Characteristics and Consequences of Thymic Involution in Inflammatory Bowel Disease.

Experimental studies in Gαi2-deficient and DSS-induced Colitis as well as in IBD patients

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ABSTRACT

Inflammatory Bowel Disease (IBD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract, comprising ulcerative colitis and Crohn's disease. Alterations in T cell subsets, an important cell type in cell-mediated immune responses in the adaptive immune system, are certainly an element contributing to disease development. The relationship between disease and T-cell maturation in the thymus is, however, poorly understood. The present study investigates intrathymic changes as well as the consequence of thymic involution by analysis of recent thymic emigrants in peripheral blood and lymphoid tissue in two different mouse models for colitis; Gαi2-deficient mice and mice with DSS-induced colitis, as well as in IBD patients.

Before the onset and during colitis, Gαi2$^{-/-}$ mice demonstrate thymic involution, whereas in DSS-induced colitis the thymic atrophy is transient, being evident during the acute phase of colitis but reversed during the chronic phase. The frequency of medullary mature thymocytes was increased in both models, but the intrathymic changes were mainly seen in the cortex and involved reduced both frequencies and absolute numbers of cortical thymocyte subsets as well as impaired chemotactic responses towards the chemokines CXCL12 and CCL25. The impaired migration was not limited to the thymus as reduced responsiveness to CXCL12 was seen also in colonic lymphocytes from Gαi2$^{-/-}$ mice. In mice with DSS-induced colitis, an increased frequency of the most immature subpopulation of double negative (DN) thymocytes and a proportional decrease in the most mature DN thymocytes correlated with the severity of colitis. These results strongly indicate that an aberrant T cell ontogeny is associated with development of colitis.

It is unknown whether thymic atrophy is evident also in IBD patients. Due to the unavailability of human thymus tissue from IBD patients for such studies, one aspect of thymus function was evaluated by analysis of the levels of T cell receptor excision circles (TRECs), a marker for recent thymic emigrants (RTEs), in T lymphocytes from peripheral blood and the intestinal mucosa. This analysis revealed reduced levels of RTEs in peripheral blood from IBD patients, irrespective of the expression of the mucosal homing receptor integrin $\alpha_4\beta_7$. In strong contrast to peripheral blood, an increased level of TRECs was found in the intestinal musosa, indicative of an instant recruitment of recent thymic emigrants into the intestine. These results were seen in both UC and CD -patients but were more pronounced in UC patients, and could not be explained by enhanced extrathymic T cell maturation within the mucosa. Preliminary data also indicate that the TRECs levels in the mucosa are not influenced by the activity of the disease.

A similar analysis of TRECs levels was performed in colitic Gαi2$^{-/-}$ mice but decreased levels were found both in peripheral blood and intestinal mucosa. However, a massive proliferation of memory/effector T cells, especially in the mucosa, disguised the true level of recent thymic emigrants in this compartment.

Thus, chronic intestinal inflammation in IBD is clearly associated with changes in T cell ontogeny and thymic output. It is likely that this influences the peripheral T cell population and further studies would reveal whether this leads to lower ability for T cell mediated immunoregulation and/or the presence of autoreactive T cell clones.

ORIGINAL PAPERS

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

I. Kristina Elgbratt, Malin Bjursten, Roger Willén, Paul W. Bland and Elisabeth Hultgren Hörnquist. Aberrant T-cell ontogeny and defective thymocyte and colonic T-cell chemotactic migration in colitis-prone Gαi2-deficient mice. *Immunology 2007, 122 (2), 199–209*.

II. Maria Fritsch Fredin, Kristina Elgbratt, David Svensson, Liselotte Jansson, Silvia Melgar and Elisabeth Hultgren Hörnquist. Dextran sulfate sodium-induced colitis generates a transient thymic involution – impact on thymocyte subsets. *Scandinavian Journal of Immunology 2007. 65 (5), 421-429.*

III. Kristina Elgbratt, Göran Kurlberg, Mirjana Hahn-Zohric, and Elisabeth Hultgren Hörnquist. Increased TRECs (T cell Receptor Excision Circle) levels in inflamed mucosa and decreased levels in peripheral blood in IBD patients indicate rapid migration of thymic emigrants to the gut. *Manuscript*

IV. Kristina Elgbratt, Sarah Peterson, and Elisabeth Hultgren Hörnquist. Thymic atrophy in Gαi2-deficient colitis is associated with reduced levels of recent thymic emigrant in the gut-associated lymphoid tissue. *Manuscript*

CONTENTS

ABBREVIATIONS

5-ASA	5-aminosalicylic acid
6-MP	6-mercaptopurine
AIRE	Autoimmune regulator
APC	antigen presenting cell
AZA	azathioprine
CARD	caspase activation and recruitment domain
CD	Crohn's disease
CD(X)	cluster of differentiation
CMJ	cortico-medullary junction
cTEC	cortical thymic epithelia cell
DC	dendritic cell
DN	double negative
DP	double positive
DSS	dextran sodium sulfate
ETCM	extrathymic T cell maturation
FACS	fluorescence activated cell sorter (FACS)
FAE	follicle associated epithelium
FCS	fetal calf serum
Gαi2	G-alpha-i-2
GALT	gut-associated lymphoid tissue
GAPDH	glyceraldehyd-phosphate-dehydrogenase
GI	gastrointestinal
GlyCAM	glycan-bearing cell adhesion molecule
HEV	high endothelial venule
IBD	inflammatory bowel disease
ICAM-1	intracellular adhesion molecule-1
Ig	Immunoglobulin
IEL	intraepithelial lymphocyte
IFN	interferon
ILF	isolated lymphoid follicle
IL	interleukin
LRR	leucine rich repeat
LPL	lamina propria lymphocyte
MACS	magnetic activated cell sorter
MDP	muramyl dipeptide
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MΦ	macrophage
mTEC	medullary thymic epithelia cell
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PP	Peyer's patch
PRR	pattern recognition receptor
PSGL	P-selectin glycoprotein ligand
RAG	recombination activating gene
RNA	ribonucleic acid
RTE	recent thymic emigrant
rt-PCR	real time-polymeras chain reaction
SCID	severe combined immunodeficency
SED	sub-epithelial dome
SP	single positive
TCR	T cell receptor
TEC	thymic epithelial cell
Th	T helper
TNF	tumor necrosis factor
TRECs	T cell receptor excision circles
Treg	regulatory T cell
tg	transgene
UC	ulcerative colitis
VCAM	vascular-cell adhesion molecule
wt	wild type
+/+	wild type
+/-	heterozygous for gene deficiency
-/-	homozygous for gene deficiency (knockout)

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract, comprising ulcerative colitis and Crohn's disease. It is the general belief that multiple factors such as genetics and environmental aspects are involved in disease pathogenesis. Alterations in T cell subsets, an important cell type in cell-mediated immune responses in the adaptive immune system, are certainly an element contributing to disease development. The relationship between disease and T-cell maturation is, however, poorly understood. The aim of the present thesis was to characterize intrathymic changes and the consequences of thymic involution by analysis of recent thymic emigrants in peripheral blood and lymphoid tissue using two mouse models for colitis; Gαi2-deficient mice and mice with DSS-induced colitis, as well as IBD patients.

INFLAMMATORY BOWEL DISEASE – IBD

Inflammatory bowel disease (IBD) is the collective name for ulcerative colitis (UC) and Crohn's disease (CD). IBD is largely a disease of the twentieth century, and is associated with the rise of the modern, westernized industrial society. The incidence of CD today ranges from 10-200 new cases per 100 000 per year, which is an 8-10-fold increased since the 1960s [1]. The incidence of UC ranges from 10-20 new cases per 100 000 per year and has been stable since the 1960s [1]. The traditional geographic picture is that of a high incidence of IBD in Northern and Western Europe as well as North America with a lower incidence recorded in Africa, South America and Asia. This picture is now slowly changing, as an increased incidence of IBD has been reported from Eastern Europe and Asia [2]. The age at onset of IBD is usually relatively low, starting at 20-30 years of age [3], and up to 25% of IBD patients are diagnosed during childhood or adolescence [4]. In younger patients, disease morbidity can be significant, with the risk of lifelong consequences related to growth, reproductive health, and psychological well-being. The disease is more or less equally distributed between males and females, even though UC is slightly dominated by men and CD by women [3]

IBD patients suffer from chronic diarrhea and weight loss, abdominal pain, fever and fatigue. Both UC and CD are characterized by mucosal ulcerations, which in CD is patchy and can start anywhere in the gastrointestinal (GI) tract, whereas it is limited to the colon and always

starts at rectum and continues throughout the colon in a continuous manner in UC. Even though CD and UC share many clinical similarities, the histopathology is most often different.

PATHOLOGY OF CROHN'S DISEASE

CD can affect different segments of the GI tract, with the terminal ileum and the proximal colon being most commonly affected, followed by the anorectal area and the colon. CD patients often have severe abdominal pain, which usually comes from formation of deep fissures where the ulcers reach or pass through the muscularis propria. These deep ulcers can form fistulas, connecting one part of the intestine to another (internal fistulas), to other internal organs (enterovesical fistulas) or to the skin (enterocutaneous fistulas). The inflamed part becomes thick and can cause obstruction. The transmural inflammation affects all layers of the bowel wall but superficial erosions known as aphthoid lesions, where small superficial ulcers are surrounded by unaffected epithelium, are also found. Formation of granulomas is a histological hallmark of CD [5], and lymphoid aggregates is very common with cell infiltration of T cells and macrophages (MΦ) [6].

PATHOLOGY OF ULCERATIVE COLITIS

The inflammation in UC starts from the rectum and spreads proximally in a continuous fashion, involving a variable length of the colon. The inflammation is limited to the mucosa and usually does not extend into the submucosa. The disease causes superficial mucosal ulcers [5] of various sizes which can become confluent. The mucus producing goblet cells are depleted, the crypt architecture is distorted and crypt abscesses are formed, the latter being one of the histological hallmarks of UC [5]. The inflammation can cause toxic dilatation which increase the colon volume and damage the peristaltic movements [7]. In the more advanced stages, the entire bowel becomes fibrotic, narrowed and shortened [8]. Intestinal perforation can lead to leakage of intestinal contents into the abdominal cavity. The cells that are infiltrating the mucosa are mainly neutrophils, eosinophils and lymphocytes. During remission, the mucosal healing often occurs in an irregular way leading to a discontinuous, heterogeneous mucosa, which sometimes can be confused with CD [6].

FACTORS CONTRIBUTING TO DEVELOPMENT OF IBD

Even though the aetiology of IBD is still unknown, several susceptibility factors have been listed that in different ways contribute to development of the diseases.

Our **intestinal microflora**, consisting of more than 500 species, with ~ 40 bacteria species comprising up to 99% of the total miroflora [9], normally lives in a complex, systemic relationship with the eukaryotic cells of the mucosa. Although no one knows exactly how, the immune cells are able to distinguish these commensal bacteria from pathogenic microorganisms. Several observations have shown that the intestinal bacteria are involved in the initiation and maintenance of IBD and that the adaptive immune system is hyper-responsive to the commensal intestinal microflora in genetically susceptible individuals [10, 11]. This hypothesis is also supported by the findings that inflammation occurs predominantly in areas with the highest density of intestinal bacteria [12], that broad spectrum antibiotics improve chronic intestinal inflammation in CD [10, 13], and that most animal models for IBD (although not DSS-induced colitis [14] fail to develop chronic intestinal inflammation when raised under germ-free conditions [15-17].

It has long been observed that cases of IBD cluster within families and among ethnic groups, suggesting that **genetic factors** contribute to disease pathogenesis. In IBD, numerous genetic linkage studies have implicated a number of susceptibility loci in various chromosomal regions, termed IBD1, 2, 3 , 4, 5 etc [18-20]. Two of the IBD susceptibility genes for which the evidence is felt to be strongest are NOD2/CARD15 in CD and MDR-1 in UC. The best characterized IBD susceptibility gene is NOD2/CARD15 (nucleotide-binding oligomerization domain 2)/(caspase activation recruitment domain 15) within the IBD1 locus on chromosome 16q12 [21-24]. Mutations in this gene are only linked to CD and not to UC. NOD2/CARD15 is a member of a family of intracellular NOD-like receptors, pattern-recognition receptors (PRRs) which recognize microbial components, also called PAMPs (pathogen associated molecular patterns). It is expressed in the cytosol of e.g. gut epithelial cells, including Paneth cells, located at the base of the intestinal crypts, macrophages and dentritic cells, and is an intracellular sensor of muramyl dipeptide (MDP), a degradation product of bacterial cell wall peptidoglycan, present in both Gram-positive and –negative bacteria [25, 26]. Three major polymorphisms have been specifically associated with ~15% of CD cases (Arg702Trp, Gly908Arg and the frameshift mutatation Leu1007fsinsC), and all are in, or around, the leucine rich repeat (LRR) region of the protein required for recognition of MDP. Activation of NOD2 leads to phosphorylation and degradation of IκB and activation of NF-κB [27]. Therefore it has been speculated that mutation in NOD2 may lead to deceased ability of kill gut bacteria [28], although it has also been implicated as a negative regulator of innate immune responses [29, 30]. Individuals who carry two copies of the risk alleles have a 20- to 40-fold increased risk of developing CD [1]. However, this association is found in CD

patients of European ancestry only, while there in no correlation in CD patients of African, American or Asian cohorts [19].

The MDR1 gene (multidrug resistance 1), encoding P-glycoprotein 170, a transmembrane efflux pump involved in drug transport, is situated in an IBD susceptibility locus on chromosome 7q21 [31] It is highly expressed at the apical surface of epithelia of the colon and distal small bowel [32]. MDR1 knockout mice are susceptible to developing a severe, spontaneous intestinal inflammation, which is preventable by and treatable with antibiotics [33] and genetic analyses in humans have demonstrated a significant association with UC, but not CD [34, 35]. One of the more recently described genetic associations to IBD is the interleukin-23 receptor (IL-23R). IL-23 amplifies and stabilizes a new $CD4^+$ T-cell subset, the Th17 cells, producing IL-17. The IL-23/Th17 axis has been shown to contribute to several immune-mediated inflammatory autoimmune diseases. In this regard, it is interesting to note that a germline variation of IL-23R recently was implicated in conferring protection to ileal CD [36]

Environmental factors have also been suggested to have a large influence on the risk of developing IBD. Use of tobacco, particularly cigarette smoking, has been shown to impact both CD and UC patients but in opposite ways: Cigarette use is an important risk factor for CD as it increases the frequency of disease relapses and need for surgery and discontinuation improves the disease course [37, 38]. In contrast, UC patients are frequently non-smokers, and cessation of smoking increases the risk of developing UC, supporting its protective role in this disease [38]. Dietary factors such as high consumptions of sugar and fat have also been associated with IBD whereas fruit, vegetable, and fiber consumption seem to decrease the risk of developing IBD [39]. Prior appendectomy is associated with a decreased the risk of UC [40]. Among women, long-term users of oral contraceptives were found to be at increased risk of developing UC as well as CD whereas long-term users of hormone replacement therapy had an increased risk of developing CD but not UC [41]

MEDICAL TREATMENT OF IBD

There is still no medical therapy that can cure IBD, but there are several medications that can increase the maintenance of remission or relieve the symptoms during a relapse. **Corticosteroids** were first introduced as therapy for IBD in the 1950s and have been the mainstay of treatment for acute relapses since then. Corticosteroids are mainly for short-time use to achieve remission and because of undesirable side effects, e.g. high blood pressure,

diabetes, osteoporosis, increased risk of infections, weight gain and mood disorders [42] they are rarely used for maintenance therapy in IBD. For treatment of mild-to-moderate episodes of UC and CD, as well as preventing relapse and maintaining remission, **Aminosalicylates** are often used. The active substance of the drug, 5-aminosalicylic acid (5-ASA), is bound to sulfapyridine by an azo-binding, a compound that delivers 5-ASA to the intestine. Sulfasalazine was discovered by Nanna Svartz, the first female professor in Sweden and was the first drug to induce remission in active UC [43]. Today there are several sulfonic free alternatives, e.g. mesalazine and olsalazin that avoid the side effects associated with the sulfonic part of the drug.

For those patients that cannot use corticosteroids or aminosalicylates, **immunomodulators** such as 6-mercaptopurine (6-MP), azathioprine (AZA) or cyclosporine are sometimes used. Immunomodulators decrease the inflammatory response and are mostly used in organ transplantation and autoimmune diseases such as rheumatoid arthritis. Since the late 1960s, they have also been used to treat people with IBD and help patients to maintain remission.

Infliximab, a chimeric monoclonal anti-tumor necrosis factor alpha (TNF-α) antibody is nowadays a common treatment for both induction and maintenance of remission in refractory and fistulizing CD [44]. It has also recently been proven efficient in inducing remission and in maintenance therapy of therapy refractory UC [45]. However, the risk of severe opportunistic infections, e.g. tuberculosis, in these patients, as well as the high immunogenicity of infliximab limits the extensive use of anti-TNF treatment [46] In contrast, etanercept and onercept, both soluble human recombinant TNF-α receptor fusion proteins, are not efficacious in IBD [47]

Novel **biological agents** include the humanized IgG1 anti-TNF monoclonal adalimumab and the humanized pegylated Fab-fragment certolizumab-pegol. Inhibition of lymphocyte trafficking to the gut through anti-adhesion molecule specific therapies (anti integrin α4, natalizumab, anti integrin α4β7, MLN-02, and anti ICAM-1, alicaforsen) have also shown promising results [48]. The use of α4-integrin blockade with natalizumab is however tempered by reports of several cases of progressive multifocal leucoencephalopathy caused by the human polyoma JC virus in patients with multiple sclerosis and Crohn's disease treated with natalizumab [49]. Visilizumab, a humanised anti-CD3 antibody, have shown clinical benefit in patients with severe steroid refractory UC [50]. Two studies with basiliximab and daclizumab, monoclonal antibodies blocking the interleukin-2 receptor α-chain (CD25) on the surface of activated T cells, and approved for prevention of graft rejection in transplantaion, have generated contradictory results. [51, 52] Another interesting concept is the blockade of

the co-stimulatory signal required for T-cell activation with CTLA-4-Ig (abatacept), which is approved for rheumatoid arthritis and phase II and III studies in patients with inflammatory bowel disease are planned. [53]

In come cases **surgery** is necessary to induce remission or to treat complications. In UC emergency surgery is indicated due to perforation, refractory rectal bleeding, and toxic megacolon not responsive to medical management [54], whereas elective surgery is indicated in patients with dysplasia or cancer, UC refractory to medical management, or intolerance to long-term immunosuppression or other medical therapies [55]. Although surgery will not cure Crohn's disease, it is indicated due to formation of fibrotic strictures leading to partial or complete bowel obstruction or fistulas [55].

DEVELOPMENT OF T LYMPHOCYTES

THYMUS; THE T CELL SCHOOL WITH VERY FEW GRADUATES

Hematopoietic progenitor cells migrate from the fetal liver and bone marrow through the bloodstream into the thymus where they are educated to become mature, functional T lymphocytes. During the maturation process the “students”, the developing pro-T cells are termed thymocytes. This is however a tough school, with only ~5 % of the students graduating.

The thymus is located in the upper anterior portion of the thoracic cavity behind the sternum, overlying the heart and major blood vessels. It has two lobes that are divided into multiple lobules separated from each other by connective tissue trabeculae. Each lobule consists of two major compartments, an outer cortex and an inner medulla. The tightly packed cortex contains the majority of the immature proliferating thymocytes whereas the medulla contains more mature cells. Besides the major composition of thymocytes, the thymic microenvironment comprises distinct cell types, including different types of thymic epithelial cells (TEC), stroma cells, macrophages, fibroblasts and dendritic cells. All these cells participate, in one way or the other, in the thymocyte maturation process. At least three types of epithelial cells can be distinguished in the thymic lobules, which have different roles for thymocyte proliferation, maturation and selection: Nurse cells in the outer cortex which stimulate proliferation of T cell progenitors through production of IL-7, cortical TECs that mediate positive selection of maturing thymocytes, and medullary TECs that mediate negative selection by displaying a large variety of self peptides (see below).

To provide protection against the multitude of different infectious agents that an individual is likely to encounter, the mature T lymphocytes have to recognize a wide variety of different antigens. To accomplish this, the genes for the T cell receptor (TCR) α and β or γ and δ chains undergo somatic recombination. During this process a relatively limited set of inherited, or germline, DNA sequences, so called V (variability), D (diversity), J (joining) and C (constant) genes - initially separated from each other - are randomly brought together by deletion of intervening sequences and religation, generating an enormous variety of TCRs with different specificities.

POSTIVE AND NEGATIVE SELECTION OF DEVELOPING T LYMPHOCYTES IN THE THYMUS

The thymocyte enters the thymus through high endothelial venules (HEVs) at the corticomedullary junction of the thymic lobules and starts to migrate across the cortex into the subcapsule. As they migrate up to the capsule the thymocytes are termed double negative (DN), referring to their lack of the T cell surface markers CD8 and CD4. At this stage the TCR genes are still in germline configuration. The DN population is subdivided on the basis of expression of CD44 and CD25. Progression from the most immature stage, $CD44^{+}CD25^{-}$ (DN1) requires the transient acquisition of CD25 so that the cell becomes $CD44^{+}CD25^{+}$ (DN2) before down regulating CD44 to become $CD44^{-}CD25^{+}$ (DN3) and then finally down regulating CD25 to become $CD44^{-}CD25^{-}$ (DN4) [56] [57, 58]. The expression of CD44 allows them to migrate to the outermost cortex where most of the proliferation takes place, whereas CD25 enables them to respond to IL-2 which will, together with IL-7, sustain their proliferation. The commitment to the T cell lineage is initiated at this stage, when the Notch-1 (named after that *Drosophila melanogaster* notched wing phenotype due to a loss-of-function mutation in the gene [59, 60] receptor on DN thymocytes binds to the Notch-1 ligand expressed on cortical thymic epithelial cells (cTEC) [61]. The Notch signal initiates transcription of the gene encoding the pre-T cell receptor (TCR) α (pTα) and the expression of pTα is considered to indicate T cell commitment [61]. The expression of pTα is believed to occur during the DN2 development stage and during the DN3 stage the thymocytes undergo extensive rearrangement of the TCR β chain genes [62]. Once the thymocytes have undergone a successful TCRβ chain rearrangement they proliferate and mature further prior to the rearrangement and expression of the TCRα chain. This process is termed β-selection and is controlled by the pre-TCR complex, consisting of the TCRβ chain and pTα [61]. Before the expression of a complete αβTCR on the thymocyte surface, they start to up regulate CD4 and CD8 and are now termed double-positive (DP) thymocytes. The DP thymocytes account for

approximately 80% of the thymocytes in a normal thymus. During this stage the genes encoding the TcR α chain are rearranged, and both chains of the αβ TcR are expressed together with CD3, which transduces activating signals into the cell. The $\alpha\beta TCR^{+}CD4^{+}CD8^{+}$ thymocytes now start to migrate from the outer cortex into the inner cortex where they will be target for positive selection. Developing thymocytes undergo extensive selection to ensure that the mature T cells that are exported from the thymus are functional, i.e. self-MHC restricted and self-tolerant. The **positive section** involves selection of those DP thymocytes with expression of a complete αβTCR with randomly generated specificity that are able to recognize self peptides bound to class I and class II MHC molecules. The peptide-loaded MHCs are expressed on cTEC and those thymocytes that bind with intermediate affinity and avidity by "dual recognition" of both the antigenic peptide and the polymorphic part of the MHC molecule are rescued from cell death and continue to mature while thymocytes displaying very high or very low receptor affinities for self MHC plus peptide will die by apoptosis. The positively selected DP thymocytes upregulate CD69 and migrate towards the medulla and become targets for the next selection. The **negative selection** is believed to take place in the medulla because of the increased number of MHC expressing APCs in this compartment, even though some evidence indicate that it takes place near the cortico-medullary junction [61] The negative selection induces apoptosis of those thymocytes that express TCRs with high affinity for self antigen expressed together with MHC molecules on the medullary TECs and macrophages. The former can express antigens whose expression is normally limited to specific organs. This ectopic expression is controlled by a gene called *AIRE* (for autoimmune regulator). By this process the potentially most harmful self-reactive T cells are eliminated and it is one of the mechanisms ensuring that the immune system does not respond to many self antigens. The DP thymocytes will now loose one of either surface marker CD4 or CD8, depending on which class of MHC molecule they bind to during the selection, and become SP for either CD4 or CD8, and will express their TCR at a high density. The SP thymocytes found in the medulla down regulate CD69 and upregulate CD62L [63] and are now ready to egress the thymus and seed the peripheral T cell pool.

In addition to the TCR α and β loci, there are also a γ and a δ loci that undergo rearrangement almost simultaneously in developing thymocytes, leading to maturation of a minor subset of mature T lymphocytes expressing the γδ form of the T cell receptor. Analysis of gene rearrangements in thymocytes and mature $TCR\gamma\delta^{+}$ and $TCR\alpha\beta^{+}$ T cells indicate that these two lineages diverge from a common precursor after certain gene rearrangements have

already occurred. Thus, mature γδ T cells can have productively rearranged β chain genes, and mature αβ T cells can contain rearranged γ chain genes. The factors regulating this lineage commitment are still unknown; in fact, commitment to either lineage might simply depend on whether productive rearrangements of the genes of the other lineage have occurred in that specific cell. In most thymocytes, however, there is successful rearrangement of a β chain gene, resulting in production of a functional β chain that can pair with the pTα chain to create a pre-TCR, before successful rearrangement of both y and d chain genes have occurred. T cells that express functional γ and δ chains do not express αβTCRs and vice versa, and the two lineages are totally independent on each other. The function of TCRγδ^{+} T cells in the periphery is described below.

Less than 5% of the thymocytes leave the thymus as mature T cells – the rest die either as the result of positive and negative selection, or failure to undergo productive rearrangements of the T cell receptor genes.

This migration, maturation and differentiation process was previously believed to take about 3 weeks before thymocytes are ready to leave the thymus [64] [65]. However, very recent data demonstrate that naïve SP thymocytes emigrate only 4-5 days after entering the SP pool [66]. Even though the number of thymocytes will decline with age, about 1-2 % of the total number of thymocytes will egress per day throughout life [65].

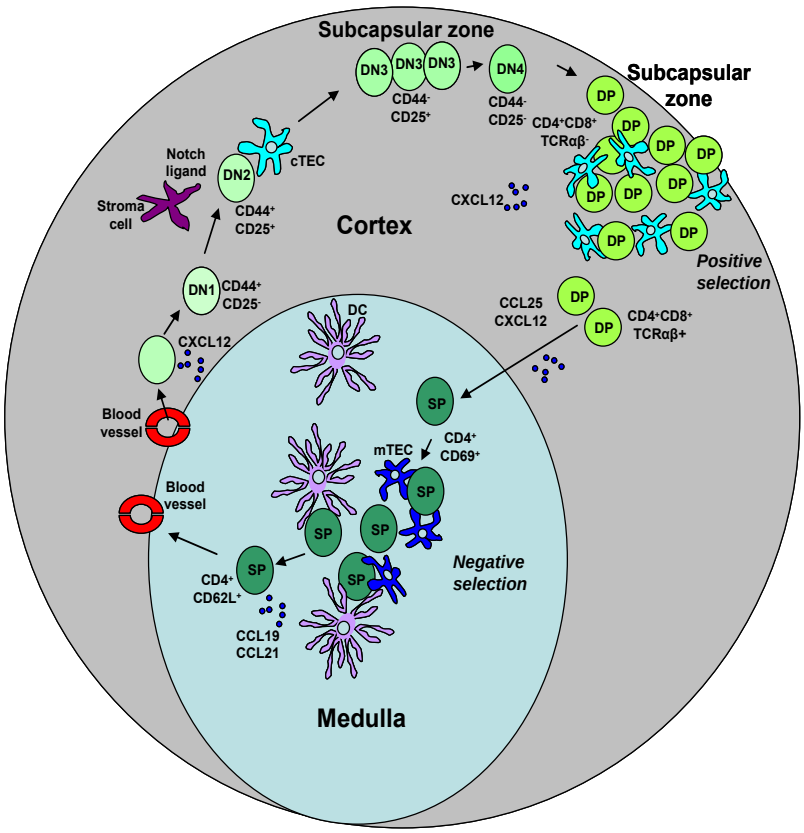

Figure 1. Thymocyte development. Progenitor T cells enter the thymus from the blood vessels in the cortico-medullary junction and bind to the stroma cells to commit to the T cell lineage. Double-negative (DN) thymocytes migrate to the subcapsular zone where an extensive proliferation takes place. Double-positive (DP) thymocytes undergo positive selection by binding to MHC expressed on cortical thymic epithelial cells (cTECs) in the cortex and migrate into the medulla were binding to MHC expressed on medullary thymic epithelial cells (mTECs) mediates negative selection. Mature single-positive (SP) thymocytes exit the thymus as naïve T cells via blood vessels. The migration within the cortex and medulla is driven by chemotactic responses, CXCL12 and CCL25 in the cortex and CCL19 and CCL21 in the medulla. Picture modified from Fu et al, 2004 [67] and Ladi et al, 2006 [68]

ADHESION MOLECULES, CHEMOKINES AND CYTOKINES IN THYMUS

The migration of hematopoetic progenitor cells from the bloodstream via post-capillary high endothelial venules into the cortico-medullary junction (CMJ) in the thymus is believed to be guided by P-selectin expressed on thymic endothelium and binding to PSGL-1 (P-selectin glycoprotein ligand-1) on early thymocytes [68]. Therefore the amount of P-selectin expressed may regulate the homing of precursors to the thymus [68]. It is also possible that the migration of hematopoietic precursor cells into the thymus is regulated by chemotactic cytokines, so called chemokines that form a concentration gradient that directs movement of cells. Chemokines are classified into four different subgroups on the basis of a conserved cysteine motif at the N-terminal end; C, CC, CXC and CXXXC ligand (L). Likewise, their respective receptors are named CC Receptor (CCR) 1-9, CXCR1-5 and so on. All chemokines act via receptors that have seven transmembrane segments linked to G proteins. They are a very complex group of cytokines, as most of them act on more than one receptor and most receptors will respond to several chemokines.

The chemokines are secreted by dendritic cell and stroma cells within the thymus and requires corresponding chemokine receptor expression on thymocytes. Even though no chemokines have been strongly linked to homing to the adult thymus so far, CXCL12, CCL21 and CCL25 have all been shown to be important in homing to the fetal thymus [69, 70]. Likewise, thymocyte movement within the thymus during maturation is not a random phenomenon but is tightly controlled by chemotactic responses. Thus, DN thymocytes express the chemokine receptor CXCR4 [71] and are able to respond to CXCL12 [72].

Chemokine signaling leads to activation of integrins, a family of heterodimeric receptors that are composed of two non-covalently linked protein chains, which bind to the cytoskeleton and thereby induce conformational changes in the cell. DN2 thymocytes have been shown to express different kinds of α and β integrins on their surface, α_4, α_5, α_6, β_1, β_4, and β_7, which gives the potential for formation of different kinds of heterodimer integrins, such as $\alpha_4\beta_1$ (VLA-4), $\alpha_5\beta_1$ (VLA-5), $\alpha_6\beta_1$ (VLA-6), $\alpha_6\beta_4$ (CD49fCD104) and $\alpha_4\beta_7$ (LPAM-1) [73]. Integrin $\alpha_4\beta_1$, VLA-4, binds to vascular-cell adhesion molecule 1 (VCAM1) expressed on thymic stromal cells which seems to support the migration of DN2 thymocytes across the cortex to the subcapsule [74]. Even though VCAM1 is currently the only confirmed ligand to participate in DN thymocyte migration [73, 75, 76], laminin and fibronectin are two extracellular proteins that have been identified within the cortex [75, 76], which potentially also could bind to the integrins and thereby facilitate the migration of DN thymocytes.

The migration of the DP and SP thymocytes in the cortex and in the medulla is, however, much more investigated. DP thymocytes express CXCR4, CCR9 and CCR4 which mediate their chemotactic movement towards CXCL12, CCL25 and CCL22 respectively [63]. The SP thymocytes in the medulla instead express CCR7 and respond to CCL19 and CCL21 [63, 77, 78].

Besides the importance of chemokines and integrins in directing the migration within the thymus there are other molecules that are equally important for thymocyte maturation and differentiation. The thymic epithelial cells (TEC) express cytokines, such as interleukin-1 (IL-1), IL-6 [79] [80] and IL-7. IL-7 is a cytokine involved in thymocyte expansion and proliferation [81] and experimental studies in mice deficient for IL-7 [82] or IL-7Rα [83] as well as anti-IL-7 mAb-treated mice [84] have a major reduction in production of both T and B cells. IL-7 seems to be especially important during the DN developmental stages as it is also involved in V(D)J rearrangement by induction of the recombination-activating genes RAG-1 and RAG-2. There are currently contradictory data on whether IL-7 gene expression is altered by age. Thus, whereas Andrew et.al. reported on reduced expression of the IL-7 gene with age, although it was not clear whether this was due to loss of the IL-7-producing thymic epithelial cells or to a decline in epithelial cell functions [85], Sempowski et.al. claimed that IL-7 remains unaltered with age [86].

THYMIC ATROPHY

At birth the thymus weighs 10-15 g in humans and it increases in size until puberty when it may weigh approximately 25-30 g, after which time atrophy starts with age. This loss of functional status is often accompanied by the deposition of fat. Although thymic involution is a phenomenon that occurs in all aging individuals, the cause of this is still unknown. It has been estimated that the thymus volume shrinks by 3 % per year until middle age and less than 1% per year for the remaining years of life, thereby reducing the capacity to develop thymocytes [87]. Besides thymic involution caused by aging, this is also a phenomenon seen e.g during a viral or bacterial infection [88], during pregnancy [89] and in mice experiencing stress due to immobilization [90]. The precise mechanism(s) responsible for the thymic atrophy seen during acute infection are not completely elucidated, and may vary with distinct diseases. One major mechanism is related to the rise in glucocorticoid hormone levels in the blood, an effect comprised within the organism's stress response to the infection [88]. It is

well known that steroids can trigger apoptosis in thymocytes, especially in DP thymocytes while SP thymocytes are much more resistant [91] [92].

T LYMPHOCYTES IN THE PERIPHERY

MIGRATION OF LYMPHOCYTES TO PERIPHERAL LYMPHOID ORGANS

The egress of mature, naïve T cells from the thymus into peripheral blood is induced by Sphingosine 1-Phosphate (S1P) [93], probably via a S1P-directed chemotactic response[94] Upregulation of the S1P-receptor on mature SP thymocytes leads to binding to S1P, predominantly secreted by platelets in blood [95]. Naïve T cells recirculate between lymph nodes, which are strategically positioned sampling stations for peripheral antigens, and the blood, in their search for their cognate antigen leading to activation and clonal expansion of T cells. It is only after activation that the T cells gain access to peripheral tissues. Naïve T cells homing to the peripheral lymph nodes express CD62L/L-selectin and the chemokine receptor CCR7, and both molecules are up regulated during their final maturation stage in the thymus [63]. This allows them to bind addressins and chemokines expressed on high endothelial venules (HEV) in the lymph node. The HEVs express the the Peripheral lymph Node Addressins (PNAds) GlyCAM-1 (Glycan-bearing cell adhesion molecule-1) and CD34, as well as the chemokines CCL19, produced by stromal cells surrounding HEV [96], and CCL21, produced by endothelial cells of HEV and by dendritic cells in lymph node [97, 98] This binding mediates rolling of the T cell on HEV and the chemokines attached to the proteoglycan on HEV activates LFA-1 (integrin $\alpha_L\beta_2$), leading to clustering of LFA-1 in the area of cell-cell contact as well as an increased affinity of LFA-1 to ICAM-1 and ICAM-2 on the endothelial surface, thereby mediating arrest and transmigration of the T lymphocyte into the lymph node. Once inside the lymph node the naïve T cells migrate towards the T-cell zone where it is exposed to dendritic cells expressing self MHC molecules loaded with antigenic peptides together with costimulatory molecules such as B7, which induce survival signals and clonal expansion of the T cell. During this process the activated T lymphocytes acquire effector and/or memory functions. Similar to the thymus, the egress of primed T cells from the lymph node is actively driven by S1P [93]. They leave the lymph node via the efferent lymphatic vessels and return to the blood stream via the thoracic duct, and aim for the site of inflammation. Those naïve T cells that fail to recognize their specific antigen migrate out of the lymph node in the same way as the activated T cells, and continue to circulate through the lymph nodes in the search for better luck. The activated effector T lymphocytes down regulate

CCR7 and CD62L and up-regulate P-selectin glycoprotein ligand (PSGL-1) and E-selectin ligand-1 (ESL-1) [99], and chemokine receptors for inflammatory chemokines, e.g. CCR5 and CXCR3. These effector T cells are now ready to home to the site of inflammation. At the inflammation site, macrophages produce cytokines, TNF-α, IL-1, IL-6, IL-8, and IL-12, that will in different ways alarm the site of inflammation and induce infiltration of immune cells, such as effector T cells. TNF-α and IL-1 induce endothelial cells to secrete Weibel-Palade bodies containing P-selectin that will simultaneously be expressed on the cell surface together with E-selectin. As E-selectin is synthesized de novo, its expression is however not evident until after a few hours. Other adhesion molecules that are expressed on the endothelial cells are ICAM-1 and VCAM that will bind to LFA-1 and VLA-4, respectively, on the effector T cell. This will induce T cell rolling and adhesion to the endothelium. Chemokines such as RANTES, MIP-1α and MIP-1β, bound to proteoglycans on the endothelial cells, will bind to chemokine receptors on the T cells and stimulate transendothelial migration of the T cells through the endothelium into the inflamed tissue.

RECENT THYMIC EMIGRANTS AND TRECS

One way of measuring thymic function and -output is by the analysis of so called recent thymic emigrants (RTE). The previous lack of specific markers to identify these human recent thymic emigrants (RTEs) was a big hurdle to accurately characterize and quantify thymic output. By a relative new method, thymic output can be measured by quantifying the amount of T cell Receptor Excision Circles (TRECs) in the peripheral T cell populations. TRECs analysis was first used to study thymic output in chickens [100] and then in humans [101].

Throughout thymocyte development, the thymocytes undergo several gene rearrangement steps of the T-cell receptor genes in order to express a complete receptor. As described above, in TCRαβ$^+$ T cells the β-chain of the TCR is rearranged first and is expressed together with the pTα chain. The rearrangements of α-chain gene starts during the double positive stage of the thymocytes, [102] and will replace the pTα chain. TRECs are formed during both the α and the β chain rearrangements, but as thymocytes with a rearranged β chain, and thus a TREC from the β chain, undergo several rounds of cell division within the thymus, the β TRECs are more dilute. Therefore the TREC resulting from the α-chain gene rearrangement is the most common molecule investigated to identify RTEs.

The *TCRD* gene segments, containing the δ-chain genes, are interspersed within the *TCRA* gene segment, containing the α-chain gene, and therefore rearrangements of the *TCRA* gene

segments will delete the *TCRD* gene [102]. The responsible segments are two so-called *TCRD* deleting elements, δRec and ΨJα, that flank the major part of the *TCRD* gene segment [103]. During the rearrangement of the α-chain, the δRec and ΨJα segments are rearranged to join each other and will thereby form an extrachromosomal circular DNA product containing the TCR δ locus. This DNA circle is known as a sjTREC, as it contains the single signal joint segment from δRec and ΨJα ligated to each other. Each $\alpha\beta TCR^+$ thymocyte leaving the thymus will contain either one or two sjTRECs, depending on whether the rearrangement has occurred in one or both alleles, provided that the cell does not proliferate following the rearrangement. The recombined δRec to ΨJα junction is removed from the signal joint when TCR Vα to Jα recombination occurs and a second TREC, termed the coding joint (cj) TREC, is formed during the Vα-Jα rearrangement.

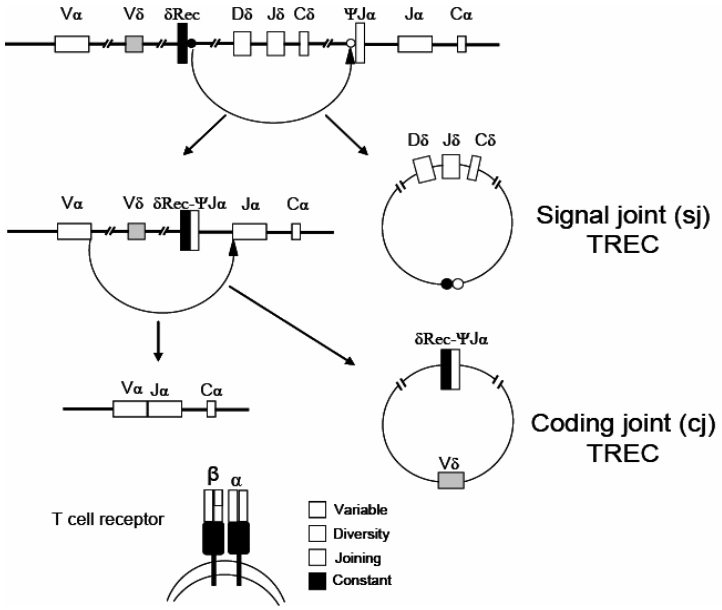

Figure 2. Formation of T cell receptor α-chain involves splicing and rearrangement of the DNA coding region. The δ-locus and the α-locus are interspersed and during α-chain rearrangement, the δ-chain is excised forming an episomal DNA circle with a signal joint DNA region (sjTRECs). Before a productive α-chain is expressed, further rearrangement forms the coding joint TRECs (cjTRECs).
Picture modified from Haynes et al 2000 [104] and Ribeiro et al 2007 [105]

The resulting TRECs are relatively stable, are not integrated into the genome and do not replicate during cell division, meaning that the proportion of cells containing TRECs is reduced in each cell division [106]. Therefore, a high proportion of newly exported RTEs will contain TRECs, compared with T cells that have undergone one or more rounds of peripheral division, where only a small fraction of the cells will contain TRECs.

Therefore a good compliment to TRECs analysis is to analyze the proliferation level within the T cell population. The proliferation can be evaluated by e.g. intracellular staining of Ki-67, a nuclear antigen that are expressed in all dividing cells and absent in resting cells [106]. Together with proliferation analysis, evaluation of TRECS in a peripheral lymphocyte

population is a good measurement on the number of T cells that have recently left the thymus and, hence, reflects thymus function.

THE GASTROINTESTINAL IMMUNE SYSTEM

The gastrointestinal (GI) tract functions not only as a major organ for uptake of fluids and nutrients, but also as a protective barrier between the body and the outside world. The epithelial layer consists of formations of millions of finger-like villi (small intestine) and crypts (large intestine), which gives a surface area expanded to the order of 400 m^2, 200 times larger than the skin. The cellular barrier of the gut consists of closely connected epithelial cells that are sealed together by tight junctions – a protein complex consisting of claudins, occludins ZO-1, ZO-2, ZO-3, cingulin and 7H6 [107, 108], forming a barrier selectively impermeable to fluids and antigens.

The GI tract contains the largest and most complex immune system of the entire body, which is able to distinguish between harmless and harmful antigen as it encounters food antigen, pathogenic bacteria and the intestine's own microflora. The gut-associated lymphoid tissue (GALT) consists of diffusely spread lymphocytes within the epithelium and the lamina propria, as well as organized lymphoid tissue; Peyer's patches (PP) in the small intestine, isolated lymphoid follicles (ILF) in the colon, and mesenteric lymph nodes (MLN), the largerst lymph nodes in the body [109]. Most of the knowledge about the intestinal immune tissue stems from studies on the small intestine.

The PPs are found predominantly in the lower ileum and are macroscopically visible lymphoid aggregates that are covered by a specialized follicle-associated epithelium (FAE), allowing transport of antigens into the lymphoid tissue. The FAE contains a number of microfold (M) cells, which are specialized epithelial cells that lack surface microvilli and the normal thick layer of mucus. The name stems from the fact that these cells have microfolds, invaginations in their basolateral plasma membrane, which form pockets containing lymphocytes and professional antigen-presenting cells (APC) such as dentritic cells (DC) and macrophages (MΦ). Antigen from the intestinal lumen enters though the M cells into the underlying subepithelial dome (SED) where it is processed by professional antigen-presenting cells (APC) such as dentritic cells (DC). From there, the DCs move to the T-cell areas and/or B-cell follicles in PP, where they can either interact with naïve lymphocytes or exit through the draining lymphatics into the MLNs.

Activated B cells undergo immunoglobulin (Ig) class switching from expression of IgM to IgA under the influence of TGF-β [110] produced by T cells. Some of the IgA produced by plasma cells in the lamina propria remains in the tissue, but large amounts of IgA are also transported across the epithelium and secreted into the lumen as the first defence to microbes and toxins. IgA binds to microbes and toxins in the lumen and neutralize them by blocking their entry into the host. Luminal IgA is secreted in the form of a dimer that is held together by the coordinately produced joining (J) chain. The transport of IgA through the epithelium is facilitated by the poly-Ig receptor (pIgr), expressed on the basolateral side of the epithelial cells. The secreted, dimeric IgA containing the J chain binds to the pIgr, and this complex is actively transported in vesicles to the luminal surface. In the lumen the pIgr is proteolytically cleaved, leaving its transmembrane and cytoplasmic domains attached to the epithelial cell, and releasing the extracellular domain of the receptor, carrying the so-called secretory IgA (sIgA) molecule, into the intestinal lumen [111]. sIgA is relatively resistant to cleavage by proteolytic enzymes resident in the intestinal lumen.

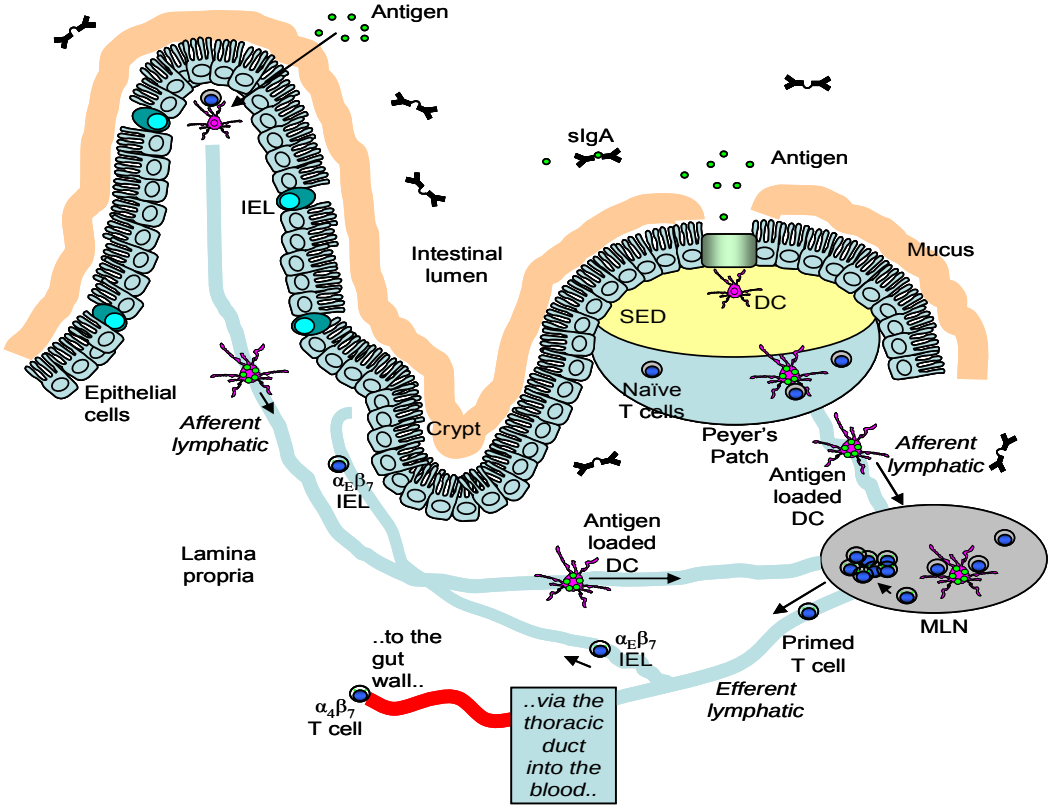

Figure 3. Antigen uptake in the intestine. Antigen enters the Peyer's patch via microfold (M) cells in the intestinal epithelial cell layer. The antigen is processed in the Peyer's patches by dendritic cells (DC) that then migrate to the mesenteric lymph node (MLN) to activate naïve T cells. Alternatively, DCs activate naïve T cells in the Peyer's patch. A possible alternative pathway for antigen entrance is through the epithelium covering the villus lamina propria, where MHC class II^+ enterocytes act as local antigen presenting cells (APCs). Activated T cells express CCR9 and integrin $\alpha_4\beta_7$ and leave the MLN through the efferent lymph via the thoracic duct into the blood and enter the lamina propria via high endothelia venules (HEV). T cells expressing integrin $\alpha_E\beta_7$ will incorporate within the epithelial cells as intraepithelial lymphocytes (IEL). Modified from Mowat Nat.2003 [109].

T LYMPHOCYTES IN THE INTESTINAL MUCOSA

In addition to the organized lymphoid tissue, a large number of lymphocytes are found diffusely spread in the mucosa of the small and large intestine, both in the connective tissue of the lamina propria and on the other side of the basement membrane, within the epithelial layer. Lamina propria lymphocytes (LPLs) are predominantly activated T cells, but numerous activated B lymphocytes and plasma cells, secreting mainly IgA, are also present. The major T cell subpopulation in the lamina propria is the $CD4^+$ T lymphocyte. Unlike conventional peripheral T cells, however, the TCR repertoire of the small-intestine LPLs, and to a lesser extent the large-intestine LPLs, is oligoclonal. Together with their antigen-experienced phenotype, this indicates that re-encounter with their specific antigens in the gut might lead to expansion of selected clones [112] Intraepithelial lymphocytes (IELs) are mostly T cells, and this population is quite different from the LPLs as it contains a high proportion of $CD8^+$ T lymphocytes and other more unusual populations. The so-called conventional $CD8^+$ T cells in the gut are $TCR\alpha\beta^+$ and express the usual heterodimer of CD8, $CD8\alpha\beta^+$. They have likely encountered antigen in the periphery and have been instructed, probably via contact with dendritic cells in the MLN and PP, to migrate to the intestine. In addition, there are populations of IELs in the mouse that express a $CD8\alpha\alpha$ homodimer. These cells can be either $TCR\alpha\beta^+$ or $TCR\gamma\delta^+$ T cells. Although the frequency of $TCR\gamma\delta^+$ IELs varies in different species, in mice they are more numerous among the IELs of the small intestine compared with the colon [113, 114]. $CD8\alpha\alpha$ SP IELs are an important T-cell population early in life, but with age, they are gradually taken over by an expanding pool of conventional IELs [115, 116]. Although potentially self-reactive $TCR\alpha\beta^+$ T cells are depleted from the peripheral T-cell repertoire during negative selection in the thymus, these TCRs accumulate among the $TCR\alpha\beta^+CD8\alpha\alpha^+$ [117]. Despite the heterogeneity of the intestinal T lymphocytes they have the common denominator of having an antigen-experienced phenotype [118]. However, despite this phenotype, most studies have demonstrated a decreased ability of LPLs to proliferate in response to TCR/CD3 stimulation, compared to other peripheral T cells [119-121] This hyporesponsiveness appears to be selective for the TcR/CD3 pathway, because CD2- or CD28-driven activation resulted in strong proliferation [120].

HOMING OF LYMPHOCYTES TO THE INTESTINAL MUCOSA

Primed lymphocytes migrate from the Peyer´s patches via the afferent lymph into the mesenteric lymph nodes, where they undergo further differentiation before migration through the efferent lymph and the thoracic duct into the bloodstream and finally accumulate in the mucosa:

Lymphocytes activated by DCs in the GALT selectively up regulate expression of two molecules that are specific for homing to the gut, integrin $\alpha_4\beta_7$ and CCR9, the receptor for CCL25 (TECK). CCR9 is highly expressed by epithelial cells, closely associated to vessels expressing mucosal addressin cell-adhesion molecule 1 (MadCAM-1), the ligand for integrin $\alpha_4\beta_7$, on the endothelium of the small bowel and directs the migration of the lymphocytes from the bloodstream into mucosal effector sites, such as the intestinal lamina propria [122]. DCs in the gastrointestinal immune system produce retinoic acid (RA) (a derivate of Vitamin A) that binds to intracellular retinoid receptors in T cells, which then activate transcription of the genes encoding CCR9 and integrin $\alpha_4\beta_7$ [123]. This characteristic is specific for gut DCs, as DCs isolated from the peripheral lymph node or spleen do not induce CCR9 and $\alpha_4\beta_7$ on responding T cells [124-126]. In parallel, $CD8^+$ intraepithelial lymphocytes (IELs) express integrin $\alpha_E\beta_7$. This integrin does not promote homing to the mucosa, [127] but rather retention of the IELs within the epithelium by binding to its specific ligand E-cadherin on epithelial cells [128]. Upregulation of this integrin is probably regulated locally, possibly under the influence of transforming growth factor-β (TGF−β) [129].

The mechanisms directing homing to the colon is much less characterised. Lymphocytes isolated from the colon express high levels of integrin $\alpha_4\beta_7$ but are mostly devoid of CCR9-expressing cells and CCL25 is not expressed in this tissue. One chemokine that seems to have the potential of mediating homing of lymphocytes to the colon is CCL28 (MEC) [130]. mRNA for CCL28 is expressed in the colon [131] and mucosa-homing IgA plasma cells express CCR10, the receptor for CCL28, and bind to CCL28 presented on epithelial cells in the colon [132]. Expression of CCL28 is however not unique to the colon, but is found also in other organs and mucosal tissues such as the salivary glands, the trachea and bronchi, the mammary glands, the stomach, the small intestine and the bone marrow [131, 132]. Other chemokines that have the possibility of influencing homing of T cells to the colon is CXCL12 binding to CXCR4 and CCL20 binding to CCR6 in mice, although neither of these chemokines are exclusive for colon as both are found also in the small intestine [133]. For entry into the MLN both CD62L as well as $\alpha_4\beta_7$-integrin are necessary [134].

LINKS BETWEEN THE GUT AND THYMUS

Several phylogenetic as well as ontogenetic facts suggest a connection between the thymus and the gastrointestinal tract. In humans, the primordial thymus develops from the anterior portion of the embryonic gut tube (the pharyngeal pouches). At one point during gestation, the third pair of pouches buds off the gut epithelium (endodermis) and forms a sac-like epithelial protrusion that bends towards the digestive tract. This becomes the thymus rudiment. Thymus epithelial cells develop and differentiate from the gut endoderm and bone marrow lymphocyte precursors interact with the epithelium. As a result, the thymus tissue becomes organized and a clear cortex and medulla are formed [135].

Other common denominators are expression of desmosomes and tight junctions in the epithelium [136-139] and the presence of chemokines and chemokine receptors selectively expressed in the thymus and intestinal mucosa, or on cells homing to the thymus or gut, i.e. CCL25/CCR9 and CXCL12/CXCR4 [63, 133, 140].

EXTRATHYMIC MATURATION OF T LYMPHOCYTES

Given the links between the thymus and the gut, it is not surprising that extrathymically derived T cells can be found in the intestinal epithelium both in mice and humans [141-143]. Extrathymic T cell maturation has been observed in different organs including lymph nodes [144], but mostly in the intestine and especially IELs [145, 146] Evidence for extrathymic T cell maturation (ETCM) in human infants has been shown by expression of $CD3^{-}CD7^{+}CD2^{+}$ and $CD3^{-}CD7^{+}CD2^{-}$ IELs and LPLs in both the small intestine and colon, indicating early linage T cells [142]. Also adults show evidence of ETCM, as mRNA expression of pTα was found in the small intestine [143]. Frequently used markers for ETCM are mRNA expression of Recombination activating gene (RAG) and pTα, two proteins involved in rearrangement of the TCR genes, where RAG1 and RAG2 are responsible for the DNA cleavage during this process, and the pre-TCR α-chain associates with the mature TCR β-chain allowing rearrangement of the α-chain gene [147], as described above. In addition extrathymically matured T cells have been reported to, similar to NK cells, express the intermediate affinity IL-2R consisting of the IL-2R β-chain only, without the high-affinity IL-2Rα chain (CD25). Conventional, thymus derived T cells have the IL-$2R\alpha^{-}\beta^{+}$ phenotype under resting conditions but acquire the IL-2R $\alpha^{+}\beta^{+}$ phenotype following activation [148]. This subset of extrathymic T cells seems to be linked to thymic involution during aging, stress, autoimmune disease, pregnancy and infections as they increase in the liver under these conditions [148].

T CELLS AND IBD

There are a number of studies demonstrating a correlation between alterations in T cell subsets and development of IBD. Several animal models with IBD-like syndrome have demonstrated the importance of T cells to induce colitis; immunodeficient SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells develop colitis [149-151] as do Tgε26 transgenic mice transgenic for the human CD3ε gene resulting in lack of a normal thymic microenvironment [152]. In addition, syngeneic bone marrow transplantation into adult Tgε26 mouse results in development of colitis associated with aberrant thymic development retarding thymic regulatory T cells ($CD4^+CD25^+$) that may contribute to chronic colitis [153]. Likewise, TCR-α chain deficient mice develop colitis [154] Aberrant thymocyte developments due to colitis have also been shown in mice deficient for IL-2 and after challenge with TNP-KLH [155]. By transferring SP thymocytes from these mice into $IL\text{-}2^{+/+}$ mice, the colitis are introduced in the $IL\text{-}2^{+/+}$ mice, demonstrating the importance and the consequence of dysregulated thymocytes on the development of colitis [155].

In humans, UC patients undergoing thymectomy was shown to enter remission more frequently than non-thymectomized patients [156] and excision of invasive thymomas was shown to cure ulcerative colitis [157]. Furthermore, a case report of a human immunodeficiency virus (HIV) infected CD patient reported on complete remission of the gastrointestinal symptoms in association with progressive immunodeficiency [158].

Over the past ten years, a large research field in aetiology of IBD has been investigations of the presence/absence or dysfunction of regulatory T cells (Tregs). Regulatory $CD4^+$ T cells represent a population of lymphocytes with the ability to suppress both adaptive and innate immune responses [159, 160] and these characteristics make them important for both maintenance of immunological tolerance and control of antimicrobial responses. Various types of regulatory T cells (Tregs) have been identified such as naturally occurring thymus induced $CD4^+CD25^+$ Tregs and two peripherally induced Treg populations, so-called adaptive Treg; Tr1 (T regulatory1) and Th3 (T helper 3) [161].

Naturally occurring $CD4^+CD25^+$ Tregs develop in the thymus and it is believed that $CD4^+$ T cells bearing receptors with a high affinity for self-peptides are positively selected in the thymus and will differentiate into Tregs following encounter with cognate peptide on thymic stromal cells [162]. The cell surface marker CD25, the IL-2 receptor α chain is often used to identify Treg cells, but it is not Treg specific, as it is also expressed on recently activated effector T cells. However, the transcription factor forkhead box protein 3 (Foxp3) has been identified, in both murine and human systems, as being necessary for both the development

and function of Tregs [163] [164]. Additional membrane expressed markers are the glucocorticoid induced TNF receptor (GITR) and cytotoxic T lymphocyte associated antigen 4 (CTLA-4), indicating that the regulatory function is mediated by cell-contact-dependent signalling [165, 166].

Peripherally induced adoptive Tregs, Th3 and Tr1 are phenotypically distinct from Tregs with an intrathymic origin. These Tregs generally do not express CD25 or Foxp3 and are characterized by the secretion of the immunosuppressive cytokines transforming growth factor-β (TGF-β) and IL-10, respectively [161]. The cytokines IL-10 and TGF- β have the ability to modulate or downregulate immune responses: IL-10 downregulate production of inflammatory cytokines such as IL-1α, IL-6 and TNF-α from activated MΦ [167] and TGF- β directly inhibits the proliferation and acquisition of effector functions of naïve T cells and also inhibits maturation of dendritic cells (DC) and thereby indirectly affects T-cell responses [168].

Colitis in mice can be induced by transfer of naïve $CD4^+CD45RB^{high}$ T lymphocytes into recombination activating gene (RAG) deficient mice or into severe combined immunodeficiency (SCID) mice [150, 169]. Cotransfer of $CD4^+CD25^+$ T cells will however prevent the induction of colitis. This can be reversed by the addition of monoclonal anti-CTLA-4, anti-IL-10R or anti-TGF-β antibodies. Not only do $CD4^+CD25^+$ T cells prevent the induction of colitis, they can also reverse established colitis and wasting disease, indicating their importance in controlling ongoing immune mediated inflammation [170].

Despite promising results of Tregs in experimental colitis, little has so far been confirmed in IBD patients. In patients with CD a fraction of lamina propria $CD4^+$ T cells with a high expression of CD25 ($CD25^{bright}$) expressing CTLA-4 and GITR was found. *Foxp3* was predominantly transcribed by lamina propria $CD4^+CD25^{bright}$ T lymphocytes and these Tregs were able to suppress the proliferation of peripheral blood $CD4^+CD25^-$ T cells [171, 172]. Similar results have been shown in CD patients, where $Foxp3^+CD4^+$ Tregs were expanded in the lamina proria and MLN, and $CD4^+CD25^+$ from MLN could efficiently suppress the proliferation of effector $CD4^+CD25^-$ T cells [173]. Likewise, $CD4^+CD25^+$ T cells isolated from human MLN in UC patients display features typical of Treg cells and possess potential suppressor activity in vitro in spite of persistent mucosal inflammation [174]. These results indicate that the inflammatory pathology in IBD patients does not result from an absence or altered functionality of the Treg populations although the Treg numbers and/or activity may be insufficient to suppress the inflammatory condition [175].

IBD MOUSE MODELS

In 1961 came the first report of an immune complex mediated colitis in rabbits and since then at least 63 experimental animal models of IBD have been described [176]. The experimental mouse models of IBD have a variable range of clinical manifestation more or less similar to those observed in human IBD. The reasons for using mouse models for IBD are many, as they give the advantage to study several parameters that comes with the inflammation that would not be possible in experimental studies of only specimens from IBD patients, for example; changes before onset of colitis in animal models spontaneously developing colitis, and investigation of alterations in other organs during the disease. In addition, the genetic and environmental similarities of an inbred mouse strain reduce the interindividual variation. In this thesis, the Gαi2 deficient mouse model of colitis and the dextran sulfate sodium (DSS)-induced colitis model have been used:

Gαi2 DEFICIENT MICE

Through the work of Rudolph et al., a mouse deficient for the heterotrimic G protein αi2 subunit was generated by homologous recombination in embryonic stem cells at the *N*coI site in exon 3 of the Gαi2 gene.

The heterotrimeric G proteins transmit signals from a diverse variety of seven-helix transmembrane receptors, the so-called G protein-coupled receptors (GPCR). Some G proteins are ubiquitous, whereas others only occur in specialized tissues. They consist of a large α subunit, an intermediate β subunit and a small γ subunit, of which the α subunit has the binding site for the transmembrane receptor as well as GTP or GDP and also carries the GTPase activity. All three subunits show great diversity, and at least 20 different genes for the α subunit are known in mammals. The Gα proteins are divided into four families based on similarities in amino acid sequences; the Gs, Gi, Gq and G12 families. The Gi (for inhibitory) family got its name because the first member of the subfamily to be discovered inhibited adenylate cyclase. However, other members signal through phopholipase C and downstream inositol triphosphate and diacylglycerol. The Gi proteins are characterized by their inhibition by pertussis toxin [177].

Mice homozygous for the Gαi2 deficiency ($G\alpha i2^{-/-}$) spontaneously develop colitis, histopathologically closely resembling ulcerative colitis in humans, at the age of 6-10 weeks, while mice heterozygous for the deficiency ($G\alpha i2^{+/-}$) remain healthy [178]. The incidence of the disease is 100% in $G\alpha i2^{-/-}$ mice on a disease susceptible background e.g. H129SvEv, while

Gαi2$^{-/-}$ mice on C57BL/6 or 129SvJBom backgrounds are resistant to colitis [179, 180]. The development of colitis in H129SvEv Gαi2$^{-/-}$ mice is however abolished in a germfree environment. The clinical symptoms of colitis debut in Gαi2$^{-/-}$ mice starts with soft faecal pellets and continue with weight loss, diarrhea, ruffled fur and a slightly passive behavior. The inflammation is limited to the colon and does not involve the small intestine. The acute and chronic inflammation is confined to the colonic mucosa without skip areas and is characterized by infiltration of lymphocytes (mostly activated/memory CD4^{+} T cells), plasma cells and neutrophils into the colonic lamina propria, leading to crypt distortion, loss of mucin producing globlet cells, crypts abscess formation and ulcerations [178, 179]. Previous studies on the Gαi2$^{-/-}$ mice by our group have demonstrated immune changes characterized by activation of proinflammatory T helper 1 cells in late stages of the disease [179]. In addition, the colitis is preceded by immunological alterations in both the small and large intestine characterized by increased spontaneous production of proinflammatory cytokines, increased frequencies of activated T and B lymphocytes homing to the intestinal mucosa and antibodies specific for normal intestinal flora as well as self-tissues, present locally in the intestine [181] [182]. Antibodies specific for dietary antigens and cytoplasmatic neutrophil proteins, giving rise to p-ANCA staining, are however not present until after onset of disease [182]. The precolitic changes also include the regression of Peyer´s patches (PP), likely caused by excessive apoptosis due to decreased levels of the anti-apoptotic protein Bcl-2 in PP lymphocytes from Gαi2$^{-/-}$ mice [183], as well as a switch from an IL-10 dominated dietary antigen T cell response in wild type mice to a T helper 1 cytokine profile in Gαi2$^{-/-}$ mice prior to colitis [184].

Already prior to colitis the Gαi2$^{-/-}$ mice have a severely impaired ability to mount a protective B cell response to an orally administered antigen, most probably due to the Th1-dominated Ag specific cytokine response failing to support B cell activation and differentiation [185]. The immunomodulatory and beneficial effect of a human vaccine on IBD was also demonstrated, in that *Bordetella pertussis* vaccine enhanced mucosal IL-10 production, induced apoptosis of activated Th1 cells and attenuated colitis in Gαi2-/- mice [186]. Whereas treatment of Crohn's patients with a single dose of Natalizumab, a recombinant mAb specific for integrin α4, one of the key cell-surface molecules that regulates the migration of lymphocytes to the mucosa, showed good therapeutic effect [187], treatment of Gαi2$^{-/-}$ mice with anti-integrin α4 mAbs over a longer period of time, aggravated colitis, possibly by blocking the ability of T-regulatory (T_{reg}) lymphocytes to home to the inflamed mucosa [188].

After adoptive transfer of Gαi2$^{-/-}$ bone marrow, severe colitis developed in irradiated wild type recipients, whereas irradiated Gαi2$^{-/-}$ mice increased their life span more than 3 times after transfer of wild type bone marrow, accompanied by significant amelioration of colitis. Neither purified Gαi2$^{-/-}$ CD4^{+}, nor CD8^{+} splenic or MLN-derived T cells could induce colitis in RAG2$^{-/-}$ recipient mice, whereas transfer of splenic, but not mesenteric lymph node, Gαi2$^{-/-}$ CD3^{+} T cells induced severe colitis [189]. The most prominent cytokine produced, together with IFN-γ in Gαi2$^{-/-}$ colitis is IL-1β. It is however not found in the circulation neither before nor after onset of colitis. Instead, serum levels of IL-18 are highly increased in established colitis. While IL-1β and IL-18 levels were not increased at the time of colitis onset, circulating IL-1Ra (IL-1 Receptor antagonist) were significantly increased both compared to age-matched control animals and to younger healthy Gαi2$^{-/-}$ mice [190]. In addition, Wu et al. showed in an in vitro study that peripheral T cells from Gαi2$^{-/-}$ mice were unable to responed to TGF-β. Lack of Gαi2 abrogated the inhibitory effect of TGF-β on IL-2 and IFN-γ production and proliferation of T cells [191]. Other organs affected by the inflammation are the thymus with an increased frequency of mature CD4^{+}CD8^{-} and CD4^{-}CD8^{+} thymocytes [178] caused by an accelerated transition from the CD4^{+}CD8^{+} DP to the CD4^{+}CD8^{-} or CD4^{-} CD8^{+} SP stage, which accounts for the high proportion of SP thymocytes [192]. An impaired development of splenic marginal zone (MZ) and transitional type 2 (T2) as well as peritoneal B-1a B cells have also been found in Gαi2$^{-/-}$ mice and a similar impaired development was seen in Rag$^{-/-}$ mice reconstituted with Gαi2$^{-/-}$ bone marrow, indicating that a Gαi2$^{-/-}$-dependent B cell development occurs after bone marrow B lymphopoiesis [193]

MICE WITH DSS-INDUCED COLITIS

The dextran sulfate sodiums (DSS) model of colitis was originally reported by Okayasu et.al. [194], Oral administration of dextran sulfate sodiums (DSS) dissolved in water for 5-7 days results in acute colits during administration of DSS, and chronic colitis a short time after removal of DSS. The concentration of DSS usually ranges from 3% to 5% (wt/vol) depending of the mouse strain.

Clinical symptoms are weight loss, diarrhea and GI bleeding. The severity of inflammation increases from the proximal to distal colon [195] and penetrates to the submucosa. Histological features are mucosal ulcerations, infiltration with mononuclear and polymorphonuclear leukocytes in the lamina propria and submucosa, crypt distortion, hyperplastic epithelium, shortening of the colon, loss of goblet cells and formation of crypt

abscesses [194-196]. The gut epithelial cells disappear in the DSS mice and it is believed that dextran sulfate sodium is toxic to these cells [197].

Similar to $G\alpha i2^{-/-}$ mice, the severity of DSS-induced colitis is strain dependent: Whereas the C3H/HeJ, C3H/HeJBir [195] and C57BL/6 strains are susceptible to DSS, Balb/c is not, and develop only a mild acute colitis during administration [196].

In a study done by Melgar et al [196], two mice strains; C57BL/6 and BALB/c mice, were analyzed kinetically for the consequences of DSS treatment. C57BL/6 mice exposed to DSS for 5 days developed acute colitis followed by severe chronic inflammation whereas BALB/c mice exposed to DSS for 5 days resolved the colitis after the acute phase [196]. In the acute phase, both stains showed loss of crypts, reduced goblet cells and focal ulcerations. C57BL/6 mice had a moderate infiltration of neutrophils whereas there was a marked increased of neutrophils in BALB/c mice. The acute colitis in BALB/c mice was accompanied by elevated plasma levels of haptoglobin and increased colonic levels of IL-1α/β, IL-6, IL-18, and granulocyte colony-stimulating factor, and the chronic inflammation in C57BL/6 mice involved infiltration of MΦ, lymphocytes and plasma cells into the mucosa and submucosa. Production of IL-1β, IL-12 p70 and IL-17 started in the acute phase and increases during the chronic inflammation whereas high IFN-γ production was mainly found late in the chronic phase [196].

Other studies have shown that DSS-induced colitis can develop in severe combined immunodeficiency (SCID) mice and in Rag deficient mice [198], demonstrating that T and B lymphocytes are not required for induction of the colits in this model.

AIMS

The overall aim of this thesis was to evaluate thymus function in inflammatory bowel disease. We had previously observed thymus atrophy in Gαi2$^{-/-}$ mice with colitis, and we therefore wanted to investigate whether this was true also for an induced mouse model of colitis. We hypothesized that the thymus atrophy might result in an aberrant T cell ontogeny and that this would also affect the egression of naïve T lymphocytes from the thymus and contribute to the intestinal inflammation. The specific aims were therefore to:

- Characterize the thymus and thymocyte subpopulation in relation to colitis in two mouse models; Gαi2-deficient mice and mice with DSS-induced colitis.

- Functionally investigate thymocytes as wells as colonic lamina propria lymphocytes regarding their responsiveness to chemokines before and during colitis in Gαi2-deficient mice.

- Investigate thymic output by studying recent thymic emigrants (RTEs) in blood and in inflamed intestine in both colitic Gαi2-deficient mice and in IBD patients with active disease and in a chronic phase.

- Analysis of possible extrathymic T cell maturation in the intestine of IBD patients and in small and large intestine as well as the mesenteric lymph nodes in colitic Gαi2-deficient

METHODOLOGICAL CONSIDERATIONS

The following section describes the experimental approaches and choice of specific materials and methods in this thesis. More detailed descriptions of specific protocols are found in paper I-IV.

Mice

Gαi2$^{-/-}$ mice (Paper I & IV)

Specific pathogen-free mice with a target deletion of the heterotrimic G proteins subunit αi2, Gαi2-deficient (Gαi2$^{-/-}$) mice were on a pure 129SvEv or a mixed 129SvEv x C57BL/6 background. Mice on a 129SvEv x C57BL/6 background was backcrossed four to five generations into the 129SvEv background and then intercrossed.

All animals were specific pathogen free and were maintained in filter top cages with forced ventilation in micro isolator racks at the Department of Experimental Biomedicine, Göteborg University, with free access to water and standard rodent pellets in accordance with local and national ethical regulations and were health screened in accordance with recommendations from the Federation of European Laboratory Animal Science Associations (FELASA).

All Gαi2$^{-/-}$ mice on the 129SvEv x C57BL/6 or the pure 129SvEv genetic background develop a lethal colitis. The incidence of colitis was 100% for both mouse strains although the onset was later for the mice on a mixed background, between 10 and 21 weeks, compared to between six and 12 weeks of age on the pure 129SvEv background.

The benefit of having the mice on a mixed 129SvEv x C57BL/6 background is the increased amount of offspring obtained with the desired Gαi2$^{-/-}$ genotype. While the mice on a mixed background are bred as Gαi2$^{-/-}$ males with Gαi2$^{+/-}$ females, the pure 129SvEv background only allows breeding of heterozygotes. However, the early onset of colitis in mice on a 129SvEv background is an obvious advantage in experimental setups.

A neo cassette has been inserted into both alleles coding for the G protein αi2 subunits in Gαi2$^{-/-}$ mice, preventing expression of the protein. Those mice with an inserted neo cassette in one or both alleles will be of a Gαi2$^{+/-}$ or Gαi2$^{-/-}$ genotype, respectively. Genotyping of the mice was performed by Polymerase Chain Reaction (PCR) on DNA extracted from the outer tail tip. The tissue was lysed with a buffer containing proteinase K, for cleavage of peptides, sodium dodecyl sulphate (SDS), for unfolding of proteins by cleavage of disulphide bonds,

and EDTA, to protect the DNA from degradation. The DNA was then precipitated with isopropanol and pelleted after which the DNA was washed in 70% ethanol and dried.
The primer pairs used for the PCR was the following: The neo cassette: 5'GCA CTC AAA CCG AGG ACT TAC AGA AC 3'and 5' CAG GAT CAT CCA TGA AGA TGG CTA C 3'and the intact Gαi2 gene: 5' CCC CTC TCA CTC TTG ATT TCC TAC TGA CAC 3' and 5' GAT CAT CCA TGA AGA TGG CTA CTC AGA AG 3'. The PCR cycle used for the genotyping was as follows: denaturation of DNA at 94°C for 4 min, followed by 35 amplification cycles including 1 min denaturation at 94°C, annealing at 61°C for 1 min and extension at 72°C for 2 min. The process was ended with a final extension at 72°C for 5 min. The genotype of individual mice was visualized by migration of both PCR products through a 1% agarose gel where the PCR for the inserted neo cassette gene or the intact Gαi2 gene generated a product of 509 or 805 base pairs, respectively.

Mice with DSS-induced colitis (Paper II)

Specific pathogen-free female mice on C57Bl/6OlaHsD and Balb/cJ backgrounds were purchased from Harlan, the Netherlands and kept at the animal facilities at AstraZeneca R&D Mölndal under standard conditions. They were acclimatized for 2 weeks prior to the start of the study and were 7-9 weeks old and weighed 20-24 g at the start of the DSS administration. To induce colitis in C57Bl/6 mice, 3% of DSS was given in the drinking water for 5 days, and after removal of DSS mice were given ordinary tap water during the rest of the study.
Balb/cJ mice are more resistant to DSS, and therefore a a higher concentration of DSS and a prolonged administration was required to induce colitis. 5% of DSS was given in the drinking water for 5, 6 or 10 days, and after removal of DSS, the mice were given ordinary tap water during the rest of the study.

All studies were approved by the Local Animal Ethical Committee at Göteborg University.

Macroscopic scoring of Gαi2$^{-/-}$ and DSS-induced colitis (Paper I-II)

In mice with DSS-induced colitis, 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 ulceration (0-1), resulting in a total score of 10.
The macroscopic scoring in Gαi2$^{-/-}$ mice used the same parameters but in some cases where an intact colon had to be used, due to subsequent isolation of lamina propria lymphocytes, the scoring was based on the evaluation of these parameters from the serosal side.

The pre-colitic Gαi2-/- mice showed no clinical symptoms and no visible inflammatory signs in the colon.

Specimens from IBD patients and uninflamed controls (Paper III)

Peripheral blood and colon or small intestinal specimens were obtained from IBD patients undergoing small intestinal resection or subtotal colectomy due to active disease that could not be solved with medical treatment and undergoing surgery with curative intent by formation of a stoma bag. These IBD patients had an active disease and the specimens from small intesine, colon and rectum were all scored as active inflammation. IBD patients with no active disease were patients undergoing proctectomy to replace a previously created colostomy bag with a pelvic pouch. These IBD specimens were only from rectum and were all scored as non-active inflammation.

The control group consisted of healthy volunteers for peripheral blood specimens and patients admitted for therapeutic bowel resection for adenocarcinomas for colonic specimens.

All studies were approved by the Local Human Ethical Committee at Göteborg University.

Histopathology (Paper I-II)

To evaluate the influence of colitis on thymic architecture in $G\alpha i2^{-/-}$ mice and in mice with DSS-induced colitis, thymi were dissected and fixed in 4% buffered formalin and three 5µm cross section per thymic lobe were prepared and stained with hematoxylin-eosin. The entire thymus was sectioned through, randomly sampling three sections evenly spread throughout the thymus.

In $G\alpha i2^{-/-}$ mice we compared colitic thymi to normal aging thymi over a time period. The cortex and medulla area from six, ten, 13, 18, and 20-21 weeks old mice were determined using Leica 1M 1000 Image Manager Software and the mean area of cortex and medulla were calculated from the tree lobe cross-sections.

To avoid artefacts due to differences in the level of sectioning of the tissue, we checked the results of the area by analysing the mean ratio of medulla:cortex. These results showed an overall larger ratio in six to 18 weeks old $G\alpha i2^{-/-}$ mice compared to age matched mice $G\alpha i2^{+/-}$ mice. We were therefore comfortable to present the results as changes in cortex area in $G\alpha i2^{-/-}$ mice compared to $G\alpha i2^{+/-}$ mice.

In C57Bl/6J mice with DSS-induced colitis cortex and medulla areas were determined at day 3, 5 (days of DSS administration), at day 5 + 7 (7days after DSS removal), and at day 5 + 21 using Picsara software. However, there was no distinct border between the cortex and the

medulla at the peak of disease (day 5 + 7), making it impossible to calculate the areas of the respective region. The results were therefore presented as representative pictures from the different time points.

Isolation of thymocytes (Paper I-II)

Thymocytes were isolated by forcing the whole thymus through a nylon net using a syringe plunger and were then washed in PBS, and put on ice.

Isolation of intraepithelial (IELs) and lamina propria lymphocytes (LPLs) (Paper III-IV)

Isolation of intraepithelial and lamina propria lymphocytes (IELs and LPLs, respectively) from human or mouse intestinal tissue were performed with principally similar methodology; using EDTA to break down the epithelial layer and thus release the IELs, followed by collagenase to break peptide bonds in collagen and thus break down the collagenous tissue of the lamina propria to release LPLs. The pre-handling of the two types of tissue was however done differently.

In intestinal resection specimens from IBD patients and colon cancer patients the mucosa was mechanically separated from the underlying fat and muscle tissue with scissors. The mucosal layer was then cut into small pieces and incubated 4 x 15 min at 37°C on a magnetic stirrer in medium containing AB-serum, EDTA and DL-Dithiothreitol, the latter to reduce the disulphide bonds. Supernatants from the three first incubations, containing intraepithelial lymphocytes, were poured over a nylon mesh, washed twice and kept on ice until further analysis. The remaining mucosal pieces were washed twice with Hank´s Balanced Salt Solution (HBSS) and then incubated at 37°C on a magnetic stirrer in medium containing AB-serum, Collagenase Type XI and DNAse 1, the latter to degrade DNA from disrupted cells whick would otherwise clog the released lymphocytes, for 1.5 to 2 hours. Cells released into the supernatant, containing lamina propia lymphocytes, were separated from mucosal pieces by 100μm pore size cell strainers and were then washed in PBS, and put on ice.

In mice, faecal contents were removed by flushing the colon with saline. The colon was then sealed at one end, turned inside out, filled with PBS and sealed at the other end to expand the crypts. The “colon tubes” were then washed extensively in Hanks balanced salt solution without calcium and magnesium (CMF-HBSS) supplemented with HEPES, followed by five 15 min incubations at 37°C on a magnetic stirrer in CMF-HBSS containing 10 % heat

inactivated horse serum and EDTA to remove epithelial cells and intraepithelial lymphocytes. The lymphocyte containing medium was collected and stored on ice, and the remaining tissue was incubated for 15 min in RPMI-1640 containing HEPES and heat inactivated fetal calf serum (FCS) to block any remaining EDTA activity, followed by three successive 60 min incubations at 37°C on a magnetic stirrer in Collagenase Type XI dissolved in RPMI-1640 containing HEPES and heat inactivated horse serum, yielding LPLs. The cells were washed twice with PBS and either placed on ice before chemotaxis analysis or flow cytometry staining, stored in lysis buffer (AL-buffer) for TRECs analysis or stored in RNAlater for analysis of extrathymic T cell maturation.

In addition, at the time of sacrifice, 0.3-0.5 cm of the small intestine, (divided into doudenum, ileum and jejenum) was snap frozen and later used for detection of analysis of extrathymic T cell maturation.

Flow cytometry/FACS analysis (Paper I-IV)

The Fluorescence Activated Cell Sorter (FACS) is a powerful instrument for characterization of cells within a mixed population by measuring cell size, granularity or irregularity and fluorescence from different fluorochrome coupled antibodies bound outside and/or inside the cells.

In short, a mixture of antibody stained cells is forced through a nozzle in a single-cell stream and as each cell passes through a laser beam it scatters the laser light, and any fluorochrome coupled antibodies bound to the cell will be excited and will fluoresce. Sensitive photomultiplier tubes detect both the scattered light, which gives information on size and granularity of the cell, and the fluorescence emissions, which give information on the binding of the labeled monoclonal antibodies and hence the expression of cell-surface or intracellular proteins by each cell.

FACS analysis have frequently been used throughout this thesis for analysis of surface expression of different cell specific markers on lymphocytes from the two mouse models of colitis (thymocytes, mesenteric lymph node lymphocytes, LPLs and peripheral blood lymphocytes) as well as IBD patients and their controls (lamina propria and peripheral blood lymphocytes), for analysis of the migrated cells in the chemotaxis assays (thymocytes and LPLs from mice) as well as analysis of proliferating cells with the intracellular proliferation marker Ki-67 (human peripheral blood lymphocytes and mouse peripheral blood and lamina propria lymphocytes). Most FACS analyses were performed by 4- or 5-color staining to separate specific cell types within a cell population. For control of these multi-stainings, two

or three different controls were used to facilitate correct gating during analysis of specific cell populations. First, unstained cells were consistently used to predict gating of cells negative for the staining. Second, one specific staining was excluded from the multi-staining to predict gating of the specific cells taking into account the influence of other fluorochromes used in the multi-staining. Third, isotype controls were used to estimate unspecific staining.

For analysis of proliferation within a cell population, cells were intracellularly stained with anti-Ki-67. The Ki-67 is a nuclear protein exclusively present in the G1, S, G2 and M phases of the cell cycle but absent in the G0 phase [199]. Thus, staining for Ki-67 provides information on the frequency of cells undergoing cell division at the time of isolation but excludes previously divided cells. To predict proliferation within a certain cell population, cells were first surface stained and then permeabilized and stained with anti-Ki-67.

The specific assays and the specific fluorochrome coupled antibodies used are described in each paper. All the analyses were carried out on a BD LSR II, using Flow-Jo software.

Chemotaxis assay (Paper I)

To assess the capacity of mouse thymocytes and LPLs to migrate in response to different chemokines compared to wild type controls, we used a chemotaxis assay. A previous study by Campbell et al [63] demonstrated that thymocytes at different maturation stages respond differently to different chemokines in wild type mice. Knowing the aberrant thymocyte subset composition in $G\alpha i2^{-/-}$ mice and DSS-induced colitic mice, we performed a series of chemotaxis assays to evaluate the ability of pre-colitic $G\alpha i2^{-/-}$ thymocytes to respond to the same chemokines that was used by Campbell et al. Due to the very limited number of thymocytes obtainable from the colitic mice, only very limited chemotaxis studies were possible on these mice. Preliminary studies were also performed on mice with DSS-induced colitis.

In short, we used twelve-well cell culture plates with polycarbonate tissue culture inserts containing a filter with 5 μm pore size, allowing transmigration of lymphocytes. A known amount of unseparated thymocytes in suspension, pre-incubated in a plastic dish for 2 × 30 min at 37°C to exclude adherent cells, was placed in the insert/upper well, while the lower well contained medium with or without the different chemokines. The whole plate with the inserts was then incubated for 90 min at 37°C in 5% CO_2 whereupon migrated cells were harvested from the lower well and counted in a microscope.

We first determined the optimal concentration of each chemokine that gave maximum migration of wild type thymocytes. The concentration of chemokines was calculated in molar,

which gives a more exact amount of each molecule needed to attract cells rather than using a concentration of μg/ml, which do not consider the size of the chemokine molecule. For CCL19, CCL21 and CXCL12 we were able to reach the concentration that gave maximal migration, while for CCL25 we had to use a concentration where the migration had reached a stable level and where higher concentrations only gave minor increases in the number of migrated cells. Different incubation times can be used, ranging from 1 to 4 h depending on the cell type and the amount of cells used in the chemotaxis assay. We incubated the thymocytes for 90 min, similar to previous chemotaxis studies on thymocytes [63, 200].

To calculate the migratory capacity of specific thymocyte subsets, we first stained unseparated thymocytes (not included in the chemotaxis assay) for flow cytometry with anti-TCRαβ, anti-CD8, anti-CD4, anti-CD69, and anti-CD62L, to know the distribution of the different subsets in the original population of a specific mouse. The migrated cells from the same mouse were then counted in a microscope and stained with the same antibodies and analyzed on a BD LSR II, using Flow-Jo software. The fraction of cells within a certain thymocyte subset that had responded to and migrated towards a specific chemokine was calculated using the following equation: **(no. migrated cells × % of subset) / (no. of cells added in the upper well × % of origin subset) × 100 = Migration (% of input)**

Auto Magnetic Activated Cell Sorting (AutoMACS) (Paper IV)

In order to analyze peripheral blood lymphocytes containing TRECs that were homing to the gut in IBD patients, the cells were presorted based on their surface expression of integrin beta$_7$ (β_7) protein.

The cells were first stained with anti-β_7–APC monoclonal antibodies and then incubated with anti-APC-conjugated beads. The separation was done on the positive selection program on an Auto-MACS, which binds the magnetic beads bound to the cells through a separation column which is placed in a strong permanent magnet where a high-gradient magnetic field is created. The cells that have not bound the anti-APC-conjugated magnetic beads will not be retained in the magnetic column but runs through the system and are collected in a tube while the cells bound to the magnetic column are subsequently collected in a different tube. The positively selected lymphocytes were 91±9% integrin β_7 positive, whereas the remaining lymphocyte population contained 36±12% integrin β_7^+ lymphocytes as judged by FACS analysis. The unseparated population contained 56±12% integrin β_7^+ lymphocytes.

Real-time Polymerase Chain Reaction, rt-PCR (Paper III-IV)

Real-time Polymerase Chain Reaction (rt-PCR) is a very useful technology for quantifying gene expression in a sample. Compared to conventional PCR, where all the amplification cycles are completed before the final products are detected, rt-PCR can detect the amount of product after each amplification cycle as it accumulates. The principle of detecting rt-PCR products is by measuring a fluorescence signal that becomes visible during cycling and correlates to the amount of PCR products in the reaction.

In this thesis, two different fluorescence-based rt-PCR analyses have been used: SYBR Green I is a sequence-independent detection assay where fluorophores binds to all double-stranded DNA (ds-DNA) molecules regardless of sequence. During annealing, PCR primers hybridize to the target DNA strand and form small regions of dsDNA where SYBR Green I intercalates. In the end of the elongation phase a maximum amount of SYBR Green I is intercalated and the fluorescence single is measured. The specific product is characterized by melting curve analysis, based on the fact that each particular dsDNA molecule has its characteristic melting temperature based on length and AT/GC content, at which 50% of the DNA is double-stranded and 50% is melted, i.e. single stranded.

The other fluorescence analysis was done with a Hydrolysis Probe assay, where fluorophores are coupled to sequence-specific oligonucleotide probes that hybridize to their complementary sequence in target PCR products. A hydrolysis probe carries two fluorescent dyes in close proximity, with a quencher dye suppressing a reporter fluorescence signal. In the annealing phase of the PCR reaction, primers and probes specifically anneal to the target sequence. As the DNA polymerase extends the primer, it encounters the probe. The polymerase then cleaves the probe with its inherent 5´-nuclease activity, displaces the probe fragment from the target, and continues to polymerize the new amplicon, i.e. new DNA products from the PCR. In the cleaved probe, the reporter dye is no longer quenched and therefore can emit fluorescent light. The increase in fluorescence from the reporter dye directly correlates to the accumulation of released reporter dye molecule and is measured at the end of the elongation phase.

The benefit of using Hydrolysis Probes compared to SYBR Green I is that both the primer and the probe have to anneal to the target sequence, which give an extra specificity of the target. As the fluorescence is detected after every completed cycle, the product fluorescence will rise above the background fluorence at a certain cycle number. This cycle number is termed the crossing point (CP). In both assays, the amplified product is visible as crossing

point (CP) after a certain number of cycles depending on the initial concentration of specific DNA or cDNA in the sample. To quantify the amplified products, a standard curve was prepared by serial dilutions of a calibrator, 1:4 or 1:10 in each step, for each primer pair. The calibrator contains high levels of the specific mRNA/DNA that was detected, and was also used as positive control in every reaction (see below). With the standard curves, the concentration of amplified product in each sample will be calculated based on the CP in each reaction. In this thesis, all the results are presented as relative quantification of each sample, i.e. the amount of amplified product in each sample is presented as percentage of the calibrator (set to 100%). This is because we were not interested in actual quantification of each sample, but rather to compare the levels in tissues or cells from healthy control mice or humans to Gαi2$^{-/-}$ mice or IBD patients.

All the analyses were carried out on a Roche LightCyler 480 (TRECs in mice and analysis of extrathymic maturation in mice and humans) or a Roche LightCyler 1.2 (TRECs in humans).

The level of T cell Receptor Excision Circels (TRECs) was analyzed in both IBD patients and in colitic Gαi2$^{-/-}$ mice as a measurement of thymic output. Purified genomic DNA from peripheral blood lymphocytes and isolated intraepithelial and/or lamina propria lymphocytes from IBD patients and colitic Gαi2$^{-/-}$ mice, as well as lymphocytes from mesenteric lymph nodes (MLNs) from colitic Gαi2$^{-/-}$ mice, was analyzed for TRECs content by rt-PCR using SYBR Green I as fluorescence reporter. TRECs primer sequences were previously published (human [201], mouse [202]).

To quantify TRECs in samples from IBD patients, cord blood DNA, known to contain high levels of TRECs, was used as a calibrator. As analysis of TRECs content is detection of genomic DNA, which is expressed in all cells, the housekeeping gene could not be specific for T cells. The primers for the housekeeping gene were therefore designed to amplify the GAPDH (glyceraldehyde phosphate dehydrogenase) gene in humans. The level of TRECs in each sample was calculated by dividing the ratio of TRECs:GAPDH of the sample by the ratio of TRECs:GAPDH of the calibrator and the relative amount of TRECs was expressed as a normalized ratio of this.

Detection of TRECs in colitic Gαi2$^{-/-}$ mice was performed using the same procedure and calculation, but DNA from mouse thymocyte was used as the calibrator and the CD3γexon4 gene was used as the housekeeping gene. The primer for the CD3γexon4 gene was designed by using the Universal Probe Library Assay Design center.

Extrathymic maturation (Paper III-IV)

To evaluate the possibility of extrathymic maturation of T cells in peripheral tissues as a result of colitis and possibly thymic atrophy in Gαi2$^{-/-}$ mice as well as IBD patients, two approaches were chosen - flow cytometric analysis of surface markers of early T cell lineage lymphocytes (III) and real time PCR for mRNA expression of pTα and RAG1, two proteins expressed in developing T cells (III-IV).

The flow cytometry staining in IBD patients and control colon cancer patients was performed in accordance with a study performed by Williams et.al. University of Bristol, UK, [142]. Lamina propria lymphocytes were stained with antibodies directed against the cell surface markers CD2, 3, 5, 7, 16 and 19. Those cells expressing CD16 (an NK-cell marker) and CD19 (a B lymphocyte marker) was excluded by gating whereas those cells that were staining positive for CD2, CD5 and CD7 but were negative for CD3 were considered to be early linage T cells. As both CD16 and CD19 postive cells were excluded by gating, the same fluorochrome was used for these markers. In order to estimate the correct gating during the FACS analsysis several isotype controls were used, and besides this, one staining was done with all mouse-anti-human-antibodies described above but without CD3, to predict the exact gating for CD3 negative cells, a control that is especially valid when staining is performed with many different fluorochromes at the same time.

During thymocyte development in the thymus, the most immature thymocytes express a pre-TCR complex consisting of a β-chain and a pTα-chain before the final α-chain are rearranged and replace the pTα-chain. Recombination-activating gene 1 and 2 (Rag-1 and Rag-2) are two proteins that participate in the rearrangement of the V(D)J gene recombination by recognizing the specific DNA sequences, forming synapses and cleaving the DNA. Analysis of mRNA expression of pre-TcRα and RAG1 or RAG2 can therefore be used to detect developing T cells in the intestinal mucosa as extrathymic T cells.

mRNA from both IBD patients and colitic Gαi2$^{-/-}$ mice was analysed for detection of preTCRα, RAG1 and the housekeeping gene CD3γ, specific for T cells, by real-time PCR. Both the mouse and human primers were designed by the Universal Probe Library Assay Design center. Purified mRNA, isolated from intestinal mucosal lymphocytes from IBD patients and their controls, as well as tissue from the small intesine and isolated lamina propria lymphocytes from colitic Gαi2$^{-/-}$ mice and controls, was reverse-transcribed to cDNA. The rt-PCR was performed using the Hydrolysis Probe assay using probes from Universal Probe Library (Roche Applied Science, Mannheim, Germany). To quantify the relative

amount of mRNA expression of RAG1 and preTCRα, purified RNA isolated from human thymus, kindly provided by Dr S. Oskarsdottir, Dept of Pediatrics, Sahlgrenska University Hospital, Göteborg, or from mouse thymocytes, respectively, was used as a calibrator. The analysis of the level of preTCRα and RAG1 was calculated in the same way as described for detection of TRECs, i.e. as a normalized ratio of the relative amount in relation to the housekeeping gene CD3γ in each sample.

Statistical analysis

For statistical analysis we used the Mann-Whitney non-parametric test, a suitable test when comparing two groups with low numbers of samples ($n<20$) and which does not require normal distribution. For correlation analysis between two variables; TRECs and age, Pearson's correlation test was used. Values of $P \leq 0.05$ were considered to be significant.

RESULTS AND COMMENTS

In this section I give a brief overview of the results in this thesis and how the results were interpreted. As paper III and IV are in the forms of manuscripts and this text will be published on the Internet the results will not be shown in detail in this section. They will, however, be generally discussed and I will refer to the figures within the manuscripts.

Intrathymic changes in Gαi2$^{-/-}$ mice with colitis and mice with DSS-induced colitis (Paper I and II)

During extensive colitis in Gαi2$^{-/-}$ mice, it was observed that the thymus was very small or sometimes even invisible to the naked eye. A reduced thymus was also seen before any clinical symptoms of colitis were evident, hereafter referred to as pre-colitic Gαi2$^{-/-}$ mice. As described in the Introduction, it is well known that T lymphocytes have a large impact on development of colitis, both in mice and humans [150-152, 154-158]. We therefore characterized the intrathymic changes during thymocyte maturation in Gαi2$^{-/-}$ mice. To distinguish alterations caused by the Gαi2 deletion itself from colitis-associated intrathymic changes, we performed the same analysis on mice with DSS-induced colitis.

Altered thymocyte subset composition and thymic architecture in colitic mice (Paper I and II)

Colitis in Gαi2$^{-/-}$ mice was associated with a significant reduction in thymus weight and thymocyte numbers, which was evident already in pre-colitic mice and continued in colitic mice, where these observations was much more pronounced. A similar reduction in the total number of thymocytes was also seen during the acute phase; seven days post DSS treatment (day 5+7) in DSS-induced colitic C57Bl/6J mice. However, the thymocytes in these mice were replenished in the chronic phase (day 5+21 and day 5+35). This was not seen in Gαi2$^{-/-}$ mice, in which the disease is progressive.

To further explore the consequence of colitis-associated thymic atrophy on thymocyte development, we carefully characterized the different thymic subsets. Thymocytes was analyzed according to a previously defined classification of maturation stages based on phenotypic characteristics, from the most immature double negative (DN) CD4^{-}CD8^{-}, divided into DN1-DN4 based on the expression of CD44 and CD25, to immature double positive (DP) CD4^{+}CD8^{+} (stage 1-3, based on the expression of TCRαβ and CD69) and finally to mature single positive (SP) CD4^{-}CD8^{+} or CD4^{+}CD8^{-} (stage 4-5, based on the expression of

CD69 and CD62L) (Table 1). In the original classification $CD4^+CD8^+TCR\alpha\beta^-CD69^-$ and $CD4^+CD8^+TCR\alpha\beta^{low}CD69^-$ thymocytes were distinguished into maturation stage 1 and 2, respectively, but as there were no clear distinctions between $TCR\alpha\beta^-$ and $TCR\alpha\beta^{low}$ in our analyses, we decided to merge these two maturations stages into stage 1-2.

Table 1. *Different phenotypes of thymocytes during intrathymic maturation*

THYMIC SUBSET	LOCATION	PHENOTYPE	MATURATION STAGE CLASSIFICATION USED IN PAPER I-II
DN1	Cortex	$CD4^-CD8^-CD44^+CD25^-$	N/A
DN2	Cortex	$CD4^-CD8^-CD44^+CD25^+$	N/A
DN3	Subcapsule (cortex)	$CD4^-CD8^-CD44^-CD25^+$	N/A
DN4	Subcapsule (cortex)	$CD4^-CD\ 8^-CD44^-CD25^-$	N/A
DP	Cortex	$CD4^+CD8^+TCR\alpha\beta^{-/low}CD69^-$	1-2
DP	Transitional	$CD4^+CD8^+TCR\alpha\beta^+CD69^+$	3
SP	Medulla	$CD4^+CD8^-TCR\alpha\beta^+CD69^+$ Or $CD4^-CD8^+TCR\alpha\beta^+CD69^+$	4
SP	Medulla	$CD4^+CD8^-TCR\alpha\beta^+CD69^-CD62L^+$ or $CD4^-CD8^+TCR\alpha\beta^+CD69^-CD62L^+$	5

In pre-colitic and colitic $G\alpha i2^{-/-}$ mice, the cortical and transitional (between cortex and medulla) DP population of thymocytes, stage 1-2 and 3, was shown to be significantly reduced in both frequency and total numbers compared to healthy $G\alpha i2^{+/-}$ mice (Fig 4a-b). Likewise, the total number of medullary SP stage 4 thymocytes was also significantly reduced in pre-colitic and colitic $G\alpha i2^{-/-}$ mice, albeit not the frequencies. In strong contrast, mature, medullary SP stage 5 thymocytes were significantly increased in frequency in pre-colitic and colitic $G\alpha i2^{-/-}$ mice compared to $G\alpha i2^{+/-}$ mice. The total number of thymocytes within this stage was however only increased in pre-colitic $G\alpha i2^{-/-}$ mice, while colitic $G\alpha i2^{-/-}$ mice showed no difference in total numbers compared to $G\alpha i2^{+/-}$ mice due to the radically reduced total number of thymocytes in the latter (Fig 4 a-b).

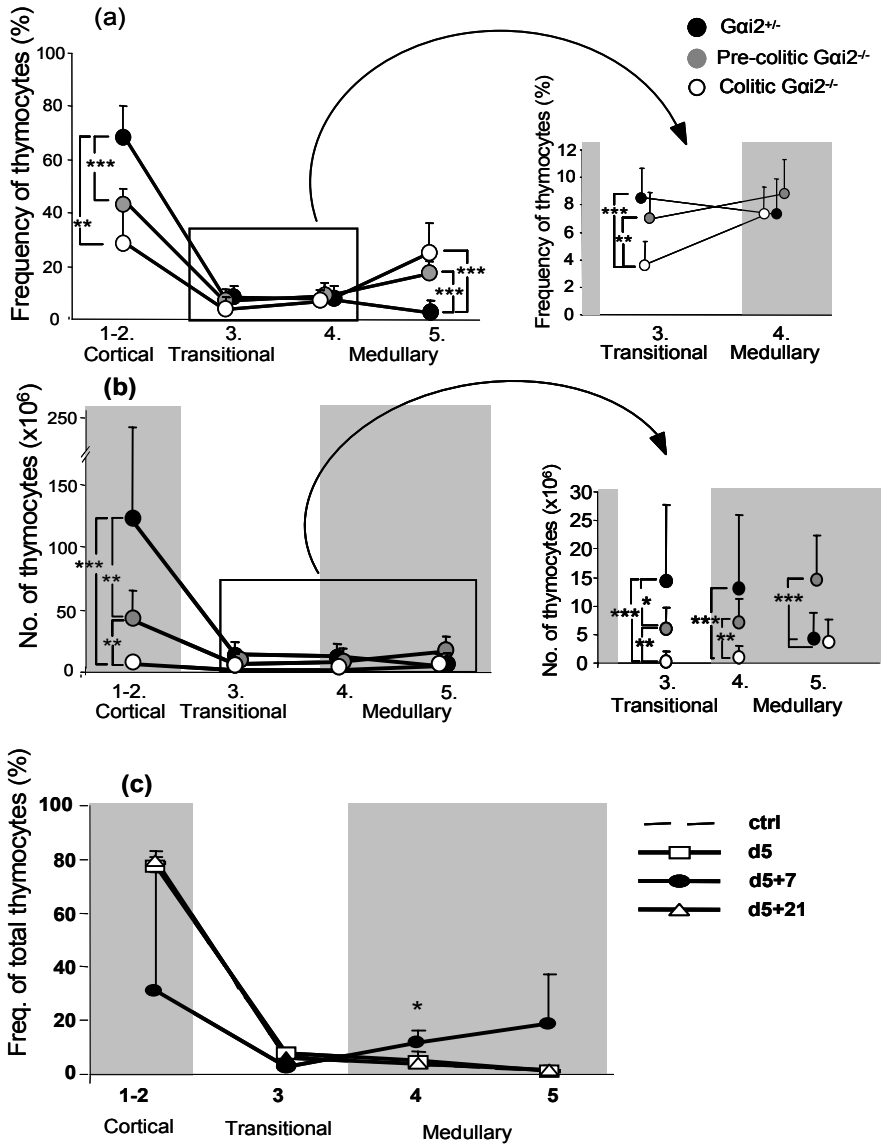

Figure 4. Frequency (a,c) and total number (b) of immature thymocytes in precolitic and colitic Gαi2$^{-/-}$ mice compared to Gαi2$^{+/-}$ mice (a-b) as well as during DSS administration (d5), in the acute phase (d5+7) and during the chronic phase (d5+21) in C57Bl/6J mice (c). Developmental stages of thymocytes are shown as follows; 1-2, CD4^{+} CD8^{+} TCRαβ $^{low/-}$ CD69^{-} (cortical); 3, CD4^{+} CD8^{+} TCRαβ^{+} CD69^{+} (transitional between medulla and cortex); 4, CD4^{+} CD8^{-} TCRαβ^{+} CD69^{+} CD62L $^{low/-}$ (medullary); and 5, CD4^{+} CD8^{-} TCRαβ^{+} CD69^{-} CD62L^{high} (medullary) as determind by FACS analysis. Results are shown as mean values of; Gαi2$^{+/-}$ (n = 14), precolitic Gαi2$^{-/-}$ (n = 10), colitic Gαi2$^{-/-}$ (n = 6) and DSS-treated (n= 5-6) mice ± SD. * = p ≤ 0.05

When comparing the results from Gαi2$^{-/-}$ mice to thymocyte maturation stages in DSS-induced colitic C57Bl/6J mice, a similar trend was found during the acute phase (day 5 + 7): The frequency of cortical DP stage 1-2 thymocytes was reduced compared to control mice (Fig 4c), and the frequency of SP thymocytes in the medulla, developmental stage 4 and 5 (Fig.4c) was increased. However, this altered thymocyte subset distribution was restricted to the acute phase, as the subpopulation frequencies were normal during the DSS administration (day 5) as well as in the chronic phase at day 5 + 21.

These results indicate that colitis has a major effect on thymic subsets, which concerns both the cortex and the medulla. However, the distribution of total thymocyte numbers indicated that the main drama was taking place in the cortex, as this compartment lost most of its thymocytes.

To investigate if this was true, thymus was analyzed histologically and the changes in cortex and medulla area were evaluated over time. To enable such a study, we used Gαi2$^{-/-}$ mice on a 129SvEv x C57BL/6 background (see Methodological Considerations) since these mice had a prolonged phase of colitis development, which gave us the opportunity to compare the results in Gαi2$^{-/-}$ mice to normal thymic involution induced by age in Gαi2$^{+/-}$ mice. The age span in Gαi2$^{-/-}$ mice represents mild colitis in 6-10-week-old mice, mild to moderate colitis in 13-to18-week-old mice and moderate to severe colitis in 21-week-old mice.

The results showed a reduction in medullary area over time in Gαi2$^{-/-}$ mice that was comparable to Gαi2$^{+/-}$ mice. In contrast, the cortex area was significantly reduced from 13- to 21-week old in Gαi2$^{-/-}$ mice as compared to age matched Gαi2$^{+/-}$ mice (Fig.5).

In contrast, the thymic cortex/medulla area in DSS-treated C57Bl/6J mice appeared to be normal during the DSS administration and during the chronic phase (day 5 + 21). Changes in thymic architecture were however seen during the acute phase (day 5 + 7), when the normally solidly packed thymocytes in the cortex appeared scattered throughout the thymus and no distinct border between the cortex and the medulla was evident.

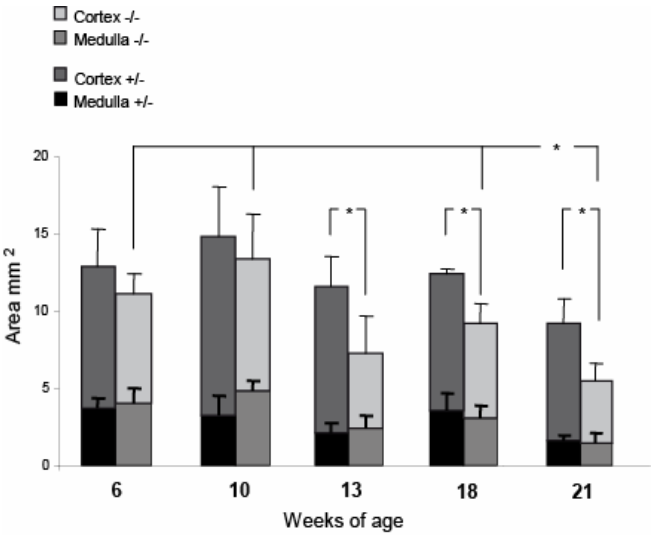

Figure 5. Changes in thymus medulla and cortex area with age. Thymi from 6-, 10-, 13-, 18- and 21-week-old Gαi2$^{-/-}$ mice (light bars) and Gαi2$^{+/-}$ mice (dark bars) mice were fixed in 4% buffered formalin and the medullary and cortical area of two to three 5-µm H&E-stained cross-sections per thymic lobe was calculated. Bars represent mean area (mm^2) of medulla or cortex in one lobe ± SD of three to five mice per group. * = $p \leq 0.05$

To summarize these results, the thymic atrophy in colitic Gαi2$^{-/-}$ and DSS-induced mice during the acute phase has a major effect on thymic subsets that mainly concern the thymocytes in the cortex.

During onset of an inflammation or infection, high levels of pro-inflammatory cytokines are produced, such as IL-1, IL-6 and TNF-α. These cytokines stimulates and activates the hypothalamus-pituitary-adrenal (HPA) axis, leading to increased levels of circulating

glucocorticoids [203, 204]. Immature thymocytes are especially sensitive to glucocorticoids and by binding to specific receptors on thymocytes it will induce apoptosis. High levels of IL-1β are expressed in both colitic Gαi2$^{-/-}$ mice and DSS-induced colitis in C57Bl/6J mice [181, 196, 205], and increased IL-1β levels are seen already before onset of colitis in Gαi2$^{-/-}$ mice [181]. However, other studies have demonstrated that thymic atrophy is independent of glucocorticoids during acute graft-versus-host disease [206] and in mice given concanavalin A [207].

Besides the impact glucocorticoid might have on reducing cortical thymocytes, other mechanisms may also contribute to the colitis-associated thymic involution. For example, in vitro experiments demonstrated that bone marrow cells from mice stressed by immobilization as well from unstressed mice both displayed a reduced migratory capacity towards thymocyte culture supernatants from stressed mice [208, 209]. This indicates that e.g. chemokines or other homing factors from the thymus that normally trigger precursor T cells from the bone marrow to enter the thymus are degraded.

Increased frequency of DN1 thymocytes correlates to severity of colitis in DSS treated Balb/cJ mice during the acute phase of colitis. (Paper II)

Besides analysis of the DP thymocyte subsets in the cortex and the SP populations in the medulla, additional analyses were performed on the most immature, double negative (DN), thymocytes (Table 1) in Balb/cJ mice during DSS administration. As Balb/cJ mice are more resistant to DSS, they only develop an acute colitis during DSS administration that is not followed by a chronic phase, and demonstrate only negligible changes in thymus size and unaltered frequencies and total numbers of the DP and SP thymocyte subpopulations compared to control mice.

Mice exposed to DSS for 5 or 6 days developed colitis with a macroscopic scoring ranging from 1.5 to 4.5. They were investigated to see whether the severity of colitis influenced the composition of the DN thymocyte subset. The majority of thymocytes within this population were either of the DN1 or the DN4 subset and the frequency of these subsets were correlated to the macroscopic scoring of colitis. Interestingly, an increased frequency of DN1 thymocytes correlated to increased colitis scoring and an increased frequency of DN4 thymocytes correlated to a decrease in colitis scoring (Fig.6a-c).

In addition, there was a significant correlation between the total number of DN1 thymocytes and increased macroscopic scoring (Fig.6d). Such a correlation was however not seen among the DN4 thymocytes (Fig.6e).

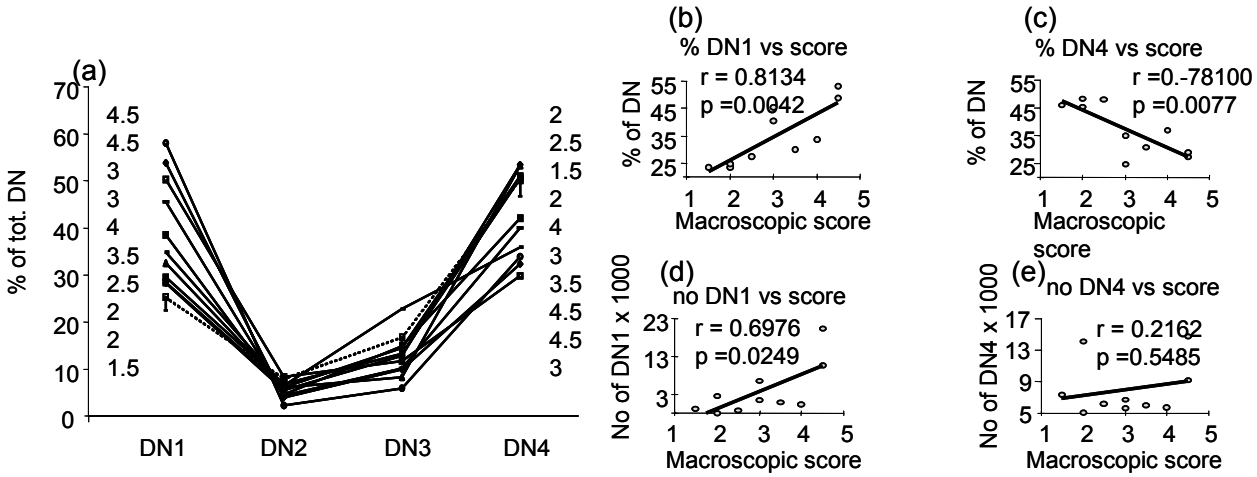

Figure 6. The fraction of the DN1 thymocytes is increased whereas the fraction of DN4 thymocytes is decreased with severity of colitis in DSS-treated Balb/cJ mice. The DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) fractions are shown for individual mice with colitis, whereas the control group (n = 6) is shown as a dashed line ± SD (a). The macroscopic colitis scoring for individual mice are depicted. The correlation between colitis score and the frequencies (b-c) as well as total numbers (d-e) of DN1 and DN4 subsets are shown. * = $p \leq 0.05$, ** = $p \leq 0.01$.

The increased frequency and total number of DN1 thymocytes suggests that increased numbers of progenitor cells from the bone marrow are entering the thymus in the acute phase of the colitis.

Development of colitis is associated with impaired chemotactic migration of thymocytes (Paper I and II)

To further investigate the aberrant thymic subsets seen in both $G\alpha i2^{-/-}$ and DSS induced colitic mice, a functional study was performed to test the responsiveness of $G\alpha i2^{-/-}$ thymocyte to chemokines. Chemokines signal through Gi-linked receptors and are known to be important in thymocyte maturation. CXCL12 (SDF-1α) and CCL25 (TECK) have previously been demonstrated to attract DP thymocytes in the cortex and transitional and early SP thymocytes in the medulla while CCL21 (SLC) and CCL19 (MIP-3β) attract only SP thymocytes in the medulla [63]. Based on the five phenotypically defined maturation stages described above (Table 1) we examined the ability of pre-colitic $G\alpha i2^{-/-}$ thymocytes to respond to these four chemokines.

Cortical, transitional and early medullary thymocytes from pre-colitic $G\alpha i2^{-/-}$ mice had a significantly lower ability to respond to CXCL12, especially during the transitional stage, compared to thymocytes from $G\alpha i2^{+/-}$ mice (Fig 7a). $G\alpha i2^{-/-}$ thymocytes also showed reduced responsiveness to CCL25 during the transitional stage (Fig 7c). In contrast, pre-colitic $G\alpha i2^{-/-}$ thymocytes had a migratory response to CCL21 and CCL19 that was similar to thymocytes

form Gαi2$^{+/-}$ mice (Fig 7b and d). The spontaneous migration (i.e. in the absence of chemokines) was equal for pre-colitic Gαi2$^{-/-}$ thymocytes and control Gαi2$^{+/-}$ thymocytes. (Fig 7e). Importantly, the reduced responsiveness of Gαi2$^{-/-}$ thymocytes could not be restored by increased concentrations of chemokines (data not shown)

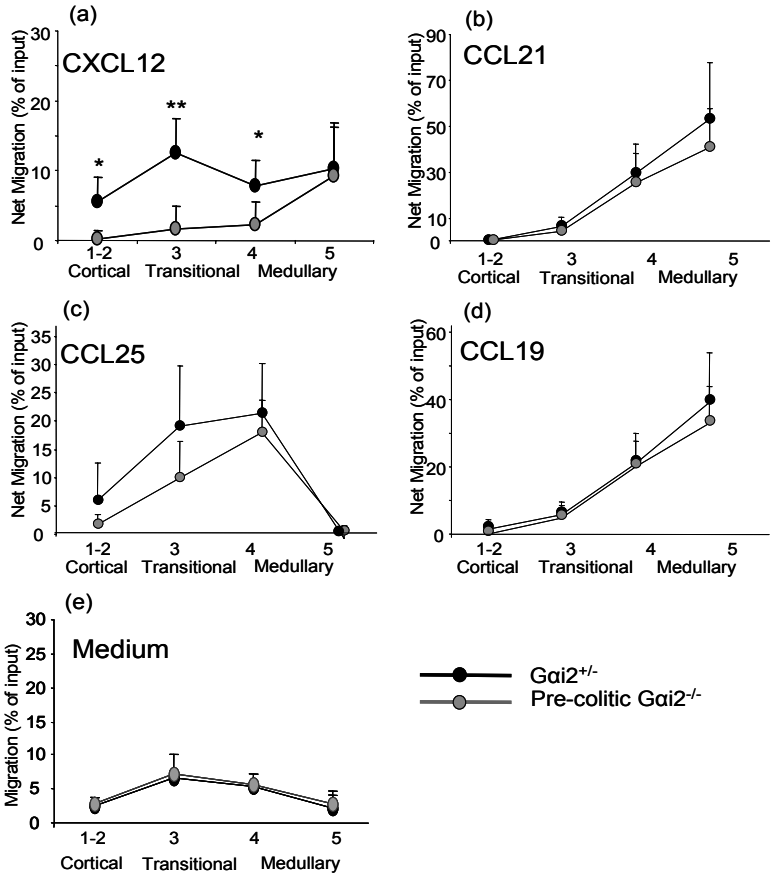

Figure 7. Migration of thymocytes from Gαi2$^{+/-}$ (n = 6) and precolitic Gαi2$^{-/-}$ (n = 6) mice in response to chemokines. Values are shown as percent migration of different thymocyte sub-populations in response to (a) CXCL12 (50nM), (b) CCL21 (100nM), (c) CCL25 (200nM) and (d) CCL19 (10nM). Spontaneous migration in the absence of chemokine (e) was similar in thymocytes from Gαi2$^{-/-}$ and Gαi2$^{+/-}$ thymocytes. Developmental stages shown are; 1-2, CD4^{+} CD8^{+} TCRαβ$^{low/-}$ CD69^{-} (cortical); 3, CD4^{+} CD8^{+}TCRαβ^{+} CD69^{+} (transitional between medulla and cortex); 4, CD4^{+} CD8^{-}TCRαβ^{+} CD69^{+} CD62L$^{low/-}$ (early medullary); and 5, CD4^{+} CD8^{-} TCRαβ^{+}CD69^{-} CD62L^{high} (late medullary) and were determined by FACS analysis. Results are demonstrated as mean value (% of input) of fluorescence-positive cells ± SD from six independent experiments. Results in a - d represent total migration minus background migration. * = p ≤ 0.05; ** = p ≤ 0.01 between migrated Gαi2$^{-/-}$ and Gαi2$^{+/-}$ thymocytes.

The results from pre-colitic Gαi2$^{-/-}$ thymocytes were compared to C57Bl/6J mice with DSS-induced colitis at day 3, 5, 5 + 7, 5 + 21 and 5 + 35.

Interestingly, our preliminary data demonstrate decreased responsiveness to CXCL12 and CCL25 in the cortical, transitional and early medullary thymocytes from DSS-treated mice compared to control thymocytes during the acute phase (day 5 + 7), but normal migration in response to CCL21 and CCL19 (Fig 8), similar to precolitic Gαi2$^{-/-}$ thymocytes. The reduced chemotactic responsiveness was however not seen at any other time point (day 3, 5, 5 + 21 and 5 + 35).

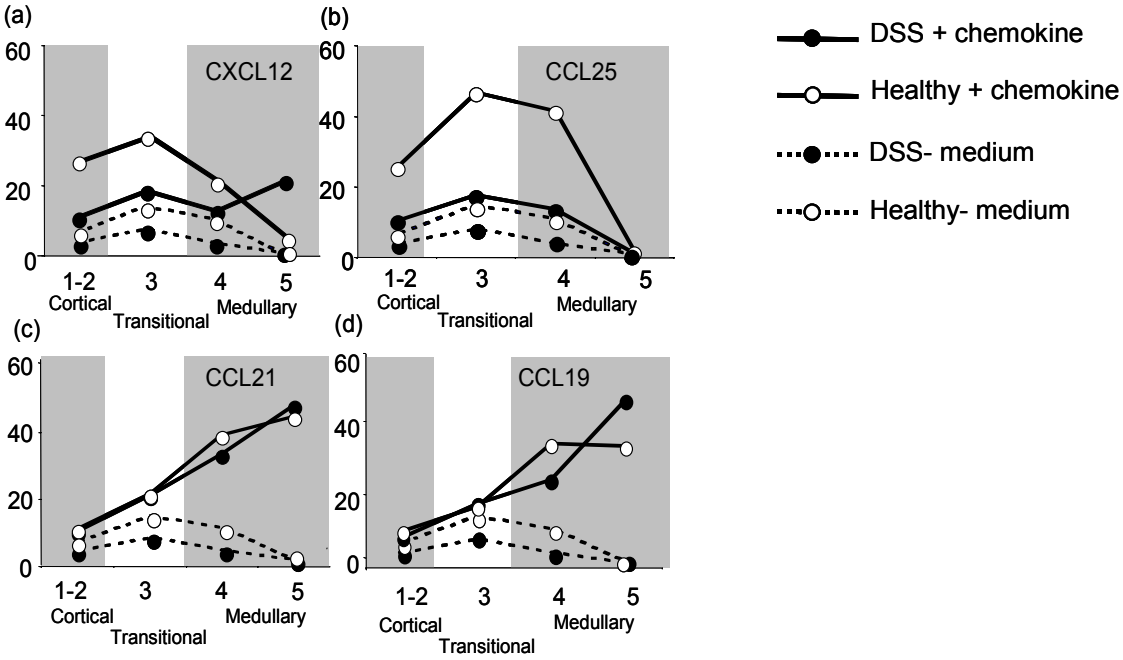

Figure 8. Migration of thymocytes from DSS-treated C57Bl/6J (n = 1; 3 mice pooled) during the acute phase (day 5 + 7) in response to chemokines during their maturation process. Values are shown as percent migration of different thymocyte subpopulations in response to (a) CXCL12 (50nM), (b) CCL21 (100nM), (c) CCL25 (200nM) and (d) CCL19 (10nM). Spontaneous thymocyte migration is shown as dashed lines. Developmental stages shown are as follows; 1-2, $CD4^{+}CD8^{+}TCR\alpha\beta^{low/-}CD69^{-}$ (cortical); 3, $CD4^{+}CD8^{+}TCR\alpha\beta^{+}CD69^{+}$ (transitional between medulla and cortex); 4, $CD4^{+}CD8^{-}TCR\alpha\beta^{+}CD69^{+}CD62L^{low/-}$ (early medullary); and 5, $CD4^{+}CD8^{-}TCR\alpha\beta^{+}CD69^{-}CD62L^{high}$ (late medullary) and were determined by FACS analysis. Results are demonstrated as mean value (% of input) of fluorescence-positive cells.

Even though these data are preliminary, it shows that the impaired responsiveness to CXCL12 and CCL25 seen in $G\alpha i2^{-/-}$ thymocytes is a result of the development of colitis and not the lack of an intact Gαi2-protein.

However, cortical thymocyte migration is not only regulated by chemokines. Thus, CXCL12 induced migration of DP thymocytes in a transwell system was enhanced if the bottom well was coated with laminin and fibronectin, two extracellular matrix proteins, either together or separately. [210, 211]. The same experiment performed on SP thymocytes gave only a minor increase in the migratory response, indicating that cortical DP thymocytes are dependent on laminin and/or fibronectin as well as CXCL12 for successful migration within the cortex and possibly from cortex to medulla. It cannot be ruled out that the reduced thymocyte migration to CXCL12 in $G\alpha i2^{-/-}$ and DSS-induced colitic mice also reflects an increased dependency on laminin and fibronectin during colitis, and that this reliance is not required to the same extent by normal thymocytes.

Gαi2$^{-/-}$ thymocytes have reduced expression of CCR9 but not CXCR4. (Paper I)

To further analyze the mechanism(s) underlying the impaired thymocyte responsiveness to CXCL12 in cortical, transitional and early medullary thymocytes in Gαi2$^{-/-}$ mice, we analyzed the expression of CXCR4, the only known receptor for CXCL12, on the thymocytes. Thymocytes in maturation stages 1-2, 3 and 4 (both CD4^{+} and CD8^{+} SP) from pre-colitic and colitic mice demonstrated a normal expression of CXCR4, both in frequency and intensity, compared to thymocytes from Gαi2$^{+/-}$ mice (Fig. 9). We also analyzed the thymocytes for their expression of CCR9, the receptor for CCL25. In contrast to CXCR4, the frequency of thymocytes expressing CCR9 was significantly reduced in Gαi2$^{-/-}$ mice with colitis compared to Gαi2$^{+/-}$ mice, irrespective of maturation stage, despite the more marginal reduction in migratory response to CCL25. The same was also true for pre-colitic Gαi2$^{-/-}$ mice, except for immature CD4^{+}CD8^{+}CD69^{-} (maturation stage 1-2) thymocytes, which demonstrated no significant difference from Gαi2$^{+/-}$ thymocytes (Fig 9). Likewise, the intensity of CCR9 expression was significantly reduced on thymocytes from both colitic and pre-colitic Gαi2$^{-/-}$ mice compared to Gαi2$^{+/-}$ mice, except on the more mature CD8^{+} SP thymocytes (Fig.9).

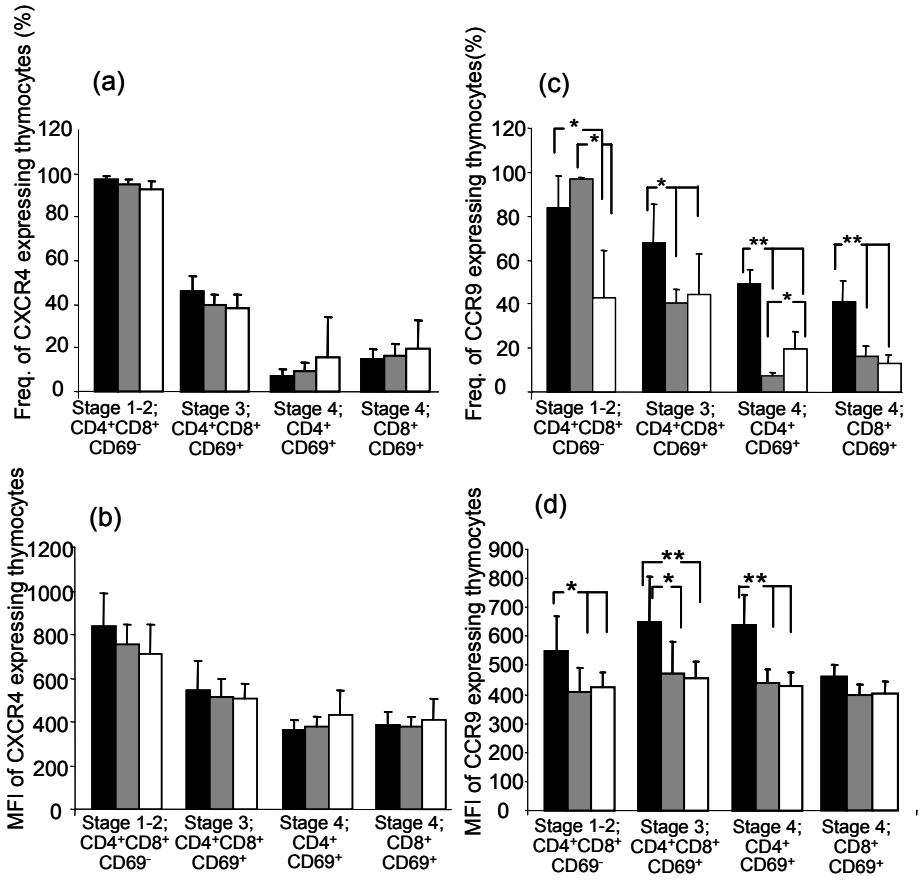

Figure 9. Frequencies (a,c) and median fluorescence intensitiy (MFI) (b,d) of CCR9 (a,b) and CXCR4 (c,d) expression on thymocytes in maturation stages 1-4. Cells were stained for four-colour flow cytometry as follows: anti-CD4-APC, anti-CD8-PerCP, anti-CD69-PE and anti-CXCR4-FITC or anti-CCR9-FITC. Gαi2$^{+/-}$ mice were used as controls (black bars)(CCR9, n = 11; CXCR4, n = 17); precolitic Gαi2$^{-/-}$ mice (grey bars)(CCR9, n = 4; CXCR4, n = 9) and colitic Gαi2$^{-/-}$ mice (white bars)(CCR9, n = 4; CXCR4, n = 6). Bars represent mean value ± SD where * = p ≤ 0.05; ** = p ≤ 0.01.

Reduced expression of CCR9 and affected migratory responses to CCL25 but unaltered expression of CXCR4 but decreased responsiveness to CXCL12 demonstrates the complexity of chemokine receptor expression and signalling. Previous studies have shown that the level of receptor expression does not necessarily correlate with the migratory potential [212] [213] [214]. Holland *et al* [213] demonstrated uniform surface expression of CXCR4 among several breast cancer cell lines, although only the receptors expressed on the invasive cells were functional. It is also possible that chemokine receptors have to form dimers to express a functional receptor [215], and induce an activation signal. A recent study by Sohy et al described heterodimers of CXCR4/CCR2 that were able to induce an active signal in response to both CXCL12 and CCL2 [216]

Aberrant migration of Gαi2$^{-/-}$ colonic lamina propria lymphocytes in response to CXCL12 (Paper I)

The chemokines CCL25 and CXCL12 are also expressed in the intestine and knowing the impaired chemotactic response in the thymus, we next analysed the chemotactic migratory

ability of colonic lamina propria lymphocytes from pre-colitic Gαi2$^{-/-}$ mice. CCR9 is predominantly expressed on lymphocytes from the small intestine while only a small subset of lymphocytes express CCR9 in the colon [217], whereas CXCR4 is found in both the small and large intestine [133]. The chemotactic analysis demonstrated that pre-colitic CD4^{+} T lymphocytes from the colonic lamina propria had a significantly reduced responsiveness to CXCL12 compared to control mice (Fig 10). Interestingly, the reduced responsiveness to CXCL12 was restricted to CD4^{+} T cells, as the migration of B220^{+} B lymphocytes was not significantly different between Gαi2$^{-/-}$ and control mice. In agreement with the previously described absence of CCL25 in the large intestine, neither Gαi2$^{-/-}$ nor control colonic LPLs were responsive to CCL25 (Fig.10).

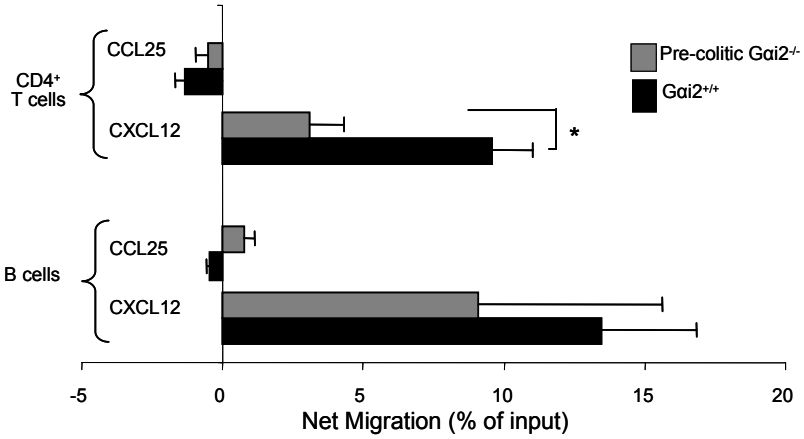

Figure 10. Migration of colonic lamina propria lymphocytes from Gαi2$^{+/+}$ (n = 3, two or three mice pooled in three different experiments) and precolitic Gαi2$^{-/-}$ (n = 3) mice in response to CCL25 and CXCL12. Values are shown as differences between total chemokine-induced migration minus spontaneous migration of CD4^{+} T lymphocytes and B lymphocytes. Cells were counted and stained for two-colour flow cytometry with anti-CD4-APC and anti-CD45R/B220-PE both before and after migration in response to the indicated chemokine. Results are demonstrated as ratio ± SD from three independent experiments. * = $p \leq 0.05$ between Gαi2$^{-/-}$ and Gαi2$^{+/+}$ colonic lamina propria lymphocytes.

Recent thymic emigrants in peripheral blood and mucosa-associated tissues from IBD patients and Gαi2-deficient mice with colitis (Paper III and IV)

The previously described aberrant thymocyte maturation and migration, with alterations in thymocyte subset composition in pre-colitic and colitic Gαi2$^{-/-}$ mice and during the acute phase of DSS-induced colitis, made us speculate that the low numbers of thymocytes is a result of a massive egression of thymocytes into the periphery, and in that case, that these so-called recent thymic emigrants (RTE) were to be found in peripheral lymphoid tissues, preferentially the colon. Knowing that colitis is associated with thymic atrophy in the two

mouse models we also speculated that the same phenomenon would be seen in patients with IBD. However, as we were unable to obtain thymi from IBD patients, we analyzed the peripheral blood and intestinal mucosa for RTEs.

To this end, we analysed the production and output of RTEs by measuring the relative amount of TCR rearrangement excision circles (TRECs) in peripheral blood and in colonic lamina propria lymphocytes in both colitic Gαi2$^{-/-}$ mice and in IBD patients, as well as in mesenteric lymph node (MLN) lymphocytes from colitic Gαi2$^{-/-}$ mice. Since TRECs are stable within the cell and do not replicate during mitosis it is instead diluted out in each cell division. The accurate TRECs level can therefore be hidden by an increased proliferation. Analysis of the relative amount of TRECs was therefore accompanied by measurements of the frequency of T lymphocytes expressing Ki-67, an intracellular protein expressed in all diving cells but absent in resting cells.

Analysis of TRECs levels and frequencies of naïve T cells in peripheral blood and the intestinal mucosa from IBD patients (Paper III)

Peripheral blood taken from IBD patients, both CD and UC, were analyzed for the relative amount of TRECs and compared to healthy controls. Besides analysis of TRECs in total peripheral blood, the lymphocytes were also divided based on their expression of the mucosal homing receptor integrin β_7 into integrin β_7 enriched and -depleted cells, in order to investigate whether RTEs found in peripheral blood were en route to the gut.

Both unseparated, integrin β_7^+ enriched and integrin β_7^+ depleted peripheral blood T lymphocytes from IBD patients had lower levels of TRECs compared to healthy controls (Fig 1, paper III). There were, however, no significant differences in TRECs levels between the unseparated, integrin β_7^+ lymphocyte enriched and integrin β_7^+ lymphocyte depleted populations.

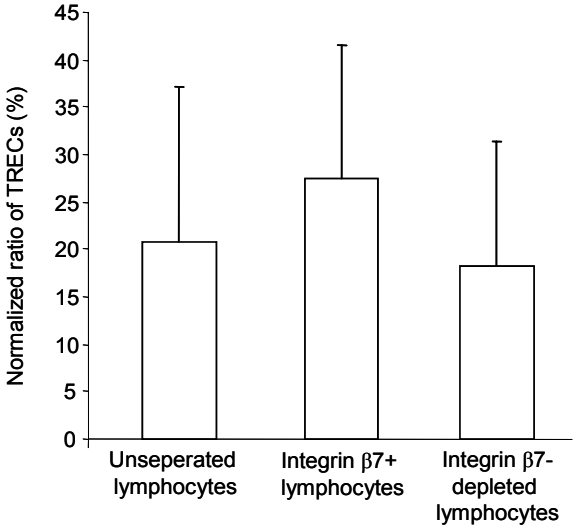

Figure 11. TRECs level in peripheral blood from healthy individuals. Relative amount of TRECs in unseparated, positively selected integrin β_7^+ and integrin β_7 depleted PBMCs from healthy controls (n = 6-7). Results are expressed as normalized ratios between relative amounts of TRECs and the reference gene GAPDH as % of positive standard (cord blood = 100%).

The reduced TRECs levels in peripheral blood of IBD patients could theoretically have three different possible explanations: Either very few of the peripheral blood T lymphocytes are recent thymic emigrants, or their vigorous proliferation dilute the TRECs to very minute amounts, or finally the recent thymic emigrants are recruited directly to the intestinal mucosa.

To examine the hypothesis that an increased proliferation are diluting the true level of TRECs, cells were analysed for Ki67 expression which provides a "snapshot" of the amount of proliferating cells at the time of isolation, a method frequently used as a compensation marker when analyzing TRECs levels in humans [106, 218, 219]. Examination of the frequency of Ki-67^+, proliferating cells within the $CD3^+$ T lymphocyte population in peripheral blood from IBD patients and healthy controls revealed no significant differences between IBD patients and healthy individuals (Fig. 2, Paper III). In addition, the frequency of naïve T cells in peripheral blood was investigated by analysis of the frequency of $CD4^+CD3^+$ T cells expressing the naïve lymphocyte markers CD62L and CD45RA, the former directing cells to the peripheral lymph node by binding to peripheral lymph node addressins (PNAds) on CD34 and GlyCAM-1 on high endothelial venules. Expression of neither marker was different between patients and controls (Fig. 3, paper III), excluding both increased frequency of proliferating T cells and decreased frequency of naïve T cells as explanations to the reduced TRECs levels in peripheral blood from IBD patients.

To test the hypothesis that recent thymic emigrants are instantly recruited to the intestinal mucosa in IBD patients and therefore reside only for a short time in peripheral blood, the relative amounts of TRECs in situ in the gut was estimated

The TRECs levels were investigated in lymphocytes in three different fractions containing IELs and in lymphocytes from lamina propria. We chose to measure the level of TRECs in the three first EDTA/DTT fractions (see Methodological Considerations), but excluded the last fraction to avoid possible contamination by LPLs. By doing this, we were able to follow the T cells containing TRECs from the epithelium at the top of the crypts down to the lamina propria, the more superficial lymphocytes being released in the first fraction, and the IELs positioned deeper into the crypts being found mostly in the third fraction.

In contrast to peripheral blood, the amount of TRECs was higher in the two latter IEL fractions and in LPLs from IBD patients, compared to controls (Fig. 5, paper III). Of note, the TRECs levels in IELs gradually increased from fraction 1 to fraction 3 in IBD patients compared to constantly very low levels in all IEL fractions from the control group.

Thymus activity is highest early in life and involution, with an associated reduced production of T cells, is seen with increasing age. To exclude the possibility that the altered TRECs levels recorded in the intestinal mucosa is a result of the age difference between IBD patients and the control group, a correlation test was performed between the levels of TRECs and the age of the individual, but no correlation was found (Fig.6, paper III), thus indicating that the TRECs levels in the intestinal mucosa are related to the inflammation rather than the age of the individual.

The reduced TRECs levels in the intestinal mucosa were also corroborated by an increased frequency of T lymphocytes expressing CD62L in IBD patients (Fig. 4, paper III).

Together these data support the hypothesis that the reduced TRECs levels seen in peripheral blood from IBD patients are due to a dramatic recruitment of recent thymic emigrants directly to the inflamed mucosa, reflected both by the increased TRECs levels as well as the increased frequencies of naïve lymphocytes in the intestinal mucosa of IBD patients.

The amount of TRECs and the frequency of proliferating cells vary between different lymphoid tissues in colitic Gαi2$^{-/-}$ mice, as well as from control mice (Paper IV)

We next analyzed the TRECs levels in colitic Gαi2$^{-/-}$ mice to see if the differences in TRECs levels found in IBD patients were present also in Gαi2$^{-/-}$ colitis.

The relative amount of TRECs was analysed in peripheral blood, lamina propria and MLN lymphocytes from colitic Gαi2$^{-/-}$ mice between 7 and 10 weeks of age and compared to wild type mice. At this age, no reduced levels of TRECs due to old age are anticipated. To investigate the proliferation rate within the T cell population, the frequencies of Ki-67^{+} cells were analyzed in peripheral blood, lamina propria and MLN lymphocytes (Fig.2, IV).

Similar to human IBD, peripheral blood mononuclear cells (PBMC) from colitic Gαi2$^{-/-}$ mice contained lower levels of TRECs compared to healthy controls. This could either be due to reduced thymic output or a consequence of rapid migration of recent thymic emigrants to the colon and/or the mesenteric lymph node. Although TRECs levels in T lymphocytes from the gut-associated lymphoid tissues differed substantially from the levels in peripheral blood T lymphocytes, with higher levels in the MLN, and lower levels in the colonic lamina propria (Fig. 13), they were lower in T lymphocytes from all three locations from colitic Gαi2$^{-/-}$ mice compared to wild type mice (Fig.1, paper IV).

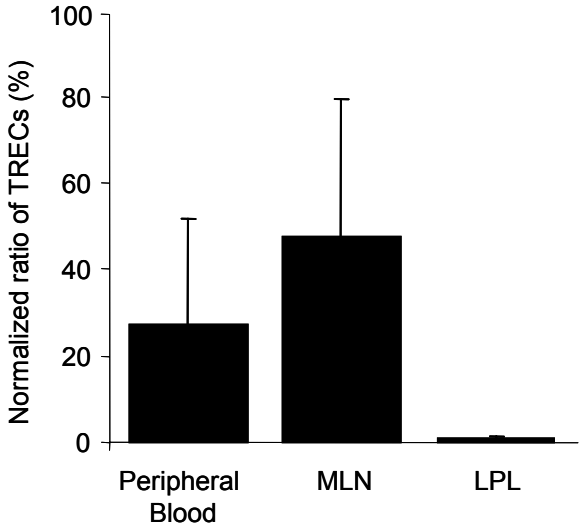

Figure 13. TRECs level in colitic Gαi2$^{-/-}$ mice in peripheral blood, MLN and lamina propria. The results are shown as the normalized ratio between the amount of TRECs and genomic reference gene CD3γ and is expressed as % of an internal standard (thymocytes = 100%) Bars represent the mean value of the frequencies of normalized ratio of TRECs ± SD in peripheral blood, MLN and lamina propria lymphocytes from colitic Gαi2$^{-/-}$ mice (n = 4-6).

However, as alluded to above, TRECs levels are to a very high degree influenced by the extent of proliferation of the T cell population being investigated. Analysis of Ki-67 expression revealed higher frequencies of Ki-67^{+}, proliferating T lymphocytes in all three locations (Fig 2, paper IV), with the highest frequency found in the colonic lamina propria (Fig. 14).

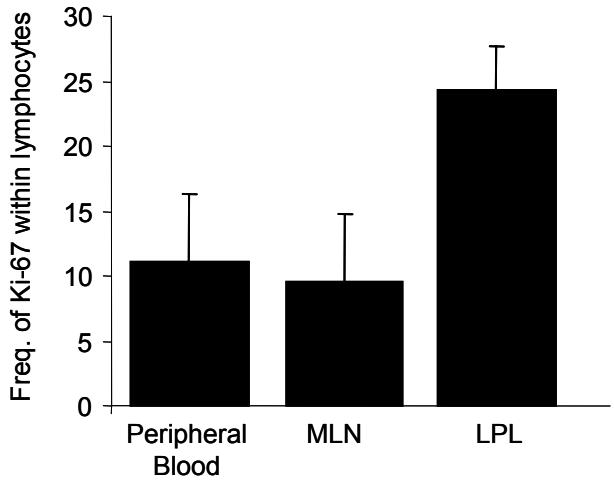

Figure 14. Frequencies of proliferating lymphocytes in peripheral blood, MLN and lamina propria. PBMC as well as lymphocytes from mesenteric lymph nodes and the colonic lamina propria were stained with Ki-67-PE for analysis of total proliferation in colitic Gαi2$^{-/-}$ mice. Bars represent the mean value ± SD of the frequency of the total lymphocyte population expressing Ki-67 (n = 5-7).

The higher frequency of proliferating T cells in the colonic lamina propria correlates well with the lower levels of TRECS in T lymphocytes from this compartment. The reduced TRECs level gives an impression of reduced RTEs but as the proliferation rate was increased, thus diluting the TRECs, the true amount of TRECs in peripheral blood, MLN and colonic lamina propria T lymphocytes from Gαi2$^{-/-}$ mice are most likely underestimated

Analysis of the frequencies of proliferating memory/effector CD4^{+} and CD8^{+} T lymphocytes in peripheral blood, MLN and lamina propria from colitic Gαi2$^{-/-}$ mice (Paper IV).

As RTEs have a naïve phenotype before they become primed by antigen and start to proliferate, we analyzed the frequency of CD4^{+} and CD8^{+} T cells expressing a naïve or memory/effector phenotype to further investigate the extent of thymocyte export in colitic Gαi2$^{-/-}$ mice. The frequencies of CD44^{+} memory/effector T cells were increased in both populations in peripheral blood, MLN and colonic lamina propria from Gαi2$^{-/-}$ mice compared to healthy control mice (Fig 3, paper IV). Supporting the Ki-67 data, the frequency of CD44^{+} lymphocytes was highest in the colonic lamina propria (Fig 15). The Ki67^{+}, proliferating T cells in colitic Gαi2$^{-/-}$ mice was found within the CD44^{+} memory/effector T cell pool in both the CD4^{+} and CD8^{+} populations in peripheral blood, MLN and especially in the lamina propria (Fig.4, paper IV).

In line with this, the frequency of CD62L^{+} naïve T cells was reduced in both peripheral blood and in MLN (not analyzed in the lamina propria due to the low cell yield), and these T lymphocytes had very low frequencies of proliferating cells (Fig 3 and 4, paper IV). Interestingly, the MLN from Gαi2$^{-/-}$ mice contained a very large fraction of naïve T cells, indeed indicating that a large amount of RTEs are likely to be found in the MLN, but that they are disguised in the analysis of TRECs levels due to the high extent of proliferation. The fact that the MLN was the tissue with highest levels of TRECs in the Gαi2$^{-/-}$ mice is also supportive of this interpretation.

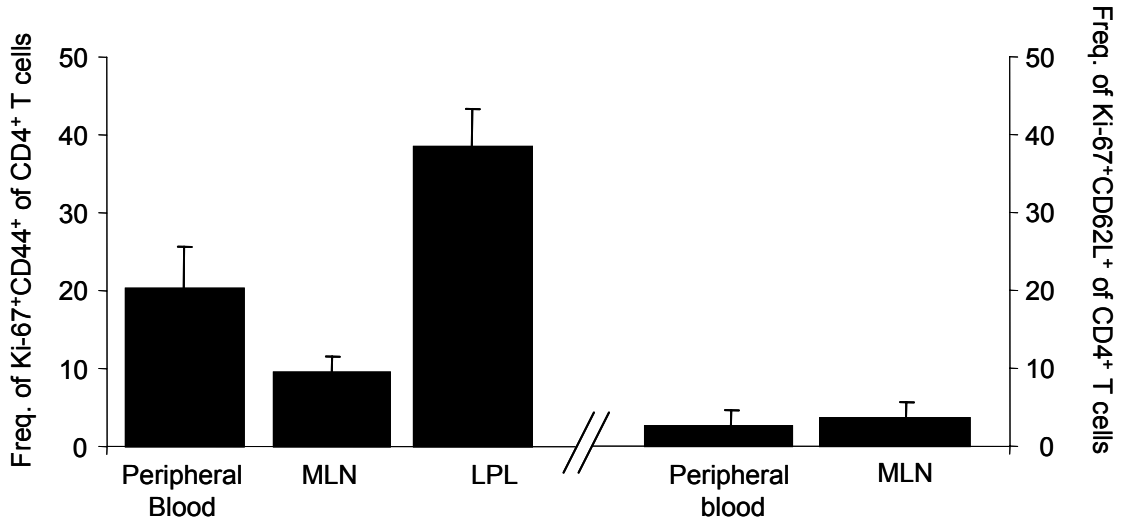

Figure 15. Frequencies of proliferating memory/effector and naïve T lymphocytes in peripheral blood, MLN and lamina propria from Gαi2$^{-/-}$ mice. The lymphocytes were stained with CD4-APC, CD8-PerCP, Ki-67-PE and CD44-FITC or CD62L-FITC for analysis of proliferating memory/effector T cells. Bars represent the mean value of the frequency ± SD of Ki-67^{+}CD44^{+} T cells (left) and Ki-67^{+}CD62L^{+} T cells (right) within the CD4^{+} population in Gαi2$^{-/-}$ mice.

There is a constant proportion of 1-2% of thymocytes exiting from the thymus at any given time point [65], and therefore the dramatic thymus atrophy in Gαi2$^{-/-}$ mice would mean that the total number of new T lymphocytes being exported from the thymus is reduced, thereby decreasing the total number of naïve T cells in peripheral blood. Due to the high variability in the yield of blood at sacrifice we were unable to calculate the total number of peripheral blood T lymphocytes, but the frequency of naïve T cells was indeed reduced.

As the increased proliferation was confined to the memory/effector T cell population, the TRECs levels in the CD62L^{+} naïve T cell population would not be diluted, and analysis of TRECs levels in sorted CD62L^{+} peripheral blood and MLN lymphocytes (excluding LPLs due to the very low frequency of naïve cells in this compartment) would therefore probably give more accurate information about the actual levels of recent thymic emigrants in the different tissues.

Analysis of extrathymic T cell maturation in the inflamed colonic intestinal mucosa (Paper III and IV).

There are several lines of evidence that T lymphocytes are able to develop in the intestinal mucosa without entering the thymus. [220-222]. In addition, Guy-Grand et al have shown that T lymphopoiesis occurs in the mesenteric lymph node and to a lesser extent in the Peyer's patches in athymic mice [146].

To evaluate the possibility that progenitor T lymphocytes are recruited from the bone marrow directly to the intestinal mucosa to undergo extrathymic maturation in IBD patients, we analyzed the intestinal T lymphocytes for subpopulations of early linage T cells, being $CD3^-CD16^-CD19^-CD2^+CD5^+CD7^+$ using five-color flow cytometry as well as by analysis of the expression of mRNA encoding RAG1 and pTα using real-time RT-PCR. Figure 12 shows a representative dot plot demonstrating the gating on $CD16^-CD19^-CD2^+$ lymphocytes and subsequently on $CD5^+CD7^+$ and $CD3^{low/-}$ lymphocytes.

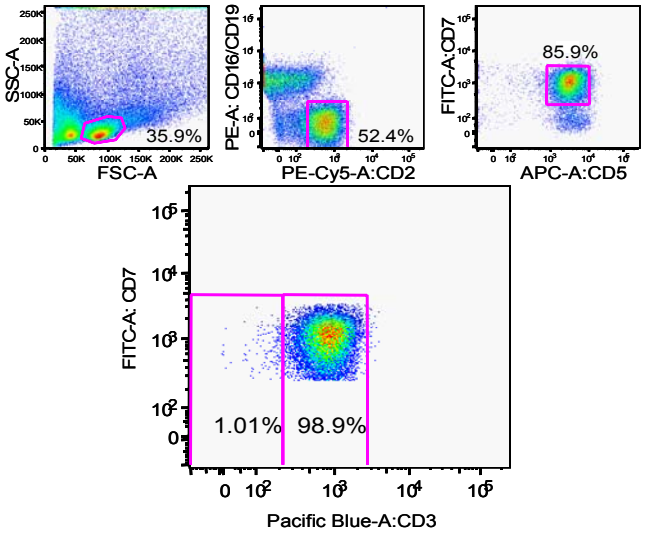

Figure 12. Representative flow cytometric analysis of extrathymic maturation in the lamina propria in one UC patient. Lamina propria lymphocytes were gated on cells staining negative for CD16-PE, CD19-PE and CD3-Pacific blue and positive for CD2-PECy5, and subsequently for CD5-APC and CD7-FITC. The $CD2^+CD5^+CD7^+CD3^-$ lymphocytes were considered to be extrathymically maturing T cell precursors. The gating was based on three control stainings in the same patients, 1) unstained cells, 2) isotype controls for each antibody, and 3) gating on $CD16^-CD19^-CD2^+CD5^+CD7^+$ lymphocytes without CD3 pacific blue staining.

The frequency of early T cell progenitors determined by FACS analysis was similar between IBD patients and controls, and the mucosal T lymphocytes from the two groups demonstrated equally low or undetectable levels of RAG-1 and pTα mRNA. Thus, the increased TRECs levels seen in IBD patients are most likely not a result of increased extrathymic maturation in the inflamed intestinal mucosa of IBD patients.

We also tested whether the marked thymus atrophy in colitic $G\alpha i2^{-/-}$ mice would lead to enhanced extrathymic maturation in the intestinal mucosa or the mesenteric lymph nodes. To this end, the expression of genes specifically expressed during rearrangement of the T cell receptor genes in T lymphocytes, RAG-1 and preTCRα, was investigated by real time-PCR. Although RAG-1 and pTα mRNA was found in low levels in the duodenum, the expression was not significantly different between colitic $G\alpha i2^{-/-}$ mice and healthy controls (Fig. 16). The ileum, jejunum and colon as well as the MLN were also analyzed for extrathymic maturation of T cells but no expression of RAG-1 and pTα mRNA was found in these tissues in either

Gαi2$^{-/-}$ nor Gαi2$^{+/+}$ mice. Thus, although thymus involution during colitis causes an impaired homeostasis of the naïve T cell pool in peripheral immune tissues in Gαi2$^{-/-}$ mice, it does not influence the extent of extrathymic maturation in the MLN or intesinal mucosa.

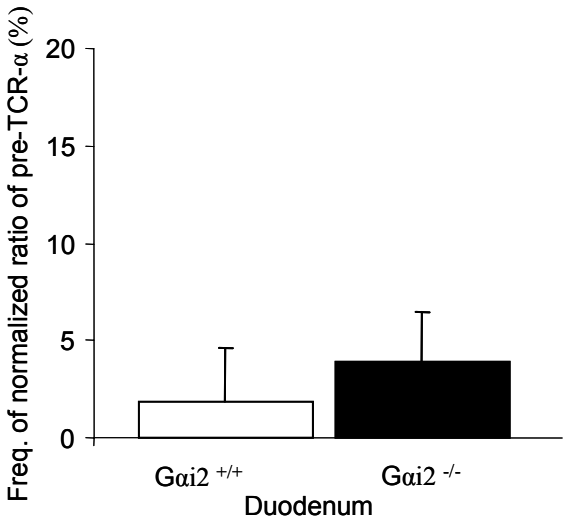

Figure 16. Frequencies of normalized ratio of pre-TcRα mRNA expression in the duodenum of Gαi2$^{+/+}$ and Gαi2$^{-/-}$ mice. Isolated RNA from the duodenum was analysed for the mRNA expression of pTα in Gαi2$^{+/+}$ (n = 4) and Gαi2$^{-/-}$ (n = 4) mice. Bars represent the normalized ratio between the amount of pre-TcRα and the reference gene CD3γ and is expressed as % of an internal standard with high levels of pre-TcRα (thymocytes = 100%) ± SD.

GENERAL DISCUSSION

During my graduate studies I have had the opportunity to work with two mouse models of IBD as well as specimens from IBD patients where I have investigated the consequence of thymic atrophy in IBD. Below I will discuss some general issues regarding this subject as well as future perspectives.

What is the biological function of thymic atrophy during inflammation or infection?

Thymus involution is a naturally occurring process that starts some time during puberty and reduces the thymocyte population with increasing age. Acute thymic atrophy due to the stress response of an inflammation or infection is a phenomenon well observed and characterized in many diseases. However, few studies have drawn the parallel between thymic atrophy, thymic output and how this event affects further progression of the diseases.

The precise mechanism(s) responsible for the thymic atrophy are not completely elucidated and it seems to vary from disease to disease. One major pathway of thymic atrophy is however related to the increasing level of glucocorticoids in the blood in response to increased secretion of cytokines due to inflammation or infection. Steroids can trigger apoptosis of thymocytes and DP thymocytes are particularly sensitive while SP thymocytes are much more resistant [91] [92]. Therefore, it seems like the biological function of thymus atrophy during the acute phase of disease is to stop further development of thymocytes, and thus the organ is "put on hold" during this time. In support of this hypothesis is the reduced thymic input of thymocyte precursors from the bone marrow demonstrated in mice stressed because of immobilization [208]. The question is why. Why do thymocytes die when they are most needed? Is it possible that immature thymocytes egress from the thymus to further develop in peripheral organs? And if so, what are the consequences of that? An interesting study done by Mendes-da-Cruz et al [223] have shown increased levels of $CD4^+CD8^+$ DP T lymphocytes in peripheral lymph nodes, accompanying the thymic atrophy observed in *Trypanosoma cruzii* infected mice. The immature $CD4^+CD8^+$ cells as well as the mature T cells was bearing "forbidden" TCRs that should normally have been deleted in the thymus [223]. This indicates that thymic atrophy can cause egression of thymocytes that have bypassed the negative selection and can therefore have the potential of being autoreactive. Although, it is not known if IBD patients have peripheral T cells bearing autoreactive TCRs, they have been demonstrated to have a skewed TCR repertoire [224]. We show here by analysis of TRECs that these patients, especially UC patients, harbor high levels of recent

thymic emigrants in the intestinal mucosa. Therefore, further evaluation of consequences of thymic atrophy in IBD is of high interest and should not be ignored as a subordinate phenomenon of the disease. Specifically, it would be very interesting to analyze the TCR repertoire in the patients with high levels of TRECs.

Another possible explanation to the thymus atrophy during inflammation could be that the immune system wants to reduce the adaptive arm temporarily. In fact, recent data indicate that T lymphocytes in general, i.e. not only regulatory T cells, dampen initial innate immune responses in an antigen unspecific manner [225].Thus, a transient thymic involution during the acute phase of an infection might be appropriate to allow a robust initial innate immune response.

What is the relevance of immunological findings in animal models for IBD compared to results from patients with IBD?

Experimental studies on animal models of IBD are invaluable tools in the patho-immunological research field of the disease. Several mouse models for IBD, be they gene knockouts, transgenics, chemically induced or induced by cell transfer, results in many similarities but also many differences of the inflammation in the intestine. The variations in IBD development are based on genetics, different immune response such as cytokine production, cell infiltration and -responses, as well as histological features of the intestine. The variation among animal models for IBD should not be considered as a disadvantage, as IBD is probably not only two diseases, i.e. CD and UC, but most likely several diseases with similar clinical and histological features. Therefore, results from experimental studies from one mouse model might not always show similar result in another mouse model or in IBD patients. The result from one specific mouse model can therefore be representative of one IBD patient subgroup.

At present, several approaches for dividing IBD patients into subclasses have been undertaken by identification of biomarkers through genetics, genomics and proteomic that can predict the disease course. Serology is also emerging as a useful tool in substratifying CD and UC phenotypes. By serologic tests specific antibodies have shown to distinguish CD form UC. The perinuclear anti-cytoplastic antibodies (pANCA) and anti-*Saccharomyces cerevisae* antibodies (ASCA) are routinely used to distinguish UC (pANCA+ASCA-) from CD (pANCA-ASCA+) [226]. Other serological markers include antibodies to the outer membrane porin type C of E coli (anti-OmpC) and antibodies to a protein expressed by Pseudomonas fluorescens, anti-12, as well as antibodies against flagellin expressed by a certain clostridial

phylum, anti-CBir1 [226]. A positive serology is associated with a more severe course of the disease, especially in CD. It is likely that in the future differences in serological responsiveness to a number of microbial components will aid in designing tailormade therapies for different subgroups of IBD, but more importantly, substratification of IBD patients in more homogenous subgroups based on antibody responses can give information on the pathophysiology and pathogenesis for different subgroups. In this respect, the many different animal models for IBD will likely be useful models for the different subgroups.

Future perspectives.

Thymic atrophy in Gαi2 deficient mice and in DSS-induced colitis mice, involves an aberrant cortical thymocyte population with decreased chemotactic responsiveness. Below I have listed some suggestion to further evaluate the consequences of thymus involution due to colitis.

Beside the analysis of RTEs that was done by measuring TRECs level, it would be of high interest to evaluate what kind of thymocytes that are egressing from the thymus in pre-colitic and colitic Gαi2$^{-/-}$ mice. Mature thymocytes respond chemotactically to S1P in peripheral blood due to their expression of the S1P receptor and thereby egress from the thymus [93]. Evaluation of thymocyte chemotactic responsiveness to S1P would therefore give an indication of the thymic output during thymic atrophy and also whether immature thymocytes are able to egress thymus and thereby enter peripheral organs. If immature thymocytes have chemotactic responsiveness to S1P, it would be very interesting to evaluate if these thymocytes have bypassed the negative selection and would be able to exit the thymus as autoreactive thymocytes.

Another way of analyzing the possibility of autoreactive thymocytes egressing from the thymus is by analysis of the expression of the transcription factor autoimmune regulator (Aire) in medullary thymic epithelial cells. During thymocyte maturation the thymic epithelial cells express a wide collection of peripheral-tissue antigens to induce central tolerance in maturing thymocytes [227]. Loss of Aire is known to cause autoimmunity [227]. Analysis of AIRE would therefore be interesting to evaluate in Gαi2$^{-/-}$ mice.

As mentioned previously, increased levels glucocorticoid in blood is one major pathway of thymic atrophy and the impact of endogenous glucocorticoids in Gαi2$^{-/-}$ mice would be interesting to evaluate. Besides measuring the glucocorticoid levels in blood, it would also be interesting to see the effect of inhibition of glucocorticoids on thymic atrophy and as well

progression of colitis. A study performed by Roggero et al [228] have shown that inhibition of endoegenous glucocorticoids by treatment with the glucocorticoid receptor antagonist RU486, prevented thymus involution but aggravate the disease.

The analysis of RTEs by measuring TRECs in Gαi2 deficient mice was difficult to evaluate due to the increased proliferation among memory/effector T cells in peripheral blood, MLN and especially in lamina propria. This analysis could be improved by separating naïve T lymphocytes by their expression of CD62L and measure the TRECs content in this subpopulation, as this would give a more accurate level of RTEs. Separation of specific T cell populations could also be done in specimens from IBD patients to further evaluate the increased TRECs level in the intestine.

Although we were unable to find any significant differences in TRECs levels in the intestine between IBD patients with active and inactive disease, it would still be interesting to follow a larger group of patients for a retrospective analysis on whether there is any correlation between TRECs levels and time span to the next relapse, as well as the course of the disease in general. In other words, could a TRECs analysis in a larger patient material aid in the substratification of IBD patients discussed above. In addition, further elucidation of T cell ontogeny in the thymus and intestinal mucosa has the potential in identifying new areas of therapeutic strategies for IBD.

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