From the Center for Infectious Medicine, Karolinska Institutet, Stockholm, Sweden

INNATE LYMPHOID CELL HETEROGENEITY IN HUMAN TISSUES AT STEADY STATE AND DURING INFLAMMATION

Marianne Forkel

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Innate lymphoid cell heterogeneity in human tissues at steady state and during inflammation
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

„Das schönste Glück des denkenden Menschen ist, das Erforschliche erforscht zu haben und das Unerforschliche ruhig zu verehren.“

Johann Wolfgang von Goethe

“A thinking man’s greatest happiness is to have fathomed what can be fathomed and to revere in silence what cannot be fathomed.”
ABSTRACT

The family of innate lymphoid cells (ILCs) is the most recently identified group of innate immune cells. The ILC classification groups the cells according to their transcription factor and cytokine profile into ILC1, ILC2 and ILC3, paralleling the classification of T cells. ILCs are increasingly recognized for their important functions in the innate immune response, largely mediated through the production of high levels of cytokines. ILCs perform a variety of protective functions in a variety of situations such as the defense against bacterial, viral or helminth infections and the direct regulation of T cell responses, especially at mucosal barrier surfaces. In addition, they are known to be important for lymphoid tissue development, tissue repair and intestinal tolerance towards commensal bacteria. However, immune responses mediated by ILCs can also cause and sustain tissue damage and ILCs are implicated in various inflammatory and autoimmune disorders of the intestine, lung and skin.

This thesis focuses on ILCs in the human body at steady state and during inflammatory diseases. In the first study, we explored the transcriptome of tonsil ILCs using single-cell RNA sequencing and for each ILC subset revealed the expression of previously unrecognized genes which indicate a number of novel functions for ILCs. Furthermore, the analysis of ILC heterogeneity identified three subpopulations of ILC3 that were confirmed by FACS analysis and showed different functionality. In the second study, we present the first detailed characterization of the ILC compartment in the human liver, revealing unique features of ILC composition in adult as well as fetal liver. Furthermore, we detected a correlation between liver fibrosis and an increased frequency of intrahepatic ILC2. In light of the involvement of ILC2 in tissue remodeling, this could indicate that deregulated ILC2 activity contributes to the development of liver fibrosis in humans. The third study addresses the role of ILCs in inflammatory bowel disease (IBD), through systematically analyzing the alterations in ILC subset frequencies in the intestinal mucosa and blood of Crohn’s disease (CD) and ulcerative colitis (UC) patients compared to non-IBD controls. Thus, we identified specific changes in the mucosal ILC composition that were unique to newly diagnosed CD and UC patients respectively. In contrast, we show that in established CD and UC, the ILC distribution is similar but significantly different when compared to non-IBD patients. Overall, a reduction of NKp44+ ILC3 correlated with disease score in IBD. In addition, we investigated the homing marker expression seen in blood ILCs and observed unique expression profiles for each ILC subset. However, treatment with vedolizumab, an anti-α4β7 antibody, did not have an effect on ILC frequencies in the blood.

In summary, the work in this thesis contributes knowledge to aid a better understanding and characterization of ILCs in the human body. The identification of novel potential functions of these cells will help to advance the ILC field. Furthermore, the studies that indicate an involvement of ILCs in liver fibrosis and IBD suggest that ILCs are worth considering when developing new treatment targets in future investigations.
LIST OF SCIENTIFIC PAPERS


Viktoria Konya, Paolo Czarnewski, Marianne Forkel, Anna Rao, Efthymia Kokkinou, Eduardo J Villablanca, Sven Almer, Ulrik Lindfors, Danielle Friberg, Charlotte Höög, Peter Bergman, Jenny Mjösberg. Vitamin D downregulates the IL-23 receptor pathway in human mucosal group 3 innate lymphoid cells. *Journal of Allergy and Clinical Immunology*. 2017


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<tr>
<td>AMPs</td>
<td>Anti-microbial peptides</td>
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<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
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<tr>
<td>CD</td>
<td>Crohn’s disease</td>
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<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<td>CRS</td>
<td>Chronic rhinosinusitis</td>
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<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>DSS</td>
<td>Dextran sulfate sodium</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<td>ieILC1</td>
<td>Intraepithelial ILC1</td>
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<td>ILCs</td>
<td>Innate lymphoid cells</td>
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<td>ILC precursor</td>
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<td>ISC</td>
<td>Intestinal stem cells</td>
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<td>GVHD</td>
<td>Graft versus host disease</td>
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<td>HBV/HCV</td>
<td>Hepatitis B/C virus</td>
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<td>Hereditary nonpolyposis colorectal cancer</td>
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<td>Hepatic stellate cells</td>
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<td>LAP</td>
<td>Latency-associated protein</td>
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<td>LT</td>
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<td>LTI cells</td>
<td>Lymphoid tissue inducer cells</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>Matrix metalloproteinase</td>
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<td>Non-alcoholic steatohepatitis</td>
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<td>Natural cytotoxicity receptor</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PGD$_2$/E$_2$</td>
<td>Prostaglandin D$_2$/E$_2$</td>
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<tr>
<td>PSC</td>
<td>Primary sclerosing cholangitis</td>
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<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
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<tr>
<td>RA</td>
<td>Retinoic acid</td>
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<tr>
<td>seRNA-seq</td>
<td>Single-cell RNA sequencing</td>
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<td>UC</td>
<td>Ulcerative colitis</td>
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1 INTRODUCTION

Traditionally, our immune system has been divided into two branches – the innate immune system and the adaptive immune system. The innate immune system is considered to be the first immunological line of defense after a pathogen has entered the body by overcoming the physical barriers that are formed by the skin, or the inner mucosal linings like the lung or the gut. It can respond immediately to invading pathogens and signals derived from the epithelium with a broad and rather non-specific immune response. In contrast, the adaptive immune system is activated through the direct interaction with innate immune cells and their secreted mediators and needs several days to mount an immune response. Furthermore, the adaptive immune response elicited through T cells and B cells is very targeted and tailored to specific antigens. Once an adaptive immune response has taken place, an immunologic memory is formed and the response of the immune cells to a second encounter with the same pathogen will be initiated more rapidly (1).

1.1 THE INNATE IMMUNE SYSTEM

The innate immune system is composed of a number of different immune cells, including dendritic cells (DCs), macrophages, granulocytes, natural killer cells (NK cells), innate lymphoid cells (ILCs) and mast cells as well as other humoral components like the complement system, anti-microbial peptides (AMPs) or acute phase proteins (2).

Cells of the innate immune system recognize many different conserved pathogen-associated molecular patterns (PAMPs), such as bacterial cell wall components or viral nucleic acids, through the interaction between PAMPs and a variety of different pattern recognition receptors expressed on their cell surface. The detection of PAMPs occurs primarily through DCs and leads to typical innate immune responses such as cytokine production, stimulation of other innate immune cells or phagocytosis, as well as antigen presentation and subsequent activation of the appropriate adaptive immune response (3).

Another well-explored innate immune system pathway is the NK cell mediated killing of infected or transformed cells via missing-self recognition. Infected cells down-regulate major histocompatibility complex (MHC)-I molecules on their surface, which normally inhibit NK cells, thereby leading to the activating of the NK cells to induce the killing of these target cells (4).

This thesis focuses on the ILC family of the human innate immune system, their general biology in homeostasis and their role in two different disease settings of chronic inflammation – inflammatory bowel disease (IBD) and liver fibrosis.
1.2 INNATE LYMPHOID CELLS

Whenever possible I will prioritize the discussion of the biology of human ILCs. However, since a lot of our knowledge concerning ILCs is derived from studies performed in mouse models and tissues, I will discuss these as and when human data is scarce.

1.2.1 Classification and biological functions

The first studies that identified ILCs in mice date back about twenty years and since then the class of ILCs has continuously grown (5-9). Currently, ILCs are divided into three groups, group 1 ILCs (ILC1), group 2 ILCs (ILC2) and group 3 ILCs (ILC3), and these follow the common T cell classification according to transcription factor profile and the effector cytokines produced by the cells (Figure 1) (10). Common hallmarks of ILCs include their lymphoid morphology, and their dependence on both IL-2Rγc-signaling and on the transcriptional repressor Id2 for their development. By borrowing the classification scheme used in the T cell field, ILCs are commonly divided into cytotoxic ILCs (NK cells) and non-cytotoxic helper ILCs (ILC1, ILC2, ILC3). All helper ILC subsets express the IL-7 receptor α-chain (CD127) on their surface. The major difference separating ILCs from T cells, is the lack of rearranged antigen receptors in ILCs (11). ILCs have been particularly recognized for their importance at mucosal surfaces where they are involved in homeostasis and disease (12). Even though ILCs account for only a small fraction of lymphocytes, they are specialized in their ability to produce large amounts of cytokines which then induce responses in a variety of stromal cells, and innate or adaptive immune cells (10, 13).

Group 1 ILCs are defined by their expression of T-bet and the production of the Th1 cytokine IFN-γ (14). They comprise of the prototypic helper ILC1 which express CD127 (10), conventional NK cells and a subset of intraepithelial ILC1 (ieILC1), although NK cells and ieILC1 do not completely fit into the description of ILC1 (15). This is due to their additionally expression of the transcription factor Eomes, the fact they do not display CD127 and that they have cytotoxic functions, the latter resulting in their designation as “cytotoxic ILCs”. Additionally, in mice, NK cells and ieILC1 are derived from a separate developmental lineage than the CD127+ ILCs (16). The discrimination of ILC1 and NK cells is challenging, as no specific ILC1 marker has been described and there is an undeniable overlap in phenotype and function between these two cell types (14). ILC1 are efficiently stimulated by IL-12 to produce IFN-γ. This can be enhanced by IL-18, even though IL-18 alone does not stimulate IFN-γ production (17). Functionally, ILC1 are important for the defense against intracellular bacteria and are suggested to be involved in anti-tumor responses (16, 18). Furthermore, they might be involved in the pathogenesis of IBD and chronic obstructive pulmonary disease (COPD) through the production of pro-inflammatory IFN-γ (15, 17, 19).

ILC2 depend on the transcription factors GATA3 and RORα for their development and maintenance, and express Th2 cytokines, mainly IL-13 and IL-5 (10, 20). They respond to stimulation with alarmins and cytokines, such as IL-33, thymic stromal lymphopoietin (TSLP) or IL-25 and they are also regulated by arachidonic acid metabolites including the
inhibiting lipoxin A₄ (LXA₄) and the stimulating prostaglandin D₂ (PGD₂). PGD₂ acts via the CRTH2 receptor, which is commonly used as a surface marker to identify ILC2 (21-23). ILC2 have been described in several human organs where they are implicated in the pathogenesis of various diseases; these include the lung (allergic inflammation) (24, 25), nasal mucosa (chronic rhinosinusitis, CRS) (22), or skin (atopic dermatitis) (26). In mice ILC2 also play a crucial role in helminth expulsion (7-9). ILC2 are additionally thought to be involved in tissue repair and wound healing through the expression of amphiregulin which acts on the epidermal growth factor receptor (24, 27). To date, this has only been shown in mice and so far, amphiregulin expression in human ILC2 has only been reported at the mRNA level (26). Interestingly, ILC2 even seem to play a role in metabolism and the development of obesity in mice. In the white adipose tissue of obese human individuals, ILC2 frequencies are reduced indicating that these findings might translate to humans (28, 29).

![ILC Classification Diagram](image_url)

Figure 1. Classification of human ILCs. Adapted from (30).
ILC3 generally express the transcription factor RORγt and produce the Th17/Th22 cytokines IL-17 and/or IL-22 in response to stimulation with IL-23 and IL-1β (10, 31). Based on their expression of the natural cytotoxicity receptor (NCR) NKp44, ILC3 are subdivided into NKp44⁺ and NKp44⁻ ILC3 (32). NKp44⁺ ILC3 express the transcription factor aryl hydrocarbon receptor (AHR) and are potent IL-22 producers, whereas NKp44⁻ cells produce rather IL-17 than IL-22. ILC3 have generally been associated with intestinal biology which will be discussed in more detail below. In brief, IL-22-producing ILC3 were found to protect the epithelial barrier and are important for the maintenance of intestinal stem cells (ISCs) in mice, thus it is likely that in humans, IL-22-producing NKp44⁺ ILC3 also act as tissue protective cells (33-35). In contrast, the IL-17-producing ILC3 have been connected with intestinal pathology in mice (36, 37). In addition to a role in the intestinal mucosa, ILC3 have been associated with psoriasis (38). Phenotypically ILC3 are identified and distinguished from other ILC subsets by the expression of c-kit (CD117) on their surface.

Lymphoid tissue inducer cells (LTi cells) which were discovered at a much earlier time point than the other helper ILCs also fall into this group of RORγt-expressing NKp44⁻ ILC3 (5, 39, 40). LTi cells play a crucial role in the development of lymphoid organs. Through lymphotxin α1β2 (LTα1β2) expressed on their cell surface, LTi cells induce the expression of adhesion molecules such as VCAM-1 and ICAM-1 and chemokines in LT-β receptor-expressing stromal cells. This, in turn leads to the recruitment and clustering of other hematopoietic cells at defined anatomical sites and ultimately the formation of secondary lymphoid organs like lymph nodes and Peyer patches (41). LTi cells are frequently present during fetal development and persist through adult life, although at lower numbers, which is probably a result of the reduced need for lymphoid organ development with age (40, 41).

A recent study using parabiotic mice kept under specific pathogen-free conditions provided the first evidence that ILCs are likely to be tissue resident cells with limited recirculation capacity (42). This is, at least in part, supported by human data from intestinal transplantations showing that donor ILCs were still found up to 8 years after transplantation in the intestinal mucosa, although they did not represent the largest cell fraction, thus indicating that ILCs are very long-lived in human tissue but nevertheless slowly renewed (43).

1.2.2 Plasticity

The current classification confines ILCs to their respective subclasses, however, this does not hold true after recent studies have shed light into the plasticity of ILCs. It now becomes more and more clear that ILC subsets exposed to certain cytokine conditions can adopt phenotypic and functional features of other ILC subsets and that this process is largely reversible (Figure 2). Considering the potential tissue residency of ILCs, plasticity would ensure the adaptation of ILC function without the need for recruitment of different cells to the tissue.

Shortly after ILC3 were first described under the name NK-22 cells, Cella et al. found considerable functional plasticity of this cell population, with production of IL-22, IL-17 or
IFN-γ depending on the cytokine combination used for culture (44). Bernink et al. later reported that the population of NKp44⁺ ILC3 from human tonsil gives rise to both RORC-expressing NKp44⁺ ILC3 and TBX21- and IFNG-expressing ILC1 when incubated with IL-2 alone (17). Preferential differentiation into NKp44⁺ ILC3 was induced by stimulation with IL-23, IL-1β and IL-2. Further investigation into the plasticity of NKp44⁺ ILC3 showed that these cells preferentially differentiate into an ILC1-like cell (termed ex-ILC3) under the influence of IL-2 and IL-12. This indicates that NKp44⁺ ILC3 can generate both NKp44⁺ ILC3 and ILC1, in line with a recent report showing that human peripheral blood NKp44⁺ ILC3 include precursors for all major groups of ILCs, which can each be specifically induced with designated cytokine combinations (45).

In a follow-up study Bernink et al. established that ILC1-ILC3 plasticity is bidirectional and reversible. ILC1 differentiate towards an ILC3 phenotype, which includes IL-22 production and up-regulation of Rorγt expression, under stimulation with IL-1β, IL-23 and IL-2, and this process is enhanced by retinoic acid (RA) (46). It is suggested that in vivo this plasticity could be driven by certain subsets of DCs, particularly CD14⁺ DCs which may drive ILC1 differentiation via IL-12 production. In contrast, CD14⁺ DCs promote an ILC3 phenotype through their secretion of IL-23, IL-1β and RA. In Crohn’s disease (CD) patients, higher frequencies of CD14⁺ DCs were found in the mucosa, arguing that they could have a role in the promotion of ILC1 accumulation in the intestine of IBD patients (46). Interestingly, no plasticity between ieILC1 and ILC3 could be observed, indicating again that there is a more distant relationship between the ieILC1 and other ILC subsets.

Figure 2. Plasticity of human ILCs
A set of studies recently revealed plasticity between ILC2 and ILC1 in humans (19, 47, 48). It has been consistently shown that IL-12 can drive the conversion of ILC2 towards an ILC1-like phenotype (termed ex-ILC2), which includes the down-regulation of GATA3, the reduction of Th2 cytokines and a simultaneous up-regulation of T-bet and IFN-γ. In addition, IL-1β plays an important role in rendering ILC2 responsive to IL-12 stimulation, whereas in the absence of IL-12, IL-1β enhances Th2 cytokine release (19, 48). Reversal of these ex-ILC2 back to their original phenotype of ILC2 could be induced by IL-4. The described plasticity of ILC2 is suggested to play a role in COPD, a disease with elevated IL-12 levels, where reduced ILC2 and increased ILC1 frequencies are found in the lung tissue. In contrast, in nasal polyps of CRS patients, elevated levels of IL-4 and the accumulation of ILC2 were found (19). Increased levels of IL-13+ IFN-γ+ double positive clones were also detected in the intestine of CD patients, supporting the theory that this type of ILC2 plasticity might occur in vivo at sites with high IL-12 expression (47).

Considering the various types of ILC plasticity discussed, it is reasonable to expect that a similar trans-differentiation occurs between ILC2 and ILC3 but published evidence for this type of plasticity in humans is, to date, lacking. However, mouse studies indicate that ILC2 to ILC3 plasticity exists. Two subsets of mouse ILC2 have been described. One subset consists of natural ILC2 (nILC2), which express the IL-33 receptor ST2 and produce IL-13 and IL-4. The other subset consists of “inflammatory” ILC2 (iILC2) which are characterized by the expression of the IL-25 receptor IL17RB but no ST2, the expression of intermediate levels of Rorc and the ability to co-produce IL-13 and the ILC3 signature cytokine IL-17 (49). It was recently shown that nILC2 can differentiate into iILC2 and that this process depends on Notch signaling which directly promotes Rorc expression and elicits IL-17 co-production alongside conservative ILC2 cytokine expression in the same cell (50).

1.3 ILCs in the Intestine

The gut lumen contains a vast number of commensal bacteria and food antigens and it is only separated from the underlying tissue by a single layer of epithelial cells. The integrity of this physical barrier is extremely important for the health of the organism and damages need to be rapidly repaired to prevent immune responses and the translocation of luminal contents to the tissue.

This thin epithelial layer comprises mainly of absorptive enterocytes and it is reinforced by specialized cells like goblet cells and Paneth cells, which secrete mucins and AMPs respectively, forming a supportive physical and biochemical barrier. This mucous layer is further armed with soluble IgA molecules secreted by sub-epithelial plasma cells and then transcytosed by intestinal epithelial cells (51).

The luminal content is constantly surveilled by mononuclear phagocytes that underlie the epithelium and receive antigens through either direct sampling via trans-epithelial dendrites
or via M cells that take up and deliver antigens (51). When there is a breach of these barriers and the pathogens are sensed in sub-epithelial compartments, potent immune responses are initiated by epithelial cell signals and mononuclear phagocytes, which involve the recruitment and activation of other immune cells (3, 51).

In this system, ILCs seem to play complex roles in maintaining the homeostasis and promoting or inhibiting immune responses during inflammation or infection.

1.3.1 ILCs in homeostasis and their tissue protective functions

Very recently, the distribution of ILCs in the healthy human intestinal mucosa was thoroughly investigated (46, 52). All subsets of ILCs are present in the healthy lamina propria with ILC3 making up the largest fraction (more than 80%), and these are mostly represented by NKp44⁺ cells, which increase in frequency from proximal to distal parts of the gastrointestinal tract. ILC2 are barely found throughout the whole gastrointestinal tract, whereas ILC1 and ieILC1 are both present with similar frequencies of around 5% throughout the intestine (46, 52). Nevertheless, there is little mechanistic insight into the functions of ILCs during homeostasis in humans. In mice though, several ways through which ILCs contribute to epithelial barrier integrity have been unraveled, mainly via the production of IL-22, and some indications exist suggesting that these mechanisms could be operative in human ILC biology as well (Figure 3).

Figure 3. ILCs in intestinal homeostasis. Adapted from (30).

The importance of the interplay between microbiota-sensing macrophages and ILCs and their resulting production of effector cytokines has been investigated in several studies, indicating a role for ILC3 in intestinal tolerance. Microbiota-induced IL-1β production by macrophages induces GM-CSF release from ILC3 and in a feedback loop, GM-CSF stimulates macrophages and DCs to release IL-10 and RA which then in turn stimulate Treg
differentiation and thereafter oral tolerance (53). Along the same lines, intestinal CX3CR1+ mononuclear phagocyes have been found to stimulate ILC3 via IL-23, IL-1β and TLA1 to produce IL-22 and GM-CSF (54). As mentioned above, it has been shown that different types of DCs are present in the healthy and the inflamed human gut which have the potential to either maintain ILC3 populations or induce ILC1 (46). Furthermore, it has been shown in mice, that IL-25 released by epithelial cells in response to microbiota, can act via DCs to indirectly inhibit ILC3 functions through a so far unknown mechanism (55).

IL-22, a major effector cytokine produced by NKp44+ ILC3, has been implicated in several tissue protective processes in the intestine. However, due to its potential pro-inflammatory effects through the induction of other pro-inflammatory cytokines and its capacity to support tumor growth, it requires tight regulation (56, 57). The IL-22 receptor is chiefly expressed on non-hematopoietic cells like intestinal epithelial cells and amongst the known IL-22 effects, there are functions such as the protection of epithelial integrity through the induction of anti-apoptotic proteins and the induction of mucus proteins and AMPs (57).

ILC3-derived IL-22 clearly plays a role in the protection and activation of intestinal stem cells (ISCs) via STAT3 activation, and thus in the regeneration of the intestinal epithelium after tissue damage (58). Such a protective capacity of ILC3 is further supported by data from graft versus host disease (GVHD), where ILC3-derived IL-22 has been found to be an important factor for intestinal stem cell protection in mice (33). In human hematopoietic stem cell transplantation, the appearance of circulating NKp44+ ILC3 seemed to correlate with a more favorable outcome, including less GVHD (59). In conclusion, it is reasonable to assume that ILC3 in humans, in an equivalent manner to the case seen in mice, also protect intestinal stem cells.

ILCs have also been shown to regulate the commensal flora. ILC3 promote IL-22-dependent containment of commensal bacteria residing in Peyer’s patches or mesenteric lymph nodes in mice, thus preventing systemic spread (60). It is well established that the maintenance of a beneficial intestinal microbiota is important for the health of the host. Fucose residues on the surface of intestinal epithelial cells are used as dietary carbohydrates by many commensal bacteria and in mice, it has been shown that this epithelial cell fucosylation is yet another process that is driven by ILC3-derived IL-22 (61). IL-22 production was induced by commensals and together with commensal-independent lymphotixin, it drives the expression of the enzyme Fut2 which is necessary for fucosylation in epithelial cells.

A cytokine-independent mechanism by which mouse ILC3 are involved in maintaining intestinal homeostasis is via their physical interaction with T cells, which leads to the induction of intestinal tolerance. ILC3 express MHC-II and present antigen but lack co-stimulatory molecules and the MHC-II-dependent ILC-T cell interaction leads to a limited response by commensal-specific T cells (62). It was later shown that this is due to commensal-specific T cell apoptosis induced by antigen presentation and the withdrawal of IL-2 mediated by ILC3. Expression of MHC-II in human intestinal ILC3 has been confirmed, thus indicating a role for human ILC3 in T cell regulation as well (62, 63).
1.3.2 ILCs in intestinal inflammation

In the inflamed mucosa of IBD patients, the frequencies of the different ILC subsets are significantly altered compared to healthy individuals. A number of consentaneous reports have been published regarding this, however, inconsistent data, especially that derived from mouse models also exists.

Intestinal inflammation in mouse models can be induced in various ways, for instance with infectious agents (e.g. *Citrobacter rodentium, Helicobacter hepaticus*), chemically (e.g. dextran sulfate sodium - DSS) or immunologically (e.g. anti-CD40 model). Therefore, the results must be interpreted with care since the models only illustrate certain aspects of IBD. Often, studies utilize mice devoid of adaptive immune cells and mechanisms found in these cases might differ from those seen in fully immune competent mice. Since the etiology of IBD is not clear, a perfect IBD mouse model that completely mirrors the human disease does not obviously exist.

It has repeatedly been shown that IFN-γ-producing cells accumulate in the inflamed intestine of IBD patients and mice (Figure 4) (15, 17, 34, 37, 46). Well-known IFN-γ mediated effects are connected to the immune response against viral and intracellular bacterial infections and to tumor control. IFN-γ induces an inflammatory response, activates macrophages and stimulates the expression of MHC-I and -II molecules on infected cells or antigen-presenting cells respectively. Furthermore it induces the differentiation of naïve T cells into Th1 cells (64). The ILC1 subsets are potent producers of IFN-γ and both CD127⁺ ILC1 and iεILC1 were found at increased frequencies in the mucosa of CD patients (15, 17). However, the accumulation of iεILC1 seems to be far less prominent than that of CD127⁺ ILC1 (46). iεILC1 appear to be more closely related to NK cells based on the expression of CD94 on their surface, and the expression of Eomes and the cytotoxic proteins perforin and granzyme B (15). In addition, the frequency of NK cells in the mucosa of CD patients is unchanged, indicating that only the CD127⁺ IFN-γ-producing ILC subsets are consistently increased in CD patients (15, 46). In contrast, data from mice lacking both adaptive immune cells and T-bet expression (and thus ILC1) argue for a protective role of IFN-γ-producing ILC1 as these mice develop spontaneous colitis (65).

Simultaneously to the increase in ILC1, a decrease in the frequency of NKp44⁺ IL-22⁺ ILC3 was consistently observed in the intestinal mucosa of CD patients and several indications for tissue-protective and anti-inflammatory roles of ILC3 in mouse models of colitis are available (17, 34, 66). Mice lacking Rorγt (and thus ILC3) developed more severe colitis in the DSS-colitis model than mice with ILC3 (67). In addition, mice lacking MHC-II expression in ILC3 developed spontaneous colitis. Paralleling this finding, MHC-II expression on ILC3 from pediatric IBD patients was reduced and inversely correlated with Th17 cell frequencies, indicating that the interaction of ILC3 with Th17 cells is important for the regulation of pro-inflammatory responses (63). Considering the aforementioned tissue protective effects of IL-22 and assuming that human ILC3 can indeed negatively regulate T cells, these findings...
could represent mechanisms through which changes in ILC frequencies contribute to the pathology of IBD and increased intestinal permeability seen in IBD. However, one study found an increase in IL-22 production by CD3\(^\text{+}\) cells from IBD versus non-IBD controls (54). This contrasting results could be due to ongoing mucosal repair as the cohort analyzed in this study had mild to moderate IBD, whereas most studies investigate resection material from patients with refractory disease.

![Figure 4. ILCs in intestinal inflammation. Adapted from (30).](image)

The plasticity described above of ILC3 towards an ILC1 phenotype is believed to account for the majority of the ILC1 accumulation and the reduction of the NKp44\(^{+}\) ILC3 seen (46). Although ILC2 only represent a negligible fraction of intestinal ILCs, Lim et al. have recently detected IFN-\(\gamma\)-producing IL-13\(^{+}\) ILC2 in the intestinal mucosa of CD patients, indicating another possible ILC-derived source of IFN-\(\gamma^{+}\) in IBD, even though their frequency was not compared to samples from healthy intestine (47). Since the process of ILC3 and ILC2 conversion towards an ILC1 phenotype was demonstrated to be reversible, it is tempting to speculate that a reversal of the skewed ILC composition and thereby an amelioration of the inflammation could be achieved in IBD patients through novel treatment regimens.

IFN-\(\gamma\)-producing ILCs were not the only cell type with increased frequencies in the mucosa of IBD patients, as greater numbers of IL-17 co-producing ILCs were also found, and furthermore, this has additionally been observed in a mouse model of Helicobacter hepaticus-induced colitis (36, 37). Typically, IL-17 induces responses against extra-cellular bacteria and fungi by instructing the epithelial cells to produce inflammatory cytokines, AMPs and chemokines, which subsequently recruit neutrophils to the site of infection (68). Moreover, it has been hypothesized that IL-17\(^{+}\) ILC3 might contribute to pathology via the
induction of the formation of isolated lymphoid follicle as IL-17^+ ILC3 contain LTi-like cells (36, 40).

Although it might seem obvious that IL-17 has pro-inflammatory effects, a clinical trial in CD patients with the monoclonal antibody secukinumab which targets IL-17 failed to show a positive effect and, in fact, led to a worsening of the disease indicating that in IBD, IL-17 could have a tissue-protective effect (69). This is supported by data showing tissue protective effects of IL-17 when released in an IL-23-independent manner from γδ-T cells (70). Furthermore, in a mouse model of DSS-independent IBD were observed (71, 73). To date, whether ILC3-derived IL-17 has a dual function according to the manner of its induction and whether it can be induced independently of IL-23 in ILC3, remain open questions.

Another antibody, ustekinumab, targeting the p40 subunit shared by IL-23 and IL-12 showed a positive effect in IBD treatment. This might be due to the neutralization of IL-12 and subsequent reduction of IFN-γ-producing ILC1. However, an involvement of a block in IL-23-dependent IL-17 release in the attenuation of disease cannot be excluded and could also be responsible for the observed effect.

1.4 INFLAMMATORY BOWEL DISEASE

1.4.1 Pathophysiology of inflammatory bowel disease

Inflammatory bowel disease (IBD) is an umbrella term for two types of chronic and progressive inflammatory disorders of the gastrointestinal tract, Crohn’s disease (CD) and ulcerative colitis (UC). These two diseases have many commonalities but also distinct separating features. Generally, they are believed to arise from an excessive immune response towards commensal bacteria in genetically susceptible individuals (73, 74). The pattern of inflammation differs between UC and CD. Inflammation seen in UC patients only affects the colon, starting from the rectum and extending proximally to varying degrees in a continuous manner and is always restricted to the mucosa. In contrast, the inflammation in CD patients can affect any part of the gastrointestinal tract from the mouth to the rectum but is predominantly detected in the colon and ileum. It shows a discontinuous pattern and is characterized by a deeper, transmural inflammation (73, 75). In IBD patients the physical barrier of the epithelium is impaired, including a disrupted mucous layer, which thus leads to increased intestinal permeability (73). The most prominent symptoms of IBD are bloody diarrhea, abdominal pain, fever and weight loss (75, 76).

IBD affects approximately 2.5-3 million people in Europe and represents a great burden to those who are affected, not only relating to symptoms and treatment but also in terms of quality of life, psychological impact and impairment of social life (77). A common complication of IBD is its association with extra-intestinal manifestations, the most prevalent being joint manifestations, such as acute arthritis. Others include cutaneous manifestations
which are predominant in women and, on rarer occasions, uveitis or primary sclerosing cholangitis (PSC) (77). Most chronic inflammatory diseases are also associated with cancer development and further to this, IBD constitutes an example of the inflammation-dysplasia-cancer sequence as it increases the risk for colon cancer (77).

The underlying causes of IBD are still enigmatic and none of the suggested susceptibility factors alone are sufficient to initiate disease. Analyses of twins and familial accumulation of cases has sparked studies that recently identified many risk genes for IBD. To date, a total of over 200 risk loci have been identified (78-80), with a number of different pathways identified that might be involved in the pathogenesis of IBD.

The strongest genetic association was observed between gene variants of the NOD2 gene and an increased risk that was specific for CD. NOD2 is important for sensing bacteria and it has been suggested to increase CD susceptibility by impairing AMP release from Paneth cells, as well as mediating inappropriate immune activation to commensals (73, 81). Other CD specific risk variants are located in the gene for ATG16L1 which is a protein essential for autophagy and might contribute to CD pathogenesis by defective microbial clearance or through Paneth cell abnormalities (81, 82). NOD2 has also been found to induce autophagy thus raising the possibility that these two genes affect the same pathway (83). Furthermore, specific variants of the IL-23R gene (IL23R) are strongly associated with IBD. Protective variants show a loss of function, indicating that deregulation of IL-23R signaling and subsequent induction of Th17 cytokine responses to commensal bacteria confer an increased risk of IBD (81).

The worldwide distribution of IBD has been changing in recent years, with an augmented incidence in emerging countries that have adopted a westernized lifestyle, leading to the conclusion that environmental factors have a great influence on the onset of IBD. Studies have identified smoking as a strong influencing factor connected with an aggravation of CD and a milder disease course in UC (84). A large number of other lifestyle factors also have adverse effects and increase the risk of developing IBD. Among others, stress and disturbed sleep, too little sunlight exposure and subsequent vitamin D deficiency, as well as the use of certain medications like non-steroidal anti-inflammatory drugs or oral contraceptives are all suggested to be associated with the development of IBD (74, 85-88).

IBD patients often show dysbiosis of the microbiota with an overall reduced diversity of commensal bacteria (89). A number of factors that can negatively influence the composition of the intestinal microbiota exist, such as the adoption of a Western diet, increased hygiene and the use of antibiotics (74, 90). In contrast, exposure to certain factors in early life is suggested to be protective for IBD through a positive influence on the diversity of the microbiota, and these include breastfeeding, contact with pets, and a larger number of siblings (74, 86, 91).
1.4.2 Treatment of inflammatory bowel disease

First-line treatment for IBD involves anti-inflammatory drugs, most commonly 5-aminosalicylates (5-ASA) for UC or glucocorticoids for CD. Refractory disease is treated with immunosuppressive drugs, most commonly the thiopurine azathioprine. For UC patients, probiotic therapy seems to be supportive in combination with conventional therapies and surgical resection can be curative of the disease in certain cases, although this is not a viable option for CD patients (76, 92).

The development of new biologic drugs has led to increased treatment options for IBD patients. Anti-TNF-α monoclonal antibodies, for instance infliximab, act by blocking the pro-inflammatory effects of this cytokine, such as macrophage activation. Anti-TNF treatment has proven to be an important addition to the standard treatment of IBD even though 30-50% of patients fail to respond (93, 94). Another anti-cytokine antibody, ustekinumab, which targets the p40 subunit shared by IL-12 and IL-23 has shown beneficial results in the treatment of CD (95). This is in agreement with genetic data revealing that mutations in the IL-23 signaling pathway are a risk factor for IBD. However, it is still unclear whether the effect of ustekinumab is based on the blocking of IL-12 and/or IL-23. This will hopefully be revealed shortly, since antibodies targeting the specific p19 subunit of IL-12 are currently in clinical trials (94). However, an antibody directly targeting IFN-γ has yielded disappointing results in a clinical trial, challenging whether there is a major role of IFN-γ in IBD pathogenesis (96).

A different strategy to alleviate IBD encompasses the blockade of lymphocyte trafficking to the intestine. The humanized monoclonal antibody vedolizumab, for example, binds specifically to the α4β7 integrin on lymphocytes and prevents their interaction with MAdCAM-1 on endothelial cells in the intestine, which is required for gut homing (97). It is thought to mainly act through blocking the infiltration of memory T cells to the intestine and treatment with vedolizumab has shown therapeutic benefits in the treatment of both CD and UC patients (97). Additional therapies utilizing similar strategies as vedolizumab are currently being tested (94).

1.5 LIVER FIBROSIS

Liver fibrosis is the excessive accumulation of extracellular matrix (ECM) and can be caused by a number of underlying diseases, which share the common hallmark of sustained inflammation. The most common causes of liver fibrosis are hepatitis B (HBV) and C (HCV) infection, alcohol abuse, non-alcoholic steatohepatitis (NASH) or autoimmune liver diseases (98). Although these diseases vary greatly in their etiologies, they all converge towards the common mechanisms of liver fibrosis.

The same mechanisms that underlie the development of liver fibrosis are indispensable for short term tissue protection, healing and regeneration in response to acute and transient tissue injury. In contrast, when they are deregulated and continuously active over a long time, these responses become detrimental. Abnormal ECM accumulation is accompanied by
progressively impaired liver function due to replacement of functional parenchymal tissue (99). Typically, fibrosis is a slowly progressing condition that remains largely asymptomatic for many years until it reaches its end stage, called cirrhosis, which is associated with portal hypertension and a high risk of liver failure and liver cancer (99). Liver cirrhosis presents a major cause of mortality worldwide with more than 1.2 million deaths in 2015 (100).

1.5.1 Basic mechanism of liver fibrosis

Hepatic stellate cells (HSCs) are localized in the sub-endothelial space (also called space of Disse) and under homeostatic conditions, they store large quantities of vitamin A in cytoplasmic droplets (Figure 5) (101). When activated, HSCs turn into proliferating, contractile myofibroblasts that produce large amounts of ECM (102). Activated myofibroblasts are the main producer of ECM although other cell types, like portal fibroblasts can contribute to its deposition as well (103, 104). In liver fibrosis, ECM quality is altered, with a dominance of collagen I and fibronectin, instead of collagen III, as the main components and extensive cross-linking of these collagen molecules (99).

Figure 5. The hepatic architecture of normal and fibrotic liver.
Excessive ECM production is observed during sustained inflammation. Damage-associated molecular patterns (DAMPs) released from hepatocytes during cell injury or mediators generated during pathogen-induced inflammation can activate HSCs. The mechanisms of HSC activation differ between underlying diseases and include for example, cytokines, adipokines, oxidative stress or TLR-signaling (105). However, the activation of HSCs is a common converging point for the different underlying diseases that cause liver fibrosis.

The most potent of the known pro-fibrotic cytokines is TGF-β as it directly induces the transcription of pro-fibrotic target genes, including collagen I, in HSCs (106). TGF-β is produced by Kupffer cells and macrophages and secreted in an inactive form bound to latency-associated protein (LAP) which becomes activated through dissociation from LAP. This dissociation can be induced, for example, through matrix metalloproteinases (MMPs). Other important pro-fibrogenic activators of HSCs include platelet-derived growth factor (PDGF), TNF-α and leptin (105, 107).

1.5.2 IL-33/IL-13 axis in liver fibrosis

Th2-associated cytokines were found to be involved in the development of liver fibrosis whereas the Th1-associated cytokine IFN-γ was demonstrated to be protective in liver fibrosis (108). Th2 cells and ILC2 are potent producers of IL-13 and are activated by IL-33, amongst other stimuli (7-9).

IL-33, an IL-1-related cytokine, is released after cell injury in the form of a DAMP which participates in the induction of inflammation and acts upstream of IL-13 release (109). Elevated IL-33 levels in serum and liver tissue of liver fibrosis patients have been recorded (110, 111) and HSCs as well as hepatocytes are the two major sources of IL-33 (111, 112). In mice, a direct effect of IL-33 in fibrosis development, via the induction of collagen and TGF-β in HSCs, has been shown (111). In addition, an indirect role of IL-33, which leads to the expansion and stimulation of intrahepatic ILC2 that in turn produce pro-fibrotic IL-13, has also been reported (110).

IL-13 acts as a pro-fibrotic cytokine in different contexts (113-116). Exactly how IL-13 mechanistically exerts its pro-fibrotic effects is not entirely clear but several pathways have been suggested. Activated human HSCs express the IL-13Rα2, which is up-regulated by TGF-β or TNF-α (117), and IL-13 directly induces collagen I expression in rat and mouse HSCs (110, 118). In mice, an indirect effect of IL-13 through the induction of TGF-β and MMPs, that thereafter cleave and activate TGF-β from LAP in macrophages, has also been observed (108, 115, 119). This indicates the possible existence of a positive feedback loop between TGF-β and IL-13 in liver fibrosis. Additionally, IL-13-induced proliferation of human lung myofibroblasts and elevated IL-13 levels were found in liver tissue of HCV patients (118, 120) supporting the important role of this cytokine in the development of liver fibrosis.
1.5.3 Treatment of liver fibrosis

It has been shown that liver fibrosis can be reversible when the factors causing fibrosis are treated. Therefore, elimination of the underlying disease or trigger (e.g. anti-viral treatment, cessation of alcohol intake etc.) is crucial and has been proven effective in initiating fibrolysis (121, 122).

No standard treatment is available for liver fibrosis and many new approaches are being tested. For instance, some of the emerging anti-fibrotic treatments aim to prevent the activation of HSCs to myofibroblasts, induce senescence or apoptosis of myofibroblasts, reduce the expression of ECM proteins or induce ECM degradation (122, 123). Others are targeting pro-inflammatory cytokines (e.g. TGF-β) or their signaling pathways (e.g. PDGF-R pathway). In addition, anti-fibrotic IFN-γ treatment is a promising strategy that is being tested (124). Nevertheless, its applicability in clinical settings still needs to be assessed. Unfortunately, when the disease has advanced to the cirrhotic stage, where little functional parenchymal tissue is left, liver transplantation becomes the only curative treatment for most patients since regression from cirrhosis is a rare event.

1.6 ILCs in tissue fibrosis

Reports on ILCs in human liver fibrosis are lacking, however direct evidence for the involvement of ILCs, especially ILC2, in tissue fibrosis has been provided by studies in different mouse models. In these models, ILC2 have been found to play a role in hepatic and pulmonary fibrosis (110, 125, 126).

In a pulmonary fibrosis mouse model, based on *Schistosoma mansoni* egg-induced fibrosis, IL-25-driven ILC2 activation was sufficient to promote fibrosis via IL-13 release, leading to subsequent collagen deposition (125). Targeted depletion of ILC2 abrogated *S. mansoni* egg-induced fibrosis, and IL-13 production by ILC2 was essential for pulmonary fibrosis as transfer of only the IL-13-expressing ILC2 could elicit fibrosis in contrast to IL-13-deficient ILC2. Additionally, Hams et al. found an increase of ILC2 and IL-25 in the bronchoalveolar lavage fluid of idiopathic pulmonary fibrosis patients, indicating that a role for ILC2 in pulmonary fibrosis is likely to be present in human disease. Moreover, in a different model of pulmonary fibrosis, which is induced by bleomycin, a role for ILC2 in fibrosis development driven by IL-33 was confirmed (126). Transfer of ILC2 into mice treated with bleomycin exacerbated lung fibrosis compared to bleomycin treatment alone.

In a model of IL-33 induced hepatic fibrosis McHedlidze et al. found that fibrosis development was IL-13-dependent since *IL13−/−* animals showed reduced hepatic collagen deposition. (110). ILC2 accumulated in fibrotic livers and were identified as the main source of the IL-13 driving fibrogenesis since transfer of ILC2 into IL-13−/− mice was sufficient to induce hepatic fibrosis. Subsequently, it was shown that IL-13 could directly act on HSCs in vitro through binding to the IL-13Rα1/IL-4Rα heterodimer which induced proliferation and
expression of pro-fibrotic genes like COL1A1 or Timp1. Thus, a first link between ILC2 and hepatic fibrosis in the mouse was discovered.

Taken together, these data strongly suggest that ILC2 play a role in fibrosis development in different tissues. Even though much can be learned from mouse studies, how these results regarding ILCs are applicable to human biology is still unclear and requires further studies on human samples.

In contrast to ILC2, the CD127+ ILC1 or ILC3 populations have not been linked to tissue fibrosis. However, a role for IL-17A in bleomycin-induced lung fibrosis was reported and the level of IL-17A in the bronchoalveolar lavage fluid of idiopathic pulmonary fibrosis patients was elevated (127). This raises the possibility that ILC3-derived IL-17A could potentially contribute to fibrosis development. In contrast, studies have shown a strong anti-fibrotic action of NK cells in hepatic fibrosis models, mediated by direct induction of apoptosis in HSCs through IFN-γ and TRAIL (128-130). Considering the high capacity of CD127+ ILC1 to produce IFN-γ it is likely that these cells also exert anti-fibrotic effects, although this remains to be formally established.
2 AIMS

The work included in this thesis aimed to investigate ILC biology in humans, including both the study of the general properties of these cells in homeostasis as well as determining a role of ILCs in two different chronic inflammatory conditions, IBD and liver fibrosis.

Specifically, the three different studies aimed to:

- Investigate the heterogeneity and identify potential novel functions of human ILCs in non-inflamed tonsil tissue through non-biased single-cell RNA sequencing and bioinformatic analysis (Paper I).
- Perform a detailed characterization of human intrahepatic ILCs in fetal and adult tissue and identify changes in the ILC compartment in fibrotic liver tissue (Paper II).
- Systematically study human ILCs in intestinal biopsies and blood from a cohort of IBD patients comparing samples from ulcerative colitis and Crohn’s disease at different disease stages (Paper III).
3 METHODOLOGICAL CONSIDERATIONS

Various standard techniques have been employed for performing the experiments in paper I-III, including real-time PCR (RT-PCR), enzyme-linked immunosorbent assay (ELISA) and multiplex immunoassay, cell isolation from various tissues and cell culture techniques. Here, I chose to only highlight the techniques that I have used extensively: namely flow cytometry analysis and flow cytometry-based cell sorting. I will also present a state-of-the-art method for single-cell RNA sequencing (scRNA-seq) that we used in paper I. In addition, I will take a closer look at the human samples used in the different studies and present some considerations.

3.1 PATIENT SAMPLES

In all the projects included in this thesis I have exclusively used fresh human samples. This material is very precious and it needs to be highly valued, even when samples arrive at unusual times thereby demanding a high degree of flexibility. It is important to not forget that behind each sample there is a real person who donated the material in order to help create and further our knowledge and move a tiny step further towards understanding biological mechanisms in the human body during health and disease. Overall, I would like to highlight the importance of following ethical guidelines when working with human patient material in order to first and foremost protect the patient’s interests.

3.1.1 Tonsils

Tonsils have served as an important source of immune cells in paper I and paper II. They are an unproblematic tissue to obtain, since they are usually discarded after a tonsillectomy and many routine operations are conducted at Karolinska University Hospital every week. Tonsils contain all of the ILC types described to date (17) and thus present an optimal material for basic ILC research. In order to investigate immune cells at steady state we have worked with non-inflamed tonsils from tonsillectomies carried out due to obstructive sleep apnea syndrome.

3.1.2 Liver tissue

The analysis of ILCs in the human liver is the topic of paper II. To analyze ILCs in the liver at steady state we collected non-fibrotic liver tissue. Access to completely healthy material when studying human tissues is generally difficult and often impossible. For a minority of samples, we had unique access to livers from healthy deceased organ donors whose livers were not suitable for transplantations. However, most samples that were used as non-fibrotic control samples are derived from tumor resection surgeries. It is currently unknown how tumor diseases affect the surrounding, macroscopically healthy, tissue. Therefore, this is always a factor to keep in mind when drawing conclusions from comparisons between diseased tissue and this type of control tissue. To investigate the possible changes in the ILC
compartment in fibrotic livers, we have collected fibrotic livers from transplantation surgeries which were most commonly due to PSC, but also from a variety of other diagnoses, such as alcoholic cirrhosis, NASH, cryptogenic cirrhosis, angiomyolipoma, leiomyosarcoma, Adams Olivier Syndrome and alpha-1 antitrypsin deficiency. This diversity of diagnoses might be a confounding factor of our study, but conversely, this situation could also have added value by contributing knowledge regarding common patterns in liver fibrosis resulting from various underlying causes.

3.1.3 Intestinal biopsies

In order to analyze the composition of ILCs in the intestinal mucosa of patients with IBD, I have worked with biopsies taken during endoscopic examination (paper III). The patients were stratified into different groups according to the diagnosis (UC or CD) and disease history; patients that were newly diagnosed on the day of examination were distinguished from those that received their diagnosed over one year prior to the examination. Even though the newly diagnosed patients are only officially diagnosed at the time they enter our study, they have often suffered from unspecific symptoms for many years, making it difficult to pinpoint a definite time of disease onset. Nevertheless, this group is closer to the initiation of disease and results obtained from this cohort can give clues regarding the role of ILCs in the onset and propagation of IBD. The control non-IBD group consisted of clinically healthy colon cancer screening patients with a hereditary predisposition for colon cancer (Lynch syndrome, also called hereditary nonpolyposis colorectal cancer, HNPCC), which is an autosomal dominantly inherited disease due to mutations in DNA mismatch repair genes (131). Although unlikely to affect ILC composition, it cannot be completely excluded that Lynch syndrome does not influence the immune cell composition in the intestine.

3.2 FLOW CYTOMETRY

The main method I have employed extensively throughout all my studies (Paper I-III) is advanced flow cytometry analysis and fluorescence-activated cell sorting (FACS).

Every cell type in the body expresses a diverse set of proteins on its surface which are there to interact with and sense the surrounding environment or other cells. This unique combination of proteins can be used to identify the cell type and to purify the cells of interest. In flow cytometry, this identification is achieved with fluorochrome-coupled antibodies that bind to the marker proteins on the cell surface, thus tagging them. This “color-coding” can be detected with a flow cytometer where after antibody labeling, the stained cells pass through laser beams of different wavelengths, with each exciting specific fluorochromes to emit light at their unique emission wavelengths. The quality and quantity of the emitted light gives information about the marker proteins that are present on and in each cell and moreover, the relative amounts. The emitted light is detected and translated into electrical signals by photomultiplier tubes and can then be displayed and analyzed on a computer.
Flow cytometry allows multi-parametric analysis of protein expression at the single-cell level at a rate of thousands of cells per second and it is routinely used in immunology to analyze a variety of immune cells from a variety of tissues. For the identification of ILCs and their respective subgroups a high number of markers are needed, due to the complex surface protein definition of these cells. Therefore, flow cytometry represents the method of choice for the investigation of ILCs, allowing both the simultaneous analysis of a combination of markers for cell identification and subsequent analysis of the proteins of interest. Additionally, flow cytometry provides a very broad spectrum of possible applications. In my work, I have routinely used and designed flow cytometry panels with up to 17 colors for the analysis of ILCs and used them to address a number of research questions, such as phenotyping immune cells ex vivo, analyzing the expression and co-expression of surface proteins of interest, analyzing transcription factor profiles of ILCs (Paper I-III), and assessing intracellular cytokine production by ILCs in stimulation cultures in vitro (Paper I and paper II).

In addition to merely analyzing cells with flow cytometry, the same methodological principle is used for cell sorting with FACS. The advantage of this method is that cells are simultaneously analyzed and viable cells are sorted without destroying them which makes FACS a very attractive tool for subsequent functional analysis (paper I and II) or other analysis methods like scRNA-seq (paper I) of pure cell subsets. Prior to paper I, we set up single-cell sorting in 96-well plates for scRNA-seq of ILCs.

### 3.3 SINGLE-CELL RNA SEQUENCING

In paper I we employed scRNA-seq using the recently developed Smart-seq2 protocol that allows for full-length characterization of the entire transcriptome. Even though the actual sequencing was performed by our collaborators, here I would like to shortly describe the basic principles of the method since it is fundamental to the study presented in paper I (132, 133).

Using flow cytometry single-cell sorting, individual ILCs were sorted straight into a mild detergent for cell lysis in 96-well plates. The lysis buffer contained free dNTPs and oligo-dT primers carrying a known anchor sequence at their 5’-end. Subsequently, all poly-adenylated RNA species were reverse transcribed using a retroviral Reverse Transcriptase, generating a complementary DNA (cDNA) strand (Figure 6). The Reverse Transcriptase used in this protocol adds 2-5 untemplated nucleotides when it reaches the 5’-end of the template mRNA, thus creating an overhang consisting mostly of cytosines. A second oligo (template switch oligo - TSO) containing the same anchor sequence at its 5’-end as the oligo-dT primer and three riboguanosines at its 3’-end, uses the untemplated nucleotides as a docking site allowing for the extension of the cDNA strand with the TSO as template. One of the riboguanosines in the TSO is a special locked nucleic acid (LNA) which increases the binding strength to the cytosine overhang and improves the thermal stability of the TSO. At
the end of the reverse transcription process, all cDNA molecules carry the same known anchor sequence and can thus be amplified in the following PCR step. Next, the amplified cDNA is fragmented by the use of a Tn5 transposase which carries partly double-stranded adaptor oligonucleotides. The Tn5 randomly fragments the DNA while it simultaneously ligates the adaptor sequences in a reaction known as “tagmentation” (tagging + fragmentation). The adaptor sequences are then used as primer binding sites for the final enrichment PCR, where barcoded adaptors are introduced to uniquely label each cell in order to increase the multiplexing capabilities. In the end, the libraries from all single-cell samples are pooled and can be sequenced on the same lane of an Illumina sequencing instrument.

Figure 6. Principle of the Smart-seq2 protocol. Adapted by permission from Macmillan Publishers Ltd: Nature Protocols (132), copyright 2014.
4 RESULTS AND DISCUSSION

4.1 PAPER I – ILC HETEROGENEITY

The work presented in paper I addresses the heterogeneity of ILCs isolated from non-inflamed human tonsils based on transcriptional expression profiles identified through single-cell RNA sequencing (scRNA-seq). Previously, ILC transcriptomes were assessed solely by bulk sequencing and only in mouse (134, 135). The high resolution offered by scRNA-seq represents an excellent tool to address, in a truly unbiased way, questions about ILC subset heterogeneity within this rare cell population in humans. We discovered three phenotypically and functionally different subsets of ILC3 in the tonsil and identified previously unknown genes expressed in ILCs, indicating new ILC functions and relevant pathways in these cells. At the same time the richness of the data raises a multitude of future research questions that will certainly spur new studies in the future.

In paper I, we analyzed the transcriptomes of 648 single ILCs (including ILC1, ILC2, ILC3 and NK cells) isolated from three different non-inflamed human tonsils. The cells were sorted by flow cytometry according to the surface marker definition of ILCs and marker expression was recorded for every cell by indexed sorting (Paper I, Figure 1a, b). The initial approach of sorting single cells from the total CD127+ population proved unfeasible due to a dominance of ILC3 in human tonsil, with very low numbers of acquired ILC1 and ILC2 (Paper I Figure 1a, c). Therefore, we additionally sorted gated ILC1 and ILC2 in order to achieve sufficient cell numbers to allow us to perform reliable analyses (Paper I, Figure 1c). All subsequent analyses of the sequencing data were performed in an unbiased manner that excluded the two different gating strategies.

t-SNE (t-distributed stochastic neighbor embedding) analysis of the transcriptome data generated four clusters of cells which corresponded to ILC1, ILC2, ILC3 and NK cells (Paper I, Figure 2a). Comparison with the recorded surface protein expression showed that these clusters correlated well with the subsets defined according to the surface phenotypes that were provided by FACS analysis. The highest frequency of mismatch was observed for the ILC1, with 18% of the cells classified as ILC1 in FACS analysis clustering with any one of the other three subsets (ILC2, ILC3 or NK cells), based on their gene expression. This higher mismatch frequency of the ILC1 is not surprising considering that no reliable surface marker for their identification exists and that these cells are only defined by the absence of surface markers used for other ILC populations. Within the four clusters, cell identity was confirmed through higher expression of known subset-specific genes in the respective clusters (Paper I, Figure 2c, d). In addition, the high resolution of the single-cell analysis disclosed that gene expression within one cell type was not homogeneous but rather highly variable between cells within each cluster. Such heterogeneity could be due to true biological variation among cells, technical failure to detect the lowly expressed transcripts or it may represent the well-described phenomenon of stochastic bursts of gene expression (136).
In analyzing the differentially expressed genes with higher expression levels in all CD127+ ILCs versus NK cells, we found 27 genes commonly expressed by all non-cytotoxic ILCs. Some of these validated the known definition of ILCs, like the IL-7 receptor gene IL7R. 14 of these genes were transcription factors, among them RORα which is known to play a role in ILC2 development and RARγ (encoding a RA receptor), indicating that all non-cytotoxic ILC subsets are regulated by RA rather than the RORγt-expressing ILC3 alone (20, 46, 137-139).

In addition, we identified 59 genes differentially expressed in NK cells compared to the CD127+ ILCs. The majority of these genes are implicated in known NK cell functions, like the cytotoxic functions (several granzyme genes and PRF1, encoding perforin) and certain well-characterized surface receptors (KLRC1, SLAMF1). Nevertheless, 20 genes with unknown function in human NK cells were revealed (Paper I, Figure 4 and Supplementary Figure 5c, d).

We further investigated differentially expressed genes in each of the CD127+ ILC subsets. For this analysis, we compared the gene expression of a subset of interest with the expression of genes in the other two subsets combined, and we excluded NK cells due to the fact that their phenotypes and functions overlap with ILC1. For each subset, we found a number of genes that were preferentially expressed. These consisted of both genes that are known and whose expression is predicted in the respective ILC groups, as well as novel genes that have not been previously described in ILCs, which we then attempted to group according to their function.

ILC1 expressed 79 genes at significantly higher levels than ILC2 or ILC3 (Paper I, Figure 5). Surprisingly, almost no expression of TBX21 (encoding T-bet), which is commonly regarded as the cardinal transcription factor of ILC1, was detected. Nevertheless, several T-bet-regulated genes, like IFNG and CXCR3 were up-regulated in ILC1 indicating that T-bet might not be constantly expressed by ILC1. In addition, proteins may be stable for a longer time than mRNA, and low detection of mRNA does not exclude the presence of the protein.

Two other transcription factors, Aiolos (IKZF3) and Blimp1 (PRDM1) which are known to be expressed in peripheral NK cells and play a role in their differentiation (140-143), were significantly up-regulated in ILC1 and therefore are also likely to play a role in ILC1 development and/or function, although this awaits further investigation.

Furthermore, a whole group of genes involved in the regulation of the expression or signaling of IFN-γ (∆IFNG-AS1), IL-18 (∆IL-18BP) or IL-6 (∆IL-6R, SOCS3) was highly expressed. ILC1 are known as potent producers of IFN-γ, the release of which is stimulated by IL-12 and can be greatly enhanced by IL-18 (17). In contrast, a role for IL-6 signaling in ILC1 has not been described before. Generally, IL-6 can have anti- or pro-inflammatory effects, either through a classical signaling pathway via the membrane-bound IL-6 receptor or through trans-signaling via the soluble IL-6R (sIL-6R) (144). Whether IL-6 can exert an anti-inflammatory effect on ILC1 or whether sIL-6R cleaved from ILC1 plays a role in inflammation has not been investigated to date, but it is tempting to speculate that influencing IL-6 signaling in ILC1
could be of importance in the pathogenesis of IBD where ILC1 accumulate (17) (and Paper III). IL-6 is increased in the mucosa of IBD patients and bulk CD3+CD127ILC1 accumulate (17) (and Paper III). IL-6 is increased in the mucosa of IBD patients and bulk CD3+CD127+ ILCs, most likely including ILC1, respond to IL-6 stimulation with increased production of IL-17A, IL-22 and IFN-γ, indicating a direct role for IL-6 stimulation of ILCs in IBD (145, 146).

ILC1 do not show cytotoxic properties and lack expression of perforin and granzyme B (GZMB) (17), which was also confirmed by our RNA sequencing data. However, ILC1 expressed several genes encoding other granzymes, namely GZMM, GZMK and GZMA. Interestingly, some perforin-independent functions of granzymes have been suggested (147). These include a contribution of granzyme A to intracellular processing of the inflammatory cytokines IL-8, IL-1β, IL-6 and TNF-α (148), as well as a role of secreted granzymes in lymphocyte migration through the cleavage of ECM proteins (147). If these granzymes are expressed in ILC1 at the protein level and which subsequent role they may play in ILC1 function is an interesting scientific question that should be addressed.

Unexpectedly, the largest group of differentially expressed genes in ILC1 comprised of T cell-related genes, including a number of genes encoding variable T cell receptor alpha- or beta-chain segments, as well as genes of the invariant CD3 receptor complex (CD3D, CD3E, CD3G). In addition, genes encoding co-stimulatory molecules that are usually expressed on T cells (CD27, CD28) were found. One could speculate that ILC1 might be related to T cells or the cells assessed were contaminated with cells originating from the T cell lineage. However, contrary to these suggestions, the ILC1 examined lacked the surface protein expression of TCRα/β, TCRγ/δ and CD3, and did not express mRNA for RAG1 or RAG2 (Figure 7).

Figure 7. Expression of transcripts for CD3ε, RAG1 and RAG2 in ILC1, ILC2, ILC3 and NK cells.

Taken together, the findings described above along with the relatively high mismatch frequency of the ILC1 between the cell definition by FACS analysis and transcriptome analysis, argue for a high degree of heterogeneity in this cell population. This was recently supported by Roan et al. who identified three subsets of ILC1 in peripheral blood on the basis of CD4 and CD8 expression (149). They further showed that all of these subsets expressed intracellular CD3ε but the expression of TCRs was lacking, thus supporting the theory that ILC1 represent a heterogeneous innate cell population distinct from T cells. Intracellular expression of CD3ε and surface expression of CD5 was recently reported for all known human ILC subsets isolated from the thymus or cord blood, and CD5 expression was suggested to serve as a marker for immature ILCs (150). As we observed a bimodal expression of CD5 on ILC1 (Paper I, Figure 5), it would be interesting to separately analyze the transcriptomes of these two subsets in a larger sample.
Despite the aforementioned results, more than 80% of the FACS-defined ILC1 actually clustered together as a cell population and no specific sub-clusters were revealed in our analysis, suggesting homogeneity within the cells we analyzed. This could be due to the low cell numbers analyzed and therefore this question will need further investigation. The heterogeneity of ILC1 is currently a topic of intense debate. One recent study stated that ILC1 represent T cell contamination rather than an individual cell population (151). However, this study was criticized for several flaws in their identification of ILCs, like the sole use of CD5 for T cell exclusion, which can also be expressed by ILC1 and no exclusion of CD34⁺ progenitor cells or CD3⁴ T cells (152, 153). These ambiguous findings indeed point towards a considerable heterogeneity of ILC1 that will need to be untangled in future studies, where scRNA-seq of large numbers of ILC1, also possibly obtained from inflamed tissues, presents a powerful tool to harness.

Analysis of the ILC2 transcriptome compared to the combined ILC1 and ILC3 transcriptomes revealed 58 differentially expressed genes (Paper I, Figure 6a). Functional classification identified a prominent set of genes involved in the metabolism of prostaglandins that were up-regulated in ILC2. These included the genes for hematopoietic prostaglandin D synthase (HPGDS), hydroxyprostaglandin dehydrogenase (HPGD) and prostaglandin E receptor 2 (PTGER2) which are involved in synthesis of prostaglandins, inhibition of prostaglandin synthesis and prostaglandin signaling, respectively. Although ILC2 are commonly defined by their expression of the PGD₂ receptor (CRTH2) (22) and previous studies have established that PGD₂ activates ILC2 and LXA₄ plays an inhibitory role (21), our findings extended this theory by suggesting that ILC2 can produce and metabolize prostaglandins, thus indicating that prostaglandins play a more important role in ILC2 biology than previously known.

This has indeed recently been investigated in a follow-up project in our group by Maric et al. where it was shown that ILC2 do produce PGD₂ in response to cytokine stimulation (154). This endogenous PGD₂ production was crucial for cytokine-stimulated activation of ILC2 and expression of IL-5 and IL-13. PGE₂ was not endogenously produced by ILC2 but in a second study, Maric et al. revealed that exogenous PGE₂ inhibits the production of IL-5 and IL-13 from ILC2 (155).

In addition to this group of prostaglandin-associated genes, a few other genes were significantly expressed by ILC2 and I will discuss one more here. This gene is IL-32, encoding interleukin 32, a pro-inflammatory cytokine with known expression in T cells, NK cells and epithelial cells (156, 157). Elevated IL-32 levels (158) and increased ILC2 frequencies have been detected in CRS (22, 159) providing a possible link between IL-32 and ILC2 in CRS. Additionally, IL-32 levels were increased in the serum of asthma patients (160) and in the skin of atopic dermatitis patients (161), two other classically Th2-associated diseases, where ILC2 were shown to play a role (25, 26). Investigating whether ILC2 produce IL-32 protein and which role it plays in ILC2 function presents an interesting question for future studies.
Lastly, we analyzed the transcriptome of ILC3 compared to ILC1 plus ILC2 and 371 genes were differentially up-regulated in ILC3. 85 of these were immune genes (Paper I, Figure 7) and a large proportion of those detected confirmed the ILC3 phenotype (e.g. RORC, IKZF2, AHR, NCR2, NCR1, KIT, IL1R1, IL23R and LTB).

One set of up-regulated genes in ILC3 was related to NKp44 signaling (e.g TYROBP, SYK and VAV3). NKp44 is a receptor found on activated NK cells and both activating and inhibiting functions have been reported, depending on the ligand involved (162). For instance, an isoform of the mixed-lineage leukemia-5 protein (MLL5), named NKp44L, activates NK cells (163) whereas proliferating cell nuclear antigen (PCNA) inhibits IFN-γ production and cytotoxicity by NK cells via NKp44 signaling (164). Under steady state these proteins are only expressed intracellularly, however, the expression of both ligands was found on the surface of tumor cells and transformed cell lines. Additionally, several viral proteins have been shown to activate NK cells via NKp44 (162). In studies examining ILC3, NKp44 is commonly used as a surface marker to discriminate two ILC3 subsets with different cytokine production profiles (10), but little is known about its physiological function in these cells. In contrast to NK cells which only express NKp44 when activated, a subset of ILC3 in tonsil and intestine constitutively expresses NKp44 (32). It has been demonstrated that the NKp44 receptor in human ILC3 is functional and induces specific cytokine responses (165), supporting our data that differentially expressed NKp44 signaling molecules are present in ILC3. Antibody-engagement of NKp44 preferentially induced TNF production, in contrast to cytokine stimulation which induced IL-22 production. Taken together, it can be speculated that ILC3 might be able to respond to tumors, DAMPs or viral infections via NKp44 signaling. Nevertheless, the ligands that activate human ILC3 via NKp44 in vivo and whether inhibitory responses can also be induced remains to be investigated.

A second group of genes identified with differential expression in ILC3 were related to Notch signaling. It is challenging to fully grasp the implications of this finding since a plethora of physiologic functions have been shown for Notch-signaling (166). However, a role for Notch-signaling in the development of human and mouse ILC2 has been reported (167, 168) and recently a role in the plasticity of mouse NCR1ILC3 to NCR-ILC3 has been revealed (169, 170). In addition, it was shown that Notch-signaling induces the plasticity of mouse ILC2 towards a more ILC3-like phenotype, characterized by up-regulated RORC expression and IL-17 production (50). This underlines the importance of Notch for the plasticity of ILCs towards ILC3-like phenotypes, which could explain its differential expression in the ILC3 subset.

In addition to defining unique gene signatures for each ILC subgroup, we analyzed the heterogeneity within each subgroup. No further sub-clusters were found within ILC1 or ILC2, possibly due to the lower number of cells available. Rare sub-clusters are more likely to become apparent in larger scale experiments only. Additionally, it is possible that more sub-clusters could be revealed in inflamed tissues where ILCs are activated.
For ILC3 we could identify three sub-clusters based on transcriptome analysis (cluster A, B and C) (Paper I, Figure 8a, b) which were later validated at the protein level by flow cytometry and shown to possess different functional properties (Paper I, Figure 8g-i). Cluster A was defined by higher expression of transcripts for NKp44 and its signaling molecules (Paper I, Figure 8b, d and Supplementary Figure 7). This subset was functionally the most diverse and produced more IL-22 and IL-2 compared to the other two subsets, which was even more pronounced under cytokine stimulation. Taken together, this subset likely represents the well-known subset of NKp44+ ILC3 in tonsils (32).

Cluster B was composed of smaller cells with fewer transcripts per cell, than those found in the other clusters. The most prominent gene with differential expression was SEL, encoding the L-selectin CD62L. CD62L+ cells expressed GM-CSF, TNF and IL-2 at the same level as the other two subsets but did not respond to cytokine stimulation. Overall these data indicate that CD62L+ cells in the human tonsil represent a more naïve, transcriptionally less active subset which is also supported by the higher expression of CD45RA, a marker for naïve T cells, in this subset. Whether this subset represents an ILC precursor (ILCP) and furthermore, whether it might be identical to a recently described precursor of ILCs which was also found amongst NKp44+ tonsil ILC3 (45) remains to be seen.

In cluster C, several genes encoding HLA class II molecules were up-regulated (Paper I, Figure 8f and Supplementary Figure 7). The HLA-DR+ subset responded to cytokine stimulation, but, in contrast to NKp44+ cells, produced far less IL-22 and was the only subset that produced low but detectable amounts of IL-17F. MHC-II expression and the absence in expression of the co-stimulatory molecules CD80 or CD86 indicate that these cells might interact and present antigen to T cells, but are unlikely to activate T cells themselves. Interestingly, the HLA-DR+ subset also expressed higher levels of TNFS10 (TRAIL), in line with mouse data showing that ILC3 can limit T cell responses via MHC-II-dependent interaction (62) and induction of T cell apoptosis (63). A recent scRNA-seq study of mouse ILCs isolated from the small intestine identified 5 subsets of ILC3 by sequencing more than 1100 ILCs in total (171). Even though these data were obtained from mouse cells of a different tissue, this report supports our data defining cluster C. They identified an ILC3 subset enriched for genes involved in antigen presentation via MHC-II which was mutually exclusive with cells expressing IL-22. The larger number of cells sequenced surely adds to a higher resolution of subsets in this later study.

Unfortunately, changes in gene expression due to sample manipulation prior to sorting can lead to undesired changes and potential artifacts (172). The time from surgery to cell sorting, via cell isolation and the staining procedure takes many hours before the cells hit the lysis buffer. How alterations in gene expression, which are induced by the stress of processing, could be reflected in the transcriptome profiles we analyzed are factors we could not control for. Nowadays, cell fixation provides a solution for the preservation of the RNA and to block unwanted changes in gene expression prior to sorting, as described in several recent papers (172-174).
Overall, **paper I** represents the first single-cell sequencing study on human ILCs which allowed us to identify a plethora of interesting differentially expressed genes in the different ILC subsets, as well as characterize new ILC3 subpopulations. This lays the foundations for deeper analysis of potentially undiscovered ILC functions in the human body and will surely serve the ILC field as an important resource.

### 4.2 PAPER II – INNATE LYMPHOID CELLS IN NON-FIBROTIC AND FIBROTIC HUMAN LIVERS

ILCs have been described in many different organs in humans, such as the gut, tonsil, skin and adipose tissue (7-9, 17, 22, 29, 38) and there are reports indicating their presence in the human liver [175], but no detailed characterization of intrahepatic non-cytotoxic ILCs had previously been performed.

The first indication of the presence of ILC2 in the mouse liver came from Price et al. in 2010 [9]. Afterwards, a number of reports confirmed the presence of ILCs in the mouse liver and a link between ILC2 and liver fibrosis in mice was established (42, 110, 176-178). However, knowledge regarding ILCs in the human liver has previously been lacking. Many studies have investigated NK cells in the human liver revealing major differences in phenotype and function of the NK cell compartment when comparing samples from mice and humans (179-181). Therefore, in order to unravel the presence and role of ILCs in the human liver, studies utilizing human material are indispensable.

In **paper II** we set out to thoroughly investigate ILCs in the human liver, comparing the distribution of ILCs in adult liver with that in fetal liver as well as fibrotic liver. Using advanced flow cytometry staining for surface markers and transcription factors, we could detect all non-cytotoxic ILC subsets, including ILC1, ILC2 and NKp44⁺/⁻ ILC3 in the adult human liver (**Paper II**, Figure 1). The pattern of subset distribution was unique in the liver compared to gut and tonsil, with the majority of ILCs being NKp44⁻ ILC3, followed by ILC1, both of which are much less frequent at mucosal sites (**Paper II**, Figure 2A-D). Surprisingly, very few NKp44⁺ ILC3 were detected in the liver, while ILC2 made up the smallest fraction. However, ILC2 were present at higher frequencies in liver than in the gut or tonsil. In contrast to adult liver, fetal liver showed a substantially different subset distribution (**Paper II**, Figure 3). Almost all of the ILCs detected were NKp44⁻ ILC3 expressing RORγt with no expression of Eomes or T-bet. These differences indicate the specialized functions of ILCs according to the particular organ where they are present and the associated environment. Although NKp44⁺ ILC3 represent the major subset at mucosal barriers where they play an essential role in epithelial barrier integrity (58), this function might not be the foremost task of ILCs in the liver.

We found a small but consistent population of Eomes⁺ ILC1 in the liver. This is in line with results in **paper III** (Figure 3F) where we show that the gated ILC1 population in blood and intestinal mucosa is composed of cells heterogeneously expressing Eomes and T-bet. This is
also likely to be the case for liver ILC1. Although we had only two samples that were simultaneously stained for Eomes and T-bet, these data confirm the heterogeneity in T-bet and Eomes expression in intrahepatic ILC1 (Figure 8) and indicate that intrahepatic ILC1 could be composed of several diverse cell subsets. A reliable phenotypic definition of bona-fide ILC1 is a topic of intense research and debate in the literature and more work is needed to unambiguously identify the real ILC1 (17, 149, 151). This is especially true for the liver, where the boundaries between NK cells and ILC1 are becoming even more blurred since the discovery of a subset of Eomes− T-bet+ liver resident NK cells (179). These cells resemble ILC1 in their lack of Eomes and dependency on T-bet as well as in their increased ability to produce cytokines and their reduced cytotoxic capabilities. However, they are distinguished from ILC1 by KIR expression and the lack of CD127 on their surface.

Figure 8. Representative plot showing Eomes and T-bet staining in intrahepatic ILC1 and NK cells.

Phenotypic analysis of intrahepatic ILCs revealed that most ILCs in the adult liver were CD69+CD62L− cells which indicates tissue resident properties (Paper II, Figure 2E). Nevertheless, the tissue residency markers CD103 and CD49a were expressed at very low levels on intrahepatic ILCs. In line with adult ILCs, fetal intrahepatic ILCs did not express CD62L but they displayed substantially lower CD69 expression (Figure 9), indicating a non-tissue-resident phenotype (Paper II, Figure 3C). Whether human ILCs are tissue-resident cells and/or how long-term their residency is, is a current pertinent topic of research. Gasteiger et al. identified ILCs in parabiotic mice that were largely tissue-resident in both lymphoid and non-lymphoid organs (42) and a study on human transplanted intestine showed that donor ILCs reside in the intestinal tissue up to 8 years after transplantation (43). Nevertheless, a commonly used marker for tissue resident cells is the integrin CD49a (179, 182) which was only expressed on a small fraction of intrahepatic CD69+ ILCs in our study. CD69 is used as a marker of tissue residency, which, in addition to preventing tissue egress, is also a sign of activation, and its expression does not necessarily implicate long-term tissue-residency. Further investigations to determine the residential or migrating behavior of intrahepatic ILCs are needed to solve this question.
Another notable difference between adult and fetal intrahepatic ILCs was the surface expression of HLA-DR on adult ILCs which was largely absent in fetal intrahepatic ILCs (Figure 9). Instead, fetal ILC3 showed neuropilin 1 (NRP1) expression which was lacking in adult ILCs (Paper II, Figure 2F and 3C-D). NRP1 was recently suggested as a surface marker for LTi cells (183). The fact that NRP1+ cells were only found among fetal liver ILCs and that they showed a non-tissue-resident phenotype is in agreement with the hypothesis that these cells represent LTi cells which will leave the liver and initiate lymphoid organ development at various sites throughout the body. In contrast to the original report that identified NRP1+ LTi cells among the NKp44+ ILC3 subset in tonsil, NRP1 expressing ILCs in the fetal liver did not express NKp44. Generally, LTi cells are considered to be NKp44- cells (10, 32) but the exact relationship between NKp44, NRP1 and LTi function remains to be determined.

On the other hand, the NRP1- fraction of NKp44- ILC3 in fetal liver could include ILCPs which are the precursors of all three ILC subsets, as shown by Lim et al. (45). Even though peripheral blood ILCPs did not express RORγt, corresponding cells in the fetal liver expressed RORγt, in agreement with the phenotype of the fetal intrahepatic NKp44- ILC3 we describe here. The intrahepatic ILCPs described by Lim et al. had multi-lineage potential even though many cells were already ILC3-committed progenitors.

![Figure 9. Representative plots comparing the expression of CD69, HLADR and NRP1 in adult and fetal intrahepatic ILC3.](image)

Several cytokines typically produced by ILCs, like IL-13 and IL-22, have been shown to play a role in liver fibrosis (184, 185) and recently a role for ILC2 in a mouse model of hepatic fibrosis was described (110). This led us to turn from the “healthy” liver setting to liver fibrosis. We discovered significant changes in the intrahepatic ILC subset distribution in fibrotic liver even though the overall ILC frequency remained unchanged (Paper II, Figure 4A-B). The frequency of ILC2 was more than two-fold higher in fibrotic liver tissue compared to non-fibrotic, with a simultaneous and significant reduction of NKp44- ILC3. The ILC2 frequency positively correlated with the severity of fibrosis and this result was replicated in a separate cohort of frozen mononuclear cells from fibrotic and non-fibrotic liver tissue (Paper II, Figure S4). Interestingly, this was an innate phenomenon as the frequency
of Th2 cells among T cells, defined as CD3⁺CD4⁺CRTH2⁺ cells, was unaffected (Paper II, Figure 4F). This points towards a unique role of innate immune cells in liver fibrosis. The reduction of Nkp44⁻ ILC3 and simultaneous increase in ILC2 in fibrotic liver is in accordance with the hypothesis that Nkp44⁻ ILC3 include an ILC precursor population (45) that has the potential to differentiate into all mature ILC subsets, including ILC2.

Using an in vitro culture system for ILCs we investigated the cytokine response of intrahepatic ILC2 to PMA/ionomycin or to the physiological stimuli IL-33 and TSLP. Intrahepatic ILC2 responded with strong IL-13 production to stimulation with either PMA/ionomycin or IL-33 plus TSLP. IL-13 is known to be a pro-fibrotic cytokine (186, 187) and this could represent a mechanism through which ILC2 actively contribute to fibrosis progression. In addition, PMA/ionomycin stimulation induced an unexpectedly strong production of IFN-γ by ILC2, which was not observed under IL-33 plus TSLP stimulation.

The strong IFN-γ response of intrahepatic ILC2 to PMA/ionomycin stimulation can be potentially explained by the findings that ILC2 show plasticity and have the potential to produce IFN-γ under IL-12 and IL-1β stimulation (19, 47, 188). It was demonstrated that irradiated PBMC are a source of IL-12 and IL-1β (47). Thus, the use of irradiated feeder cells in our 2-weeks ILC2 expansion cultures could have stimulated the differentiation of ILC2 towards ILC1-like cells, as indicated by the IFN-γ production from intrahepatic ILC2.

Additionally, we observed slight IL-22 production by intrahepatic ILC2 after culturing. This had been previously described in cultured ILC2 from fetal gut which also expressed the transcription factor AHR (22). In light of the growing body of literature on ILC plasticity, this could represent an additional type of potential ILC plasticity from ILC2 towards an ILC3-like phenotype in response to environmental stimuli. However, this would need to be formally investigated as ILC2 to ILC3 plasticity is yet to be proven.

We detected expression of IL-33 in whole liver lysates (Paper II, Figure 5A-B) and further investigated which cell types are the possible sources of IL-33 in liver fibrosis. To mimic viral infection (a common cause of liver fibrosis), we used the TLR3 ligand poly(I:C) to stimulate different hepatic cell types (189) and measured induced IL-33 and TSLP production. We found that primary hepatocytes, HSCs as well as Kupffer cells were able to produce IL-33 in response to TLR3 stimulation (Paper II, Figure 5C-E). Very recently Tan et al. observed increased IL-33 levels in cirrhotic liver tissue (111) which supports our hypothesis of ILC2 activation in human liver fibrosis.

In contrast to a study in mice where McHedlidze et al. showed that ILC2-derived IL-13 could directly activate HSCs and induce pro-fibrotic gene expression (110), we were not able to confirm this mechanism of IL-13 in the human setting. We stimulated cells of the human HSC line LX-2 with IL-13 and measured expression of the collagen encoding gene COL1A1 but did not observe an induction of gene expression (Figure 10). This discrepancy could either be due to real differences in mouse and human HSC biology or could be caused by the use of a cell line in place of fresh primary HSCs. Whether ILC2 exert pro-fibrotic functions
in the human liver and if this effect is mediated by IL-13 alone, or whether other ILC2-derived factors are involved remains to be explored. Since IL-13 plays a well-known profibrotic role in fibrosis it could serve as a potential treatment target. In a mouse model of S. mansoni-induced hepatic fibrosis, IL-13 inhibition by a soluble IL-13 receptor molecule (sIL-13Rα2-Fc) reduced collagen deposition (114). A clinical trial with an anti-IL-13 antibody for the treatment of idiopathic pulmonary fibrosis has been completed, but the data is yet to be released (NCT00532233).

Figure 10. Expression of COL1A1 in HSCs after stimulation with IL-13, TGF-β or the two combined for either 12h or 24h compared to the non-stimulated (NS) condition.

In conclusion, in paper II we provide the first insights into the distribution of intrahepatic ILCs in adult and fetal liver and reveal a possible role of ILC2 in human liver fibrosis.

4.3 PAPER III – INNATE LYMPHOID CELLS DURING THE COURSE OF CROHN’S DISEASE AND ULCERATIVE COLITIS

The role of ILCs in IBD has been addressed in several studies and alterations in the ILC frequencies in the mucosa of IBD patients are a well-known feature. However, the results are not always consistent and most studies have investigated end-stage material from CD patients, while no systematic side-by-side studies of CD and UC material have been performed. Due to the increasingly refined definition of ILCs, comparisons made between findings from the early days of ILC research and those from later studies are challenging.

In paper III we set out to investigate ILCs isolated from mucosal biopsies of CD and UC patients, who were divided into those with established disease that had been diagnosed over one year ago (eCD, eUC) and those that were newly diagnosed for IBD (nCD, nUC) on the
day of examination. Our control group consisted of subjects with Lynch syndrome which underwent screening colonoscopies and were found to be healthy and without tumors at the time of examination. This enabled us to compare shifts in ILC composition between non-IBD controls, CD and UC and additionally between long-standing disease and disease that is closer to its onset. Moreover, we compared ILCs in matched blood samples from all non-IBD and IBD patients, while in a separate cohort of IBD patients, we analyzed blood ILCs before and after treatment with vedolizumab, a monoclonal antibody targeting the gut-homing integrin α4β7.

Through the analysis of ILCs isolated from different sites of the intestine (ileum, caecum, colon ascendens, colon descendens) and from blood from non-IBD control patients by flow cytometry analysis, we established the typical distribution of ILCs at these sites (Paper III, Figure 1). NKp44− ILC3 represented the majority of ILCs in the intestine, whereas they were almost undetectable in blood (Paper III, Figure 1C). In contrast, ILC2 were barely present in the non-inflamed intestine but were readily found in blood. ILC1 and ieILC1 were found at low frequencies throughout the colon with the highest representation in the ileum. NKp44+ ILC3, the largest ILC fraction in blood was detectable at intermediate levels throughout the intestine. The distribution of ILCs in the intestine at steady state was recently analyzed in detail by Krämer et al. who examined samples taken during colon cancer screening examinations (52). Our data is in good agreement with their study thus supporting that the established proportions of ILCs in the intestine of Lynch patients that we report, do indeed represent the typical ILC distribution in the healthy intestine.

ILCs in the intestine from IBD patients showed an altered distribution (Paper III, Figure 2), whereas blood ILCs remained unchanged when compared to non-IBD controls (Paper III, Figure 3A). Combined analysis of intestinal samples from all IBD patients showed that NKp44+ cells were substantially reduced compared to non-IBD controls, whereas all other CD127− ILC subsets, including NKp44+ ILC3, ILC2 and ILC1, were significantly expanded at inflamed sites. In contrast to published reports that documented an increase in ieILC1 in intestinal samples from CD patients (15, 46), we found a tendency towards a reduction of the ieILC1 and NK cell frequencies. Due to the fact we analyzed pooled IBD samples here, it is possible that the increase in ieILC1 is a CD specific feature, as earlier reports only investigated tissue samples from CD patients. Furthermore, none of the observed changes were present at non-inflamed intestinal sites of IBD patients, indicating that the observed alterations are specifically induced and locally restricted to the sites of inflammation and leave the non-inflamed areas unaffected, even at the cellular level. The decrease in NKp44+ ILC3 correlated with the severity of the disease in CD and UC and additionally, the increase in NKp44+ ILC3 correlated with disease severity in UC (Paper III, Figure 2B). Whether these changes underlie the pathogenesis of the disease or if they are rather mere consequences of an ongoing inflammation process is still not clear. It would, however, be crucial to investigate since it could provide important information on the types of cells or the mediators that might represent promising targets for novel treatment regimens.
When the IBD patient cohort was stratified according to the different patient groups and compared to the non-IBD controls, we found common but also distinct alterations in ILC frequencies between CD and UC patients, as well as differences when comparing cells from patients with varying time spans since diagnosis (Paper III, Figure 2C). Consistently, NKp44+ ILC3 were reduced in inflamed samples from all IBD patient groups indicating that this change occurs early and is sustained throughout the disease. These results fit well with a presumed function of NKp44+ ILC3 in the protection of the epithelial barriers via their production of IL-22 (58). A reduction in their frequencies could potentially be one of the contributing factors leading to the “leaky gut” phenomenon in IBD and the perpetuation of inflammation, especially if it occurs early in the disease process.

In contrast to other studies, no changes in the ieILC1 fraction were observed even when we analyzed UC and CD samples separately (15, 46). This discrepancy could be attributable to a number of technical differences such as different starting material, varying cell isolation methods, alternative control groups or different analytical methods. Fuchs et al. separated intraepithelial lymphocytes from the lamina propria lymphocytes of resection specimens, while we isolated cells from whole biopsies. In addition, the increased frequency of ieILC1 was observed when the ratio of ieILC1 to CD3+ cells in the intraepithelial cell fraction was compared to samples from non-IBD conditions. However, it remains unclear if CD3+ cell frequencies were unchanged in these patient groups. Even when displaying our data as ratio of the CD3+ cells present, we did not detect an increase of ieILC1 in our cohort (Figure 11). Bernink et al. reported a less notable accumulation of ieILC1 in the ileum of CD patients with a less than 2-fold increase when compared to non-IBD controls (15, 46). This indicates that the accumulation of ieILC1 is not a consistent feature of CD and further work is needed to reveal the specific circumstances under which it occurs.

![Figure 11. ieILC1 isolated from biopsies of CD patient and non-IBD controls displayed as a ratio of CD3+ cells within CD45+ cells.](image_url)

Interestingly, CD127+ ILC1 were only increased in newly diagnosed CD patients but not those with UC, while conversely, ILC2 were increased in those newly diagnosed with UC but not in CD patients. However, in established disease, ILC1 and ILC2 frequencies were
similarly elevated in both CD and UC patients. This indicates that differences at the onset of inflammation exist but that the ILC profiles of the two diseases converge during later time points of the disease process. It is commonly stated in the literature that CD is a Th1-driven disease on the basis of increased IFN-γ levels, whereas UC is Th2-driven based on increased IL-5 and IL-13 levels in the colon derived from non-classical NKT cells (190-193). IL-13 has been shown to impair the epithelial barrier by inducing epithelial cell apoptosis and changes in tight junction composition (194). Our observation of altered ILC1 and ILC2 frequencies in the earlier stages of the disease favors this theory. However, the convergence at later disease states suggests that these separate patterns of ILC composition are most likely not conserved throughout the disease course. Interestingly, a study of CD-like colitis in IL-10 deficient mice found that increased IL-13 levels are a hallmark of later stages of disease (195). However, two clinical trials investigating the efficiency of anti-IL-13 antibodies in UC treatment did not show any improvement and another report refuted a role for IL-13 in IBD (196-198). Further, it has been reported that IL-13 and TGF-β synergize in intestinal fistula development in CD patients but a phase II study examining a monoclonal IL-13 antibody for the treatment of fistulizing CD has been discontinued due to slow recruitment (199, 200). Complications due to fibrosis and stricture development have been primarily reported for CD but were also recently described for UC (201, 202). Therefore, it would be an interesting question to investigate whether higher ILC2 levels could be connected to stricture or fistula development in IBD, especially in light of the possible role of ILC2 in liver fibrosis as suggested in paper II.

It would also be interesting to analyze how different treatment regimens influence the temporal changes in ILC composition in the intestine of IBD patients in a larger cohort. It is possible, that the assimilation of changes in ILC frequencies in samples from CD and UC patients with established disease are induced by treatment that redirects the immune system towards certain immune responses.

Our classification of patients into groups with newly diagnosed versus established disease was an attempt to stratify them for disease duration. Unfortunately this is not perfect, since the disease could have developed long before a diagnosis was made and thus the time between disease onset and diagnosis will vary greatly between patients. Nevertheless, the group of patients with established disease will have most likely had the disease for a longer time on average than those labeled as newly diagnosed, and they are not treatment naïve as the group of newly diagnosed patients are. Therefore, I believe this approach can give valuable indications regarding the distinct temporal roles of ILCs in disease onset and persistence.

In paper III, we additionally investigated whether differences in the expression of the transcription factors Helios, AHR, RORγt, Eomes and T-bet in ILCs from IBD and non-IBD patients could be detected. If the alterations in ILCs in the intestinal mucosa of IBD patients were due to locally induced ILC plasticity, one would expect higher RORγt levels in the ILC1 compartment with a simultaneous up-regulation of T-bet in ILC3 from IBD patients,
assuming that cells undergoing differentiation are present in both cell populations. However, we did not detect any such changes in the transcription factor profiles of IBD versus non-IBD patients, as they differed neither in blood ILCs nor in intestinal ILCs. These data showcase the challenges present in unraveling the underlying processes of ILC frequency changes in inflamed tissue.

We also investigated in which aspects the transcription factor profiles of blood ILCs and intestinal ILCs differ (Paper III, Figure D, E). Intestinal ILC3 expressed significantly higher levels of RORγt, AHR and Helios in both the NKp44− and the NKp44+ subset, although NKp44+ ILC3 in the blood were extremely rare. However, the few NKp44+ ILC3 that were found in blood displayed a typical ILC3 transcription factor profile that was similar to intestinal ILC3. These data are in line with the recently proposed concept that blood NKp44+ ILC3, that do not express RORγt, contain immature ILCs which represent progenitors of all other ILC subsets (45).

Furthermore, we analyzed the expression of T-bet and Eomes in blood and intestinal ILC1 and found a remarkable heterogeneity (Paper III, Figure 3E, F). Some cells within the ILC1 gate only expressed T-bet, some only Eomes and some showed co-expression. Interestingly, cells lacking both Eomes and T-bet made up the largest fraction of ILC1 in blood. This was even more pronounced in the gut where T-bet expression was markedly lower than in the blood. This was surprising, considering the potential of intestinal ILC1 to produce IFN-γ and the elevated IFN-γ levels seen in the mucosa of IBD patients (46, 190, 192). T-bet has been shown to be a major inducer of IFN-γ production, which in a positive feedback loop, induces T-bet expression (203, 204). T-bet is known to mediate responsiveness to IL-12 via the induction of IL-12Rβ expression in T cells, thereby contributing to IFN-γ induction (205). Thus, low T-bet expression in intestinal ILC1 is challenging to reconcile with high IFN-γ levels being produced. Whether T-bet is specifically regulated in the intestine or in tissues and therefore found at lower levels is unknown.

Moreover, the high degree of heterogeneity within the ILC1 subset is not without precedent. Roan et al. have reported three different subsets of ILC1 in the blood. These were based on CD4 and CD8 expression and comprised of CD4+CD8−, CD4−CD8+ and double negative ILC1. The CD4− ILC1 co-expressed Eomes and T-bet, whereas the CD4+ ILC1 did not express Eomes. Notably, a large percentage of each of these subsets expressed neither T-bet nor Eomes (149). Whether these subsets are present in the intestine is yet to be reported and would be interesting to explore. We also observed Eomes expression within the ILC1 population in the human liver (Paper II, Figure 1B) and when we investigated TBX21 and EOMES mRNA expression in ILC1 from tonsil, we found that only very few ILC1 actually expressed the corresponding transcripts (Paper I, Figure 2C and Supplementary Figure 5C). In conclusion, it is highly likely that the cells defined as an ILC1 population comprise of a collection of several diverse subsets of cells which require further exploration.
One possible explanation for the changes in intestinal ILC frequency in IBD is increased tissue homing of mature or immature blood ILCs that differentiate in the tissue to mature ILCs. We addressed this possibility by analyzing the expression pattern of homing markers on peripheral blood ILCs (Paper III, Figure 4). Surprisingly, ILCs from IBD patients showed the same pattern of homing marker expression as ILCs from non-IBD patients, strengthening the theory that the altered intestinal frequencies of ILCs are due to local plasticity rather than peripheral recruitment. Furthermore, the analysis confirmed a naive state of blood ILCs, marked by CD62L expression and lack of the activation marker CD69. Despite these immature features, analysis of homing marker combinations revealed unique homing marker profiles for each ILC subset. For instance, almost all ILC2 expressed CCR4, involved in skin homing, and a large proportion additionally expressed CCR6, which is involved in the recruitment of cells to sites of inflammation. Contrastingly, only about half of the cells in the other ILC subsets expressed CCR4, possibly indicating a special role of ILC2 in skin homeostasis. NKp44+ ILC3 showed a very heterogeneous homing marker profile which could support the presence of diverse progenitors in this subset. The expression of the various homing markers on the different ILC subsets was in good agreement with the recently published data from Roan et al. (152). ILC1 expressed the highest levels of α4β7, a gut homing molecule, and CXCRL3, which is involved in the recruitment of cells to sites of inflammation, compared with ILC2, ILC3 and NK cells. Although this expression was similar in ILC1 from IBD patients and non-IBD NK cells, it indicates that ILC1 do indeed have the potential to home to the inflamed intestine. Whether this homing potential is involved in the accumulation of ILC1 in the inflamed mucosa remains unclear.

Vedolizumab, a monoclonal antibody targeting α4β7, is efficiently used in the treatment of IBD. To address whether treatment of IBD patients with vedolizumab affects ILC frequencies and phenotypes, we analyzed blood ILCs before and 10-14 weeks into vedolizumab treatment (Paper III, Figure 5). The fraction of α4β7+ ILCs or CD3+ cells was significantly reduced after treatment which was most likely due to blocking of α4β7 which prevented detection by our FACS-antibody because vedolizumab contains the same binding domain. In addition, α4β7 may also possibly be internalized after vedolizumab binding, as has been reported in in vitro studies (97, 206). Even though α4β7 was efficiently bound by vedolizumab the frequency of ILCs in the blood were not reduced, as could for instance be mediated through antibody-dependent cell-mediated cytotoxicity. This result was somewhat expected as it has been shown that vedolizumab does not induce cytotoxicity in vitro due to a mutated Fc portion (206). In contrast, we expected an accumulation of CD3+ cells and ILC1 in the blood of vedolizumab treated patients due to reduced gut homing, which has been observed for T-cells in nonhuman primates (207). However, cell frequencies in our cohort were unchanged after 10-14 weeks of vedolizumab treatment. Whether these diverse observations can be explained by the rather small cohort size, the used drug concentrations, the length of treatment or other reasons remains to be investigated. In addition, whether the binding of vedolizumab to α4β7+ ILCs contributes to its disease ameliorating effect is unclear at the moment. It is possible that changes in ILC frequencies in the blood are too small to be
detected in our cohort and would only become apparent in a larger cohort or at a time point later in treatment. The mechanisms behind ILC1 accumulation in the inflamed intestine of IBD patients remain to be unraveled. As the immune system often works through complementary mechanisms, a combination of local plasticity as well as recruitment of peripheral cells probably accounts for the observed alterations in ILC distribution.
5 CONCLUDING REMARKS

The ILC field is a very young area of research as these cells were only discovered about 20 years ago and particular interest in ILC biology has only risen in the last decade. Even though our knowledge regarding ILCs is constantly increasing and important discoveries are regularly made, it is only now that we are beginning to understand the functional role of ILCs in the human body. Many questions can only be addressed in mice and translation to the human setting is not always straightforward. I believe that my work adds a small, but fundamental piece of the puzzle to further our understanding of the role of ILCs in the human body. The key findings of my work are summarized as follows:

- In paper I we have identified a large set of differentially expressed genes present in each of the known ILC subsets. This offers valuable clues about possible, and so far unknown, functions of ILCs. In addition, the results provide a base for many follow-up studies investigating in detail the roles and functions of the indicated pathways and proteins in ILCs.

- We have, for the first time, proven how valuable scRNA-seq is for ILC research. It allowed us to uncover in an unbiased way different subpopulations of ILCs based on their transcriptome. We detected three subpopulations of ILC3 with functionally diverse properties. One of them is likely to represent the well-known NKp44+ subset in tonsil, the second a novel and potentially naïve subset, while the third possibly represents an antigen-presenting subset previously unknown in humans.

- In paper II we characterized for the first time in detail the ILC compartment in the adult, fetal and fibrotic human liver. We described the unique composition of ILC subsets in the liver compared to gut and tonsil ILCs and revealed distinct patterns of ILC frequencies in the non-fibrotic adult liver compared to the fetal liver, indicating that LTi cells are only present in the fetal liver. Furthermore, we observed an increase in ILC2 frequency in fibrotic livers which correlated with the severity of the disease. Since ILC2 are capable of producing large amounts of the pro-fibrotic cytokine IL-13, these data indicate that ILC2 might contribute to the pathogenesis of liver fibrosis.

- The work in paper III revealed significant differences in ILC composition between mucosal biopsies from CD patients, UC patients and non-IBD controls. In biopsies from newly diagnosed CD patients the ILC1 frequency was increased, while this was the case for ILC2 in UC patients. These observations indicate possible differential mechanisms in the onset of these two diseases. On the contrary, in patients with established disease the ILC profile was similar between CD and UC but still distinct from non-IBD controls, with an increase in ILC1 and ILC2 and a decrease in NKp44+ ILC3. In both diseases, the decreased frequency of NKp44+ ILC3 correlated with severity of inflammation, indicating that deregulation of ILCs could be involved in the pathogenesis of IBD.
5.1 FUTURE DIRECTIONS

To uncover the diversity and understand the functions of ILCs, many questions still need to be addressed. However, based on the work included in this thesis, a few questions would be of particular interest to follow up in future work.

- scRNA-seq of greater numbers of cells from diverse tissues in homeostasis and inflammation would surely unravel the diversity of ILCs and shed light on the emerging concept of plasticity within the ILC family. The recent drop in sequencing costs and the possibility offered by automation now makes it feasible to sequence a much larger number of cells than the number we where able to assess when preparing paper I two years ago. The creation of enormous datasets requires expert knowledge of bioinformatic tools and will be the bottleneck for exploring and extracting comprehensive information from these datasets. In particular, the issue of ILC1 heterogeneity, which repeatedly emerged in all of our studies, could be addressed by scRNA-seq of large numbers of ILC1 from different inflamed and non-inflamed tissues.

- The follow-up investigation of genes, potential pathways and ILC subsets identified in our scRNA-seq study will certainly be of great interest. In particular, investigations into the effect of IL-6 on ILC1 function and plasticity in the light of increased IL-6 concentrations in the mucosa of IBD patients, the detailed exploration of a potential antigen-presenting capability of ILC3 and the expression of IL-32 by ILC2 and its possible role could be exciting questions for future research.

- It would be of great value to extend the study of ILCs in IBD to a much larger cohort and thus add statistical power to our initial findings. Moreover, it would be interesting to further explore the observed convergence of diverse ILC distributions in newly diagnosed CD and UC patients towards similar distributions seen in later stages of the diseases. In addition, whether this convergence could be possibly driven by treatment is an exciting theory to investigate. Furthermore, a strategy of new treatment regimens could include the induction of ILC plasticity in ILC1-like cells towards protective sub-types, like NKP44⁺ ILC3, thus reversing the ILC distribution in inflamed tissue to mirror that seen in a steady state distribution. Testing the feasibility of this approach in mouse models of IBD would be an intriguing study.
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