Dynamic changes in T cell compartments and new approaches in evaluating DSS induced and Gαi2 deficient colitis

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Printed by Vasastadens Bokbinderi,
Göteborg, Sweden 2007

ISBN 978-91-628-7279-3

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The overall aim of this thesis was to increase the understanding of the immunopathology of Inflammatory Bowel Disease (IBD). The first aim was to elucidate how the thymus and the gut epithelium were affected by colitis. The second aim was to investigate new ways of assessing and monitoring colitis. Two mouse models of colitis were used, the dextran sodium sulfate (DSS) induced model and the Gαi2 deficient (Gαi2$^{-/-}$) mouse model, which spontaneously develops colitis. These two models were compared throughout the study.

Colitis-induced changes were analysed in thymocytes and intestinal intraepithelial lymphocytes (IEL). To monitor and evaluate colitis, cultures of mouse and human colonic tissue were set up and the colon wall thickness was measured by micro-Computed Tomography (micro-CT).

During acute DSS induced colitis, the thymocytes were shifted towards a more mature phenotype, with loss of double positive (DP) thymocytes, paralleled by an increase in the absolute number of double negative (DN1) thymocytes. These changes were transient and returned to normal as the mice recovered or progressed into the chronic phase. In colitic Gαi2$^{-/-}$ mice, CD4^{+} IELs increased in the large intestine, while CD4^{+}CD8αα^{+} DP IELs increased in the small intestine. The dynamic changes in thymocyte and IEL composition demonstrates that colitis affect other T cell compartments than the colon.
Thymic involution and the increase in immature DN1 thymocytes during acute colitis may result in an increased export of immature T cells to the gut. The different responses in the small and large intestine during colitis suggest that the two microenvironments induce either an uncontrolled inflammation in the large intestine or suppression in the small intestine.

Approximately 75% of the genes detected in DSS induced and Gαi2$^{-/-}$ colitic mice were similarly regulated in *ex vivo* cultures and *in vivo*, and belonged to cytokines and T and B cell markers. A similar gene profile was obtained in human UC *ex vivo* cultures compared to mouse. Measurements of the colon wall in DSS treated mice demonstrated a significantly thicker colon wall during the acute phase of colitis compared to healthy controls, and correlated to the macroscopic scoring of colitis. The similar gene expression profile in mouse and human cultures and the finding that colon wall thickness can be used to identify responding animals support the relevance of these systems in monitoring colitis and evaluating new substances for the treatment of IBD.

Finally, this study points to the fact that chemically induced and spontaneously developing mouse models of colitis have several characteristics in common, such as thymic involution and expression of similar immune-related genes during colitis.

Key words: colitis, Gαi2$^{-/-}$ mice, dextran sodium sulfate, *ex vivo* cultures, micro-Computed Tomography, IEL, thymus, Inflammatory Bowel Disease

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):

I. **Maria Fritsch Fredin, Kristina Elgbratt, David Svensson, Liselotte Jansson, Silvia Melgar and Elisabeth Hultgren Hörnquist,** 2007
Dextran sulfate sodium induced colitis generates a transient thymic involution - impact on thymocyte subsets. *Scand J Immunol 2007;65:421-429.*

II. **Maria Fritsch Fredin, Roger Willén, Liselotte Jansson and Elisabeth Hultgren Hörnquist**
Regional alterations in intraepithelial cells in Gαi2 deficient colitis and $RAG^{-/-}$ recipients of peripheral T cells from colitic donor mice.
Manuscript

III. **Maria Fritsch Fredin*, Alexander Vidal*, Helena Utkovic, Yu-Yuan Götlind, Roger Willén, Liselotte Jansson, Elisabeth Hultgren Hörnquist and Silvia Melgar**
Ex vivo cultures and its relevance for assessment of treatment of inflammatory bowel disease: Comparative studies in DSS induced and Gαi2 deficient colitis and human ulcerative colitis. *Submitted*
**Both authors contributed equally*

IV. **Maria Fritsch Fredin, Leif Hultin, Gina Hyberg, Erika Rehnström, Elisabeth Hultgren Hörnquist, Silvia Melgar and Liselotte Jansson**
Predicting and monitoring colitis development in mice by Micro-Computed Tomography. *Accepted for publication in Inflammatory Bowel Diseases*

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LIST OF ABBREVIATIONS

7AAD	7-amino-actinomycin D
AZ	AstraZeneca
CD	Crohn's disease
CD(X)	cluster of differentiation (X)
CRP	C-reactive protein
CT	computed tomography
DC	dendritic cell
DN	double negative
DP	double positive
DSS	dextran sodium sulfate
FACS	fluorescence activated cell sorter
Gαi2	G-alpha-inhibitory-2
GC	glucocorticoid
GU	Göteborg University
HPA	hypothalamic-pituitary-adrenal
IBD	inflammatory bowel disease
IEL	intraepithelial lymphocyte
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLN	mesenteric lymph node
PP	Peyer's patch
RAG	recombinant activating gene
RNA	ribonucleic acid
RT-PCR	reverse transcriptase - polymerase chain reaction
SCID	severe combined immunodeficiency
SED	sub-epithelial dome
SP	single-positive
TCR	T cell receptor
Th	T helper
TNBS	tri-nitro benzene sulfonic acid
T_R	regulatory T cell
tg	transgene
UC	ulcerative colitis
wt	wild type
+/+	wild type
+/-	heterozygous for gene deficiency
-/-	homozygous for gene deficiency (knockout)

INTRODUCTION

THE IMMUNE SYSTEM

The immune system has evolved to protect the host from invading pathogens like viruses, bacteria and parasites. At the same time it has to allow uptake of food antigens and accept the presence of commensal bacteria in the gut. The immense task of discriminating between beneficial and harmful components has resulted in a complicated network of cells and organs keeping the immune system at equilibrium. In addition, the powerful immune responses towards pathogenic insults, often aiming at killing the enemy has to be balanced back to a normal "peaceful" state, not harming endogenous cells or organs.

The immune system can be divided into the innate and adaptive immune system (reviewed in (1)). The innate and the adaptive immune systems were for a long time considered to be two separate arms of immunity, independent of each other, but more recent research have shown that they are closely linked (2). The innate system consist of e.g., macrophages, dendritic cells, neutrophils and mast cells responding quickly to pathogenic insults in a rather non-specific manner (3). These cells produce high levels of cytokines and chemokines with one of the important tasks being to attract cells of the adaptive immune system, i.e., T and B cells (lymphocytes), to sites of inflammation (4). The adaptive immune system develops in primary lymphoid organs (thymus and bone marrow). Each T and B cell is unique in that the genes encoding T cell receptors (TCR) (on T cells) and immunoglobulins (Ig) (on B cells) are rearranged to generate an enormous amount of unique receptors, each recognising different antigens. It has been estimated that the total potential of the TCR and Ig repertoire are 10^{11} and 10^{16}, respectively (5). T cells migrate to the lymph nodes and there use their TCR to specifically recognise antigens which have been processed and presented as peptides bound to major histocompatibility (MHC) complex molecules on the surface of antigen-presenting cells (APCs), e.g., dendritic cells, macrophages and B cells. T cells can stimulate B cells to secrete immunoglobulins with the same specificity as the membrane-bound Ig molecules upon activation. Different classes of antibodies exist, where e.g., IgG is the most common class in the serum involved in recognition and clearance of pathogens, while IgA is secreted into the lumen of the gastrointestinal and respiratory tract. IgA binds to microbes and toxins present in the lumen and neutralise them by blocking their entry into the host (5). After the first encounter of a given antigen, memory T and B cells are formed that can generate a more rapid and enhanced secondary immune response during reinfection.

It is necessary to mount an immune response towards invading pathogens like viruses or bacteria but it is equally important that once the pathogen is cleared from the host, the activated state of the immune system subsides and returns to its normal, resting state. The delicate task of keeping the immune system in balance sometimes fail (6), resulting in uncontrolled immune responses, sometimes chronic such as in inflammatory bowel disease (IBD). The cause of the aberrant immune response

resulting in IBD is not fully understood and several animal models have been developed to elucidate these events (7).

This thesis focuses on the thymus, where T cells develop, and the intestinal immune system, a site where both maturation and effector functions of T cells take place. Two mouse models of colitis with different aetiological origin have been used, the chemically induced dextran sodium sulfate (DSS) model (8-10) and the gene targeted Gαi2 deficient ($G\alpha i2^{-/-}$) model that spontaneously develop colitis (11, 12). Despite very dissimilar causes of colitis, our current data suggest that once the inflammation is established the two models share many interesting similarities in respect to cellular and tissue changes and ability to respond to certain anti-inflammatory drugs.

T CELL DEVELOPMENT

INTRATHYMIC T CELL DEVELOPMENT

The thymus gland is situated above the heart and is the organ responsible for T cell development (13). T cell precursors arise in the bone marrow and migrate in to the thymus via the corticomedullary junction (reviewed in (14)). During the intrathymic journey the T cells are termed thymocytes and learn how to recognize components of the host and discriminate between "friend or foe" among e.g., bacteria and nutritional antigens and also how to recognise damaged or transformed (cancer) cells. The thymus is a tough school and only around five percent of the thymocytes survive the selection process and leave the thymus as mature, naïve CD4 or CD8 single positive (SP) T cells.

Well inside the thymus, the most immature precursors can still differentiate into T, B or myeloid cells (15, 16). Thymocytes differentiate through a series of events leading to the expression of a TCR with the ability to tolerate the host and foreign beneficial proteins and at the same time react against unwanted agents (e.g., bacteria, viruses, malignancies).

The invariant pre-TCRα chain (pre-Tα) chain (17) pairs with a rearranged TCRβ chain, whereafter the TCRα chain gene rearrangement starts and a complete TCRαβ receptor is expressed on the surface of the thymocyte. T cells can express two different types of TCR, either αβ or γδ and the commitment into $TCR\alpha\beta^+$ or $TCR\gamma\delta^+$ lineages is thought to occur before or during TCRβ chain rearrangements (Figure 1 and (18)). Both $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells develop inside the thymus but it is the development of $TCR\alpha\beta^+$ cells that is the most well known as described below. Many $TCR\gamma\delta^+$ cells are exported early during ontogeny to populate epithelial layers throughout the body (19).

The TCRs that recognize a self MHC + antigenic peptide complex within a certain affinity range are positively selected, whereas up to 95% of the thymocytes die from neglect (due to a too low affinity) or negative selection (due to too high affinity) (20, 21).

During the process of intrathymic T cell differentiation the thymocytes pass through a number of strictly controlled phenotypically defined maturation stages as outlined in Figure 1. The $CD4^-CD8^-$ double negative (DN) thymocytes become $CD4^+CD8^+$ double positive (DP) thymocytes and at last $CD4^+$ or $CD8^+$ single positive (SP) mature T cells (22). DN thymocytes can be further divided into four different maturation stages based on the expression of CD25 and CD44 (23) going from $CD44^+CD25^-$ (DN1) through $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3) and finally $CD44^-CD25^-$ (DN4) (23).

DP and SP thymocytes can be divided into five defined maturation stages as outlined in Figure 1, based on the expression of CD4, CD8, T cell receptor (TCR) αβ, L-selectin (CD62L) and CD69 (24, 25). Stage 1-2 are DP $CD4^+CD8^+$,$TcR\alpha\beta^{-/lo}CD69^-$ $CD62L^-$, stage 3 are DP,$TcR\alpha\beta^+CD69^+CD62L^-$, stage 4 are SP $CD4^+$ or $CD8^+$,$CD69^{hi}CD62L^{lo}$ and stage 5 are SP,$CD69^{lo}CD62L^{hi}$. Stage 5 defines fully mature naïve cells ready to leave the thymus. The CD62L marker expressed on the newly exported T cells function as a receptor for homing to the lymph nodes where they encounter dendritic cells that present antigens to the T cells (26).

The chemokines (described in the chapter "Chemokines in IBD – fatal attraction" below) shown to direct intrathymic migration from the corticomedullary junction to the outer cortex and further to the medulla during this maturation process are CXCL12 (SDF-1α), CCL25 (TECK), CCL21 (SLC) and CCL19 (MIP-3β) (27, 28).

Mature T cells are also believed to be able to recirculate through the thymus, and a possible function of the recirculating cells is that they instruct the development of new thymocytes (29).

EXTRATHYMIC T CELL MATURATION

The general opinion today is that the vast majority of T cells are dependent on the thymus to develop (30). However, some T cells differ from conventional $CD4^+$ and $CD8\alpha\beta^+$ T cells in phenotype and function e.g., $TCR\gamma\delta^+$ cells, DN cells (both $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$) and cells expressing CD8αα homodimer instead of the CD8αβ heterodimer. Although much debated, the unconventional T cells found in the mucosal layers throughout the body are believed to pass through the thymus at some point of maturation. There is evidence that some thymocytes are exported from the thymus at an earlier time point from the thymus than T cells found in the classical (or circulating) immune system (31) and that those early emigrants further mature in the gut. Some thymocytes destined to the mucosal immune system e.g., $CD8\alpha\alpha^+$ T cells, are subjected to agonist selection with a high TCRαβ-MHC affinity normally leading to negative selection, and as a result requires a higher threshold of activation in the mucosa (32).

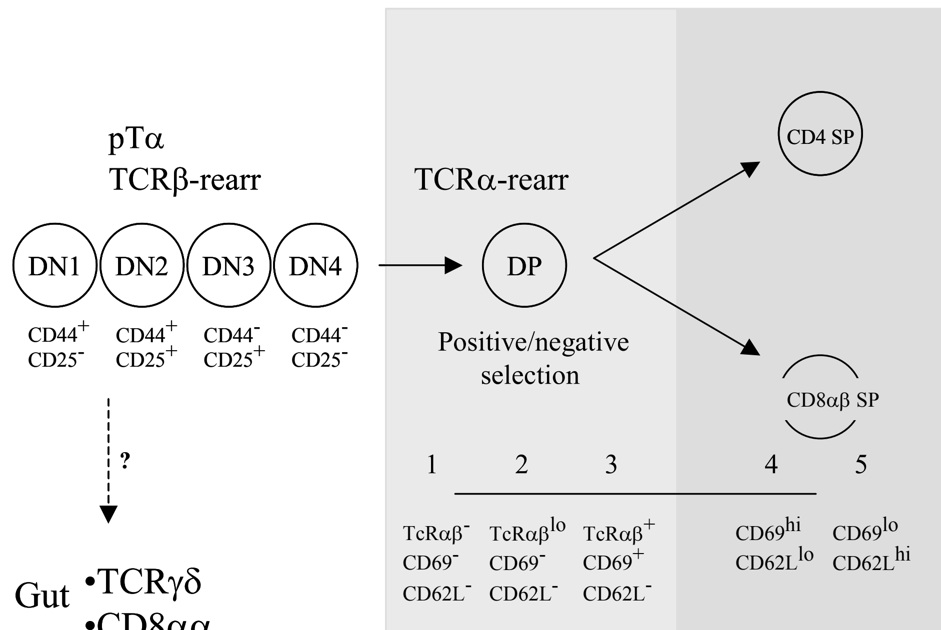

Figure 1: Intrathymic T cell development

T CELL SUBSETS

Mature TCRαβ$^+$ cells can be divided into CD4$^+$ and CD8αβ$^+$ T cells and the CD4$^+$ T cells can be further divided into different subsets of T helper (Th) cells, Th0, Th1, Th2, Th3 and Th17. Th0 cells are CD4$^+$ T cells that have the ability to differentiate into Th1, Th2, Th3, or Th17 types. Generally, CD4$^+$ cells recognise extracellularly derived antigens presented in the context of MHC class II molecules expressed on the surface of APCs and can regulate the activity of other T cells as well as other cell types by the production of different cytokines. CD4$^+$ cells can also provide help to B cells and stimulate them to produce antibodies and attract and activate other immune cells (e.g., macrophages, neutrophils, B cells).

Under the influence of IL-12 T cells can differentiate into Th1 cells (33) that secrete IL-2, IFN-γ and TNF-α, thereby inducing cell mediated immune immunity e.g., macrophage activation and cytotoxic T cells (CTL). IL-4 induce the development of Th2 cells (34) that secrete IL-4, IL-5, IL-10 and IL-13 thereby induce B cell growth and differentiation (humoral immunity). The original Th1/Th2 nomenclature described in 1986 by Mosmann et al (35) was used for almost 20 years before it was starting to be revised. However, the cardinal cytokines secreted by Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) cells are still valid.

Th3 cells were named in 1996 (36) prior to the discovery of the intracellular marker FOXP3, but seem to be the same T cell subsets as TGFβ-induced CD4CD25$^{+/-}$ FOXP3$^+$ regulatory (T_R) cells (37). The naturally occurring CD4$^+$CD25$^+$ T_R generated in the thymus does however not belong to the former Th3 lineage (see below under “Regulatory T cells”).

The exact function of the newest member of the Th family, Th17 cells, (named by their ability to secrete IL-17), is not yet known but they are rapid responders in the immune response (38). Th17 cells differentiate in response to IL-6 and TGF-β, and IL-23 mediate the expansion and maintains the function of already differentiated Th17 cells (39). Interestingly, both Th1 and Th2 secreted cytokines (IFN-γ and IL-4) inhibit the Th17 response. The Th17 response is thought to play a role in the beginning of an immune response and a sustained Th17 response is associated with pathogenic inflammation and autoimmunity (40).

$TCR\alpha\beta^+CD8\alpha\beta^+$ T cells are the classical cytotoxic T lymphocytes (CTL) recognising antigenic peptides presented by MHC class I molecules. MHC class I molecules can be expressed on all cells in the body and present intracellular antigens. Cells that have been infected with viruses or malignant cells present viral or tumour antigens in the context of MHC class I. The killing of the target cells through induction of apoptosis is mediated by Fas-FasL crosslinking or through release of perforin and serine proteases, e.g., Granzyme B (41). Cytokines secreted by CTLs are mainly IFN-γ, TNF-α and IL-2 (42).

$TCR\gamma\delta^+$ T cells mature before $TCR\alpha\beta^+$ T cells in the thymus. $TCR\gamma\delta^+$ T cells are normally found in very low numbers in the blood and spleen (2-5%) while in epithelial linings throughout the body they can sometimes be more numerous than $TCR\alpha\beta^+$ T cells (43). The distribution of $TCR\gamma\delta^+$ cells also differ between species and e.g., sheep contain a high fraction of $TCR\gamma\delta^+$ T cells in the blood compared to mouse and human (44). Human $TCR\gamma\delta^+$ IELs are mostly $CD4^+$ or $CD8^+$ whereas in mice the majority of $TCR\gamma\delta^+$ IELs are DN (45). Strain differences in $TCR\gamma\delta^+$ compositions have been observed in mice (46).

REGULATORY T CELLS

There are T cells specifically devoted to regulate the immune system and especially to turn off the immune response after clearance of pathogens, which would otherwise result in a chronic inflammation and tissue damage. These regulatory cells are $CD4^+$ regulatory (T_R) cells and suppress inflammation through the secretion the anti-inflammatory cytokines such as TGF-β and IL-10 (47).

$CD4^+$ T_R cells can be divided into naturally occurring T_R cells that develop in the thymus and adaptive T_R cells that are induced in the periphery (48). $CD4^+CD25^+$ T_R cells were originally described to protect against colitis by transfer of $CD45RB^{hi}$ T cells into severe combined immuno deficient (SCID) mice lacking T and B cells (49, 50) and more recently $CD4^+CD25^+$ T_R cells were shown to be able to prevent and cure established colitis (48). T_R cells are dependent on the transcription factor, FOXP3, for their development and function (51). There is no specific marker for T_R but in mice T_R have been shown to be $CD45RB^{low}$ and can express CD25, CD122, CD69, CD44, GITR, CD103, CD134 (OX-40), CD54 (ICAM), CTLA-4 and FOXP3.

$CD8^+CD28^-$ T cells with regulatory functions have also been described (52). The frequency of $CD8^+$ T_R cells have been shown to be decreased in the lamina propria of IBD patients (53).

T CELLS ARE EASILY STRESSED

70 years ago, Hans Selye demonstrated thymic involution as a cardinal sign of stress or "the general alarm or adaptation syndrome" (54). This was long before the thymus was known to be the site of T-cell development. Actually it was not before the early 1960's that scientists started to acknowledge the thymus as a non-redundant organ and realised that it was the site of T lymphopoiesis (13, 55).

Thymic involution, resulting from massive apoptosis of DP thymocytes, has been demonstrated in a number of physiological and pathological situations such as glucocorticoid treatment, microbial sepsis (56), restraint stress (57), malnutrition (58), malignancies (59) and intraperitoneal injections of LPS (lipopolysaccharide) (60). Thymic involution also is also seen during pregnancy (61), aging (62) and inflammatory conditions like IBD (63). Stress factors such as academic examinations have also been shown to result in decreased cellular immune function (64). In this particular study, performed on healthy students, memory T-cell proliferative response to Epstein-Barr virus polypeptides significantly decreased during examination compared to one month before examination.

The immediate set of reactions following a trauma such as acute inflammation is referred to as the acute phase response, where IL-1, IL-6 and TNF-α are the most prominent cytokines produced. IL-1, IL-6 and TNF-α stimulates the hypothalamic-pituitary-adrenal (HPA) axis and induce the release of endogenous glucocorticoids (GC) (65). GCs in turn down regulate the secretion of inflammatory cytokines like IL-1, IL-6 and TNF-α (66). In addition, GCs have been shown to be a main mediator of DP thymocyte induced apoptosis, and thymic involution does not occur in adrenalectomized animals (57). The mobilization of immune cells to the site of an extensive inflammation such as colitis causes stress to the organism resulting in production of glucocorticoids (67).

IL-4 and IL-10 are not negatively affected during the activation of the HPA-axis and can thereby result in a shift towards a Th2 response (68). As a result, the HPA axis can act as a regulatory feedback loop that reduce the inflammatory responses to invading antigens after the initial response or in a state of stress.

THE INTESTINAL IMMUNE SYSTEM

THE INTESTINAL MUCOSA

A thin layer of epithelial cells separates the intestinal lumen from the sterile environment of the body, forming a first line of defence against pathogenic intruders. The epithelial cells contain tight junction proteins that form a barrier regulating the permeability between the cells (Figure 2B and (69)). In addition to the regular intestinal epithelial cells, goblet cells produce a protective barrier of mucus, preventing bacteria to reach the epithelial surface and Paneth cells residing in the crypts produce antimicrobial peptides, e.g., defensins (70). Situated between the epithelial cells are the intraepithelial lymphocytes (IEL), T cells participating in the maintenance of the epithelial homeostasis and surveillance of the epithelium for pathogenic encounters (43). Beneath the epithelium is the lamina propria (LP), containing a variety of immune cells, such as T and B lymphocytes, macrophages, neutrophils, mast cells and dendritic cells. The mucosa and the sub mucosa are separated by a thin muscle layer, the muscularis mucosa, and beneath the submucosa are two thicker muscle layers; one inner circular and one outer longitudinal responsible for peristaltic movements. Outside the muscle layers is the connective tissue serosa (Figure 2). B cells in the LP secrete large amounts of IgA that is transported into the lumen (Figure 2A). IgA binds to microbes and toxins and neutralizes them by blocking their entry into the host (71).

The small and the large intestine differ in function and architecture; the small intestine being the main site for nutritional and antigen uptake, while the responsibility of the large intestine lies more in the uptake of salt and water. The small intestine contains crypt and villus structures whereas the large intestine contains mostly crypts. Furthermore, the composition of the mucus differs between the small and large intestine. Peyer's patches (PP), the main site of antigen entrance in the gut, are only found in the small intestine. Each PP consists of several aggregated B cell follicles with intervening T cell areas (72). The PP's are overlaid with follicle-associated epithelium (FAE) interspersed with micro-fold cells (M cells) through which antigens are transported from the intestinal lumen into the subepithelial dome (SED) and taken up by dendritic cells (DCs). Lamina propria harbours several structures of cluster cells, e.g., isolated solitary lymphoid follicles, with a structure similar to the follicles found in PPs (73, 74) and cryptopatches (CP), small clusters of immature cells localized at the base of intestinal crypts which have been suggested to be the site of IEL development in mice (75). However, more recent data have demonstrated that CPs lack recombinant activating gene (RAG) activity (76) and IELs are found in the absence of CPs (77), making this hypothesis less likely. Additional clusters of lymphocytes are found in the villi of the small intestine, called lymphocyte-filled villi (78).

The mesenteric lymph nodes (MLN) are the largest lymph nodes in the body and are situated in close vicinity of the gut (reviewed in (79)). Dendritic cells are thought to recognize mucosal antigens expressed in PPs, solitary lymphoid follicles, or lamina

propria and transport the antigens to the mesenteric lymph nodes. Within a few hours after oral antigen exposure, antigen recognition occurs in MLNs (80).

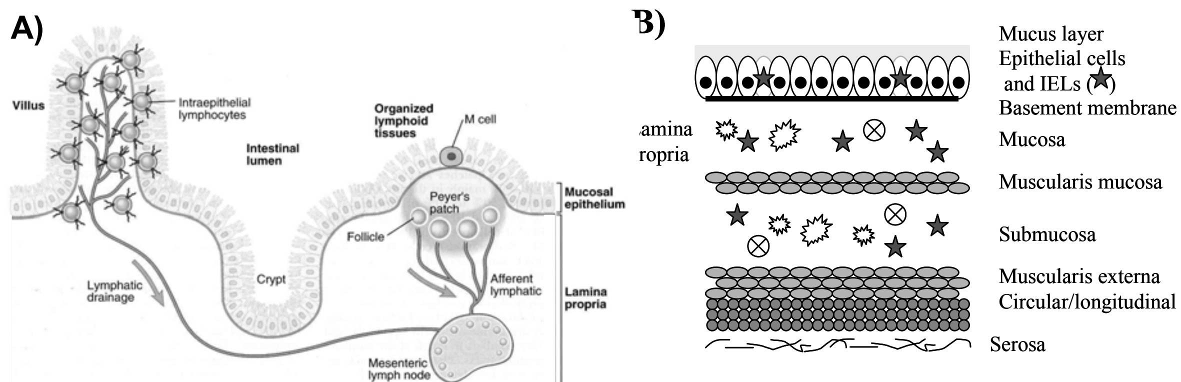

Figure 2: Anatomical overview of the small intestine. A) Villi structure, PPs and connection to the MLNs. B) Cross-section of the gut wall. (Figure 2A from "Cellular and Molecular Immunology" fifth edition, by AK Abbas and AH Lichtman, used with permission).

INTESTINAL T CELLS

As opposed to classical T cells, T cells of the intestinal mucosal immune system are not easily activated through the TCR. The mucosal immune system is in close vicinity to the massive bacterial load in the intestine and cannot be allowed to mount an inflammatory response against commensal bacteria or nutritional antigens and this is thought to be one reason why mucosal T cells are not easily activated by bacterial antigen stimuli (81).

The intestine contains classical T cells as well as unconventional subsets normally found in very low numbers in the classical compartments. Some T cells found in of the intestine e.g., CD8$\alpha\alpha^+$ and TCR$\gamma\delta^+$ cells are more sessile than other T cells such as CD4$^+$ and CD8$\alpha\beta^+$ SP TCR$\alpha\beta^+$ lymphocytes which circulate between the intestine and other immune compartments such as the mesenteric lymph nodes (CD4$^+$ and CD8$\alpha\beta^+$ SP TCR$\alpha\beta^+$). Sessile T cells are found both within the epithelium and in the LP but in larger fractions in the epithelium. IELs and lamina propria lymphocytes (LPL) differ from each other, with LPLs contains a higher percentage of CD4 SP T cells than CD8 SP IELs in most mouse strains examined (81, 82). The majority of LPLs display an activated phenotype and are sensitive to Fas-induced apoptosis (83).

Gut-specific CD8$\alpha\alpha^+$ and TcR$\gamma\delta^+$ T cells, especially IELs have been shown to play a role in the epithelial cell turnover and homeostasis and to protect against colitis (84, 85). The antigenic load in the intestine are thought to drive the development of some of these cells since germ-free mice contain reduced number of TCR$\alpha\beta^+$ but normal numbers of TCR$\gamma\delta^+$ IELs (86).

Similar to the classical immune system, both naïve, effector and memory T cells are found within the mucosa (87). Memory T cells are clonally expanded upon secondary

antigen challenge, thus mediating a faster and stronger immune response than during the first antigen encounter.

Natural Killer (NK)T cells recognise antigens that are presented in the context of the non-classical MHC I molecule CD1d expressed on epithelial cells (88) and sustained activation of NKT cells in the gut are thought to contribute to the inflammation in UC by production of IL-4 and IL-13 (89).

INTRAEPITHELIAL LYMPHOCYTES

Small round cells in the small intestinal epithelium were described already in 1847 (90). The absolute majority of IELs are T cells which are interspersed among the intestinal epithelial cells. There is approximately one IEL for every 4-10 epithelial cells (EC) in the small intestine and one IEL for every 30-50 EC in the large intestine (91). IELs are heterogeneous and in mice they have been classified into two major subpopulations based on the TCR and co-receptor type they express. Classical (or conventional) 'type a' $CD4^+$ or $CD8\alpha\beta^+$ SP $TCR\alpha\beta^+$ cells, and nonconventional 'type b' $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells which are normally found in low numbers in the classical immune compartments (92).

Type a mucosal T cells are $CD4^+$ or $CD8\alpha^+$ $TCR\alpha\beta^+$ cells and among the type b T cells $CD8\alpha\alpha^+$, DN and $TCR\gamma\delta^+$ IELs are found. Further, type b cells are negative for CD2, CD28 and Thy-1, surface markers present on conventional T lymphocytes, (reviewed in (93)). Type a IELs differ in some respect from classical T cell in that the $CD8\alpha\alpha$ homodimer can be co expressed on both $CD8\alpha\beta^+$ and $CD4^+$ T cells (94, 95). The composition of IELs in the small and large intestine have been shown to differ from each other. IELs from the colons of C57BL/6, Balb/c and C3H mice were shown to harbour mostly $TCR\alpha\beta^+$ SP and $TCR\gamma\delta^+$ DN IELs, whereas the small intestine harboured a higher proportion of $CD8^+$ than $CD4^+$ $TCR\alpha\beta^+$ and most $TCR\gamma\delta^+$ IELs were CD8+ (96). In addition, IELs from the small intestine were less cytolytic than IELs from the large intestine. A subset of naïve $CD8^+$ T cells have been shown to home directly to the mucosa without prior activation, so-called recent thymic emigrant (RTE) (97). These RTEs express the integrins $\alpha4\beta7$ and $\alpha E\beta7$ and the chemokine receptor CCR9, making them gut-tropic cells.

Other cells found in the epithelial compartment are NKT cells and NK cells. Both $TCR\gamma\delta^+$ IELs and NKT cells can recognise NKG2D (MICA/B) that is expressed by damaged or transformed epithelial cells (98, 99). NK cells within the epithelial layer are not well characterised but both NKT and NK cells are believed to participate in the maintenance of intestinal homeostasis.

The seemingly diverse functions of IELs have not been entirely elucidated. The subsets found almost exclusively in the epithelium; $CD8\alpha\alpha^+$, $TCR\gamma\delta^+$ and DP IELs have all been shown to possess protective functions against colitis in transfer experiments into immunodeficient mice (43, 81). Other IELs such as $CD8\alpha\beta^+$ are

thought to be able to circulate between the epithelium and the classical immune compartments (100). A recent study also revealed also that IELs express tight junction proteins (101). TCRγδ$^+$ but not TCRαβ$^+$ IELs have a constitutively activated profile, as they are CD69$^+$ and express high levels of cytotoxic genes, e.g., granzymes A and B and are cytotoxic (102-104).

The differences between the small and large intestine is also reflected in the composition of IELs. Thus, the small intestine harbours more DP IELs but less DN IELs than the large intestine, reviewed in (93) (Table 1), reflecting the two different environments in the two compartments.

*Table 1: Different compositions of IELs in the small and large intestine**

	Small intestine (total cell no 5.4 ± 1.4 x 10^6 cells, %)		Large intestine (total cell no 4.3 ± 1.8 x 10^5 cells, %)	
T cell subset	Among total IELs	Among the subset	Among total IELs	Among the subset
CD8αα	62.7 ± 2.5		4.7 ± 0.6	
αβTCR		35.7 ± 3.1		67.3 ± 2.5
γδTCR		64.0 ± 6.5		32.7 ± 2.5
No TCR		N.D.		N.D.
CD8αβ	15.6 ± 2.0		7.3 ± 1.2	
αβTCR		84.6 ± 3.1		95.6 ± 0.6
γδTCR		N.D.		N.D.
No TCR		15.3 ± 3.0		4.3 ± 0.6
CD4	9.0 ±1.7		31.0 ± 5.6	
αβTCR		87.3 ± 2.1		98.6 ± 0.6
γδTCR		N.D.		N.D.
No TCR		12.7 ± 2.1		1.7 ± 0.6
DP	7.3 ± 0.6		<0.1%	
αβTCR		52.0 ± 6.1		N/A
γδTCR		2.0 ±1.0		N/A
No TCR		46.1 ± 5.3		N/A
DN	5.4 ± 1.2		57.3 ± 5.2	
αβTCR		2.7 ± 0.6		5.3 ± 0.6
γδTCR		20.7 ± 3.1		4.3 ± 0.6
No TCR		76.6 ± 3.2		90.4 ± 1.0

N.D., not detectable; N/A, not applicable, type b IEL in italics.

*The data were obtained from female BALB/c mice (7-10 weeks) and represent means ± SD (n=5)

Data in this table was adapted from Kunisawa, J "Intraepithelial cells: their shared and divergent immunological behaviours in the small and large intestine" Immunol Rev 215:136-153, 2007, used with permission.

HOMING OF T CELLS – A MATTER OF MOLECULES

During its development and maturation into effector or memory cells the T cell navigate within and between organs via complicated routes. In each station, the cell receives instructions on how to proceed its destiny, and these instructions are mediated by a number of molecules, mainly chemokines, selectins and integrins (105-107).

Chemokines are molecules that stimulate leukocyte migration. The chemokines are classified into families on the basis of the number and location of N-terminal cysteine residues. The two major families are CC and CXC chemokines, in CC chemokines the cysteine residues are adjacent and in the CXC chemokines the residues are separated by one amino acid. The CXC chemokines act mainly on neutrophils, and the CC chemokines act mainly monocytes, lymphocytes and eosinophils. Chemokines act as chemoattractants for various cells and can be divided into homeostatic (e.g., CCL14, 15, 16, 18, 19, 21, 25, 27 and CXCL12, 13) and inflammatory (e.g., CCL1, 2, 3, 4, 5, 7, 8, 11, 13, 23, 24, 26 and CXCL1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 14, 16) chemokines although some chemokines can belong to both classes (e.g., CCL17, 20, 21, 22, 28 and CX3CL1) (108, 109). Chemokines are promiscuous in that they bind one or several receptors and their receptors bind more than one chemokine ligand (108). As this is not enough to confuse the field of chemokines, small changes like alterations in only one amino acid can transform the chemokine into an antagonist, and heterodimers (e.g., CCL2/CCL8) can be formed with unknown functions (110). Further, it has been demonstrated that chemokine receptors can exert their functions via different signalling pathways. For example, CCR7 regulates the survival and chemotaxis of DCs through Gi signalling while migratory speed is regulated by other mechanisms (111).

The selectin CD62L together with the chemokine receptor CCR7 are the primary homing molecules regulating the entrance of T cells into peripheral lymph nodes via the high endothelial venules (HEV) (105-107). CCR7, which is expressed on all naïve and subsets of memory T cells, bind to its ligands CCL19 and CCL21 (reviewed in (112)). HEVs in the MLNs but not PPs express peripheral node addressin (PNAd), the receptor for CD62L, while mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) which binds to integrin $\alpha 4\beta 7$ and CD62L is expressed in both MLN and PPs (112).

Homing of T cells to the gut has been studied extensively in the small intestine. T cells are primed in MLNs and PPs and then start to express CCR9. Its ligand CCL25 is expressed in the small intestinal epithelium and attract $CCR9^+$ T cells (113). The CCR9/CCL25 interaction are believed to promote the induction of integrin αE (CD103) on newly recruited IELs retaining the T cell in the epithelium through the binding of its ligand E-cadherin (114). Interestingly, $CCR9^{-/-}$ mice still contain IELs and recent research suggest that $CD8^+$ T cells can home to the epithelium in a CCR9 independent fashion (113). In the latter study, the authors also found that CCR9-independent $CD8\alpha\beta^+$ T cell entry was pertussis toxin-sensitive, suggesting a role for additional Gαi-linked G protein-coupled receptors.

Much less is known about the homing of T cells to the colon. CCR9 is expressed at very low levels in the colon and do not seem to be involved in the homing of IELs in the colon (112). Instead, CCR10/CCL28 have been suggested to be involved based on its abundant expression in the colon (109).

MUCOSAL TOLERANCE

The mucosal immune system needs to be wide-ranging and selective due to the constant potential for pathogen exposure among the commensal flora. The commensal flora has to be protected as it shelters the host from pathogen colonisation and helps the host to build the intestinal immune system and metabolise nutrients. It is also necessary to avoid inflammatory responses to dietary antigens. Keeping this balance is known as mucosal homeostasis or tolerance (115).
Oral tolerance can be described as a state where mature cells in the local and peripheral lymphoid tissues are rendered hyporesponsive to previously orally administered antigens. It has been shown to occur in the absence of CD8+ but not CD4+ cells (116) and in IFN-γ deficient animals (117). It has also been reported in mice that lack PPs but not in mice that lack both PPs and MLNs (118). Isolated lymphoid follicles are generated in response to mucosal challenges and may in that way contribute to the mucosal homeostasis (118, 119).

$CD4^+CD25^+FOXP3^+$ T_R cells are found within the mucosa of healthy mice. In colitic mice T_R accumulate in the intestine (120), suggesting that the chronic inflammation is not a result from the lack of T_R, but rather an impaired function of these cells in suppressing pathogenic T cells. Also in human UC regulatory T cells have been shown to increase with disease activity (121).

Other cells than $CD4^+$ T_R have also been suggested to participate in the regulation of the mucosal immune system. $TCR\gamma\delta^+$, $CD8\alpha\alpha^+$ and DP IELs have all been shown to protect against colitis in several mouse models of colitis (85, 122, 123). LP T cells, NKT cells, IL-10 producing B cells and plasmacytoid DCs have also been suggested to play a regulatory role in the intestinal mucosa (reviewed in (124)).

When the equilibrium of mucosal tolerance is disturbed food hypersensitivities or IBD can develop.

INFLAMMATORY BOWEL DISEASE

IBD is traditionally divided in two entities, Crohn's disease (CD) and ulcerative colitis (UC) and is manifested by chronic inflammation, characterised by acute flares followed by remission. The prevalence of IBD is increasing and it has been estimated that up to 1.4 million people in the United States and 2.2 million people in Europe are affected by the disease (125).

UC is confined to the large intestine with superficial inflammation of the mucosa that extends proximally from the rectum sometimes the entire colon is affected and is associated with an increased risk for colorectal cancer. Neutrophils are enriched within the lamina propria and the crypts. Depletion of goblet cell mucin is also common. (126). The inflammation in CD is transmural but not restricted to the colon but can

affect any part of the gastrointestinal tract, from the mouth to the anus, but involvement of the terminal ileum is most common. Unlike ulcerative colitis, Crohn's disease can be patchy and segmental. Extra intestinal manifestations, such as inflammation of the joints, eyes and skin are much more common in CD than in UC. Crohn's disease is characterized by aggregation of macrophages that can form granulomas (126).

The aetiology of IBD is unknown, but the development of both CD and UC are thought to be the result of an uncontrolled or insufficiently suppressed immune response. Components of the commensal flora have been suggested to contribute to the sustained inflammation (127), as well as a defective mucosal barrier (128). Genetic, environmental and immunological factors have all been suggested as factors initiating IBD. The first gene to be linked to increased risk of IBD was NOD2 (129), a gene encoding an intracellular receptor for a bacterial cell wall component called muramyl dipeptide. Family studies have shown that the risk of inheriting IBD seem to be stronger in CD than UC (130, 131). Many of the developing countries with historically low rates of IBD, have experienced an increasing incidence during the past one to two decades (132), suggesting that environmental factors are also involved in the predisposition of individuals. It has also been shown that smoking can protect against UC whereas it exacerbates CD (133). Finally, stress is a factor that have been shown to induce disease relapse (134).

T CELL RESPONSES IN IBD

In IBD, an excess of dysregulated $CD4^+$ T cells is thought to contribute to the chronicity of the inflammation. However, the profile of T cell cytokines is not the same in UC and CD. Both UC and CD contain increased levels of TNF-α, IFN-γ, IL-1 and IL-6 (135, 136). In addition UC, is characterised by production of Th2 associated cytokines like IL-4, IL-5 and IL-10 (137, 138). Therefore, CD have been considered to be a Th1 mediated disease whereas UC have been considered to be Th2 mediated (139).

IL-23 have been shown to expand Th17 cells and both IL-17 and IL-23 have been demonstrated to be increased in IBD (140, 141). The Th17 cells are thought to be beneficial in the initiation of an immune response but may lead to enhanced inflammation if over expressed (142).

Dysregulated apoptosis have been demonstrated in IBD. The Bcl-2/Bax protein family consists of several proteins with opposing activity, such as Bcl-2, which protects from apoptosis, and Bax, which promotes apoptosis (83). The relative balance between the agonist and the antagonist proteins affects how well a cell responds to apoptotic signals, thereby determining the degree of cell survival of T cells. The Bcl-2/Bax ratio is elevated in CD (143), a finding that suggests increased resistance of T cells in the mucosa of patients with CD Levels of expression of Bax protein are markedly reduced

in inflamed UC colonic epithelium (144), demonstrating the down regulation of Bax in inflamed colonic epithelium.

The Fas/FasL system is another important apoptosis pathway that induce apoptosis in activated T cells (145). FasL is expressed on cytotoxic T cells and Fas is expressed on the target cells (146). Normally, pro-inflammatory cytokines IFN-γ and 124 and IL-2 seem to play a central role in activating the Fas–FasL system (147). Decreased apoptosis of activated mucosal T cells during IBD contributes to the perpetuated inflammation (148). It have been suggested that T cells in UC are less sensitive to apoptosis than T cells from healthy patients (149)

Homing of leukocytes from the circulation into the lymphoid tissue (or Peyer's patches) of the intestine are altered during IBD, e.g., MAdCAM-1 has been linked to inflammation in CD and in UC, where MAdCAM-1 was increased within venular endothelium in the lamina propria of inflamed intestinal tissue (150).

Increased expression of a number of chemokines have been reported in IBD (reviewed in (151)). Chemokine expression has been investigated both on the transcriptional and translational level and many chemokines, such as CCL2, 3, 4, 5, 7, 8, 19, 21, CXCL-5, 8, 10, 12, and CXC3CL1 have been reported to be up regulated in both UC and CD as assessed by immunohistochemistry or ELISA (152-161). Analysis of RNA (ribonucleic acid) expression of chemokines in UC (162) and CD (163) have also been performed, reporting enhanced levels of CXCL1, 2, 3, 5, 8, 9 and 10 and CCL2, 4, and 5. CCR2 and CCR5 deficient mice were protected from DSS-induced colitis (164), and an increase in CCR2 and CCR5 have been observed in human IBD(165, 166).

DIAGNOSING AND TREATING IBD

Endoscopic evaluation of the colon with multiple biopsies has been the prevailing method for diagnosing UC and CD. Recent technological advances have greatly improved several imaging technologies, such as computed tomography (CT) and magnetic resonance imaging (MRI). All three techniques are useful as tools in narrowing the differential diagnosis of inflammatory conditions of the gut. Monitoring IBD with these imaging techniques offers objective and non-invasive methods with relatively little discomfort for the patients (167, 168). A typical feature of colitis detected by CT and MRI technologies is intestinal mural thickening and the target or "halo" sign (169) indicating submucosal oedema or fat deposition. Additional characteristics that can be detected by CT and MRI are e.g., luminal narrowing, mesenteric hypervascularization accompanied by associated mesenteric lymphadenopathy and fibrofatty proliferation (reviewed in (170)). C-reactive protein (CRP) levels and Crohn´s disease activity index (CDAI) are other non-invasive ways of diagnosing IBD.

Most therapies currently used in the treatment of IBD such as 5-aminosalisylic acid (5-ASA), antibiotics, corticosteroids and immunomodulators have been used for some

decades, whereas antibodies against TNF-α (e.g., Infliximab) is a recently developed therapy that has become a routinely used drug in CD (171).

Corticosteroids are frequently used to treat active IBD being effective at inducing remission. However, the use of corticosteroids are often associated with adverse effects and resistance to the drug (172). Corticosteroids are used to induce, but not to maintain remission (173). The most common steroids used are budesonide and prednisolone (174) and much work is focused on creating high first-pass metabolism and controlled-released formulations. One of the adverse effects of steroids is the suppression of the HPA axis (reviewed in (175)).

5-ASA compounds are used in the first-line therapy for primarily UC, but also CD. The mechanisms of action are thought to be mediated by induction of and binding to the peroxisome proliferator-activated receptor-γ (PPAR-γ) expressed on epithelial cells (171). Antibiotics are commonly used with good results, and probiotics may also prove useful in IBD by competitive exclusion of pathogenic bacteria, immunomodulation, antimicrobial activity and enhancement of barrier function (reviewed in (7)). Immunomodulation achieved by azathioprine, 6-mercaptopurine, or methotrexate is increasingly used to treat moderate-to-severe IBD. These agents are generally well tolerated but severe toxicity may occur with these medications (reviewed in (7)). Infliximab neutralizes soluble TNF-α and induce apoptosis of activated inflammatory cells. It is mostly used in moderate-to-severe CD although some patients suffer from severe adverse effects as toxicity and infections. New studies have also shown efficacy of Infliximab in UC patients (176). New biological treatments tested in patients are antibodies against integrin α4 (Natalizumab), CD3 (visilizumab), IFN-γ (fontolizumab) and IL-12 (177-180).

MOUSE MODELS OF COLITIS

Already in 2002, 63 animal models of colitis was described (181) but far from all are widely used (182, 183). The models can be divided in different subgroups depending on the cause of colitis. Table 2 lists some of the most frequently used mouse models for intestinal inflammation (182).

One aim of this thesis was to compare the Gαi2 deficient and the DSS models of colitis. Despite different aetiology and cause of colitis the results herein show that genetically deficient and chemically induced models may have more in common than generally anticipated. The similarities and differences between the models are outlined in Table 3 and described in the text below.

Table 2: Mouse models of intestinal inflammation

Induced	Gene targeted	Cell transfer models	"Naturally occurring"
DSS TNBS DNBS Oxazalone Acetic acid Indomethacin	**Gαi2$^{-/-}$** TCRα$^{-/-}$ TCRβ$^{-/-}$ MHC II$^{-/-}$ Mdr1a$^{-/-}$ IL-2$^{-/-}$ IL-2Rα$^{-/-}$ IL-10$^{-/-}$ TGF-β$^{-/-}$	CD4+CD455RB^{hi} → SCID or RAG$^{-/-}$ Bone marrow → Tgε26 **Gαi2$^{-/-}$ CD3^{+} → RAG2$^{-/-}$****	C3H-HejBir mice SAMP1/Yit mouse *

Bold, models used in this thesis, *ileitis, **Reference # (184)

DSS INDUCED AND Gαi2 DEFICIENT MOUSE MODELS OF COLITIS

BACKGROUND AND GENETIC INFLUENCE

The DSS induced model is today one of the most commonly used models of colitis. Addition of 3-10% of 30–50 kDa DSS, (polymers of sulfated dextran molecules) to the drinking water causes colitis in a variety of animals, including hamsters, rats, and mice (185). Colitis in these animals is generally manifested by bloody diarrhoea, weight loss, shortening of the colon, neutrophilic infiltration, epithelial loss, fibrosis, crypt loss, goblet cell emptying, and focal ulceration (8). In the IBD group at AZ the DSS model is used both in acute and chronic settings (Table 3). Five days of 3% DSS to C57BL/6 mice induce an acute colitis that progresses into a chronic inflammation after DSS withdrawal. On the other hand, in Balb/c mice, 5% DSS for five or seven days produce an acute inflammation mice that resolves within four weeks post DSS (9, 10, 67, 186, 187). Thus, mice with different genetic background respond differently to DSS.

Mice deficient for the Gαi2 protein spontaneously develop a pancolitis which is usually more severe in the distal colon (11, 12, 27, 184, 188-196). Colitis in these animals is generally manifested by mucus filled diarrhoea, weight loss, shortening of the colon, infiltration of lymphocytes and neutrophils, crypt loss, goblet cell depletion, ulceration and colonic adenocarcinomas (11, 197) (Table 3). The genetic background has a strong influence: the Gαi2 deficiency bred on the 129SvEv background have an onset of colitis between 4-8 weeks of age, while mice bred onto the backgrounds 129SvBom or C57BL/6 fail to develop colitis (188). Gαi2$^{-/-}$ 129SvEv mice cross-bred 5-6 generations to the C57BL/6 background develop colitis between 12-20 weeks of age (193). In this thesis, the majority of Gαi2$^{-/-}$ animals used were on the 129SvEv background (papers II and III), except for two-three control experiments on crossbred animals.

MECHANISMS

The exact mechanism(s) by which DSS induces colitis is not known but the initial injury was suggested to be due to DSS acting as a toxic agent, damaging the epithelium (8). As a consequence the mucosa is exposed to bacterial antigens, generating an inflammatory response. IELs have been shown to aggregate within the damaged epithelium during DSS-induced colitis (198). Neither bacteria nor cells seem to be necessary for the initiation of colitis since germ-free mice, nude or SCID mice develop colitis upon DSS exposure (199-202). The first cells to infiltrate the mucosa and submucosa after DSS challenge are large numbers of neutrophils and macrophages, followed by T and B cells (8, 187), indicating that these cells may be involved in the modulation of the disease. Macrophages has been demonstrated to engulf DSS that can later be detected in the MLN and liver (203).

Guanosine triphophate (GTP)-binding proteins (G-proteins) are a family of heterotrimeric proteins consisting of an α, a β and a γ chain. Upon activation the α chain binds GTP instead of GDP and dissociates from the βγ complex, shifting the effector pathways inside the cell (204). The αi subunit inhibit adenylate cyclase, that converts ATP to cyclic AMP (cAMP), thereby activating protein kinases like mitogen-activated protein kinase (MAPK) networks.

Gαi2 proteins are found in many cell types, including immune cells and gastrointestinal epithelial cells. It has been shown that Gαi2$^{-/-}$ mice have impaired marginal zone and B-1 B cell development (205). PPs are found to be smaller in size in pre-colitic Gai2-/- mice and disappear during colitis (194). In addition, chemokines use Gai2 proteins in directing cell migration (206) e.g., in the exit of T cells from the thymus. Transgenic *Lck*-Pt mice have inactivated Gαi proteins in thymus and as a result contains abnormal numbers of CD3^{+} T cells, and a heavily impaired peripheral pool of T cells (207). Gαi2 proteins are also important for the maintenance and development of tight junctions (208). In my group it has previously been shown that colitic and precolitic Gαi2$^{-/-}$ mice contain activated T cells and aberrant migration of T cells in the thymus and the mucosa (11, 27, 184, 188, 193, 194).

HISTOPATHOLOGY

The first histopathological changes caused by DSS are shedding of epithelial cells. As the acute phase peaks at day 12 of inflammation in C57BL/6 mice (9) large areas of epithelial denudation occurs mixed with almost normal epithelium, resulting in a patchy appearance of the inflammation (Table 3 and Figure 3). Infiltration of cells is frequent both in the mucosa and submucosa and is most severe in the distal colon. Lymphoid follicles and crypt distortion is also evident. Mice in the chronic phase present a large infiltration of inflammatory cells and a restored but irregular epithelial structure.

The histopathology in colitic Gαi2$^{-/-}$ mice closely resembles the inflammation seen in UC and is associated with frequent adenocarcinomas (197). The infiltration of cells is confined to the mucosa (and not the submucosa) with crypt distortion, loss of goblet cells and crypt abscesses (Table 3 and Figure 3).

Table 3: Comparison of the DSS and Gαi2$^{-/-}$ mouse models of colitis

	DSS model	**Gαi2$^{-/-}$ model**
Kinetics	Acute model (Balb/c): Clinical resolution after 7 days post DSS. Histological and biomarker recovery approximately 28 days after DSS removal (9) Chronic model (C57Bl/6): clinical symptoms fading, remaining histopathology (9)	Progressive (no remissions) Mice die within two weeks after onset of clinical symptoms (12)
Clinical disease	Acute: Weight loss, diarrhoea, GI bleeding Chronic: Recovered bodyweight, soft faeces (9)	Weight loss, diarrhoea, prolapse (12)
Diarrhoea	Blood-filled after 3-4 days Relatively “grainy” compared to Gαi2$^{-/-}$diarrhoea (9)	Watery, mucus-filled diarrhoea (rarely blood-filled)
Localisation	Entire colon, worst distally, patchy epithelial damage	Entire colon (no skip areas) (12)
Small intestine	No pathology Increase of TCR$\gamma\delta$ cells in PPs (210)	Mild pathology in some mice, with a histological inflammatory score up to 2 (Table 4) in the distal ileum
Histopathology (Figure 3)	Infiltration of polymorpho-nuclear and mononuclear cells both in mucosa and submucosa Branched crypts Lymphoid follicles Epithelial shedding (8)	Infiltration of polymorpho-nuclear and mononuclear cells. Inflammation only in mucosa. Branched crypts Crypt abscesses Invasive adenocarcinoma(197)
Genetic influence	Yes (9)	Yes (11, 188)
Gender differences	C57BL/6: males more sensitive than females	Female Gαi2$^{-/-}$ colitic mice generally smaller than male Gαi2$^{-/-}$ colitic mice.
Dependent on flora	Not for initial epithelial injury	Yes
Cytokines/chemokines	IFN-γ, TNF, IL-1, IL-4, IL-6, IL-10, IL12, IL-17, IL-18, CCL2, 3, 4, 5, 17, 22, CXCL1, 2, 3 10 (187)	IFN-γ, TNF, IL-1β, IL-1Rα IL-6, IL12p40, IL-17, IL-18 (193)
Thymic involution	Yes (10)	Yes (27)
IEL alterations	TCR$\gamma\delta^+$ T cells in colon (211) IEL-like TcR$\gamma\delta^+$ in PPs (210)	Yes (paper II)

CELLS AND SOLUBLE SIGNALS

The colonic cytokine profile in DSS-induced colitis contains high levels of Th1 cytokines e.g., IFN-γ, TNF, IL-1α/β, IL-6, IL-12p40, IL-12p70, IL-17 and IL-18 and

low levels of Th2 cytokines IL-4, IL-10 (9, 209) as well as a number of chemokines e.g., CCL2, 3, 4, 5, 17, 22, CXCL1, 2, 3 and 10 (187).

One of the hallmarks of the colitis in $G\alpha i2^{-/-}$ mice is the production of high levels of IFN-γ. Other Th1 cytokines produced are TNF, IL-1β, IL-6 and IL12p40, IL-18 and IL-1Rα but not IL-4, IL-5 and IL-10 (11). Mucosal T cells in colitic $G\alpha i2^{-/-}$ mice have an activated phenotype; $CD44^{high}$, $CD45RB^{low}$ and $CD62L^{low}$. Precolitic $G\alpha i2^{-/-}$ mice display signs of an activated mucosa including increased numbers of activated $CD4^{+}$ T cells in the LP and increased levels of immunoglobulins against normal flora in the large intestine (193). The $G\alpha i2^{-/-}$ mice have been shown to display a deficiency of marginal zone cells, that in normal mice produce IL-10, in the mesenteric lymph nodes (205).

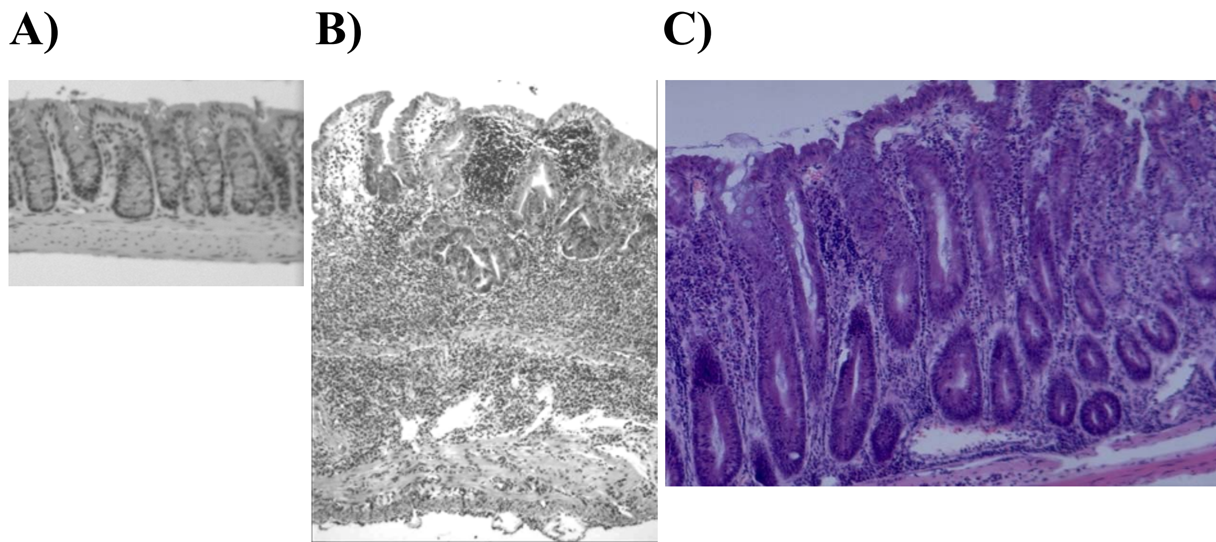

Figure 3: Representative histological H&E sections of colons from A) healthy wild type, B) chronic colitic mice in the chronic phade of DSS induced colitis (day 5+21) and C) colitic $G\alpha i2^{-/-}$ mice. Epithelial reconstitution occur in the chronic phase of DSS induced colitis, with infiltration of polymorpho-nuclear and mononuclear cells both in mucosa and submucosa. In the $G\alpha i2^{-/-}$ model, infiltration of polymorpho-nuclear and mononuclear cells is limited to the mucosa.

AIMS OF THE THESIS

The overall aim of this thesis was to increase the understanding of the immunopathology of Inflammatory Bowel Disease. I had the great opportunity to examine two very different mouse models of colitis: a chemically induced model, the DSS induced model and a spontaneous model, the Gαi2 deficient mouse. Through the work in this thesis I brought the two models closer to each other and also to findings in human IBD.

The first more specific aim of the thesis was to elucidate how two of the main T cell compartments in the body, the thymus and the gut epithelium are affected by colitis. Therefore, I wanted to test the hypotheses that:

- The changes in thymocyte subsets during thymic involution observed in Gαi2$^{-/-}$ colitic mice may not be unique to the Gi protein deficiency but to the colitis, and is thus found also in the DSS induced colitis model.

- T lymphocytes not only in the colon but also in the small intestine are affected by the immunological alterations leading to colitis (in Gαi2$^{-/-}$ mice).

The second aim was to investigate new ways of assessing and monitoring colitis in a way that hopefully can make drug testing more efficient with fewer animals being used. Therefore, I wanted to test the hypotheses that:

- The gene expression profile in ex vivo cultured colonic tissue from healthy and inflamed mice reflects the in vivo profile, and that murine colon culture systems are relevant to validate future therapies for IBD.

- Measurements of colon wall thickness can be used to identify mice that have responded to DSS, similar to the use of this methodology to monitor the extent of intestinal inflammation in human IBD.

METHODOLOGICAL COMMENTS

This sections aims to give a brief overview over the methods used in paper I-IV, but more importantly why those particular methods were used. (For a more detailed description of the methods, see paper I-IV.)

ANIMALS AND ANIMAL MATERIAL (paper I-IV)

Animals on different genetic backgrounds were used in the study. C57BL/6 and Balb/c female mice were used for the DSS-model whereas the Gαi2 deficient mice were bred on the 129SvEv background.
DSS-colitis: Specific pathogen free female C57BL/6JOlaHsD or Balb/c mice, 7-9 weeks old, weighing 20-24g, were used. Animals were kept in the animal facilities at AstraZeneca R&D Mölndal under standard conditions. In vivo treatment with methyl-prednisolone started the same day as the animals started to receive DSS.
Gαi2-deficient colitis: Specific pathogen free female and male Gαi2$^{-/-}$ mice on a 129SvEv background were bred as heterozygotes at the animal facilities at the Department of Experimental Biomedicine, Göteborg University. Mice were kept in filter top cages with forced ventilation, otherwise under standard conditions. In vivo treatment started the same day as the onset of diarrhoea.
The Local Animal Ethical Committee at Göteborg University approved all studies.

Comments: C57BL/6 mice have been used extensively to study the DSS model and previous studies from the IBD group at AstraZeneca (AZ) established a chronic inflammation in this strain by administrating 3% DSS in the drinking water for five days, followed by tap water (9). In contrast, Balb/c mice recover to normal within a few weeks after DSS-withdrawal. In paper I C57BL/6 and Balb/c mice were used to study the thymic alterations during chronic and acute colitis during DSS induced colitis. Thymic involution is irreversible as the Gαi2$^{-/-}$ mice die from the colitis, the events during acute colitis was found to be very similar between the models (27).
The DSS model on the C57BL/6 background was also employed in paper III and IV. The C57BL/6 strain is the “standard” strain for testing substances in the DSS model at AZ, while the aim of paper IV was to monitor the DSS induced colitis in the chronic settings of the DSS induced colitis in C57BL/6 mice.
The Gαi2$^{-/-}$ mice do not develop colitis on a pure C57BL/6 background (11, 193), which is the rationale for using 129SvEv mice in paper II and III, despite 129SvEv mice being notorious bad breeders and less well characterised immunologically than C57BL/6 mice.

HUMAN MATERIAL (paper III)

Surgical specimens from colons of two male patients with diagnosed active UC were analysed. Informed consent was obtained from the patients. The local Human Ethical Committee at Göteborgs University approved the studies.

Comments: Colonic tissue from UC patients was cultured *ex vivo* to compare the responses towards treatment in cultures in the DSS induced and Gαi2$^{-/-}$ models. The main scope of paper III was to compare the expression profile between the two mouse models. As the opportunity to assess human material appeared we performed the very interesting comparison that proved to strengthen the data obtained in the animal cultures.

SCORING OF COLITIS (paper I-IV)

It is of profound importance to evaluate the severity of the intestinal inflammation in all possible ways when performing research on colitic mice. Subjective parameters as ocular scoring are complementary to “hard data”, like cytokine levels and body weight loss. In my groups at AZ and GU, both macroscopical and microscopical scoring systems have been developed to assess the severity of colitis. Upon termination the colon was dissected, opened and the mucosal side was judged for inflammation according to a system that was developed and improved during several years within the IBD group at AZ. The system originally developed for the DSS and TNBS (tri-nitro benzene sulfonic acid) models was adapted to the Gαi2$^{-/-}$ model. The inflammatory macroscopic score reflecting the degree of inflammation in the colon at sacrifice was based on the extent of oedema (0-3), thickness (0-4), stiffness (0-2) and ulcerations (0-1), resulting in a total score of 10 (10).

Colonic tissue were also routinely collected for histological scoring of colitis and two different scoring systems had been developed prior to this thesis, one for the DSS model and one for the Gαi2$^{-/-}$ model. The inflammatory histological score reflecting the degree of inflammation within the intestinal tissue for the DSS model was based on the extent of cellular infiltrates, ulcerations, oedema and other signs of damage, and tissue sections were scored from 0 (no signs of damage) to 6 (severe inflammation and ulcerations) (Table 4B). A scale ranging from 1-5 originally developed for UC (Table 4A) was used to grade the inflammation in colitic Gαi2$^{-/-}$ mice (212).

Comment: The appearance of an inflamed colon from DSS treated animals differs in some aspects from inflamed Gαi2$^{-/-}$ colons. In both models the inflamed colon is thicker than in normal mice. DSS-exposed colons also appear stiff and “bumpy” or rough with frequent blood in the diarrhoea and sometimes small ulcers appear on the mucosal surface. The colons from colitic Gαi2$^{-/-}$ mice are generally not as stiff as DSS treated colons but contain frequent ulcers transforming into necrotic areas and sometimes perforation of the colon wall is found in terminally ill mice.

Further, the appearance of DSS treated colons and inflamed colons from Gαi2$^{-/-}$ mice differ in that the Gαi2$^{-/-}$ mice have seemingly more indifferent signs of inflammation. In DSS treated mice the inflammation starts with mild symptoms and gradually the colon becomes stiff, oedemic and thick, sometimes with small ulcers; parameters that can be graded relatively easy according to the 10 point macroscopic inflammation

Table 4A: Histological grading of colitis in the Gαi2$^{-/-}$ model

Grade		*Characteristics*
1	Normal mucosa	
2	Mild inflammation	Enhanced glands with intraepithelial granulocytes, enhancement of cells and/or eosinophils in the stroma
3	Intermediate inflammaton	Goblet cell depletion, loss of tubular parallelism and reduced mucin production in some glands. Marked increase of inflammatory cells in the stroma.
4	Severe inflammation	Marked gland and mucosal atrophy. Evident crypt abscesses and pus on the surface. Massive increase of acute inflammatory cells and follicle formation in deeper cell layers
5	Fulminate inflammation	Ulcerations with pus, gland and mucosal atrophy, crypt abscesses, extensive stromal inflammation and deep follicles

Table 4B: Histological grading of colitis in the DSS model

Grade	*Characteristics*
0	No signs of damage
1	Few inflammatory cells, no signs of epithelial degeneration
2	Mild inflammation, some signs of epithelial degeneration
3	Moderate inflammation, some epithelial ulcerations
4	Moderate to severe inflammation
5	Moderate to severe inflammation, large ulcerations of more than 50% of the tissue section
6	Severe inflammation and ulcerations of more than 75% of the tissue section

score. On the other hand, once the inflammation starts in Gαi2$^{-/-}$ mice the surface of the colon appear smoother and often not so stiff as in the DSS model. This appearance is very similar in mild and moderate Gαi2$^{-/-}$ colitis. When the colitis develops into severe stages, the colon rapidly develops ulcers and denudation of the mucosa, the latter visible as "whiteish" areas. The ulcers grow in size and depth and result in necrosis of the tissue and perforation of the colon wall, ultimately leading to the death of the animal. Interestingly, the site of the necrosis/perturbation is almost always located in the same place, approximately two centimetres proximal of anus. The phenomenon with the "indifferent" signs of inflammation is also observed in IL2$^{-/-}$ and IL10$^{-/-}$ mice (own unpublished observations) and this might be a reason why macroscopic scoring systems are more seldom used in knockout compared to induced models of colitis. DSS treated animals are rarely scored 8 or higher, whereas terminally ill Gαi2$^{-/-}$ mice often get 10 points.

Similar to the macroscopic scoring, the histological appearance differs between the two models. The histopathology in the DSS model is characterised by loss of epithelial cells and infiltration of immune cells in the mucosa and submucosa (9). In contrast, the inflammation in the Gαi2$^{-/-}$ model is confined to the mucosa and does not involve the submucosa and is also frequently associated with adenocarcinomas (11).

The histological scoring of DSS treated mice was developed at AZ by Dr Erika Rehnström and is routinely used in all studies performed at AZ. The system used for the assessment of the histological inflammation in the $G\alpha i2^{-/-}$ model was originally developed by Professor Roger Willén for grading of the inflammation in UC patients (212). The reason for using this system on the $G\alpha i2^{-/-}$ model is the remarkable resemblance of the histology between colitic $G\alpha i2^{-/-}$ mice and UC patients. This scoring system has been used for an extended period of time and in several earlier published studies from the Elisabeth Hultgren-Hörnquist group at GU (27, 184, 189, 196).

EX VIVO CULTURES (paper III)

Inflamed colons from colitic mice and UC patients were used in the study in paper III. Total mouse colon was cut into 1 mm "tubes", while the human mucosa was separated from the submucosa and muscle layers with a scalpel before culture. The tissue was placed in cultures with or without compounds and cultured overnight at 37˚C, 5% CO_2. The tissue was then snap-frozen for RNA analysis and the supernatant was analysed for IL-6 protein by ELISA and lactate dehydrogenase content for tissue viability control.

Comments: Tissue was placed in cultures with or without compounds and after six hours the medium was replaced with new medium with or without anti-inflammatory compound and cultured for another 18 hours. The medium replacement was done to detect the changes in protein levels upon treatment, i.e. the excess protein synthesized within the cell prior to treatment was excluded from the analysis.

The work to develop faster and more efficient methods of testing substances is beneficial in many aspects, not the least the chance to reduce the amount of animals used in the pre-clinical studies. Central to this study was to mimic the *in vivo* situation as close as possible, thus culturing the tissue without prior activation. In general, in *ex vivo* culture systems reported previously, the tissue have often been pre-activated with inflammatory agents e.g. PMA (Phorbol 12-myristate 13-acetate) or LPS or bacterial ligands (213, 214). By treating inflamed tissue *ex vivo* directly taken from colitic animals, we aimed at reflecting an *in vivo* treatment effect as close as possible. Furthermore, a direct comparison to human tissue cultured under the same conditions can be obtained.

MICRO-COMPUTED TOMOGRAPHY (paper IV)

The aim of paper IV was to evaluate colon wall thickness as a non-invasive marker in the DSS model using micro-CT. Mice were examined by micro-CT one, two or four times between day 0 (d0) and d26 after induction of colitis to document the kinetics of changes in colon wall thickness and its relation to colitis development. This provides

an important tool in stratifying responding and non-responding animals. Potential impacts of handling and anesthesia were considered upon repeated examinations.

Comment: We and others have extensively characterized the DSS model. However, the problem of anticipating the severity of colonic inflammation without sacrificing the animal remains an open issue. Therefore, several methods have been evaluated to assess the severity of the intestinal inflammation, ranging from blood and urine tests to advanced imaging methods such as endoscopy, MRI and CT. The challenge of evaluating the utility of CT in the DSS model was a part of a larger assessment of imaging methods, such as endoscopy and MRI. CT is a relatively quick and inexpensive method with a higher image resolution compared to MRI and reveals the colon wall thickness rather than the surface of the mucosa as in endoscopy.

INFLAMMATORY MARKERS AND PHENOTYPIC CHARACTERISATION OF T CELLS (paper I-IV)

Blood was routinely collected from the DSS model and sometimes from the Gαi2 model and analysed for the acute phase protein, haptoglobin (9). In some cases, the blood was also analysed for cytokines using the xMAP technology developed by Luminex Corporation (Austin, Texas, USA) (9, 215). Colonic tissue was snap-frozen and analysed for protein content – up to eight different markers were simultaneously analysed from the same tissue homogenate; IL1-β, IL-6, IL-12p40, IL-17, CXCL1/KC, CCL2/MCP-1, CCL5/RANTES and TNFα. In paper III colonic tissue were analysed using low density TaqMan array and RT-PCR (reverse transcriptase - polymerase chain reaction) in addition to IL-6 protein content from *ex vivo* cultures by ELISA. In paper I and II extensive characterisation of T cells was performed using FACS analysis.

Comment: One of the problems with using mice is the limited amount of colonic tissue. A common method to analyse colonic protein is multiple enzyme-linked immunosorbent assay (ELISA) (216). Colons were homogenised, yielding approximately 150-300 µl supernatant. Since 50 µl is used for one traditional ELISA, only three separate assays can be performed on tissue from each colon. Using a fluorescent-bead-based technology (xMAP technology developed by Luminex Corporation (Austin, Texas)) it is possible to detect multiple markers in the same sample volume (215). Special polystyrene beads prefilled with varying concentrations of two fluorescent dyes is used, and each bead is prelabelled with a specific antibody towards the molecule that is to be analyzed. The beads are then pooled and mixed with the sample and biotinylated antibodies. After incubation and washing, the beads are mixed with e.g., Strept-Avidin phycoerytrin (SA-PE). The sample is then passed through two lasers, one laser measuring the intensity of the SA-PE while the other laser measures the fluorescent content of the beads. Theoretically, with this technique, 100 different markers can be identified within one sample.

RT-PCR reactions measure the level of the protein on the transcriptional level, and gene arrays have been developed to measure many genes simultaneously (217). When assessing the production of proteins on the transcriptional level the amount of RNA transcribed from the genome is measured. Since RNA is very sensitive to degradation it is convenient to copy the RNA back to the more stable DNA form (cDNA). Thereafter, the cDNA is multiplied through the PCR reaction making it possible to quantitatively measure the actual amount of DNA transcribed. The gene arrays performed in paper III produced an "on-the-spot" picture of a 93 gene panel, in DSS treated mice and one Gαi2$^{-/-}$ mouse with pronounced colitis. Due to small sample volumes, array results generally need to be confirmed by a separate RT-PCR for each gene. More common is to select a small panel of genes for confirmation. In this study arrays were performed on three different colitis situations, DSS treated and Gαi2$^{-/-}$ mice in addition to one UC patient. Separate RT-PCRs for IL-1β, IL-6, NOS2 (mouse) and IL-1β, IL-6 TNF-α (human) were performed in paper III.

Proteins can also be detected on the surface of the cell using Fluorescence Activated Cells Sorter (FACS) analysis. Cells are incubated together with antibodies that are labelled with different fluorochromes. The cells are then passed through a laser that excite the fluorochromes and emit light in specific wavelengths. In this way up to nine different "colours" can be used to identify the different markers. However, more than four or five colours are difficult to operate.

STATISTICS (paper I-IV)

When calculating the significance of the results various statistical methods were used in the studies. An extended ANOVA test was used in paper I, while unpaired two-way t-tests were employed in paper II and for mRNA expression in paper III. The Mann-Whitney U test was used for in vivo data in paper III and IV. For correlation analysis the Pearson correlation was used in both paper I and IV.

RESULTS AND DISCUSSION

This section aims to give a brief overview of the results in this thesis and how the results were interpreted. Also, some results that were not published are shown and discussed. Results from paper II (manuscript), III (submitted) and IV (submitted) will not be shown in details in this section, as this text will be published on the Internet. The results will, however, be generally discussed and referred to by their figures within the manuscripts.

The first part of the section deals with alterations in T cell compartments during colitis. The second part of the thesis searches for new methods in evaluating and monitoring colitis and how these techniques can be used in the evaluation of human IBD. Comparisons of the DSS induced and Gαi2$^{-/-}$ models are discussed through out the section.

ORGANISATIONAL CHANGES IN T CELL COMPARTMENTS DURING COLITIS

T cells in the colon are affected during colitis. However, forceful inflammation in the colon usually causes the animal or the patient to suffer from symptoms such as body weight loss and diarrhoea. The aim of this first section was to elucidate to what extent T cells in the thymus and the small intestine are affected during colitis.

COLITIS-INDUCED THYMIC INVOLUTION (Paper I, published)

Thymic involution has been observed during various inflammatory conditions (27, 56, 60, 218, 219) and paper I examines the effect of DSS-induced colitis on the thymus in C57BL/6 and Balb/c mice. Both strains develop an acute colitis with similar clinical symptoms and histopathological changes (9). However, whereas the acute colitis completely resolves in BALB/c mice, in C57BL/6 mice it progresses into a chronic inflammation subsequent to the acute inflammatory response.

Balb/c mice displayed an increased colon inflammation score and elevated haptoglobin levels at day 5 of colitis (Figure 1D-F, paper I) and at that time-point no effects on the thymocyte composition was observed (Figure 4B and 5C-D, paper I). However, 6 days of DSS exposure resulted in transient alteration in plasma haptoglobin levels and thymocyte subset distribution (Figure 1F and 5C-D, paper I). Four weeks later, at the time which Balb/c mice were recovered from clinical or histopathological signs of colitis the thymus was restored to a normal size and phenotypic distribution.

During the thymic involution in the acute phase of DSS-induced colitis in C57BL/6 mice DP thymocytes disappeared from the thymic cortex, which is though to be the result of massive apoptosis (57, 220). The cortex, normally filled with DP thymocytes

was almost absent in the acute phase whereas the medulla did not decrease (Figure 4). A closer analysis of the maturation stages during thymocyte maturation (outlined in Figure 1) revealed that in addition to the reduction in DP thymocytes, the composition of SP thymocytes was also affected. Seven days after the 5-day administration of DSS (d5+7) the fraction of DP thymocytes being in maturation stage 1-2 was only 30% as compared to 80% of total thymocytes in C57BL/6 control mice. The $CD4^+$ SP thymocytes were instead increased, and thymocytes in maturation stage 4 were increased from a mean value of 4% to 12% in DSS-induced colitic mice compared to controls and from 1% to 19% in stage 5 (Figure 4, paper I). An increased frequency in $CD8^+$ SP thymocytes was also seen (data not shown). Surprisingly, the subset distribution in DSS treated C57BL/6 mice were restored to normal levels three weeks post DSS administration (d5+21), despite the chronic intestinal inflammation. In Balb/c mice receiving DSS for 6 days, a significant reduction in the frequency of thymocytes in maturation stage 4 within both the $CD8^+$ and the $CD4^+$ SP thymocyte populations were observed, whereas no significant changes could be detected in maturation stage 5 (Figure 5C and D, paper I).

After having analysed the DP and SP thymocytes we examined the composition of the DN thymocytes. This analysis was performed on Balb/c mice, since we observed that these Balb/c mice contained a somewhat larger proportion of DN thymocytes compared to C57BL/6 mice. Interestingly, an increase in both the proportion and absolute number of DN1 thymocytes among total DN thymocytes were observed during acute colitis (Figure 6, paper I). The fraction of DN4 thymocytes was decreased in percentage but not absolute number. The increase in percentage and absolute number of DN1 thymocytes possibly reflects an enhanced influx of precursor T cells into the thymus or possibly a compensatory increase in proliferation of immature DN thymocytes during colitis-induced thymic involution.

A very interesting finding in C57BL/6 mice was the complete restoration of the thymus with respect to size, numbers and phenotype within two weeks after the severe involution in the acute phase of colitis. The cortex/medulla ratio was also normalised in these mice (Figure 4). At day 5+35 of colitis, a tendency towards a reduction in the ratio was observed, which could possible reflect an accelerated thymic involution due to ageing.

The immediate set of reactions following a trauma such as acute inflammation is referred to as the acute phase response, where acute phase proteins (e.g., haptoglobin, CRP, serum amyloid A (SAA)) and the cytokines IL-1β, IL-6 and TNF-α are produced. These cytokines stimulate the hypothalamic-pituitary-adrenal (HPA) axis and induce the release of glucocorticoids (GC) (220). Balb/c mice have been shown to have a higher concentration of circulating endogenous steroids than C57BL/6 mice and upon infection, C57BL/6 mice develop a more extensive thymic involution than Balb/c mice (221). C57BL/6 mice have also been shown to be more susceptible to infections by e.g., *Trypanosoma cruzi* and *Plasmodium berghei* than Balb/c mice (222). In this study, endogenous glucocorticoids were elevated in both strains upon infection but to a higher extent in the less susceptible Balb/c strain (223, 224).

Glucocorticoid receptor antagonist RU486 treatment of infected mice aggravated the disease but prevented thymic involution (224).

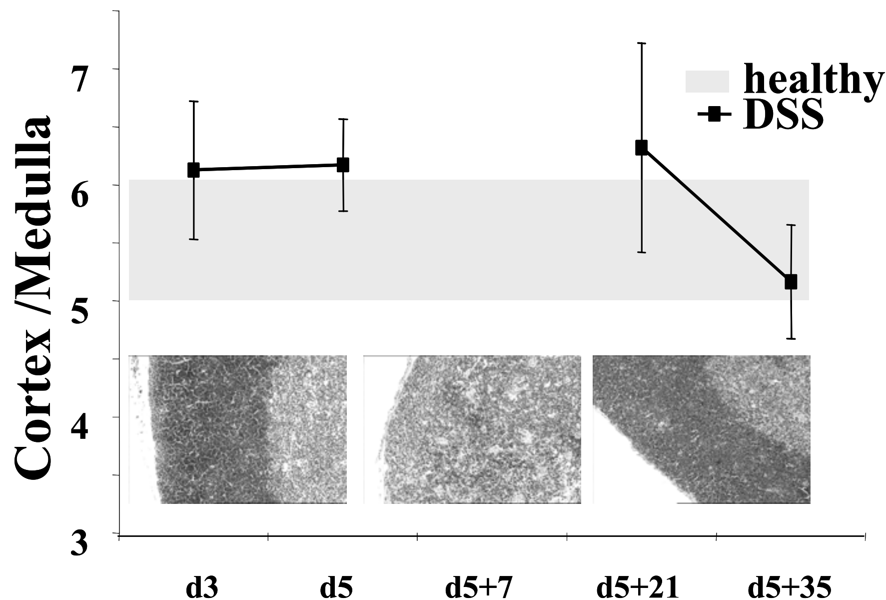

Figure 4: Cortex/medulla ratio in healthy and DSS induced C57BL/6 mice. Inserted in the figure are representative histological pictures demonstrating the lack of a distinguishable cortex in the acute phase of colitis. Cortex; dark grey, medulla; light grey, original magnification 10x. Data are shown as mean ± S.D. n=5-6/time point.

The apoptosis of DP thymocytes during thymic involution is thought to be mediated by endogenous steroids and thymic involution does not occur in adrenalectomized animals (54, 57). It is not known why the C57BL/6 mice but not Balb/c mice develop a chronic colitis in response to DSS. It has been shown that Balb/c mice display higher levels of endogenous corticosteroids than C57BL/6 mice (222). This could possible suppress the inflammation in Balb/c mice and in turn result in a milder thymic involution. Also, compared to C57BL/6 mice, Balb/c mice develop a more limited intestinal inflammation in response to DSS, and this may not affect the systemic parameters to such extent that it severely affect the thymus.

The thymic involution in the DSS model was found to be very similar to the thymic involution observed in colitic Gαi2$^{-/-}$ mice (27). However, in contrast to the transient thymic alterations in the DSS-induced colitis in both Balb/c and C57BL/6 mice the thymic alterations were irreversible in the Gαi2$^{-/-}$ model.
Thymocytes from Gαi2$^{-/-}$ mice with colitis displayed a defective chemotactic migration towards chemokines known to be involved in thymocyte maturation, mainly CXCL12 and CCL25 (27). Interestingly, preliminary data from DSS treated C57BL/6 mice indicated similar effects on the migration of DP thymocytes towards CXCL12 and CCL25 during the acute phase of colitis (day 5+7) (Figure 5). This effect was not seen at any of the other time-points of colitis (day 3, 5, 5+21 or 5+35) (data not shown). During these chemotactic experiments we found that thymocytes from these Balb/c mice were more sensitive to cell death compared to C57BL/6 thymocytes. We

were therefore unable to draw any conclusions from the chemotaxis experiments performed on Balb/c thymi.

Thus, the observations in paper I suggest that the thymic involution and alterations in thymocyte subset composition as well as thymocyte chemotactic responsiveness are intimately connected to the intestinal inflammatory response in colitis regardless of the inflammatory stimuli.

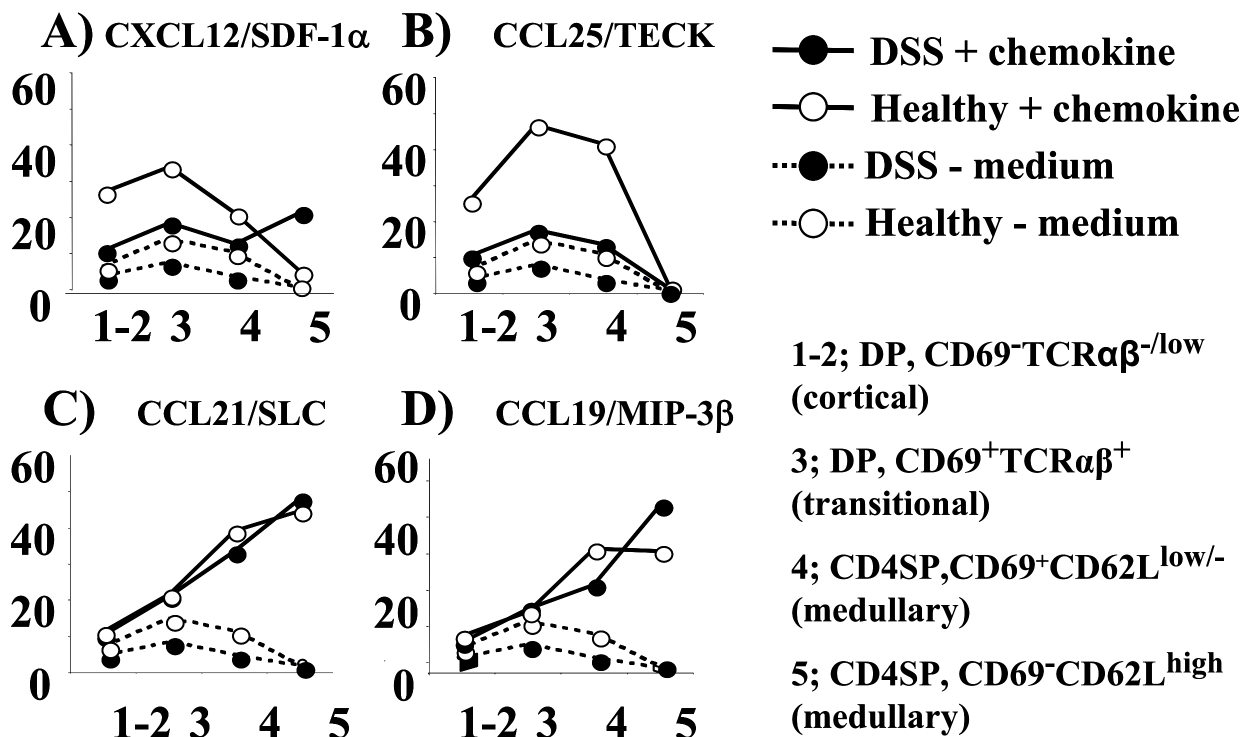

Figure 5: Migration of thymocytes from healthy and DSS induced colitic mice at day 5+7 in response to CXCL12 (A), CCL25 (B), CCL21 (C) and CCL19 (D). Data were generated from one experiment with duplicate wells, using pooled material from two and three mice, respectively.

COLITIS-INDUCED IEL ALTERATIONS (Paper II, manuscript)

It is natural to expect that the T cell populations in the colon are affected during the prominent intestinal inflammation in $G\alpha i2^{-/-}$ colitic mice. The observation in paper I, that colitis affected thymocytes as well as similar results from studies in $G\alpha i2^{-/-}$ mice (27), led us to speculate that this could be the case also in other extracolonic sites, e.g., the small intestine. The analyses in paper II were performed on IELs from both the large and small intestine from $G\alpha i2^{-/-}$ colitic mice and healthy $G\alpha i2^{+/-}$ controls.

Upon termination of the mice, the colon was dissected and macroscopically scored for inflammation. Some mice were also scored for signs of histological inflammation, and the relationship between these two scoring systems was found to be low (Figure 2, paper II). Interestingly, a similar pattern was found also in the DSS model (Figure 6). A likely explanation to this is the fact that whereas the macroscopical scoring system

grades the surface of the entire intestine, while the histological scoring take into account other features such as inflammatory cell infiltration, crypt abscesses, epithelial damage and distortions of glands often grading a more limited area of the intestine. In fact, this phenomenon is also seen in human intestinal inflammation (Prof. Roger Willén, Uppsala University). As shown in one clinical study (212) the opposite can also be the case, i.e., the histopathological grading detected a more advanced inflammatory distribution than the endoscopic evaluation revealed.

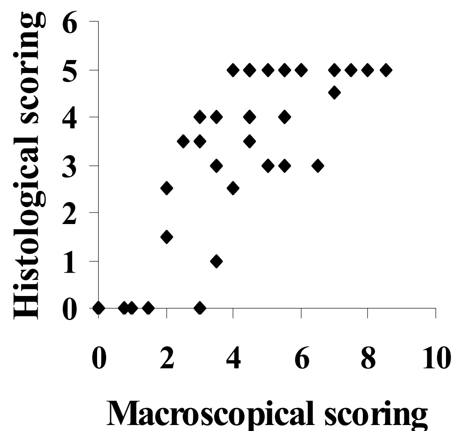

Figure 6: Macroscopical versus histological inflammatory score of colons from DSS-treated mice. Colons scoring maximum on histology score (five) corresponded to a Macroscopical scoring ranging from four to nine on a scale to ten.

IELs were isolated as described in paper II by EDTA treatment followed by separation using a 66.7/50/40/20/0 % Percoll gradient. The 66.7/50 % and 50/40 % interphases that contained IELs were pooled before FACS analysis. A higher yield of small and large IELs can in this way be extracted than using a gradient of 66.7/40 % only. In addition to the expected position of IELs in the forward/side scatter plot (Figure 8), a population of smaller cells were found to express T cell markers. Therefore, they were included in the analysis.

As expected, an increase in the total number of IELs was found in the colon of colitic Gαi2$^{-/-}$ mice compared to healthy Gαi2$^{+/-}$ mice (Figure 1, paper II). Interestingly, the number of IELs was also increased in the small intestine, despite the absence of histopathological inflammation, indicating that the small intestine as well as the colon was affected by the colitis.

The distribution of CD4$^+$ and CD8$\alpha\alpha^+$ SP and DP IELs in the small and large intestine were first examined and it was found that colitis caused changes in both compartments (Figure 4C and D, paper II). However, the alterations in CD4/CD8 distribution during colitis were not the same in the two compartments, CD4$^+$ IELs increased in the large intestine whereas an increase in DP IELs were observed in the small intestine. The findings possibly reflect the presence of IELs with inflammatory properties in the large intestine during colitis, while IELs in the small intestine may even harbour protective properties. This is interesting considering that it is the large and not the small intestine that is subjected to pathological inflammation in Gαi2$^{-/-}$ colitic mice (12).

DP IELs are found in the small intestine during ageing and infections (225, 226) and we found that this population was increased also in Gαi2$^{-/-}$ colitic mice. We phenotypically characterized these cells for the expression of CD44, CD62L, CD69, CCR9 and integrins β7, α4 and αE (Figure 9 A and B, paper II). In parallel, CD4 and CD8α SP IELs were analysed for the same markers. The presence of TCRγδ^{+} IELs was also assessed.

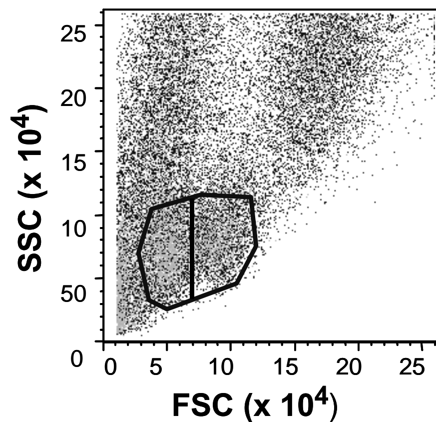

Figure 7: Forward and side scatter (FSC/SSC) plot, demonstrating the gating of small and large IELs. Large IELs were found within the normal cell gate whereas small IELs were found to be approximately half the size of the large IELs. FSC denotes the size of the cell whereas SSC denotes granularity. Large IELs are found in the right gate, and small IELs are found in the left gate.

Interestingly, several observations within paper II indicate that IEL populations are different between various strains of mice, such as the proportions of CD4^{+} and CD8^{+} of IELs. For example, tissue distribution and function of TCRγδ^{+} cells have been shown to differ between species and between different mouse strains (46, 227). It is tempting to speculate that these strain differences contributes to the fact that some strains develop colitis and other do not when made deficient for the Gαi2 protein (188).

In this paper, we also investigated the differentiation of IELs after transfer of CD3^{+} (total T cells) splenic lymphocytes from wt Gαi2$^{+/+}$ or colitic Gαi2$^{-/-}$ mice into lymphocyte-deficient RAG2$^{-/-}$ mice on a 129Sv/Ev background.

Contrary to the frequently used SCID or RAG transfer model where naïve wild type CD45RB^{hi} T cells induce colitis upon transfer (228), the RAG$^{-/-}$ mice on a 129SvEv background that we are using are resistant to colitis induction by wild type cells in the absence of infection (229). Thus, they make up a perfect model for the studies of the colitis-inducing properties of Gαi2$^{-/-}$ cell populations. Previous studies from my group have shown that colitis could be transferred to RAG2$^{-/-}$ recipients from spleen cells of colitic Gαi2$^{-/-}$ donors (184). In the current study, CD3^{+} spleen cells were transferred from pre-colitic Gαi2$^{-/-}$ donors to RAG2$^{-/-}$ recipients and the population of IELs in the recipient animals were phenotypically analysed. It has previously been shown that classic T cells can repopulate the epithelium in SCID mice, and that the IEL population in the small intestine contains a high fraction of DP IELs (230). In line with this, we also found a large fraction of DP IELs within the recipient mice, regardless of the donor phenotype (Gαi2$^{+/+}$ or Gαi2$^{-/-}$) or colitic status of the donor (pre-colitic or colitic).

In DSS induced colitis, aggregation of $TCR\gamma\delta^+$ cells have been observed at sites of DSS-induced epithelial cell damage in the colons of DSS-exposed mice (211). Previous studies in my group at AZ have also demonstrated increased numbers of $CD3^+$ lymphocytes in the colon during DSS-induced colitis (187). Interestingly, as observed in the $G\alpha i2^{-/-}$ model, changes in T cell subsets within the small intestine have been detected also in DSS treated mice (210). During DSS induced colitis $TCR\gamma\delta^+$ T cells accumulated within the PPs. These $TCR\gamma\delta^+$ T cells were thought to originate from the epithelium, since they have a shared $TCR\gamma\delta$ repertoire with the IELs.
IELs have never been characterised in colons of DSS treated mice. The shedding of epithelial cells in the DSS model will most certainly decrease the yield of IELs from the colon. A part of the accumulated $TCR\gamma\delta^+$ cells seen in the colon of DSS treated mice might derive from IELs escaping the epithelium in an attempt to avoid shedding together with damaged epithelial cells. DN T cells have also been detected in the PPs of autoimmune lpr mice (210) in addition to the accumulation in liver, spleen, MLNs (231) but not among IELs, suggesting that the accumulation of cells in the PPs during disease is not specific for $TCR\gamma\delta^+$ cells.

CCR9 plays an important role in the homing of IELs to the small intestine (107). Its ligand CCL25 is abundantly expressed on epithelial cells in the small intestine but in low amount in the colon (113, 232-234).

In paper II we stained IELs with antibodies against CCR9 and surprisingly, we found a low expression on the small IELs in both the large and small intestine (Figure 6, paper II). However, we could not detect any CCR9 expression on the “large” IELs (IELs found within the normal lymphocyte gate), not even in the small intestine (Figure 8). The CCR9 staining was not affected by the addition of FcR-block (2.4G2), and isotype control antibody stained negative as shown in Figure 8.

Upon staining with 7-amino-actinomycin D (7AAD), a marker for cells in late stage of apoptosis, proportionally more small IELs than large IELs were $7AAD^+$. In addition, the proportion of $7AAD^+$ IELs was higher in the large intestine than the small intestine (Figure 9). Interestingly, only the cells that were $7AAD^{hi}$ stained positive for CCR9 (Figure 9). In contrast to the antibody 7E7 used in the reports that describe CCR9 and its function in the small intestine (235), the antibody used in this study (242503 from R&D) (236) do not seem to detect CCR9 in the small intestine, although both antibodies stained thymocytes. This is not easily explained, but CCR9 in the intestine could possibly differ from that in the thymus in the sterical formation in some way, preventing clone 242503 to bind to intestinal CCR9. The explanation to the observation that this clone stains $7AAD^+$ IELs remains elusive.

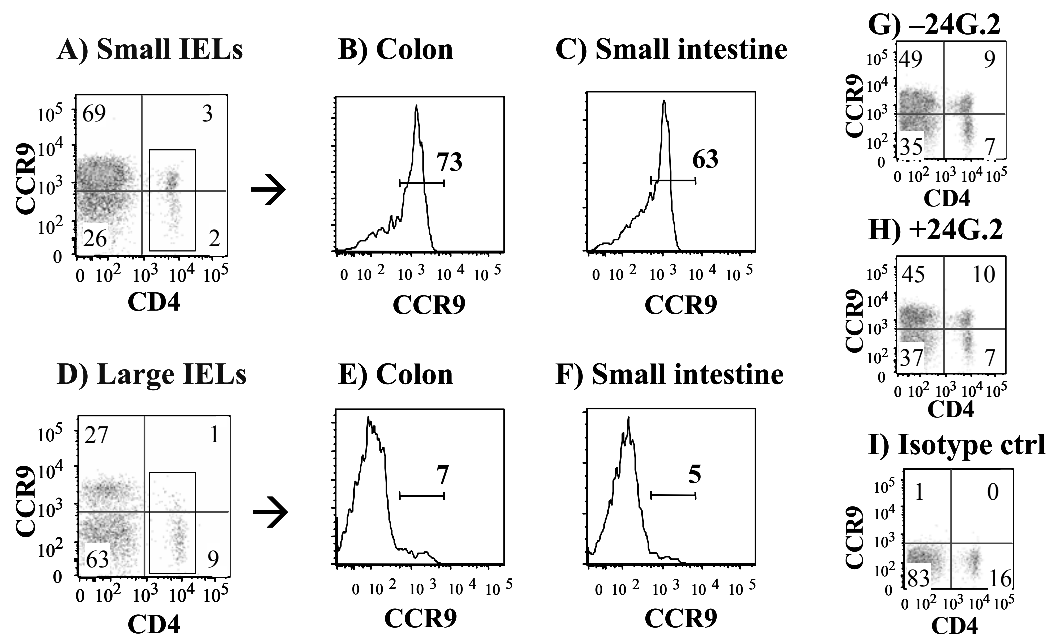

Figure 8: Small (A-C) and large (D-F) size IELs were stained for CCR9 and CD4 and gated according to the FSC/SSC plot. CCR9 histograms for $CD4^+$ IELs show that the majority of small IELs were $CCR9^+$ (B, C) while the large IELs were $CCR9^-$ (E, F). The majority of the $CD4^-$ cells that stained positive for CCR9 were non-T cells. The CCR9 expression could not be abolished by blocking with FC-receptors (24G.2) (G, H) and isotype control stained negative for CCR9 (I). Representative plots from one out of three experiments are shown. Figure A, D, G, H and I show staining from small intestine, but similar results were obtained from colon and $CD8\alpha^+$ IELs.

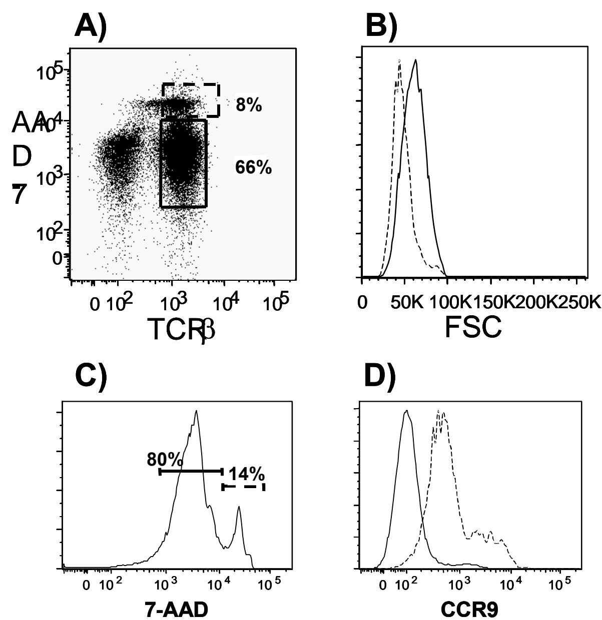

Figure 9: Small IELs are $7AAD^+$ and $CCR9^+$. A) $TCR\alpha\beta^+$ IELs were gated for $7AAD^-$ and $7AAD^+$ cells and B) histograms shows that $7AAD^+$ IELs are smaller (dotted line) than $7AAD^-$ IELs (solid line). C) $CD4^+$ and $CD8\alpha^+$ IELs were gated and analysed for the expression of 7AAD and D) shows that $7AAD^-$ IELs are completely negative for CCR9 whereas a fraction $7AAD^+$ IELs are $CCR9^+$

We detected a higher proportion of $7AAD^+$ cells among small IELs although their expression of CD4 and CD8 were normal. However, no DP IELs were found within the $7AAD^+$ population. $7AAD^+$ cells were also mostly TCR^{low} (data not shown).

In conclusion, the observations in paper I and II suggest that the inflammatory response in the colon have impact not only on the large intestine, but also on the

thymus. The altered T cell phenotype in the small intestine might partly be an effect of the colitis but may also be an inherent capacity of the small intestine to deal with mucosal challenges in a controlled way without pathological manifestations.

NEW WAYS OF ASSESSING AND MONITORING COLITIS

As discussed in the methodological section, it is important to assess the severity of colonic inflammation upon termination of the animal. However, even mice that have been inbred for decades do not respond identical to mucosal inflammatory challenges. This phenomenon is seen to a varying extent in all animal models tested so far. The only certain way to assess the severity of the colonic inflammation has so far been to dissect the colon. However, when treating the mice with anti-inflammatory substances it is desirable to find tools of how to assess the colonic inflammation in the animal prior to treatment, without having to terminate the mouse. This was the aim of paper IV.

A way of assessing the severity of colitis before *in vivo* treatment is to treat the colonic tissue *ex vivo* as in paper III. Since approximately 30 mg colonic tissue is needed per culture, 5-10 cultures can be generated from one mouse colon. An ethical bonus of using these kinds of systems is that more than one compound can be tested on each animal. Substances that show efficacy in the cultures can then be selected for testing *in vivo* and monitored by micro-CT in long-term studies.

MODULATING INFLAMMATORY PARAMETERS IN *EX VIVO* CULTURES (paper III, submitted)

The aim of this study was to investigate the relevance of *ex vivo* cultures of inflamed colonic tissue in the assessment of treatment of IBD using tissue from DSS-induced and Gαi2 deficient colitis as well as human ulcerative colitis (UC). First, the *ex vivo* mouse colonic tissue culture system was established. Total colonic tissue from DSS and Gαi2 colitic mice were cultured in the absence or presence of anti-inflammatory compounds (methyl-prednisolone or the proteasome inhibitor MG132). The viability of the cultures was confirmed using a lactate dehydrogenase assay (LDH) and IL-6 protein was measured in the supernatant. To assess the mRNA expression, TaqMan low-density 93 genes arrays (Table 5) and separate RT-PCR reactions were used to confirm the expression of IL-1β, IL-6 and NOS2 (mouse) and IL-1β, IL-6 and TNF-α (human).

For a general comparison of the two models, we compared the expression of cultured colonic tissue from healthy control mice (C57BL/6 and Gαi2$^{+/+}$ 129SvEv) against DSS-induced and Gαi2$^{-/-}$ colitic mice, respectively. A threshold of two-fold up or down regulated expression compared to healthy control mice was used to define gene response. Twenty-seven genes were excluded because they were not detected neither in the DSS nor in the Gαi2$^{-/-}$ model, i.e., they were not amplified after 35 cycles. Of

Table 5: Mouse and human genes assayed with TaqMan low-density arrays.

Name protein	Mouse gene	Human gene
angiotensin receptor II	Agtr2-Mm00431727_g1	AGTR2-Hs00169126_m1
Bax	Bax-Mm00432050_m1	BAX-Hs00180269_m1
Bclx	Bcl2l1-Mm00437783_m1	BCL-XL-Hs00169141_m1
Bcl2	Bcl2-Mm00477631_m1	BCL2-Hs00153350_m1
C3	C3-Mm00437858_m1	C3-Hs00163811_m1
CCL19/MIP-3β	Ccl19-Mm00839967_g1	Mip-3c-Hs00171149_m1
CCL2/MCP-1	Ccl2-Mm00441242_m1	MCP-1-Hs00234140_m1
CCL3/MIP-1α	Ccl3-Mm00441258_m1	Mip-1a-Hs00234142_m1
CCL5/RANTES	Ccl5-Mm01302428_m1	Rantes-Hs00174575_m1
CCR2	Ccr2-Mm99999051_gH	CCR2-Hs00174150_m1
CCR4	Ccr4-Mm00438271_m1	CCR4-4324386-CCR4
CCR7	Ccr7-Mm00432608_m1	CCR7-Hs00171054_m1
CD19	Cd19-Mm00515420_m1	CD19-Hs00174333_m1
CD28	Cd28-Mm00483137_m1	CD28-Hs00174796_m1
CD34	Cd34-Mm00519283_m1	CD34-Hs00156373_m1
CD38	Cd38-Mm00483146_m1	CD38-Hs00233552_m1
CD3ε	Cd3e-Mm00599683_m1	CD3-Hs00167894_m1
CD40L	Cd40lg-Mm00441911_m1	CD154-Hs00163934_m1
CD40	Cd40-Mm00441895_m1	CD40-Hs00374176_m1
CD4	Cd4-Mm00442754_m1	CD4-Hs00181217_m1
CD68	Cd68-Mm00839636_g1	CD68-Hs00154355_m1
CD80	Cd80-Mm00711660_m1	CD80-Hs00175478_m1
CD86	Cd86-Mm00444543_m1	CD86-Hs00199349_m1
CD8α	Cd8a-Mm01182107_g1	CD8-Hs00233520_m1
collagen type IV alpha 5	Col4a5-Mm00801606_m1	COL4A5-Hs00166712_m1
M-CSF	Csf1-Mm00432688_m1	CSF-1-Hs00174164_m1
GM-CSF	Csf2-Mm00438328_m1	CSF-2-Hs00171266_m1
G-CSF	Csf3-Mm00438334_m1	CSF-3-Hs00357085_g1
CTLA-4	Ctla4-Mm00486849_m1	CD152-Hs00175480_m1
CXL10/IP10	Cxcl10-Mm00445235_m1	IP10-Hs00171042_m1
CXL11/ITAC	Cxcl11-Mm00444662_m1	ITAC-Hs00171138_m1
cytochrome p450, family 1	Cyp1a2-Mm00487224_m1	CYP1A2-Hs00167927_m1
cytochrome p450, family 7	Cyp7a1-Mm00484152_m1	CYP7A1-Hs00167982_m1
endothelin converting enzyme	Ece1-Mm01187091_m1	ECE-1-Hs00154837_m1
endothelin 1	Edn1-Mm00438656_m1	EDN1-Hs00174961_m1
FasL	Fasl-Mm00438864_m1	FasL-Hs00181225_m1
Fas	Fas-Mm00433237_m1	Fas-Hs00163653_m1
fibronectin 1	Fn1-Mm01256734_m1	FN-Hs00365052_m1
GADPH	Gapdh-Mm99999915_g1	GAPDH-Hs99999905_m1
glucuronidase, beta	Gusb-Mm00446953_m1	GUSB-Hs99999908_m1
Granzyme B	Gzmb-Mm00442834_m1	GZMB-Hs00188051_m1
MHC class II Ea	H2-Ea-Mm00772352_m1	HLADR-4328304-HLADRB1
MHC class II Eb	H2-Eb1-Mm00439221_m1	HLA-DRA-Hs00219575_m1
Heme oxygenase-1	Hmox1-Mm00516004_m1	HO-1-Hs00157965_m1
ICOS	Icos-Mm00497600_m1	ICOS-Hs00359999_m1
IFN-γ	Ifng-Mm00801778_m1	IFN-g-Hs00174143_m1
IκB	Ikbkb-Mm00833995_m1	IkB2-Hs00395088_m1
IL-10	Il10-Mm00439616_m1	IL-10-Hs00174086_m1
p35	Il12a-Mm00434165_m1	IL-12p35-Hs00168405_m1
p40	Il12b-Mm00434170_m1	IL-12p40-Hs00233688_m1
IL-13	Il13-Mm00434204_m1	IL-13-Hs00174379_m1
IL-15	Il15-Mm00434210_m1	IL-15-Hs00174106_m1

IL-17	Il17-Mm00439619_m1	IL-17-Hs00174383_m1
IL-18	Il18-Mm00434225_m1	IL-18-Hs00155517_m1
IL-1α	Il1a-Mm00439620_m1	IL-1a-Hs00174092_m1
IL-1β	Il1b-Mm00434228_m1	IL-1b-Hs00174097_m1
IL-2	Il2-Mm00434256_m1	IL-2-Hs00174114_m1
IL-2-Rα	Il2ra-Mm00434261_m1	CD25-Hs00166229_m1
IL-3	Il3-Mm00439631_m1	IL-3-Hs00174117_m1
IL-4	Il4-Mm00445259_m1	IL-4-Hs00174122_m1
IL-5	Il5-Mm00439646_m1	IL-5-Hs00174200_m1
IL-6	Il6-Mm00446190_m1	IL-6-Hs00174131_m1
IL-7	Il7-Mm00434291_m1	IL-7-Hs00174202_m1
IL-9	Il9-Mm00434305_m1	IL-9-Hs00174125_m1
TNF-β	Lta-Mm00440227_m1	Lta-Hs00236874_m1
NFκB2	Nfkb2-Mm00479807_m1	NFKB2-Hs00174517_m1
iNOS	Nos2-Mm00440485_m1	Nos2A-Hs00167248_m1
perforin	Prf1-Mm00812512_m1	PRF1-Hs00169473_m1
COX	Ptgs2-Mm00478374_m1	Ptgs2-Hs00153133_m1
CD45	Ptprc-Mm00448463_m1	CD45-Hs00365634_g1
CD62E	Sele-Mm00441278_m1	CD62E-Hs00174057_m1
CD62P	Selp-Mm00441295_m1	CD62P-Hs00174583_m1
Sloan-Kettering viral oncogene homologue	Ski-Mm00448744_m1	SKI-Hs00161707_m1
Smad3	Smad3-Mm00489637_m1	MADH-3-Hs00232219_m1
Smad7	Smad7-Mm00484741_m1	MADH-7-Hs00178696_m1
STAT3	Stat3-Mm00456961_m1	Stat3-Hs00234174_m1
T-bet	Tbx21-Mm00450960_m1	TBX21-Hs00203436_m1
transferrin	Tfrc-Mm00441941_m1	CD71-Hs99999911_m1
TGF-β	Tgfb1-Mm00441724_m1	TGF-b-Hs00171257_m1
TNF-α	Tnf-Mm00443258_m1	TNF-a-Hs00174128_m1
GITR	Tnfrsf18-Mm00437136_m1	TNFRSF18-Hs00188346_m1
Vasc. endoth.growth factorA	Vegfa-Mm00437304_m1	VEGF A-Hs00173626_m1
β2-microglobulin	B2m-Mm00437762_m1	Na
low density lipoprotein receptor-related protein 2	Lrp2-Mm01328172_g1	Na
NFκB	Nfkb1-Mm00476361_m1	Na
Phosphoglycerate kinase 1	Pgk1-Mm00435617_m1	Na
SOCS1	Socs1-Mm00782550_s1	Na
SOCS2	Socs2-Mm00850544_g1	Na
STAT1	Stat1-Mm00439518_m1	Na
STAT4	Stat4-Mm00448890_m1	Na
STAT6	Stat6-Mm00447411_m1	na
VCAM-1	Vcam1-Mm00449197_m1	na
CXCR3	Cxcr3-Mm00438259_m1	na
HPRT	Hprt1-Mm00446968_m1	na
Angiotensin I-conv. enzyme	na	ACE-Hs00174179_m1
AGTR1	na	AT1R-Hs00241341_m1
β-actin	na	Beta Actin-Hs99999903_m1
CCR5	na	CCR5-Hs00152917_m1
ICAM	na	CD54-Hs00164932_m1
granulysin (a lymphokine)	na	GNLY-Hs00246266_m1
IL-8	na	IL-8-Hs00174103_m1
megalin	na	MEGALIN-Hs00189742_m1
myosin	na	MYH6-Hs00411908_m1
renin7angiotensin	na	RENIN-Hs00166915_m1
ribosomal protein	na	RPL3L-Hs00192564_m1
ribosomal protein	na	18S-Hs99999901_s1

na, not assayed Adapted from htpp://www.appliedbiosystems.com

the remaining 66 genes 23 genes were up regulated more than two-fold in both models. Eight genes were up regulated in the DSS model but not in the Gαi2$^{-/-}$ model, and 13 genes were up regulated in the Gαi2$^{-/-}$ model but not in the DSS model. Two genes were down regulated in both models whereas the remaining genes were not regulated in either model. In summary, 85% of the detected genes (57/66) were similarly regulated in both models compared to controls. Genes regulated by inflammation in both models belonged to cytokines, chemokines/chemokine receptors, T and B cell marker, co-stimulatory markers and oxidative stress mediators.

Next, we treated the cultures with either the steroid methyl-prednisolone or the proteasome inhibitor MG132. In addition to the mouse cultures, colonic tissue from human UC patients was treated with the same substances. We chose these compounds since methyl-prednisolone belongs to a class of substances with clinically documented efficacy in IBD (237, 238), while proteasome inhibitors are a relatively new class of anti-inflammatory compounds (239). Both compounds suppressed several genes (Table 6) including chemokine/chemokine receptors, cytokine/cytokine receptor, co-stimulatory or adhesion molecules and oxidative stress mediators. Neither methyl-prednisolone nor MG132 up regulate any gene more than two-fold. In summary, both substances were suppressive, although MG132 suppressed more genes than did methyl-prednisolone in both mouse and human cultures.

Table 6: Number of genes down regulated in DSS, Gαi2$^{-/-}$ or UC cultures after treatment with MG132 or methyl-prednisolone

Model/substance	*MG132*	*Methyl-prednisolone*	*MG132 and methyl-prednisolone (separate cultures)*
DSS	34	31	17
Gαi2$^{-/-}$	49	20	17
UC	39	8	7
In both DSS and Gαi2$^{-/-}$ models (separate cultures)	27	16	14
In both DSS, Gαi2$^{-/-}$ and UC models (separate cultures)	17	8	7

As described above, the DSS induced and Gαi2$^{-/-}$ colitic mice generated similar response upon *ex vivo* anti-inflammatory treatment of colonic tissue. Next, we compared the anti-inflammatory responses in ex *vivo* cultures to *in vivo* treatment to further strengthen the results achieved in the cultures. Therefore, we treated DSS induced and Gαi2$^{-/-}$ colitic mice with methyl-prednisolone *in vivo* and performed the same analysis of mRNA expression as on the *ex vivo* cultures. The expression of IL-1β, IL-6 and NOS2 was found to be similarly regulated *ex vivo* and *in vivo*. For a more detailed analysis we performed *in vivo* array analysis on colonic tissue from healthy C57BL/6 control mice and DSS colitic mice before and after treatment with methyl-prednisolone and compared that to the responses obtained in the cultures. A highly

similar profile of genes being expressed were observed in the inflamed tissue *in vivo* and *ex vivo* compared to healthy controls; 37 genes were similarly regulated (higher than or lower than two-fold) and 14 genes were differently regulated. Of these 14 genes, 13 genes were up regulated *in vivo* but not *ex vivo* and one gene was up regulated *ex vivo* but not *in vivo*. Also, methyl-prednisolone treatment generated a highly similar response *in vivo* and *ex vivo*. 51 genes were similarly regulated *in vivo* or *ex vivo*, and 14 differed in regulation. Of these 14 genes, 8 genes were down regulated *in vivo* but not *ex vivo* and 6 genes were down regulated *ex vivo* but not *in vivo*.

In line with earlier studies, no obvious beneficial treatment effect of methyl-prednisolone was seen on macroscopic or histological inflammation scoring of colitis (240, 241). Steroids in general are potent anti-inflammatory agents acting on the transcriptional level but can induce severe side effects, such as body weight loss, osteoporosis and susceptibility to infections, if used over a long time period (242). This phenomenon is in agreement with the findings in animal models where the animals sometimes actually display worsened clinical (i.e., body weight loss) and histological parameters upon steroid treatment (240). Steroids have been used to treat inflammatory conditions for 60 years (237) and are still one of the largest classes of anti-inflammatory compounds, which is one of the best proofs that steroids actually possess anti-inflammatory properties. The need for new substances is obvious and proteasome inhibitors have shown promising results. Proteasome inhibitors act by stabilizing IκB, thereby preventing the activation of NfκB and the subsequent production of proinflammatory cytokines (243). Proteasome inhibitors have so far been tested in animal models of bacterial infections (244, 245) and has also been discussed as a potential cancer therapy (246). Its efficacy in IBD remains to be investigated, although the present study demonstrate promising results.

In conclusion, we have validated a murine *ex vivo* culture system, which reflects the situation *in vivo* on the basis of regulation of genes in inflamed tissue. We have also demonstrated similar anti-inflammatory responses by methyl-prednisolone in murine cultures as compared to *in vivo* treatment of colitis and our preliminary findings indicate a good relationship to *ex vivo* cultures from human UC. Thus, we conclude that the murine culture system can be used as a first screening model to validate future therapies for IBD.

COLON WALL THICKNESS AND ITS RELEVANCE TO COLITIS (Paper IV, accepted)

All animal models of colitis developed this far contain a certain degree of variability between individuals with respect to the induction and severity of colitis. None of the non-invasive parameters used today, e.g., body weight, diarrhoea, blood or urine biomarkers such as haptoglobin or nitrite have a perfect correlation to the degree of colitis (9, 247). It is thus highly desirable to find parameters that enable determination of the grade of inflammation before start of drug treatment. It was recently

demonstrated that colon wall thickness can be measured using magnetic resonance imaging (MRI) in healthy and DSS treated mice (186, 248). Computed Tomography (CT) is a relatively quick and inexpensive method with a higher image resolution compared to MRI. Micro-CT has never, to our knowledge, been used to monitor the colon wall thickness during colitis development in mice. Therefore, the aim of this study was to investigate whether it was possible to measure the colon wall thickness in mice using micro-CT and to predict and monitor the changes in colon wall thickness by repeated imaging. Also, the relation between colon wall thickness and the severity of inflammation was examined.

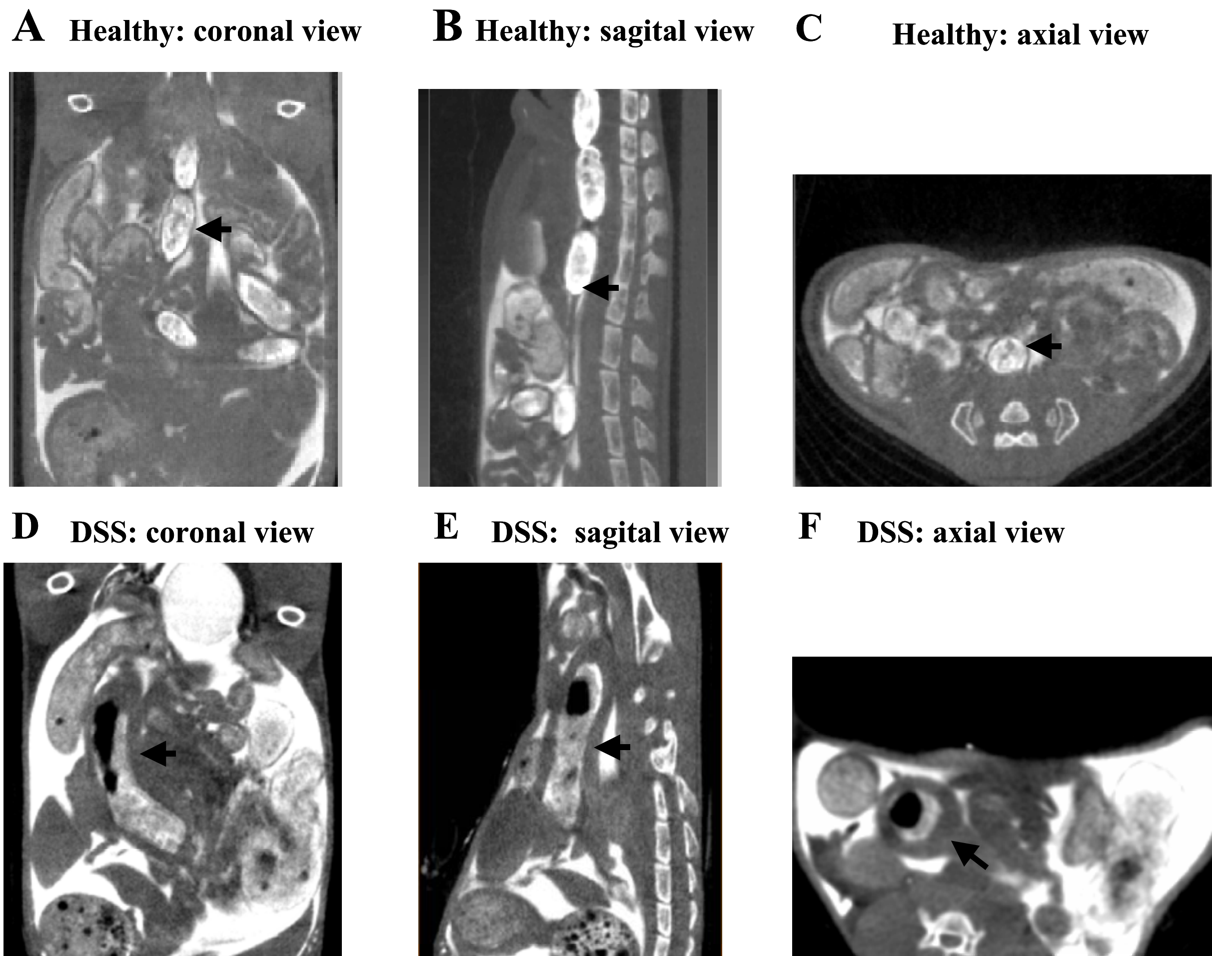

Figure 10: Representative micro-CT images visualizing the colon of healthy C57BL/6 mice (A-C) and DSS treated mice at day 5+21 (chronic phase) of colitis (D-F). Coronal (A, D), sagital (B, E) and axial (C, F) views. In A, B, D and E, the mouse is shown "upside down", with the tail in the upper part of the images. Contrast agents in the feaces and in the peritoneum are seen as light areas, black areas inside the colon is air or gases. In the upper part in D, the urine bladder is seen as a large light "bubble".

Mice were examined by micro-CT one, two or four times between day 0 and day 26 of colitis to document the kinetics of colon wall thickness and its relation to colitis development. First, we confirmed that it was possible to detect the colon wall in healthy and colitic mice using micro-CT (Figure 10).

After having established the kinetics of the colon wall thickening in individual mice, it was clear that the increase in colon wall thickness was relatively predictable and occurred mainly during the acute phase of colitis (up to day 12) (Figure 3A, paper IV). The colon wall thickness was not enhanced much further in the chronic phase of colitis. Importantly, the difference in response to DSS between different mice was manifested already during the first two weeks of colitis, and mice that did not develop a colon wall exceeding 300 μm in thickness were demarcated as non-responders (Figure 4A–B, paper IV). However, this cutoff probably need be adjusted in each study, depending on the nature of the study. Importantly, once the colon wall thickness reached its maximum thickness at day 12, it was stabilized and remained thickened for at least two more weeks. Consequently, mice that do not develop a significant colon wall thickness at day 12 of colitis should be excluded from an imminent drug treatment. The colon wall thickness as measured by micro-CT correlated well with the measurements performed on histological sections (R=0.81, Figure 3B, paper IV). Further, the macroscopical inflammation score increased with the colon wall thickness. Interestingly, it seems that below the cut-off value (in this study 300 μm), the association between micro-CT and colon wall thickness is different to above the cut-off. This is most likely due to that the superficial damage of the mucosa occur prior to the events that cause the colon wall thickening (e.g., infiltration of cells and oedema).

At the day of examination, the mice were brought to the examination room and kept anesthetisized for approximately 20 minutes, contrast agents were injected intraperitoneally (i.p.) and micro-CT examination was performed (125mGy irradiation). To examine potential effects of repeated micro-CT (four times/animal), a control experiment was performed (study outline in table 7). We did not find any

Table 7: *Influence of multiple CT on the development of colitis; study design*

Group	*Treatment*
1- DSS	Entire CT protocol
2 - DSS	Sham CT; no irradiation exposure
3 - DSS	Sham CT; no irradiation exposure, no buscopan
4 - DSS	Housed in the same cage as group 1-3; not anaesthetized but moved to separate cages during 15 minutes
5 - DSS	Housed in separate cage; Handled in parallel to group 4.
6 - DSS	DSS controls, remained in the animal facility until the day of termination
7 – no DSS	Water controls, remained in the animal facility until the day of termination

Animals were examined/handled four times; at d5, d8, d12 and d19. Animals from group 1-4 were housed together, i.e. two mice from each group was housed in cages containing eight mice each (a total of four cages) receiving $BaSO_4$ containing diet at least 12 hours prior to experiments, groups 1-3 were anaesthetizised. Groups 5-7 was housed in separate cages and were given $BaSO_4$ only the day before termination of the study – all mice were examined by CT on the day of termination.

significant alterations in any parameter tested (body weight loss, inflammatory scoring of the colon, plasma haptoglobin, IL-6 or stress hormones (adrenalin and noradrenalin)) when comparing one and four micro-CT examinations (Table 3, paper

IV). However, animals subjected to four micro-CT displayed a tendency to lower macroscopical inflammatory scoring and higher plasma adrenalin compared to animals subjected to one CT examination. This emphasizes the impact of handling and anaesthetization of the animals during the study. Importantly, irradiation exposure during micro-CT did not affect the development of colitis.

In conclusion, the results predict that micro-CT is a highly relevant technique for evaluating the onset of disease in other IBD models such as the $G\alpha i2^{-/-}$ mouse that spontaneously develops colitis.

GENERAL DISCUSSION

Animal models are used as tools to understand human diseases, including IBD. To translate findings in mice to human, an impressive number of animal models of colitis have been developed, each representing different aspects of the human disease. As a complement to *in vivo* studies, *ex vivo* and *in vitro* models has been developed, which often permits testing of different substances on almost identical material. In the section below, I will discuss some of the questions that were considered already before or during the work in this thesis.

Why is the colon but not the small intestine inflamed in most colitis models, and why are there so few animal models of small intestinal inflammation?

Up to date, more than 60 animal models of colitis have been described (181, 249). When this year's Nobel Prize winning technique of generating transgenic and knockout mice was developed, immunologists started to create knockout and transgenic mice for many of the proteins known to play central roles in the immune system, such as IL-2, IL-4, IL-7, MHC I and MHC II, TCRα, β, γ or δ (250). People asked questions as "what will happen to the immune system if we removed "the" Th1 cytokine IL-2 (251) or "the" Th2 cytokine IL-4 (252)". Surprisingly, many of the deficient mice developed a colonic inflammation, e.g., IL-$2^{-/-}$ (253) and IL-$10^{-/-}$ (254) mice. Interestingly, mice that were made deficient for other molecules known to be involved in other cell signalling pathways, for example the Gαi2 protein also developed colitis (197). Almost all colitis models tested so far fail to develop colitis during germ-free conditions, demonstrating the delicate balance between the colonic mucosal immune system and the continuous presence of the normal bacterial flora in the intestinal lumen (255). It has been estimated that 10^{14} bacteria of more than 400 different species reside in the intestine (256) and this has to be dealt with every day. We now know that removing only one piece of the puzzle, such as IL-2, IL-10 or Gαi2, in many cases results in an uncontrolled immune response in the colon but not in the small intestine. The intrinsic functions of the small and large intestines, to mediate nutrient/antigen uptake and regulate salt/water balance, respectively (257), may render the small intestine better equipped to maintain the immunologic homeostasis during mucosal challenges. As demonstrated in paper I and II, non pathogenic alterations in extracolonic tissues such as the thymus and small intestine occur during colitis. Subsets of IELs mainly found in the small intestine (DP, TCR$\gamma\delta^+$) have been demonstrated to protect against colitis in the presence of IL-10 (258). If the presence of these cells or their functions are induced due to the micro environment in the small intestine, it would at least partly explain why so few models of small intestinal inflammation has been described. Interestingly, the ileitis in SAMP-1/Yit mice was shown to be associated with a proportional decrease in CD8$\alpha\alpha^+$ and TCR$\gamma\delta^+$ IELs while the frequency of CD8$\alpha\beta^+$ IEL increased (259). It is possible that if the composition of IELs in the small intestine is altered to a more "colon-like" distribution, as for example in the SAMP-1/Yit mice, it may render also the small intestine susceptible to inflammation.

What is the thymus-gut connection?

The thymus is the site of T cell origin maturation and, although much debated, it is currently believed that all T cells pass through the thymus at some point of its life. Classical T cells leave the thymus as fully mature $CD4^+$ or $CD8\alpha\beta^+$ SP T cells. However, it has been suggested that some thymocytes can leave the thymus at an early time point of maturation and give rise to $CD8\alpha\alpha^+$ gut IELs (260). It has also been suggested that T_R cells are educated through agonist selection, that is through a higher affinity of the TCR-MHC/peptide binding, during thymocyte development (261). In normal situations, DP thymocytes have been suggested to influence DN thymocytes to become effector T cells in a process called trans-conditioning (262). The mechanism by which trans-conditioning induce differentiation of thymocytes into regulatory T cells is unclear, but experiments have shown that DP thymocytes can act in trans on $TCR\gamma\delta^+$ progenitors by lymphotoxin (263). It has been shown that regulatory T cells can increase during inflammatory conditions (121, 264). When the acute inflammation becomes chronic in the DSS model, the DP thymocytes increase (10). It is possible that the restoration of DP thymocytes may suppress the development of regulatory T cells. The activation of the HPA-axis during an acute inflammation and the resulting thymic involution may be another way for the peripheral immune system to transmit emergency signals to the thymus to direct additional T cells to the gut. In this way, the thymus may feel the status of the intestinal immune system, and production of T_R cells may be one way to balance the acute inflammation. The seemingly normal or increased input of T cell progenitors during DSS-induced colitis (10) implies that the early thymocyte development is only marginally affected and could still give rise to normal numbers of IEL progenitors.

Is thymic involution a mistake or a cunning way to fight enemies?

Thymic involution was observed already in the 1930's, as a response to events ranging from physiological stress, ageing, pregnancy, infections or inflammation (54). This was termed "the general alarm syndrome" or " the general adaptation syndrome", and was believed to be a way of accommodating to new physiological situations (54). It may be beneficial for the organism to produce endogenous glucocorticoids during the acute response, thereby activating the innate immune system to a rapid first defence by production of proinflammatory cytokines and acute phase proteins. The suggested export of IEL precursors from DN thymocytes from the thymus (262) could then contribute to the fine-tuning of the mucosal immune system. This theory is also supported by a study demonstrating that blocking of corticosteroids prevented thymic involution but aggravated disease in an infectious model (224). It would not be surprising if the thymus were involuted several times during childhood due to infections. Indeed, a number of infections (possibly with concomitant thymic involution?) have been considered beneficial for shaping the immune system (265) and early infections have been shown to decrease the risk of asthma and allergies (266, 267). However, it is not known whether there are any direct beneficial effects of thymic involution on the immune response. The acute phase reaction results in the production of proinflammatory cytokines that stimulate cells of the innate immune system, e.g., macrophages, which can rapidly fight pathogens and attract cells of the adaptive immune system. In this way, the acute phase response may stimulate a rapid

first defence against invading pathogens. The quick restoration of the thymus as observed in paper I (10) occur within the estimated time of thymocyte development (268). This suggests that the thymic involution during an acute inflammation affect the mature peripheral pool of T cells to a limited extent.

Chemicals or genetics – does it really matter?

There is no definitive answer to which animal model for IBD, chemically induced or genetically engineered mice that spontaneously develop colitis, is better. Chemically induced models (e.g., DSS, TNBS, Oxazalone, HAc) have the advantage of having a known time of onset of disease (249). The kinetics of colitis progression can therefore be studied in detail and there is no limit to the numbers of mice that can be used – most wild type mice can be easily bred or purchased. However, the initial damage producing the inflammation in these models may not reflect the human situation. For instance, early phases in the DSS model are characterised by epithelial shedding (8) and TNBS is an agent that have erosive qualities on the mucosa (269).

The other side of the coin are the genetically engineered models (250, 270, 271). No exogenous agents are needed to initiate colitis in these models, even though bacteria seem to be crucial for the onset of disease. However, some of the genetic models need to be bred from heterozygotes and are sometimes not available to the public.

The endeavour towards finding animal models of colitis that perfectly match the human situation will probably continue to be utopian. However, both induced and spontaneous animal models of colitis have made their contribution in the understanding of the complexity of the mucosal immune system and IBD. The results in this thesis show that very diverse ways of initiating intestinal inflammation generate common denominators, e.g., regarding thymic alterations and colonic gene expression. Once the immunological homeostasis is broken either "mechanically" (DSS) or by aberrant cell signalling in the epithelial layer and/or underlying tissue ($G\alpha i2^{-/-}$) (8, 11, 12, 194, 198, 199, 211), the immune system respond by trying to correct the imbalance. When the immune system is activated, cells start to express activation markers such as cytokines, chemokines/receptors and T and B cell markers (paper III). Neutrophils may arrive in larger numbers as in the DSS model whereas lymphocytes are seen more frequently in early stages of $G\alpha i2^{-/-}$ colitis, but the events during the early immune response may result in a similar response later during the inflammation aiming at restoring the immunologic balance. In one study it was suggested that various initial damage in induced models of colitis may generate an unspecific, stereotype response, thus serving as an initiator of a final common immunologic pathway (272). The results in this thesis show that induced and spontaneous models of colitis also display common features, such as similar gene expression and thymic alterations.

Is there a relation between colon wall thickness and inflammation?

2000 years ago Celsus defined swelling of the tissue as one of the cardinal signs of inflammation. Today, this feature is used in the diagnosis of IBD through e.g., MRI and CT. Despite numerous studies, the exact relationship between the thickness of the colon wall and the activity of inflammation remains unclear (273). CD is associated with intestinal fibrotic tissue (274) that contributes to the thickened colon wall. The

intestinal wall remain thickened for a prolonged time during patient remission, when the inflammatory activity decrease (168). This results in a different correlation between colon wall thickness and severity of intestinal inflammation in the acute versus the chronic phase. No animal models of intestinal fibrosis exist today, but collagenous depositions have been observed in colitis models induced by repeated TNBS administration (275) and chronic DSS colitis (9). Another clinical marker to diagnose inflammation is CRP, but its relation to the activity of disease in IBD is controversial. Whereas one study showed that CRP levels correlated to active CD (276), another study found that CRP correlated to disease activity in UC but not CD (277). However, patients with high CRP levels have been shown to respond better to anti-inflammatory drugs, such as anti-TNF (278).

The kinetics of colon wall thickening in the DSS model strongly suggest that this technique would be useful also in spontaneous models, with unknown onset of disease. Clinical parameters, such as diarrhoea, which is currently used to define the onset of colitis in $G\alpha i2^{-/-}$ mice (193) occur subsequent to the initiation of inflammation and sometimes also to the initiation of colon wall thickening (own observation). Thus, the study in paper IV makes a contribution on how to diagnose and stratify animals with colitis especially before start of treatment.

FUTURE DIRECTIONS

The consequences of thymic involution during an acute inflammation are not known. The DN1 thymocytes were increased during DSS colitis (paper I) but this study did not give the answer to whether the increase was due to an augmented influx from the bone marrow or augmented proliferation of immature thymocytes. It would also be interesting to investigate what impact thymic involution may have on the peripheral TCR repertoire.

The observation that the composition of small intestinal IELs was altered in $G\alpha i2^{-/-}$ colitic mice may not be unique to this model. If the DP and $CD8\alpha\alpha$ SP IELs prove to increase also in other models it may be a general way of the small intestine to avoid pathological inflammation during colitis. This would also strengthen the hypothesis that the colon may not have the ability to mount sufficient numbers of IELs with a profile that prevents pathological inflammation.

The *ex vivo* culture system (paper III) needs to be further evaluated. The arrays presented a momentary picture of the DSS induced and $G\alpha i2^{-/-}$ colitis at given time points. Upon analysis of the kinetics of colitis in the $G\alpha i2^{-/-}$ model, precolitic mice will also be included considering earlier findings in pre-colitic mice (193). Colonic tissue from more UC and CD patients is also needed to confirm and extend the findings in paper III.

I would like to breed the $G\alpha i2$ deficiency onto a pure C57BL/6 or 129SvBom background, since mucosal immune activation occur also in at least 129SvBom mice (188). It would also be interesting to resolve the seemingly contradictory findings that $G\alpha i2^{-/-}$ thymocytes have defective migratory responses to chemokines (27) versus the recent findings by Pero et al that $G\alpha i2$ deficiency in the endothelium but not in the leukocytes resulted in impaired migration of eosinophils and neutrophils (279). The different results could either be due to the genetic background or the inflammatory status of the cells. Using colitis-resistant animals as controls to colitis-prone animals would lead to a better understanding of the events leading to colitis. Colitis in the $G\alpha i2^{-/-}$ mice can be transferred by T cells to immunodeficient mice (184). The results in paper III suggest that the colitic status of the donor T cells influence the development of colitis in the recipient mice. To compare T cells from colitic, precolitic and colitis resistant animals belongs to future projects. Breeding of the $G\alpha i2^{-/-}$ on to a RAG background will generate additional information as to whether the lymphoid or the myeloid cells are responsible for the colitis development.

Prediction of colon wall thickening in spontaneously developing models could be used to map the kinetics of colitis in these models and perhaps also predict the development of colitis in individual animals. However, the process of intestinal fibrosis is relatively unknown and need to be further explored.

ACKNOWLEDGEMENTS

Under resan på väg mot målet har jag mött otroligt många människor som har inspirerat, uppmanat och inte minst utmanat mig! Jag har de senaste tre åren spenderat ungefär halvtid på GI-sektionen på AstraZeneca och halvtid på Göteborgs universitet. Och, känns det som, halvtid med min underbara och tålmodiga familj…

Först av allt vill jag tacka mina handledare – Bettan, Lotta och Silvia. Det har varit väldigt kul och spännande att få jobba med er alla tre. Bettan, du har varit min akademiska handledare och delat med dig av dina djupa kunskaper inom immunologi och IBD men också på ett väldigt nära sätt givit mig utrymme och förtroende att utvecklas.
Lotta, att gå in på ditt rum för att lösa ett problem är för evigt förknippat med att komma ut med hundra nya idéer. Samtidigt har du visat mig många aspekter om kulturskillnaderna mellan akademi och företag och låtit mig hitta min egen väg mellan dessa olika världar.
Silvia, du kom i min grupp som post-doc innan jag började doktorera. Vilken tur jag har haft att du har funnits vid min sida och alltid delat med dig av dina kunskaper. Dessutom har du på något magiskt sätt alltid kunnat hjälpa mig med allt från att skapa nya idéer till att genomföra experiment och skriva. Och hur har du kunnat stå ut att dela rum med mig så länge…?

Jag vill tacka Peter Thorén och Per-Göran Gillberg för att ni har gett mig arbetstid att genomföra avhandlingen samt dessutom visat stort intresse på vägen.

Min IBD grupp på AZ: Erik, Silvia, Agneta, Helena, Lisa, Malin och Erika – tack för allt stöd ni har gett mig under åren. Ni har ibland gått på knäna så mycket ni har haft att göra och ändå så har ni aldrig begärt att jag ska rycka in – ni anar inte hur mycket ni har betytt för mig även om det ibland känns som om jag sprungit bredvid er och inte med er.

Min gamla GI-sektion – tack för alla fikastunder och glada skratt! Även om sektionen inte längre finns så kommer alltid gemenskapen att finnas kvar!

Anders och innan dess Lennart och HB2 gruppen som har analyserat biomarkörer i vått och torrt. Alltid när jag har haft bråttom så har ni varit där och fixat analyserna med glatt humör., Anna, Charlotte, Anne-Christine, Gisela, Marie-Louise, Britt-Marie och speciellt Lena som blivit något av min koordinator och stöttepelare där nere.

Lise och djurgruppen på HG2, Anett, Anette, Marie, Marie, Rebecka och Monica, ni är sanslösa med ert engagemang – jag har jobbat med er länge innan jag började doktorera. Ni lyckas alltid ”klämma in” en extra studie i ett redan mäckfullt djurhus. Lotta och gänget nere på HF1, ni har tagit hand om många av mina DSS möss och verkligen engagerat er i dom små liven – tack ni också!

Även min grupp på avdelningen för Mikrobiologi och Immunologi på GU har betytt jättemycket för mig. Kristina, Yu-Yuan, Martin och Maria som håller labbet på fötter. Tack för alla glada stunder och ibland vansinnigt roliga laborationer! Tanya, som blev klar för ett halvår sedan, med ett alltid lika glatt humör, till och med när du försökte vara arg på oss andra slarviga doktorander…

Paul vill jag alldeles speciellt tacka för alla i sanning intressanta journal clubs. Aldrig har jag behövt gå därifrån med känslan av att inte ha lärt mig något.

Alla glada doktorander på avdelningen som kämpar på med sina egna arbeten – jag har hunnit dela rum eller kurslabbar med dom flesta av er! Även alla andra på avdelningen som alltid är lika beredda att låna ut hjälp eller antikroppar. Pia på EBM, tack för all hjälp med skötseln av mössen.

Professor Roger Willén vill jag tacka för ett gott samarbete med histologisk utvärdering av Gαi2 mössen, samt en spännande diskussion kring histologi, vilken har pågått under ett par års tid via email.

Alexander, vi har haft många spännande och glada stunder i labbet, både innan och under avhandlingen. Tack för din ständiga entusiasm och all hjälp som anländer nästan innan jag har hunnit ställa frågorna!

Leif och Gina, många svettiga timmar blev det framför CT-apparaten. Att genomföra stora studier samtidigt med att utveckla både lab och analysmetoder kräver stort engagemang. Tack också Lennart för en lysande insats när Gina äntligen fick hämta sin Simon.

Gourmetklubben – Marie, Anna, Josefina och Niklas! Ni blev mina första vänner i ett Göteborg jag aldrig satt foten i förut.

Marie, tack för allt stöd du har gett mig efter att du själv levererade din avhandling i våras. Skönt att ha någon som precis har gått igenom hela processen…

Alla ni kompisar runt om i landet som har fått se lite mindre av mig dom sista åren, mina äldsta vänner Erika, Maria och Sissa – I'll be back! Och hela Skånegänget med alla glada fester som förgyller livet med regelbundna mellanrum!

Till alla i familjen som hjälpt till under det senaste året, min pappa och min svärmor som kommit hit en vecka i taget för att ta hand om barnen. Lillebror Simon och Emma som också har ställt upp och hjälpt till när det behövs.

Till sist, min egen lilla älskade familj, Johan som alltid har funnits vid min sida, och Felix och Emil, dom två största guldklimparna i världen. Den största påminnelsen om att det finns annat än jobb och forskning här i världen.

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