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IMMUNOLOGICAL MECHANISMS UNDERLYING INFLAMMATORY BOWEL DISEASE

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I came up with a good idea… see-through skin!

- Karl Pilkington

ABSTRACT

Ulcerative colitis (UC) and Crohn‘s disease (CD), collectively referred to as inflammatory bowel diseases (IBD), are characterized by aberrant immune responses in the gut, resulting in chronic intestinal inflammation. There is no cure for IBD, and the disease is increasing world-wide. The pathogenic mechanisms that drive disease onset and chronicity are currently unclear but seem to include a complex mixture of heritable pre-disposition and acquired factors.

The inflammation during IBD is characterized by a large influx of immune cells from the circulation to the inflamed intestine. This is mediated by the expression of chemokine receptors on the surface of the leukocytes, allowing them to migrate towards gradients of chemokines produced in the intestinal tissues. However, the mechanisms behind chemokine receptor-mediated leukocyte infiltration to the gut are poorly understood. The papers included in this thesis investigate the roles of $CD4^+$ T helper cells and monocytes, focusing on chemokine receptor interactions, in mediating intestinal inflammation during IBD.

Whereas the inductive mechanisms of chemokine receptors during colitis have mainly been studied in murine T cells, we set out to investigate blood monocytes in IBD patients. We found that the chemokine receptor CCR9, important for gut-homing in T cells, is expressed on a subset of monocytes that is increased during active IBD. Furthermore, we could show that a large number of chemokine receptors are up-regulated on IBD monocytes compared to healthy controls, as well as being differentially expressed between ulcerative colitis and Crohn’s disease. As UC and CD may be clinically similar, they are often difficult to distinguish. However, as optimal therapy choices for the respective disorders differ, correctly diagnosing IBD is crucial and thus, chemokine receptor profiling on blood monocytes constitutes a potential diagnostic approach.

In T cells, murine data suggests that CCR9 interactions are important for small intestinal homing whereas their role during colitis remains unclear. We have investigated $CD4^+$ T helper cells infiltrating the colonic mucosa during inflammation, and found that CCR9 is widely expressed on these cells, indicating importance role during human colitis. Furthermore, CCR9 expression levels were higher on T cells derived from un-affected compared to inflamed specimens, which might suggest that CCR9-positive cells have a regulatory function.

In conclusion, we have shown that chemokine receptor interactions are important for colonic immune responses during IBD. Understanding the complexity of the chemokine receptor system is fundamental to successfully targeting leukocyte migration pathways for therapeutic purposes.

LIST OF SCIENTIFIC PAPERS

I. Linton L, Karlsson M, Grundström J, Hjalmarsson E, Lindberg A, Lindh E, Glise H, Befrits R, Janczewska I, Karlén P, Winqvist O, Eberhardson M. HLA-DR(hi) and CCR9 Define a Pro-Inflammatory Monocyte Subset in IBD. Clin Transl Gastroenterol. 2012 Dec 20;3:e29.

II. Linton L, Jonsson Rolandsdotter H, Jones M, Jones P, Glise H, Eberhardson M, Finkel Y, Winqvist O. Chemokine receptor expression profiling of human blood monocytes in juvenile patients with inflammatory bowel disease. *Manuscript*

III. Janson PC, Linton LB, Bergman EA, Marits P, Eberhardson M, Piehl F, Malmström V, Winqvist O. Profiling of CD4+ T cells with epigenetic immune lineage analysis. J Immunol. 2011 Jan 1;186(1):92-102.

IV. Linton L, Jonsson Rolandsdotter H, Glise H, Finkel Y, Eberhardson M, Winqvist O. Promoter demethylation is associated with CCR9 expression on colon-infiltrating CD4+ T cells during juvenile inflammatory bowel disease. *Manuscript*

List of publications not included in the thesis:

Pieper J, Johansson S, Snir O, Linton L, Rieck M, Buckner JH, Winqvist O, van Vollenhoven R, Malmström V. Peripheral and site-specific CD4(+) CD28(null) T cells from rheumatoid arthritis patients show distinct characteristics. Scand J Immunol. 2014 Feb;79(2):149-55.

Karlsson M, Linton L, Lampinen M, Karlén P, Glise H, Befrits R, Janczewska I, Carlson M, Winqvist O, Eberhardson M. Naïve T cells correlate with mucosal healing in patients with inflammatory bowel disease. Scand J Gastroenterol. 2014 Jan;49(1):66-74.

Grundström J, Linton L, Thunberg S, Forsslund H, Janczewska I, Befrits R, van Hage M, Gafvelin G, Eberhardson M. Altered immunoregulatory profile during anti-tumour necrosis factor treatment of patients with inflammatory bowel disease. Clin Exp Immunol. 2012 Aug;169(2):137-47.

Eberhardson M, Karlén P, Jones P, Linton L, Jones Kostalla M, Lindh E, Odén A, Glise H, Winqvist O. Randomized, double-blind, placebo-controlled trial of CCR9-targeted leukapheresis in the treatment of ulcerative colitis patients**.**
Submitted manuscript

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
BAC	Bacterial artificial chromosome
CCR	Chemokine receptor
CD	Crohn's disease
CD-	Cluster of differentiation
CpG	Cytosine-guanine
CNS	Conserved nucleotide sequence
DC	Dendritic cell
DNA	Deoxy-ribonucleic acid
EILA	Epigenetic immune lineage analysis
FACS	Fluorescence-activated cell sorting
FOXP3	Forkhead box transcription factor P3
GALT	Gut-associated lymphoid tissue
GI	Gastro-intestinal
GMA	Granulocyte monocyte apheresis
HBI	Harvey-Bradshaw index
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IEL	Intra-epithelial cell
IFN-	Interferon
IL-	Interleukin
LFA-	Lymphocyte function-associated antigen
MACS	Magnetic-activated cell sorting
MAdCAM	Mucosal vascular addressin cell adhesion molecule
MALT	Mucosal-associated lymphoid tissue
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MS-SNUPE	Methylation sensitive single nucleotide primer extension
dNTP / ddNTP	deoxy-/dideoxy-nucleotide triphosphate
PBMC	Peripheral blood mononuclear cells
PCDAI	Pediatric Crohn's disease activity index
PCR	Polymerase chain reaction
PUCAI	Pediatric ulcerative colitis activity index

RA	Rheumatoid arthritis
RNA	Ribo-nucleic acid
TCR	T cell receptor
Th	T helper
TREC	T cell receptor excision circle
Treg	T regulatory cell
TSS	Transcription start site
UC	Ulcerative colitis

1 INTRODUCTION

The main function of the human immune system is to protect the host from disease, which may be brought upon by external pathogens such as bacteria, viruses or parasites. When triggered, the immune system may launch a broad repertoire of immune responses of varying complexity and specificity, which aim to eliminate the pathogen while minimizing damage to host tissue, and form an immunological memory that allows for a faster response upon pathogen re-encounter. The human immune system is generally divided into two arms, innate and adaptive immunity, that represent different layers of function as well as evolutionary origin. The role of the innate immune system is to quickly initiate a primary response upon the encounter of broad molecular structures that are expressed on pathogens (pathogen-associated molecular patterns; PAMPs) or debris derived from dead cells or dying tissue (danger-associated molecular pattern; DAMPs). These structures are recognized by pattern-recognition receptors (PRRs) expressed on the surface of the immune cell. Conversely, the cells of the adaptive immune system react towards peptide antigens in a highly specific manner, creating an immunological memory, which forms the basis for vaccinations. The adaptive immune system has been fine-tuned through evolution and follows a later time course than the innate arm – whereas inflammatory mediators are released by cells of the innate immune system within minutes after injury, the adaptive response generally takes days to become effective. Although the cellular composition, means of pathogen recognition as well as response time differ between the arms, they do act in close collaboration and the adaptive arm is highly dependent on the innate system in order to fully respond. The functional maturation of adaptive cells relies upon signals from the innate arm that are derived through the process of antigen presentation that typically occurs in the lymph nodes where cells from the innate and adaptive arms physically interact. The net result of the immune response, with regards to variables such as cellular composition and duration, is defined by a number of factors; including the surrounding cytokine milieu, the specific molecules that take part in the cell-cell interactions during antigen presentation, as well as the affinity/avidity of these interactions.

Many disease factors alter either the onset or the regulation of an immune response, resulting in impaired pathogen clearance and/or increased tissue damage. Inflammatory bowel disease (IBD), a chronic disorder of the gastrointestinal system, results from a complex combination of inherited and acquired traits. This thesis focuses on the role of innate monocytes (papers I and II) and adaptive T cells (papers III and IV) in mediating intestinal inflammation during IBD.

1.1 THE BIOLOGY OF THE T CELL

The basis of T lymphocyte immune responses lies on the diverse recognition repertoire of the T cell receptor pool to specifically eliminate peptide antigens, followed by the formation of an immunological memory.

Naïve T lymphocytes become activated during the antigen presentation process in the lymph node, where an antigen presenting cell (such as a macrophage or a dendritic cell) presents its bound antigen to the T cell which, upon recognition of the antigen by its T cell receptor (TcR) may clonally expand and thus, a population of T effector cells that specifically recognizes the initially presented antigen may exit the lymph node and travel the circulation in search for its cognate antigen. In the following sections, the fundamentals of $CD4^+$ T cell responses are outlined.

1.1.1 T cell development

1.1.1.1 Thymic maturation

Thymocytes, which are the progenitors to T lymphocytes, develop in the bone marrow but migrate through the blood stream to the thymus while still retaining some ability to differentiate to other hematopoietic cell types. In the thymus, the lymphoid progenitor cell matures to become a $CD4^+CD8^-$ or $CD4^-CD8^+$ naïve cell through the highly organized, step-wise thymic selection process, which is here described briefly. In the thymus, the T cell receptor is re-arranged on the DNA level through recombination of V(D)J segments, an enzymatic process that aims at altering the recognition sequence of the TcR to produce a T cell pool that theoretically recognizes any potential protein antigen.

The human T cell receptor repertoire is extremely diverse and may recognize an astonishing 10^9-10^{15} peptide sequences. Thus, it is of greatest importance to eliminate any T cell receptors that recognize endogenous antigens to prevent autoimmunity. During thymic maturation, only T cell progenitors that are non-reactive to self-antigens are permitted to enter circulation whereas self-reactive clones are dismissed through programmed cell death. This process, referred to as central tolerance development (as opposed to peripheral tolerance development that occurs outside the thymus) is for T lymphocytes composed of two steps, positive and negative selection.(Mueller) The purpose of positive selection is to only select T cells that recognize antigens presented in the context of MHC expressed on cortical thymic epithelial cells (cTECs). Based on the TcR-MHC binding affinity, as well as whether the TcR recognizes MHC class I or II, CD4 or CD8 (respectively) will be down-regulated and the T cell fate as a $CD4^+CD8^-$ or a $CD4^-CD8^+$ has now been determined. Following migration to the thymic medulla, autoimmunity is evaded during the negative selection process, where cells that recognize MHC-self peptide complexes are eliminated. Here, the affinity of the functional TcR is tested against MHC molecules expressed on medullary thymic epithelial cells (mTECs) and the naïve T cells that survive the selection processes may enter the circulation as recent thymic emigrants (RTEs).(Starr, Jameson et al. 2003)

1.1.1.2 Effector maturation

Given that the T cell encounters its antigen, the lifespan of a T cell will involve a series of functional and phenotypic changes, which are here described briefly. Recent thymic emigrants (RTEs) express markers such as CCR7 and CD62L that allow them to enter secondary lymphoid organs (SLOs) in search for their cognate antigens. During this time, survival signals may induce homeostatic proliferation of the RTE which is then considered a mature naïve T cell (T_{MN}), characterized by proliferative experience and reduction of T-cell receptor excision circles (TRECs) (Fink) The T cell will become activated in the lymph node upon encounter with an antigen presenting cell (APC) carrying its cognate antigen on an MHC class I (for $CD4^+$ cells) or II (for $CD8^+$) molecule. In order to clonally expand, the T cell needs at least three distinct signals: 1) the peptide presented in the context of MHC; 2) co-stimulation such as binding of CD28 on the T cell to B7 molecules of the APC; and 3) stimulation by external cytokines. The third signal plays a fundamental role in shaping the differentiation of the activating T cell into functionally distinct lineages (discussed in greater detail in section 1.1.2).

Upon clonal expansion, the daughter cells will give rise to a heterogeneous population consisting of highly proliferative central memory cells (T_{CM}) as well as effector-memory cells (T_{EM}; Figure 1).(Sallusto, Lenig et al.) T_{CM} express adhesion molecules (such as α4β7) and chemokine receptors (such as CCR9) allowing for blood vessel extravasation and entry into the tissues while retaining CCR7 and CD62L expression and are thus also capable of circulating SLOs; whereas T_{EM} cells are solely tissue bound and thus do not express lymph node homing markers. A terminal, tissue-resident differentiation stage referred to as T effector-memory RA-expressing (T_{EMRA}), characterized by low homeostatic proliferation and re-expression of the naïve T cell marker CD45RA have been described as well, although primarily within the CD8 compartment.(Sallusto, Geginat et al. 2004)

1.1.2 T helper cell lineages

Originally, T helper cell responses (investigated in papers III and IV of the thesis) were modeled as a dichotomy of either Th1 or Th2, described as separate lineages with distinct function as well as cytokine secretion profiles. In this model, the Th1 response (important for the cellular defense against intracellular bacteria) is characterized by production of IFN-γ whereas Th2 cells mainly produce the cytokines IL-4, IL-5 and IL-13 and play a role in the humoral response against extracellular parasites.(Mosmann and Coffman) Subsequent research has revealed additional phenotypes within the T helper cell lineage, such as T regulatory cells (Tregs) for suppressing immune responses, IL-17-producing Th17 cells, involved in host response against extracellular bacteria; as well as the comparatively recently discovered Th9 subset (characterized by IL-9 production) that has been attributed a role in mucosal immune responses.(Geginat, Paroni et al.) As the mechanisms behind T helper cell differentiation have unraveled, studies have challenged the initial notion that lineage commitment represents end-stage differentiation with no possibility to revert to opposing commitment pathways. Indeed, it has become increasingly evident that the majority of human

T effector cells are plastic in the sense that they, during optimal cytokine conditions, may co-produce cytokines of an opposing lineage or even switch commitment pathway.(Geginat, Paroni et al.) However, most studies investigating the potential of an effector T cell to switch commitment pathway have been carried out *in vitro* and/or in the murine setting, and the relevance of these investigations for T cell responses in the human tissues need to be specifically addressed.

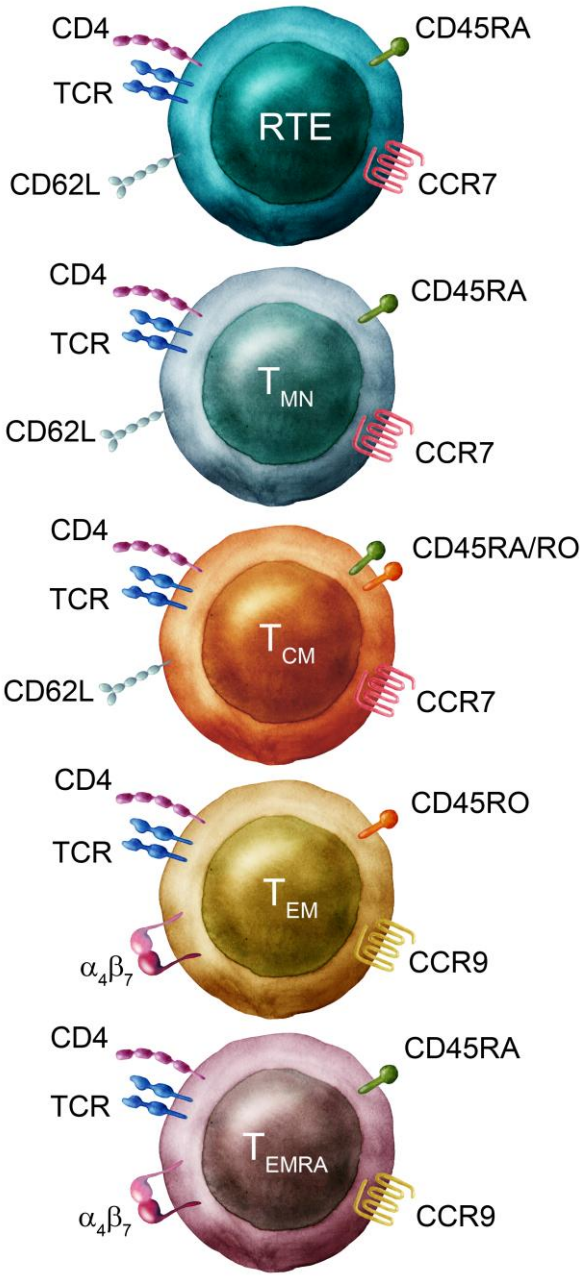

Figure 1. T helper cell maturation stages. Adapted and modified from Smids and colleagues, 2015.

1.1.2.1 T helper cell lineage stability

Whereas the ability of an antigen-experienced T helper cell to switch differentiation pathways during optimal conditions is now an established feature, the extent of lineage plasticity seems to vary between pathways as well as stage of differentiation.(Sallusto, Geginat et al.) Grogan and colleagues elegantly demonstrated that a naïve T cell co-expresses *Th1* and *Th2* cytokines (*Ifng* and *Il4*, respectively) and that several replication cycles are needed for the expanding cell to become completely selective for either *Th1* or *Th2*. During stable commitment observed at later cell divisions, the silenced alleles were physically repositioned to the heterochromatin and thus un-accessible for the transcription machinery.(Grogan, Mohrs et al.) The lineage stability of regulatory T cells is debated, much owing to difficulties in separating thymic Tregs (tTregs), that constitute a separate thymic lineage, from Tregs that are induced following antigen presentation in secondary lymphoid tissues of the periphery (pTregs). Several studies have demonstrated co-expression of FOXP3 and lineage-specific cytokines. Blood Tregs isolated from patients with multiple sclerosis may co-express FOXP3 and IFN-γ although with reduced suppressive capacity that was restored by antibody blockade of IFN-γ *in vitro*, indicating lineage plasticity of a mixed population of tTregs and pTregs.(Dominguez-Villar, Baecher-Allan et al.) Voo and colleagues demonstrated that circulating $FOXP3^+$ Tregs from healthy donors express the Th17 transcription factor RORγt, and readily produce IL-17 upon activation.(Voo, Wang et al. 2009) Although there is not, as of yet, any well-established markers that may be used for isolating tTregs, there is evidence that FOXP3-expressing cells derived from the naïve T cell population maintain FOXP3 expression during *in vitro* culturing to a higher extent than those derived from the effector population, these findings may indicate discrepancies in stability between tTregs and pTregs.(Hoffmann, Eder et al. 2006)

1.1.3 T cell epigenetics

Eukaryotic DNA contains several billions of nucleotide residues. In order to fit all this genetic information into a nucleus, DNA molecules are tightly packed onto nucleosomes which are composed of ~146 base pairs of DNA wrapped around a core of four pairs of histone proteins designated H2A, H2B, H3 and H4.(Kornberg 1977; Richmond and Davey 2003) Nucleosomes, in turn, are packed in a higher order of dense chromatin fibers. This organization makes it impossible for the transcription machinery to access the genome without structural changes of the relevant loci whereby the DNA may be unwound and transcription initiated. Such remodeling events include nucleosome repositioning, post-translational modifications of histone tails as well as DNA methylation; which all may account for tissue- and/or cell-specific gene expression and heritability and are thus by definition referred to as epigenetic events. This thesis discusses the use of DNA methylation as markers for T cell lineage (paper III), as well as its role in regulating the chemokine receptor CCR9 (paper IV).

1.2 THE BIOLOGY OF THE MONOCYTE

Blood monocytes are innate cells that circulate the blood, bone marrow and spleen. They represent about 10% of the total blood leukocyte population and derive from a common macrophage- and DC progenitor (MDP) cell in the bone marrow.(Akashi, Traver et al. 2000) Besides from being able to readily migrate to the tissues and differentiate to macrophages or myeloid dendritic cell, monocytes also perform scavenging as well as inflammatory functions in the blood vessels during inflammation and homeostasis.(Auffray, Fogg et al. 2007)

1.2.1 Monocyte development

Monocytes develop from hematopoietic stem cell-derived common myeloid precursors (CMPs) in the bone marrow that in turn give rise to granulocyte/macrophage precursors (GMPs). In turn, GMPs differentiate to macrophage/DC precursors (MDPs) Apart from giving rise to monocytes and tissue macrophages, these cells may directly, without monocyte intermediates, give rise to conventional as well as plasmacytoid dendritic cell (cDC and pDC) subsets. Through CCR2-dependent mechanisms, the mature monocytes may enter the circulation to carry out their effector functions.

1.2.2 Monocyte subsets in mouse and man

Human blood monocytes are sub-divided into two main subsets based on their expression of the pattern recognition/TLR4 co-receptor CD14 and the IgG receptor CD16, namely the $CD14^{+}CD16^{-}$ and the $CD14^{lo}CD16^{+}$ subsets. Much of our understanding regarding monocyte function and migration is derived from studies in mice. The main murine circulating monocyte subsets, the $Ly6C^{hi}$ and $Ly6C^{lo}$ subsets, overlap phenotypically and functionally with their human counterparts (the $CD14^{+}CD16^{-}$ and the $CD14^{lo}CD16^{+}$ subsets, respectively). (Geissmann, Jung et al. 2003; Cros, Cagnard et al. 2010; Ingersoll, Spanbroek et al. 2010) In mice, $Ly6C^{hi}$ monocytes readily migrate to the gut mucosa through CCR2-CCL2-interactions whereas $CCR2^{-}CX3CR1^{hi}Ly6C^{lo}$ cells do not migrate to the tissues but rather represent a circulation-restricted, terminally differentiated monocyte subset (originating from $Ly6C^{hi}$ cells) that may play a role in maintenance of the vasculature.(Yona, Kim et al. 2013) In contrast with these results, *Ccr2*$^{-/-}$ mice have little or no circulating $Ly6C^{hi}$ monocytes. The same study shows that *Ccr2*$^{-/-}$ monocytes readily migrate to sites of infection but also that these cells accumulate in the bone marrow after infection with *L. Monocytogene*s, indicating that CCR2 plays an important role in monocyte egress from the bone marrow rather than tissue migration during inflammation.(Serbina and Pamer 2006) In the human setting, the CD16-expressing population has been shown to be able to roll and attach under shear flow using CX3CR1-CX3CL1 interactions as well as migrate towards ligands of CX3CL1 and CXCL12, the ligand for CXCR4.(Ancuta, Rao et al. 2003)

The human colonic mucosa has been shown to mainly harbor $CD14^{hi}$ macrophages, with a phenotype resembling the $CD14^{+}CD16^{-}$ subset with regard to the low expression of CD16 and CX3CR1 as well as ability to migrate towards CCL2.(Thiesen, Janciauskiene et al. 2014)

The roles of the respective human monocyte subsets during inflammatory bowel disease are discussed at greater detail in papers I and II of this thesis.

1.3 LEUKOCYTE TISSUE MIGRATION

During inflammation, inflammatory cytokines produced by structural cells in the tissue (such as epithelial cells and stromal cells) as well as resident or migratory immune cells activate the endothelium, which responds by up-regulating the expression of selectins, integrins and chemokines that are presented on the cell surface.(Campbell, Qin et al. 1996)

In order to mediate their effector functions of pathogen eradication and tissue repair, circulating leukocytes are required to extravasate through the endothelium and migrate through the tissues to the site of inflammation. This is carried out by the induction of signaling molecules on the endothelial surface as a result of tissue injury, enabling interactions with circulating cells.

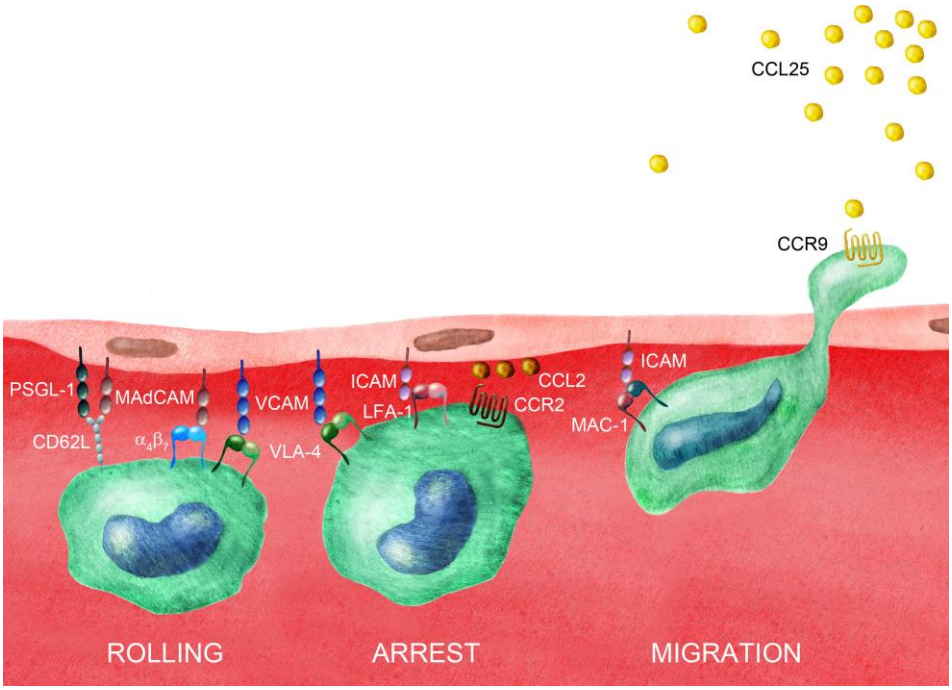

Figure 2. The leukocyte adhesion cascade. Adapted from Ley and colleagues, 2007

1.3.1 Extravasation

Leukocyte extravasation is mediated through sequential but overlapping steps referred to as the leukocyte adhesion cascade, which is mainly carried out by selectins (expressed on leukocytes and endothelial cells) and integrins (expressed on leukocytes).(Ley, Laudanna et al. 2007) The included steps are slow rolling, adhesion strengthening, intraluminal crawling, paracellular or intracellular migration, and finally migration through the basement membrane.(Ley, Laudanna et al. 2007) The leukocyte adhesion cascade is outlined below.

1.3.1.1 Slow rolling

The retention and rolling of the leukocyte on the endothelial surface is carried out by L-selectin, P-selectin and E-selectin which all pre-dominantly interact with the P-selectin glycoprotein ligand 1 (PSGL1).(Kansas 1996; McEver and Cummings 1997) L-selectin (CD62L) is expressed by most leukocytes whereas E-selectin and P-selectin is expressed by the endothelium. The binding properties of selectins with ligands (referred to as catch bonding) require shear flow to properly adhere; rolling cells actually detach when flow is stopped. (Marshall, Long et al. 2003) Apart from selectin interactions, members of the integrin family may play a part in leukocyte rolling as well. In gut homing, interactions between the $\alpha_4\beta_7$ integrin expressed on leukocytes, and MAdCAM-1 expressed on the endothelium are involved in mediating intestinal inflammation.(Berlin, Berg et al. 1993) Phase III-studies addressing the use of vedolizumab, a humanized antibody targeting $\alpha_4\beta_7$, have shown promising results in ulcerative colitis.(Lobaton, Vermeire et al. 2014)

1.3.1.2 Adhesion and activation

Following slow rolling, interactions between leukocyte integrins (such as VLA4 and LFA1, members of the β_1- and β_2-families, respectively) and immunoglobulin superfamily members such as ICAM1 and VCAM1 mediate firm arrest on the endothelial surface.(Campbell, Qin et al. 1996) In addition, chemokines that bind with high affinity to chemokine receptors expressed on leukocytes are produced by endothelial cells in response to cytokines derived from the inflamed tissue. Besides from constituting an additional arrest signal, chemokine interactions provides cell type selectivity based on the differential expression of chemokine receptors among leukocyte subsets.(Middleton, Patterson et al. 2002; Ley, Laudanna et al. 2007) Chemokines that are presented on the endothelial surface may also play a crucial part in controlling leukocyte extravasation by altering the affinity of endothelial integrins.(Shamri, Grabovsky et al. 2005)

1.3.1.3 Transendothelial migration (TEM)

Transmigration through vessel walls occurs following MAC1-ICAM1-dependent crawling, which has primarily been observed in monocytes and neutrophils, is a process where leukocytes are guided to the optimal transmigration site.(Schenkel, Mamdouh et al. 2004; Phillipson, Heit et al. 2006) Subsequently, endothelial redistribution of junctional molecules actively promotes paracellular transendothelial migration.

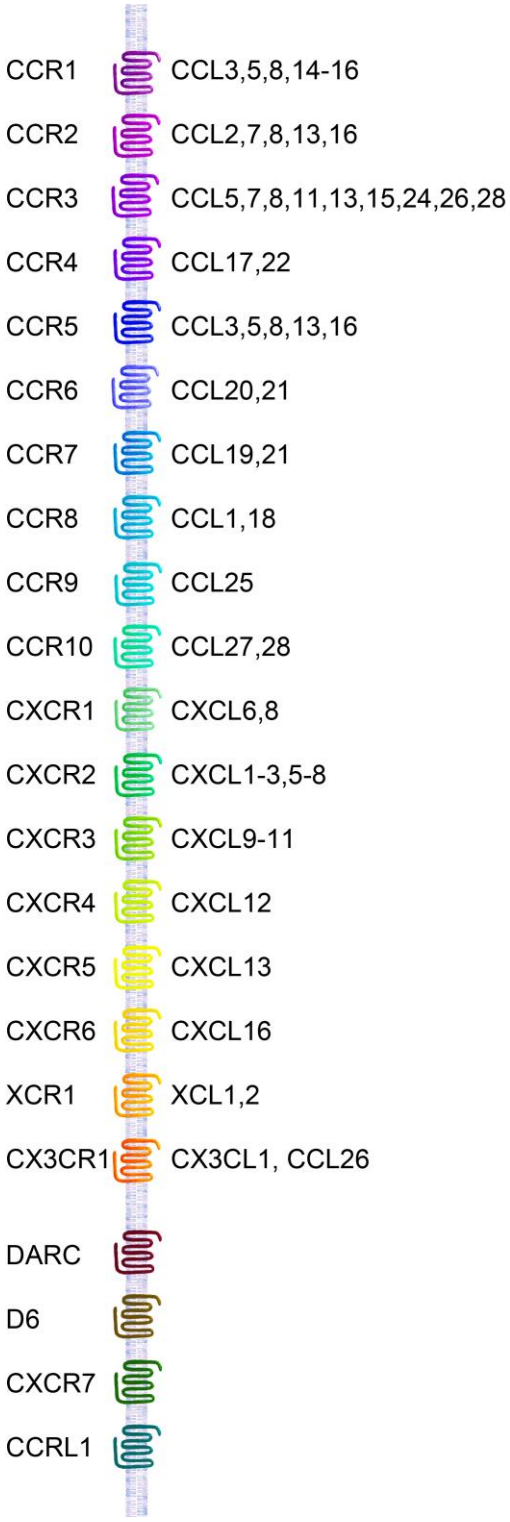

Figure 3. Human chemokine receptors and their ligands. The four bottom receptors represent atypical chemokine receptors that primarily carry out scavenging functions. Adapted from Griffith and colleagues, 2014

An alternative route where leukocytes migrate through the body of endothelial cells has also been described but seems to mainly apply within the central nervous system.(Muller 2003; Nourshargh and Alon 2014)

1.3.1.4 Interstitial migration

In order to enter the interstitium, the migrating leukocyte need to pass the venular basement membrane, a protein network mainly composed by collagen type IV and laminins, as well as the pericyte sheath, a mural cell wrapping that is embedded in the basement membrane of most venules.(Nourshargh, Hordijk et al. 2010) Subsequent interstitial migration is classically mediated by chemokine interactions, resulting in biochemical asymmetry and cellular polarization. Cytoskeleton rearrangements, in particular actin polymerization, at the leading edge of the cell protrudes the cell through the interstitium in response to chemokine ligands, discussed below in greater detail.(Lämmermann and Germain 2014)

1.3.2 The chemokine receptor system

Apart from playing a major role in the activation of leukocytes and endothelial cells during the extravasation process, as discussed above, and the circulation and tissue homing of naïve and effector leukocyte populations, the chemokine system is fundamental in homeostatic organization of immune cells, exemplified by the importance of CXCR4-CXCL12 interactions in hematopoietic stem cell trafficking in the bone marrow; as well as CCR7, CXCR4 and CXCR5 in navigating the microenvironment of secondary lymphoid tissues.(Campbell, Kim et al. 2003) Furthermore, chemokines receptor interactions are implicated to play a highly important role during disease and thus constitute interesting potential therapeutic targets for many inflammatory disorders. The role of the

chemokine receptor system during inflammatory bowel disease will be discussed in the following sections in greater detail.

The chemokine superfamily induces migration of their target cell by binding with varying affinity to one or several of their cognate chemokine receptors, which belong to the class A G-protein coupled 7-TM (GPCR) superfamily and signal through phosphoinositide 3-kinase-dependent- or –independent pathways.(Heit, Liu et al. 2008; Germena and Hirsch 2013) Classically, chemokine binding will induce asymmetric distribution of intracellular cytoskeletal regulators such as the Rho family of GTPases which will coordinate contractile and polymerizing effects on actin and myosin filaments, resulting in rapid cell shape changes

which will drive the cell forward.(Lämmermann and Sixt 2009)

To date, 19 chemokine receptors that confer chemotactic activity have been identified, together with some 50 chemokine ligands. The established nomenclature divide chemokines into four subgroups based on the relative location of the N-terminal cysteine residues (CXC-, CC-, CX3C- and C-). Four atypical receptors, carrying out non-chemotactic functions such as scavenging and recycling of chemokines have been identified as well. Chemokine receptor interactions are involved in directed migration of specific leukocyte subsets, both during steady-state conditions and in inflammation.(Griffith, Sokol et al. 2014)

1.3.2.1 Biased agonism

Within the chemokine receptor system, an in-built element of tissue specificity with regard to that certain chemokine receptor axes apply for specific bodily tissues has been assumed. This notion has been exploited by the pharmaceutical industry as a rationale to therapeutically target single chemokine receptors in tissue-specific disease.(Schall and Proudfoot 2011) However, apart from maraviroc (a CCR5 modulator used for blocking HIV-1 host cell entry) and plerixafor (a CXCR4 antagonist used for mobilizing stem cells for cancer therapy), most chemokine receptor antagonists have failed to show clinical efficacy.(Proudfoot, Bonvin et al. 2015) The concept of chemokine receptor redundancy has often been cited to explain this fact; most CCRs are promiscuous with regard to that a single receptor has multiple chemokine ligands whereas a single chemokine may bind several receptors.(Mantovani 1999) It was originally believed that this phenomenon reflects an in-built redundancy of the system where several chemokine ligands have identical effects on their receptor. The concept of chemokine receptor redundancy has been widely cited by the pharmaceutical industry in order to understand why chemokine receptor antagonists often fail to show efficacy.(Mantovani 1999) However, recent evidence suggests that CCRs may activate multiple down-stream signaling pathways with different efficacies depending on the bound ligand, thus shaping the immune response by means of mechanisms known as biased agonism.(Rajagopal, Bassoni et al. 2013) These findings bring the concept of chemokine receptor redundancy into question and rather suggest a fine-tuning role for the broad receptor-ligand specificities within the system. Therefore, in order to have a deeper apprehension of the migration potential of a cell population during health or disease, there is need for studies

investigating the full chemokine receptor landscape rather than the expression of isolated CCRs.

1.4 THE INTESTINAL IMMUNE SYSTEM

Although the human gastro-intestinal (GI) system ranges from the mouth to the anus, the compartments above the stomach (generally referred to as the upper GI) are often overlooked in studies addressing GI immunology due to the low prevalence of lymphoid tissue here. The small intestine constitutes the first part of the lower GI, and is divided into three parts; the duodenum starts at the pylorus, followed by the jejunum and the ileum which ends at the ileocaecal valve, which is the starting point of the large intestine. The caecum is followed by the ascending colon, the transverse colon, the descending colon and finally the rectum which ends at the anus.

Whereas the main function of the human gastrointestinal system (GI) is to metabolize and absorb food nutrients and expel waste, it is also highly immunologically active. The extreme exposure to commensal bacterial antigens as well as dietary compounds, of which many are potentially pathogenic, requires precise immunological control to evade local and systemic disease development and to establish oral tolerance.

The small and large intestines (lower GI) are structurally similar in the sense that they are continuously formed by a single layer of columnar epithelium but display large differences with regard to length, morphology, physiological role and immunological function. The small intestine is packed in a highly coiled pattern and amounts to a length of 6-7 m. It is characterized by projections known as villi, which protrude into the lumen and significantly increases the surface area of epithelial cells. The luminal sides of these cells, in turn, are covered by microvilli, which harbor digestive enzymes important for nutrient digestion and absorption. The enzymatic activity of the brush border, as well as the length of the villi, gradually decreases throughout the ileum and are completely missing in the large intestine which is shorter in length (~1.5m), wider in diameter and lesser coiled in relation to the small intestine. Thus, the large intestine has little digestive function and its main physiological roles lie in the re-absorption of fluids and waste elimination. It is also the main reservoir for the trillions of commensal bacteria that harbor the human intestine.

The need for immune responses in meeting the constant antigenic challenge throughout the GI tract is naturally large, and those are carried out by a vast number of effector cells that are located within the epithelium or in the underlying lamina propria. In the small intestine, anti-microbial responses may be elicited by Paneth cells, which are concentrated in the ileum. Activation of these cells in response to toll-like receptor (TLR) activation or IL-22 signaling results in the production of anti-microbial peptides such as defensins or lysozyme.(Clevers and Bevins) From a disease development perspective, Paneth cells are interesting in the sense that they express several genes that have been associated with the onset of Crohn's disease, such as *ATG16L1* or *NOD2*.(Clevers and Bevins) Paneth cells are completely lacking in the colon, wherefore a different anti-microbial strategy is employed to protect the epithelium

from the high caecal bacterial load. Here, Goblet cells produce a thick mucus layer that is impenetrable for bacteria and has shown to be compromised in murine models of ulcerative colitis.(Van der Sluis, De Koning et al. 2006; Johansson, Phillipson et al. 2008)

With regard to intestinal adaptive responses, the lower GI is densely populated by intraepithelial lymphocytes (IELs), which are located in the lamina propria or at the base of the epithelial cells at a ratio of 10-15 IELs per 100 epithelial cells.(Ferguson) Gut T cells are derived from conventional T cells and are thus primed in secondary lymphoid organs, and the CD4/CD8 ratio is typically 2:1(Mowat and Agace). Although the majority of IELs throughout the intestines are T cells, a higher prevalence of non-T cell IELs have been observed in the lower GI.(Selby, Janossy et al. 1981) In the small intestine, adaptive responses are generally initiated in specialized gut-associated lymphoid tissue (GALT) which mainly consists of macroscopically visible congregations of lymphoid follicles known as Peyer's patches. Here, luminal antigens may be transcytosed to the underlying lamina propria by specialized epithelial cells (M cells) that are located within the crypts of the villi and in direct association with GALT, resulting in B-cell activation and production of IgA, and/or antigen presentation and T cell responses.(Mabbott, Donaldson et al. 2013) Structures that are functionally similar to Peyer's patches exist in the colon as well and are known as colonic patches.(Mowat and Agace)

1.4.1 Inflammatory bowel disease (IBD)

Ulcerative colitis (UC) and Crohn's disease (CD), collectively referred to as inflammatory bowel diseases, are often described as a breach of tolerance to commensal flora resulting from a combination of inherited and acquired immunological traits, leading to chronic intestinal inflammation. As of current, there is no cure for IBD; instead, therapeutical strategies aim at relieving inflammatory symptoms. IBD is increasing world-wide, especially among the pediatric population where disease-associated malnutrition may result in severe developmental disturbances such as growth retardation. The peak age of disease onset is 20-30 years for CD and 30-40 years for UC, but 7-20% of all IBD cases are found in the pediatric population.(Cosnes, Gower–Rousseau et al. 2011) In Sweden, the prevalence of IBD has been increasing the last decades, now reaching 0,65%.Thus, IBD is rapidly becoming a problem for industrialized societies, and there is great need for increased understanding regarding the pathogenesis of IBD as well as developing novel treatment regimes.

1.4.1.1 Epidemiology

Although IBD can occur at any age, UC patients are usually diagnosed during the third or fourth decade of life, whereas CD patients are generally 5-10 years younger. No particular gender differences are observed among UC patients; however, CD is slightly more common in women, especially in high-incidence areas.(Burisch and Munkholm 2015)

In Sweden, the frequency of pediatric Crohn's disease has increased sharply over the last decades (Malmborg, Grahnquist et al. 2013)

The prevalence and incidence of IBD continues to increase world-wide, but the highest worldwide incidence of both UC and CD is found in industrialized countries of North America and Northern Europe.(Molodecky, Soon et al. 2012) In Europe, IBD incidence numbers have increased dramatically since 1962 and currently reach 9.8 and 6.3 per 100000 person-years for UC and CD, respectively.(Burisch and Munkholm 2015) Illustrating the great variations that are often observed between cohorts when studying IBD epidemiology, the prevalence of UC currently varies between 2.4 and 294 cases per 100000 persons compared to 1.5 to 213 for CD.(Gheorghe, Pascu et al. 2004; Jacobsen, Fallingborg et al. 2006; Lapidus 2006) The reason for this increase (that is observed particularly in the occurrence of Crohn's disease) is not entirely understood but the fact that IBD is increasing in regions that were once considered low-incidence areas (such as Asia and Eastern Europe) indicate the importance of acquired factors such as life-style and diet in the pathogenesis of IBD. However, increased disease awareness and improved diagnostics are factors that may partly explain the measured increase and should thus not be neglected.

Apart from family history, risk factors for developing Crohn's disease include cigarette smoking and previous appendectomy while the relationship to ulcerative colitis is inverse and thus protective.(Andersson, Olaison et al. 2001; Andersson, Olaison et al. 2003; Mahid, Minor et al. 2006) In Crohn's disease, but not ulcerative colitis, the mortality risk is slightly increased compared to the general population, due to post-operative complications as well as intestinal cancer.(Burisch and Munkholm 2015) The risk of developing colorectal cancer (CRC) is, however, increased in both CD and UC and correlates positively with inflammatory burden and duration of immunosuppressive treatment.(Beaugerie 2011; Ullman and Itzkowitz 2011) However, the increased risk of dying from IBD-associated CRC as well as other disease factors is expected to decrease due to better treatment alternatives and disease monitoring as well as improved patient care.

1.4.1.2 Genetics

The causative mechanisms of IBD remain unclear, but it is generally established that disease results from a complex mix of environmental factors and heritability. Genome-wide analysis studies (GWAS) lay the foundation of IBD genetic research.(van Heel, Fisher et al. 2004) To date, some 160 IBD risk loci have been identified, containing an average of five genes each.(Jostins, Ripke et al. 2012) Whereas two thirds of these loci display overlap between UC and CD, 30 are specific for Crohn's disease and 23 for ulcerative colitis. These findings indicate that the genetic component is stronger for CD than for UC, which is in line with results from twin studies that have established a concordance in the range of 20-50% for CD and 16-19% for UC.(Halme, Paavola-Sakki et al. 2006)

So far, the strongest contributor to disease that have been discovered through GWAS is loss-of-function mutations in the gene encoding the pattern-recognition receptor NOD2, associated with Crohn's disease.(Hugot, Chamaillard et al. 2001) Its discovery led to a significant shift in focus of IBD research towards the innate side. Other important polymorphisms that have been linked to disease through GWAS are mutations in the *IL10RA*

gene, encoding the IL-10 receptor, that are particularly associated with early-onset IBD; and mutations in the autophagy gene *ATG16L1*, connected to Crohn's disease.(Hampe, Franke et al. 2007; Glocker, Kotlarz et al. 2009) Interesting to note is that only 11% of disease-associated loci found through GWAS are located in exons, whereas 57% are found in open chromatin regions. This represents a dramatic enrichment, as only 1% of the total DNA is protein-encoding, but still means that a majority of potentially causable variants are located in non-coding regions that may have regulatory or enhancing properties.

1.4.1.3 Clinical features

Both Crohn's disease and ulcerative colitis follows a clinical course that is characterized with bouts of active inflammation, with remission periods of greatly varying length in between. Most patients debut with moderate-to-severe disease, but the severity seems to decrease over time. The sub-diagnoses of IBD are empirically defined through clinical presentations and differ with regard to pathophysiology, epidemiology and optimal choice of therapy. Although clinical symptoms to a large extent overlap between ulcerative colitis and Crohn's disease, the fundamental clinical differences lie in the location and extent of the inflammation. Unlike ulcerative colitis, which by definition is restricted to the colon, Crohn's disease may affect any part of the GI between the mouth and the anus, but inflammation is rarely seen proximally of the terminal ileum where inflammatory patches often debut, located in conjunction with Payer's patches. Whereas UC is confined to the superficial mucosal layer, the inflammation during Crohn's disease may affect the full thickness of the mucosal wall.

Endoscopically, the inflammatory pattern of Crohn's disease is patch-like, and affected sections may be abruptly skipped with healthy tissue in between, a phenomenon referred to as skip lesions and that generates the cobble-stone appearance that is often associated with CD. Furthermore, in Crohn's disease there is often presence of macroscopically visible scarring known as strictures that arise as a result of the transmurality of the inflammation associated with CD. This may lead to intestinal blockade and subsequent abdominal pain, which is often seen at the onset of Crohn's disease. Conversely, ulcerative colitis may debut with bloody diarrhea owing to extensive superficial mucosal ulcerations resulting in impaired absorptive functionality of the colon.(Xavier and Podolsky 2007)

Histologically, small abscesses (known as crypt abscesses) formed by neutrophil congregates are often observed in ulcerative colitis, as well as diminished goblet cells within the crypts. When neighboring crypts are simultaneously affected, the crypt walls will eventually disrupt resulting in an ulcer.(Danese and Fiocchi 2011) The histological hallmark of Crohn's disease is instead the presence of non-caseating granulomas, histiocyte congregations located in conjunction with the epithelium.(Chambers and Morson 1979)

2 AIMS OF THE THESIS

The overall of aim of this thesis was to investigate the driving mechanisms of inflammatory bowel disease from two perspectives; the first one being exploring the contributing role of circulating leukocytes infiltrating the intestinal mucosa; the second one being defining methylation markers for inflammation.

The specific aims were:

Paper I. To investigate a subset of HLA-DR^{hi}-expressing circulating monocytes in patients with inflammatory bowel disease with regard to their disease-contributing properties.

Paper II. To characterize the expression pattern of chemokine receptors on four subsets of circulating monocytes in inflammatory bowel disease.

Paper III. To develop a combinatorial assay for the investigation of T helper cell lineage commitment by using methylation markers in inflammatory disease.

Paper IV. To investigate the maturation stage of T cells infiltrating the colonic mucosa during inflammatory bowel disease. Furthermore, the role of methylation in regulating the expression of CCR9 in these cells was investigated.

3 MATERIALS AND METHODS

This section contains a summary of the patient cohorts and the methodology used in the studies included in this thesis. A detailed account of this information is included within the materials and methods section of each paper (I-IV).

3.1 PATIENTS

3.1.1 Cohorts

3.1.1.1 Paper I

In this study, 51 IBD patients were included (UC=31; CD=20). As the cohort consisted of adults (mean age=37,9 years) diagnosed prior to inclusion, 21 patients received baseline azathioprine treatment. At the study start, patients were divided into three groups receiving mechanistically distinct IBD therapies: corticosteroids (n=16), anti-TNF-α (infliximab or adalimumab; n=17) or granulocyte-monocyte apheresis (GMA; n=18). Patients were monitored for five weeks and clinically assessed for the duration of the study.

3.1.1.2 Paper II

16 juvenile patients with inflammatory bowel disease (UC=8; CD=8) as well as 20 healthy controls were included. Mean ages were 13,5 for patients and 36 for healthy controls. All patients were newly diagnosed, and thus fully treatment-naïve, at the initiation of the study.

3.1.1.3 Paper III

Two separate cohorts were investigated in this study; one consisting of 13 patients with established arthritis (seropositive RA=9; seronegative RA=3; undifferentiated polyarthritis=1); and a second one of 17 patients diagnosed with multiple sclerosis (MS). The MS cohort received concomitant therapy with natalizumab (n=10); conventional immunomodulatory treatment (glatiramer acetate or IFN-1β; n=5). 2 MS patients were untreated.

3.1.1.4 Paper IV

10 newly diagnosed juvenile IBD patients were included (UC=5; CD=5; mean age=13,4). All patients were treatment-naïve at the time of sampling.

3.1.2 Healthy controls

In paper I, 14 patients that had undergone gastroenterological evaluation but were determined non-IBD were used as controls. In paper II, 20 healthy controls were randomly recruited during blood donation. Leukocyte enrichments (referred to as buffy coats) derived from whole blood of healthy blood donors were used in paper I-IV.

3.1.3 Clinical assessment

All patients (papers I-IV) were clinically assessed at the time of study inclusion. In IBD, combinatorial disease activity indices that take factors such as stool pattern, general well-being and subjective pain into account are often used to assess disease severity. The cohort of adult IBD patients investigated in paper I were evaluated using the Harvey-Bradshaw (HBI) or the ulcerative colitis disease activity index (UC-DAI) indices for CD and UC, respectively.(Harvey and Bradshaw 1980; Sutherland and Martin 1987). Juvenile patients in papers II and IV were assessed using the pediatric variants PUCAI (UC) and PCDAI.(Hyams, Ferry et al. 1991; Turner, Otley et al. 2007)

3.2 CELL CULTURING

All *in vitro* cultures were carried out in RPMI-1640 (Sigma) or AIM-V (Life technologies) medium in the presence of 10% fetal calf serum (only included in RPMI-1640 cultures), 2 mM of L-glutamine, 100 U/mL penicillin and 100µg/mL streptomycin (all from Sigma). T cells (papers III and IV) were cultured in the presence of 180 U/mL rhIL-2 (Sigma).

3.2.1 Leukocyte isolation

In preparation for flow cytometry analyses in papers I and II, leukocytes were isolated from whole blood by lysing red blood cells by means of incubation in hypotonic buffer (160 mM NH_4Cl, 10 mM Tris-HCl). Erythrocyte removal from buffy coats (papers I, II and III) was carried out through density gradient centrifugation (Ficoll-Paque PLUS, Amersham Biosciences).

From intestinal samples (papers I and IV), leukocytes were isolated by mechanical homogenization using a glass homogenizer.

Magnetic cell sorting (MACS) was used to isolated CD14+ monocytes (papers I and II) and CD4+ T cell populations (paper III and IV) on a AutoMACS cell separator (Miltenyi).

3.2.2 T cell differentiation

Naïve T cells may be differentiated *in vitro* to either of the described T effector lineages by stimulating with polarizing cytokines while blocking reciprocal pathways. In paper III, naïve T cells were differentiated into Th1, Th2, Th17 and iTreg by stimulation with cytokine cocktails during polyclonal activation with plate-bound anti-CD3 and soluble anti-CD28 (eBioscience). The protocol is described in more detail in the methods section of paper III.

3.2.3 5-aza cultures

The DNMT1 methyltransferase may be covalently linked to the DNA molecule by including nucleoside analogues such as 5-aza-2'-deoxycytidine (5-aza) in the medium of cultured cells, resulting in random genomic de-methylation and expression of genes that are normally silenced by CpG methylation.(Christman 2002) In papers III and IV, T cells were activated using anti-CD3/anti-CD28 beads (Dynabeads; Life Technologies) and 5-aza (Sigma) was

added to the proliferating cells after 24 hours. Following culturing for 48 hours, 5-aza was removed from the culture medium and cells were harvested after an additional 24 hours and the expression of IL-17A (paper III) or CCR9 (paper IV) was measured using PCR (paper III) and flow cytometry (papers III and IV).

3.3 FLOW CYTOMETRY (FACS)

3.3.1 Surface stainings

Isolated populations of monocytes (papers I and II) or T cells (papers III and IV) were blocked for unspecific Fc-receptor interactions using phosphate-buffered saline supplemented with 10% human serum (Sigma), followed by surface staining using combinations of fluorochrome-labelled antibodies. Isotype- and fluorochrome matched control antibodies were used to define marker positivity. For a detailed account of FACS antibody panels, refer to the methods section of the corresponding papers I-IV.

3.3.2 Intracellular staining

In paper III, intracellular analyses of T helper cell-associated cytokines were carried out subsequent to surface staining by permeabilizing cell membranes followed by antibody incubations.

3.3.3 Sorting

Flow cytometry sorting was carried out to analyze monocyte subpopulations for PCR experiments (paper I) and T cells for methylation analyses (papers III and IV). All sorting were carried out on a FacsARIA (BD Biosciences).

3.4 POLYMERASE CHAIN REACTION (PCR)

3.4.1 RNA isolation and cDNA synthesis

RNA was isolated using TRIzol reagent (papers I and III; Life Technologies) or using RNeasy kits (papers I, II and III; Qiagen). RNA quality was spectrometrically measured using a NanoDrop instrument (Thermo Scientific) and only samples that displayed ratios of 260/280 nm and 260/230 nm of >1.9 (ratios indicate RNA purity and presence of phenol contaminants, respectively) were used for downstream applications.(Glasel 1995) On-column DNase digestions were performed on all RNA used for PCR arrays (papers I and II; Qiagen) Subsequent cDNA syntheses were carried out using iScript reverse transcriptase (Bio-Rad) or RT^2 First Strand kit (for PCR arrays in papers I and II; Qiagen).

3.4.2 PCR run and data analysis

Subsequently, single gene real time PCR was carried out on a CFX96 thermal cycler (Bio-Rad) using SYBR Select mastermix (Applied Biosystems). Real-time PCR arrays (papers I and II) were performed on a 7900HT or on a QuantStudio 7 Flex PCR system (Applied Biosystems). Run data was subsequently analyzed using software provided by each PCR

system manufacturer, and data was normalized to the mean expression of the housekeeping genes described in the methods section of the respective study.

3.5 EPIGENETICS

3.5.1 Genomic DNA isolation

Genomic DNA was isolated from T cell populations isolated from whole blood, *in vitro* cultures or synovial fluid (paper III); or from intestinal biopsies (paper IV). In paper III, isolation was carried out using phenol:chloroform:isoamyl alcohol (25:24:1; Sigma) on proteinase-K (Qiagen) digested cell lysates. DNA isolation in paper IV was done as an on-column step on the EZ DNA Direct- Methylation kit (ZYMO research).

3.5.2 Bisulfite conversion

Treatment of genomic DNA with bisulfite results in the conversion of un-methylated cytosine residues to uracil, which are recognized as thymines in subsequent PCR amplification. Cytosines that were originally methylated remain cytosines, thereby enabling distinction between methylated and un-methylated residues.(Frommer, McDonald et al. 1992) Following DNA isolation, bisulfite conversion was carried out using the EZ DNA Methylation Gold kit (paper III) or the EZ DNA Direct-Methylation kit (paper IV; all bisulfite conversion reagents purchased from ZYMO research).

3.5.3 Bisulfite sequencing

Sequencing of bisulfite converted DNA may be carried out from sorted cell population through classical Sanger sequencing of PCR products representing individual cells, following cloning of PCR products into a bacterial vector. Alternatively, the cloning step can be skipped and sequencing carried out directly on PCR products using pyrosequencing. The advantages and dis-advantages of the respective methods are discussed by Reed and colleagues.(Reed, Poulin et al. 2010)

3.5.3.1 TOPO cloning and Sanger sequencing

In paper III, bisulfite sequencing of the *IL-17A* locus was carried out by cloning PCR product from sorted IL-17A-positive and –negative populations into a TOPO vector (Life Technologies) followed by Sanger sequencing carried out on an ABI Prism 310 genetic analyzer (Applied Biosystems). All reagents for Sanger sequencing were purchased from Applied Biosystems.

3.5.3.2 Pyrosequencing

In paper IV, pyrosequencing of the *CCR9* locus was carried out by binding of biotinylated PCR product onto sepharose beads that were subsequently cleaned using the PyroMark Q96 vacuum workstation (Qiagen). Following sequencing primer annealing, PCR product was sequenced using the PyroMark Q96 ID sequencer (Qiagen). All reagents were purchased

from Qiagen. Primers design and data analysis were performed using the PyroMark Assay Design v2.0 and PyroMark CpG v1.0 software packages, respectively (Qiagen).

3.5.4 Epigenetic immune lineage analysis (EILA)

The EILA method was specifically developed for paper III in order to define T cell lineage commitment on a population of isolated cells using methylation markers.

3.5.4.1 MS-SNuPE

Each T helper cell locus (*IFN-γ, FOXP3, IL-17A and IL-13*) was PCR amplified from bisulfite converted DNA, and PCR products were treated with exonuclease I and calf intestinal phosphatase (CIP; New England Biolabs) in order to remove fragments that may disturb the downstream application. Subsequently, PCR products were mixed at equal volumes followed by annealing of oligonucleotides of varying length, neighboring each reporter CpG. Subsequently, methylation-sensitive single nucleotide primer extension (MS-SNuPE) reactions with fluorochrome-labelled ddNTPs (SnapShot Multiplex kit; Applied Biosystems) complementary to methylated or non-methylated DNA were carried out. Subsequent fragment analysis carried out on an ABI Prism 310 genetic analyzer (Applied Biosystems) thus enabled distinction between locus (by probe length) and methylation status (by fluorescence). Fragment analyses were performed using GeneScan v3.7 software (Applied Biosystems).

4 RESULTS AND DISCUSSION

4.1 HLA-DR^{HI} AND CCR9 DEFINE A PRO-INFLAMMATORY MONOCYTE SUBSET DURING IBD

Originally, blood monocytes were believed to possess little phenotypic heterogenetity. The first study attempting to sub-divide circulating monocyte subsets was published as late as in 1989, with the introduction of the CD16+ subset.(Passlick, Flieger et al. 1989) Since then, studies have arrived at the current consensus that the numerically dominant CD14+CD16- subset readily migrates to the tissues and differentiates to effector macrophages and DCs, whereas the main function of the CD16-positive subset is to carry out scavenging within the circulation, and to maintain vessel integrity.(Cros, Cagnard et al. 2010; Shi and Pamer) In patients with inflammatory bowel disease, it has been shown that monocyte migration into the intestinal mucosa correlates with inflammatory load, and that polymorphisms in the *NOD2* gene, which is abundantly expressed in monocytes, constitute the strongest genetic correlation with Crohn's disease.(Hugot, Chamaillard et al. 2001; Ogura, Inohara et al. 2001) These findings have led to a considerable interest in studying monocytes from a disease-causing perspective, as well as therapeutically. In treatment of IBD patients with corticosteroids as well as with granulocyte-monocyte apheresis (GMA), a column-therapy selectively removing Fcγ-receptor-positive leukocytes from the circulation, CD16+ monocytes are selectively removed.(Saniabadi, Hanai et al. 2003; Hanai, Iida et al. 2008) Although this would indicate that a proportional decrease of CD16+ monocytes correlates with reduced inflammatory activity in IBD, the mechanisms directing monocytes to the inflamed mucosa during IBD are unclear.

In this study, we have identified a sub-population of monocytes expressing high levels of HLA-DR while being positive for the chemokine receptor CCR9, previously reported to be involved in gut-homing of T cells in the murine setting.(Koenecke and Förster) The proportion of these cells, that are CD14-positive and express intermediate levels of CD16, are significantly increased in patients with active IBD ($p=0.0006$). However, no differences between Crohn's disease and ulcerative colitis were shown. Furthermore, these cells are differentially affected by IBD therapy with distinct working mechanisms. Whereas both cortisone and GMA therapy results in a therapy-induced decrease of these cells, the reduction associated with GMA is immediate and highly significant only after one week of therapy (consisting of one single treatment session; $p<0.001$), which was expected due to the immediate physical removal of these cells, probably by means of CD16/FcγRIII-binding to the matrix of the GMA column.<Saniabadi, Hanai et al. 2003> Whereas a large number of studies have reported changes in the relative proportions of blood monocyte subsets during IBD, the exact working mechanisms of monocyte-mediated inflammatory responses during IBD remain unclear. Interestingly, the HLA-DR^{hi} subset expresses low but detectable levels of the gut-homing receptor CCR9, posing a potential level of selectivity for the intestinal compartment for these cells. Studies addressing the mechanistic properties of CCR9 interactions in monocytes are needed to fully understand the extent of chemokine receptor

interactions in human myeloid cells; and whether these interactions merely constitute markers of activation, or functionally relevant gut-homing interactions remain to be elucidated.

We conclude from this study that HLA-DR^{hi} monocytes, further defined by their CCR9 expression, are differentially affected, kinetically as well as proportionally, by conventional and biological IBD therapy. Furthermore, we demonstrate that these cells readily produce inflammatory cytokines and chemokine upon stimulation with bacterial products. The selective expression of CCR9 on this subset underlines the relevance in investigating chemokine receptors in monocyte sub-populations, in addition to established sub-population markers. Our finding that circulating pro-inflammatory TNF-α producing CCR9^{+} monocytes are increased in IBD patients has led to us to develop a CCL25-containing apheresis column. In addition, a double-blind placebo-controlled clinical study on patients with UC with promising results has been carried out (submitted manuscript).

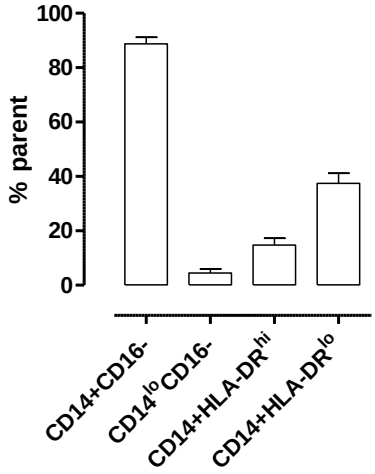

Figure 4. Frequencies of blood monocyte subset in healthy controls. Graph shows flow cytometry data from 20 healthy controls (mean age = 36, range 23-66; bars represent +SD). Data is presented as proportions of the parent CD14^{+} population.

4.2 CHEMOKINE RECEPTOR EXPRESSION PROFILING OF HUMAN BLOOD MONOCYTES IN JUVENILE PATIENTS WITH INFLAMMATORY BOWEL DISEASEI

In line with the classical notion that blood monocytes follow a rigid and predictable phenotypic and migratory pathway, studies addressing monocyte chemokine receptor expression beyond their use as population markers are scarce. In humans, CD14^{+}CD16^{-} (classical subset) and the CD14^{+}CD16^{+} (non-classical subset) monocytes are characterized by their expression of CCR2 and CX3CR1, respectively.(Geissmann, Jung et al. 2003; Linton, Karlsson et al. 2012) A majority of the understanding regarding the physiological roles of these CCRs in monocyte migration are derived from the murine setting where the equivalents to the human monocyte populations, the Ly6C^{hi} and Ly6C^{lo} subsets, largely overlap with their human counterparts with regard to migratory behavior, chemokine receptor expression and transcriptional profiles.(Geissmann, Jung et al. 2003; Cros, Cagnard et al. 2010; Ingersoll, Spanbroek et al. 2010) Even though the inflammatory importance of CCR2 and CX3CR1 interactions in monocyte migration is now well established, it is not clear whether the actions

of additional chemokine receptor interactions, induced by an ongoing inflammatory process, might provide additional support in the recruitment process during inflammation.

In paper II, we continue to investigate the importance of migratory blood monocytes in the disease process during IBD. By comparing the protein expression of 19 chemokine receptors on four monocyte subsets ($CD14^{+}CD16^{-}$, $CD14^{lo}CD16^{+}$, $CD14^{+}HLA\text{-}DR^{hi}$ and $CD14^{+}HLA\text{-}DR^{lo}$) between IBD patients and healthy controls, we set out to gain an understanding as to whether the CCR profile of these cell populations would reflect the disease phenotype of the patient. By exclusively including treatment-naïve juvenile patients, we come to terms with the potential problem of anti-inflammatory treatment affecting chemokine receptor expression. As expected, the pan-monocyte population displayed large differences in CCR expression between IBD patients and healthy controls (14 out of 19 CCRs were significantly differentially expressed. Although our control population represented adults (n=20; mean age=36) whereas the patients were all juvenile (n=16; mean age=13.5), the age difference did not seem to affect our read-out as no correlations were observed between age and CCR expression. Among the chemokine receptors that displayed the highest relevant differences between patients and controls were CCR3, CCR4 and CCR9; wherefore these were further investigated in subset analyses. When comparing ulcerative colitis with Crohn's disease, the median expression levels of both CCR3 and CCR4 were consistently higher in UC whereas CCR9 was higher in CD. It should be noted, however, that the differences between UC and CD were generally non-significant (with the exception of the expression of CCR3 on the $CD14^{lo}CD16^{+}$ subset; p=0.0007), possibly reflecting the relatively small patient cohort (nUC=8; nCD=8). Nevertheless, this pattern was observed in all four monocyte subsets, indicating that monocyte sub-populations, in response to systemic inflammatory signals, up-regulate CCRs similarly, which is in line with the notion that monocyte subset represents a continuum of differentiation rather than absolutely functionally distinct subsets. In order to understand the mechanisms behind the differential CCR profiles observed between IBD patients and healthy controls, we carried out *in vitro* stimulations of blood monocytes, isolated from buffy coats of healthy blood donors, using inflammatory cytokines that had previously been shown to be differentially produced between UC and CD.(Dionne, Hiscott et al. 1997; Martínez-Borra, López-Larrea et al. 2002; Melgar, YEUNG et al. 2003) Interestingly, we could find that, whereas CCR9 was primarily induced by TNF-α and IL-1β, both CCR3 and CCR4 was up-regulated by IL-10. This suggests that these CCRs are controlled by separate induction pathways that might extrapolate to the respective inflammatory milieus of UC and CD.

In conclusion, the results of this study suggest that local intestinal inflammation is reflected upon circulating monocytes during IBD.

4.3 PROFILING OF $CD4^{+}$ T CELLS WITH EPIGENETIC IMMUNE LINEAGE ANALYSIS

The intestinal epithelium and the lamina propria constitute the largest reservoir of T cells in the human body.(Mowat and Agace 2014) Thus, T cell responses are fundamental in oral

tolerance development as well as mounting immune responses against invading pathogens. T cell immunity during IBD were originally described as a dichotomy of either Th1 or Th2, where CD4+ IELs in Crohn's disease were skewed towards Th1 and IFN-γ production; whereas ulcerative colitis represented Th2 and IL-5.(Fuss, Neurath et al. 1996) However, the fact that IL-4, the signature cytokine of Th2 commitment, is not increased during ulcerative colitis, disturbs the Th1/Th2 paradigm of IBD.(Fuss, Neurath et al. 1996) In addition, reports on increased plasticity with regard to cytokine profile changes among CD4+ T cells makes lineage commitment difficult to study by means of transcription profiling or protein markers.(Geginat, Paroni et al. 2014) Instead, the use of epigenetic markers has been proposed as a solution to that issue, taking advantage of the transcriptional stability of the epigenome. Members of our group have previously identified a CpG site in an enhancer region in the 5' CNS of the *IFNG* gene to constitute a reporter for stable Th1 commitment; and the *IL-13* gene promoter has previously been shown to display de-methylation selectively in Th2 cells.(Santangelo, Cousins et al. 2002; Janson, Marits et al. 2008) Furthermore, we and others have shown that suppressive Treg phenotype correlates with de-methylation of CpG sites in the promoter region or in conserved nucleotide sequences of the *FOXP3* gene.(Baron, Floess et al. 2007; Janson, Winerdal et al. 2008) In this study, we set out to develop a method to assess T cell lineage commitment based on methylation marks. ‘

First, we wanted to determine whether stable Th17 phenotype could be associated with de-methylation of the *IL-17A* gene. We identified six well-conserved CpG sites within a 100 bp stretch of the promoter region by performing m-Vista alignments comparing the murine and human gene sequences. Upon bisulfite sequencing of IL-17A-producing $CD4^+CD45RO^+$ memory cells in comparison with $CD4^+CD45RO^+IL17^-$ cells, a differentially methylated reporter site located 122 bp down-stream of the *IL-17A* TSS was identified. It is interesting to note that our Th17 skewings of naïveCD4+ T cells *in vitro* did not result in de-methylation of the promoter in spite of inducing *IL-17A* transcription, pointing the physiological disparities that might result from the many protocols that exist for Th17 induction.(De Jong, Suddason et al. 2010)

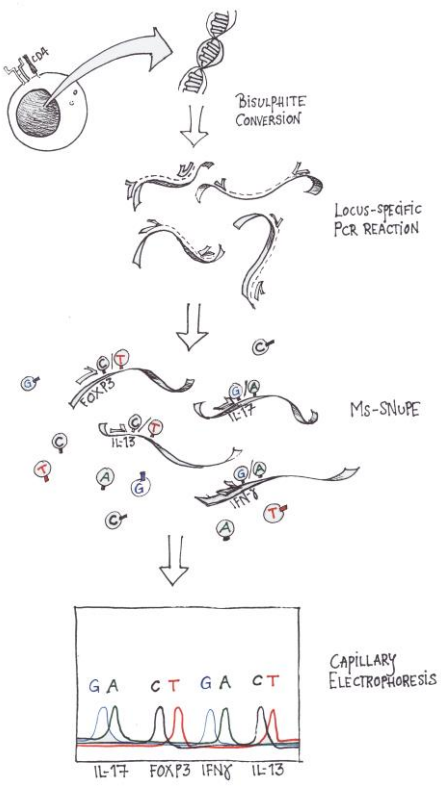

Figure 5. Schematic overview of the EILA method.

Next, we developed a PCR-based assay (referred to as EILA; Figure 5) based on the annealing of differentially lengthed oligos adjacent to the respective reporter CpGs of Th1, Th2, Th17 and Treg. By subsequent methylation-specific single nucleotide primer extension (MS-SNuPE) of fluorochrome-labelled ddNTPs spanning the differentially methylated CpGs, the methylation status of each locus could be

measured through fragment analysis (GeneScan, Applied Biosystems).

The EILA method was subsequently validated on *ex vivo*-isolated or *in vitro*-differentiated cell populations by correlating methylation status to mRNA data for each gene product. The accuracy of the method was evaluated by titrating *in vitro*-methylated bacterial artificial chromosomes (BACs) in 2% increments, followed by EILA analysis. To verify that the method is clinically applicable, we analysed memory ($CD45RO^+$) $CD4^+$ T cells, isolated *ex vivo* from peripheral blood and synovial fluid of patients with rheumatoid arthritis (RA), as well as from the circulation of multiple sclerosis (MS) patients. In extensively used murine models of RA and MS, the importance of Th17 for disease development is highlighted by the fact that deficiency of IL-23, a cytokine important for Th17 commitment, protects from disease development.(Cua, Sherlock et al. 2003; Murphy, Langrish et al. 2003) In contrast, our methylation data from the human setting rather suggests that Th1, and not Th17, is the pre-dominant T cell lineage in the inflamed joints of RA patients. Also, we confirm these results on the protein level using intracellular flow cytometry, where significant IFN-γ production is observed in synovial fluid-infiltrating $CD4^+$ cells. However, in line with the murine data, circulating $CD4^+$ T cells from patients with multiple sclerosis display commitment towards Th17.

Although not included in this publication, preliminary data from methylation analysis of T_{EM} cells (the current model of T helper cell maturation stages are outlined in section 1.1.2) infiltrating the colonic mucosa during juvenile IBD reveal that these cells are predominantly de-methylated towards Th1/*IFN-γ*, whereas the *IL-17A* and *FOXP3* loci were epigenetically closed (Figure 6). Interestingly, we observed significantly lower *IFN-γ* de-methylation levels in inflamed samples compared to non-inflamed, reflecting the effector role of Th1 cells during ongoing inflammation (n=5; p=0.042). A corresponding shift in methylation levels (although not significant) was observed in the *IL-13* locus, indicating Th2 commitment, but

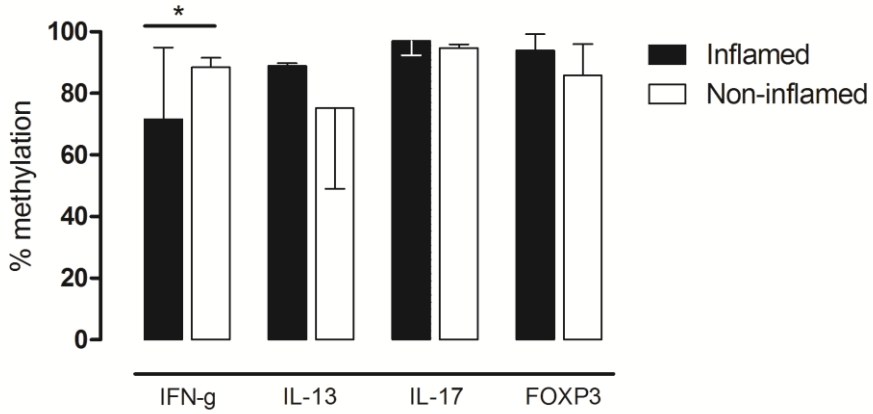

Figure 6. Lineage commitment of T_{EM} cells infiltrating the colonic mucosa during juvenile IBD, as defined by EILA. Each bar represents at least five individual samples. * indicates p=0.042 (Mann-Whitney U test); error bars represent +/- SD.

no Th1/Th2 predominance could be observed when analyzing UC and CD samples separately (data not shown). However, this is an ongoing study and a larger cohort will potentially reveal any disease-specific methylation characteristics.

From this study, we conclude that *IL-17A* is regulated by promoter methylation, and that *in vitro*-induced IL-17A production is not necessarily associated with de-methylation of the promoter. Furthermore, the EILA method may be used to accurately assess lineage commitment of $CD4^+$ T cells in clinical samples, taking advantage of the transcriptional stability of methylation markers.

4.4 PROMOTER DEMETHYLATION IS ASSOCIATED WITH CCR9 EXPRESSION ON COLON-INFILTRATING CD4+ T CELLS DURING JUVENILE INFLAMMATORY BOWEL DISEASE

Lymphocytes are classical mediators of chronic intestinal inflammation, and are found at high numbers throughout the lower gastro-intestinal tract, where they infiltrate the epithelium and the underlying lamina propria. Although separated by only a thin basement membrane, the epithelium and the lamina propria represent separate immunological compartments. For example, whereas $CD4^+$ T cells represent only a minor fraction of the intraepithelial lymphocyte (IEL) pool, they exist at profoundly higher ratios in the lamina propria (at a $CD4^+/CD8^+$ ratio of approximately 2:1).(Mowat and Agace 2014) These $CD4^+$ T cells are derived from conventional, naïve T cells that were primed in secondary lymphoid organs followed by seeding into the lamina propria. Thus, these cells are mainly of the T_{EM} phenotype, having down-regulated lymph node homing markers in exchange for markers for tissue migration and retention.(Smids, Horje et al. 2014)

However, recent reports suggest that intestinal non-lymphoid tissues may also be populated by naïve cells. Based on the expression of the naïve CD45RA marker, several studies have demonstrated naïve T cells infiltrating the intestinal mucosa during active IBD.(ten Hove, Berkhout et al. 2004; Linton, Karlsson et al. 2012) As discussed in section 1.1.2, defining T cell naivety solely based on CD45RA expression may result in the inclusion of the T_{EMRA} subset, a terminal, homeostatic T cell lineage that was originally discovered in the $CD8^+$ population.(Sallusto, Lenig et al.)

In order to elucidate the composition of the $CD4^+$ T cell pool of the colonic mucosa during IBD, we included 10 juvenile IBD patients (UC=5; CD=5) with the purpose of studying the lymph node homing markers CD62L and CCR7 in addition to the memory versus naïve T cell markers CD45RA and CD45RO, as well as the tissue marker CCR9.

Whereas no naïve T cells were detected, the T_{EM}:T_{EMRA} was approximately 3:1 We observed no changes in the relative proportions between the T_{EMRA} and T_{EM} subsets depending on whether the T cells were isolated from a colonic section with macroscopically high

inflammatory burden This indicates that inflammation has no impact on the rate of T cell maturation in this context, or that T_{EMRA} and T_{EM} are recruited to the colon at similar proportions regardless of inflammation. CCR9 was abundantly expressed in both subsets, levels are similar between the subsets supports the latter idea.

Interestingly, our data shows that the proportion of colon-infiltrating CD4+ T cells expressing CCR9 was significantly higher in non-affected mucosa. When studying the T_{EMRA} and T_{EM} separately, we found that this difference mainly reflects the T_{EM} subset, as no significant difference was observed within the T_{EMRA} population. These findings were surprising considering that murine studies have shown that infiltration of Ccr9-expressing T cells to the ileal mucosa correlates with inflammatory burden, thereby attributing a pro-inflammatory role to CCR9-CCL25 interactions.(Rivera–Nieves, Ho et al. 2006) Conversely, *Ccr9*$^{-/-}$ mice display more severe inflammation and slower recovery from DSS colitis compared to wild-type, and it has been shown that CCR9 is required for oral tolerance induction in mice.(Cassani, Villablanca et al. 2011; Wurbel, McIntire et al. 2011) In light of the findings in mice in relation to our data, it is interesting to speculate that CCR9 has a role in the healing process from colitis, or that the function or kinetics of the CCR9-CCL25 axis during colitis differs from that of small intestinal inflammation (Figure 2). These issues could help to explain the difficulties in pharmaceutically targeting CCR9-CCL25 interactions during IBD and should therefore be specifically addressed in the human setting.(Saruta and Papadakis 2014)

We have shown that the colonic CD4+ T cell pool of treatment-naïve juvenile patients with inflammatory bowel disease mainly consists of the T_{EM} and T_{EMRA} maturation stages, and that these cells express high levels of the gut-homing receptor CCR9. Furthermore, we show that CCR9 is significantly decreased on T_{EM} during active inflammation. In conclusion, these data increase the understanding on local T cell maturation during colonic inflammation, and provide evidence of the involvement of the CCR9-CCL25 axis during juvenile colitis.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Even though chemokine receptors have been widely studied on all leukocyte subsets with regard to homeostatic organization, the in-built plasticity and diversity of the system has only begun to unravel. The concept of biased agonism, which proposes differential signaling outcomes (rather than redundancy) for every chemokine ligand that has affinity for a receptor, brings a whole other level of complexity into perspective when it comes to fine-tuning inflammatory responses.(Rajagopal, Bassoni et al. 2013) Thus, a complete understanding of the targeted pathway as well as its specific role in the investigated disease setting will be necessary in order to successfully target chemokine receptor interactions for therapeutic purposes.

In comparison with T cells, which have been widely addressed in studies investigating homeostatic as well as inflammatory chemokine receptor interactions, monocytes have a shorter life span (1-3 days compared to 90 days for T cells) accompanied by less complex migration routes.(Whitelaw 1972; Hellerstein, Hanley et al. 1999) These facts may partly explain why monocytes are often overlooked in studies investigating plasticity and induction mechanisms of chemokine receptors. Another reason might be due to the notion that blood monocytes do not extensively traffic secondary lymphoid organs, which are important sites for induction of inflammatory (such as CCR9 for gut-homing) as well as homeostatic chemokine receptors in T cells.(Mora, Bono et al. 2003; Smids, Horje et al. 2014) However, it was recently shown that classical monocytes do traffic lymph nodes in the murine setting during steady-state by means of CD62L-mediated interactions, and thus it is interesting to speculate whether these findings also apply for the human $CD14^{+}CD16^{-}$ subset, which has been shown to express high levels of CD62L.(Geissmann, Jung et al. 2003; Jakubzick, Gautier et al. 2013)

The chemokine receptor CCR9 has been shown to play an important role in directing activated T cells to the small-intestinal compartments in mice, but reports on its functional role in inflammation are inconclusive. Rivera-Nives and colleagues showed that antibody blockade of CCR9-CCL25 interactions resulted in less severe ileitis, whereas another study has shown that CCR9 is mainly responsible for Treg recruitment to the small intestine.(Rivera–Nieves, Ho et al. 2006; Wermers, McNamee et al. 2011) As for its role in murine colitis, CCR9-CCL25 seems to mainly confer protection.(Wurbel, McIntire et al. 2011; Wurbel, Le Bras et al. 2014) In human IBD, CCL25 has been shown to be primarily expressed in the small intestine, although increased colonic mRNA levels have been observed during active colitis.(Papadakis, Prehn et al. 2001; Linton, Karlsson et al. 2012; Zhu, Bing et al. 2014) Adopting the notion derived from murine studies of CCR9-CCL25 interactions mainly representing small intestinal pro-inflammatory elements, therapeutical blockade of

CCR9 is currently being investigated in a phase III-trial on patients with Crohn's disease. However, the trial was halted in August 2013 due to the primary endpoint (clinical remission at week 12) not being met, but was continued in 2014 after dosage adjustments.(Arseneau and Cominelli 2014; ChemoCentryx 2014)

Thus, questions remain unanswered as to whether the CCR9-CCL25 axis is preferentially used by suppressive cells, or whether its functional impact on inflammation may be context-dependent. Our data from paper IV, demonstrating decreased levels of colonic CCR9-positive $CD4^+CD45RO^+$ effector memory cells only in un-affected mucosa compared to inflamed specimens, rather support the suppression model. Characterization of the proteome as well as the transcriptome of these cells would reveal whether the roles of colon-infiltrating CCR9-expressing $CD4^+$ T cells are primarily in suppressing or promoting inflammation.

There are few studies investigating CCR9 on the transcriptional level. In 2000, Yu and colleagues demonstrated that two *CCR9* splice variants exist, differing in their sensitivity to CCL25. The larger *CCR9A* isoform is expressed in human lymphocytes at 10-fold higher ratios compared to the *CCR9B* variant, as well as being more responsive to CCL25.(Yu, Peden et al. 2000) The clinical relevance of these isoforms has not been investigated, and thus it remains to be seen whether shifting of isoform balance is involved in fine-tuning CCR9-mediated immune responses within the gut.

In paper IV, we demonstrate that colon-infiltrating $CD4^+$ T cells are all CCR9-positive, that CCR9-expressing $CD4^+$ T cells of the colon are HLA-DR-positive (indicating long-term activation) and that CCR9 expression levels are similar between the T_{EM} and the terminally differentiated T_{EMRA} subsets.(Chadburn, Inghirami et al. 1991; Hislop, Annels et al. 2002) Based on these findings, we speculate that CCR9, besides from being involved in colonic migration, is also needed for colonic retention of the infiltrating T cells. Thus, there is a requirement for transcriptional stability of *CCR9* expression, possibly conferred through epigenetic changes of regulatory regions within the *CCR9* gene. However, preliminary data from our lab shows that CCR9 may be transiently up-regulated on $CD4^+$ T cells following TCR-dependent and –independent stimulation. Thus, although our bisulfite sequencing of CCR9-expressing compared to non-expressing cells revealed profound de-methylation in the $CCR9^+$ cells (thus indicating stable phenotype), gut-infiltrating CCR9 cells should be monitored *ex vivo* over time to establish whether de-methylation of *CCR9* is involved in long-term transcriptional stability. Furthermore, the role of retinoic acid in the imprinting of intestinal phenotype is well established in the murine setting. In mice, both CCR9 and $\alpha_4\beta_7$ have been shown to be dependent on retinoic acid produced in secondary lymphoid tissues of the gut for their induction.(Mora, Bono et al. 2003; Iwata, Hirakiyama et al. 2004)

The papers included in this thesis have discussed the role of T helper cells and monocytes in conferring intestinal inflammation in the setting of IBD. With regard to taking advantage of the chemokine receptor system in designing novel treatment strategies for IBD, much work remains in order to fully understand the complex mechanisms orchestrating intestinal immune responses. Also, the immunological heterogeneity of IBD patients constitutes a

therapeutical challenge that may be overcome by individualizing diagnostic and therapeutic approaches.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunförsvarets främsta uppgift är att skydda kroppen från bakterier, virus och parasiter. Detta möjliggörs genom olika fysiska barriärer i kroppen, men också med hjälp av immunceller i blodet. Immunförsvaret brukar delas upp i två delar som representerar olika celltyper med skiljda angreppsstrategier mot sjukdomsalstrande ämnen (så kallade patogener): det medfödda (innata) och det specifika (adaptiva) immunförsvaret. De immunceller som ingår i den innata delen (såsom monocyter och granulocyter) är konstruerade för att snabbt och brett regera mot ämnen som ofta återfinns på ytan av bakterier, virus och parasiter – detta sker oftast inom någon eller några minuter efter exponeringen. Däremot kan det dröja dagar innan det specifika immunförsvaret når sin fulla potential, vilket dels beror på att det specifika immunförsvaret är beroende av det medfödda för att aktiveras, men också på att cellerna som ingår i den specifika delen (såsom T- och B-lymfocyter) styrs av omfattande kontrollmekanismer för att förhindra överaktivering. Detta är speciellt viktigt på grund av att cellerna i det specifika immunsvaret kan ge upphov till immunologiskt minne (vilket är anledningen till att vaccinering kan ge ett livslångt sjukdomsskydd) – en felaktig aktivering av en T- eller B-lymfocyt kan leda till att immunförsvaret angriper kroppens egna celler, vilket i värsta fall kan ge upphov till livslång sjukdom (autoimmunitet).

Denna avhandling behandlar inflammatorisk tarmsjukdom (eng. inflammatory bowel disease; IBD), en sjukdom som inte alltid räknas som autoimmun men likväl orsakas av en överaktivering av tarmens immunförsvar i kombination med svårigheter att tolerera de godartade bakterier som ständigt återfinns i höga antal i tarmsystemet. IBD är egentligen ett samlingsbegrepp för ett flertal sjukdomar med likartade manifestationer (såsom magsmärtor och blodiga diarréer), varav de två vanligast förekommande är ulcerös kolit (eng. ulcerative colitis; UC) och Crohn’s sjukdom (eng. Crohn’s disease; CD). Det finns inga botemedel mot IBD utan behandlingen syftar endast till att lindra patientens smärtor och dämpa inflammationen i tarmen. IBD är vanligast förekommande i den industrialiserade västvärlden (främst i Nordamerika och i norra Europa). De immunologiska mekanismer som orsakar IBD är inte klarlagda, och sjukdomen ökar ständigt över hela världen. Således är IBD viktig att studera ur ett immunologiskt perspektiv.

Det första arbetet handlar om en specifik typ av monocyter, som kännetecknas av höga ytnivåer av proteinerna HLA-DR och CCR9. HLA-DR spelar en viktig roll i sammankopplandet av det medfödda med det specifika immunförsvaret, och är en väletablerad markör för funktionellt aktiva immunceller. CCR9, en så kallad kemokinreceptor, har tidigare visats ha betydelse för inflödet av T-lymfocyter till tarmslemhinnan men har aldrig studerats på monocyter. I arbetet visar vi att förekomsten av dessa blodmonocyter (som vi benämner HLA-DR^{hi}-monocyter) är ökad hos patienter med

inflammatorisk tarmsjukdom i jämförelse med friska kontroller, samt att nivåerna återställs vid behandling med kortikosteroider eller granulocyt-monocyt-aferes (GMA), en kolonnbehandling som fysiskt filtrerar blodet från bland annat monocyter. Vidare visar vi att nivåerna av HLA-DR^{hi}-monocyter korrelerar med patienternas sjukdomsaktivitet, varför vi föreslår att denna celltyp kan användas som en blodmarkör för inflammation vid IBD. Vi föreslår även att CCR9 kan spela en roll i att styra blodmonocyter till tarmslemhinnan, något som dock behöver utredas i en mer specifikt riktad frågeställning.

I det andra arbetet utredde vi hur förekomsten av 19 kemokinreceptorer (inklusive CCR9 som nämndes i arbete I) varierar mellan tre grupper: barnpatienter med ulcerös kolit, Crohn's sjukdom samt vuxna friska kontroller. Studien syftar primärt till att öka förståelsen kring hur kemokinreceptorer, som kan liknas vid adresslappar som cellerna använder för att ta sig till olika platser i kroppen, används av olika monocytpopulationer samt hur de återspeglar inflammation i tarmen. Ett sekundärt syfte är att utveckla en icke-invasiv kombinatorisk markör med förmågan att åtskilja mellan UC och CD, vilket skulle vara av stor betydelse eftersom det ofta är svårt att skilja mellan de båda diagnoserna kliniskt. Resultaten från studien visar att skillnader i nivåer mellan IBD-patienter och friska kontroller förekommer hos 14 av 19 studerade kemokinreceptorer. Vid jämförelse mellan ulcerös kolit och Crohn's sjukdom fann vi däremot endast en receptor, CCR3, som åtskiljde de båda diagnoserna. Sammantaget tyder dessa fynd på att förekomsten av kemokinreceptorer på monocyter återspeglar inflammationsprocessen vid IBD. Eftersom endast åtta patienter studerades i varje grupp (samt 20 friska kontroller) bör en större upprepning av denna studie utföras för att fastställa huruvida dessa resultat kan ligga till grund för utvecklandet av ett diagnostiskt test för IBD.

Vår arvsmassa (DNA), som återfinns i exakta kopior i nästan varje cell i kroppen, är uppdelad i gener som i sin tur ger upphov till proteiner. Eftersom alla gener inte ska översättas till proteiner samtidigt, och vissa proteiner bara ska finnas i vissa delar av kroppen, krävs olika former av reglering. En sådan mekanism kallas för DNA-metylering, och innebär att små molekyler (metylgrupper) kopplas fast på generna. Detta medför oftast att genen stängs av och inte kan översättas till protein. Det tredje arbetet syftade till att utveckla en metod för att mäta graden av DNA-metylering hos fyra gener som är viktiga för aktiveringen av T-lymfocyter. Metoden, som vi kallar för EILA (eng. Epigenetic Immune Lineage Analysis) är nyskapande så till vida att den möjliggör metyleringsanalys av dessa fyra gener i en enstaka läsning, vilket är tidsbesparande samt gör resultaten mer jämförbara och tillförlitliga. Efter att ha validerat metoden tillämpade vi den på T-lymfocyter från patienter med reumatoid artrit och multipel skleros varefter vi kunde konstatera att dessa lymfocyter hade utvecklats mot en speciell variant av T-lymfocyter (Th1 respektive Th17). Denna immunologiska förståelse är viktig för att kunna utveckla effektiva behandlingar mot sjukdomarna.

I det sista arbetet studerade vi T-lymfocyter som vi isolerat ur tarmslemhinnan hos barnpatienter med IBD. Det primära syftet var att utreda huruvida kemokinreceptorn CCR9 förekom på dessa lymfocyters cellyta, något som fastställts i musstudier men inte kunnat

påvisas i människa. Resultaten visade att CCR9 förekom i höga nivåer hos aktiverade T-lymfocyter, vilket tyder på att CCR9 är ett viktigt protein för transporten av T-lymfocyter till tarmen även hos människa. Vi kunde även visa att metylering av CCR9-genen kan påverka genens funktion. Detta arbete ökar således förståelsen kring CCR9 och dess roll vid inflammation vid tarmen.

Sammanfattningsvis har denna avhandling behandlat inflammatorisk tarmsjukdom med fokus på kemokinreceptorernas roll, och då främst CCR9, i att föra monocyter och T-lymfocyter till den inflammerade tarmslemhinnan vid IBD. Flertalet större läkemedelsbolag har på sin agenda att blockera kemokinreceptorer för att förhindra inflödet av immunceller till vävnaden vid olika sjukdomstillstånd men få preparat finns ännu på marknaden. Ökad förståelse kring hur de komplexa kemokinreceptorerna uttrycks och regleras på olika celltyper är således viktigt för behandlingsutveckling och för att bromsa den globala framfarten för inflammatorisk tarmsjukdom.

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