Defensins and Cytokines in Inflammatory Bowel Disease

Arman Rahman

Umeå 2007
The published articles have been reproduced with permissions from Inflammatory Bowel Disease (Wiley InterScience) and Immunology (Blackwell Publishing Ltd).

Copyright ©2007 by Arman Rahman
Printed by Solfjädern Offset AB,
This thesis is dedicated in loving memory of
my father Mahbubur Rahman
and my teacher Takeshi Matsunaga
# TABLE OF CONTENTS

Abstract .................................................................................................................................................. 1  
Abbreviations ......................................................................................................................................... 2  
List of original papers ............................................................................................................................. 3  
1. Introduction ......................................................................................................................................... 4  
   1.1 Immune system .............................................................................................................................. 4  
      1.1.1 Innate immune system .......................................................................................................... 5  
      1.1.2 Adaptive immune system ..................................................................................................... 5  
         1.1.2.1 T cells ............................................................................................................................ 6  
            1.1.2.1.1 T helper cells ............................................................................................................ 7  
            1.1.2.1.2 Cytotoxic T lymphocytes .......................................................................................... 8  
            1.1.2.1.3 Regulatory T cells .................................................................................................... 8  
               1.1.2.1.3.1 Treg cells (CD4^CD^{25}bright) ................................................................. 8  
               1.1.2.1.3.2 Type 1 Treg cells .............................................................................................. 11  
               1.1.2.1.3.3 Th3 cells ........................................................................................................... 11  
      1.1.2.2 B cells ............................................................................................................................... 12  
      1.1.2.3 Antigen presenting cells .................................................................................................... 14  
   1.1.3 Antimicrobial peptides .............................................................................................................. 15  
      1.1.3.1 Defensins .......................................................................................................................... 16  
      1.1.3.2 Mechanism of action ......................................................................................................... 18  
      1.1.3.3 Human defensins ............................................................................................................. 19  
      1.1.3.4 Defensin expression in IBD ............................................................................................. 23  
      1.1.3.5 Mouse defensins .............................................................................................................. 24  
1.2 Inflammatory bowel disease .......................................................................................................... 26  
   1.2.1 Clinical features and treatment modalities .............................................................................. 28  
   1.2.2 Pathogenesis of IBD .............................................................................................................. 29  
1.3 Animal models of IBD .................................................................................................................... 31  
   1.3.1 Colitis in IL-2 knockout mice ................................................................................................. 32
1.3.2 Colitis in DSS induced mice .................................................. 33

2. Aims of the thesis ............................................................................. 34

3. Methodology .................................................................................. 35

4. Results and discussion .................................................................. 36
   4.1 Paper I ...................................................................................... 36
   4.2 Paper II .................................................................................. 38
   4.3 Paper III .................................................................................. 40
   4.4 Paper IV .................................................................................. 41

5. Conclusion ..................................................................................... 43

6. Acknowledgements ........................................................................ 44

7. References ..................................................................................... 46

8. Papers I-IV
ABSTRACT

Ulcerative colitis (UC) and Crohn’s disease (CD) constitute the two major inflammatory bowel diseases in man. Both are serious chronic illnesses of the intestine with severe debilitating effects. The etiology of the diseases is unknown, but involvement of both adaptive and innate immune reactions seems to be major factors in the pathogenesis. In this thesis the roles of key molecules of the adaptive immunity, i.e. interleukin-2 (IL-2), and innate immunity, i.e. β-defensins, were studied both in human inflammation of the large intestine and in mouse colitis models.

β-defensins are small endogenous peptides with antimicrobial activity. Previous studies showed that expression of human β-defensin-2 (hBD-2), hBD-3, and hBD-4 is induced in colonic epithelial cells of UC patients. Here we demonstrate that cells expressing these three β-defensins are present also in the colonic lamina propria of UC patients and less frequently in CD patients, and controls. These cells were identified as mature plasma cells by the highly specific CD138 marker, by their prominent IgA or IgG expression, and by their ultrastructural characteristics. Immunoelectron microscopy analysis of the hBD-2 peptide demonstrated synthesis and transport for secretion. Defensin producing plasma cells were 2-3 times more abundant in UC colon than in control and CD colon. Additionally, hBD-2 mRNA expression was demonstrated in 3 out of 4 well-characterized plasma cell lines.

Defensin expression was studied in large intestinal mucosa before and after onset of colitis in two colitis models - the IL-2 KO mouse and the dextran sulphate sodium (DSS) induced colitis mouse. Mouse β-defensin-3 (mBD-3) and mBD-4 mRNA was expressed in colonic epithelial cells of homozygous IL-2 KO (IL-2−/−) mice with established colitis (15 weeks old) and at significantly higher levels than in apparently healthy wild type mice, heterozygous (IL-2−/+), mice, and 5 weeks old IL-2−/− mice. Similarly mBD-3 was expressed in epithelial cells of DSS treated mice with chronic colitis but not in DSS treated mice with acute inflammation. Cells expressing mBD-3 mRNA were seen also in colonic lamina propria of diseased animals. Thus, expression of β-defensins in the colonic epithelium seems to be a consequence of the chronic inflammation.

IL-2−/+ mice have reduced levels of IL-2 in the intestinal mucosa but are clinically healthy. IL-2−/− mice showed markedly reduced susceptibility to DSS induced colitis. This was associated with a significantly reduced infiltration of both CD4+ and CD8+ T cells in the colonic mucosa and lower expression levels of the cytokines IL-2, IL-4, and IL-10 in colonic T cells compared to DSS treated wild type mice. These results suggest that reduced level of IL-2 leads to attenuated activation and function of colonic T cells in turn causing a milder colitis in response to DSS challenge.

Interestingly, IL-2−/+ mice had a reduced frequency of regulatory T cells (CD4+CD25+) in both small and large intestine compared to wild type mice. As the small intestine of IL-2−/− and IL-2−/+ mice appear normal, small intestinal T cells have never been critically analyzed in these mice. The cytokine profile of small intestinal T cells in IL-2−/+ and IL-2−/+ mice was changed compared to wild type control mice (IL-2−/+ with significantly elevated expression levels of IL-10 and IL-4. DSS treatment of IL-2−/+ mice caused a marked reduction in cytokine expression levels in small intestinal T-cells. These results suggest that lack of IL-2 and even the partial decrease seen in IL-2−/+ mice influence T cell function locally in the intestinal mucosa and cause a skewed cytokine milieu also in the small intestine despite its normal histology.

In this thesis we demonstrate the pivotal importance of different T-cell subsets in intestinal inflammation. The upregulation of intestinal antimicrobial peptides seems to be a consequence of chronic inflammation in an effort to minimize intestinal damage.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cell mediated cytotoxicity</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BcR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte associated antigen-4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cells</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>hBD</td>
<td>Human beta defensin</td>
</tr>
<tr>
<td>HNP</td>
<td>Human neutrophil peptide</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IEC</td>
<td>Intestinal epithelial cells</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-2^-/-</td>
<td>Interleukin 2 gene knockout homozygous</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LPL</td>
<td>Lamina propria lymphocyte</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mBD</td>
<td>Mouse beta defensin</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>M cell</td>
<td>Microfold cell</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated molecular pattern</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma cell</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patches</td>
</tr>
<tr>
<td>PRP</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory IgA</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PAPERS

This thesis is based on the following articles and manuscripts, which are cited in the text by their roman numerals ( I-IV ):


1. INTRODUCTION

1.1 The Immune system

The immune system has developed to protect us against invading micro-organisms and parasitic worms. The invaders include viruses, bacteria, protozoa, and fungi. Possibly the immune system also protects us from “internal enemies” like cancer cells. Like all biological systems the immune system is not perfect. When the immune response is directed against our own bodily components disease may develop i.e. autoimmune disease.

Two different, but collaborating, arms of the immune system, the innate and the adaptive arms execute the protective functions. The innate immune system is an evolutionarily ancient form of host defence found in most multi-cellular organisms and serves as a first line of defence against invading microbes.

1.1.1 The innate immune system:

Innate immunity is triggered upon pathogen recognition by any of a set of pattern recognition receptors (PRRs), one important example being the family of Toll-like receptors (TLRs), recognizing conserved molecular patterns shared by groups of micro-organisms. In humans there are 11 known TLRs recognizing different pathogen-associated molecular patterns (PAMPs). For example TLR4 binds to bacterial lipopolysaccharide (LPS) found in the cell wall of Gram-negative bacteria and TLR7 and TLR8 to single-stranded RNA in viruses.[1] TLRs reside in the plasma membrane (TLR1, 2, 4, 5, and 6) or in internal membranes (TLR3, 7, 8, and 9) of different immune cells notably macrophages and neutrophils but also on epithelial cells.

One of the major contributions of the innate immune system is the barrier function of the epithelial surfaces preventing entry of microbes into our body, another being mucin production by certain epithelia, a third, release of anti-microbial factors such as defensins, lysozyme, lactoferrin, NO etc. Mucins act as a protective layer on the apical surface of
epithelial cells entrapping micro-organisms in this highly viscous layer. Antimicrobial peptides can be produced constitutively or induced by microbial binding to TLRs (see below).

If an infectious agent crosses the epithelial barrier it will be recognized by TLR on resident macrophages inducing a cascade reaction leading to elimination of the invading pathogen through various mechanisms including phagocytosis and intracellular killing of the micro-organism by macrophages and neutrophils, direct killing by the defensins, lysozyme etc. Another important innate immunity mechanism is activation of the complement system via the alternative or lectin pathways. This leads to deposition of C3b on the surface of the micro-organism, which acts as an opsonizing agent promoting microbial uptake by phagocytic cells. A further consequence of complement activation is the formation of the membrane-attack complex resulting in lysis of the pathogen. Phagocytic cells release different cytokines, which in turn can induce the mobilization of antigen-presenting cells (APCs), which are important for the induction of the adaptive immune system. [2]

1.1.2 The adaptive immune system:

The central cell of adaptive immunity is the lymphocyte. There are two major divisions of lymphocytes: B lymphocytes (B cells), responsible for humoral immunity, and T lymphocytes (T cells) responsible for cell-mediated immunity and for immune regulation. In contrast to innate immunity adaptive immunity is highly specific recognizing small structural details (epitopes) on foreign macromolecules (antigens) for example surface proteins on a bacterium. It is also highly efficient resulting, in most cases, of the complete elimination of the invading micro-organism. The effectors in humoral immunity are circulating antibodies, which bind to epitopes on foreign antigens. In cell-mediated immunity the effectors are cytotoxic T cells and cytokine producing T cells. The main drawback with the adaptive immune system is that it takes 5-7 days before it is fully operative if it is the first time the individual meets the antigen. This is probably why innate immunity is of great importance also for mammals although they are equipped with the highly sophisticated adaptive immune system. Innate immunity will keep micro-organisms at bay while adaptive immunity develops. If the infected individual has met the micro-organism before he/she has developed memory cells which shortens the response time to 3-4 days.

Adaptive immunity is mediated by B cells and T cells through their highly specific receptors. B cells use cell-surface bound immunoglobulin (Ig) molecules as receptors and
upon activation B cells differentiate into plasma cells that secrete the immunoglobulin as soluble antibody providing defence against pathogens in the extra-cellular spaces of the body. T-cells have receptors that recognize short peptides of protein antigens of pathogens presented on the surface of APCs on a special type of molecules called major histocompatibility complex (MHC) molecules. The MHC molecules appear in two different forms: MHC class I molecules and MHC class II molecules. Similarly the T cells are of two types with respect to interaction with MHC-peptide complex: T helper cells equipped with the CD4 molecule that interacts with a constant region on MHC class II molecules and cytotoxic T cells equipped with the CD8 molecule interacting with a constant region on MHC class I molecules. Depending on which type of T cells that are activated the effect could be killing of infected target cells (cytotoxic CD8\(^+\)T cells), activation of macrophages and B cells (CD4\(^+\) T helper cells) or inhibition of an immune response (T regulatory cells).

The adaptive immune system is highly specific and each encounter with a new foreign antigen will induce long-lived specific memory cells that will protect individuals from re-infection with the same pathogen. Presumably the memory cell repertoire changes during our life span affording increased protection as we grow older up to a time when the immune system starts to senesce. Innate immune responses recognize generic targets on pathogens using germ line encoded receptors, whereas adaptive immune response recognises specific targets using randomly generated receptors which have an virtually unlimited recognition repertoire.

Interplay between innate and adaptive immune recognition is increasingly being recognized as essential for the effective functioning of an immune response.

Some of the major components of adaptive and innate immune system that have been studied in this thesis are being described here.

### 1.1.2.1 T cells:

T cells are lymphocytes that mainly develop in the thymus. This organ is seeded by lymphocytic precursor cells from the bone marrow. In the thymus, thymocytes develop their T cell antigen receptors (TCRs), which are of two major types-one is a heterodimer of two disulphide-linked polypeptides (\(\alpha\) and \(\beta\)); the other is structurally similar, but is built up of two different polypeptide chains termed \(\delta\) and \(\gamma\). Both of these receptors are associated with a set of five polypeptides, the CD3 complex and together they form the T cell receptor complex. The CD3 components show no amino acid variability on different T cells.
and thus cannot generate diversity associated with the TCRs. Rather, they are required for the signal transduction following antigen recognition by the TCR heterodimer. Two very important selection processes take place in the thymus: positive and negative selection. In the positive selection process only those αβ T cells with ability to recognize MHC molecules are allowed to live. Positive selection seems not to apply to γδ T cells since they recognize foreign antigen without the need for MHC presentation. In the negative selection process T cells with TCR recognizing peptides from the individuals own proteins are eliminated. The αβ T cell comprises the majority of the T cells (90-95%) in periphery, whereas the γδ T cells comprise a minor population (5-10%) of total T cells, but constitute a greater proportion in particular sites, including the gut, skin and vagina.

T cells can also develop extra-thymically notably in the small intestine. αβ T cells are subdivided into two distinct non-overlapping populations: a subset that carries the CD4 marker (CD4^+ T cells). The main function of these cells is to ‘help’ or ‘induce’ immune responses (T_h). Another subset carries the CD8 marker (CD8^+ T cells), and they are cytotoxic (T_c). As mentioned above CD4^+ T cells recognize their specific antigens in association with MHC class II molecules, while CD8^+ T cells recognize their antigens in association with MHC class I molecules. Thus the CD4 or CD8 restricts the type of cells with which the T cell can interact. [2]

1.1.2.1.1 T helper cells: CD4^+ T cells can be subdivided into different subtypes depending upon their cytokine production. Mouse Th1 cells produce interleukin-2 (IL-2), interferon-γ (INF-γ) whereas Th2 cells produce IL-4, IL-6, IL-9, IL-10 and IL-13. Human Th1 and Th2 cells produce the same types of cytokines as the mouse, although the synthesis of IL-2, IL-6, IL-10 and IL-13 is not as strictly restricted to a single subset as in mouse T cells. [3] There are other cytokines, for example IL-12 which promotes the development of either Th1 or Th2 cells, not necessarily secreted only by T cells, but considered to be a Th1 cytokine. [4] T cells that produce both Th1 and Th2 cytokines are referred to as Th0, while T cell that produce high amount of transforming growth factor β (TGF-β) have been termed Th3 cytokine.[3]

The Th1 cell provides help to the cytotoxic precursor T cell to develop it into a cytotoxic T lymphocyte (CTL) and plays a role in local inflammatory reactions and hence is considered to contribute to cell mediated immunity. Th2 cells on the other hand contribute in the humoral immunity by stimulating B cells to proliferate and develop into plasma cells, which produce antibodies and Th3 cells are involved in down regulation of immune reaction. [2] Th17 - a
recently discovered effector CD4 T cell lineage, distinct from Th1 and Th2 cells, secrete IL-17A, IL-17F, IL-6, and TNF-α. Development of Th17 cells share a common cytokine TGF-β with some regulatory T cells, while IL-23 promotes the expansion of Th17 cells. These cells are involved in tissue destruction in many diverse pathological conditions including IBD.

1.1.2.1.2 Cytotoxic T lymphocytes (CTLs): These are CD8⁺ T cells especially effective in killing target cells infected by intracellular bacterial pathogens and by viruses. When activated they can kill their target cells by releasing cytotoxic effector molecules like perforin and granzymes, which form pores in the target cell plasma membrane destroying the membrane integrity leading to cell death. They can also induce apoptosis via binding of FasL to Fas on the target cell. Moreover, cytotoxic T cells can release cytokines like IFN-γ and TNF-α, which contribute to host defence by activating macrophages and increase the expression of MHC class I molecules.

1.1.2.1.3 Regulatory T cells: It is long known that most high-affinity self-reactive T cells are clonally deleted within the thymus, but this system is leaky and by itself insufficient to prevent auto-reactivity. Regulatory T cells prevent auto-reactivity thus maintaining peripheral immune tolerance. Absence of regulatory T cells leads to hyperproliferation of normal lymphocytes causing destruction of various tissues. Although it is well established that regulatory T cells act via cell-cell interactions and/or the production of cytokines, many aspects behind these mechanisms remain to be revealed. A number of subpopulations of regulatory T cells, with different modes of operation, have been described (Table 1). The three most common subtypes of regulatory T cells are discussed below.

1.1.2.1.3.1 Treg cells (CD4⁺CD25bright) is a subset of thymus derived T cells, also known as the natural Tregs, accounts for 5–10% of mice and 1-2% of human peripheral CD4⁺ T cells. They were the first type of regulatory T cells to be shown to inhibit the activation and proliferation of effector T cells where the main mechanism of suppression seemed to be inhibition of the transcription of IL-2 in the responder population. The phenotype of these cells is not only characterised by the expression of the surface proteins CD4 and CD25 (IL-2R α-chain), but also by the expression of the transcription factor Foxp3 (X-linked fork head/winged helix transcription factor, scurfin), and the surface molecules CTLA-4 and GITR. The FOXP3 gene was identified as a master regulatory gene which is constitutively
expressed in natural Treg cells and plays an indispensable role in their development and function. FOXP3 contains all the sequences that are necessary to inhibit transcriptional activation through nuclear factor of activated T cells (NFAT) and foxp3 dependent inhibition of T cell activation with down-regulation of cytokine expression and upregulation of immunosuppressive cell surface molecules is shown. A recent report described regulatory T cell mediated suppression of IL-2, INF-γ production, upregulation of Treg associated surface molecules such as CTLA-4, GITR is due to Foxp3 binding with the transcription factor AML1/Runx1 (acute myeloid leukaemia 1/Runt-related transcription factor 1). Upregulation of expression of homing receptors that direct effector T cells to effector sites is closely related to their functional differentiation. It is believed that Foxp3 mediates this function in Treg cells and their ultimate homing at different effector sites, for example expression of α4β7-integrin and CCR9 for homing to the intestine. Mutation of the FOXP3 gene results in Scurfy and IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome in mouse and human, respectively.  

### Table 1. Types of regulatory T cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Mediators/Effector molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>nTreg (CD4⁺ CD25⁺bright FOXP3⁺)</td>
<td>Cell/cell contact</td>
</tr>
<tr>
<td>Treg (CD4⁺CD25⁺bright)</td>
<td>IL-10, TGF-β, CTLA-4, GITR</td>
</tr>
<tr>
<td>Tr1</td>
<td>IL-10, TGF-β</td>
</tr>
<tr>
<td>Th3</td>
<td>TGF-β</td>
</tr>
<tr>
<td>CD8⁻CD25⁺CD28⁻</td>
<td>IL-10, TGF-β, CTLA-4, GITR</td>
</tr>
<tr>
<td>*Qa-1-dependent CD8⁺</td>
<td>Qa specific TCR (cell/cell contact)</td>
</tr>
<tr>
<td>CD4⁺CD8⁻ TCRαβ⁺.</td>
<td>Induction of apoptosis by Fas-FasL pathway.</td>
</tr>
<tr>
<td>TCRγδ⁺</td>
<td>IL-10, TGF-β</td>
</tr>
</tbody>
</table>

Adapted from Taylor A et al 2005, [22] and Roncarolo MG et al. 2007, [23] * Mouse only.

Intracellular and cell surface expression of CTLA-4 (Cytotoxic T lymphocyte-associated antigen 4) is another marker for regulatory T cells which is expressed by a high percentage of natural Tregs. This molecule belongs to the same family as CD28 and binds to the same ligands, i.e. B7-1 (CD80) and B7-2 (CD86). CTLA-4 deficient mice rapidly develop a lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction.
Treatment with anti-CTLA-4 antibody abolish all the symptoms strongly demonstrating that CTLA-4 plays a critical role in down-regulating T cell activation and maintaining immunologic homeostasis. [25, 26] CTLA-4 can exert its immunosuppressive function in several different ways. A direct engagement of CTLA-4 on the Treg cells with CD80/CD86 molecules on APCs transducing a co-stimulatory signal to activate Treg cells. [24, 27] This cross linking of CTLA-4 concomitant with the TCR signalling exerts suppressive functions by inhibiting IL-2 gene expression and cell cycle progression, [28, 29] induction of the immunosuppressive enzyme IDO (indoleamine 2,3-digoxigenase) from DCs [30] and leads to secretion of TGF-β by the effector T cells. [31]

GITR (Glucocorticoid-induced TNF receptor), a member of the TNF receptor superfamily, was first reported to be a suppressor molecule on Treg cells. [14] But significant development in recent years in Treg research shows that this molecule has many other functions than previously believed. Although highly expressed in both human and mouse CD4⁺CD25⁺ cells, GITR has been detected in NK cells, PMN cells, monocytes, macrophages, B cells, DCs where the expression level can be increased upon activation. [32, 33] The use of GITR as a Treg marker is further complicated by the fact that GITR can be expressed and upregulated in activated effector CD4⁺ and CD8⁺ T cells as comparable to Tregs, so that evaluation of GITR positive cells ends up in measuring both effector and Treg cells. [34]

Probably Foxp3 is a superior marker to identify regulatory T cell population than CTLA-4 and GITR. Foxp3-expressing CD4⁺CD25⁺ cells have been identified in both B7-1/B7-2/CTLA-4 KO and B7-1/B7-2 KO mice and they were clearly demonstrated to have retained their functional activity by preventing colitis in the experimental animal used. [35] It is even further strengthened by the finding that CTLA-4 deficient CD4⁺CD25⁺ T cells can be converted to Treg like cells by retroviral transduction with Foxp3 but not with CTLA-4 [24] and transgenic overexpression of Foxp3 in CTLA-4 deficient mice ameliorates their debilitating systemic effect and prolong survival. [36]

Besides all the known markers for regulatory T cell, Yamaguchi et al 2007 has described a yet another Treg surface marker-folate receptor 4, which is highly expressed by the natural treg cells in mouse alone with CD25 and foxp3. [37] Confirmation of this receptor present in the human regulatory T cell is yet to come.
1.1.2.1.3.2 Type 1 Treg cells (Tr1) also known as adaptive or inducible Tregs are defined by their ability to be induced in the periphery as a regulatory cell and clonally suppress naïve T cells in order to induce peripheral tolerance. IL-10 and INF-α are needed to induce the differentiation of CD4^+ cells into Tr1 cells. [38] Cloned Tr1 cells produce significant levels of IL-10, IFN-γ, TGF-β and IL-5, low levels of IL-2 and no IL-4. [39] The suppressive activity of Tr1 is mediated by IL-10 and TGF-β and Tr1 cells are capable of preventing disease in experimental colitis in mice.[39] IL-10 producing Tr1 cells can also be induced with pharmacological immunosuppressive drugs using a combination of Vitamin D3 and dexamethasone and these cells could prevent autoimmune disease when adoptively transferred into mouse model of central nervous inflammation. [40] Dendritic cells, both mature (CD11c^+) and immature (CD83^-) have the capacity to differentiate naïve T cells into non-proliferating IL-10 producing Tr1 cells. [41, 42] Evidently Foxp3^+ cells can be generated in the periphery from Foxp3 negative CD4^+ cells that appear to function in a similar manner as natural Treg cells, in other words CD4^+CD25^+Foxp3^+ cells can be of both natural and adaptive origin. [43, 44]

1.1.2.1.3.3 Th3 cells are characterised by their prominent production of TGF-β. They are class II MHC restricted and can have identical αβ TCR as Th1 and Th2. [45] The important role of TGF-β in down regulating T cell mediated immune response and control of peripheral tolerance has been established since long, although a clear understanding of their mechanism of action is still under investigation [46]

Studies have shown that TGF-β induces not only expression of Foxp3 upon activation of thymus derived CD4^+CD25^- cell that leads to generation of CD4^+CD25^+ Tregs in vitro, but it also induces their expansion in the periphery. [43, 47] Carrier et al 2007 recently demonstrated a new lineage of peripheral Tregs in the IL2^-/- mice which are TGF-β derived Foxp3^+ Th3 cells contributing to the peripheral Foxp3^+ Treg pool which is essential to maintain and re-establish peripheral tolerance. [48]

On top of the above mentioned CD4^+ innate and acquired regulatory T cells, there are other T cell clones which have regulatory properties. Of them, CD8^+ Tregs are of particular interest because they have been shown to prevent inflammation in experimental colitis by inhibiting INF-γ production by the CD4^+ responder cells. [49] Brimnes J et al 2005 showed that this CD8^+ T cells have no regulatory activity in IBD patients, whereas they retain their immune
suppressive function in control individuals suggesting that the absence of regulatory CD8\(^+\) cell may be an important contributor to the pathogenesis of IBD. [50]

1.1.2.2 B cells: B cells develop from the haematopoietic stem cells in the adult bone marrow and fetal liver. From birth and onwards the entire antigen-independent maturation process of B cells takes place in the bone marrow. The role of B cells is to interact with B cell epitopes on foreign antigen via their B-cell receptors (BCR) and as a consequence of this interaction develop into plasma cells, which produce antibodies with the same specificity as the BCR. Plasma cells can be considered as an “antibody factory” releasing large amounts of antibodies that enter the circulation and bind the specific antigen on for example an invading micro-organism. Antibodies protects the host by different means, for example by neutralising microbial toxins, by preventing adhesion of pathogenic bacteria to mucosal surfaces, by activating the complement system, which in turn kills micro-organisms via a lytic complex and deposit C3b on the microbial surface making them more susceptible to phagocytosis by neutrophils or macrophages. The latter process is called opsonization. Antibodies may also participate in antibody dependent cell mediated cytotoxicity (ADCC).

The activation of B cells and their differentiation to antibody-secreting plasma cells is triggered by antigen binding to the BCR. This process usually requires T cell help through CD40-CD40L interaction and through stimulation by cytokines produced by the T cell. [2] A naïve B cell express BCR of the IgM (monomeric) and IgD isotypes on its surface, while B cells which have encountered the antigen previously have undergone so called isotype switching. This means that the BCR on these cells include other isotypes like IgG, IgA or IgE. When these cells mature to plasma cells IgG, IgA or IgE antibodies are released. This is biologically very useful because the different isotypes have different effector functions.

Apart from conventional B cells (so called B-2 cells), there is evidence for another type of B cell (so called B-1 cells) originally discovered in the mouse peritoneal cavity. These cells follow a separate differentiation pathway, have a different surface phenotype and localize preferentially to other anatomical sites than B-2 cells. Major differences between B-1 and B-2 cells are:

1. B-1 and B-2 cells arise from distinct progenitors. Progenitors in the fetal liver are the main source of B-1 cells, whereas progenitors from the adult bone marrow give rise to B-2 cells.
2. The antibody repertoire of B-1 cells tends to be more restricted than the B-2 repertoire. [52]
3. B-2 cells undergo somatic hyper-mutation of their Ig genes, leading to affinity maturation of the antibody response. In contrast, B-1 cells are largely responsible for “innate type” of immune response where they respond rapidly to a variety of T-independent antigens. [53]

B cells can also function as antigen presenting cells (APC) although not as efficient as professional APC. When the B cell binds to a specific antigen via BCRs, after processing it leads to presentation of antigen peptide on MHC class II molecules on the surface of B cell together with low expression of co-stimulatory molecules like CD86. The TCRs of the T cell specific for the antigen bind to the peptide/MHC complex on the B cell and to CD86 by its receptor CD28. These signals induce expression of low levels of CD40L on the T cell which binds to the CD40 on the B cell leading to higher expression of costimulatory molecules on the B cell. These interactions lead to an exponential growth of the signals exchanged by CD4⁺ T cells and B cells and terminate resulting in the complete activation of both cells. [54]

Major sites of B cell maturation are in the mucosa associated lymphoid tissue (MALT), lymph nodes and spleen. MALT can be divided according to different anatomical sites such as GALT (gut-associated lymphoid tissue), BALT (bronchus-associated lymphoid tissue), NALT (nasopharynx-associated lymphoid tissue). GALT comprises of Payer’s patches (PPs), the appendix, and scattered solitary or isolated lymphoid follicles. In GALT, antigen sampling from the gut lumen takes place through M cells (membraneous or microfold) situated in the follicle associated epithelium (FAE). It has been shown in both germ free and conventional rats that bacterial colonization drives accumulation and differentiation of T and B cells under the M cell pockets, where with the help of antigen transporting dendritic cells the germinal centre (GC) is formed. [55] GCs are specialized areas in the follicle where B cells undergo rounds of proliferation, which is accompanied by affinity maturation and class switch recombination of immunoglobulin. Antigen specific T helper cells and follicular dendritic cells are key component of the GC response. B lymphocytes that survive germinal centre reaction are destined to become plasma cells or memory cells. [56] Memory B cells have the intrinsic ability to respond more rapidly than naïve B cells and they show a proliferative burst on secondary encounter with antigens. Complement receptors expressed by the stromal cells are required for this rapid recall response.

Most plasma cells precursors quickly dispersed form the GC and migrate via blood or lymph to effector sites and undergo terminal differentiation. This dispersion is thought to be mediated by chemokine receptors especially CCR7 which is upregulated on the GC B cells.
Before differentiation into plasma cells, B cells undergo a proliferative burst which is enhanced by cytokine secreted by T cells. Ultimately proliferation ceases and non-dividing plasma cells are formed. Upon plasma cell differentiation there is a marked increase in steady state amount of Ig heavy and light chain mRNA and J chain mRNA when Ig M and Ig A secretion is required. It is not clear whether the increase Ig mRNA is the result of an increased transcription, increased mRNA stability or as proposed combination of both mechanism. To accommodate translation and secretion of the abundant Ig mRNA, plasma cells have an increased cytoplasmic to nuclear ratio and prominent amount of endoplasmic reticulum and secretory vacuoles. At least 80% of the body’s Ig-producing plasma cells are located in the intestinal mucosa making it the largest effector organ of the adaptive humoral immunity. Most mucosal plasma cells (70-90%) produce preferentially dimers and some trimers of IgA (collectively called pIgA), which contain J chain and therefore can bind to the epithelial secretory component (SC). This transmembrane glycoprotein functions as pIg receptor (pIgR) that also translocate pentameric IgM to the epithelial surface. Thus, plasma cells with high level of J-chain expression and pIg-pIgR interaction at mucosal surface are the key components of secretory antibodies (SIgA and SIgM) which provide non-inflammatory first line defence by preventing microbial colonization and noxious antigens penetrating the epithelial barrier. A failure in maintaining the homeostasis of these secretory antibodies has dire consequences leading often to intestinal pathology.

1.1.2.3 Antigen presenting cells: Adaptive immune response initiate primarily by the recognition of foreign peptide bound to MHC molecules on the surface of an antigen presenting cell (APC). MHC class 1 molecules are present in nearly all nucleated cells in the body and present peptides from intracellular proteins whereas MHC class II molecules present processed extracellular antigens and expressed by the APCs. Dendritic cells, monocyte/macrophages, activated B cells and follicular dendritic cells belong to this category of cells. Dendritic cells (DCs) are the most potent antigen presenting cells which are a group of bone marrow derived leukocytes that are specialized for the uptake, transport and presentation of antigens to T cells. DCs are characterized by long, branched cytoplasmic processes making the surface area in contact with the environment very large. DCs have been suggested to sample luminal antigen from the small intestine by trans-epithelial extension of
their cytoplasmic process. [62] In the organized mucosal tissue, such as Peyer’s patches (PP) and colonic follicles, so called M cell deliver antigen from the lumen to the underlying dendritic cells. M cells are characterized by a very thin cytoplasm separating outside from inside and an apical surface with microfolds and short microvilli. In human colon the apical surface expresses carcinoembryonic antigen (CEA) and CEACAM1 potential receptors for microbial adhesion. [63] In addition to MHC class II molecule, dendritic cells also express CD80 and CD40, which can interact with their respective ligands CD28 and CD40L on the surface of T cells. Dendritic cells are present in increased numbers in the inflamed mucosa of both patients with IBD and in the animal models of IBD. [64]

Follicular dendritic cells (FDC) are special stromal cells residing in the primary and secondary lymphoid follicles in lymph nodes, spleen, tonsil and mucosa associated follicles. FDC are thought to be important in the initiation and maintenance of secondary antibody response and formation of germinal centres. [65] Monocytes/macrophages, important players in innate immunity (see above), represent different developmental stages of the same cell type. Monocytes circulate in the blood and in the lymph and when they settle in the tissue develop into macrophages. It is thought that these cells also have some antigen presenting capacity. In case of infection, macrophages move to the site of infection and they are capable of releasing a number of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, IL-12 and TNF-α.

1.1.3 Antimicrobial peptides:

Antimicrobial peptides are widely distributed in nature and are found not only in vertebrates, but also in invertebrates and even in the plant kingdom. Today about 800 different antimicrobial sequences have been identified. [66] They are short polypeptides containing less than 100 amino acid residues, and display a broad spectrum of antimicrobial activity. They are cationic molecule as the molecules are rich in histidine, lysine and arginine and also amphipathic, containing hydrophobic and hydrophilic regions. [67] Based on their composition and structure they are classified into four catagories: [68-70]

1. Cysteine-rich amphiphilic β-sheet peptides-such as α and β defensins.
2. Amphipilic α-helical peptides-such as secropins and LL-37.
3. Cysteine-disulphide ring peptides-such as renalexin, brevinine.
4. Linear peptides with one or two predominant amino acids—such as dipterin, apidaecin.

In mammals there are two main groups of antimicrobial peptides: defensins and cathelicidins. [71, 72] Other mammalian antimicrobial peptides, including histatins, dermicidin and ‘anionic peptides’ are restricted to a few animal species only. [72]

1.1.3.1 Defensins:

Defensins belong to a family of vertebrate antimicrobial peptides comprised of α-defensins, β-defensins and θ-defensins. [73, 74] The mature peptides share common features, including short polypeptide sequence (ranging from 18 to 45 amino acids), three intra-molecular disulphide bridges, a cationic net charge (ranging from +1 to +11) and a tertiary structure that is composed of three anti-parallel β sheets. All defensins are synthesized as prepropeptides and are processed to various degrees depending on the site of expression. [75] Based on their adjacent chromosomal location, similar peptide precursor and gene structure, it is likely that all vertebrate defensins arose from a common gene precursor. [76] The α- and β-defensins are distinguished from each other by the pairing of the six conserved cystein residues to form the three disulphide bridges. [73] Whereas in α-defensins the six cysteins are linked in a 1-6, 2-4, 3-5 pattern, in β-defensins the pattern is 1-5, 2-4, 3-6 (fig 1A-B). [77] Despite their differing covalent structures, the tertiary structure of α- and β-defensins are strikingly similar.

α-defensin genes are expressed in cells of myeloid origin and in Paneth cells. Human neutrophil granulocytes express four α-defensins (HNP1 to 4) and other two α-defensins (HD5 and HD6) are expressed by Paneth cells. The myeloid α-defensin genes are made up of three exons whereas the Paneth cell defensin genes are made up of two (fig 1-A). In Paneth cells α-defensin genes, the 5' untranslated region and the pre-prosegment is coded by exon 1 while in myeloid defensin genes this region is interrupted by an intron. The most distal exon encodes the functional peptide (fig 1A). The inactive precursors are activated by the post
**Figure 1.** Defensin genes and peptides. Left, alignment of α-defensin and β-defensin genes. Boxes marked with different filling represent signal peptide, propieces (pro-segment) and mature peptide. Right, three different disulphide ‘schemes’. Numbers above the diagrams indicate the disulphide connections in each defensin class. Adapted from Selsted et al. Nature Immunology 2005;6(6):551-7. [71]

Translational proteolytic removal of the anionic pro-segment, creating a cationic peptide that is microbiocidal. [78]

Generally, the β-defensin gene precursors are simpler, consisting of two exons and one intron only (fig 1-B). However, the hBD-5 gene is an exception consisting of three exons and two introns. In all but the hBD-1 gene, the first exon encodes the signal peptide and the second exon encodes the mature peptide preceded by a short anionic pro-peptide. In the hBD-1 gene, the first exon encodes the signal peptide and the pro-peptide segment.[76] The circular peptide, θ-defensins apparently evolved in primates, but are inactivated in human due to mutations that encode premature stop codon. [79]
Figure 2. Different models of antimicrobial killing of organism. (A) shows Barrel-stave model, (B) shows toroidal pore model and (C) shows carpet model. Hydrophilic regions of the peptide are shown in red colour; hydrophobic regions of the peptides are shown in blue. Modified from Brogden et al 2005 [81] with the permission of nature publishing group.

1.1.3.2 Mechanism of action:

Although all antimicrobial peptides have the same basic capability to interact with the bacterial membrane, they are known to inactivate bacteria by means of two different mechanisms, membrane disruption and non-membrane disruption. [70,80-81] Three mechanistic models, the ‘barrel stave’, ‘thoroidal pore’ or ‘micellar aggregate’ and ‘carpet’ models have been developed to explain membrane disruption.[72, 80-81]

In the barrel-stave model, (fig2-A) the attached peptides aggregate and traverse the membrane bilayer in a way that the hydrophobic peptide regions align with the lipid core region and the hydrophilic peptide regions form the interior region of the pore, which allows water and electrolyte leakage through the membrane. In the toroidal pore model, (fig2-B) the killing mechanism is similar to the barrel stave pore model; however, here the peptides are suggested to aggregate and induce the lipid monolayer to bend continuously thought the pore causing the water core to be lined by both the inserted peptides and the lipid head groups.

In the carpet model, (fig2-C) the bacterial membrane is disrupted by the peptides sitting parallel to the surface of the lipid bilayer forming an extensive layer of carpet. The positively charged peptides neutralize anionic lipid head groups of the membrane over a wide area.
around the peptide. This orientation leads to a local disturbance in membrane stability causing the formation of large cracks, leakage of cytoplasmic components, disruption of the membrane potential and ultimately disintegrate the membrane. [72, 81, 82] Figure 1 shows three different models of membrane disruption described here.

The observation that a proline-rich peptide Bac7 can kill the bacteria without disrupting the membrane initiated the possibility that AMPs might use other mechanisms rather than membrane disruption. [83,84] Brogden K et al 2005 nicely summarized the recent mechanisms described for the non-membrane disruption of antimicrobial killing.

1. Alters cytoplasmic membrane (inhibits septum formation)-PR-39, PR-26
2. Inhibit cell wall synthesis-Mersacidin
3. Inhibit DNA, RNA and protein synthesis-HNP-1, HNP-2, Buforin-II
4. Inhibit enzymatic activity-Histatins
5. Activation of autolysin-Pep5

A number of studies indicate that β defensin molecule binds to the negatively charged cytoplasmic membrane and disrupt their integrity, leading to a leakage of intracellular components and inhibition of the DNA, RNA and protein synthesis. [85-87] Although it has been clearly demonstrated how α-defensins can produce multimeric pores in artificial lipid bilayers, [88, 89] nowhere it is discussed which model explains the creation of these pores. The β defensin, hBD-2, disrupts the bacterial membrane via an electrostatic interactions with the polar head groups of the membrane probably destroying the membrane according to the carpet model.[85, 86] Supportive evidence that β defensins kill bacteria by a “carpet-like” mechanism came from a recent study by Bohling et al 2006 where they elegantly showed how hBD-3 molecules aggregated on the membrane to initiate the killing mechanism. [90]

1.1.3.3 Human defensins:

α-defensins:
The α-defensin family in human contains six members. These are the human neutrophil peptides 1-4 (HNP1-4), which are expressed predominantly by the circulating neutrophil, and the human defensins 5 and 6 (HD5-6) which are expressed by the Paneth cells in the small intestine. [91-93]. The HNP1-3 are constitutively expressed, and besides neutrophils they have been shown to be expressed by other leukocyte subsets such as NK cells, γδ T cells, B
cells, monocytes, macrophages and dendritic cells.[94, 95] HNP1-3, which differ from each other only in the first few amino acid residues account for 5-7% of the total neutrophil protein, whereas HNP-4 which has an amino acid sequence distinct from the other three comprises less than 2% of the total neutrophil proteins. [73] HNP1-4 are stored as mature peptides in the dense azurophilic granules of neutrophils where it constitute 30-50% of all azurophilic granules of neutrophils. [96] Besides being microbicidal, HNPs have potent antitoxin and antiviral activity and have been shown to be particularly effective against HIV-1 infection. [91, 97]

As mentioned above HD5 and HD6 are produced mainly by intestinal Paneth cells in small intestine but are also found in other tissues such as salivary glands, the female genital tract, and the inflamed large bowel.[98, 99] Unlike the HNPs, HD5 and presumably also HD6 are stored as proforms in the secretory granules of Paneth cells. Once they are secreted into the lumen, they are processed and cleaved into the active form by trypsin, also released from the Paneth cells. [100] Recombinant forms of the human enteric α-defensin have been shown to be active against variety of bacteria and fungi. [101, 102] Most functional studies of α-defensins have been conducted in vitro or in the mouse model. However, a recent paper described a cohort study of African adults where they showed that decreased HD-5 and HD-6 expression levels in jejunal biopsies were associated with an increased risk of infectious diarrhoea. [103]

**β-defensins:**

The first human β-defensin, hBD-1, was described in 1995. It was isolated and purified from hemofiltrates of patients with end stage renal disease. [104] hBD-2 and hBD-3 were first isolated from psoriatic skin lesions almost simultaneously, whereas hBD-4, -4, -6 were identified through genome analysis. [105-107] A recent comprehensive genome analysis revealed almost 40 potential coding regions for β-defensins. [108]

Table 2 lists major expression sites of human β-defensins (modified from Pazgier et al 2006)
Table 2. Human β defensins and their expression sites.

<table>
<thead>
<tr>
<th>Human β defensin</th>
<th>Expression Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The epithelial cells of respiratory and urogenital tract, trachea, uterus, pancreas, kidney, lung, prostate, placenta, thymus, testis, vagina, ectocervix, endocervix, fallopian tubes, gingival tissue, buccal mucosa and tongue, salivary gland, small intestine, conjunctiva, cornea, lacrimal gland, mammary gland, limb joints, astrocytes, microglia, meningeal fibroblast.</td>
</tr>
<tr>
<td>2</td>
<td>Skin, oral and pulmonary epithelia, conjunctiva, cornea, astrocytes, gut epithelia, epidermal and gingival keranocytes.</td>
</tr>
<tr>
<td>3</td>
<td>Oral, respiratory, gastrointestinal, urinary epithelia, fetal thymus, placenta, testis, esophagus, heart, neutrophils, trachea, skeletal muscle, jejunum, tonsils, skin</td>
</tr>
<tr>
<td>4</td>
<td>Testis, epididymis, gastric antrum, uterus, neutrophils, thyroid gland, lung, kidney, gingival tissues and primary keratinocytes</td>
</tr>
</tbody>
</table>

**hBD-1:** Six isoforms, ranging in size from 36 to 47 amino acid residues, were isolated from urine and hBD-1 consisting of 36 amino acid residues was found to be most biologically active. [109] A naturally occurring 36 amino acid hBD-1 peptide showed antibacterial activity at micromolar concentrations against Gram-negative bacteria (i.e. *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*), as well as against yeast *Candida albicans*. hBD-1 was less potent against the gram-positive bacterium, *Staphylococcus aureus*. [108-110] hBD-1 is constitutively expressed in small intestine and colonic epithelial cells and is not upregulated by proinflammatory cytokines and bacterial stimuli.[97, 111] hBD-1 is chemotactic for memory T cells and immature dendritic cells and this activity is provided by the binding of hBD-1 to a receptor, CCR6, present on these cells. [109, 112]

**hBD-2:** hBD-2, isolated from skin, was highly effective in killing Gram negative bacteria. [40] hBD-2 is not constitutively expressed in normal intestinal mucosa, but is strongly induced in response to infection or proinflammatory stimuli and under conditions of intestinal inflammation. [97, 111, 113] Upregulation of hBD-2 is thought to be mediated by binding of
transcription factors to binding sites at the promoter region of the hBD-2 gene; namely NF-κB, activator protein (AP-1), AP-2, NF-IL-6. [115, 116] It has been demonstrated that upregulation of hBD-2 in the intestine is mediated by Toll-like receptors (TLRs), especially by TLR4 and TLR2 through their interaction with PAMPs on micro-organisms. [117] Several studies have confirmed that bacteria-induced upregulation of hBD-2 in the intestinal epithelium is mediated via NK-κB and AP-1 pathway, [112, 114] while corticosteroid mediated upregulation of hBD-2 is NF-κB independent implying that hBD-2 has the potential to be regulated by different activation pathways. [118] Similar to hBD-1, hBD-2, posses the capacity to chemoattract memory T cells, immature dendritic cell and mast cells [119]

Moreover hBD-2 can stimulate mast cells to release histamine and to generate prostaglandin. [119, 120] Human defensins have different capacity to induce cytokines. hBD-2 is the most potent inducer of IL-6, IL-8 and IL-10 from the peripheral blood mononuclear cells. [121]

**hBD-3:** The hBD-3 gene was described by two different groups simultaneously. [122, 123] The tissue expression is listed in Table 2. Unlike hBD-2, hBD-3 shows salt-insensitive broad-spectrum antimicrobial activity killing both Gram-positive and Gram-negative bacteria and the yeast *Candida albicans*. Moreover, hBD-3 is highly effective against multi-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium* making it the most interesting β-defensin described so far. [105, 123] hBD-3 forms a dimer in the solution that possibly contribute to its potent biological activity. hBD-3 is induced by both Gram-positive and Gram-negative bacteria and by different cytokines including TNF-α, IL-1, IFN-γ. [105, 123, 124] The 5’ untranslated region of hBD-3 contains several consensus sequences for activator protein AP-1 response element, gamma interferon response element, and NF-IL-6 response elements, but not NF-κB sites. [105, 122, 123] In addition to its antimicrobial activities, hBD-3 has been shown to be a chemoattractant for T cells, immature dendritic cells, monocytes and mast cells. hBD-3 induces mast cell degranulation, mediates tissue remodelling and plays a role in fertilization. [123, 125-127]

**hBD-4:** hBD-4 is a salt-sensitive, inducible, broad spectrum antimicrobial peptide. Although selectively expressed in testis, it is present in other sites as well (Table 2). It is particularly effective against *Pseudomonas aeruginosa*, [128] and has been shown to be upregulated by bacteria. [128] Although proinflammatory cytokine induced upregulation of hBD-4 in colonic epithelial cell line used by our group and airway epithelia cell line used by Garcia JR et al
2001 did not show any upregulation of this defensin. [124, 128] A slight induction has been observed by Harder J et al 2004 in the primary keratinocytes when stimulated with IL-1β, IFN-γ and TNF-α. [129] It is chemotactic for monocyte and mast cells, but its action in mast cell degranulation is not as potent as that of hBD-3. [125, 128]

**Other human defensins:**

hBD-5 and hBD-6 were identified through the human genome sequence project and their expression were shown to be epididymis specific. [107] Both of these genes are situated at a site termed ‘epididymis-specific β defensin’ region on chromosome 8 adjacent to all other β-defensin genes. hBD-6 gene contains NF-κB consensus sequence and it was later identified in the lung. Synthetic hBD-6 was shown to have a strong antimicrobial activity against *E. coli* [130, 131] Apart from a growing number of proteins with strong homology to known defensins, called epididymis-specific secretory proteins (EP2/HE2), there are several other β defensins identified in the male reproductive tract. Although misleadingly called ‘epididymis specific’-they were later shown be expressed at other anatomical sites as well. Little is known about their biological function(s). [85]

**1.1.3.4 Defensin expression in IBD:**

hBD-1 is constitutively expressed in the normal intestinal epithelium and the expression is reported to be reduced [132, 133] or unchanged [97] in the colonic mucosa of ulcerative colitis (UC). In contrast, the epithelial expression of inducible hBD-2, 3 and 4 is heavily upregulated in inflamed colonic mucosa of UC. [98, 124, 134] The Paneth cell associated α-defensins, HD5 and HD6, were detected in a minority of healthy control colons, but were significantly increased and readily detectable in the UC colon samples. The source of these defensins is the metaplastic Paneth cells in the inflamed colon. [98, 135]

The expression pattern of intestinal α- and β-defensins in Crohn’s disease (CD) appears to be more complex than in UC. Colonic expression of HD5 and HD6 was slightly increased in patients with CD compared to controls, [98, 133] while their expression was unchanged or reduced in ileal epithelial cells of patients suffering from ileal CD. [98, 136] As Paneth cells
are the main source of these defensins, the results are consistent with the finding that the number of Paneth cell is increased in colon but decreased in ileum of CD patients. [137] According to Wehkamp and associates hBD-1 expression in colon is decreased in CD patients, and this reduction is more pronounced in patients with a NOD2 mutation.[132, 133] However, our group did not find a significant decrease in hBD-1 in colon of CD patients compared to controls. [98] The inducible hBD-2 defensin was expressed to a lesser extent in colon of CD patients in comparison to UC patients. [98, 134] A similar weak or reduced expression of hBD-3 and hBD-4 mRNA in both colon and ileum samples of CD patients was seen in our lab. Recently, it was discovered that the copy number of genes in the β−defensin cluster on chromosome 8p23.1 was highly polymorphic within the healthy population containing from 2 to 10 hBD-2 copies per genome (mean value ≈ 4). [138] Interestingly, colonic CD patients were shown to have on average three copies of this gene. [138] Thus, reduced β-defensin expression in colon of CD patients compared to colon of UC patients could be due to low copy number of the β-defensin gene. Furthermore, it was demonstrated that low gene copy number was associated with reduced production of hBD-2 mRNA in the colonic mucosa. [138] In a recent study, Nuding et al demonstrated that cationic extract from the colonic CD patients show diminished functional antimicrobial activity against intestinal bacteria which is consistent with the reported low β defensin expression in this disease. [139]

1.1.3.5 Mouse defensins:

One of the major differences in terms of defensin expression between mice and men is that mice do not possess neutrophil defensins. Instead mice have increased numbers of α-defensins in their Paneth cells. Moreover, the processing of the α−defensins is different. These differences are probably a consequence of differences in evolutionary pressure related to micro-organism exposure through food intake. [140]

**Mouse α-defensin:** Mouse α-defensins are called cryptdin (‘crypt defensin’), first described by Oullette et al in 1989. Seventeen different isoforms are known to be present in small intestinal Paneth cells. [141] Cryptdins appear to be equally active against both Gram-positive and Gram-negative bacteria with cryptdin-4 having the strongest antimicrobial activity *in vitro* among all mouse cryptdins investigated. [142] The cryptdin composition differs along the small intestine of the mouse. Interestingly, the most potent form is most abundantly
expressed in the distal ileum where there is greater exposure of bacteria due to reflux of colonic contents. [143] Mouse cryptdins are processed to their active form during granulogenesis and matrix metalloproteinase-7 (MMP-7, also called matrilysin) was found to be the convertase. [144] Knockout mice lacking MMP-7 cannot produce mature cryptdins and as a consequence succumb more rapidly and to lower doses of virulent *Salmonella typhimurium* than wild-type mice. [144] In contrast to human α-defensins, mouse cryptdins are processed to mature active form within the Paneth cells. [144]

**Mouse β-defensins:** *In silico* sequence analysis of the Celera mouse genome database identified 23 potential β-defensin genes. [145] Among them, five fully characterized murine β-defensins (mBD-1 to 4 and mBD-6) with different, but partially overlapping tissue distribution, have been described. [146] β-defensins are expressed in epithelial cells of various tissues. mBD-1 and mBD-3 were demonstrated in the intestine. mBD-1 is expressed constitutively, while the other mBDs were shown to be induced by bacteria, lipopolysaccharide or cytokines. [147-151] Consistent with this finding—the 5’UTR region of mBD-3 have been shown to possess NF-κB, interferon-γ and IL-6 binding site whereas in mBD-4, the NF-κB site is missing but they have IFN-γ and IL-6 binding sites intact. [149,152]

**Table 3. Percent amino acid homology of mouse and human β defensins.**

<table>
<thead>
<tr>
<th></th>
<th>hBD1</th>
<th>hBD2</th>
<th>hBD3</th>
<th>hBD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>mBD1</td>
<td>57.3</td>
<td>39.6</td>
<td>40.0</td>
<td>30.0</td>
</tr>
<tr>
<td>mBD2</td>
<td>41.1</td>
<td>34.3</td>
<td>35.8</td>
<td>25.8</td>
</tr>
<tr>
<td>mBD3</td>
<td>40.9</td>
<td>52.3</td>
<td>50.7</td>
<td>35.0</td>
</tr>
<tr>
<td>mBD4</td>
<td>42.6</td>
<td>57.1</td>
<td>42.8</td>
<td>27.4</td>
</tr>
</tbody>
</table>

Mouse defensins, especially mBD-3, display a broad-spectrum antimicrobial activity against gram-positive bacteria, gram-negative bacteria and fungi. [153-154] Moreover, mBD-2 and
mBD-3, like human β-defensins possess the ability to chemotactically attract immature dendritic cells and memory T cells through the chemokine receptor CCR6. [155-56] Moreover, mBD-2 has also been shown to activate immature dendritic cells into mature professional antigen presenting cells, these immunomodulatory functions of mBDs made them potent candidate for vaccine development to combat cancer and other relevant diseases. [155, 157]

1.2 Inflammatory bowel diseases

Inflammatory bowel disease refers to the two major chronic diseases that cause inflammation of the intestines: ulcerative colitis (UC) and Crohn's disease (CD). They are chronic, relapsing inflammatory diseases of the gastrointestinal tract. An estimated 1.4 million individuals in the United States and 2.2 million individuals in Europe suffer from IBD. [158] In Sweden the point prevalence for UC is around 250/100 000 inhabitants, and that of CD is 150/100 000. The aetiology of IBD is still unknown, but both conditions seem to be the result of still mostly uncharacterized environmental insult(s) in the intestine and the immunological response to these insults in genetically predisposed individuals.

The importance of genetic factors became evident when it was discovered that first-degree relatives have a 4-20 times higher risk of developing IBD than the general population. It was also found that disease concordance was higher in monozygotic twins than in dizygotic twins. [159] The concordance was most marked in CD, suggesting an important role of genetics in the development of this disease. Several IBD susceptibility genes has been identified (Table 5) [160-161] The first gene discovered to be associated with increased risk to develop CD was the NOD2/CARD15 (nucleotide binding oligomerization domain 2/caspase recruitment domain family member 15) gene. [162] The relative risk of developing CD in homozygous or in compound heterozygous individuals is 10 to 40 times higher than in the general population. [163] NOD2/CARD15 is a cytosolic protein involved in intracellular recognition of microbes by sensing a peptidoglycan fragment, muramyl dipeptide (MDP), present in the cell wall of bacteria. NOD2 is expressed in APC such as dendritic cells and macrophages and in intestinal epithelial cells. [164-165] The binding of MDP to NOD2/CARD15 activates the transcription factor NF-κB, which stimulates the transcription of multiple genes that encodes proinflammatory molecules. Mutations in the NOD2 gene cause defective binding of MDP resulting in reduced ability to
clear invasive bacteria [164] and reduced production of \( \alpha \)-defensins in both ileum and colon [166-167]

### Table 4. Major susceptibility loci showing linkage to IBD

<table>
<thead>
<tr>
<th>IBD locus designation</th>
<th>Cromosomal location</th>
<th>Diagnosis</th>
<th>Candidate gene within or near locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBD1</td>
<td>16q12</td>
<td>CD</td>
<td>NOD2/CARD15</td>
</tr>
<tr>
<td>IBD2</td>
<td>12q13</td>
<td>UC</td>
<td>VDR, IFN-( \gamma )</td>
</tr>
<tr>
<td>IBD3</td>
<td>6p13</td>
<td>CD, UC</td>
<td>MHC I and II, TFN-( \alpha )</td>
</tr>
<tr>
<td>IBD4</td>
<td>14q11</td>
<td>CD</td>
<td>TCR ( \alpha/\delta ) complex</td>
</tr>
<tr>
<td>IBD5</td>
<td>5q31-33</td>
<td>CD</td>
<td>IL-3, IL-4, IL-5, IL-13, CSF-2</td>
</tr>
<tr>
<td>IBD6</td>
<td>19p13</td>
<td>CD, UC</td>
<td>ICAM1, C3, TBXA2R, LTB4H</td>
</tr>
<tr>
<td>IBD7</td>
<td>1p36</td>
<td>CD, UC</td>
<td>TNF-R family, CASP9</td>
</tr>
<tr>
<td>IBD8</td>
<td>16p12</td>
<td>CD, UC</td>
<td>MUC-3</td>
</tr>
<tr>
<td>IBD9</td>
<td>3p26</td>
<td>CD, UC</td>
<td>HGFR, EGFR, GNAI2</td>
</tr>
</tbody>
</table>

Adapted from Newman B et al 2005, Bonen DK et al 2003, [160-161]

There are three mutations affecting a leucine rich repeat in NOD2 responsible for the bacterial recognition. The three mutations seem to have different effects on the risk of developing CD. One of the mutations is found in 25-35% of CD patients in the US of European origin and not at all in CD patients of Japanese- or other Asian origin. [161] The frequency of NOD2 mutations in Swedish Crohn-patients is lower that other parts of Europe. Mutations in the NOD2/CARD15 gene are particularly associated with Crohn’s disease in distal ileum and mostly with strictureting and penetrating disease. [168]

Significant differences in mRNA expression profiles compared to healthy individuals were found for 170 genes, 20% of which were common to both UC and CD. Among these genes, scaffolding proteins involved in epithelial integrity (DLG5), epithelial transporters (SLC22A4/5 and MDR1) and pattern recognition molecule such as TLRs together with NODs (CARD15 and more recently CARD4), are involved in host-microbe homeostasis and maintenance of intestinal barrier function. These findings together with studies in animal models where inflammation does not occur in the ‘germ free’ animals support the hypothesis that IBD might be the result of abnormal immune response towards commensal bacteria in genetically susceptible individuals.

Several environmental factors have been identified in relation to IBD development. For instance, use of anti-inflammatory drugs (NSAIDs) can lead to disease flare, early
appendectomy reduces the incidence of UC and smoking has been found to protect against UC but increases the risk of CD. [159]

In UC, the inflammatory process invariably involves the rectum and extends proximally in a continuous fashion but remaining restricted to the colon. Sometimes UC is limited to rectum only known as ulcerative proctitis. UC is essentially a disease of the mucosa. Macroscopic features of UC are a granular mucosal surface with occasional ulcerations. Early microscopic features of UC include mucosal congestion, oedema, microscopic haemorrhages, and diffuse inflammatory infiltrate in the lamina propria and variable loss of surface epithelium with ulcer formation. Suppurative necrosis of the crypt epithelium gives rise to the characteristic crypt abscesses, which appears as a dilated, degenerated crypts filled with neutrophils. As the disease progresses, the epithelium is disrupted, crypts are elongated and branched, goblet cells are depleted and in some case Paneth cell metaplasia is induced. In the advanced cases, UC is characterized by mucosal atrophy and heavy infiltration of inflammatory cells into both mucosa and submucosa. [169]

In CD, the affected area is commonly the ileum or ileocaecal region but CD can occur in any part of the digestive tract. One of the hallmarks of CD-pathology is the transmural inflammation involving all layers of the intestine. The inflammation is often patchy leaving healthy tissue between inflamed areas. Nodular swellings, fibrosis and ulceration of the mucosa lead to a ‘cobblestone’ appearance. Fistula and abscess cavities may form from the inflamed area into the peritoneal cavity, mesentery, or in retroperitoneal structures. Perianal fistula - a common presenting feature - may develop if the lesion involves distal rectum. Small superficial mucosal ulceration (apthous ulcers), mucosal and submucosal oedema, increased number of immune cells are the early microscopic features of CD. Key microscopic features are transmural nodular lymphoid aggregates accompanied by proliferative changes of the muscularis mucosa and nerves of the submucosal and myentric plexus. Noncaseating granulomas mostly in the submucosa are often present as well. [169]

**1.2.1 Clinical features and treatment modalities:**

*Ulcerative colitis:* The most characteristic clinical symptoms are frequent diarrhoea, gradually increasing amounts of fecal blood, and abdominal cramps. In severe cases general malaise, weight loss, and wasting is observed. A serious complication in acute colitis is ‘toxic
megacolon’, which may lead to perforation of colon if left untreated. In long-standing UC, progressive loss of peristaltic function and shortening of the colon often leads to impairment of normal intestinal function. Extra-intestinal manifestations include inflammation in the joints, eyes, skin, mouth and liver. In longstanding UC there is an increased risk to develop colon cancer. UC, depending on severity of the disease is treated with 5-ASA compounds, corticosteroids, azathioprine/6-mercaptopurin, intravenous cyclosporine, anti-TNF-antibodies or colectomy.

_Crohn’s disease:_ The symptoms of Crohn’s disease are highly variable depending on disease localisation, extent, and complications. The clinical features can be subdivided into inflammatory, stricturing and penetrating disease. The major symptoms are diarrhoea, mostly non-bloody, and abdominal pain. Narrowing of the gut lumen often leads to strictures, bowel obstruction, fistulisation and abscess formation. Extra-intestinal manifestations are similar to those of UC but more frequent. CD is treated with 5-ASA compounds, corticosteroids, azathioprine/6-mercaptopurin, Methotrexate, anti-TNF-antibodies or by surgical resection of obstructing segments.

### 1.2.2 Pathogenesis of IBD:

One of the major hypotheses in the pathogenesis of inflammatory bowel disease contends that primary dysregulation of the mucosal immune system leads to excessive immunologic responses to normal microflora. The other hypothesis postulates that changes in the composition of gut microflora and/or deranged epithelial barrier function elicit pathological response from the normal mucosal immune system. [170] Intestinal pathology in both UC and CD is caused by the overactivated innate (macrophage, neutrophil) and adaptive (T and B cells) immune responses due to the loss of tolerance to enteric commensal bacteria. [171-172]

The rapid response mediated by the innate immune molecules by releasing different cytokines initiates the development of adaptive immune response exerted by the T and B lymphocytes in IBD. T cells functions in the disease process can be classified as their excessive secretion of proinflammatory cytokines, defects in T cell programmed cell death (apoptosis) and defects in regulatory T cell function. [173]
Although there are increased number of activated T cells in blood and intestinal mucosa of both UC and CD patients, cytokine production profile is different in these two groups of diseases. The Th1 cytokine profile is prominent in Crohn’s disease which is associated with an increased production of INF-$\gamma$, and TNF-$\alpha$, IL-2, as well as increased Th1-inducing cytokine IL-12 production. [174] In contrast, mucosa of UC patients showed decreased Th1 cytokines. [175] Th2 immune response includes cytokines such as IL-4, IL-5, IL-10, IL-13 which support humoral immune responses. [176] The cytokine profile in UC has been more difficult to classify as a typical Th2 response, one of the reasons being the absence of an increased or even decreased IL-4 levels in UC which is a key member of Th2 cytokines. [173,175,177] In comparison to CD patients, T cells (CD4$^+$) secreted IL-10 has been shown to be heavily increased in UC. [175] In the colon, majority of these cells has been shown to be located in the basal lymphoid aggregates in the UC patients. [178] IL-10 is a regulatory cytokine and apparently it fails to regulate the proinflammatory cytokine secretion in the colon which is supported by a finding in animal model where IL-10 secreted by the regulatory T cells failed to inhibit IFN-$\gamma$ and TNF-$\alpha$ production in the colon. [179] Th2 cells provide more efficient help in the induction of humoral immune response than Th1 cells. As a result, increased amount of plasma cells and the presence of auto-antibodies is more prominent in UC than CD. [173,180] IgG2 antibodies, which are increased in CD is thought to be mediated by Th1 response whereas IgG1, IgG4 antibodies which are predominant in UC are a result of Th2 cytokine response. [181-182] IgA production is also increased in IBD but not as pronounced as IgG production. The percentages of IgA1 producing plasma cells were increased in both UC and CD, but the proportions of mucosal IgA1 secreting cells were significantly higher in UC than CD. [183] 50% of these IgA1 lacks the J chain, a hallmark for secretary immunoglobulins and significant down regulation of pIgA secretion is another feature of IBD. [180] Perineutrophilic cytoplasmic antibody (pANCA) is present in up to 70% of UC patients but its role in the pathogenesis of UC remains controversial. [174, 180] Other antibodies against microbial products such as anti-\textit{Saccharomyces cerevisiae} (ASCA), anti OmpC (directed against outer membrane porin C of E coli), anti flagellin antibody (CBir1) has been detected in Crohn’s disease patients but exactly how they cause tissue damage is not fully understood. [174] Oxidative agents produced by the respiratory burst have long been known to cause tissue damage in IBD. [184] Furrie et al 2004 demonstrated that the increased IgG produced in the IBD binds to the Fc receptors of PMN cells driving them into enhanced respiratory bursts releasing free radicals, which in turn cause extensive tissue damage. [185, 186]
Regulatory T cell mediated suppression of immune reaction and its absence or reduction leading to various autoimmune diseases is a well accepted immunological phenomenon. [187] Recently Makita et al 2007 showed the potency of CD4\(^+\)CD25\(^+\) Treg cells isolated from the lamina propria of mouse colon and how they suppressed the development of the CD4\(^+\)CD45RB\(^{\text{high}}\) T cell-transferred colitis in mice. [188] In human, it has been suggested by two different groups that the frequency of Tregs in the peripheral blood is significantly decreased in active UC in comparison to controls. [189-190] These cells were CD4\(^+\)CD25\(^{\text{high}}\) Foxp3\(^+\), the natural Tregs. In contrast to UC, this reduction was not observed in CD. [189] On top of that FOXP3 gene mutations have not been associated with CD when four mutations were analyzed in 93 CD patients and 108 normal individuals. [191] Although the decreasing Treg population was shown to be directly associated with the enhancement of colonic inflammation several studies including observation in our group describes a manifold increase of Foxp3\(^+\)CD4\(^+\)Treg population in the colonic tissue of UC patients. (Basel Sitohy, unpublished data) [176,188] These cells have been isolated from the colonic samples of UC patients and shown to have suppressive activity both on effector T cell proliferation and cytokine secretion by them. [176,188] It has also been shown that this increase in Treg cells in the UC colon is significantly lower compared with diverticulitis or infectious enteritis colon. [190] May be that the Tregs are not sufficiently increased in the UC colon in relation to the effector cells to suppress an immune reaction. Other possibility is that these Tregs are functionally impotent in the intestinal milieu where proinflammatory cytokines secreted by the effector T cells inhibit these cells to exert their function.

1.3 Animal models of IBD:

Animal models allow studies of disease mechanisms and have a major advantage that it gives the opportunity to investigate a particular molecule or disease process involved at any stage of disease especially in the early preclinical stage. IBD animal models can be subdivided in four categories: spontaneous models, inducible models in mice with normal immune system, adoptive transfer models in immuno-compromised hosts, and genetically engineered models (transgenic mice, knock-out mice). [192-193] Some of the commonly used mouse models of the different categories are presented in Table 5.
Either Th1-cytokines (IL-12, IFN-γ/TNF-α) or Th2-cytokines (IL-4/IL-5) invariably inflicts inflammation in these mice. With the exceptions of TCR-α chain deficiency colitis, TNBS colitis and oxazolone colitis the other colitis models seem to be inflicted by Th1-cytokines. [194]

Table 5. Some commonly used mouse colitis models

<table>
<thead>
<tr>
<th>Model</th>
<th>Pathology</th>
<th>Affected sites</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spontaneous model</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3H/HeJ Bir</td>
<td>Acute/chronic</td>
<td>Cecum, right colon</td>
</tr>
<tr>
<td></td>
<td>transmural</td>
<td></td>
</tr>
<tr>
<td><strong>Inducible model</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextran sulphate sodium (DSS)</td>
<td>Acute/chronic</td>
<td>Colon</td>
</tr>
<tr>
<td></td>
<td>mainly mucosal</td>
<td></td>
</tr>
<tr>
<td>Trinitrobenzene sulfonic acid (TNBS)</td>
<td>Acute/chronic</td>
<td>Colon</td>
</tr>
<tr>
<td></td>
<td>transmural</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acute/chronic</td>
<td>Distal colon</td>
</tr>
<tr>
<td>Oxazolone</td>
<td>Mainly mucosal</td>
<td></td>
</tr>
<tr>
<td><strong>Adoptive transfer model models</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD45RB hi transfer into SCID or RAG deficient mice</td>
<td>Acute/chronic transmural</td>
<td>Colon, duodenum</td>
</tr>
<tr>
<td><strong>Genetically engineered or transgenic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 knockout mice</td>
<td>Acute/chronic</td>
<td>Colon</td>
</tr>
<tr>
<td></td>
<td>mucosal</td>
<td></td>
</tr>
<tr>
<td>IL-10 knockout mice</td>
<td>Chronic transmural</td>
<td>Colon, sometimes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>jejunum/ileum</td>
</tr>
<tr>
<td>STAT4 transgenic</td>
<td>Acute/chronic</td>
<td>Colon, ileum</td>
</tr>
<tr>
<td>TCR-α knockout</td>
<td>Chronic transmural</td>
<td>Colon</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Summarized from</strong> [193-194]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Regardless of which type of cytokines that are involved in the disease process, an important driving force of the inflammation is micro-organisms of the commensal flora since disease is not developed in most models if the animals are kept under germ-free conditions. [194-195]

**1.3.1 Colitis in IL-2 mice:**

IL-2 is an important growth and activation factor for T cells following stimulation by antigens and also functions to promote T cell survival. [196] It also function to limit the immune response by promoting growth and survival of regulatory T cells. IL-2−/− mice accumulate
activated T cells and when raised in a conventional environment develops a haematopoietic-
and immune system disorder characterised by lymphadenopathy, bone marrow infiltration, splenomegaly and haemolytic anaemia. A significant fraction of the mice dies at an early age
due to this autoimmunity. [197] IL-2\(^{-/-}\) mice that survive this initial phase develop a colitis
that is highly reminiscent of UC in humans with diarrhoea, rectal bleeding, and generalized
wasting. [198] Progressive colitis develops between 6-15 weeks of age. [196]

Colitis in IL-2\(^{-/-}\) mice shows the following characteristics:
1. The peripheral T cell response is dysregulated and it is CD4\(^{+}\) T cells producing Th1
cytokines that mediate disease. CD8\(^{+}\) T cells and B cells seem not to be involved in the
disease process. [199-210]
2. IL-2\(^{-/-}\) mice fail to limit any given immune response. [202-203]
The importance of Tregs in keeping the immune reaction under control has been discussed
earlier in this thesis. It has been shown that: a) IL-2 is needed for the development of Tregs in
thymus; b) IL-2 is required for peripheral expansion and maintenance of Tregs; c) IL-2 is
critical for the function of Tregs.
3. Activated T cells in IL-2\(^{-/-}\) mice fail to undergo activation induced cell death (AICD), a
process that limits the magnitude of T cell expansion through programmed cell death. [204]
4. The intestinal micro-flora is important for developing colitis in IL-2\(^{-/-}\) mice [205] but
specific-pathogen-free (SPF) IL-2\(^{-/-}\) mice still develop colitis. A milder and delayed form of
colitis has been reported. [206] The authors hypothesized that non-viable luminal bacterial
fragments present in the food might have caused the disease. Commensals appear to differ in
their colitogenic capacity. Thus, E. coli mpk generated colitis but B. vulgaris mpk did not. [189]

1.3.2 Colitis in DSS-induced mice:
Among the chemically induced mouse models of colitis, DSS-induced colitis appears to be
the favoured model. An important feature of DSS is that it disrupts the epithelial cell barrier
and thereby increases host cell exposure to the normal mucosal micro-flora. [207] Acute and
chronic DSS colitis models can be reproducibly induced depending on dose and duration of
DSS treatment. [208-210] Clinical signs of disease are: diarrhoea, gross rectal bleeding, and
weight loss. Microscopic changes in DSS colitis include epithelial erosion, mucosal
inflammation, and ulcers with subsequent destruction of crypt architecture of the colonic
mucosa. [208,211] It is known that factors like sex, genetic background of the mice and
exposure to intestinal micro-flora affects the site and level of inflammation in DSS-induced mice. [212-213] Kitajima et al have shown that intestinal mucosal permeability increases after DSS treatment. Moreover, administration of DSS to mice lacking intestinal trefoil factor, a protein important for maintenance and repair of the epithelial layer, leads to a far more severe colitis than observed in normal mice. [214] A similar result has been reported for villin, a protein required for the structural integrity of brush border. [215]

The pathogenesis of DSS-induced colitis is still unclear, but several factors including direct toxicity to the epithelium, invasion of commensal bacteria, mucosal inflammation with an influx of macrophages and granulocytes, changes in the T cell activation status and immunoglobulin production are currently believed to be important in causing disease. [208,216] It is believed that macrophage derived cytokines (TNF-α and IL-6) inflict the preliminary damages as colitis can be induced in mice lacking T cells, B cells, and NK cells. [217-218] In the later phase of the disease process, CD4+ T cells, secreting Th1/Th2 cytokines, play an important role. [218-219] As so elegantly put by Strober et al: “DSS induced colitis is a T cell mediated inflammation superimposed on macrophage induced inflammation” [194]

Both mouse models point to key roles of the colonic micro-flora, the integrity of the epithelial barrier, and an unbalanced hyperactive T lymphocyte in the pathogenesis of mucosal inflammation.

2. AIMS OF THE THESIS

The general aim of this thesis is to gain further insight into the patho-physiology of IBD. The role of defensins in IBD is still not clear. Significant improvement has been achieved in this field over the last few years, but there is still a lot more to be learnt. Identification of increased expression of defensins in the epithelial cells of UC patients in our lab led us to ask questions like: Are the other cellular sources of defensins in the intestine? Is the expression related to the level of inflammation? Furthermore we asked the question: what is the role of IL-2 for immune regulation and for establishment of inflammation in the intestinal mucosa?
Specific aims:

- To characterize the defensin-expressing cells in lamina propria of colon mucosa in IBD patients and controls
- To investigate the expression pattern of α- and β-defensins in three murine IBD models (IL-2 KO and acute and chronic DSS-induced colitis) in order to determine the relationship between defensin expression and intestinal inflammation
- To study the effect of IL-2 gene dosage on intestinal T cell functions and establishment of inflammation in small and large intestine

3. METHODOLOGY

Methods used for paper I-IV are described in detail in the respective “Material and Methods” sections. The following methods were used in this thesis work:

1. Isolation of epithelial cells, intraepithelial lymphocytes and lamina propria lymphocytes from intestinal tissues (I-IV)
2. Extraction of RNA, DNA, in vitro transcription, cloning and sequencing (I-II)
3. Real time quantitative RT-PCR (I-IV)
4. Generation of DIG-labelled probes and in situ hybridization (I, II)
5. Immunohistochemistry (I-III)
   A) Two-color immunofluorosence staining (I, IV)
   B) Indirect immunoperoxidase staining (I-IV)
6. Immunoelectron microscopy (I)
7. Two-color immunoflow cytometry (III-IV)
4. RESULTS AND DISCUSSION

4.1 Paper I. Human colonic plasma cells produce β-defensins

Epithelial cells have long been known as the major source of β-defensins. Previous finding in our laboratory showed increased mRNA expression of hBD-2, hBD-3 and hBD-4 in colonic epithelial cells of UC patients. [98, 124]

Question: Are there other cellular sources than epithelial cells for β-defensins in the human intestine?

Answer: In situ hybridization using antisense probes for hBD2, hBD-3 and hBD-4 mRNAs on colonic tissue sections of UC patients, CD patients and controls revealed positive staining of cells in the lamina propria. These individual positive cells were located outside of the lymphoid aggregates [178] in UC colon or the lymphoid follicles. The numbers of positive cells were higher in UC colon than the control colon.

Question: Which specific cell type(s) expressed β-defensins?

Answer: To answer this question, we performed in situ hybridization and immunohistochemistry on consecutive tissue sections of UC colon using: a) anti-CD138 mAb for plasma cells; b) a mixture of anti-CD19/anti-CD20/anti-CD22 mAbs for identification of B cells; c) anti-CD3 mAb for T cells; d) KiM4 mAb for follicular dendritic cells; e) anti-CD68 mAb for macrophages. Figure 1 in paper I shows the typical staining pattern with these reagents, where it is obvious that only the plasma cells staining corresponded to the in situ hybridization with the antisense reagents for hBD-3 and hBD-4 mRNAs.

Question: Can β-defensin peptides be identified in plasma cells?

Answer: To address this question we performed two-color immunofluorescence on UC colonic tissue sections with immunoabsorbant-purified anti-hBD-2 antibodies and anti-CD138 mAb. Figure 2 in paper I shows that such double stained cells indeed are present in lamina propria of UC colon. We then performed immunoelectron microscopy (IEM) using the anti-hBD-2 antibodies and anti-CD138 mAb. The IEM with anti-hBD2 antibodies revealed staining of rough endoplasmic reticulum, the Golgi complex, and small secretory vesicles indicative of an active process of synthesis and transport of this molecule inside plasma cells. IEM with anti-CD138 mAb showed staining of cells with typical plasma cell appearance i.e. increased
cytoplasm to nuclear ratio, prominent amount of rough endoplasmic reticulum and secretory vacuoles (Figure 4 in paper I)

Question: What types of plasma cells secrete \( \beta \)-defensins? Is it IgA-secreting or IgG-secreting plasma cells or both types?

Answer: We performed immunofluorescence double staining using antibodies against IgA or IgG and hBD-2. These experiments revealed that most of the plasma cells of both categories (IgA and IgG) could produce the \( \beta \)-defensin. There was no statistically significant difference in the fraction of defensin positive plasma cells irrespective of whether the colonic plasma cells were from UC patients, CD patients or controls (Figure 3 and 5 in paper I). The major difference between the three groups were that plasma cells were clearly more abundant in UC colon compared to CD or control colon; thus indirectly making \( \beta \)-defensins more prominent in UC colon.

Question: Do human plasma cell lines express hBD-2?

Answer. Quantitative RT-PCR revealed that three out of four well-established myeloma cell line expressed mRNA for hBD-2 (Table 1 in paper I).

\( \beta \)-defensins are potent antimicrobial agents. Having \( \beta \)-defensins also in the lamina propria must be considered as an advantage in fighting microbial attack. From our study, it is evident that \( \beta \)-defensins are more abundant in UC colon than in CD or control colon. In UC, luminal micro-organisms most likely manage to reach underneath the epithelial cell layer due to leakage of the epithelium. Probably, \( \beta \)-defensins produced by plasma cells in lamina propria constitute an additional line-of-defence.

All three \( \beta \)-defensins identified in plasma cells are inducible. However, it is not clear how they are induced in colonic plasma cells, whether induction is mediated by the cytokines or by bacterial products or both as these defensins posses different activator sites at their 5´UTR. We speculate that the hBD-2 expression in plasma cells of the UC colon is mediated by the TLRs signalling pathway. TLR activation of NF-\( \kappa \)B and subsequent upregulation of hBD-2 is well documented in the intestinal context as well as in other epithelial sites of the body. [220] A recent paper describes multiple TLRs expressed in CD138\(^+\) plasma cells isolated from bone marrow aspirates of multiple myeloma patients as well as in the three different human plasma cell lines that we have investigated. [221] Taken together, these data are highly indicative of similar TLR positive plasma cells presence in colon.
From the immunoelectron micrographs, we found evidence for β-defensin synthesis in colonic plasma cells. Furthermore, the specific staining of small vesicles in the cytoplasm is indicative of an active transport process suggesting that the β-defensins are released from the cells into the surrounding intercellular space. Presently, we do not know if the synthesized defensins are ‘ready to use’ molecules or if they need further processing to become active mature forms. As discussed in a previous section, post-translational modification of β-defensins is not as well understood as for α-defensins. In β-defensins it has been shown that a variable number of amino acid residues are removed from the proform probably in order to create diversity. [75] The finding that both salt-sensitive defensins (hBD-2, hBD-4) and a salt-insensitive defensin (hBD-3), having diverse antimicrobial activities, are elaborated by colonic plasma cells, probably make plasma cells excellent defenders of the mucosa. In this context it is worth remembering the plasma cells also produce antibodies and that in UC much of the antibody activity is probably directed against microbial components. An interesting question posed by our work is: Do β-defensins and antibodies cooperate in some way to augment microbial defense efficacy?

Phagocyte recruitment to the site of inflammation is an important and early event that occurs in the innate defense of the host against microbes. hBD-3 and hBD-4 have been shown to chemoattract monocytes/macrophages and hBD-2 to chemoattract neutrophils to the site of inflammation. In the latter case the effect is indirect and appears to work through factors released by activated mast cells. [75]

4.2 Paper II. Increased expression of β-defensins is a consequence of chronic inflammation

Both α- and β-defensins are expressed in increased amounts at the epithelial surface of human intestine in patients suffering from UC. The question arose whether increased defensin expression is a consequence of chronic inflammation or whether it precedes intestinal inflammation. To dichotomize this issue, we analyzed β-defensin (mBD-3, mBD-4) mRNA expression in three mouse models of colitis, the IL-2 knockout mouse and the DSS-induced colitis mouse. In the case of the DSS-mouse model both an acute and a chronic form were studied.

We divided the IL-2 knockout mice into four different categories based on the level of intestinal inflammation. The first group was 5 weeks old IL-2−/−, which had not yet developed
any symptom of disease. The second group was 15 weeks old IL-2^{−/−} mice, which had pronounced symptom of disease. The third and the fourth groups of animals were IL-2^{+/−} and IL-2^{+/+} mice. These mice were apparently healthy. In case of DSS-induced colitis we generated two separate groups of animals one with acute inflammation and another with chronic inflammation according to standard protocols.

The questions that we answered in this works were:

1) What are the mRNA expression levels of defensins in acute and chronic colitis models of inflammation? Does it differ in acute and chronic colitis?

2) Is the finding of increased level of defensin expression observed at mRNA level can be confirmed at the protein level in both of this mouse models?

*In situ* hybridization using antisense probes for mBD-3 and mBD-4 mRNAs showed a strong positive signal in colonic epithelial cells of IL-2^{−/−}15w sick mice. In contrast, no or very faint hybridization signals were seen in the other three groups of animal *i.e.* mice with no visible colonic inflammation (IL-2^{−/−}5w, IL-2^{+/−}, IL-2^{+/+} mice) (*Figure 1 in paper II*). To obtain a quantitative estimate of the defensin mRNA expression levels in the different groups, we performed qRT-PCR analysis on mRNA extracted from freshly isolated epithelial cells from the colon of all four groups of mice. We found a significantly higher level of mRNA expression in the sick IL-2^{−/−}15 w mice in comparison to the other groups of animals (*Figure 2 in paper II*). These findings were confirmed by immunohistochemistry at the protein level using an anti-mBD-3 antibody. Thus, prominent staining of epithelial cells were seen in the diseased IL-2^{−/−}15 w mice but no or only faint staining in the epithelium of the other groups of mice (*Figure 4 in paper II*).

To investigate whether the β-defensin expression indeed was a consequence of chronic inflammation we performed the same type of experiments as described above quantifying mBD-3 mRNA and also investigated expression of mBD-3 protein in DSS-induced acute- and chronic colitis. We found a progressive increase of mBD-3 mRNA from acute to chronic colitis. Thus, in acute DSS-colitis model there was moderately increased expression of mBD-3 in comparison to controls, in contrast to a highly significant increase in chronic DSS-colitis. In line with this mBD-3 protein was prominently expressed in epithelial cells in chronic DSS-colitis (*Figure 3 and 4 in paper II*).
Taken together, these data indicate that the expression of β defensins is indeed related to the chronicity of inflammation. We found a gradual increase in β-defensin expression in mice from acute to chronic inflammation. The increase of mBD-3 and mBD-4 expression in chronic disease shown in our study could be the consequence of either binding of microbial components to TLRs on the epithelial cells or the effect of induction by proinflammatory cytokines or both. The 5’UTR region of mBD-3 has NF-κB, IFN-γ and IL-6 binding sites and mBD-4 has interferon-γ, IL-6 binding sites but no NF-κB site. [149-150] We believe that upregulation of β-defensin expression via the NF-κB pathway initiated by binding of microbial products to TLRs on epithelial cells would be a fast process. However, we did not observe significant upregulation of mBDs in the acute DSS colitis or in the 5 weeks old IL-2−/− mice. In contrast, significant upregulation of defensin expression was seen in animals suffering from long-standing inflammation. Moreover, it has been shown both in the chronic DSS model and in the IL-2−/− mice, that proinflammatory cytokines (IL-1, IL-6, TNF−α and IFN-γ) from macrophages and T cells progressively increase in the colonic tissue. [201,210] Therefore it is more likely that the observed increased expression of both mBD-3 and mBD-4 is the consequence of duration of inflammation and increased expression of these cytokines.

4.3 Paper III. Colonic T cells in IL-2+/− mice show attenuated function and reacts inadequately in DSS challenge

Despite their apparent health, IL-2+/− mice express reduced amounts of IL-2 in their peripheral T cells, thymocytes and large intestine. [222] We have analyzed intestinal T cell function in this group of mice after challenging with DSS. When IL-2+/− and IL-2+/+ mice were exposed to 3.5% DSS in their drinking water, the IL-2+/− mice displayed reduced and delayed clinical symptoms in comparison to IL-2+/+ mice. Clinical symptoms were measured as rectal bleeding, loose stools and diarrhoea (Figure 2 in paper III). The histological changes at day 6 of DSS-treatment were in agreement with the clinical symptoms. Thus the total histological score in IL-2+/− mice was significantly lower than in IL-2+/+ mice affirming that overall inflammation is less pronounced in IL-2+/− mice group (Figure 4 in paper III).

That IL-2+/− mice are less susceptible to DSS than IL-2+/+ mice was puzzling keeping in mind that these mice produce reduced level of IL-2. We assumed that it must be the T cells that are not functioning properly. So several questions popped up after this finding namely, is the T cell less proliferative in IL-2+/− mice, does the total number of T cell reduces due to the lack of
IL-2 or is there any alteration in the regulatory T cell number. To study this phenomenon further we analyzed the proliferative response of splenic T cells in untreated IL-2<sup>+/−</sup> mice and found that it was reduced compared to wild-type mice (Figure 1 in paper III). Furthermore, the number of regulatory T cell (CD4<sup>+</sup>/CD25<sup>+</sup>) in the intestine was reduced in IL-2<sup>+/−</sup> mice compared to IL-2<sup>+/+</sup> mice (Table 1 in paper III). After DSS exposure, both groups of mice had increased level of macrophages and granulocytes but the frequency of total number of T cells, both CD4<sup>+</sup> and CD8<sup>+</sup>, were significantly reduced in the colon of IL-2<sup>+/−</sup> mice as compared to IL-2<sup>+/+</sup> mice (Figure 5 in paper III). We then measured IL-2-, INF-γ-, IL-4-, and IL-10- mRNA expression levels in colonic T cells isolated from the two different groups of mice before and after DSS-treatment. qRT-PCR analysis revealed that all four cytokines were expressed at much lower level in the IL-2<sup>+/−</sup> mice than the IL-2<sup>+/+</sup> type after 6 days of DSS exposure. In particular, the IL-4 expression in the T cells of IL-2<sup>+/−</sup> mice was heavily upregulated but there was no change in this cytokine expression in the IL-2<sup>+/−</sup> mice.

Taken together, these data indicate that T cell functions are “crippled” in IL-2<sup>+/−</sup> mice making the animals less susceptible to DSS exposure. Since the net result of an immune response at a given time point is determined by the balance between positive and negative forces e.g. by active immune cells producing cytokines, cytotoxic cells and regulatory T cells we believe that low IL-2 levels preferentially affect effector cells.

4.4 Paper IV. Influence of IL-2 gene dose levels on T cell functions in the small intestine

The observation that the small intestine appears macroscopically and histologically normal in IL-2<sup>−/−</sup> mice despite the well-documented findings that colon is severely inflamed lead us to investigate the T cell status in small intestine in relation to gene dosage of IL-2. In particular, we wanted to know if the cytokine profile was altered and if the T cell response to DSS-treatment was affected. We also measured the length and weight of the small intestine of IL-2<sup>−/−</sup>, IL2<sup>+/−</sup>, and IL-2<sup>+/+</sup> mice.

IL-2<sup>−/−</sup> mice had on average 15% longer small intestine than the wild type mice. Heterozygous (IL-2<sup>+/−</sup>) mice also had a longer small intestine, although not as long as the IL-2<sup>−/−</sup> group. In contrast there was no difference in small intestinal weight between the three groups. There was a statistically significant linear trend in increase of intestinal length relative to dysfunctional IL-2 alleles. The same was true when the ratio of length/weight was calculated in relation to dysfunctional IL-2 alleles (Figure 1 in paper IV). However, histological
examination of these small intestines revealed that all of them including samples from the IL-2−/− mice were normal in appearance (Figure 1 in paper IV). Quantitative mRNA analysis of purified T cells from the small intestine revealed that the IL-10 mRNA levels were increased with a statistically significant linear trend relative to number of dysfunctional IL-2 alleles with a mean level 17 times higher in IL-2−/− mice compared to wild type mice (Figure 2 in paper IV). As expected, IL-2 mRNA levels decreased with number of dysfunctional IL-2 alleles and IL-2−/− mice expressed no IL-2 mRNA. For IL-4 mRNA and IFN-γ mRNA we found no clear trends.

Previous studies have shown that short time DSS-treatment of IL2+/− mice gives a milder colonic inflammation compared to wild type mice treated in exactly the same way (Sund et al., 2005). While in wild type mice DSS-treatment increased the frequency of T cells in the intestinal leukocyte fraction no such increase was seen in IL2+/− mice after DSS-treatment (Sund et al., 2005). Moreover, in that study it was found that isolated colonic T cells from DSS-treated heterozygous mice expressed lower levels of cytokine mRNA than wild type mice exposed to DSS. Interestingly, however, the decrease was small and not statistically significant for IL-10 mRNA. Performing the same type of cytokine analysis on isolated small intestinal T cells from DSS-treated heterozygous and wild type mice we again found decreased mRNA expression levels in the heterozygous mice compared to wild type mice and for all four cytokine mRNAs the decrease was statistically significant.

IL-10 is a potent immunoregulatory cytokine. To ascertain that the CD4 positive T cells were producing IL-10 we performed two color immunofluorescence staining using antibodies against IL-10 and CD4. We found that the majority of the IL-10 producing cells were CD4 positive T cells (Figure 3 in paper IV). Both natural Treg and Tr1 cells can secrete IL-10. To find out which type of regulatory T cells produce IL-10, we performed dual color immunoflow cytometry using monoclonal antibody against Foxp3 and CD4 (Figure 4 in paper IV). We found a small proportion of Foxp3+CD4+ T cells in the wild type mice that was even less frequent in the IL-2+/− mice, and absent in IL-2−/− mice. This is consistent with previous finding that IL-2 is needed for the maintenance of the Foxp3 positive regulatory T cells population in the periphery.
Decrease or absence of regulatory T cells, is likely to lead to uncontrolled immune reactions causing pathology especially in organs such as colon which normally is exposed to a potentially harmful microflora. Why is the small intestine not diseased in IL-2 KO mice or in IL-2 heterozygous mice? One reason is probably that the small intestine only contains low numbers of microorganisms and therefore is exposed to a weaker immunological insult. Another may be that the effect of IL-2 gene dosage on T cells producing proinflammatory cytokines is more pronounced than on T-cells producing down-regulatory cytokines giving down-regulative forces the upper hand.

5. CONCLUSIONS

- Mature IgA and IgG producing colonic plasma cells can express at least three different β-defensins, hBD-2, HBD-3 and hBD-4
- Due to the increased number of plasma cells in colonic lamina propria of patients with ulcerative colitis the tissue will be particularly rich in defensins probably representing an innate immune response to invading microorganisms
- Chronic intestinal inflammation leads to increased expression of inducible β-defensins in epithelial cells
- β-defensin expression in chronic intestinal inflammation is probably induced by immune cell cytokines
- Inactivation of the IL-2 gene induces a T-cell dependent colitis. Unexpectedly loss of one IL-2 allele leads to decreased sensitivity to the noxious agent DSS and a milder colitis than in wild type animals exposed to DSS. T cells play an important role in these phenomena and we found that the regulatory T cell subtype composition appears to be tilted towards IL-10 producing Tr1 cells probably prohibiting strong proinflammatory effects particularly in the small intestine.
6. ACKNOWLEDGEMENTS

I would like to thank all present and former colleagues who have helped me to accomplish this thesis and all my friends and family for always being there for me during these years. In particular, I would like to pay my tribute to my former supervisor, Takeshi Matsunaga—a fine scientist and wonderful person—who taught me how to think science.

Åke Danielsson, my supervisor. Thank you for all the support that you have provided throughout these years. It is a privilege and I feel honoured knowing a man of your class. You are the greatest supervisor any student can ever have.

Sten Hammarström, my supervisor. I thank you from the bottom of my heart for all these years. I would not be here without your support. I am truly in debt to you for the support that you have provided for preparation of this thesis. Thank you for everything.

Marie-Louise Hammarström, my supervisor. Thank you for your endless support and enthusiasm. I never knew that you were ‘not’ my supervisor on papers. I am so grateful to you that this was never an issue in our lab. This is a great feeling that I can come to you at any time knowing your busy schedules.

Vladimir Baranov, I have always considered you as a great teacher. I thank you so much for all the practical helps that I got from you during staining. It is great to have you around when ever one needs scientific advice. I thank you for always listening to me.

Ivor Tittawella, thank you for the moral support that you provided me during the difficult times. I still remember with fond memories you discussing scientific matters with Takeshi—it seems only yesterday.

I thank my friend and co-author Malin Sund. Did I ever thank you before for bringing me back to immunology—perhaps not? I came to know Åke through you—and I thank you for that. It’s great to have a friend like you.

I thank my good friend and co-author Silvia Melgar. You are a great personality, I consider my self lucky to have a friend like you. For all the tips on DSS experiments and the samples that you sent—I am in debt to you. Not to mention the hospitality that you showed on our Gothenburg tour.

Anna Fahlgren, a former colleague and co-author, I thank you so much. My life in laboratory became easier, thanks to your perfectionism. Just that you know—every time I found the samples or other reagents in right place with proper marking—I thanked you.

Anne Israelsson and Marianne Sjöstedt, I do not have words to describe how grateful I am to you. For all these years, for all the support and help in the laboratory that I got from you—I thank you. You never let me feel like an ‘out sider’ although I come from a different culture. Marianne, special thanks to you for the last minute help with the experiments. Life could be miserable at the lab if you two were not here. Thanks for everything.

Lena Hallström-Nylen, thank you for your help with the administrative affair.
Prof. Torgny Stigbrand and his group members-I have always being jealous on you because of your relations to one another. Can it be any better? Thank you Torgny for your hospitality and all the good foods that we had at your place. I thank my good friend David Eriksson, a wonderful personality and also for keeping that part of the lab alive (and also Therese Lindgren).

Lucia Mincheva Nilsson, thank you for letting me use your microscope. I thank Anna Patoka, Ignacio and Olga at the clinical Immunology for being friendly with the neighbours at work.

To all the new members of Immunology, Kristina Lejon, Mia Sundström and Rifat Ekici-it is great to have you here so we can share our laughter during coffee breaks.

I thank Gangwei Ou; soon it will be your turn to write an acknowledgement.

I thank Lina, Veronika and Lotta, my labmates. I wish you good luck with your studies.

I am also grateful to my colleagues in cardiothoracic surgery for a wonderful time. My special appreciation to Hans Peter Ildgruben and Fredrik Holmner for their kind support during my tenure in thorax.

I thank all my friends at badminton. Guys, I would never tell it to you-but off the record, you are one of the few good reasons that keep me going on here in this exile.

To all my Bangladeshi friends-especially Niamat and Rupak with their families-it’s great to have good friend like you.

Dr. Aijaz Farooqi, whom I consider as my brother, thank you for all these years. I consider myself privileged to have you and your family as a friend.

Finally I would like to thank my family. Without their support and understanding-it would have been difficult to peruse a ‘doktorands’ life here. To my lovely wife Rimi-do they give a Nobel Prize for being a good companion….

My son Adnaan and my daughter Arminta-my life. Every time I look at you-I thank almighty for blessing me with a lovely family.
6. REFERENCES

5. Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nature medicine 2007;13(2):139-45.


49

50


