that acted selectively on Gram positive bacteria. This finding suggests that intestinal microbes may employ innate immune elements in their internal colonization competition (Brandl et al., 2008). In another model, an innate immune-deficient T-bet$^{-/-}$Rag2$^{-/-}$ double knock out mouse developed spontaneous colitis which was transmittable to immune-competent mice (Garrett et al., 2007). It was later showed that the model favored colonization of the opportunistic pathogens *K. pneumonia* and *P. mirabilis,* which again were responsible for driving an UC-like disease (Garrett et al., 2010).

## 2.4 IBD etiology and pathogenesis

For long IBD has been considered a multifactorial disease occurring in genetically predisposed individual as a response to one or more environmental factors (Xavier and Podolsky, 2007; Baumgart and Carding, 2007). Although the full etiology of IBD has not been unveiled there has been a tremendous improvement in the understanding of etiologic and pathogenic mechanisms over the last 15 years.

Among patients with CD and UC up to 22% and 15% of cases, respectively, have affected first-degree relatives (Baumgart and Carding, 2007). The concordance in monozygotic twins is 37% in CD and 10 % in UC which indicates that the genetic component is greater in CD than in UC (Baumgart and Carding, 2007). Almost 80% of affected families are concordant for one disease type (CD vs. UC) while the remaining 20% of families have a mixed disease phenotype (CD and UC). This indicates that there should be genes that predispose to only one of the disease types while other predisposing genes should be shared (Cho, 2008). Genome-wide association studies have confirmed these predictions. Currently 71 gene loci are associated with CD and about half of these are shared with UC (Franke et al., 2010; McGovern et al., 2010).

The genes associated with the highest odds ratios (OR) for developing IBD are the *NOD2* gene and a number of genes associated with autophagy and with the IL-23/Th17 axis. Mutations in *NOD2*, whose association is restricted to CD, confer loss of function in response to its ligand, muramyl dipeptide, which is present in peptidoglycan of both Gram positive and Gram negative bacteria (Cho, 2008). The close association between *NOD2* mutations and CD strongly suggest disturbed host microbe interactions as an important cause of CD. The effect comes about through altered tolerance to chronic bacterial stimulation, impaired clearance of pathogens, or altered microbial colonization of mucosal surfaces (Cho, 2008). *NOD2* mutations are also associated with reduced expression of α-defensins in affected ileum of CD patients (Wehkamp et al., 2008). After *NOD2* the innate immunity related genes with highest OR for CD are the two genes *ATG16L1* and *IRGM* that both are important for autophagy. Autophagy is an essential cellular process in which the cell degrades its own components and foreign bodies (e.g. microbes) and has recently been linked to NOD2 function (Travassos et al., 2010). Impaired autopahgy in CD patients further emphasizes the importance of the host's proper handling of microbes at the mucosal surface.

The overall closest association (OR 2.7 (Franke et al., 2010)) between gene mutations and IBD is the IL-23 receptor (R) gene which is associated with both CD and UC and implies a role for adaptive immunity in IBD pathogenesis. There are multiple susceptibility alleles within the *IL23R*, of which some also confer protection from IBD (Cho, 2008). Furthermore, several genes encoding signaling molecules downstream of IL23R are also associated with IBD (Cho, 2008). IL-23 is produced by activated DCs and is important for stabilization of Th17 cells (Bettelli et al., 2008) (Figure 6). Experimental mouse models have demonstrated IL-23's importance in driving colitis (Sarra et al., 2010; Maloy and Kullberg, 2008). Th17 cells are increased in IBD lesions and considered to drive the mucosal inflammation (Sarra et al., 2010; Maloy and Kullberg, 2008).

Experimental animal models for IBD are generally dependent on the presence of intestinal microbiota (Uhlig and Powrie, 2009) and there is overwhelming clinical evidence that the intestinal microbiota is important for IBD pathogenesis (reviewed in (Sartor, 2008)). *E. coli* strains are more adherent and invasive in human IBD lesions. Antibiotics cure disease flares and probiotics may ameliorate the disease. IBD patients demonstrate serologic and cellular immune responses against gut microbes. New 16S rRNA phylogenetic microarrays and bacterial sequencing technology have also demonstrated a reduced diversity and a shift in the composition of the intestinal microbiota where Proteobacteria (e.g. Enterobacteriaceae) are increased at the expense of the phyla Firmicutes and Bacteriodetes (Frank et al., 2007; Qin et al., 2010). However, whether this shift is a cause or a consequence of the intestinal inflammation is still unknown. One recent report identified *Faecalibacterium prasnitzii* as a commensal that protect against CD (Sokol et al., 2008), but the full picture of key species that protect from or precipitate IBD remains to be determined.

IBD has several mucosal immunopathological features (reviewed in (Baumgart and Carding, 2007; Xavier and Podolsky, 2007). IBD patients have reduced epithelial barrier function and downregulated tight junctions proteins, the mechanism (s) being unknown. Impaired mucosal barrier function precedes the clinical symptoms and has been demonstrated also in non-inflamed areas of the gut and in unaffected first-degree relatives of IBD patients. Reduced AMP production by Paneth cells in the small intestines of CD patients have made some researchers claim that CD is primarily due to reduced microbial killing by AMPs (Wehkamp et al., 2008). Also, epithelial cells have altered expression of PRRs in IBD patients. These observations all point to the importance of intestinal epithelial cell functions in the development of IBD.

As mentioned above, the gut mucosa of IBD patients has an increase of Th17 cells and their signature cytokine IL-17 (Sarra et al., 2010). Differentiation of Th17 cells is delicately balanced with peripheral induction of Tregs (see section 2.3.5) and dependent on the cytokines TGF-β and IL-6. The close relationship with Th17 cells have suggested that Tregs may play an important role in IBD immune pathology (Xavier and Podolsky, 2007). Tregs are also increased in IBD lesions (Kelsen et al., 2005; Makita et al., 2004; Holmen et al., 2006; Maul et al., 2005; Saruta et al., 2007; Yu et al., 2007). Mice deficient in the immunosuppressive cytokine IL-10 develop colitis in the presence of commensal microbes. Recently it was also reported that a cohort of children with IBD had increased frequency of mutations in the *IL-10R* gene, which underscore the significance of endogenous immune suppression for the prevention of IBD (Glocker et al., 2009).

As described in section 2.1.1 pediatric onset IBD differs clinically from adult onset IBD. For this reason it has been hypothesized that the etiology and pathogenesis of this entity differ from adult onset disease (Kugathasan and Cohen, 2008; Kugathasan and Fiocchi, 2007). So far studies on pediatric onset IBD have only recapitulated etiology and pathogenesis already demonstrated in adults (Kugathasan and Fiocchi, 2007; Henderson et al., 2011). However, there is a lack of studies that have directly compared the immunopathology of pediatric and adult IBD patients, and such studies have been requested (Kugathasan and Cohen, 2008).

# 3 AIMS OF STUDY

**Main aim:**

To explore mucosal immunopathogenic mechanisms in inflammatory bowel disease.

**Aims :**

1. To identify putative disparities between human pediatric onset and adult onset disease, focusing on distribution of macrophages and regulatory T cells.

2. To elucidate interactions between secretory immunoglobulins and intestinal epithelial cell function and the intestinal microbiota in experimental animal models.

# 4 SUMMARY OF RESULTS

## 4.1 Paper I

**Increased number and activation of colonic macrophages in pediatric patients with untreated Crohn's disease.**

G. Perminow, D. H. Reikvam, L.G. Lyckander, P. Brandtzaeg, M. Vatn, and H. S. Carlsen.

Colonic and ileal biopsy specimens from untreated pediatric-onset IBD patients and pediatric non-IBD controls and colonic biopsies from untreated adult-onset IBD patients and their non-IBD controls were examined by immunohistochemistry for the density of macrophages *in toto* ($CD68^+$) and for the density of activated macrophages ($CD40^+$). Non-IBD adults had significantly higher density of colonic $CD68^+$ macrophages than non-IBD children. Pediatric CD patients had significantly higher density of $CD68^+$ macrophages in colon than pediatric UC patients and non-IBD controls. The density of activated ($CD40^+$) macrophages was significantly elevated in both the colon and the ileum of CD children compared with non-IBD controls.

Conclusion: Histologically normal colonic mucosa has a higher density of macrophages in children than in adults. Activated mucosal macrophages are increased in untreated pediatric IBD regardless of inflammatory grade. This may reflect innate mucosal immune activation differences that contribute to different disease phenotypes in children and adults.

## 4.2 Paper II

**Increase of Regulatory T Cells in Ileal Mucosa of Untreated Pediatric Crohn's Disease Patients**

D. H. Reikvam, G. Perminow, L. G. Lyckander, J. M. Gran, P. Brandtzaeg, M. Vatn, and H. S. Carlsen.

Biopsy specimens obtained from colon and ileum of untreated pediatric-onset and adult-onset CD patients and from non-IBD controls were examined by immunohistochemistry with regard to markers associated with Tregs. The density of mucosal T cells displayed only small variations, while those of markers associated with Treg, $Foxp3^+$ cells and $CD25^+$ cells, were increased in CD patients. Multicolor immunofluorescence showed that most $CD25^+$cells were macrophages. In the ileum of pediatric CD patients the density of $Foxp3^+$ cells was significantly higher than in adult CD patients. Co-expression of Foxp3 and CD25, as well as Foxp3 and CTLA-4, indicated that the $Foxp3^+$ cells were Tregs. .

Conclusion: Densities of mucosal $Foxp3^+$ Tregs and $CD25^+$ cells, the latter representing mostly macrophages, are elevated in both pediatric and adult ileal CD. The greater increase of ileal $Foxp3^+$ Tregs in pediatric than in adult CD might contribute to the relatively less frequent phenotype of isolated ileal enteritis in CD children.

## 4.3 Paper III

**Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression**

D. H. Reikvam*, A. Erofeev*, A. Sandvik, V. Grcic, F. L. Jahnsen, P. Gaustad, K. McCoy, A. Macpherson, L. A. Meza-Zepeda, and F-E. Johansen.

* These authors contributed equally to this paper.

Germ-free animals represent a powerful approach to study bacterial-host interaction but are not readily accessible to the wide scientific community. This study aimed at refining a protocol that would deplete the cultivable intestinal microbiota of conventionally raised mice as previously published protocols which administered broad-spectrum antibiotics in drinking water were difficult to reproduce. We show that twice daily delivery of antibiotics by gavage depleted mice of their cultivable fecal microbiota and reduced the fecal bacterial DNA load by 400 fold while ensuring the animals' health and producing a macroscopic immune phenotyping resembling germ-free mice. The antibiotic treatment significantly reduced the

expression of antimicrobial factors to a level similar to that of germ-free mice and altered the expression of 517 genes in total in the colonic epithelium

Conclusion: We presented a robust protocol for depleting conventionally raised mice of their cultivatable intestinal microbiota with antibiotics by gavage and showed that the biological effect of this depletion phenocopies physiological characteristics of germ-free mice.

## 4.4 Paper IV

**Altered mucosal homeostasis in mice lacking secretory antibodies**

D. H. Reikvam, A. Erofeev, R. Islam, M. Derrien, V. Grcic, A. Sandvik, P. Gaustad, L. A. Meza-Zepeda, F.L. Jahnsen, H. Smidt, and F-E. Johansen**.**

PIgR KO mice, which lack SIgs as a consequence of deleted pIgR gene and consequently abrogated epithelial transport of Igs to mucosal lumens, have no overt pathology but are thought to have an impaired epithelial barrier function. This genotype was used to study the significance of SIgs for colonic epithelial cell (EC) gene expression profile, intestinal microbial composition, and for induced experimental colitis. Microarray analyses of isolated colonic ECs revealed that 208 genes were >2-fold differentially expressed between pIgR KO and wt mice, and this number was reduced to 27 when the intestinal microbiota of both genotypes were suppressed by long term treatment with oral antibiotics. Phylogenetic microarray analyses of fecal samples and cecal biopsies showed that pIgR KO had a significantly altered composition of the intestinal microbiota compared with wt. PIgR KO had increased susceptibility to DSS-induced colitis compared with wt, but the morbidity of both genotypes were alleviated when the mice were depleted of their cultivable intestinal microbiota by antibiotic treatment, thus demonstrating that colitis was dependent on the presence of the microbiota.

Conclusion: PIgR and/or SIgs are crucial to maintain mucosal homeostasis and the microbial composition in the gut. In case of an epithelial injury, the absence of pIgR/SIgs compromises the host's ability to control inflammation and recover from colitis.

## 4.5 Paper V

**Absence of the polymeric immunoglobulin receptor protects B cell deficient mice from colitis.**

D. H. Reikvam, A. Erofeev, A. Sandvik, C. Bang, E. Bækkevold, F. L. Jahnsen, and F-E. Johansen

The physiological significance of unbound pIgR and free SC has not been determined. To assess pIgR's role for colonic mucosal homeostasis independent of its SIg transportation properties pIgR KO mice were crossed with B cell-deficient ($J_H$ KO) mice to obtain a novel pIgR; $J_H$ double knock out (DKO) mouse. DKO mice proved viable. After seven days of DSS induction $J_H$ KO mice showed aggravated morbidity compared with both the pIgR KO and DKO strains. Untreated $J_H$ KO mice developed subtle spontaneous colitis with significant influx of $Ly6G^+$ neutrophils at week 12 while this was not observed in pIgR KO or DKO mice and could be reversed by concurrent oral treatment with broad spectrum antibiotics. In untreated mice, 8 out of 9 $J_H$ KO mice were euthanized due to weight loss by 22 weeks of age while the other strains remained healthy.

Conclusion: Absence of pIgR protects B cell-deficient mice from colitis and death. Studies to explore the mechanisms behind this observation are warranted.

# 5 METHODOLOGICAL CONSIDERATIONS

This chapter discusses the main methods that have been used in the experimental work that is the basis for the submitted thesis. For a descriptive presentation of the applied materials and methods I refer to the methods' sections of the individual manuscripts.

## 5.1 Human material

### 5.1.1 Patients

**Papers I** and **II** are studies based on patients included in the study Inflammatory Bowel Disease South-East Norway II (IBSEN II) (Perminow, 2010).

Patients in the pediatric arm of the IBSEN II study were all recruited at Ullevål University Hospital or Akershus University Hospital. The pediatric clinics of these two hospitals have well-defined catchment areas, consisting of the city of Oslo and the northern and eastern part of the county of Akershus, respectively. This implies that all patients from these defined geographical areas referred to a pediatric hospital for assessment of IBD were evaluated at these two hospitals only. As all the pediatric patients consecutively included in the study between May 2005 and December 2006 were included in **Papers I** and **II** sampling bias should not be an issue. Moreover, all but three patients included were clinically and endoscopically evaluated by one physician (Dr. Gøri Perminow), and accordingly inter-individual variation of patient and tissue specimen handling were minimized.

The adult patients, from whom tissue specimens for **Paper I** and **II** were obtained, were randomly selected from the study database of patients included within the same time period as the pediatric patients. The numbers of patients specimens selected was based on a rational estimation of the numbers of patient necessary to demonstrate possible difference between the pediatric and adult patient groups in the immunohistochemistry (IHC) investigations. Sampling bias in the adult patients groups cannot be ruled out and type II errors due to inadequate sample sizes are possible.

The symptomatic non-IBD control patients in the IBSEN II study have been subjected to scrutiny. They were all initially referred to a gastroenterological specialist evaluation due to suspected IBD and consequently were not healthy at the time of examination. This fact

provided both strengths and weaknesses to our investigations. As the control patients had IBD-like symptoms at the time of diagnosis they could potentially help reveal disease-specific IBD characteristics as opposed to pathogenic characteristics associated with diarrhea, abdominal pain, and other IBD-like symptoms. On the other hand, the symptomatic control group is a possibly heterogeneous group with concurrent gastrointestinal pathology that might have caused increased random bias. For adults one might have recruited healthy volunteers as controls. However, as pediatric patients have to be endoscoped under general anesthesia, a sound clinical indication for the procedure had to be presented in order for the procedure to be ethically justified. Symptomatic patients in whom IBD was later excluded were therefore the only pediatric control group accessible for mucosal biopsy specimens.

The symptomatic non-IBD controls were diagnosed as irritable bowel syndrome or recurrent abdominal pain. Could these diagnoses have been preliminary, and could some of the control patients eventually have developed IBD at later time point? Excluding the possibility that study patients have moved out of the initial catchment area, none of the pediatric control patients were diagnosed with IBD as of March 2010. (Personal communication, Dr. Gøri Perminow.)

### 5.1.2 Tissue specimen collection

Mucosal tissue specimens were collected with a forceps at the diagnostic endoscopy of each included patient. For diagnostic purposes multiple biopsies were collected according to a predefined protocol. Tissue specimens specifically intended for the immunohistochemical investigations (**Paper I** and **II)** were collected and preserved additionally along with the diagnostic biopsies. The endoscopic appearance (inflamed vs. non-inflamed) and the gut location of each biopsy site was recorded by the examiner. When the immunohistochemistry studies started we decided not take this information into consideration for two reasons. First, the number of patients and tissue specimens were considered not large enough to justify comparisons between different regions of the colon, and therefore the only topical distinction made was terminal ileum versus colon. Second, as the endoscopic appearance of the mucosa does not fully correlate with the histological degree of inflammation (Hommes and van Deventer, 2004), we decided to disregard the endoscopic inflammatory evaluation of each biopsy and only take into consideration the histological inflammation assessment.

## 5.2 Animal models

**Paper III, IV** and **V** of this thesis are based on laboratory animal studies. Animal models are of great value to explore the biology of an integrated organism and the pathogenesis of a disease as well as to test new treatments for human disorders. Although *in vitro* studies are valuable for research on biologic mechanisms, they cannot recapitulate the full complexity of an integrated organism.

Scientists are allowed to carefully inflict pathology on to animals and manipulate animals in ways that are considered unethical in humans. However, the ethics of laboratory animal research have received increasing attention over the last 50 years and can be summed up by the three R's (Flecknell, 2002). *R*eplacement: always consider other methodological approaches than animal studies to assess a scientific problem. *R*eduction: reduce the number of animals to the minimum. At the same time, scale the animal experiments big enough to allow robust statistical conclusions to be drawn; if not, the experiment and the animals used are wasted. *R*efinement: always make an effort to improve the conductance of the animal experiments in order to minimize the discomfort of the animals. It is recognized that adoption of the three R's can improve the quality of the science (Flecknell, 2002). We strived to adhere to the three R's in all animal studies performed for this thesis. All animal experiments were approved by the National Animal Research Authority (Forsøksdyrutvalget) and conducted in accordance with the Norwegian Animal Protection Act of 1974 (repealed by the Animal Welfare Act Jan 1$^{st}$ 2010) and the Norwegian Regulation on Animal Experimentation. (2010)

Rodents in general, and mice in particular, are the most widely used laboratory animals as they are small, which make them easy to handle and transport and which minimize space requirements. Furthermore, they reproduce at high rate and are accessible for genetic modifications. Mice are clearly valuable as model organisms for human biology and pathology. However, all scientists should keep in mind that mice are not men and numerous biological differences between the two species have been demonstrated (Mestas and Hughes, 2004). Research results obtained in mice may not apply to man in general and must be validated in humans. Laboratory animal disease models generally do not recapitulate the full complexity of human disease.

### 5.2.1 pIgR KO

To explore the role of SIgs in host-microbial cross talk and in experimental colitis (**Paper IV** and **V**) we based our studies on the pIgR KO mouse (Johansen et al., 1999). This genetically modified mouse was generated by Dr. Finn-Eirik Johansen and colleagues by disrupting exon 3 of the *pIgR* gene, which encodes for the Ig binding site in D1. Analysis of mRNA from *pIgR*$^{-/-}$ mice indicated that a truncated receptor lacking D1 may be produced, but this does not possess any Ig-transporting properties (Johansen et al., 1999). Validation of the pIgR KO demonstrated that all epithelial transport of polymeric IgA and IgM was abrogated and that the pIgR KO had elevated serum IgA (mostly polymeric) and IgG (Johansen et al., 1999). Further studies have shown that the pIgR KO have increased numbers of IgA secreting cells in lamina propria (Uren et al., 2003) and in the MLNs, PPs, and spleens (Sait et al., 2007). Oral tolerance could be induced in the pIgR KO to the same degree as WT controls and initial tests of inducible immune functions was unaltered (Uren et al., 2003; Karlsson et al., 2010). Several studies have later demonstrated that the pIgR KO elicits strong serum responses to various microbial antigens (Wijburg et al., 2006; Uren et al., 2005; Cunningham et al., 2008; Maaser et al., 2004; Sun et al., 2004; Davids et al., 2006; Sait et al., 2007).

Bacterial cultivation (Sait et al., 2007) and denaturing gradient gel electrophoresis (Sait et al., 2003) of ileal biopsies from co-housed pIgR KO and C57BL/6 controls did not reveal any differences in microbiota composition. However, use of newer gene sequencing and microarray-based techniques would have provided superior resolution to these assessments.

The initial characterization of the pIgR KO revealed that it had no overt pathology and was of normal size and fertility. Histopathologic evaluation of the intestines showed no pathology. However, it had increased IgG and albumin in mucosal secretion compared with wild type (WT) controls, which is interpreted as increased epithelial protein leakage (Johansen et al., 1999). Furthermore, the pIgR KO had increased titers of *E. coli*-specific IgG indicating systemic immune stimulation in the abscense of SIgs (Johansen et al., 1999). This finding was later reproduced and the notion was reinforced with the observation that MLN of pIgR KO harbor more viable bacteria than WT controls (Sait et al., 2007). In summary, it is believed that the pIgR KO has an epithelial membrane barrier defect due to its lack of SIg that renders it vulnerable to microbial or toxic stress and more susceptible to develop colitis.

As experiments reported in **Paper IV** were launched, a paper comparing conventionally raised pIgR KO with IgA KO and WT C57BL/6 in the DSS colitis model was published (Murthy et al., 2006). This article showed that the pIgR KO was more susceptible to DSS than both IgA KO and WT controls.

The pIgR KO has been employed in various infection models. It has been shown to be more susceptible to *Vibrio cholera* toxin challenge than C57BL/6 controls, even after vaccination, while vaccination against *Salmonella typhimurium* offered protection against recurrent infection equally well in both the pIgR KO and the C57BL/6 (Uren et al., 2005). Unvaccinated pIgR KO had a lower threshold dose for surviving infection with *Salmonella typhimurium* and shed more bacteria than C57BL/6 pointing at a putative role for SIg in herd immunity (Wijburg et al., 2006). In contrast, two reports found that pIgR KO cleared *Citrobacter rodentium* and an attenuated *Salmonella* sp., respectively, at the same rate as WT controls (Maaser, 2004; Endt et al., 2010). The inconsistency of these results may reflect differences in the size of the enterobacterial inoculums. The pIgR KO had also increased susceptibility to *Giardia muris*, but not *Giardia lamblia* (Davids et al., 2006). Outside the gut the pIgR KO has been shown to be more susceptible to chlamydial prostatitis (Cunningham et al., 2008) and to nasal colonization by *Streptococcus pneumonia* (Sun et al., 2004).

In parallel to and independent of Dr. Johansens development of the pIgR KO, a Japanese group made another pIgR KO by deleting exon 2 of the pIgR gene (Shimada et al., 1999). This Japanese pIgR KO confirmed the findings in Dr. Johansens pIgR KO with abrogated transcellular transport of dIgA with subsequent increased levels of serum dIgA. In the Japanese pIgR KO some of the results obtained in Dr. Johansen's pIgR KO have also been recapitulated such as increased susceptibility to cholera toxin challenge (Tokuhara et al., 2010), reduced cross-protection between a selection of influenza viruses (Asahi et al., 2002; sahi-Ozaki et al., 2004), and increased susceptibility to *Mycobacterium tuberculosis* (Tjarnlund et al., 2006) All of these features occurred despite robust responses to vaccination in terms of antigen specific serum IgA and IgG levels.

In conclusion, with its assumed leaky epithelial barrier and its reduced ability to cope with microbial pathogens in spite of good inductive immune responses, we believed that the pIgR KO would be of interest to study in a colitis model, and, furthermore, we considered this approach to be valid for investigating SIgs and pIgR/SCs role in protection against colitis.

### 5.2.2 Genetic background

The pIgR KO was originally created by injection of embryonic stem cells from the inbred 129 strain into C57BL/6 and then backcrossed to C57BL/6. It was later backcrossed over 10 generations to the inbred albino strain BALB/c by Denis Meztger (pers.comm. F-E. Johansen) and transferred to Taconic Farm Inc's emerging models program from where it is now commercially available. Several of the pilot studies for **Papers III** and **IV** were performed on C57BL/6. However, we eventually decided to convert the main experiments to BALB/c for three reasons. First, the BALB/c background was chosen for other projects that required a Th2 skewing, which the BALB/c strain provides. Applying the same strain for the colitis experiments would therefore reduce the breeding colonies. Second, as DSS experiments with the pIgR KO on C57BL/6 background had already been published (Murthy et al., 2006), conversion to BALB/c background would provide novelty to our colitis experiments. Third, the $J_H$ KO (see section 5.2.3) that we intended to cross the pIgR KO onto was readily available on BALB/c background.

The albino trait is caused by spontaneous mutation affecting tyrosin kinase, an enzyme required for melanin production. The mutated enzyme may also be involved in numerous other physiological functions (Hem A et al., 2004). Of note for immunologists, BALB/c mice are well known for their strong development of Th 2 type responses (Chen et al., 2005). Important to our colitis experiments, BALB/c have been shown to be susceptible to DSS (Melgar et al., 2005) and consequently provided a suitable genetic background for studying the pIgR KO in the DSS colitis model.

BALB/c mice are commercially available in several substrains. All our BALB/c were provided by Taconic Farms' plants in New York and in Tornbjerg, DK. For our studies any further substrain specifications were disregarded.

### 5.2.3 $J_H$ KO and DKO

In **Paper V** we wanted to assess the innate significance of pIgR uncoupled from its Ig transporting properties and the role of SC without luminal Igs. $J_H$ KO was purchased from Taconic Farms and crossed with the pIgR KO to produce a pIgR/$J_H$ double KO (DKO). To our knowledge this crossing has previously not been described. The $J_H$ KO was developed by deleting the four J segments of the Ig heavy chain locus (Chen et al., 1993). This causes

aborted V (D)J recombination of the Ig variable region, and consequently no functional B cell receptors or Igs are produced.

The $J_H$ KO has been employed in numerous studies on B cell dynamics but very few studies on B cell deficiency and colitis have been reported. One study investigated the colon of $J_H$ KO up to 40 weeks of age and found macroscopically and histologically normal colons and recorded no weight loss, diarrhea or premature death (Ma et al., 1995). This same study crossed the $J_H$ KO onto IL-2 KO, which aquire spontaneous colitis, and showed that the $J_H$/IL-2 double KO got colitis to the same degree as the IL-2 single KO. Another paper reported normal litter sizes, body weight, and no protein-losing enteropathy in $J_H$ KO (Slack et al., 2009). By employing another B cell-deficient mouse (*Igμ*$^{-/-}$) in the spontaneous *TCRα*$^{-/-}$ colitis model it was shown that B cells had a protective role (Mizoguchi et al., 1997; Mizoguchi et al., 2000). In summary, experimental colitis studies have shown that the role of B cells, if any, is a beneficial one.

The target genetic model that we looked for when we acquired the $J_H$ KO was one that did not produce any Igs, but elsewise was as immunologically normal as possible. B-cell development is disrupted at an early stage in the $J_H$ KO, which may result in abrogation of other functions of B cells than differentiation to plasma cells and Ig production (Lund and Randall, 2010). As alternatives to the $J_H$ KO we considered to acquire the *RAG2*$^{-/-}$ to cross breed with the pIgR KO. However, the *RAG2*$^{-/-}$ also have an absent T cell compartment, and we therefore considered it less suitable. We also discussed to cross breed the pIgR KO with the J chain KO. This model has mature B cells, plasma cells, and produces Igs of all classes but has impaired ability to polymerize IgA and IgM and therefore lacks the ligands for pIgR (Hendrickson et al., 1995). However, the J-chain KO model is shown to be “leaky” in the way that it has some IgA and IgM in feces and bile (Hendrickson et al., 1995), and it would not rule out the potential contribution of luminal IgG, either by passive diffusion or facilitated by the FcRn transporter (Baker et al., 2009). All together, we judged the $J_H$ KO as the most specific model of the available alternatives.

### 5.2.4 Breeding strategy

Breeding colonies for pIgR KO and BALB/c mice for the DSS experiments in **Paper IV** were kept in one SPF facility at Taconic Farms, Ejby, DK and adult age-matched male mice were shipped from those colonies. We emphasized that the pIgR KO and BALB/c controls should

be bred within the same barrier facility to avoid systemic bias in terms of different microbial environment.

All other animal experiments (**Papers III-V)** were performed on in-house bred mice. Twelve JH KO females from Taconic Farms, Germantown, USA and four pIgR KO males from Taconic Farms, Ejby, Denmark, both on BALB/c background, were purchased as founders and mated to produce F1 hybrids that were double heterozygotes with regard to *pIgR* and $J_H$. The F1 hybrids were then sibling mated and the four different double homozygote variants $pIgR^{-/-}$ $JH^{+/+}$ (termed pIgR KO), $pIgR^{+/+}JH^{-/-}$ (termed JH KO), $pIgR^{-/-}JH^{-/-}$ (termed DKO), and $pIgR^{+/+}JH^{+/+}$ (termed WT or BALB/c) were selected and continuously bred as homozygotes over ten generations. The breeding took place in filter top cages with enclosed ventilation placed in one rack in a minimal disease barrier facility, which was under strict health monitoring and free of FELASA-defined pathogens in sentinel animals. All animals employed in our experiments were within 10 generation of the founders. Outside a gnotobiotic facility we believed this was the best effort to conserve a uniform microbial environment for the breeding colony as a whole.

The breeding strategy for obtaining the theoretically optimal control animals, in terms of genetic and environmental uniformity to the study animal, would be to mate double heterozygotes and use offspring littermates in experiments. However, as only 1/16 offspring from a double heterozygote mating would be double homozygotes and the average litter size was approximately six, this breeding strategy was not feasible. As we wanted to use gender matched animals in experiments comparing pIgR KO and WT controls only, mating $pIgR^{+/-}$ for these experiments was also considered not feasible.

### 5.2.5 DSS colitis model

In **Paper IV** and **V** we applied the DSS colits model, an experimental animal model for ulcerative colitis. Several animal models of IBD have been published. These animal models fall into four categories: spontaneous development of intestinal inflammation, induction of colitis by genetic manipulation, induction of colitis by administration of exogenous chemical compounds, and finally transfer of defined cell population into an immunodeficient host (Blumberg et al., 1999). Of note, with a few exceptions, all experimental colitis models require the presence of an intestinal microbiota. Some models even have specific microbiological requirements for colitis to occur (Uhlig and Powrie, 2009). As the pIgR KO

does not develop spontaneous colitis, we had to find a model based on administration of a chemical compounds. Of the two most frequently applied models within this category, the DSS and the trinitrobenzene sulfonic acid (TNBS) models, we chose the DSS model due to its technical simplicity. DSS can be administered in drinking water as opposed to administration of TNBS which is dissolved in ethanol and applied through enema and consequently requires sedation of the mice. Moreover, the validity of the DSS model is better than the TNBS model as a study of the gene expression profile of the distal colon showed that 15 out of 32 genes that are known to be differentially expressed in IBD were also differentially expressed in the DSS colitis model while the same fraction for the TNBS model was 2 out of 32 (te Velde et al., 2007).

DSS is a sulfated synthetic polysaccharide and is produced in different dextran sizes. For all our experiments we used the 40 kD dextran purchased from the same vendor, which is the widely used product for the rodent colitis model, and which is shown to induce the most sever colitis (Kitajima, 2000). DSS ability to induce colitis may be related to the total sulphur contents of each dextran molecule (Kitajima, 2000). Our experiments were performed with DSS with a sulfur content of 17% according to the manufacturer. Larger dextran molecules do not produce colitis, presumably due to reduced ability for the larger dextran molecules to penetrate to the mucosal membrane (Kitajima, 2000).

The DSS colitis model was first described by Okayasu et al in 1990. It was considered a valid model for UC due to its clinical and pathological resemblance to the human disease. Mice subjected to the protocol suffer weight loss, diarrhea, and occult and gross blood in feces, which correlate with multiple mucosal erosions and inflammatory infiltrates, all confined to the colon and more prominent in the distal part (Cooper et al., 1993; Okayasu, I, 1990). The relevance of the model has also been demonstrated by the fact that drugs of all classes used in the treatment of human IBD also ameliorates DSS-induced colitis (Murthy et al., 1999; Axelsson et al., 1998; Bjorck et al., 1997; Melgar et al., 2008).

The molecular and cellular mechanisms for how DSS induces colitis have been explored, but are still not clear. It has been shown that DSS can induce colitis in the absence of adaptive immune cells (Dieleman et al., 1994; Axelsson et al., 1996), and it has further been reported that DSS affect the function of macrophages (Okayasu, I, 1990; Ohkusa et al., 1995) and dendritic cells (Berndt et al., 2007), pointing at a role for innate immunity. However, the mantra has been that DSS exerts its pathogenic effect by disrupting the

epithelial barrier. Early studies pointed to a direct cytotoxic effect of DSS on the epithelial cells and that DSS interfered with the normal interactions between IECs and intraepithelial lymphocytes (Ni et al., 1996). In line with this notion, it has been demonstrated that increased mucosal permeability can be observed prior to erosions and inflammatory changes (Kitajima, 1999) and this coincides with an alteration of tight junction proteins in the epithelium (Poritz et al., 2007). A recent report demonstrated that DSS after 12 hours' exposure compromised the inner mucus layer's ability to prevent luminal bacteria from reaching the epithelial cells, suggesting that the mucosal inflammation is driven by commensal intestinal bacteria rather than a direct toxic effect of DSS on the epithelium (Johansson et al., 2010).

The significance of the intestinal microbiota in the propagation of DSS colitis has been demonstrated at several levels. The original presentation of the DSS colitis model reported a shift in the composition of the gut microbiota following DSS colitis (Okayasu, I, 1990). Several reports have later demonstrated the protective effect of probiotics (Schultz, 2004; Fujiwara, 2003; Osman et al., 2004; Herias et al., 2005; Mennigen et al., 2009) or sonicates of intestinal microbiota (Verdu et al., 2000) on DSS colitis. Systemic administration of antibiotics is reported to attenuate DSS-induced colitis (Hans et al., 2000) while full depletion of the intestinal microbiota by orally administered antibiotics increased the mortality after DSS exposure (Rakoff-Nahoum et al., 2004; Hudcovic et al., 2001). Reports on DSS administration in germ-free animals are somewhat conflicting as some have reported decreased susceptibility (Hudcovic et al., 2001) while others have reported increased susceptibility (Bylund-Fellenius et al., 1994) to DSS. This may be due to differences in the concentration of the administered DSS. Also, the phenotype of the morbidity related to the increased mortality in germ-free mice is different from that in conventional mice (Kitajima et al., 2001). It is therefore proposed that DSS may induce two different types of intestinal pathology: i) colitis that is dependent on the presence of bacteria; and ii) a hemorrhagic-type mucosal destruction, in which the intestinal microbiota has a protective role.

Another intriguing feature of the DSS colitis model is the variation of DSS concentrations used by different researchers, ranging from 1.5% to more than 5%. To some extent this may be due to the fact that different experimental approaches demand different immediacy and severity of colitis. The concentration, but not the consumed dose, of the administered DSS correlated with the histological severity in one report (Egger et al., 2000). Animal strain, sex, and age may also affect the susceptibility to DSS. However, frequently conveyed by word between researchers but rarely reported in manuscripts is the variable

susceptibility to DSS also within research labs, both in space and time. We have experienced that performing identical DSS experiments in two different corridors within the same animal barrier facility gave different results, and that the response to DSS in the same animal room fluctuated over time. We therefore believe that all DSS experiments should be interpreted with a spatial and temporal specificity. All the DSS experiments included in this thesis were performed with the same DSS concentration in same animal barrier within one year of time. Though difficult to assess, most researchers believe the variable response to DSS is caused by variations in the intestinal microbiota.

Mice employed in the DSS experiments were intended to be stabled two and two in each cage due to animal welfare. As we used males only it restricted the possibility to regroup mice from different litters, with the consequence that a mouse from a litter of odd numbered males was stabled alone. Each animal was defined as an experimental unit, but this could be debated considering environmental confounders within in a cage. Defining each cage as one experimental unit would have required a doubling of the number of animals used.

Mice from litters were stratified to the different experimental groups in order to minimize systematic bias. The individual mouse was randomly picked to the experimental group that an animal from its litter was stratified to.

We considered it not feasible to blind the performing researchers to the genotype or experimental treatment of each cage. The daily read-out of the experiments was weighing of mice, which should not be subject to examiner bias. The other parameters evaluated by the researchers were diarrhea and gross blood from anus. Coherent definitions were made for these assessments in order to reduce examiners bias. All organ evaluation upon sacrifice (e.g. colon length, Payer's patches assessment) was performed by a technician who was kept blinded for the genotype and the treatment of the individual mouse.

## 5.3 Histopathology

**Papers I, II, IV, and V** all includes histopathological assessments of intestinal mucosal tissue. Biopsies were cut with a predefined thickness and stained with hematoxylin and eosin before being evaluated by a trained histopathologist. Histopathological evaluation of tissue morphology is by nature a qualitative assessment. To compare the morphology of different biopsies we applied previously published scoring systems, which quantified the degree of

inflammation of the individual biopsies. We applied the scoring systems in the same species and for the same diagnosis and experimental approach as the ones they were based upon (Siegmund et al., 2001; Rugtveit et al., 1997).

All slides were blinded and assigned a random examination number to mask the identity and diagnosis of the slides and to scramble the order in which they were examined. This should prevent systemic bias of the scoring due to unconscious drift of the scoring throughout the examination.

To assess the intra-individual variability of histopathologic scoring the slide sets were in part reevaluated in a blinded fashion by the same histopathologists. For the scoring described in **papers I** and **II** the histopathologist had a diagnostic reproducibility of 83%. For the inflammation score in the DSS treated mice in **paper V**, the histopathologist had a 79% reproducibility rate for the individual scoring factors. Both numbers are considered acceptable for intra-individual variability with regard to a clinical histopathologist's assessment of inflammation (personal communication, Lars Gustav Lyckander).

## 5.4 Immunohistochemistry

**Papers I** and **II** are entirely based on data obtained by immunohistochemistry (IHC) and **papers III** and **V** presents some data based on this method. IHC takes advantage of the specific recognition of an antigen by an antibody (immunoglobulin). As the target protein – the antigen - is fixed in its natural place in the tissue, IHC provides spatial information on the distribution of the antigen, which is the major advantage of IHC compared with ELISA or Western blotting technices. The principles for IHC have been extensively described (Brandtzaeg, 1994) and are outlined in figure 7 and 8.. However, a few key issues deserve consideration.

Although antibodies used in IHC bind specifically to a target antigen, unspecific staining does occur. This can be caused either by specific antibody recognition of an antigen present on a protein other than the target protein (cross reaction), or by unspecific binding of any elements in the IHC protocol to the target tissue (noise). To assess the contribution of noise in the IHC staining we always included a control staining that was incubated with an irrelevant primary antibody but otherwise was treated identical as the target staining.

There are two principles for production of antibodies. Polyclonal antibodies are purified from serum of immunized animals and will generally possess reactivity to several epitopes on the target protein. Monoclonal antibodies are produced by a clonal population of hybrodoma cells generated from fusion of a single B cell with an immortal myeloma cell. Polyclonal antibodies are considered to be more sensitive than a monoclonal antibody, but they are also more prone to react with epitopes shared by other proteins than the target protein, and are therefore more susceptible to give unspecific staining (cross reaction) in IHC.

Monoclonal antibodies only react with one epitope on the target protein. Naturally they are therefore not as sensitive as polyclonal antibodies, but their specificity is generally better. However, the epitope recognized by a monoclonal antibody may be shared by other proteins so the specificity of new monoclonal antibodies should always be validated on tissue specimens by comparing the staining pattern with that of other well-tested antibodies. Monoclonal antibodies will also be of a specific IgG subclass (or other isotype), which widens the range of possibilities for multistaining on one slide as secondary antibodies targeting a specific IgG subclass have become available.

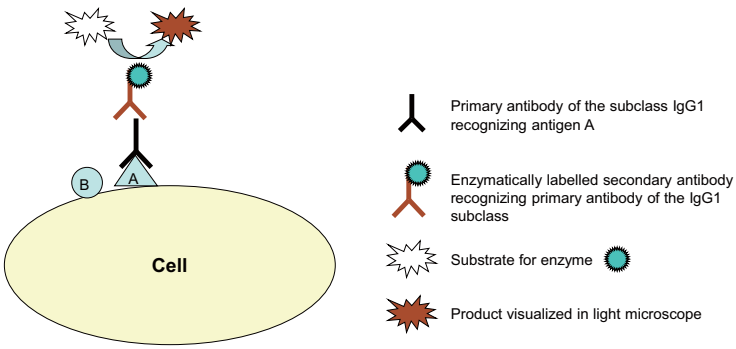



**Figure 7. Immunohistochemistry 1.** Principle of enzymatic detection of immunostaining method applied in papers I and II. The primary antibody specifically recognizes antigen A and not antigen B. (Adapted from I. Heier)

Several aspects of the examined tissue specimens are important for the accessibility of the antigen-antibody immune reaction that forms the basis of IHC. Traditionally, IHC has easiest been performed on cryopreserved specimens, which are fixed in acetone or alcohols after sectioning. This tissue preparation retains most epitopes available for antibody detection, but does not preserve tissue morphology as well as formaldehyde-based fixatives. Formaldehyde forms protein-protein and protein-nucleic acid methylene cross bridges, which ensures good tissue morphology but often masks epitopes. This can be overcome by immersing the formaldehyde-fixed tissue section in buffers at boiling temperatures prior to the IHC (Montero, 2003). The acetone or alcohol fixatives offer another advantage over formaldehyde as they disrupt the lipid bilayers of cell membranes, which open up for antibody penetration and intra-cellular immunostaining (e.g. Foxp3). Formaldehype-fixed section must be treated with a detergent to allow for intra-cellular staining.

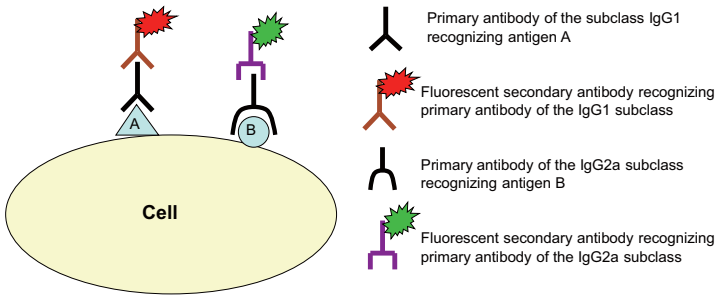



**Figure 8. Immunohistochemistry 2.** Principle of fluorescent detection of immunostaining applied in papers I-III and V. One primary antibody specifically recognizes antigen A and not antigen B, and the other primary antibody specifically recognizes antigen B but not antigen A. By conjugating different fluorescent dyes to the specific secondary antibodies multiple antigens can be detected simultaneously. (Adapted from I. Heier)

For all formaldehyde-fixed tissue we carefully tested antigen retrieval protocols. Primary and secondary antibodies were titrated to determine the maximum signal-to-noise

ratio, which is the concentration of antibody that provides the least unspecific background staining (noise) without reducing the signal from the target antigen (signal).

Detection of bound target is mediated either by an enzymatic reaction (as in figure 7) or by fluorescence (as in figure 8). In **papers I** and **II** we used horseradish peroxidase (HRP) enzyme polymers conjugated to the secondary antibody. These HRP polymers produced a brown color when substrate was added. Enzyme-based detection techniques offer the advantage that the sections can be counterstained with eosin, which gives a better orientation of the morphology. These techniques are generally more accessible as they are evaluated by light microscopy. A disadvantage of HRP-based detection is that tissue may contain endogenous peroxidase activity, but this can be overcome by blocking agents. However, the blocking agent may also destroy the target epitope. We therefore applied two alternative blocking agents for the HRP-based IHC. All papers except paper IV also include IHC experiments that applied fluorescence as the detection mode. Apart from reducing the need for blocking of endogenous enzyme, fluorescence-based detection more readily offers the possibility of detecting several antigens simultaneously by multi-color fluorescence (Figure 8) and still allowing the individual target antigens to be evaluated separately by filtering the fluorescent emission in the microscope. Disadvantages of fluorescence-based detection are that it requires special microscopes and that the fluorescent dye inevitably will fade, which reduces the longevity of the IHC fluorescent staining.

IHC is by nature a qualitative method. However, by counting the number of positive signals and relating that to a fixed measure one can obtain quantitative data from IHC. We generated quantitative continuous data from IHC by relating positive signals (cells) to the area of the examined section (**paper I** and **II**), to a defined micro-anatomical structure (e.g. crypt or colon cross section) (**paper III** and **V**), or as a fraction of positive cells co-expressing the target marker in two-color staining (**paper II**). All quantitative evaluations of the IHC stainings were performed with the examiner blinded to the specimen identity and in a randomized order.

The following paragraphs provide a brief presentation and validation of the markers used for IHC.

*CD68*. CD68 is a highly glycosylated transmembrane protein belonging to the lamp family of proteins and located in the endosomal compartment of the cytoplasm (Holness and Simmons, 1993). Unlike other lamp family proteins, which are ubiquitously expressed in all

cell types, CD68 has been considered a marker restricted to the monocyte/macrophage lineage (Pulford et al., 1990). For several years it has been known that some monoclonal antibodies against CD68 may cross-react with other myeloid cells (Pulford et al., 1990) and over the years some clones have even showed reactivity to non-hematopoietic cells. Accordingly scientists have questioned the specificity of several anti-CD68 antibodies as macrophage markers (Gottfried et al., 2008; Kunisch et al., 2004). However, no other marker has been proposed as the pan-macrophage marker. The function of CD68 is not known.

*CD40.* CD40 is a receptor located on the plasma membrane of B cells, APCs including macrophages, and has also been described on non-hematopoietic cells like epithelial, endothelial, and mesenchymal cells. Its ligand is CD40L (CD154), which is preferentially expressed on activated $CD4^+$ T cells and on activated platelets. Engagement of the CD40/CD40L system on APCs activates these cells to express other co-stimulatory molecules and to produce pro-inflammatory cytokines. Expression of CD40 is shown to be upregulated in several mucosal cell types in IBD (Carlsen et al., 2006; Danese et al., 2004; Hori et al., 2003; Fontenot et al., 2003).

*CD3.* As a protein complex required for the cell surface expression and signaling of the T cell receptor, CD3 is the undisputed restricted T cell pan-marker.

*Foxp3.* Several publications from 2003 showed the transcription factor Foxp3 to be linked to $CD4^+CD25^+$ T cells with immunosupressive capacities (Fontenot et al., 2003; Hori et al., 2003). However, intense research on human Tregs over the last decade has demonstrated the complexity of Foxp3 and Tregs. On the one hand peripheral blood in humans contains $Foxp3^+$ T cells that do not confer suppressive capacities, but if this is the case in intestinal tissue is unknown (Sakaguchi et al., 2010). Also, *in vitro* stimulation of naïve T cells has produced transient expression of Foxp3 in cells that eventually display a pro-inflammatory phenotype, but if this has relevance for *in vivo* T cell dynamics is debated (Sakaguchi et al., 2010). Recent reports point out that high expression of Foxp3 in T cells confer high suppression *in vitro* compared with low Foxp3 expression (Miyara et al., 2009). On the other hand, $Foxp3^-$ T cells, termed Tr1 and Th3, may have suppressive properties. Thus, identification of $Foxp3^+$ cells is not synonymous with identification of immunosuppressive T cells, but no other single marker has proved to be better. The crucial importance of this transcription factor for immunosuppressive function is demonstrated clinically by the fact that mutations in the *FOXP3* gene results in a multiorganic

inflammatory syndrome (Wildin et al., 2002; Walunas et al., 1996). For further discussion on Tregs see section 2.3.5.

*CTLA-4.* CTLA-4 (CD152) may be expressed on the plasma membrane of T cells and is a structural homologue of the co-stimulatory molecule CD28. CTLA-4 binds to CD80 and CD86 located on APCs in competition with CD28 and prevents CD28-CD80/CD86-induced T cell activation (Walunas et al., 1996; Krummel and Allison, 1996). CTLA-4-CD80/CD86 interaction transduces suppressive signals to the T cell leading to cell cycle arrest and aborted IL-2 secretion (Krummel and Allison, 1996). Mice lacking CTLA-4 display immune-mediated multiorgan failure (Tivol et al., 1995). CTLA-4 may be expressed on effector T cells, but concomitant expression on $Foxp3^+$ cells is highly suggestive of active Tregs (Sakaguchi et al., 2010; Malek and Castro, 2010).

*CD25.* CD25 is the α chain of the high-affinity IL-2 receptor complex located on the plasma membrane. It is transiently expressed upon activation of all T cells, but constitutive and high expression marks Tregs (Malek and Castro, 2010). As proposed by Sakaguchi, concomitant high expression of CD25 and Foxp3 is confined to Tregs (Sakaguchi et al., 2010). The suppressive significance of signaling through the IL-2 receptor is demonstrated both by deletion of IL-2 and by deletion of CD25 in mice, which both lead to lethal autoimmune syndromes (Malek and Castro, 2010). CD25 may also be expressed by blood monocytes or activated macrophages (Smith et al., 2005).

*Ly-6G.* Ly-6G is surface molecule used as a restricted marker for murine granulocytes (Lai et al., 1998).

*Ki-67.* Ki-67 is a nuclear protein always present during all phases of cell cycle but never present in resting cells (Scholzen and Gerdes, 2000). It is therefore considered an excellent marker for proliferating cells. Although Ki-67 appears to be required for cellular proliferation, its molecular function is still unknown (Scholzen and Gerdes, 2000).

## 5.5 Bacteriological assays

### 5.5.1 Bacteriological cultivation

Bacterial cultivation assays were performed (**papers III** and **IV**) in relation to the microbiota depletion treatment. The assays were performed at Peter Gaustad's laboratory at the Institute for Microbiology.

First, feces were assessed for a numerical estimate of cultivable bacteria. To minimize the risk of contamination, mice were let to defecate directly into clean Eppendorf tubes prefilled with ice cold sterile phosphate buffered saline (PBS), and the wet weight of the pellets were calculated. Dry weight would have been more valid for estimation of fecal mass, but removing water from the pellets would have killed most of the bacteria present.

The selection of agar plates was made to detect all major groups of cultivable bacteria. Colonies detected by this assay were identified down to genus or family level based on the selective media they were collected from, colony morphology, and Gram staining. Most of the intestinal microbiota is as yet not cultivable *ex vivo* as these microbes are fastidious and often highly susceptible to oxygen toxicity (Duncan et al., 2007; Peterson et al., 2008). The cultivation assay was not performed in order to give an exact number or identification of the intestinal microbes, but to provide a reference that later could be used in the evaluation of the effect of the antibiotic treatment.

After antibiotic treatment fecal pellets were collected and weighted in the same manner as above. However, to optimize the sensitivity of the assay we plated all fecal suspensions on agar plates nutritionally sufficient for all cultivable intestinal aerobic and anaerobic bacteria and for yeasts. The incubation time was extended to 48 hours for the aerobic agar plates and 72 hours for the anaerobic agar plates. As the median wet weight of the fecal pellets collected was 46 mg (ranging from 17 to 120 mg) and 1/10 of the mass was plated on each agar plate, we reasoned that viable bacteria present in the fecal pellets at a concentration lower than 1 cfu/mg would remain undetected. Theoretically the cultivation assay may have been affected by remnant antibiotics in the fecal samples, but we judged this unlikely because of dilution of the inoculated antibiotics, first in the gut fluids and feces and then in the PBS of the fecal sample.

### 5.5.2 Molecular biological microbial analyses

As mentioned above only a fraction of the intestinal microbiota is generally accessible for *ex vivo* cultivation. Over the last two decades new culture independent technologies have been developed to explore the microbial communities of the gut (Peterson et al., 2008). Until recently these technologies have been based on 16S rRNA gene analysis of bacteria. 16S rRNA is a subunit of the rRNA of all bacteria. As the ribosome is essential for the life of the bacteria, the 16S rRNA gene is generally highly conserved making it a target for evolutionary and taxonomic studies (Clarridge, III, 2004). Also, the 16S rRNA gene contains hypervariable regions making it useful for identifying bacteria down to strain level by comparing sequences with gene databases (Peterson et al., 2008). In **paper III** and **IV** we applied two different 16S rRNA gene analyses.

In **paper III** primers were designed for the conserved regions flanking variable regions V2 and V6 of the 16S rRNA gene. Fecal samples were analyzed by quantitative polymerase chain reaction (qPCR) in order to quantify the bacterial load in the fecal pellets after antibiotic treatment. Two issues regarding this assay deserve a brief discussion. First, the qPCR assay would amplify and detect all target genes present in the samples regardless of the viability of the bacteria. Thus, the amount of 16S rRNA gene present in the sample does not directly correlate to the number of live bacteria present in the gut. Second, by comparing the gut bacterial DNA load of mice between different mice the mass of detected bacterial DNA had to be normalized to a fixed measure. The fecal samples were weighted, but as the water content of the pellets may vary the wet weight was not considered to be an adequate measure. As described in **paper III** we normalized the bacterial DNA to genomic mouse DNA. This analysis is based on the assumption that all mice have the same mass of epithelial cells in their gut and shed intestinal epithelium at the same rate. The added advantage of comparing DNA (bacterial) with DNA (murine) is identical purification procedures, molecular stability etc., thus reducing the influence of differences in yield and quality of different samples.

In **paper IV** the composition of the intestinal microbiota of pIgR KO mice and wt counterparts was assessed by phylogenetic microarray. Snap frozen fecal pellets and cecal samples were shipped to Hauke Smidt's laboratory in Wageningen, the Netherlands where the processing of the samples, microarray and bioinformatics were performed. The principle of the technology is to extract DNA from the samples, amplify the bacterial 16S rRNA DNA by PCR, and detect target sequences by nucleotide probes printed on a glass slide. The technology, which is called the mouse intestinal tract chip (MITChip), is newly designed and

as of today not yet published, but is based on the technology of a human intestinal tract chip (HITChip) (Rajilic-Stojanovic et al., 2009). The MITChip/HITChip technology offers excellent reproducibility and specificity, and with a resolution comparable to FISH and superior to DGGE (Rajilic-Stojanovic et al., 2009). The MITChip/HITChip is a semi-quantitative analysis that offers information about the abundance of the target sequence. The phylogenetic microarray provides accurate information of the taxonomic composition of the intestinal microbiota ("who is there?") but does not offer any information on functional disparities between different samples ("what are they doing?"). As bacteria extensively transfer genes horizontally one cannot infer that differences in 16S rRNA gene analyses implies functional differences of the microbial community as a whole. Recently published reports on sequencing the combined genome of the intestinal microbial community ("microbiome") may provide such information (Nelson et al., 2010; Qin et al., 2010).

## 5.6 Statistics

As in all experimental biomedical research the numbers of individuals (persons or animals) or samples, termed the experimental units, examined in this thesis are relatively small. Still, proper statistical handling of the data is crucial to draw scientific conclusions. The applied statistical methods are described in the individual papers, but a few key issues regarding the choice of the methods are discussed here.

As the experimental units are relatively few and consequently the sample sizes of each experimental group are small, a decision has to be made whether to apply parametric statistical tests or non-parametric tests. If all assumptions for parametric tests are met, parametric tests offer greater ability to detect a true significant difference between groups (Lamb et al., 2008). The main assumption for using parametric tests is a normal distribution of data within each sample group. There is no clear definition of when to regard a data set normally distributed. Formal statistical tests for normality exist, but these are considered unnecessary (Lamb et al., 2008). Based on literature and consultations with biostatisticians we considered data set normally distributed if the visual impression of symmetry around the mean in columnar scatter plots was evident; and if mean and median values were approximately equal; and if the nature of data indicated normality. No minimal number of experimental units per experimental group was set as a requirement for assuming normality. Other assumptions on which the applied statistical tests are based are: that the experimental

units are either randomly selected from a population or randomly allocated to the experimental group; that the experimental units are independent of each other both within groups and across the groups; and that the variances between groups are relatively equal (Lamb et al., 2008). Except for the procedure of the stabling of mice which could be questioned with regard to independency (see section 5.2.5), these assumptions were met for all our analyses where parametric statistics where applied.

A disparity exists between **papers I** and **II** in terms of applied statistical method considering that assessments performed on partially the same specimens. When designing the project resulting in papers I and II we regarded the sample sizes too small for parametric tests and we therefore initially performed non-parametric tests. However, when analyzing data for **paper II** we considered the possibility of adjusting the cell density counts for inflammation score. We discussed this with a statistician (co-author J.M.G), and the data were judged to be eligible for parametric statistics.

Comparisons between two independent groups were analyzed with independent (a.k.a unpaired) tests (e.g. Student t test, Mann-Whitney). When comparing more than two groups these were analyzed with analysis of variances (ANOVA) rather than multiple t tests. The ANOVA tests reduce the chances of statistically significant results to erroneously occur by chance (type I error). Relevant post-tests were performed with Bonferroni adjustment. ANOVA tests require assumptions of normality to be met.

In **paper I** and **II** we performed multiple tests and not ANOVA. The choice of test was discussed with a statistician and considered valid because a number of null hypotheses were defined and decided to be tested prior to the acquisition of data, and because not all comparisons between various groups were considered relevant (e.g. adult colon vs. pediatric ileum).

All tests were performed as two-tailed tests and the level of significance ($\alpha$ level) was consistently set to the conventional 0.05.

# 6 DISCUSSION

Discussions of the isolated results of the individual papers are provided in the respective manuscripts. This chapter provides an integrated discussion of the results in relation to the aims of the thesis.

This thesis aimed at providing new information on mucosal immunopathogenic mechanisms in IBD. **Papers I** and **II** present novel observations on the distribution of macrophages and Tregs cells in the mucosa of recent onset pediatric IBD patients compared with recent onset adult IBD patients. The study design founding the basis for these papers is original, as at the time of diagnosis biological material from untreated patients were collected. This provided insight into the early pathogenesis of IBD. Most investigations of biologic material from IBD patients have been based on collections from patients with long-standing disease and concurrently varying medication. To our knowledge the two papers included here are the first to have investigated mucosal specimens from exclusively untreated pediatric patients at the time of diagnosis and having compared them with specimens from untreated adult patients at the time of diagnosis.

**Papers I** and **II** report on differential distribution of macrophages and Tregs, both important players in intestinal mucosal homeostasis (Hooper and Macpherson, 2010). We suggest that these differences may contribute to phenotypic differences in pediatric and adult onset IBD. This hypothesis is based on the assumption that there is no qualitative difference in the function of these cell types in pediatric versus adult patients. Regarding macrophages, researchers have proposed that CD is the result of impaired macrophage function (Casanova and Abel, 2009). Dysfunction of Tregs has not been linked to development of IBD, but the increasing knowledge of plasticity between Tregs and Th17 effector cells makes it difficult to assess the exact role of $Foxp3^+$ Tregs in the etiology and pathogenesis of IBD (Sakaguchi et al., 2010).

**Papers I** and **II** describe that the pediatric control patients have fewer macrophages in their colon and that the pediatric CD patients have more Tregs in their ileum compared with adults, but no mechanistic explanations are provided. As we have demonstrated in the included **Papers IV** and **V** and consistent with the current literature, nearly all experimental IBD animal models depend on the presence of intestinal microbes (Uhlig and Powrie, 2009). Furthermore, both experimental and human IBD are associated with alterations in the

composition of the intestinal microbiota (Sartor, 2008). Is it possible that the altered distribution of immune cells described in **Papers I** and **II** has been caused by or is associated with microbial perturbations? Few investigations have been performed on the microbiota in pediatric IBD and none have identified alterations specific to the pediatric segment (Conte et al., 2006; Schwiertz et al., 2010). However, as recent animal studies have identified key phylotypes responsible for the induction of Tregs (see section 2.3.5), the hypothesis that the differential distribution of macrophages and Tregs is instigated by pediatric-specific microbes, which ultimately may cause the pediatric-specific IBD phenotypes, deserves to be explored.

The methodology of **papers I** and **II** did not allow functional assessments of the role of macrophages and Tregs in the IBD lesions. It is well documented that activated ($CD40^{+}$) macrophages contribute to drive inflammation (Mosser and Edwards, 2008; Smith et al., 2010). It is therefore likely that the increased density of activated macrophages leads to increased inflammatory potential in the IBD lesion. Regarding Tregs, it may seem counterintuitive that there are many studies along with ours that report that Tregs are generally increased in numbers in all kinds of inflammatory lesions. Our analysis of the distribution of Tregs in the intestinal mucosa indicated that there are spatial differences in the distribution of Tregs regardless of the inflammatory status of the examined biopsy. Experimental studies on the inflammatory potential in lesions with or without Tregs are not possible to perform in humans. However, humans with mutations in *FOXP3* (IPEX) have IBD-like enteropathy, and experimental animal studies have demonstrated that Tregs are able to both prevent colitis and attenuate established disease (Fantini et al., 2006; Mottet et al., 2003). Thus, the functional role of the cell types studied in **papers I** and **II** should not be controversial. The applied immunohistochemistry methods provide a snapshot of real life distribution of key players in mucosal homeostasis.

**Papers III-IV** focus on the roles of colonic epithelial cells and the transport of SIg in laboratory animal models. These are essential components of mucosal homeostasis along with macrophages and Tregs. The homeostatic function of IEC, IgA, macrophages, and Tregs all pivot around a few key cytokines such as TGF-β, IL-10, TSLP and RA. Recently, experimental studies have presented evidence for Tregs' promotion of IgA production, a.k.a as a Treg-IgA axis (Feng and Elson, 2011).

The second aim of this thesis was to elucidate interactions between secretory immunoglobulins and intestinal epithelial cell function on one hand and the intestinal

microbiota on the other. **Paper IV** shows how lack of SIg makes mice more susceptible to experimental colitis and that this is associated with alterations of the composition of the intestinal microbiota and of the colonic IEC gene expression profile. These results are consistent with previously published data in mice (Murthy et al., 2006). However, as previously mentioned, data recorded in mice cannot automatically be extrapolated to humans. There are well known differences in the IgA system between mice and men (reviewed by Gibbons and Spencer (Gibbons and Spencer, 2011). In humans, no mutation resulting in aborted function of pIgR is known. From an evolutionary point of view, this may indicate that lack of pIgR is incompatible with life in humans as opposed to in mice. Alternatively, a defective pIgR may have little consequence in healthy humans living in a clean environment and therefore not have been identified. There are known single nucleotide polymorphisms in the human *PIGR* gene (2007). Some of these correlate with IgA nephropathy and with Epstein-Barr virus diseases, but none have been shown to correlate with IBD or other intestinal diseases.

On the other hand, selective IgA deficiency is well described in humans with a prevalence of 1:200 to 1:1000 in western societies. IgA deficiency leaves most of the affected persons asymptomatic, but it is still associated with increased incidence of IBD (Cunningham-Rundles, 2001). The reason for the mild disease phenotype in selective IgA deficiency is not established, but one hypothesis suggest that increased levels of SIgM could compensate for the lack of SIgA (Cunningham-Rundles, 2001). However, also patients with X-linked agammaglobulinemia, which lack all classes of immunoglobulins, rarely present with gastrointestinal disorders (Agarwal and Mayer, 2009). This suggests that isolated loss of immunoglobulins in humans is not critical to gut homeostasis, and consequently that there is redundancy to humoral immunity for maintenance of mucosal homeostasis. In humoral immunodeficencies such as common variable immunodeficiency, it is believed that intestinal inflammation is dependent on concurrent impairment of T cell function (Agarwal and Mayer, 2009). Also, mice gut homeostasis is dependent on T cell function (Gibbons and Spencer, 2011). One study has demonstrated the importance of Tregs for mediating OVA specific tolerance in pIgR KO (Karlsson et al., 2010), but further reports on T cell functions in the pIgR KO and $J_H$ KO mouse models are lacking.

**Paper IV** outlines changes in the composition of the intestinal microbiota in mice deficient in SIgs. As previously mentioned, several new pieces of evidence indicate how the intestinal microbiota is shaped by SIgA. Reports on how humoral immunodeficiency affects

the intestinal microbiota in humans are sparse, and to our knowledge the microbiota of these patients have not been assessed with modern phylogenetic tools. Such studies would be of great interest as they could validate the relevance of the observations in mice and help with identifying defined phylotypes that exploit the lack of SIgs to establish their niche.

To what extent do the results from this thesis provide information that can be implemented into efforts to prevent IBD or improve the care of affected patients? Immunosuppressive therapy by enhancing Treg function based on principles of *ex vivo* expansion and reinfusion is under intense research (Sakaguchi et al., 2010). The finding of increased density of Tregs in an intestinal segment of pediatric intestines with reduced occurrence of CD lesions (**paper II**) should encourage continuation of this line of research.

Our results in the mouse models (**papers III-V**) confirm previous reports that colitis is dependent on presence of intestinal microbes, and we showed that mice with increased susceptibility to colitis had an altered composition of the microbiota. However, since these mice were lacking SIgs and pIgR-mediated mucosal protection, we cannot determine whether enhanced susceptibility to DSS-induced colitis was due to altered microbiota or altered handling of the present microbes. Key phylotypes that potentially cause IBD have yet to be identified. The idea of modulating the intestinal microbiota to treat IBD has been pursued. Probiotics have shown promising results in ulcerative colitis, but larger clinical trials are still lacking. Recent reports on specific phylotypes responsible for Treg induction in the gut of mice may give a clue to key species that a probiotic concoction should contain (Atarashi et al., 2011). Allogenic fecal transplantation, which is becoming established therapy for resistant *C. difficile* infections, has been tested in IBD patients with good results (Khoruts and Sadowsky, 2011). Recent data in an experimental mouse model demonstrated that colitogenic bacterial strains can be transferred vertically (mother to pup) and horizontally (between litter mates) in mice (Garrett et al., 2007; Garrett et al., 2010). Fecal transplantation from a healthy to an affected individual is the therapeutic analogue to those observations and should be explored more thoroughly despite esthetical concerns. Our results along with other experimental data on the interaction between IgA and intestinal microbiota (Peterson et al., 2007; Suzuki et al., 2004) suggest that IBD patients with impaired humoral immunity should in particular benefit from restoration of a healthy gut microbiota.

# 7 CONCLUSIONS

Intensive research over the past few decades has demonstrated that IBD is associated with disrupted homeostasis in the intestinal mucosa with alterations in both host immunity and in the intestinal microbiota. This thesis provides the following new information to the ever increasing knowledge on disrupted intestinal mucosal homeostasis:

- Histologically normal colonic mucosa has a higher density of macrophages in children than in adults.
- Activated mucosal macrophages are increased in untreated pediatric IBD.
- The majority of $CD25^+$ cells in intestinal mucosa are macrophages.
- Densities of mucosal $Foxp3^+$ Tregs and $CD25^+$ cells (activated macrophages) are elevated in both pediatric and adult ileal CD regardless of histological inflammation.
- Cultivatable intestinal microbiota can be depleted by gavaging antibiotics, and the biological effect of this treatment phenocopies physiological characteristics of germ-free mice.
- Mice lacking SIg because of abrogated transport of pIg have altered composition of the intestinal microbiota, and this is associated with increased susceptibility to experimental colitis and differential gene expression profile of colonic IECs.
- Absence of pIgR protects B cell-deficient mice from microbiota-dependent colitis.

# 8 REFERENCES

(2007). Mucosal Immune Defense: Immunoglobulin A. Springer US.

(2010). http://oslovet.veths.no/fag.aspx?fag=64..
Ref Type: Internet Communication

Abreu,M.T. (2010). Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat. Rev. Immunol. *10*, 131-144.

Abreu,M.T., Vora,P., Faure,E., Thomas,L.S., Arnold,E.T., and Arditi,M. (2001). Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J Immunol *167*, 1609-1616.

Agarwal,S. and Mayer,L. (2009). Pathogenesis and treatment of gastrointestinal disease in antibody deficiency syndromes. J Allergy Clin. Immunol *124*, 658-664.

Akbar,A.N., Vukmanovic-Stejic,M., Taams,L.S., and Macallan,D.C. (2007). The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. Nat. Rev. Immunol. *7*, 231-237.

Artis,D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat. Rev. Immunol. *8*, 411-420.

Asahi,Y., Yoshikawa,T., Watanabe,I., Iwasaki,T., Hasegawa,H., Sato,Y., Shimada,S., Nanno,M., Matsuoka,Y., Ohwaki,M., Iwakura,Y., Suzuki,Y., Aizawa,C., Sata,T., Kurata,T., and Tamura,S. (2002). Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. J Immunol *168*, 2930-2938.

Asquith,M. and Powrie,F. (2010). An innately dangerous balancing act: intestinal homeostasis, inflammation, and colitis-associated cancer. J. Exp. Med. *207*, 1573-1577.

Atarashi,K., Tanoue,T., Shima,T., Imaoka,A., Kuwahara,T., Momose,Y., Cheng,G., Yamasaki,S., Saito,T., Ohba,Y., Taniguchi,T., Takeda,K., Hori,S., Ivanov,I.I., Umesaki,Y., Itoh,K., and Honda,K. (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. Science *331*, 337-341.

Axelsson,L.G., Landstrom,E., and Bylund-Fellenius,A.C. (1998). Experimental colitis induced by dextran sulphate sodium in mice: beneficial effects of sulphasalazine and olsalazine. Aliment. Pharmacol. Ther. *12*, 925-934.

Axelsson,L.G., Landstrom,E., Goldschmidt,T.J., Gronberg,A., and Bylund-Fellenius,A.C. (1996). Dextran sulfate sodium (DSS) induced experimental colitis in immunodeficient mice: effects in CD4 (+) -cell depleted, athymic and NK-cell depleted SCID mice. Inflamm. Res. *45*, 181-191.

Baker,K., Qiao,S.W., Kuo,T., Kobayashi,K., Yoshida,M., Lencer,W.I., and Blumberg,R.S. (2009). Immune and non-immune functions of the (not so) neonatal Fc receptor, FcRn. Semin. Immunopathol. *31*, 223-236.

Barnes,M.J. and Powrie,F. (2009). Regulatory T cells reinforce intestinal homeostasis. Immunity. *31*, 401-411.

Baumgart,D.C. and Carding,S.R. (2007). Inflammatory bowel disease: cause and immunobiology. Lancet *369*, 1627-1640.

Baumgart,D.C. and Sandborn,W.J. (2007). Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet *369*, 1641-1657.

Benchimol,E.I., Guttmann,A., Griffiths,A.M., Rabeneck,L., Mack,D.R., Brill,H., Howard,J., Guan,J., and To,T. (2009). Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: evidence from health administrative data. Gut *58*, 1490-1497.

Berndt,B.E., Zhang,M., Chen,G.H., Huffnagle,G.B., and Kao,J.Y. (2007). The role of dendritic cells in the development of acute dextran sulfate sodium colitis. J. Immunol. *179*, 6255-6262.

Bettelli,E., Korn,T., Oukka,M., and Kuchroo,V.K. (2008). Induction and effector functions of T (H)17 cells. Nature *453*, 1051-1057.

Bjorck,S., Jennische,E., Dahlstrom,A., and Ahlman,H. (1997). Influence of topical rectal application of drugs on dextran sulfate-induced colitis in rats. Dig. Dis. Sci. *42*, 824-832.

Blumberg,R.S., Saubermann,L.J., and Strober,W. (1999). Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. Curr. Opin. Immunol. *11*, 648-656.

Bouskra,D., Brezillon,C., Berard,M., Werts,C., Varona,R., Boneca,I.G., and Eberl,G. (2008). Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature *456*, 507-510.

Brandl,K., Plitas,G., Mihu,C.N., Ubeda,C., Jia,T., Fleisher,M., Schnabl,B., DeMatteo,R.P., and Pamer,E.G. (2008). Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature *455*, 804-807.

Brandl,K., Plitas,G., Schnabl,B., DeMatteo,R.P., and Pamer,E.G. (2007). MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection. J Exp. Med. *204*, 1891-1900.

Brandtzaeg,P. (1994). [Immunohistochemistry--more than a staining method]. Tidsskr. Nor Laegeforen. *114*, 2381-2385.

Brandtzaeg,P. (2007). Induction of secretory immunity and memory at mucosal surfaces. Vaccine *25*, 5467-5484.

Brandtzaeg,P. (2009). Mucosal immunity: induction, dissemination, and effector functions. Scand. J. Immunol. *70*, 505-515.

Brandtzaeg,P. and Johansen,F.E. (2005). Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. Immunol Rev. *206*, 32-63.

Buckner,J.H. and Ziegler,S.F. (2008). Functional analysis of FOXP3. Ann. N. Y. Acad. Sci. *1143*, 151-169.

Bylund-Fellenius,A.C., Landstr+Âm,E., Axelsson,L.G., and Midtvedt,T. (1994). Experimental Colitis Induced by Dextran Sulphate in Normal and Germfree Mice. Microbial Ecology in Health and Disease *7*, 207-215.

Cario,E., Gerken,G., and Podolsky,D.K. (2007). Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology *132*, 1359-1374.

Carlsen,H.S., Yamanaka,T., Scott,H., Rugtveit,J., and Brandtzaeg,P. (2006). The proportion of CD40+ mucosal macrophages is increased in inflammatory bowel disease whereas CD40 ligand (CD154)+ T cells are relatively decreased, suggesting differential modulation of these costimulatory molecules in human gut lamina propria. Inflamm. Bowel. Dis. *12*, 1013-1024.

Casanova,J.L. and Abel,L. (2009). Revisiting Crohn's disease as a primary immunodeficiency of macrophages. J Exp. Med. *206*, 1839-1843.

Cash,H.L., Whitham,C.V., Behrendt,C.L., and Hooper,L.V. (2006). Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science *313*, 1126-1130.

Cerutti,A. and Rescigno,M. (2008). The biology of intestinal immunoglobulin A responses. Immunity. *28*, 740-750.

Chen,J., Trounstine,M., Alt,F.W., Young,F., Kurahara,C., Loring,J.F., and Huszar,D. (1993). Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus. Int. Immunol. *5*, 647-656.

Chen,X., Oppenheim,J.J., and Howard,O.M. (2005). BALB/c mice have more CD4+CD25+ T regulatory cells and show greater susceptibility to suppression of their CD4+. J Leukoc. Biol. *78*, 114-121.

Chervonsky,A.V. (2010). Influence of microbial environment on autoimmunity. Nat. Immunol. *11*, 28-35.

Chieppa,M., Rescigno,M., Huang,A.Y., and Germain,R.N. (2006). Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp. Med. *203*, 2841-2852.

Cho,J.H. (2008). The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev. Immunol *8*, 458-466.

Clarridge,J.E., III (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. *17*, 840-62, table.

Conte,M.P., Schippa,S., Zamboni,I., Penta,M., Chiarini,F., Seganti,L., Osborn,J., Falconieri,P., Borrelli,O., and Cucchiara,S. (2006). Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease. Gut *55*, 1760-1767.

Coombes,J.L. and Powrie,F. (2008). Dendritic cells in intestinal immune regulation. Nat. Rev. Immunol. *8*, 435-446.

Cooper,H.S., Murthy,S.N., Shah,R.S., and Sedergran,D.J. (1993). Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest *69*, 238-249.

Corthesy,B. (2007). Roundtrip Ticket for Secretory IgA: Role in Mucosal Homeostasis? J Immunol *178*, 27-32.

Cunningham,K.A., Carey,A.J., Finnie,J.M., Bao,S., Coon,C., Jones,R., Wijburg,O., Strugnell,R.A., Timms,P., and Beagley,K.W. (2008). Poly-immunoglobulin receptor-mediated transport of IgA into the male genital tract is important for clearance of Chlamydia muridarum infection. Am. J Reprod. Immunol *60*, 405-414.

Cunningham-Rundles,C. (2001). Physiology of IgA and IgA deficiency. J Clin. Immunol *21*, 303-309.

Curotto de Lafaille,M.A. and Lafaille,J.J. (2009). Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? Immunity. *30*, 626-635.

Dallas,S.D. and Rolfe,R.D. (1998). Binding of Clostridium difficile toxin A to human milk secretory component. J. Med. Microbiol. *47*, 879-888.

Danese,S., Sans,M., and Fiocchi,C. (2004). The CD40/CD40L costimulatory pathway in inflammatory bowel disease. Gut *53*, 1035-1043.

Dann,S.M. and Eckmann,L. (2007). Innate immune defenses in the intestinal tract. Curr. Opin. Gastroenterol. *23*, 115-120.

Davids,B.J., Palm,J.E., Housley,M.P., Smith,J.R., Andersen,Y.S., Martin,M.G., Hendrickson,B.A., Johansen,F.E., Svard,S.G., Gillin,F.D., and Eckmann,L. (2006). Polymeric immunoglobulin receptor in intestinal immune defense against the lumen-dwelling protozoan parasite Giardia. J. Immunol. *177*, 6281-6290.

de,O., I, de Araujo,A.N., Bao,S.N., and Giugliano,L.G. (2001). Binding of lactoferrin and free secretory component to enterotoxigenic Escherichia coli. FEMS Microbiol. Lett. *203*, 29-33.

Dieleman,L.A., Ridwan,B.U., Tennyson,G.S., Beagley,K.W., Bucy,R.P., and Elson,C.O. (1994). Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology *107*, 1643-1652.

Donaldson,D.S., Tong,K.K., and Williams,N.A. (2011). Mucosal administration of the B subunit of E. coli heat-labile enterotoxin promotes the development of Foxp3-expressing regulatory T cells. Mucosal. Immunol *4*, 227-238.

Duerkop,B.A., Vaishnava,S., and Hooper,L.V. (2009). Immune responses to the microbiota at the intestinal mucosal surface. Immunity. *31*, 368-376.

Duncan,S.H., Louis,P., and Flint,H.J. (2007). Cultivable bacterial diversity from the human colon. Lett. Appl. Microbiol. *44*, 343-350.

Eckburg,P.B., Bik,E.M., Bernstein,C.N., Purdom,E., Dethlefsen,L., Sargent,M., Gill,S.R., Nelson,K.E., and Relman,D.A. (2005). Diversity of the human intestinal microbial flora. Science *308*, 1635-1638.

Egger,B., Bajaj-Elliott,M., Macdonald,T.T., Inglin,R., Eysselein,V.E., and Buchler,M.W. (2000). Characterisation of acute murine dextran sodium sulphate colitis: cytokine profile and dose dependency. Digestion *62*, 240-248.

Endt,K., Stecher,B., Chaffron,S., Slack,E., Tchitchek,N., Benecke,A., Van,M.L., Sirard,J.C., Mueller,A.J., Heikenwalder,M., Macpherson,A.J., Strugnell,R., von,M.C., and Hardt,W.D. (2010). The microbiota mediates pathogen clearance from the gut lumen after non-typhoidal Salmonella diarrhea. PLoS Pathog. *6*.

Fagarasan,S., Muramatsu,M., Suzuki,K., Nagaoka,H., Hiai,H., and Honjo,T. (2002). Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. Science *298*, 1424-1427.

Fantini,M.C., Becker,C., Tubbe,I., Nikolaev,A., Lehr,H.A., Galle,P., and Neurath,M.F. (2006). Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. Gut *55*, 671-680.

Feng,T. and Elson,C.O. (2011). Adaptive immunity in the host-microbiota dialog. Mucosal. Immunol *4*, 15-21.

Flecknell,P. (2002). Replacement, reduction and refinement. ALTEX. *19*, 73-78.

Fontenot,J.D., Gavin,M.A., and Rudensky,A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. *4*, 330-336.

Frank,D.N., St Amand,A.L., Feldman,R.A., Boedeker,E.C., Harpaz,N., and Pace,N.R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc. Natl. Acad. Sci. U. S. A *104*, 13780-13785.

Franke,A., McGovern,D.P.B., Barrett,J.C., Wang,K., Radford-Smith,G.L., Ahmad,T., Lees,C.W., Balschun,T., Lee,J., Roberts,R., Anderson,C.A., Bis,J.C., Bumpstead,S., Ellinghaus,D., Festen,E.M., Georges,M., Green,T., Haritunians,T., Jostins,L., Latiano,A., Mathew,C.G., Montgomery,G.W., Prescott,N.J., Raychaudhuri,S., Rotter,J.I., Schumm,P., Sharma,Y., Simms,L.A., Taylor,K.D., Whiteman,D., Wijmenga,C., Baldassano,R.N., Barclay,M., Bayless,T.M., Brand,S., Buning,C., Cohen,A., Colombel,J.F., Cottone,M., Stronati,L., Denson,T., De Vos,M., D'Inca,R., Dubinsky,M., Edwards,C., Florin,T., Franchimont,D., Gearry,R., Glas,J., Van Gossum,A., Guthery,S.L., Halfvarson,J., Verspaget,H.W., Hugot,J.P., Karban,A., Laukens,D., Lawrance,I., Lemann,M., Levine,A., Libioulle,C., Louis,E., Mowat,C., Newman,W., Panes,J., Phillips,A., Proctor,D.D., Regueiro,M., Russell,R., Rutgeerts,P., Sanderson,J., Sans,M., Seibold,F., Steinhart,A.H., Stokkers,P.C.F., Torkvist,L., Kullak-Ublick,G., Wilson,D., Walters,T., Targan,S.R., Brant,S.R., Rioux,J.D., D'Amato,M., Weersma,R.K., Kugathasan,S., Griffiths,A.M., Mansfield,J.C., Vermeire,S., Duerr,R.H., Silverberg,M.S., Satsangi,J., Schreiber,S., Cho,J.H., Annese,V., Hakonarson,H., Daly,M.J., and Parkes,M. (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet *42*, 1118-1125.

Fujiwara,M. (2003). Inhibitory effects of Bifidobacterium longum on experimental ulcerative colitis induced in mice by synthetic dextran sulfate sodium. Digestion *67*, 90-95.

Gaboriau-Routhiau,V., Rakotobe,S., Lecuyer,E., Mulder,I., Lan,A., Bridonneau,C., Rochet,V., Pisi,A., De,P.M., Brandi,G., Eberl,G., Snel,J., Kelly,D., and Cerf-Bensussan,N. (2009). The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity. *31*, 677-689.

Garrett,W.S., Gallini,C.A., Yatsunenko,T., Michaud,M., DuBois,A., Delaney,M.L., Punit,S., Karlsson,M., Bry,L., Glickman,J.N., Gordon,J.I., Onderdonk,A.B., and Glimcher,L.H. (2010). Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. Cell Host. Microbe *8*, 292-300.

Garrett,W.S., Lord,G.M., Punit,S., Lugo-Villarino,G., Mazmanian,S.K., Ito,S., Glickman,J.N., and Glimcher,L.H. (2007). Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell *131*, 33-45.

Geissmann,F., Manz,M.G., Jung,S., Sieweke,M.H., Merad,M., and Ley,K. (2010). Development of Monocytes, Macrophages, and Dendritic Cells. Science *327*, 656-661.

Gewirtz,A.T., Navas,T.A., Lyons,S., Godowski,P.J., and Madara,J.L. (2001). Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J. Immunol. *167*, 1882-1885.

Gibbons,D.L. and Spencer,J. (2011). Mouse and human intestinal immunity: same ballpark, different players; different rules, same score. Mucosal. Immunol *4*, 148-157.

Gill,S.R., Pop,M., Deboy,R.T., Eckburg,P.B., Turnbaugh,P.J., Samuel,B.S., Gordon,J.I., Relman,D.A., Fraser-Liggett,C.M., and Nelson,K.E. (2006). Metagenomic analysis of the human distal gut microbiome. Science *312*, 1355-1359.

Glocker,E.O., Kotlarz,D., Boztug,K., Gertz,E.M., Schaffer,A.A., Noyan,F., Perro,M., Diestelhorst,J., Allroth,A., Murugan,D., Hatscher,N., Pfeifer,D., Sykora,K.W., Sauer,M., Kreipe,H., Lacher,M., Nustede,R., Woellner,C., Baumann,U., Salzer,U., Koletzko,S., Shah,N., Segal,A.W., Sauerbrey,A., Buderus,S., Snapper,S.B., Grimbacher,B., and Klein,C. (2009). Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N. Engl. J Med. *361*, 2033-2045.

Gordon,S. and Taylor,P.R. (2005). Monocyte and macrophage heterogeneity. Nat Rev. Immunol *5*, 953-964.

Gottfried,E., Kunz-Schughart,L.A., Weber,A., Rehli,M., Peuker,A., Muller,A., Kastenberger,M., Brockhoff,G., Andreesen,R., and Kreutz,M. (2008). Expression of CD68 in non-myeloid cell types. Scand. J Immunol *67*, 453-463.

Griffiths,A.M. (2004). Specificities of inflammatory bowel disease in childhood. Best. Pract. Res. Clin. Gastroenterol. *18*, 509-523.

Hans,W., Scholmerich,J., Gross,V., and Falk,W. (2000). The role of the resident intestinal flora in acute and chronic dextran sulfate sodium-induced colitis in mice. Eur. J. Gastroenterol. Hepatol. *12*, 267-273.

Hapfelmeier,S., Lawson,M.A., Slack,E., Kirundi,J.K., Stoel,M., Heikenwalder,M., Cahenzli,J., Velykoredko,Y., Balmer,M.L., Endt,K., Geuking,M.B., Curtiss,R., III, McCoy,K.D., and Macpherson,A.J. (2010). Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science *328*, 1705-1709.

He,B., Xu,W., Santini,P.A., Polydorides,A.D., Chiu,A., Estrella,J., Shan,M., Chadburn,A., Villanacci,V., Plebani,A., Knowles,D.M., Rescigno,M., and Cerutti,A. (2007). Intestinal bacteria trigger T cell-independent immunoglobulin A (2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity. *26*, 812-826.

Hem A, Eide DM, Engh E, and Smith A (2004). Kompendium i forsøksdyrlære.

Henderson,P., van Limbergen,J.E., Wilson,D.C., Satsangi,J., and Russell,R.K. (2011). Genetics of childhood-onset inflammatory bowel disease. Inflamm. Bowel. Dis. *17*, 346-361.

Hendrickson,B.A., Conner,D.A., Ladd,D.J., Kendall,D., Casanova,J.E., Corthesy,B., Max,E.E., Neutra,M.R., Seidman,C.E., and Seidman,J.G. (1995). Altered hepatic transport of immunoglobulin A in mice lacking the J chain. J Exp. Med. *182*, 1905-1911.

Herias,M.V., Koninkx,J.F., Vos,J.G., Huis,i., V, and van Dijk,J.E. (2005). Probiotic effects of Lactobacillus casei on DSS-induced ulcerative colitis in mice. Int. J. Food Microbiol. *103*, 143-155.

Holmen,N., Lundgren,A., Lundin,S., Bergin,A.M., Rudin,A., Sjovall,H., and Ohman,L. (2006). Functional CD4+CD25high regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. Inflamm. Bowel. Dis. *12*, 447-456.

Holness,C.L. and Simmons,D.L. (1993). Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood *81*, 1607-1613.

Hommes,D.W. and van Deventer,S.J.H. (2004). Endoscopy in inflammatory bowel diseases. Gastroenterology *126*, 1561-1573.

Hooper,L.V. and Macpherson,A.J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat. Rev. Immunol. *10*, 159-169.

Hooper,L.V., Midtvedt,T., and Gordon,J.I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu. Rev. Nutr. *22*, 283-307.

Hooper,L.V., Wong,M.H., Thelin,A., Hansson,L., Falk,P.G., and Gordon,J.I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. Science *291*, 881-884.

Hori,S., Nomura,T., and Sakaguchi,S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science *299*, 1057-1061.

Hudcovic,T., Stepankova,R., Cebra,J., and Tlaskalova-Hogenova,H. (2001). The role of microflora in the development of intestinal inflammation: acute and chronic colitis induced by dextran sulfate in germ-free and conventionally reared immunocompetent and immunodeficient mice. Folia Microbiol. (Praha) *46*, 565-572.

Ivanov,I.I., Frutos,R.L., Manel,N., Yoshinaga,K., Rifkin,D.B., Sartor,R.B., Finlay,B.B., and Littman,D.R. (2008). Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host. Microbe *4*, 337-349.

Janeway,C.A. (2005). Immunobiology
the immune system in health and disease. (New York: Garland Science).

Jiang,H.Q. (2004). Interactions of commensal gut microbes with subsets of B- and T-cells in the murine host. Vaccine *22*, 805-811.

Johansen,F.E., Braathen,R., and Brandtzaeg,P. (2000). Role of J chain in secretory immunoglobulin formation. Scand. J Immunol *52*, 240-248.

Johansen,F.E. and Brandtzaeg,P. (2004). Transcriptional regulation of the mucosal IgA system. Trends Immunol *25*, 150-157.

Johansen,F.E., Pekna,M., Norderhaug,I.N., Haneberg,B., Hietala,M.A., Krajci,P., Betsholtz,C., and Brandtzaeg,P. (1999). Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. J. Exp. Med. *190*, 915-922.

Johansson,M.E., Phillipson,M., Petersson,J., Velcich,A., Holm,L., and Hansson,G.C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc. Natl. Acad. Sci. U. S. A *105*, 15064-15069.

Johansson,M.E.V., Gustafsson,J.K., Sj+Âberg,K.E., Petersson,J., Holm,L., Sj+Âvall,H., and Hansson,G.C. (2010). Bacteria Penetrate the Inner Mucus Layer before Inflammation in the Dextran Sulfate Colitis Model. PLoS ONE *5*, e12238.

Josefowicz,S.Z. and Rudensky,A. (2009). Control of regulatory T cell lineage commitment and maintenance. Immunity. *30*, 616-625.

Kappelman,M.D., Rifas-Shiman,S.L., Porter,C.Q., Ollendorf,D.A., Sandler,R.S., Galanko,J.A., and Finkelstein,J.A. (2008). Direct Health Care Costs of Crohn's Disease and Ulcerative Colitis in US Children and Adults. Gastroenterology *135*, 1907-1913.

Karlsson,M.R., Johansen,F.E., Kahu,H., Macpherson,A., and Brandtzaeg,P. (2010). Hypersensitivity and oral tolerance in the absence of a secretory immune system. Allergy *65*, 561-570.

Kelsall,B. (2008). Recent progress in understanding the phenotype and function of intestinal dendritic cells and macrophages. Mucosal. Immunol *1*, 460-469.

Kelsen,J., Agnholt,J., Hoffmann,H.J., Romer,J.L., Hvas,C.L., and Dahlerup,J.F. (2005). FoxP3 (+)CD4 (+)CD25 (+) T cells with regulatory properties can be cultured from colonic mucosa of patients with Crohn's disease. Clin. Exp. Immunol. *141*, 549-557.

Khoruts,A. and Sadowsky,M.J. (2011). Therapeutic transplantation of the distal gut microbiota. Mucosal. Immunol *4*, 4-7.

Kitajima,S. (1999). Changes in colonic mucosal permeability in mouse colitis induced with dextran sulfate sodium. Exp. Anim. *48*, 137-143.

Kitajima,S. (2000). Histological analysis of murine colitis induced by dextran sulfate sodium of different molecular weights. Exp. Anim. *49*, 9-15.

Kitajima,S., Morimoto,M., Sagara,E., Shimizu,C., and Ikeda,Y. (2001). Dextran sodium sulfate-induced colitis in germ-free IQI/Jic mice. Exp. Anim *50*, 387-395.

Kobayashi,K.S., Chamaillard,M., Ogura,Y., Henegariu,O., Inohara,N., Nunez,G., and Flavell,R.A. (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science *307*, 731-734.

Koloski,N.A., Bret,L., and Radford-Smith,G. (2008). Hygiene hypothesis in inflammatory bowel disease: a critical review of the literature. World J Gastroenterol. *14*, 165-173.

Krummel,M.F. and Allison,J.P. (1996). CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. J Exp. Med. *183*, 2533-2540.

Kugathasan,S. and Cohen,S. (2008). Searching for new clues in inflammatory bowel disease: tell tales from pediatric IBD natural history studies. Gastroenterology *135*, 1038-1041.

Kugathasan,S. and Fiocchi,C. (2007). Progress in basic inflammatory bowel disease research. Semin. Pediatr. Surg. *16*, 146-153.

Kunisch,E., Fuhrmann,R., Roth,A., Winter,R., Lungershausen,W., and Kinne,R.W. (2004). Macrophage specificity of three anti-CD68 monoclonal antibodies (KP1, EBM11, and PGM1) widely used for immunohistochemistry and flow cytometry. Ann. Rheum. Dis. *63*, 774-784.

Lai,L., Alaverdi,N., Maltais,L., and Morse,H.C., III (1998). Mouse cell surface antigens: nomenclature and immunophenotyping. J Immunol *160*, 3861-3868.

Lamb,T.J., Graham,A.L., and Petrie,A. (2008). T testing the immune system. Immunity. *28*, 288-292.

Ley,R.E., Hamady,M., Lozupone,C., Turnbaugh,P.J., Ramey,R.R., Bircher,J.S., Schlegel,M.L., Tucker,T.A., Schrenzel,M.D., Knight,R., and Gordon,J.I. (2008). Evolution of mammals and their gut microbes. Science *320*, 1647-1651.

Ley,R.E., Peterson,D.A., and Gordon,J.I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell *124*, 837-848.

Loftus,E.V., Jr. (2004). Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology *126*, 1504-1517.

Lotz,M., Gutle,D., Walther,S., Menard,S., Bogdan,C., and Hornef,M.W. (2006). Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. J Exp. Med. *203*, 973-984.

Lund,F.E. and Randall,T.D. (2010). Effector and regulatory B cells: modulators of CD4 (+) T cell immunity. Nat. Rev. Immunol. *10*, 236-247.

Ma,A., Datta,M., Margosian,E., Chen,J., and Horak,I. (1995). T cells, but not B cells, are required for bowel inflammation in interleukin 2-deficient mice. J. Exp. Med. *182*, 1567-1572.

Maaser,C. (2004). Clearance of Citrobacter rodentium requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. Infect. Immun. *72*, 3315-3324.

Maaser,C., Housley,M.P., Iimura,M., Smith,J.R., Vallance,B.A., Finlay,B.B., Schreiber,J.R., Varki,N.M., Kagnoff,M.F., and Eckmann,L. (2004). Clearance of Citrobacter rodentium requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. Infect. Immun. *72*, 3315-3324.

Macpherson,A.J., Gatto,D., Sainsbury,E., Harriman,G.R., Hengartner,H., and Zinkernagel,R.M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science *288*, 2222-2226.

Macpherson,A.J., McCoy,K.D., Johansen,F.E., and Brandtzaeg,P. (2008). The immune geography of IgA induction and function. Mucosal. Immunol *1*, 11-22.

Macpherson,A.J. and Uhr,T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science *303*, 1662-1665.

Makita,S., Kanai,T., Oshima,S., Uraushihara,K., Totsuka,T., Sawada,T., Nakamura,T., Koganei,K., Fukushima,T., and Watanabe,M. (2004). CD4+CD25bright T cells in human intestinal lamina propria as regulatory cells. J. Immunol. *173*, 3119-3130.

Malaty,H.M., Fan,X., Opekun,A.R., Thibodeaux,C., and Ferry,G.D. (2010). Rising incidence of inflammatory bowel disease among children: a 12-year study. J. Pediatr. Gastroenterol. Nutr. *50*, 27-31.

Malek,T.R. and Castro,I. (2010). Interleukin-2 receptor signaling: at the interface between tolerance and immunity. Immunity. *33*, 153-165.

Maloy,K.J. and Kullberg,M.C. (2008). IL-23 and Th17 cytokines in intestinal homeostasis. Mucosal. Immunol. *1*, 339-349.

Marshall,L.J., Perks,B., Ferkol,T., and Shute,J.K. (2001). IL-8 released constitutively by primary bronchial epithelial cells in culture forms an inactive complex with secretory component. J Immunol *167*, 2816-2823.

Maslowski,K.M. and Mackay,C.R. (2011). Diet, gut microbiota and immune responses. Nat Immunol *12*, 5-9.

Maul,J., Loddenkemper,C., Mundt,P., Berg,E., Giese,T., Stallmach,A., Zeitz,M., and Duchmann,R. (2005). Peripheral and intestinal regulatory CD4+ CD25 (high) T cells in inflammatory bowel disease. Gastroenterology *128*, 1868-1878.

Mazmanian,S.K., Round,J.L., and Kasper,D.L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. Nature *453*, 620-625.

McGovern,D.P., Gardet,A., Torkvist,L., Goyette,P., Essers,J., Taylor,K.D., Neale,B.M., Ong,R.T., Lagace,C., Li,C., Green,T., Stevens,C.R., Beauchamp,C., Fleshner,P.R., Carlson,M., D'Amato,M., Halfvarson,J., Hibberd,M.L., Lordal,M., Padyukov,L., Andriulli,A., Colombo,E., Latiano,A., Palmieri,O., Bernard,E.J., Deslandres,C., Hommes,D.W., de Jong,D.J., Stokkers,P.C., Weersma,R.K., Sharma,Y., Silverberg,M.S., Cho,J.H., Wu,J., Roeder,K., Brant,S.R., Schumm,L.P., Duerr,R.H., Dubinsky,M.C., Glazer,N.L., Haritunians,T., Ippoliti,A., Melmed,G.Y., Siscovick,D.S., Vasiliauskas,E.A., Targan,S.R., Annese,V., Wijmenga,C., Pettersson,S., Rotter,J.I., Xavier,R.J., Daly,M.J., Rioux,J.D., and Seielstad,M. (2010). Genome-wide association identifies multiple ulcerative colitis susceptibility loci. Nat. Genet. *42*, 332-337.

Melgar,S., Karlsson,A., and Michaelsson,E. (2005). Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. Am. J. Physiol Gastrointest. Liver Physiol *288*, G1328-G1338.

Melgar,S., Karlsson,L., Rehnstrom,E., Karlsson,A., Utkovic,H., Jansson,L., and Michaelsson,E. (2008). Validation of murine dextran sulfate sodium-induced colitis using four therapeutic agents for human inflammatory bowel disease. Int. Immunopharmacol. *8*, 836-844.

Melmed,G., Thomas,L.S., Lee,N., Tesfay,S.Y., Lukasek,K., Michelsen,K.S., Zhou,Y., Hu,B., Arditi,M., and Abreu,M.T. (2003). Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. J Immunol *170*, 1406-1415.

Mennigen,R., Nolte,K., Rijcken,E., Utech,M., Loeffler,B., Senninger,N., and Bruewer,M. (2009). Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. Am. J Physiol Gastrointest. Liver Physiol *296*, G1140-G1149.

Mestas,J. and Hughes,C.C.W. (2004). Of Mice and Not Men: Differences between Mouse and Human Immunology. J Immunol *172*, 2731-2738.

Meyer-Hoffert,U., Hornef,M.W., Henriques-Normark,B., Axelsson,L.G., Midtvedt,T., Putsep,K., and Andersson,M. (2008). Secreted enteric antimicrobial activity localises to the mucus surface layer. Gut *57*, 764-771.

Miyara,M., Yoshioka,Y., Kitoh,A., Shima,T., Wing,K., Niwa,A., Parizot,C., Taflin,C., Heike,T., Valeyre,D., Mathian,A., Nakahata,T., Yamaguchi,T., Nomura,T., Ono,M., Amoura,Z., Gorochov,G., and Sakaguchi,S. (2009). Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity. *30*, 899-911.

Mizoguchi,A., Mizoguchi,E., Smith,R.N., Preffer,F.I., and Bhan,A.K. (1997). Suppressive role of B cells in chronic colitis of T cell receptor alpha mutant mice. J. Exp. Med. *186*, 1749-1756.

Mizoguchi,E., Mizoguchi,A., Preffer,F.I., and Bhan,A.K. (2000). Regulatory role of mature B cells in a murine model of inflammatory bowel disease. Int. Immunol. *12*, 597-605.

Montero,C. (2003). The antigen-antibody reaction in immunohistochemistry. J Histochem. Cytochem. *51*, 1-4.

Mosser,D.M. and Edwards,J.P. (2008). Exploring the full spectrum of macrophage activation. Nat Rev. Immunol *8*, 958-969.

Mottet,C., Uhlig,H.H., and Powrie,F. (2003). Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J. Immunol. *170*, 3939-3943.

Murai,M., Krause,P., Cheroutre,H., and Kronenberg,M. (2010). Regulatory T-cell stability and plasticity in mucosal and systemic immune systems. Mucosal. Immunol. *3*, 443-449.

Murthy,A.K., Dubose,C.N., Banas,J.A., Coalson,J.J., and Arulanandam,B.P. (2006). Contribution of polymeric immunoglobulin receptor to regulation of intestinal inflammation in dextran sulfate sodium-induced colitis. J. Gastroenterol. Hepatol. *21*, 1372-1380.

Murthy,S., Cooper,H.S., Yoshitake,H., Meyer,C., Meyer,C.J., and Murthy,N.S. (1999). Combination therapy of pentoxifylline and TNFalpha monoclonal antibody in dextran sulphate-induced mouse colitis. Aliment. Pharmacol. Ther. *13*, 251-260.

Nair,M.G., Guild,K.J., Du,Y., Zaph,C., Yancopoulos,G.D., Valenzuela,D.M., Murphy,A., Stevens,S., Karow,M., and Artis,D. (2008). Goblet cell-derived resistin-like molecule beta augments CD4+ T cell production of IFN-gamma and infection-induced intestinal inflammation. J. Immunol. *181*, 4709-4715.

Nelson,K.E., Weinstock,G.M., Highlander,S.K., Worley,K.C., Creasy,H.H., Wortman,J.R., Rusch,D.B., Mitreva,M., Sodergren,E., Chinwalla,A.T., Feldgarden,M., Gevers,D., Haas,B.J., Madupu,R., Ward,D.V., Birren,B.W., Gibbs,R.A., Methe,B., Petrosino,J.F., Strausberg,R.L., Sutton,G.G., White,O.R., Wilson,R.K., Durkin,S., Giglio,M.G., Gujja,S., Howarth,C., Kodira,C.D., Kyrpides,N., Mehta,T., Muzny,D.M., Pearson,M., Pepin,K., Pati,A., Qin,X., Yandava,C., Zeng,Q., Zhang,L., Berlin,A.M., Chen,L., Hepburn,T.A., Johnson,J., McCorrison,J., Miller,J., Minx,P., Nusbaum,C., Russ,C., Sykes,S.M., Tomlinson,C.M., Young,S., Warren,W.C., Badger,J., Crabtree,J., Markowitz,V.M., Orvis,J., Cree,A., Ferriera,S., Fulton,L.L., Fulton,R.S., Gillis,M.,

Hemphill,L.D., Joshi,V., Kovar,C., Torralba,M., Wetterstrand,K.A., Abouellleil,A., Wollam,A.M., Buhay,C.J., Ding,Y., Dugan,S., FitzGerald,M.G., Holder,M., Hostetler,J., Clifton,S.W., len-Vercoe,E., Earl,A.M., Farmer,C.N., Liolios,K., Surette,M.G., Xu,Q., Pohl,C., Wilczek-Boney,K., and Zhu,D. (2010). A catalog of reference genomes from the human microbiome. Science *328*, 994-999.

Ni,J., Chen,S.F., and Hollander,D. (1996). Effects of dextran sulphate sodium on intestinal epithelial cells and intestinal lymphocytes. Gut *39*, 234-241.

Nieuwenhuis,E.E. and Escher,J.C. (2008). Early onset IBD: what's the difference? Dig. Liver Dis. *40*, 12-15.

Ochoa-Reparaz,J., Mielcarz,D.W., Ditrio,L.E., Burroughs,A.R., Begum-Haque,S., Dasgupta,S., Kasper,D.L., and Kasper,L.H. (2010). Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. J Immunol *185*, 4101-4108.

Ohkusa,T., Okayasu,I., Tokoi,S., Araki,A., and Ozaki,Y. (1995). Changes in bacterial phagocytosis of macrophages in experimental ulcerative colitis. Digestion *56*, 159-164.

Okayasu,I., I (1990). A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology *98*, 694-702.

Orel,R., Kamhi,T., Vidmar,G., and Mamula,P. (2009). Epidemiology of pediatric chronic inflammatory bowel disease in central and western Slovenia, 1994-2005. J. Pediatr. Gastroenterol. Nutr. *48*, 579-586.

Osman,N., Adawi,D., Ahrne,S., Jeppsson,B., and Molin,G. (2004). Modulation of the effect of dextran sulfate sodium-induced acute colitis by the administration of different probiotic strains of Lactobacillus and Bifidobacterium. Dig. Dis. Sci. *49*, 320-327.

Otten,M.A. and van,E.M. (2004). The Fc receptor for IgA (FcalphaRI, CD89). Immunol Lett. *92*, 23-31.

Park,K.T. and Bass,D. (2010). Inflammatory bowel disease-attributable costs and cost-effective strategies in the United States: A review. Inflamm. Bowel. Dis.

Perminow, G. IBD in Children: Epidemiology and Mucosal Immunopathology. Inflammatory Bowel South Eastern Norway-II (IBSEN-II). 2010. University of Oslo.
Ref Type: Thesis/Dissertation

Perminow,G., Brackmann,S., Lyckander,L.G., Franke,A., Borthne,A., Rydning,A., Aamodt,G., Schreiber,S., and Vatn,M.H. (2009). A characterization in childhood inflammatory bowel disease, a new population-based inception cohort from South-Eastern Norway, 2005-07, showing increased incidence in Crohn's disease. Scand. J. Gastroenterol. *44*, 446-456.

Peterson,D.A., Frank,D.N., Pace,N.R., and Gordon,J.I. (2008). Metagenomic approaches for defining the pathogenesis of inflammatory bowel diseases. Cell Host. Microbe *3*, 417-427.

Peterson,D.A., McNulty,N.P., Guruge,J.L., and Gordon,J.I. (2007). IgA response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host. Microbe *2*, 328-339.

Phalipon,A. and Corthesy,B. (2003). Novel functions of the polymeric Ig receptor: well beyond transport of immunoglobulins. Trends Immunol *24*, 55-58.

Poritz,L.S., Garver,K.I., Green,C., Fitzpatrick,L., Ruggiero,F., and Koltun,W.A. (2007). Loss of the tight junction protein ZO-1 in dextran sulfate sodium induced colitis. J. Surg. Res. *140*, 12-19.

Pulford,K.A., Sipos,A., Cordell,J.L., Stross,W.P., and Mason,D.Y. (1990). Distribution of the CD68 macrophage/myeloid associated antigen. Int. Immunol *2*, 973-980.

Qin,J., Li,R., Raes,J., Arumugam,M., Burgdorf,K.S., Manichanh,C., Nielsen,T., Pons,N., Levenez,F., Yamada,T., Mende,D.R., Li,J., Xu,J., Li,S., Li,D., Cao,J., Wang,B., Liang,H., Zheng,H., Xie,Y., Tap,J., Lepage,P., Bertalan,M., Batto,J.M., Hansen,T., Le,P.D., Linneberg,A., Nielsen,H.B., Pelletier,E., Renault,P., Sicheritz-Ponten,T., Turner,K., Zhu,H., Yu,C., Li,S., Jian,M., Zhou,Y., Li,Y., Zhang,X., Li,S., Qin,N., Yang,H.,

Wang,J., Brunak,S., Dore,J., Guarner,F., Kristiansen,K., Pedersen,O., Parkhill,J., Weissenbach,J., Bork,P., Ehrlich,S.D., and Wang,J. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature *464*, 59-65.

Rajilic-Stojanovic,M., Heilig,H.G., Molenaar,D., Kajander,K., Surakka,A., Smidt,H., and de Vos,W.M. (2009). Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environ. Microbiol. *11*, 1736-1751.

Rakoff-Nahoum,S., Paglino,J., Eslami-Varzaneh,F., Edberg,S., and Medzhitov,R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell *118*, 229-241.

Rescigno,M. and Di,S.A. (2009). Dendritic cells in intestinal homeostasis and disease. J. Clin. Invest *119*, 2441-2450.

Robinson,J.K., Blanchard,T.G., Levine,A.D., Emancipator,S.N., and Lamm,M.E. (2001). A mucosal IgA-mediated excretory immune system in vivo. J Immunol *166*, 3688-3692.

Rugtveit,J., Nilsen,E.M., Bakka,A., Carlsen,H., Brandtzaeg,P., and Scott,H. (1997). Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. Gastroenterology *112*, 1493-1505.

sahi-Ozaki,Y., Yoshikawa,T., Iwakura,Y., Suzuki,Y., Tamura,S., Kurata,T., and Sata,T. (2004). Secretory IgA antibodies provide cross-protection against infection with different strains of influenza B virus. J Med. Virol. *74*, 328-335.

Sait,L., Galic,M., Strugnell,R.A., and Janssen,P.H. (2003). Secretory Antibodies Do Not Affect the Composition of the Bacterial Microbiota in the Terminal Ileum of 10-Week-Old Mice. Appl. Environ. Microbiol. *69*, 2100-2109.

Sait,L.C., Galic,M., Price,J.D., Simpfendorfer,K.R., Diavatopoulos,D.A., Uren,T.K., Janssen,P.H., Wijburg,O.L.C., and Strugnell,R.A. (2007). Secretory antibodies reduce systemic antibody responses against the gastrointestinal commensal flora. Int. Immunol. *19*, 257-265.

Sakaguchi,S., Miyara,M., Costantino,C.M., and Hafler,D.A. (2010). FOXP3+ regulatory T cells in the human immune system. Nat. Rev. Immunol. *10*, 490-500.

Salzman,N.H., Hung,K., Haribhai,D., Chu,H., Karlsson-Sjoberg,J., Amir,E., Teggatz,P., Barman,M., Hayward,M., Eastwood,D., Stoel,M., Zhou,Y., Sodergren,E., Weinstock,G.M., Bevins,C.L., Williams,C.B., and Bos,N.A. (2010). Enteric defensins are essential regulators of intestinal microbial ecology. Nat. Immunol. *11*, 76-83.

Sarra,M., Pallone,F., Macdonald,T.T., and Monteleone,G. (2010). IL-23/IL-17 axis in IBD. Inflamm. Bowel. Dis. *16*, 1808-1813.

Sartor,R.B. (2008). Microbial influences in inflammatory bowel diseases. Gastroenterology *134*, 577-594.

Saruta,M., Yu,Q.T., Fleshner,P.R., Mantel,P.Y., Schmidt-Weber,C.B., Banham,A.H., and Papadakis,K.A. (2007). Characterization of FOXP3+CD4+ regulatory T cells in Crohn's disease. Clin. Immunol. *125*, 281-290.

Sauer,C.G. and Kugathasan,S. (2009). Pediatric inflammatory bowel disease: highlighting pediatric differences in IBD. Gastroenterol. Clin. North Am. *38*, 611-628.

Schneeman,T.A., Bruno,M.E., Schjerven,H., Johansen,F.E., Chady,L., and Kaetzel,C.S. (2005). Regulation of the polymeric Ig receptor by signaling through TLRs 3 and 4: linking innate and adaptive immune responses. J Immunol *175*, 376-384.

Scholzen,T. and Gerdes,J. (2000). The Ki-67 protein: from the known and the unknown. J Cell Physiol *182*, 311-322.

Schultz,M. (2004). Preventive effects of Escherichia coli strain Nissle 1917 on acute and chronic intestinal inflammation in two different murine models of colitis. Clin. Diagn. Lab. Immunol. *11*, 372-378.

Schwiertz,A., Jacobi,M., Frick,J.S., Richter,M., Rusch,K., and Kohler,H. (2010). Microbiota in pediatric inflammatory bowel disease. J Pediatr. *157*, 240-244.

Scoville,D.H., Sato,T., He,X.C., and Li,L. (2008). Current view: intestinal stem cells and signaling. Gastroenterology *134*, 849-864.

Shale,M. and Ghosh,S. (2009). How intestinal epithelial cells tolerise dendritic cells and its relevance to inflammatory bowel disease. Gut *58*, 1291-1299.

Shevach,E.M. (2009). Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity. *30*, 636-645.

Shimada,S., Kawaguchi-Miyashita,M., Kushiro,A., Sato,T., Nanno,M., Sako,T., Matsuoka,Y., Sudo,K., Tagawa,Y., Iwakura,Y., and Ohwaki,M. (1999). Generation of polymeric immunoglobulin receptor-deficient mouse with marked reduction of secretory IgA. J. Immunol. *163*, 5367-5373.

Siegmund,B., Lehr,H.A., Fantuzzi,G., and Dinarello,C.A. (2001). IL-1 beta -converting enzyme (caspase-1) in intestinal inflammation. Proc. Natl. Acad. Sci. U. S. A *98*, 13249-13254.

Slack,E., Hapfelmeier,S., Stecher,B., Velykoredko,Y., Stoel,M., Lawson,M.A., Geuking,M.B., Beutler,B., Tedder,T.F., Hardt,W.D., Bercik,P., Verdu,E.F., McCoy,K.D., and Macpherson,A.J. (2009). Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. Science *325*, 617-620.

Smith,P.D., Ochsenbauer-Jambor,C., and Smythies,L.E. (2005). Intestinal macrophages: unique effector cells of the innate immune system. Immunol. Rev. *206*, 149-159.

Smith,P.D., Smythies,L.E., Shen,R., Greenwell-Wild,T., Gliozzi,M., and Wahl,S.M. (2010). Intestinal macrophages and response to microbial encroachment. Mucosal. Immunol.

Sokol,H., Pigneur,B., Watterlot,L., Lakhdari,O., Bermudez-Humaran,L.G., Gratadoux,J.J., Blugeon,S., Bridonneau,C., Furet,J.P., Corthier,G., Grangette,C., Vasquez,N., Pochart,P., Trugnan,G., Thomas,G., Blottiere,H.M., Dore,J., Marteau,P., Seksik,P., and Langella,P. (2008). Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc. Natl. Acad. Sci. U. S. A *105*, 16731-16736.

Sonnenburg,J.L., Angenent,L.T., and Gordon,J.I. (2004). Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nat. Immunol. *5*, 569-573.

Spor,A., Koren,O., and Ley,R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. Nat Rev. Microbiol. *9*, 279-290.

Stecher,B. and Hardt,W.D. (2008). The role of microbiota in infectious disease. Trends Microbiol. *16*, 107-114.

Strugnell,R.A. and Wijburg,O.L. (2010). The role of secretory antibodies in infection immunity. Nat. Rev. Microbiol. *8*, 656-667.

Sun,K., Johansen,F.E., Eckmann,L., and Metzger,D.W. (2004). An important role for polymeric Ig receptor-mediated transport of IgA in protection against Streptococcus pneumoniae nasopharyngeal carriage. J Immunol *173*, 4576-4581.

Suzuki,K. and Fagarasan,S. (2009). Diverse regulatory pathways for IgA synthesis in the gut. Mucosal. Immunol. *2*, 468-471.

Suzuki,K., Meek,B., Doi,Y., Muramatsu,M., Chiba,T., Honjo,T., and Fagarasan,S. (2004). Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc. Natl. Acad. Sci. U. S. A *101*, 1981-1986.

Talham,G.L., Jiang,H.Q., Bos,N.A., and Cebra,J.J. (1999). Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. Infect. Immun. *67*, 1992-2000.

te Velde,A.A., de,K.F., Sterrenburg,E., Pronk,I., ten Kate,F.J., Hommes,D.W., and van Deventer,S.J. (2007). Comparative analysis of colonic gene expression of three experimental colitis models mimicking inflammatory bowel disease. Inflamm. Bowel. Dis. *13*, 325-330.

Tivol,E.A., Borriello,F., Schweitzer,A.N., Lynch,W.P., Bluestone,J.A., and Sharpe,A.H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. *3*, 541-547.

Tjarnlund,A., Rodriguez,A., Cardona,P.J., Guirado,E., Ivanyi,J., Singh,M., Troye-Blomberg,M., and Fernandez,C. (2006). Polymeric IgR knockout mice are more susceptible to mycobacterial infections in the respiratory tract than wild-type mice. Int. Immunol *18*, 807-816.

Tokuhara,D., Yuki,Y., Nochi,T., Kodama,T., Mejima,M., Kurokawa,S., Takahashi,Y., Nanno,M., Nakanishi,U., Takaiwa,F., Honda,T., and Kiyono,H. (2010). Secretory IgA-mediated protection against V. cholerae and heat-labile enterotoxin-producing enterotoxigenic Escherichia coli by rice-based vaccine. Proc. Natl. Acad. Sci. U. S. A *107*, 8794-8799.

Travassos,L.H., Carneiro,L.A., Ramjeet,M., Hussey,S., Kim,Y.G., Magalhaes,J.G., Yuan,L., Soares,F., Chea,E., Le,B.L., Boneca,I.G., Allaoui,A., Jones,N.L., Nunez,G., Girardin,S.E., and Philpott,D.J. (2010). Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol *11*, 55-62.

Turnbaugh,P.J., Hamady,M., Yatsunenko,T., Cantarel,B.L., Duncan,A., Ley,R.E., Sogin,M.L., Jones,W.J., Roe,B.A., Affourtit,J.P., Egholm,M., Henrissat,B., Heath,A.C., Knight,R., and Gordon,J.I. (2009). A core gut microbiome in obese and lean twins. Nature *457*, 480-484.

Turner,J.R. (2009). Intestinal mucosal barrier function in health and disease. Nat. Rev. Immunol. *9*, 799-809.

Turunen,P., Kolho,K.L., Auvinen,A., Iltanen,S., Huhtala,H., and Ashorn,M. (2006). Incidence of inflammatory bowel disease in Finnish children, 1987-2003. Inflamm. Bowel. Dis. *12*, 677-683.

Uhlig,H.H. and Powrie,F. (2009). Mouse models of intestinal inflammation as tools to understand the pathogenesis of inflammatory bowel disease. Eur. J Immunol *39*, 2021-2026.

Uren,T.K., Johansen,F.E., Wijburg,O.L., Koentgen,F., Brandtzaeg,P., and Strugnell,R.A. (2003). Role of the polymeric Ig receptor in mucosal B cell homeostasis. J. Immunol. *170*, 2531-2539.

Uren,T.K., Wijburg,O.L., Simmons,C., Johansen,F.E., Brandtzaeg,P., and Strugnell,R.A. (2005). Vaccine-induced protection against gastrointestinal bacterial infections in the absence of secretory antibodies. Eur. J. Immunol. *35*, 180-188.

Vaishnava,S., Behrendt,C.L., Ismail,A.S., Eckmann,L., and Hooper,L.V. (2008). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc. Natl. Acad. Sci. U. S. A *105*, 20858-20863.

Van der,S.M., De Koning,B.A., De Bruijn,A.C., Velcich,A., Meijerink,J.P., Van Goudoever,J.B., Buller,H.A., Dekker,J., Van,S., I, Renes,I.B., and Einerhand,A.W. (2006). Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology *131*, 117-129.

Verdu,E.F., Bercik,P., Cukrowska,B., Farre-Castany,M.A., Bouzourene,H., Saraga,E., Blum,A.L., Corthesy-Theulaz,I., Tlaskalova-Hogenova,H., and Michetti,P. (2000). Oral administration of antigens from intestinal flora anaerobic bacteria reduces the severity of experimental acute colitis in BALB/c mice. Clin. Exp. Immunol. *120*, 46-50.

Walunas,T.L., Bakker,C.Y., and Bluestone,J.A. (1996). CTLA-4 ligation blocks CD28-dependent T cell activation. J Exp. Med. *183*, 2541-2550.

Wehkamp,J., Koslowski,M., Wang,G., and Stange,E.F. (2008). Barrier dysfunction due to distinct defensin deficiencies in small intestinal and colonic Crohn's disease. Mucosal. Immunol *1 Suppl 1*, S67-S74.

Wen,L., Ley,R.E., Volchkov,P.Y., Stranges,P.B., Avanesyan,L., Stonebraker,A.C., Hu,C., Wong,F.S., Szot,G.L., Bluestone,J.A., Gordon,J.I., and Chervonsky,A.V. (2008). Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature *455*, 1109-1113.

Wijburg,O.L., Uren,T.K., Simpfendorfer,K., Johansen,F.E., Brandtzaeg,P., and Strugnell,R.A. (2006). Innate secretory antibodies protect against natural Salmonella typhimurium infection. J. Exp. Med. *203*, 21-26.

Wildin,R.S., Smyk-Pearson,S., and Filipovich,A.H. (2002). Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J. Med. Genet. *39*, 537-545.

Willing,B.P., Russell,S.L., and Finlay,B.B. (2011). Shifting the balance: antibiotic effects on host-microbiota mutualism. Nat Rev. Microbiol. *9*, 233-243.

Xavier,R.J. and Podolsky,D.K. (2007). Unravelling the pathogenesis of inflammatory bowel disease. Nature *448*, 427-434.

Yu,Q.T., Saruta,M., Avanesyan,A., Fleshner,P.R., Banham,A.H., and Papadakis,K.A. (2007). Expression and functional characterization of FOXP3+ CD4+ regulatory T cells in ulcerative colitis. Inflamm. Bowel. Dis. *13*, 191-199.

Zhou,L., Chong,M.M., and Littman,D.R. (2009). Plasticity of CD4+ T cell lineage differentiation. Immunity. *30*, 646-655.

## ERRATUM

In **paper IV** "Altered mucosal homeostasis in mice lacking secretory antibodies", page 5, bottom paragraph, the stated number of genes differentially expressed between conventional pIgR KO mice and pIgR KO that have been gavaged antibiotics is wrong:

> "Strikingly, the number of differentially expressed genes between pIgR KO and wt mice was reduced to 27 when the conventional microbiota was suppressed by antibiotic treatment (Fig. 1A). We also compared differential gene expression between antibiotic-gavaged pIgR KO and pIgR KO with a conventional intestinal microbiota and found 208 genes more than 2-fold differentially expressed."

In **paper IV's** figure 1A, page 45, the number for the comparison between antibiotic-gavaged pIgR KO and pIgR KO with a conventional intestinal microbiota is given as 296. There is therefore a discrepancy between the results section, page 5, and the figure 1A that is referred to in the results section. The number in the figure is correct.

The second last sentence on page 5 should read:

> "We also compared differential gene expression between antibiotic-gavaged pIgR KO and pIgR KO with a conventional intestinal microbiota and found 296 genes more than 2-fold differentially expressed."

I

# Paper I

**Increased number and activation of colonic macrophages in pediatric patients with untreated Crohn's disease.**

G. Perminow, D. H. Reikvam, L.G. Lyckander, P. Brandtzaeg, M. Vatn, and H. S. Carlsen.

*Inflamm Bowel Dis*. 2009;15:1368-1378.

II

# Paper II

**Increase of regulatory T cells in ileal mucosa of untreated pediatric Crohn's disease patients.**

D. H. Reikvam, G. Perminow, L. G. Lyckander, J. M. Gran, P. Brandtzaeg, M. Vatn, and H. S. Carlsen.

*Scand J Gastroenterol*. 2011;46 (5):550-60. Epub 2011 Feb 1.

# Paper III

**Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression.**

D. H. Reikvam*, A. Erofeev*, A. Sandvik, V. Grcic, F. L. Jahnsen, P. Gaustad, K. D. McCoy, A. J. Macpherson, L. A. Meza-Zepeda, and F-E. Johansen.

*PLoS One.* 2011 Mar 21;6 (3):e17996.

III

OPEN ACCESS Freely available online

PLoS one

# Depletion of Murine Intestinal Microbiota: Effects on Gut Mucosa and Epithelial Gene Expression

**Dag Henrik Reikvam[1*☯], Alexander Erofeev[1☯], Anders Sandvik[1], Vedrana Grcic[1], Frode Lars Jahnsen[1,2], Peter Gaustad[3], Kathy D. McCoy[4], Andrew J. Macpherson[4], Leonardo A. Meza-Zepeda[5,6], Finn-Eirik Johansen[1,2]**

**1** Department of Pathology and Centre for Immune Regulation, University of Oslo, Oslo, Norway, **2** Department of Pathology, Oslo University Hospital Rikshospitalet, Oslo, Norway, **3** Institute of Microbiology, University of Oslo, Oslo, Norway, **4** Maurice Müller Laboratories, Department of Clinical Research, University of Bern, Bern, Switzerland, **5** Department of Tumor Biology, the Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway, **6** Norwegian Microarray Consortium, Department of Molecular Biosciences, University of Oslo, Oslo, Norway

## Abstract

***Background:*** Inappropriate cross talk between mammals and their gut microbiota may trigger intestinal inflammation and drive extra-intestinal immune-mediated diseases. Epithelial cells constitute the interface between gut microbiota and host tissue, and may regulate host responses to commensal enteric bacteria. Gnotobiotic animals represent a powerful approach to study bacterial-host interaction but are not readily accessible to the wide scientific community. We aimed at refining a protocol that in a robust manner would deplete the cultivable intestinal microbiota of conventionally raised mice and that would prove to have significant biologic validity.

***Methodology/Principal Findings:*** Previously published protocols for depleting mice of their intestinal microbiota by administering broad-spectrum antibiotics in drinking water were difficult to reproduce. We show that twice daily delivery of antibiotics by gavage depleted mice of their cultivable fecal microbiota and reduced the fecal bacterial DNA load by 400 fold while ensuring the animals' health. Mice subjected to the protocol for 17 days displayed enlarged ceca, reduced Peyer's patches and small spleens. Antibiotic treatment significantly reduced the expression of antimicrobial factors to a level similar to that of germ-free mice and altered the expression of 517 genes in total in the colonic epithelium. Genes involved in cell cycle were significantly altered concomitant with reduced epithelial proliferative activity *in situ* assessed by Ki-67 expression, suggesting that commensal microbiota drives cellular proliferation in colonic epithelium.

***Conclusion:*** We present a robust protocol for depleting conventionally raised mice of their cultivatable intestinal microbiota with antibiotics by gavage and show that the biological effect of this depletion phenocopies physiological characteristics of germ-free mice.

**Citation:** Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, et al. (2011) Depletion of Murine Intestinal Microbiota: Effects on Gut Mucosa and Epithelial Gene Expression. PLoS ONE 6(3): e17996. doi:10.1371/journal.pone.0017996

**Editor:** Markus Heimesaat, Charité, Campus Benjamin Franklin, Germany

**Received** October 12, 2010; **Accepted** February 21, 2011; **Published** March 21, 2011



**Funding:** The project was funded by the Norwegian Cancer Society and the Research Council of Norway. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: dag.henrik.reikvam@rr-research.no

☯ These authors contributed equally to this work.

## Introduction

The human gut harbors a microbiological community of $10^{14}$ bacteria consisting of more than a 1000 species whose collective genome (''microbiome'') outnumbers the human genome by more than a 100 fold[1,2]. The cross talk between the host and its intestinal microbiota is of obvious significance for the function of the intestines[3], and has also been shown to be important for immune-mediated diseases with extra-intestinal manifestations such as in allergy and asthma[4]. More recently the influence of the intestinal microbiota in experimental models for both type 1 diabetes, obesity, and multiple sclerosis has been demonstrated[5–7]. Microbe-associated molecular patterns (MAMPs) use an array of germ-line encoded pattern recognition receptors (PRRs) to activate signaling pathways in host cells [8,9]. Importantly, signaling via these receptors drive host inflammatory responses to microbes[10–12], but can also mediate mucosal homeostasis and integrity when the microbe or microbial product is delivered via the intestinal lumen to the intact epithelium[8,9]. Functional assessments of the intestinal microbiota's impact on the host is thus of great interest.

The intestinal epithelium, consisting of a single cell layer, composes the barrier between the host sterile environment and the bacteria-rich intestinal lumen. Having only a partially bacteria-free mucus layer as a mechanical shield[13], the intestinal epithelial cells (IECs) are in direct contact with the highly diverse microbiota[14,15]. The enterocytes express a wide range of PRRs whose functions and engagement by MAMPs are essential for the homeostasis of the intestinal mucosa[8,9]. The other way around, IECs are shown to modulate the microbiota through the secretion

of antimicrobial peptides[16] (and reviewed in ref[15]) and by facilitating the transport of secretory IgA[17,18]. Even though the close and mutual relationship between the intestinal microbiota and the IECs is demonstrated, very little knowledge exists about how the full gene expression repertoire in the colonic IECs is regulated by the presence of a complete conventional microbiota.

Germ-free animals bred in sterile environment may potentially be used for such comparative studies. However, establishing and running germ-free facilities is expensive and requires special expertise and infra-structure. Compared with animals living in a conventional microbiological environment, germ-free animals display an immature and underdeveloped lymphoid system[19].

A generally accessible alternative to using germ-free animals for studying host-microbe interaction *in vivo* is to deplete animals of their intestinal microbiota by using a combination of broad spectrum antibiotics administered *per os*. In a hallmark paper, Rakoff-Nahoum and colleagues delivered broad-spectrum antibiotics in the drinking water to demonstrate that intestinal MAMPs signaling via Toll-like receptors (TLRs) supported gut homeostasis[20]. However, many have found it difficult to reproduce this protocol, and researchers who have succeeded in making mice drink the antibiotics *ad libitum* have experienced increased baseline morbidity and mortality among some strains and genotypes of mice subjected to this protocol (W. Garrett, personal communication). Importantly, several published papers that report to have applied this intestinal microbiota depletion protocol describe incomplete depletion of the cultivable bacteria[21–23].

In order to compare the gene expression profiles of colonic IECs in the presence and absence of a complete intestinal microbiota, we determined to refine a protocol that would deplete mice of their cultivable intestinal microbiota in a predictable and reproducible manner while ensuring the health of the animals. We demonstrate that applying this refined protocol we obtain mice which resemble germ-free mice in terms of hypoplastic lymphoid tissue. Importantly, antibiotic-gavaged mice have significantly altered gene expression profiles of colonic IECs resembling those of germ-free mice and affecting a wide range of pathways.

## Results

### Delivering antibiotics by gavage ensures health of the mice

We attempted to deplete mice of their intestinal microbiota by providing ampicillin, vancomycin, neomycin, and metronidazol *ad libitum* in drinking water according to the previously published protocol[20]. However, BALB/c mice consistently refrained from drinking the concoction, presumably due to the foul taste of metronidazol (Figure 1A). Respecting the ethical standards of our animal facility and the humane end point of >20% loss of baseline body weight, we had to euthanize all mice receiving the full antibiotic combination (Figure 1B). Adding low caloric aspartame-based sweetener with the concoction in an attempt to mask the taste of the antibiotics also failed (Figure 1C, D). C57BL/6 mice displayed the same unwillingness as BALB/c mice to drink the antibiotic concoction provided *ad libitum* (data not shown).

To ensure a safe and stable delivery of the antibiotic concoction to every mouse subjected to the protocol we administered it by gavage every 12 hours. Due to occasional overgrowth of *Candida* spp. in pilot experiments (data not shown), we initiated our protocol with 3 days treatment of gavaging the antifungal substance amphotericin-B (1 mg/kg bodyweight (BW)) attempting to suppress fungal growth prior to starting the antibacterial treatment (Figure 2A). From day 3, ampicillin 1 mg/ml was added to drinking water and mice were gavaged every 12 hours with the antibiotic concoction consisting of vancomycin 50 mg/kg BW, neomycin 100 mg/kg BW, metronidazol 100 mg/kg BW, and amphotericin-B 1 mg/kg BW.

Gavaging mice did not inflict detectable distress or pain. Six weeks old mice given water gavage of a volume and frequency identical to the mice receiving the antibiotic concoction displayed unaltered weight gain compared with mice receiving water *ad libitum* only (Figure 2B). Some mice gavaged with antibiotic concoction experienced a transient weight loss on day 4–5 after introduction of the antibacterial therapy (Figure 2B). This transient weight loss we believe was caused by mice adjusting to ampicillin in drinking water rather than the gavaged concoction as we observed that mice receiving ampicillin in drinking water as only therapy had the same transient drop in body weight (Figure 1B). Within a week mice receiving the full antibiotic therapy had regained their weight compared with their untreated peers and appeared healthy.

### Antibiotic therapy by gavage effectively depletes cultivable microbiota and reduces fecal bacterial DNA load

Fecal bacteria of untreated mice were enumerated by cultivations of serial dilutions of resuspended fecal pellets on differential media (Figure 3A). Validation of successful depletion after the antibiotic gavage treatment was performed by cultivation of fecal pellets collected and handled aseptically on day 13 and 24. Due to limitations in the quantity of fresh fecal pellets that could be collected, the detection limit for this assay was set to 1 cfu/mg feces and mice with < 1cfu/mg feces were defined as successfully depleted. Only animals successfully depleted by this criterion were included in further characterization of antibiotic treated mice. At day 13, 86% of the mice subjected to the protocol displayed successful depletion of their cultivable aerobic and anaerobic fecal microbiota (Figure 3B). At day 24, the corresponding fraction was 74%. Thus, in depleted mice (<1 cfu/mg feces), we obtained a minimum of 100-fold reduction of cultivable aerobic Gram negative rods and $10^6$-fold reduction of cultivable aerobic Gram positive cocci as well as cultivable anaerobic fecal bacteria. A similar depletion efficacy was obtained in C57BL/6 mice as in BALB/c mice (data not shown).

As the majority of the intestinal microbiota is not cultivable[1] we estimated the load of bacterial DNA in feces by 16S rRNA gene quantitative polymerase chain reaction (qPCR). DNA from fecal pellets was isolated and the V2 and the V6 region of bacterial 16S rRNA genes amplified with degenerate primers targeted to conserved flanking sequences. Due to shedding of epithelial cells, feces contain host DNA. Taking advantage of this we quantified mouse DNA by qPCR and used this value to normalize the amount of bacterial 16S DNA present (Figure 3C). We found that all mice subjected to the depletion protocol had significantly reduced copy number of 16S rRNA genes in their feces: the level of bacterial DNA was similar in all samples of antibiotic treated mice and, on average, more than 400 fold less than the level in untreated mice demonstrating the effect of the microbiota depletion protocol.

### Depletion of gut microbiota produces a macroscopically germ free-like phenotype

Mice born and raised in a germ-free environment possess numerous characteristics distinguishing them from mice living in a conventional microbiological environment. Macroscopically, germ-free mice display hypoplastic secondary lymphoid organs, enlarged ceca, and reduced epithelial cell turnover[19].

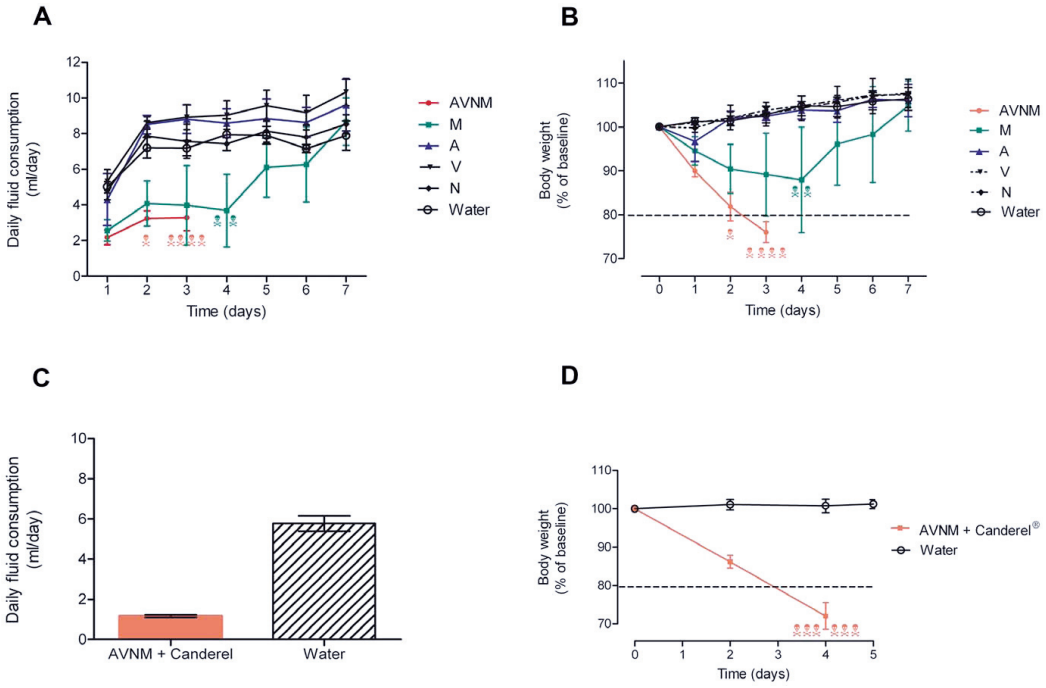



**Figure 1. Mice refrain from drinking antibiotics *ad libitum*.** Experiments attempting to administer mice ampicillin 1 g/l (A), vancomycin 500 mg/ml (V), neomycin 1 g/l (N), and metronidazol 1 g/l (M) in drinking water *ad libitum*. Water indicates mice receiving regular drinking water. Skulls represent euthanized animals. Stapled lines indicate humane endpoint of 20% loss of body weight. Treatment groups presented as mean ± SD. Deceased mice excluded from subsequent time points after death. (**A**) Daily fluid consumption estimated by daily weighting of flasks and (**B**) bodyweight presented as percent of baseline (= Day 0) for mice receiving the full AVNM concoction (red) in drinking water as well as for mice receiving the individual antibiotics as single solutions in drinking water, n = 5 for all groups. (**C**) Daily fluid consumption and (**D**) bodyweight presented as percent of baseline (= Day 0) attempting to mask the foul taste of the antibiotic concoction AVNM in drinking water by adding the aspartame-based sweetener Canderel® 1.5% weight/volume, n = 6 for AVNM + Canderel® 1.5% group, n = 4 for water control group.
doi:10.1371/journal.pone.0017996.g001

Mice verified to be successfully depleted after treatment with the antibiotic concoction by gavage for 17 days displayed significantly fewer Peyer's patches, smaller spleens and enlarged ceca (Figure 4A–D), macroscopically phenocopying germ-free mice.

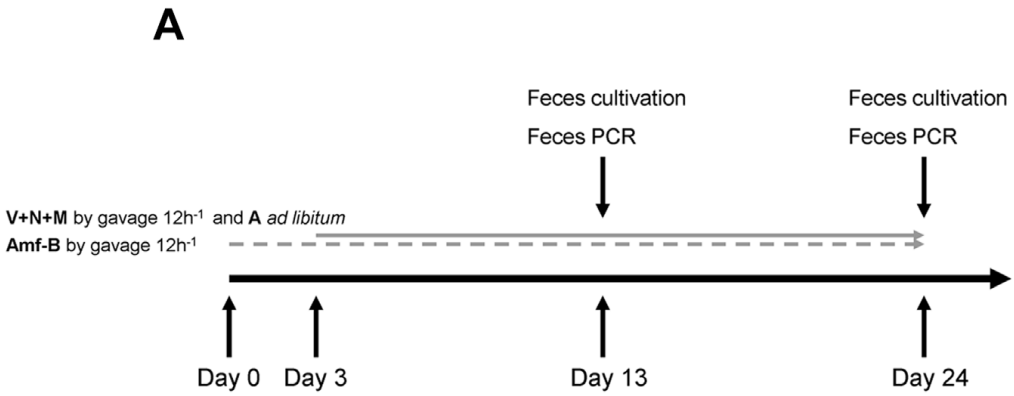
Immuno luorescent staining of sections from the colon of these same animals demonstrated that they had acquired a reduced epithelial regenerative activity in terms of fewer $Ki67^+$ cells than what was observed in mice with an intact intestinal microbiota (Figure 4E, F).

## Depletion of gut microbiota alters gene expression in colonic epithelium illustrative of germ-free mice

As we observed that the refined depletion protocol affected such a basic function of IECs as the regenerative activity, we wanted to study how the intestinal microbiota affected IECs globally by performing gene expression profile analysis on isolated colonic IECs of mice successfully depleted by the refined microbiota depletion protocol compared with that of sham treated control mice. We identified a total of 517 differentially expressed genes. However, many of these genes only showed a small alteration in expression level between antibiotic treated and control animals.

Focusing on genes with at least a 2-fold altered expression 36 genes were higher expressed and 70 genes showed reduced expression upon antibiotic treatment (Figure 5A). Gene ontology analysis revealed that expression of genes related to cell cycle and lipid biosynthesis were the most affected of the antibiotic treatment but also inflammatory response genes were significantly altered (Figure 5B). Strikingly, 5 of the 7 genes that had more than 4-fold reduced expression in antibiotic treated mice encode known antimicrobial factors[24–29] (Table 1).

To validate the gene expression profile and its relevance to germ-free mice we performed quantitative reverse transcriptase (qRT)-PCR on all nine genes that showed more than 4-fold differential expression by microarray analysis (Table 1). For all nine genes tested, the qRT-PCR confirmed the differential expression between antibiotic-gavged and sham-treated mice discovered in the array (Figure 6). In all cases, the fold difference between the two groups was greater when gene expression was determined by qRT-PCR than by micro array. Moreover, differential gene expression of colonic IEC from germ-free mice versus conventional age- and gender matched controls showed similar pattern as the antibiotic-gavaged versus sham treated mice. For most of the genes the antibiotic treated and germ-free mice showed similar expression levels (Figure 6).



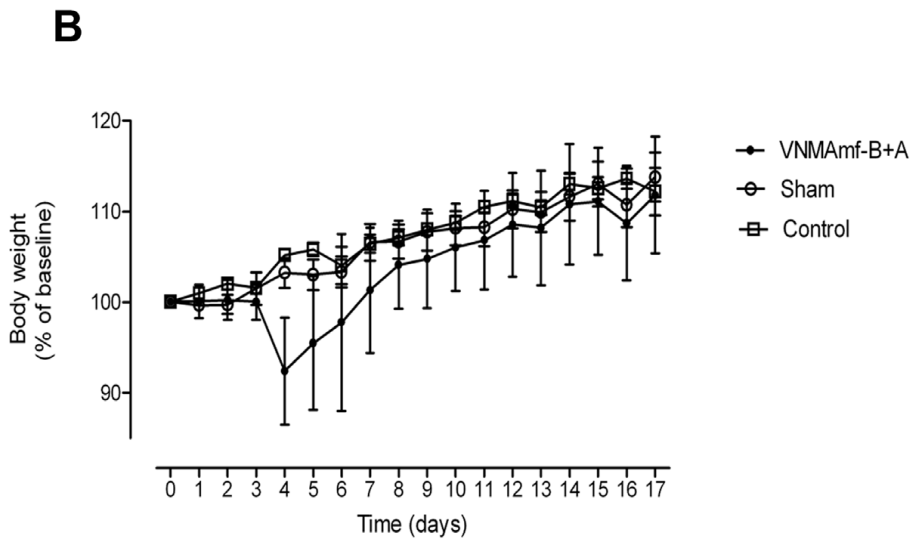
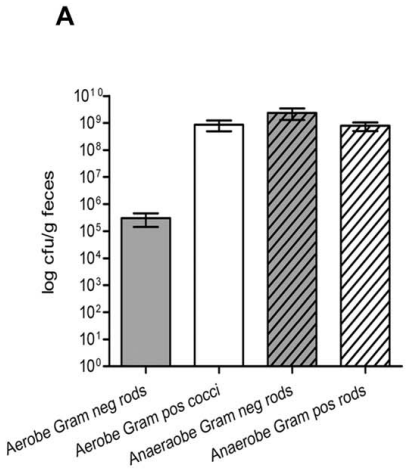
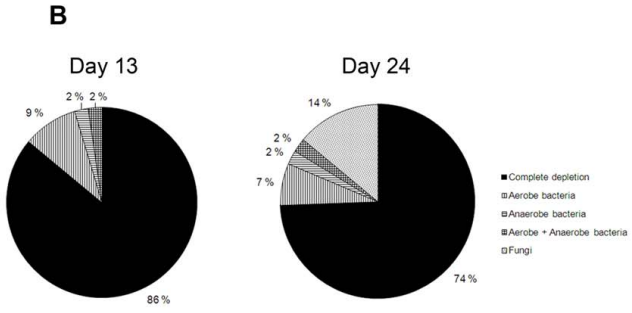




**Figure 2. Protocol for administering antibiotics by gavage ensures mice health.** (**A**) Sketch of protocol for gavage administration of vancomycin 50 mg/kg (V), neomycin 100 mg/kg (N), metronidazol 100 mg/kg (M), and amphotericin-B 1 mg/kg (Amf-B) every 12 hours with ampicillin 1 g/l *ad libitum* in drinking water (A). (**B**) Body weight, presented as percent of base line (= Day 0), of mice successfully depleted of cultivable fecal microbiota by gavage (VNMAmf-B+A) or gavaged with water in equivalent volume and frequency (Sham). Control mice received water *ad libitum* only
doi:10.1371/journal.pone.0017996.g002

## Discussion

Recently, there has been an increased understanding of the importance of intestinal microbiota for human physiology, evidenced by large endeavors such as the MetaHIT project[2] and the Human Microbiome Project[30]. However, simple protocols for manipulation of intestinal microbiota in experimental animals are also needed. Thus, the seemingly simple method of depleting mice of their cultivatable microbiota by adding a concoction of antibiotics in drinking water has gained a lot of


A
log cfu/g feces
Aerobe Gram neg rods
Aerobe Gram pos cocci
Anaeraobe Gram neg rods
Anaerobe Gram pos rods


B
Day 13
Day 24
9 %
2 %
2 %
86 %
14 %
2 %
2 %
7 %
74 %
Complete depletion
Aerobe bacteria
Anaerobe bacteria
Aerobe + Anaerobe bacteria
Fungi

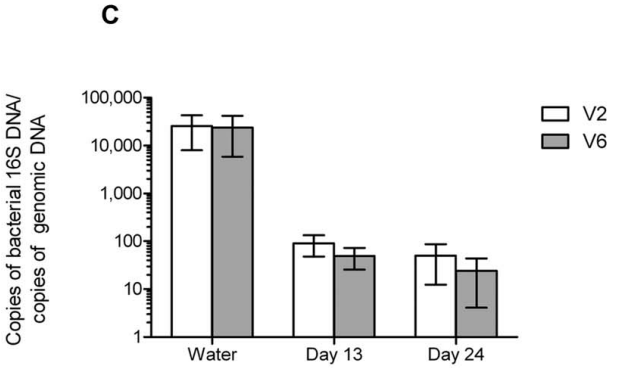

C
Copies of bacterial 16S DNA/ copies of genomic DNA
100,000
10,000
1,000
100
10
1
Water
Day 13
Day 24
V2
V6

**Figure 3. Protocol for administering antibiotics by gavage effectively reduces intestinal bacterial contents.** (**A**) Enumeration of cultivable fecal bacteria (mean ± SEM) in untreated mice. (**B**) Efficacy of successful depletion (<1 cfu/mg feces) of cultivable gut microbiota and distribution of aerobe, anaerobe, and fungal overgrowth (def.: ≥1 cfu/mg feces) after 13 and 24 days of treatment with vancomycinm (V), neomycin (N), metronidazol (M), and amphotericin-B (Amf-B) every 12 hours by gavage with ampicillin *ad libitum* in drinking water (A), n = 43. (**C**) Bacterial 16S DNA load (mean ± SD) in fecal pellets. 16S DNA determined by quantitative PCR for the V2 and V6 regions and normalized to total mouse genomic DNA in the same pellets of mice treated 13 and 24 days with VNMAmf-B +A compared with water controls, n = 7 for water controls, 40 for 13 days, and 35 for 24 days.
doi:10.1371/journal.pone.0017996.g003

attention, but also caused a lot of frustration among immunologists. Although some labs have successfully used the *ad libitum* protocol to obtain valuable data, it appears that it is not applicable to all mouse strains or genotypes and that it is affected by the general conditions in the vivarium. Here we have presented and validated an alternative method which deliver the antibiotics in a safe and predictable manner without inflicting any morbidity on the animals. Furthermore, we demonstrated that mice subjected to this depletion protocol acquire a germ free-like phenotype.

Our protocol for depletion of intestinal microbiota is based on the same composition of antibacterial agents as others have applied for *ad libitum* administration in drinking water. Ampicillin, vancomycin, neomycin and metronidazol offer in combination bactericidal activity against the full spectrum of bacteria and, notably, dual activity against both Gram positive (ampicillin and vancomycin) and Gram negative (ampicillin and neomycin) aerobic and facultative strains, which is potentially important for preventing antibiotic resistance [31]. We therefore used this combination of antibiotics and overcame the delivery problems by administering the antibiotics by gavage twice daily which the mice tolerated well.

In mice treated with the gavage protocol for 17 days we observed a reduction in the bacterial DNA load that was substantial though numerically not as great as the reduction observed in cultivation assays. This may reflect that the 16S DNA qPCR detect DNA from dead as well as viable bacteria. Also, a recent report demonstrated that bacterial DNA in the animal chow contributes to the 16S DNA load in fecal pellets[32]. However, the fact that the few fecal samples in our assays that contained cultivable bacteria did not distinguish themselves in terms of higher 16S DNA load than the successfully depleted mice (not shown) indicates that the qPCR assay is a more crude analysis.

Fecal pellets were used for evaluating the efficacy of the microbiota depletion protocol. Microbes that preferentially adhere to the mucosa of the mice are also affected by antibiotics[32] but would not be thoroughly assessed by analysis of fecal pellets. Even though the fecal pellets were devoid of cultivable bacteria and had greatly reduced amount of bacterial DNA we acknowledge that the intestines of the mice likely harbor residual bacteria and should not be regarded as sterile, as in true germ-free mice. However, collecting fecal pellets is non-invasive and allows for longitudinal studies. Contrary to this, collecting intestinal tissue from mice to confirm depletion of adherent bacteria would not be compatible with having the same mice entering functional experiments. Applying the gavage antibiotic depletion protocol had a profound effect on the phenotype of the mice as they developed several characteristics typical of germ-free mice. The reduced spleen, number of Peyer's patches, the enlarged ceca, and reduced epithelial proliferative activity in our treated mice is also observed in germ-free mice[19]. This suggests that these features are typical of under-stimulation by commensals and that they are reversible effects rather than a consequence of growing up germ free. Furthermore, both mucosal and systemic secondary lymphoid organs were affected by commensal depletion.

We assessed the impact of the intestinal microbiota on the colonic IEC's gene expression profile, which revealed that more than 500 genes were differentially expressed as a consequence of the antibiotic by gavage treatment. A number of genes related to cell cycle were differentially regulated between untreated and antibiotic treated mice, suggesting that the commensal microbiota regulate epithelial proliferation in the colon. Furthermore, pathways regulating lipid biosynthesis, specifically production of arachidonic acid, appeared to be affected by the commensal microbiota. This was due to reduced expression of several small secreted phospholipases from the phospholipase A2 family after antibiotic treatment. Recent reports suggest that these secreted phosphoslipases are not primarily involved in arachidonic acid production. Although they have enzymatic activity, they show no substrate specificity for arachidonic acid release[26,33]. Furthermore, secreted phospholipases have been shown to have antimicrobial activity[26,34].

In colonic IEC from germ free mice and matched conventional mice we determined the expression level of nine genes strongly affected by our antibiotics by gavage treatment. In eight out of nine cases, differences between antibiotic and sham treated mice were nearly identical to differences between germ-free and conventional mice strongly suggesting that the altered gene expression profile is a consequence of the highly reduced microbial stimulation of the colonic IECs. For these eight genes we rule out that altered gene expression due to antibiotic treatment was a consequence of a direct pharmacologic effect of the antibiotics. These comparisons support the validity of the antibiotic treatment as an analogue to germ-free status in mice.

The greatest fold change was seen in genes coding for antimicrobial factors, some of which (*Ang4*[24], *Retnlb*[25] *Reg3g*[28] and *Reg3b*[25]) have been demonstrated to be induced by microbial stimuli through mono-associating or conventionalizing germ-free mice or by comparing germ-free and conventionally bred mice. *Retnlb, Reg3g,* and *Reg3b* have also previously been shown to be down-regulated after antibiotic treatment[25,35]. These studies have been performed on samples from the small intestines and we now complement these by demonstrating the identical effect in the colon. We believe we are also the first to demonstrate that in the colon high expression of the antimicrobial factor *Pla2g2a* is dependent on the presence of the intestinal microbiota, which is incongruent to previous observations in the small intestines[24]. The reduced expression of antimicrobial factors in IEC from mice depleted of commensal microbiota (to levels similar to that of germ free mice) demonstrate that continuous presence of microbial stimuli is required to maintain normal expression level of these genes.

To our knowledge only one study has performed gene expression profile comparison on colonic IECs from germ-free and conventionally bread mice. In the infancy of array-based gene expression assays Fukushima *et al.* compared mRNA expression profiles of colonic IEC from germ-free mice with mice with conventional non-pathogenic microbiota and confirmed two genes downregulated and five genes upregulated in germ-free mice[36]. Of these seven genes four were similarly differentially expressed in our microbiota depleted mice. This comparison lends further support to our hypothesis that the differentially expressed genes in

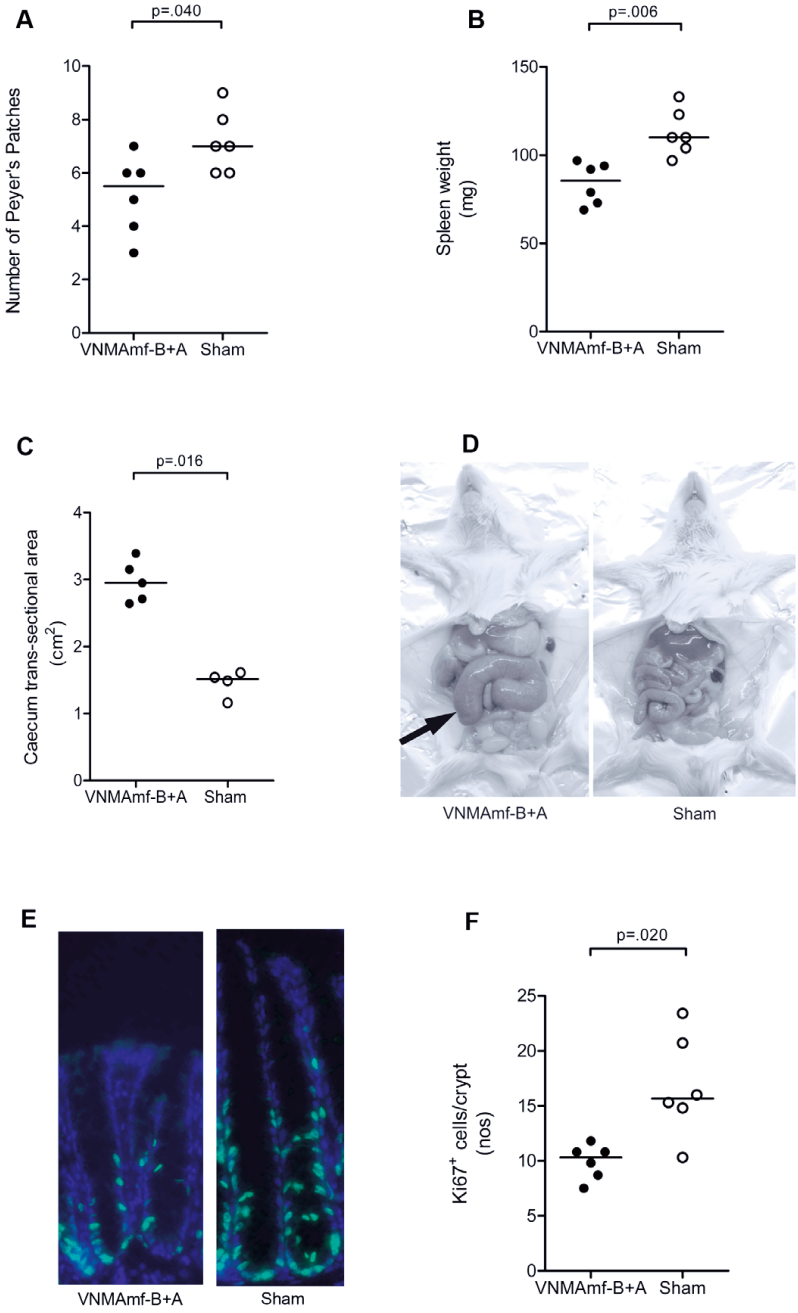

A
p=.040
Number of Peyer's Patches
10
8
6
4
2
0
VNMAmf-B+A
Sham
B
p=.006
Spleen weight (mg)
150
100
50
0
VNMAmf-B+A
Sham
C
p=.016
Caecum trans-sectional area (cm2)
4
3
2
1
0
VNMAmf-B+A
Sham
D
VNMAmf-B+A
Sham
E
VNMAmf-B+A
Sham
F
p=.020
Ki67+ cells/crypt (nos)
25
20
15
10
5
0
VNMAmf-B+A
Sham

**Figure 4. Mice subjected to antibiotic gavage treatment alters macroscopic phenotype and epithelial proliferative activity.** Numbers of macroscopically visible Peyer's patches (**A**), spleen weight (**B**), and cecal longitudinal cross section area (**C**), after 17 days of treatment with vancomycin (V), neomycin (N), metronidazol (M), and amphotericin-B (Amf-B) every 12 hours by gavage with ampicillin *ad libitum* in drinking water (A) compared to mice gavaged with water only (Sham). All VNMAmf-B+A treated mice exhibited successful depletion of cultivable intestinal microbiota (<1 cfu/mg feces). (**D**) Photograph of VNMAmf-B+A and sham fed mice. Note the enlarged cecum (arrow) of the antibiotic treated mouse. (**E**) Immunofluorescent staining for Ki67 (green) and DNA (Hoechst dye; blue) of cross sections of colons from VNMAmf-B+A fed mice and sham fed mice with (**F**) enumeration of $Ki67^+$ cells per crypt. In all graphs each symbol represents one animal and horizontal bars represent medians. Statistical differences calculated by Mann-Whitney two-tailed test.
doi:10.1371/journal.pone.0017996.g004

our antibiotic treated mice are caused by greatly reduced microbial stimuli.

Two recent publications reported to having gavaged mice with ampicillin, vancomycin, neomycin and metronidazol[7,32]. In line with our experiments, Hill *et al.* demonstrated the pitfall of the previously reported antibiotics *ad libitum* protocol and showed that gavaging mice with antibiotics once daily gave a significant reduction in bacterial 16S DNA load and altered gut phenotype[32]. Ochoa-Reparaz *et al.* demonstrated that mice gavaged with the antibiotics tolerated that treatment better than the *ad libitum* approach[7]. Our protocol applied a gavage frequency of twice daily and we used higher antibiotic concentrations than Hill *et al.* and Ochoa-Reparaz *et al.* Thus, we were able to reduce the fecal 16S DNA load by 400 fold while Hill *et al.* reported a 10-fold reduction. We have furthermore validated the gavage protocol demonstrating acquisition of a "germ free-like" phenotype of the subjected mice. We believe that our assessments of the macroscopic appearance of lymphoid organs and colonic IEC gene expression profile provide additional knowledge on how the intestinal microbiota affects a mammalian organism.

In conclusion, we present a generally accessible protocol for depleting mice of their cultivable intestinal microbiota by administering an antibiotic concoction by gavage every 12 hours. Our protocol showed increased feasibility compared with previous protocols, provided a predictable delivery of the antibiotics and at the same time ensuring the health of the animals. We have verified the depletion efficacy of our protocol both in terms of cultivable microbes and in terms of bacterial DNA load in feces. Finally, our protocol proved to produce a germ free-like phenotype of the animals subjected to the protocol, suggesting it to be a valid and accessible way to perform experiments on the host-bacteria cross talk in mice.

## Materials and Methods

### Ethics statement

All use of laboratory animals was approved by the National Animal Research Authority (Forsøksdyrutvalget) (approval IDs: 48/05, 1468, and 1734) and conducted in accordance with the Norwegian Animal Welfare Act and the Norwegian Regulation on Animal Experimentation. Humane endpoint was set to loss of >20% body weight compared with the starting weight in each experiment.

### Mice

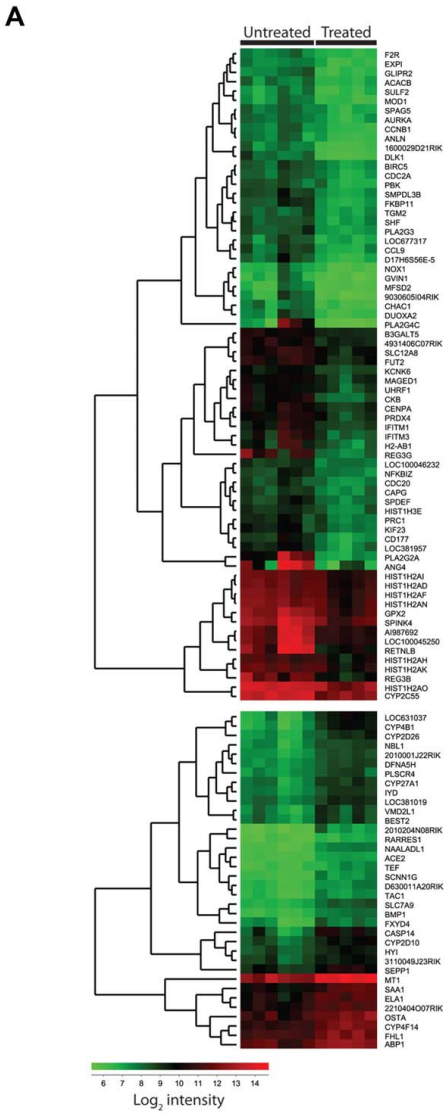
BALB/c mice were bred and kept in a conventional laboratory animal facility at Centre for Comparative Medicine, University of Oslo with temperatures maintained at 21°C and with 55% relative humidity, 12 hour light and darkness cycles with 1 hour of dusk and dawn. The mice received regular chow No. 3 (801080, Special Diets Services, Witham, England) and water purified by reverse osmosis and ionic exchange. Males 6–10 weeks of age were used in experiments, age and weight matched for each individual experiment to have an age span of maximum 2 weeks and a weight range of maximum ±20% of medians.

For reference controls in quantitative gene expression analyses 6 month old female BALB/c were obtained from the Clean Mouse Facility (CMF), Department of Clinical Research, University of Bern. Germ-free mice were bred and maintained in flexible film isolators and germ-free status was routinely confirmed by aerobic and anaerobic culture as well as DNA (sytox green; Invitrogen) and gram staining (Harleco) of cecal contents to detect unculturable contamination. Germ-free BALB/c mice were sacrificed under sterile conditions and tissue collection was performed at the University of Bern. Age- and gender matched conventionally kept controls were from Centre for Comparative Medicine, University of Oslo.

**Table 1.** Genes ≥ 4-fold differentially expressed in colonic epithelium of untreated conventional and antibiotic treated mice.

| Gene symbol | Product | Function[Reference] | Conventional/Antibiotic treated |
|---|---|---|---|
| *Ang4* | Angiogenin, ribonuclease A family, member 4 | AMF[24] | 17.8 |
| *Pla2g2a* | Phospholipase A2, group IIA | AMF[26,34] | 14.4 |
| *Retnlb* | Resistin like beta | AMF[25,27,40] | 8.8 |
| *Pla2g4c* | Phospholipase A2, group IVC | AA metabolism?[41] | 8.6 |
| *Reg3g* | Regenerating islet-derived 3 gamma | AMF[25,28] | 7.6 |
| *Reg3b* | Regenerating islet-derived 3 beta | AMF[25] | 5.1 |
| *Gsdmc2* | Gasdermin C2 | Unknown[42] | 4.1 |
| … | … | … | … |
| *Casp14* | Caspase 14 | Proteinase?[43] | 0.23 |
| *Cyp4b1* | Cytochrome P450, family 4, subfamily b, polypeptide 1 | Metabolism of carcinogens?[44] | 0.16 |

AMF, antimicrobial factor. AA, arachidonic acid.
doi:10.1371/journal.pone.0017996.t001


A
Untreated
Treated
F2R
EXPI
GLIPR2
ACACB
SULF2
MOD1
SPAG5
AURKA
CCNB1
ANLN
1600029D21RIK
DLK1
BIRC5
CDC2A
PBK
SMPDL3B
FKBP11
TGM2
SHF
PLA2G3
LOC677317
CCL9
D17HIS56E-5
NOX1
GVIN1
MFSD2
9030605I04RIK
CHAC1
DUOXA2
PLA2G4C
B3GALT5
4931406C07RIK
SLC12A8
FUT2
KCNK6
MAGED1
UHRF1
CKB
CENPA
PRDX4
IFITM1
IFITM3
H2-AB1
REG3G
LOC100046232
NFKBIZ
CDC20
CAPG
SPDEF
HIST1H3E
PRC1
KIF23
CD177
LOC381957
PLA2G2A
ANG4
HIST1H2AI
HIST1H2AD
HIST1H2AF
HIST1H2AN
GPX2
SPINK4
AI987692
LOC100045250
RETNLB
HIST1H2AH
HIST1H2AK
REG3B
HIST1H2AO
CYP2C55
LOC631037
CYP4B1
CYP2D26
NBL1
2010001J22RIK
DPYASH
PLSCR4
CYP27A1
IYD
LOC381019
VMD2L1
BEST2
2010204N08RIK
RARRES1
NAALADL1
ACE2
TEF
SCNN1G
D630011A20RIK
TAC1
SLC7A9
BMP1
FXYD4
CASP14
CYP2D10
HYI
3110049J23RIK
SEPP1
MT1
SAA1
ELA1
2210404O07RIK
OSTA
CYP4F14
FHL1
ABP1
6 7 8 9 10 11 12 13 14
Log2 intensity

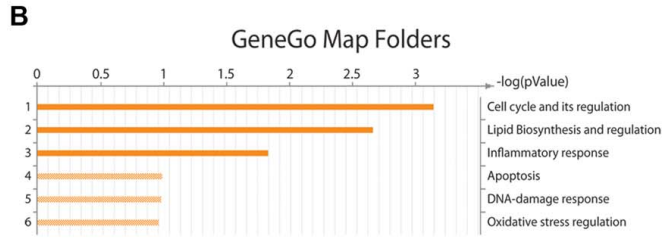

B
GeneGo Map Folders
0 0.5 1 1.5 2 2.5 3
-log(pValue)
1 Cell cycle and its regulation
2 Lipid Biosynthesis and regulation
3 Inflammatory response
4 Apoptosis
5 DNA-damage response
6 Oxidative stress regulation

**Figure 5. Antibiotic gavage treatment alters gene expression profile of the colonic epithelium.** Gene expression profile of colonic epithelial cells isolated from mice subjected to 17 days of treatment with vancomycin, neomycin, metronidazol, and amphotericin-B every 12 hours by gavage with ampicillin *ad libitum* in drinking water (Treated) compared to mice gavaged with water only (Untreated). All Treated mice exhibited successful depletion of cultivable intestinal microbiota (<1 cfu/mg feces). (**A**) Heat-map analysis of significantly ($p<0.05$) differentially expressed genes with >two-fold change organized in dendrogram according to Eukledian relation of the differentially expressed genes. Colors indicate absolute expression of one gene estimated by mean values of the multiple probes detecting each gene on the chip. Each row represent one gene, each column represent one mouse. (**B**) Gene ontology map folder enrichment analysis of differentially expressed genes in MetaCore (GeneGo, St Joseph, MI).
doi:10.1371/journal.pone.0017996.g005

## Antibiotic treatment protocol

Antibiotic treatment started with three days of amphotericin-B (Bristol Meyers Squibb, New York City, NY) 0.1 mg/ml, administered by gavage $12h^{-1}$ (Figure 2A). From day three, water flasks were supplemented with ampicillin (Bristol Meyers Squibb, New York City, NY) 1 g/l and antibiotic concoction consisting of vancomycin (Abbot, Abbot Park, IL) 5 mg/ml, neomycin (Invitrogen, Carlsbad, CA) 10 mg/ml, metronidazol (Actavis, Hafnarfjordur, Iceland) 10 mg/ml, and amphotericin-B (Bristol Meyers Squibb) 0.1 mg/ml was administered by antibiotic gavage $12h^{-1}$. Gavage volume of 10 ml/kg body weight was delivered with a stainless steel tube without prior sedation of the mice. Fresh antibiotic concoction was mixed every day and ampicillin and water was renewed every 7th day.

## Bacterial cultivation of feces

Day 13 and day 24 of the antibiotic treatment mice were fixed to defecate directly into a pre-weighted 2 ml capped microtube (Sarstedt, Nümbrect, Germany) prefilled with 1 ml sterile ice-cold phosphate-buffered saline (PBS). Tubes with fecal pellets were kept on ice, weighed and the weight of the pellets calculated (median 46 mg, range 17–120). Fecal pellets were resuspended in the 1 ml PBS by vortexing and by bashing with a sterile bacteriological loop. The fecal suspension was then plated on blood agar, anaerobic blood agar (hemin – vitamin K agar), and yeast agar (Sabouraud agar) in doubles with 100 µl suspension on each plate. Blood agar and Sabouraud agar plates were incubated aerobically at 37°C with 5% $CO_2$ for 72 hours, while anaerobic blood agar plates were incubated at 37°C in anaerobic conditions for

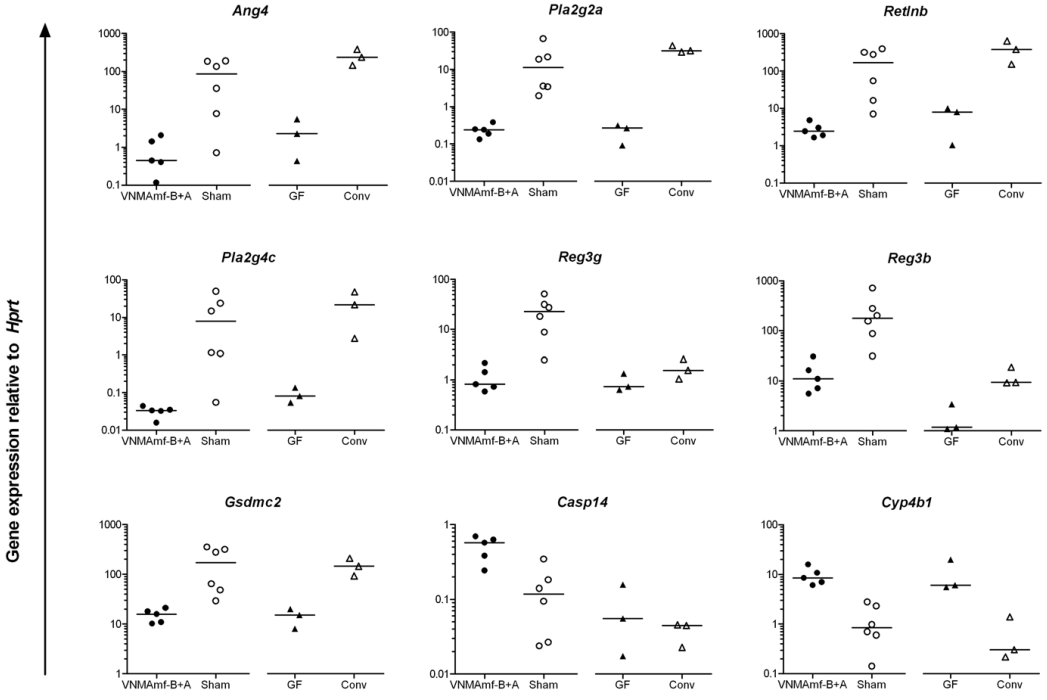



**Figure 6. Quantitative PCR validates colonic IEC gene expression profiles of antibiotic treatment in relation to germ-free mice.** Quantitative reverse transcriptase PCR of colonic intestinal epithelial cells (IEC) isolated from mice subjected to 17 days of treatment with vancomycin (V), neomycin (N), metronidazol (M), and amphotericin-B (Amf-B) every 12 hours by gavage with ampicillin (A) *ad libitum* in drinking water (VNMAmf-B+A) compared to mice gavaged with water only (Sham) and from colonic IEC of untreated germ-free mice (GF) and their conventional controls (Conv). Target gene listed on top of each panel and plotted in relation to the house-keeping gene *Hprt*. Each symbol represents one mouse, horizontal bars represent medians. Mann-Whitney analyses between VNMAmf-B+A and Sham, $p<0.05$ for all genes.
doi:10.1371/journal.pone.0017996.g006

96 hours. At the end of incubation the numbers of colonies on the plates were counted and the number of bacteria per mg of feces calculated. Evaluation of cultivated agar plates was performed by an experienced bacteriologist (P.G.) The detection limit of the assay was defined as 1 cfu/mg feces. Only mice successfully depleted (<1 cfu/mg feces) were included in phenotypic and gene expression analyses.

As a positive control for the depletion verification assay, and to enumerate cultivable microbes with the fecal collection procedure, fecal pellets from untreated mice were collected with the above described procedure. Serial dilutions made in sterile PBS and suitable dilutions were plated on selective media for intestinal Gram negative rods, enterococci, anaerobic Gram negative rods (*Bacteroides* spp), *Clostridium* spp, *Lactobacillus* spp and *Bifidobacterium* spp. The aerobic agar plates were incubated in 37°C with 5% $CO_2$ for 48 hours while anaerobic agar plates were incubated in 37°C for 48 hours. After incubation the numbers of colonies on the plates were counted and the number of bacteria per mg of feces was calculated.

## 16S rRNA gene quantification

In the same procedure as fecal pellets were collected for cultivation, the mice were let to defecate directly into a dry 1.5 ml capped microtube (Sarstedt, Nümbrect, Germany) which was snap frozen in liquid nitrogen and stored at −70°C until use.

DNA was isolated from bacterial fecal pellets with QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) and quantified by spectrophotometry at 260 nm. Degenerate primers for V2 and V6 region of bacterial 16S genes were as previously described[37,38] and primers for mouse genomic DNA was designed with Primer3 (frodo.wi.mit.edu) (Table 2). All PCR reactions were carried out with 50 ng of DNA, EvaGreen® (Biotium, Hayward, California) and Taq2000 in a Stratagene MX3000P with a 15 min activation step (95°C); then 40 cycles of 30 s denaturation (95°C), 30 s annealing (60°C for 16S-V2 and mpIgR-genomic, 55°C for 16S-V6), and 30 s extension (72°C). The efficiency of each PCR was determined by dilution series of template. The number of 16S DNA copies was related to the number of mouse genomic DNA copies for each sample. Samples with a threshold crossing point >32 cycles for the genomic PCR were deemed poorly amplifiable and excluded from analysis.

## Organ evaluation

After 17 days of antibiotic treatment mice were anaesthetized with 150 µl Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanison

**Table 2.** PCR primers used for amplification of bacterial 16S, mouse genomic DNA, and colonic IEC target genes.

| Target | Primer name | Sequence |
|---|---|---|
| 16S V2 region | 16S-V2-101F[37] | AGYGGCGIACGGGTGAGTAA |
| | 16S-V2-361R | CYIACTGCTGCCTCCCGTAG |
| 16S V6 region | 16S-V6-784F[38] | AGGATTAGATACCCTGGTA |
| | 16S-V6-1061R | CRRCACGAGCTGACGAC |
| Mouse pIgR genomic region | mpIgRgenomic.for | TTTGCTCCTGGGCCTCCAAGTT |
| | mpIgRgenomic.rev | AGCCCGTGACTGCCACAAATCA |
| Angiogenin, ribonuclease A family, member 4 | Ang4.for | TCTCCAGGAGCACACAGCTA |
| | Ang4.rev | AAGGACATGGGCTCATTGTC |
| Phospholipase A2, group IIA | Pla2g2a.for | CTGTTGCTACAAGAGCCTGG |
| | Pla2g2a.rev | TTTTCTTGTTCCGGGCGAAA |
| Resistin like beta | Retnlb.for | AGGATCAAGGAAGCTCTCAGTC |
| | Retnlb.rev | ATTTCCATTCCGGATATCCCA |
| Phospholipase A2, group IVC | Pla2g4c.for | AAGGCTCTCAGACTGTGGAG |
| | Pla2g4c.rev | GCCCACAGTACCCTGAAAAC |
| Regenerating islet-derived 3 gamma | Reg3g.for | ACATCAACTGGGAGACGAATC |
| | Reg3g.rev | TTTGGGATCTTGCTTGTGGCTA |
| Regenerating islet-derived 3 beta | Reg3b.for | CCTTAGACCGTGCTTTCTGTG |
| | Reg3b.rev | GTCCATGATGCTCTTCAAGACA |
| Gasdermin C2 | Gsdmc2.for | GGACCTGGAGGCTAACTTGA |
| | Gsdmc2.rev | CCTTTCCATCCGGCAAAACT |
| Caspase 14 | Casp14.for | ATCTCAGGAGAAGCTTGGGG |
| | Casp14.rev | TCTGGCTTTCAGCACCTTTG |
| Cytochrome P450, family 4, subfamily b, polypeptide 1 | Cyp4b1.for | TGATCCTGATGGTAACCGTCC |
| | Cyp4b1.rev | CATAGGGGAACTGTTCGGTC |
| Hypoxanthine guanine phosphoribosyl transferase | HPRT.for | TGATCAGTCAACGGGGGACA |
| | HPRT.rev | TTCGAGAGGTCCTTTTCACCA |
| Beta 2 microglobulin | B2m.for | TGACCGGCCTGTATGCTATC |
| | B2m.rev | GCAGTTCAGTATGTTCGGCT |

Target region, name and sequence of primers used in this study. The matching forward and reverse primers targeted to mouse cDNA lie in separate exons of each gene.
doi:10.1371/journal.pone.0017996.t002

10 mg/ml, VetaPharma Ltd, Leeds, UK) and midazolam (5 mg/ml, B. Braun Melsungen AG, Melsungen, Germany) subcutaneously and sacrificed by cardiac puncture. Abdominal organs were swiftly dissected and evaluated by a technician blinded to the treatment of the individual animal. Macroscopically visible Peyer's patches along the entire length of the small intestines from the ventricle to the ileocecal junction were counted. Cecum was gently put on its side along a ruler, digitally photographed and with the software analySISpro (Olympus Europa, Hamburg, Germany) the longitudinal cross section area of the cecum was calculated. The intact spleen was excised, abdominal fat gently removed, and the spleen was put in 10% formalin for 24 h, and then removed from the formalin, excess liquid wiped off with a filter paper, and the spleen was weighted.

### Immunohistochemistry

Colons of sacrificed mice were swiftly dissected and flushed with 2× 10 ml of ice cold PBS. A 1.5 cm piece of the middle colon was cut off and instantly fixed in 10% formalin for 24 h at 4°C before being automatically processed (TP 1050, Leica Microsystems, Wetzlar, Germany) and embedded in paraffin. Sections were cut at 4 µm thickness, deparaffinized and incubated 20 minutes in preheated 0.05% citraconic anhydride pH 7.4 at 98°C. After cooling to room temperature the sections were incubated 18 h with rabbit anti-Ki67 (Abcam, Cambridge, UK) or isotype control (rabbit anti-hemocyanin, Sigma, St. Louis, MR) at 4°C. After washing with PBS (pH 7.4) sections were incubated 90 minutes with Alexa Fluor 488 conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) at room temperature. After another washing in PBS sections were stained with Hoechst dye before being rinsed in water and mounted with PVA under cover slips.

Slides were evaluated by one examiner (D.H.R) a blinded fashion with the help of microscope (Eclipse E800, Nikon, Tokyo, Japan) fitted with a digital camera and imaging software (AnalySISpro 3.2, Olympus Soft Imaging System GmbH, Münster, Germany). Enumeration of $Ki67^+$ cells were performed by calculating the mean number of positive cells in 5–8 crypts in each transverse section ensuring crypts were cut to display the full crypt heights.

### Isolation of colonic IECs and microarray

After 17 days of antibiotic treatment mice were anaesthetized with 150 µl Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanison 10 mg/ml, VetaPharma Ltd) and midazolam (5 mg/ml, B. Braun Meslungen AG) subcutaneously and bled to death by cardiac puncture. Colon was swiftly excised and flushed with 2× 10 ml ice cold PBS (w/o $Mg^{2+}$ and $Ca^{2+}$) and kept moist. Mesenteric and adipose tissue was removed from the colon which was subsequently opened longitudinally, then cut transversally in 5 cm long pieces and incubated 25 min in 25 ml 20 mM EDTA (Sigma-Aldrich, St. Louis, MO) at room temperature on a shaker before being vigorously hand shaken for 5 minutes. The colonic IECs were harvested in a fresh tube, washed twice in ice cold PBS, resuspended in TRI Reagent® (Ambion Applied Biosystems, Foster City, CA), and stored at −70°C until RNA isolation according to manufacturer's instructions.

Gene expression profiling was performed at the Microarray Core Facility at The Norwegian Radium Hospital (Oslo, Norway). Total RNA corresponding to samples of 5 microbiota depleted mice and 6 control mice were labeled and hybridized to Illumina's MouseWG-6 v2.0 Expression BeadChips (Illumina, Inc., San Diego, CA) according to manufacturer's protocol. Normalization and statistical analysis of gene expression was performed in R (www.r-project.org) using Bioconductor packages and differentially expressed genes were identified using moderated t-statistics with an adjusted p-value $<0.05$. Differentially expressed genes were further analyzed in MetaCore (GeneGo, St Joseph, MI) to identify functional enrichment. Process and pathways were selected based on a p-value $< 0.05$. The complete gene expression dataset can be viewed in the Gene Expression Omnibus (GEO) repository accession number GSE22648 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE22648). Data submitted complied with MIAME standards.

### Quantitative RT-PCR

Epithelial cells and mRNA were isolated as described above. cDNA was synthesized with Superscript™ III Reverse transcriptase (Invitrogen) and 20 pmol/µl oligo(dT) according to manufacturer's protocol. The PCR primers were selected with the "Pick PCR primer" tool available from NCBI (http://www.ncbi.nlm.nih.gov) making sure each primer pair was separated by an intron on genomic DNA. The optimal $Mg^{++}$ concentration was determined empirically for each primer pair and PCR performed with EvaGreen® (Biotium, Hayward, California) and Taq2000 in a Stratagene MX3000P with a 15 min activation step (95°C); then 35 cycles of 30 s denaturation (95°C), 30 s annealing (60°), and 30 s extension (72°C). The median value of triplets were used to calculate relative expression of each gene according to the ΔΔCt method[39]. Normalization to the housekeeping genes *hprt* or β2-microglobulin gave similar results.

### Statistics

Where appropriate, statistical differences were calculated by Mann-Whitney two- tailed test using the software Prism5 (GraphPad Softvare Inc., La Jolla, CA).

## Acknowledgments

We thank Linda Manley for technical assistance with the laboratory animals, Linda Solfjell for molecular bioengineering, Aaste Aursjø for immunohistochemistry handcraft, Anne Kilsund Axelssen for all bacteriological work, and the FUGE funded Microarray Core Facility for providing gene expression services.

## Author Contributions

Performed the experiments: DHR AE AS VG PG KM AM LAM-Z F-EJ. Analyzed the data: DHR LAM-Z F-EJ. Contributed reagents/materials/analysis tools: FLJ PG KM AM LAM-Z F-EJ. Edited manuscript: DHR AS VG FLJ PG KM LAM-Z F-EJ.

## References

1. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, et al. (2006) Metagenomic analysis of the human distal gut microbiome. Science 312: 1355–1359.
2. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, et al. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464: 59–65.
3. Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 9: 313–323.
4. Noverr MC, Huffnagle GB (2004) Does the microbiota regulate immune responses outside the gut? Trends Microbiol 12: 562–568.
5. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, et al. (2008) Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 455: 1109–1113.
6. Backhed F, Manchester JK, Semenkovich CF, Gordon JI (2007) Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A 104: 979–984.

7. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, et al. (2009) Role of gut commensal microflora in the development of experimental autoimmune encephalomyelitis. J Immunol 183: 6041–6050.
8. Abreu MT (2010) Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol 10: 131–144.
9. Lavelle EC, Murphy C, O'Neill LA, Creagh EM (2010) The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. Mucosal Immunol 3: 17–28.
10. Rhee SH, Im E, Riegler M, Kokkotou E, O'brien M, et al. (2005) Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. Proc Natl Acad Sci U S A 102: 13610–13615.
11. Khan MA, Ma C, Knodler LA, Valdez Y, Rosenberger CM, et al. (2006) Toll-like receptor 4 contributes to colitis development but not to host defense during Citrobacter rodentium infection in mice. Infect Immun 74: 2522–2536.
12. Fukata M, Vamadevan AS, Abreu MT (2009) Toll-like receptors (TLRs) and Nod-like receptors (NLRs) in inflammatory disorders. Semin Immunol 21: 242–253.
13. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, et al. (2008) The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A 105: 15064–15069.
14. Artis D (2008) Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol 8: 411–420.
15. Duerkop BA, Vaishnava S, Hooper LV (2009) Immune responses to the microbiota at the intestinal mucosal surface. Immunity 31: 368–376.
16. Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, et al. (2010) Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol 11: 76–83.
17. Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, et al. (2004) Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A 101: 1981–1986.
18. Peterson DA, McNulty NP, Guruge JL, Gordon JI (2007) IgA response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host Microbe 2: 328–339.
19. Smith K, McCoy KD, Macpherson AJ (2007) Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin Immunol 19: 59–69.
20. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118: 229–241.
21. Fagarasan S, Muramatsu M, Suzuki K, Nagaoka H, Hiai H, et al. (2002) Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. Science 298: 1424–1427.
22. Garrett WS, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, et al. (2007) Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell 131: 33–45.
23. Bollyky PL, Bice JB, Sweet IR, Falk BA, Gebe JA, et al. (2009) The toll-like receptor signaling molecule Myd88 contributes to pancreatic beta-cell homeostasis in response to injury. PLoS One 4: e5063.
24. Hooper LV, Stappenbeck TS, Hong CV, Gordon JI (2003) Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol 4: 269–273.
25. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV (2008) Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc Natl Acad Sci U S A 105: 20858–20863.
26. Birts CN, Barton CH, Wilton DC (2010) Catalytic and non-catalytic functions of human IIA phospholipase A2. Trends Biochem Sci 35: 28–35.
27. Herbert DR, Yang JQ, Hogan SP, Groschwitz K, Khodoun M, et al. (2009) Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection. J Exp Med 206: 2947–2957.
28. Cash HL, Whitham CV, Behrendt CL, Hooper LV (2006) Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science 313: 1126–1130.
29. Dann SM, Eckmann L (2007) Innate immune defenses in the intestinal tract. Curr Opin Gastroenterol 23: 115–120.
30. Nelson KE, Weinstock GM, Highlander SK, Worley KC, Creasy HH, et al. (2010) A catalog of reference genomes from the human microbiome. Science 328: 994–999.
31. Kollef MH (2005) Bench-to-bedside review: antimicrobial utilization strategies aimed at preventing the emergence of bacterial resistance in the intensive care unit. Crit Care 9: 459–464.
32. Hill DA, Hoffmann C, Abt MC, Du Y, Kobuley D, et al. (2010) Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol 3: 148–158.
33. Balsinde J, Winstead MV, Dennis EA (2002) Phospholipase A(2) regulation of arachidonic acid mobilization. FEBS Lett 531: 2–6.
34. Nevalainen TJ, Graham GG, Scott KF (2008) Antibacterial actions of secreted phospholipases A2. Review. Biochim Biophys Acta 1781: 1–9.
35. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, et al. (2008) Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature 455: 804–807.
36. Fukushima K, Ogawa H, Takahashi K, Naito H, Funayama Y, et al. (2003) Non-pathogenic bacteria modulate colonic epithelial gene expression in germ-free mice. Scand J Gastroenterol 38: 626–634.
37. Sundquist A, Bigdeli S, Jalili R, Druzin ML, Waller S, et al. (2007) Bacterial flora-typing with targeted, chip-based Pyrosequencing. BMC Microbiol 7: 108.
38. Andersson AF, Lindberg M, Jakobsson H, Backhed F, et al. (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One 3: e2836.
39. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.
40. Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, et al. (2004) RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. Proc Natl Acad Sci U S A 101: 13596–13600.
41. Underwood KW, Song C, Kriz RW, Chang XJ, Knopf JL, et al. (1998) A Novel Calcium-independent Phospholipase A2, cPLA2-+???, That Is Prenylated and Contains Homology to cPLA2. J Biol Chem 273: 21926–21932.
42. Tamura M, Tanaka S, Fujii T, Aoki A, Komiyama H, et al. (2007) Members of a novel gene family, Gsdm, are expressed exclusively in the epithelium of the skin and gastrointestinal tract in a highly tissue-specific manner. Genomics 89: 618–629.
43. Van de CM, van LG, Pype S, Van CW, Van db, I, Molemans F, et al. (1998) Identification of a new caspase homologue: caspase-14. Cell Death Differ 5: 838–846.
44. Baer BR, Rettie AE (2006) CYP4B1: an enigmatic P450 at the interface between xenobiotic and endobiotic metabolism. Drug Metab Rev 38: 451–476.

# Paper IV

**Altered mucosal homeostasis in mice lacking secretory antibodies**

D. H. Reikvam, A. Erofeev, R. Islam, M. Derrien, V. Grcic, A. Sandvik, P. Gaustad, L. A. Meza-Zepeda, F. L. Jahnsen, H. Smidt, and F-E. Johansen.

*Submitted*. 2011

IV

# Paper V

**Absence of the polymeric immunoglobulin receptor protects B cell-deficient mice from colitis.**

D. H. Reikvam, A. Erofeev, A. Sandvik, C. Bang, E. Bækkevold, F. L. Jahnsen, and F-E. Johansen.

*Manuscript.* 2011

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