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Intraepithelial Lymphocytes in
Refractory Celiac Disease:
Lost in Transition

Intraepithelial Lymphocytes in Refractory Celiac
Disease:
Lost in Transition

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Also, the NCV organizes contacts amongst fellow-sufferers, offers and spreads information, informs and is active in advocacy for persons with coeliac disease or dermatitis herpetiformis (www.glutenvrij.nl).

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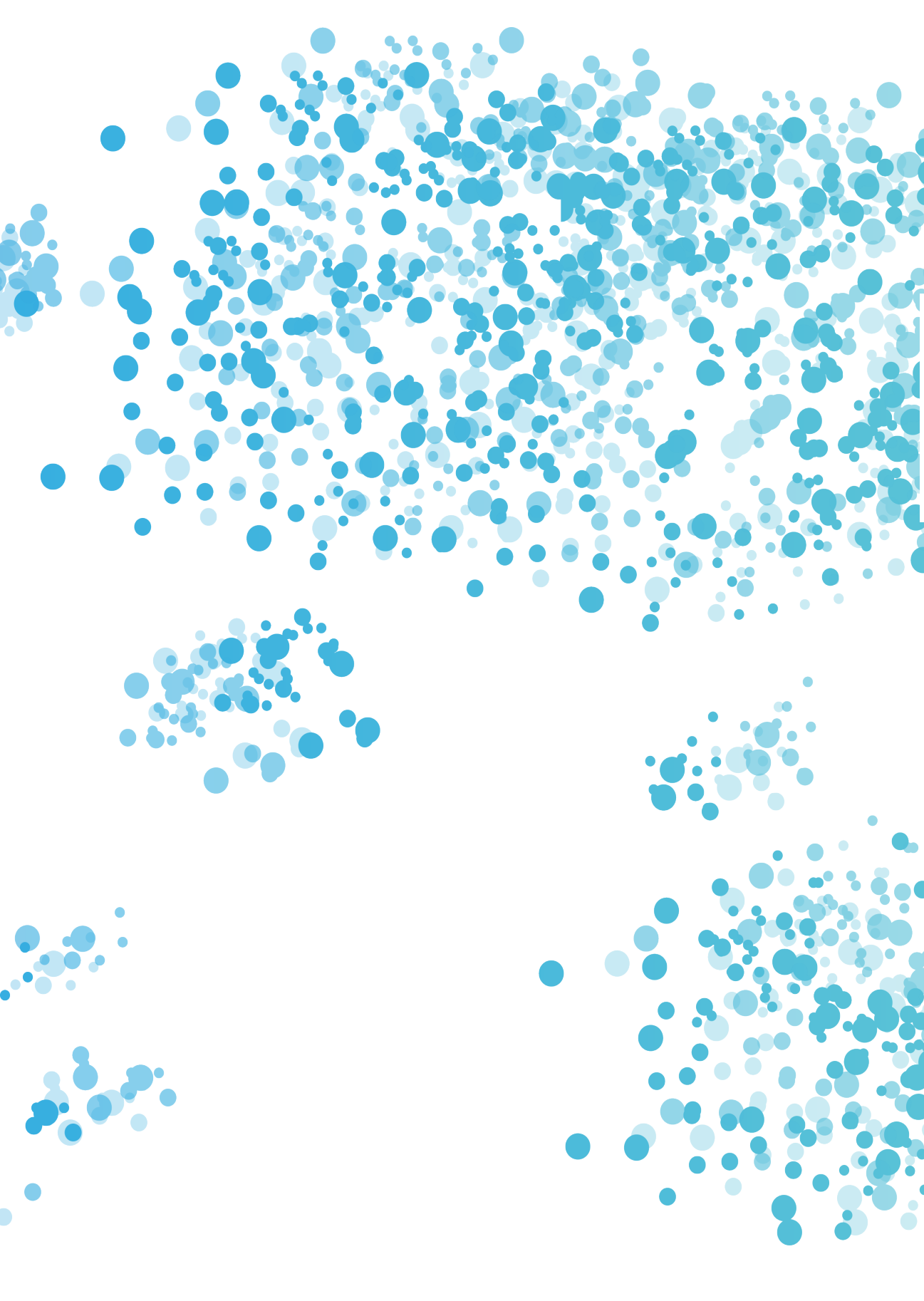
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HARBOR BIPOTENTIAL T/NK CELL PRECURSORS

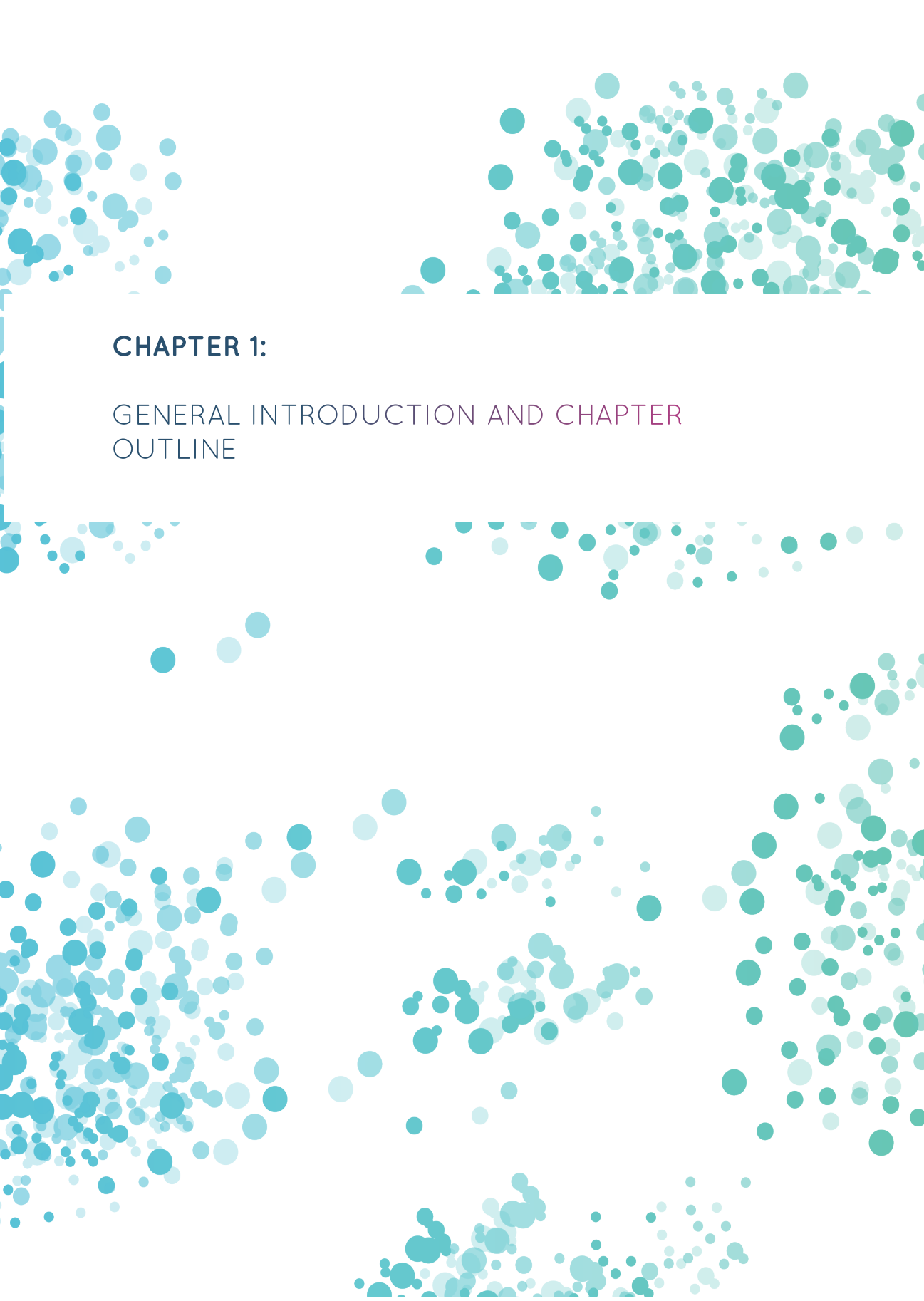
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CHAPTER 1:

GENERAL INTRODUCTION AND CHAPTER OUTLINE

CHAPTER 1: GENERAL INTRODUCTION AND CHAPTER OUTLINE

THE IMMUNE SYSTEM

The immune system has evolved to protect the body against pathogens. It comprises both humoral and cellular components that act in concert to eliminate pathogens. Cells of the immune system are classically divided into innate and adaptive immune cells. Innate immune cells are regarded as a first line of defense because they can immediately respond to pathogens in a generic way, whereas adaptive immune cells recognize individual antigens with specialized antigen receptors, leading to a delayed but highly specific immune response. Innate immune cells comprise macrophages, dendritic cells (DC), neutrophils, eosinophils, basophils, mast cells and natural killer (NK) cells. T and B cells are part of the adaptive immune system, each cell carrying a unique T cell receptor (TCR) or B cell receptor (BCR), respectively. While the BCR can directly interact with the antigen, the TCR recognizes sequences derived from foreign molecules bound to major histocompatibility complex (MHC) proteins. T cells can carry either CD4 or CD8 as a co-receptor for the TCR. CD4⁺ T cells respond to peptides bound to MHC-class II and CD8⁺ T cells are targeted towards MHC-class I-peptide complexes. In general, CD8⁺ T cell responses lead to elimination of (infected) target cells while CD4⁺ T cells provide help for such cytotoxic CD8 T cells and facilitate the production of immunoglobulins by antigen specific B cells. In addition, subsets of T cells, such as natural killer T cells (NKT) and mucosa-associated invariant T (MAIT) cells, can respond to non-peptide antigens bound to non-classical MHC molecules. A unique feature of the adaptive immune response is immunological memory: once exposed to a certain pathogen, pathogen-specific T and B cells expand and form a memory pool that can mediate accelerated and stronger responses towards the same pathogen upon re-exposure.

T CELLS

Leukocytes originate in the bone marrow and either develop and mature there or migrate to the thymus for further differentiation. Whereas NK cells mainly complete their differentiation in the bone marrow, T cells generally mature in the thymus. Nevertheless, recent evidence for NK and T cell development in secondary lymphoid organs such as the spleen, lymph nodes or the liver have also been presented^{1,2}. In the thymus distinct T cell lineages are generated: T cells carrying a TCR $\alpha\beta$ or TCR $\gamma\delta$, which can express the co-receptors CD4 or CD8. Through somatic recombination T cells randomly rearrange TCR genes, creating a unique and diverse TCR repertoire. Recombination is carried out by enzymes encoded by recombination activating genes (RAG), which cut the DNA flanked by recombination signal sequences (RSS), and by terminal

deoxynucleotidyl transferase (TdT), which randomly inserts nucleotides during the recombination event, generating further diversity in the T cell receptor repertoire. Developing T cells go through a series of developmental stages, which have been thoroughly characterized. Immature T cells sequentially pass through a CD3⁺CD4⁺CD8⁻ double-negative (DN), a CD3^{low} double-positive (DP), and a CD3⁺ single-positive CD4 or CD8⁺ stage, which eventually yields committed and fully matured specialized T cell subsets. Moreover; in the thymus four stages of DN cells (DN1-4) can be discriminated by a combination of markers, including CD25, CD44, c-kit (CD117) and CD34. While DN1 and DN2 cells still have the potential to differentiate into other lineage such as DC or NK cells (Figure 1), at the DN2 and DN3 stages T cell commitment is initiated and completed. It is generally believed that upon commitment to a certain lineage, the cell fate is set due to the expression of particular transcription factors and epigenetic changes (T cell differentiation is reviewed in ^{1,3}). Throughout the T cell maturation process, the expression of the CD3ε, CD3γ, CD3δ and ζ chains are gradually upregulated resulting in the protein expression of all CD3 chains intracellularly before their association with the T cell receptor dimer and translocation to the surface of mature T cells ⁴. The expression of the T cell receptor on the surface is therefore dependent on the presence of the CD3 chains.

During the past few years several factors that are essential for thymic T cell maturation have been characterized. Receptor-ligand interactions such as notch and notch-receptors are now recognized to be crucial in T cell commitment (reviewed in ⁵). Knowledge about such factors has laid the groundwork for the development of *in vitro* T cell differentiation culture systems, such as the generation of the mouse bone-marrow cell line OP9 transduced with the notch-ligand delta-like 1 (OP9DL1). Progenitor cells that are seeded on these OP9DL1 stroma cells (with the addition of cytokines such as FLT3L, SCF and IL-7) can develop into mature TCRαβ or TCRγδ T cells ⁶.

To effectively recognize pathogen-derived antigens while maintaining tolerance to self-antigens, T cells that have rearranged the T cell receptor (TCR) undergo an extensive selection process in the thymus. To ensure that the TCR generated will recognize foreign but not self-antigens presented by MHC, T cells with a TCR capable of recognizing self-antigens with a low affinity are positively selected in the thymus while T cells carrying a T cell receptor recognizing MHC/self-peptide with too strong of an affinity are deleted (negative selection). This process results in CD8⁺ T cells, able to detect 'non-self' peptides in the context of MHC class I and CD4⁺ T cells recognizing 'non-self' peptides on MHC class II. Despite this strict selection process, not all TCR with high affinity for self-antigens are successfully eliminated. T cells carrying an autoreactive TCR can still be deleted or silenced in the periphery. Failure of central (thymic) and peripheral regulatory mechanisms contributes to the development of autoimmunity in diseases like rheumatoid arthritis (RA), type 1 diabetes (T1D) and multiple sclerosis (MS).

Only a minority of T cells survives the positive and negative selection in the thymus and subsequently leaves the thymus in anticipation of its specific antigen. During an infection, activation of these naïve T cells occurs in lymph nodes, where recirculating T cells come in close contact to antigen-presenting cells (APC) such as DC, that have encountered pathogens in the periphery. Before moving to draining lymph nodes, these DC have been activated by pathogens via conserved receptors recognizing damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) and engulfed the pathogen in order to present pathogen-derived peptides via MHC. Upon MHC-restricted recognition of the presented antigens on APC in lymph nodes, T cells are either silenced or turned into effector cells, depending on the level of costimulation provided by the APC and the local cytokine milieu.

Upon activation effector T cells clonally expand and most cells die once the pathogen is cleared, but a small fraction survives as memory cells, responsible for immunological memory. Effector CD8⁺ T cells are cytotoxic T cells lymphocytes (CTL), capable of lysing infected cells, while CD4⁺ T cells (T-helper cells) produce various cytokines, help B cell antibody class switching and activate other immune cells. In contrast to other tissues, the intestine is overrun with foreign antigens from food, pathogenic or harmless commensal bacteria, emphasizing the need to preserve tolerance and prevent systemic immune reactions.

NK CELLS

NK cells are cytolytic effector cells that play an essential role in immune defense, particularly in the first week of primary infections with intracellular pathogens. Individual NK cells express multiple inhibitory and activating receptors and depending on the triggering either become activated or not (reviewed in ⁷). Inhibitory receptors, such as a subset of killer-cell immunoglobulin-like receptors (KIR) and the CD94/NGK2A heterodimer, recognize MHC class I and suppress NK cell responses upon recognition. Cells that have downregulated MHC-I either as a result of virus infection or malignant transformation are targets for activated NK cells, which then lyse these cells. This mechanism has been described as ‘missing-self’. NK cells also express activating receptors, such as CD16, Nkp46, Nkp44, NKG2D, CD244 (2B4) and several activating KIR, which tend to recognize ligands that are upregulated on infected and transformed cells ⁸. Upon activation NK cells can kill target cells and produce pro-inflammatory cytokines such as IFN γ and TNF α . In addition, they are able to activate DC.

NK cells develop in the bone marrow and in secondary lymphoid organs. As for T cells, distinct NK cell developmental stages have been defined in detail (reviewed in ²). NK and T cells share a common CD34⁺ T/NK cell progenitor that is found in the bone marrow and also in the thymus. In line with this, several reports have provided evidence for NK cell development in the thymus.

Nevertheless, it is generally believed that the thymic microenvironment favors T over NK cell differentiation⁹. In humans, committed NK cell precursors are defined as CD34⁺CD7⁺CD3⁻ cells that express CD122 (IL-15/IL-2R β) transcripts, an essential component of the IL-15-receptor¹⁰. Upon IL15-dependent maturation, NK cell progenitors lose CD34 expression and mature NK cells are characterized by the expression of CD56 and NKp46, among others (Figure 1).

Occasionally individual lymphocytes can undergo malignant transformation and develop into systemic leukemias or tissue-resident lymphomas. Based on morphological criteria, genetic evaluation and immunophenotyping, it has become clear that such malignancies can arise at any stage of lymphocyte development. They are classified accordingly in the WHO classifications of leukemias and lymphomas¹¹. Examples of immature T cell leukemias are the common acute lymphoblastic leukemia, or the acute lymphoblastic leukemia (T-ALL) and more mature T cell leukemias include Sezary syndrome or the adult T cell leukemia. The distinct types of lymphomas are subdivided according to the cell type a lymphoma arises from (B, T, NK cells) and the grade of proliferation (low or high grade). Examples of high-grade lymphomas are peripheral T cell lymphomas including anaplastic large cell lymphoma and enteropathy-associated T-cell lymphoma (EATL).

THE INTESTINAL IMMUNE SYSTEM

Mucosal surfaces of the gastrointestinal, respiratory or urogenital tract, but also their associated glands (such as salivary glands) or the lactating breast are in close contact to both harmful invading pathogens and commensal bacteria from the environment. At the same time they serve essential functions, such as nutrient absorption in the intestine or exchange of oxygen and carbon dioxide in the lung. Altogether mucosal surfaces form an enormous surface area to protect. To meet these demands, the intestine possesses specialized anatomical features and harbors an elaborate compartment of innate and adaptive immune cells, with distinct features compared to their counterparts in other organs or the blood. Immune cells from mucosal sites can circulate from any mucosal site to the other via the expression of specific epithelial adhesion and chemokine receptors, a mechanism referred to as lymphocyte homing. This facilitates the coordinated immune responses towards pathogens at various mucosal sites and the protection of the entire mucosal surfaces. Even though immune cells at mucosal sites show an activated phenotype, inhibitory mechanisms downregulate the local immune response to non-pathogenic antigens such as food-derived antigens or commensal bacteria. In fact, oral administration of antigens leads to the peripheral suppression of immune responses against these same antigens at non-mucosal sites—a principle known as oral tolerance¹². The intestine for instance harbors trillions of microbes, which are introduced early in childhood during birth and by weaning, as

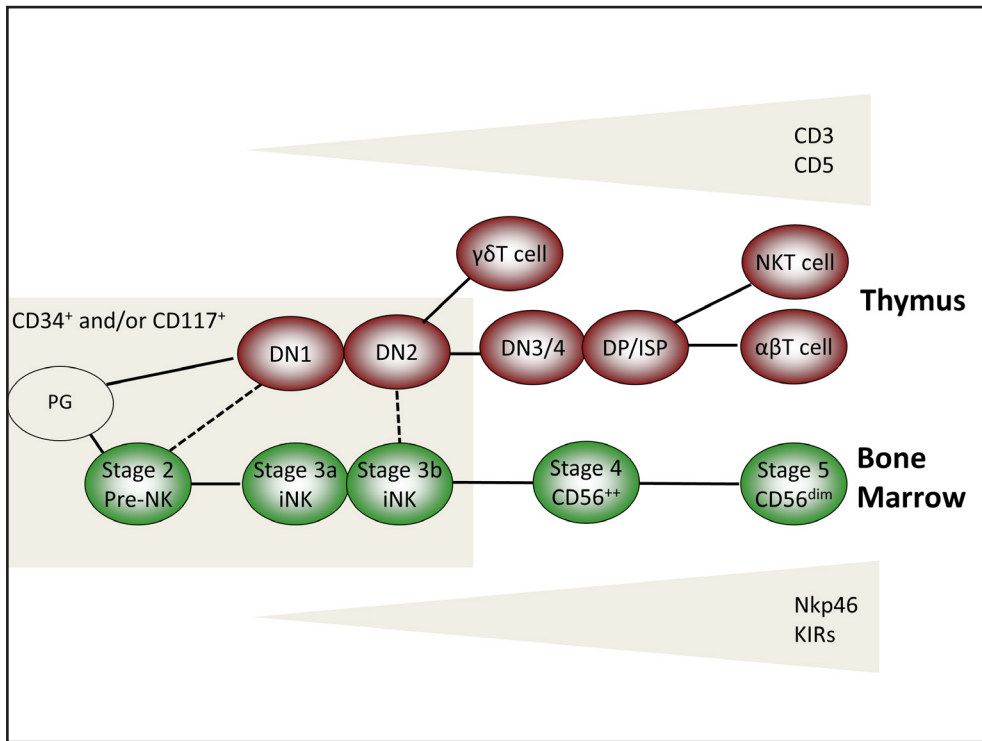


FIGURE 1. HUMAN T AND NK CELL DEVELOPMENT. The cartoon shows the main developmental stages of T and NK cells, which for NK cells mainly take place in the bone marrow and for T cells mainly in the thymus ^{1,2}. Nevertheless, NK and T cell differentiation has been described for secondary lymphoid organs such as lymph nodes, spleen, liver ^{1,2} or NK cell differentiation for the thymus ⁹ (not depicted here). At immature developmental stages (marked by the grey box), cells can still switch developmental paths either towards T or NK cells and a common T/NK cell progenitor (PG-progenitor) is characterized by the expression of CD34 and/or CD117. Upon maturation NK cells express CD56, Nkp46 and KIR, whereas mature T cells are characterized by surface CD3 (along with the TCR) and CD5 expression (grey triangle indicates upregulation). DN- double negative, DP- double positive, iNK- immature NK cell

food may carry microbes. The presence of such enormous amounts of microbes in the intestine renders it the most densely populated organ in the body. Yet, under physiological conditions, a mutual beneficial interplay between microbes and the host persists (reviewed in ¹³).

Anatomically, the intestine is composed of the small and the large intestine. The small intestine consists of the duodenum, which connects to the stomach, the jejunum and the ileum, which attaches to the large intestine. The main function of the small intestine is the digestion of food and the absorption of nutrients and water. At the same time the small intestine is the first line of defense against various pathogens entering via the same route. The small and the large intestine show large structural and functional differences with regards to the composition and size of the microbial community-of which the vast majority is present in the large intestine-, ion and nutrient concentration, pH, oxygen tension and retention time or composition of the surface structure, among others. The small intestine has a larger surface area compared to the large intestine as it is composed of finger-like structures which protrude into the lumen, shaping a landscape of villi (extrusions) and crypts (intrusions). This network of villi and the expression of additional microvilli on enterocytes increases the overall surface for optimized absorption of nutrients and results in an immense surface area of 200-400m² ¹⁴. Even though the barrier between the lumen-the content of the gut- and the body consists only of a single layer of epithelial cells, this barrier has to maintain both absorption of essential nutrients and contribute to the defense against pathogens. To perform these tasks, the intestinal epithelium consists of enterocytes which absorb nutrients, while secretory cells, such as goblet cells produce a sophisticated matrix of mucus supporting the barrier function against pathogens. Furthermore, the epithelium consists of hormone-producing (endocrine) cells, anti-bacterial lysozyme-producing paneth cells and stem cells replenishing these specialized cell types. These stem cells provide continuous, rapid cell renewal of the entire epithelial population, with a turnover rate of approximately 60h and a strict regulation of cell birth, differentiation and cell death. As proliferating stem cells differentiate, they move upwards from the crypt towards the villi, where they are shed into the lumen and are discarded with the feces (reviewed in ¹⁵). These epithelial cell types are situated on a basal membrane which serves as a boundary to the underlying lamina propria (LP).

The epithelium and the lamina propria contain distinct populations of immune cells. CD8⁺ T cells comprise the major proportion of the intraepithelial lymphocytes (IEL) which are located between the enterocytes in the epithelium while in the lamina propria both CD8 and CD4 positive T cells are present, as well as B cells and an abundance of antibody secreting plasma cells. In addition dendritic cells reside mainly in the lamina propria, but can sample luminal antigens via protrusion of dendrites through the epithelium ¹⁶ or via interaction with goblet cells ¹⁷. In addition, various innate immune cell populations are located in the lamina propria, such as macrophages,

mast cells, basophils, eosinophils and innate lymphoid cells (ILC), which play important roles in tissue remodeling and immunity against viruses and bacteria (reviewed in ¹⁸). These innate immune cells, which also include NK cells, rapidly sense and initiate immune reactions towards pathogens while tolerance to harmless antigens is maintained. It is becoming increasingly clear that also enterocytes and goblet cells actively participate in these processes by expressing pattern-recognition receptors (PRRs) amongst others, which can detect conserved motives from pathogens (reviewed in ¹⁹). Furthermore, organized lymphoid structures in the LP harbor various lymphocyte subsets whose function it is to induce and synchronize a tailored immune response. These structures are classified as gut-associated lymphoid tissue (GALT) and include peyer's patches with microfold cells (M cells), which are specialized in antigen-uptake, cryptopatch (CP)-like structures ²⁰ and isolated lymphoid follicles (reviewed in ²¹).

Deregulation of intestinal immune responses can contribute to a variety of inflammatory bowel diseases like Crohn's disease, ulcerative colitis and celiac disease. Whereas Crohn's disease can affect the entire intestine, ulcerative colitis only affects the large intestine (colon) and celiac disease generally affects the small intestine.

INTRAEPITHELIAL LYMPHOCYTES

Intraepithelial lymphocytes (IEL) are situated at the basolateral side of the epithelium, dispersed in between the epithelial cells. In the small intestine IEL are mainly located in the crypts, with decreasing frequencies of IEL towards the tip of the villi. The healthy small intestine displays a characteristic IEL distribution pattern ^{22, 23}. As IEL are in close contact with enterocytes, they are generally believed to contribute in preserving the mucosal barrier integrity, as well as inhibiting pathogen entry and distribution by lysing infected epithelial cells. Furthermore, IEL possess immune-regulatory functions, dampening unwanted immune reactions and limiting potent immune responses directed against pathogen entry to the site of infection (reviewed in ²⁴).

The majority of small intestinal IEL in humans and mice are TCR $\alpha\beta$ and TCR $\gamma\delta$ T cells (T-IEL). Most of these express CD8, whereas CD4⁺ T-IEL form a minority population in the epithelium. All IEL constitutively express the epithelial adhesion marker CD103 (α E chain) in association with the β 7 chain, thus forming the α E β 7 heterodimer. CD103 expression distinguishes IEL from blood-derived lymphocytes, where only a minority is CD103 positive. The α E β 7 heterodimer specifically interacts with E-Cadherin which is expressed on epithelial cells and is therefore essential to the gut homing and retention of IEL ²⁵⁻²⁷. Furthermore, the majority of IEL express antigen-experienced/memory T cell markers, the T/NK cell marker CD7 ²⁸ (Thy-1 in mice ²⁴) and a variety of both activating and inhibitory NK cell receptors ²⁹⁻³⁴. Studies on the TCR repertoire of T-IEL in

mice and men demonstrate that the repertoire is polyclonal after birth but becomes oligoclonal in adults (reviewed in ³⁵), likely the result of the expansion of particular T-IEL.

MICE

In mice the majority (60%) of T-IEL is composed of TCR $\gamma\delta^+$ cells ^{36,37} which express CD8 $\alpha\alpha$ homodimers. CD8 $\alpha\alpha$ serves as an activation marker, being expressed either by itself or in combination with CD4 or CD8 $\alpha\beta$ and binds to thymus leukemia antigen (TLA), a non-classical MHC-I molecule which is abundantly expressed in the small intestine ²⁴. The composition of IEL can be influenced by the intestinal environment such as by colonization with microbes or by dietary antigens. Studies in mice for example, show that the IEL pool is reduced in an antigen-deprived environment ^{38,39} and is influenced by dietary compounds, such as aryl hydrocarbon receptor (AhR)-ligands in cruciferous vegetables ⁴⁰ and vitamin D ⁴¹. As the small intestine harbors considerably fewer microbes than the colon, but contains many more IEL, it is therefore possible that the response to dietary antigens in the small intestine is more pronounced than in the colon.

In mice two subtypes of IEL can be classified according to their phenotype and origin of development: induced (type a) and natural (type b) T-IEL. Whereas induced IEL in mice are derived from conventional T cells, which become activated by peripheral antigens, natural IEL are assumed to develop in the thymus in the presence of self-antigens (referred to as agonist selection) or complete their development in the intestine ²⁴. In fact, pre-committed T cell precursors have been found in the thymus, which can complete their maturation under the influence of the local intestinal cytokine IL-15 in the gut ^{42,43}. If extrathymic T cell differentiation from early progenitor cells exists in the intestine, is a subject of a long-standing debate (reviewed in ⁴⁴). Evidences against and in favor of intestinal T cell development have been adduced for mice and humans. The absence of detectable RAG proteins in the intestine ⁴⁵ and the complexity of the thymic environment necessary for TCR selection are prominent reasons to oppose extrathymic T cell development. Nevertheless, athymic mice show reduced, but non eliminated TCR $\alpha\beta$ and TCR $\gamma\delta$ T-IEL populations, which increase with age ^{46,47}. This could indicate that the majority of T-IEL develops in the thymus, but a minority could develop locally. Furthermore, in the intestine evidence of ongoing T cell differentiation has been presented for lineage-negative (Lin⁻) cells, thus for cells lacking surface markers of all known lineages. Molecular evidence of T cell development in Lin⁻ cells include preT α expression ^{48,49}, initiation of TCR rearrangements and the presence of intracellular CD3 chains ⁵⁰. Moreover, CD8⁺ T-IEL could be reconstituted from Lin⁻ cells in athymic RAG2/ γ c- mice ⁵⁰ as well as CD8 $\alpha\alpha^+$ T-IEL from Lin⁻ cells derived from CP ^{51,52}. It is therefore hypothesized that in mice CD8⁺ T-IEL can develop extrathymically.

HUMAN

Considerably less is known about human IEL development. In humans only a minority of the T-IEL express CD8 $\alpha\alpha$ ^{28, 53, 54} and due to the limitations of studies with human primary tissue only indirect evidence for *in situ* IEL development is available^{55, 56}. Nevertheless evidence for the presence of T cell precursors within a CD3⁺ IEL population exists^{55, 56}. Others have shown that enzymes necessary for TCR rearrangements such as RAG-1 are expressed in the small intestine⁵⁷⁻⁵⁹, while only a minor proportion of cells were found positive for TdT⁵⁴. The exact phenotype of the putative T cell precursors was not determined. It is also unknown if these cells have a thymic origin and which factors are crucial for their local development.

In humans IEL are composed of about 70-80% of TCR $\alpha\beta$ ⁺ CD8 $\alpha\beta$ ⁺ and the remaining are TCR $\gamma\delta$ ⁺ (either CD4⁺CD8⁻ or CD8⁺) (5-20%), or CD4⁺ TCR $\alpha\beta$ ⁺ (10-15%)⁶⁰. Human TCR $\gamma\delta$ ⁺ predominantly express V δ 1⁶¹⁻⁶³ which recognizes non-classical MHC-I-related molecules such as CD1c⁶⁴ and MIC proteins⁶⁵⁻⁶⁷. Furthermore, 10% of the IEL are CD3⁺ IEL, some of which are functional NK cells^{68, 69}. In fact, T-IEL also express several NK cell receptors such as NKG2D^{31, 32, 70}, CD94^{34, 69, 71}, CD56^{34, 72} and CD161^{34, 69}. Furthermore, T-IEL can be activated without engagement of the TCR^{31, 32, 70}.

Thus, in mice and men T-IEL show an activated-yet resting- innate-like phenotype. IEL therefore represent unique types of activated lymphocytes, which are tightly regulated under physiological conditions and likely play an important role in the maintenance of intestinal homeostasis.

CELIAC DISEASE

Celiac disease (CD) is a gluten induced enteropathy of the small intestine, caused by the ingestion of gluten compounds in genetic susceptible individuals. The prevalence of CD is around 1 % in the western population and is therefore one of the most common intestinal disorders⁷³. CD can affect both children and adults. It is still unclear why some individuals develop CD during infancy or childhood, while others acquire the disease at a later stage in adulthood. Classical clinical manifestations such as diarrhea, failure to thrive and abdominal distention are mostly found in children. CD can also present with diverse symptoms such as anemia, osteoporosis, dermatitis herpetiformis and neurological symptoms which are part of a complex spectrum of clinical manifestations associated with celiac disease due to malabsorption and autoimmune phenomena^{73, 74}.

Celiac disease is generally diagnosed by the detection of auto-antibodies against tissue transglutaminase (tTG) in the serum⁷⁵. If tTG auto-antibodies are detected, duodenal biopsies are taken and analyzed by histology. Celiac lesions demonstrate typical histological features, such as villous atrophy, crypt hyperplasia and increased counts of intraepithelial lymphocytes (IEL) in

the epithelium. Collectively these histological abnormalities are included in a diagnostic scoring system for disease severity which is reflected in the Marsh criteria ^{76,77}. After diagnosis patients are treated with a life-long gluten free diet (GFD), avoiding any gluten intake from wheat, barley and rye.

Celiac disease has a strong autoimmune component, as auto-antibodies against tTG are detected in active CD patients. Furthermore, epidemiological studies show a strong link between CD and other autoimmune diseases such as type-1 diabetes and autoimmune thyroiditis ⁷⁸. In fact, such autoimmune comorbidities affect about 20% of adult CD patients ⁷⁹. In the last decades it has been demonstrated that HLA-DQ2 and HLA-DQ8 are the major genetic predisposing factors in CD, as 90-95% of CD patients express HLA-DQ2 and the remaining 5-10% HLA-DQ8 (reviewed in ⁸⁰). Besides HLA, other genes have been linked to CD by genome-wide association studies (GWAS), but these explain only approximately 5% of the genetic heritability ⁸¹. Therefore HLA itself is the strongest disease-associated gene in CD, accounting for about 35% of the genetic heritability ⁸². The nature of the remaining genetic heritability still has to be determined and could be explained by gene-gene interactions, or rare genetic variants (reviewed in ⁸⁰). As about 25% of the western population is HLA-DQ2⁺, but only 4% of DQ2 carriers develop the disease, additional environmental or genetic factors also play a crucial role in disease development that still need to be determined ⁸³.

Environmental factors influencing CD development could be the amount of gluten intake, the age of gluten introduction into the diet of an infant ⁸⁴, or viral infections, such as rotaviruses which lead to an increased risk of CD development ⁸⁵. Nevertheless, more work is needed in order to understand the complex interplay of gene variations, gene networks and gene-environmental interactions, ultimately leading to CD development.

The molecular basis for the association of CD with HLA-DQ2 and HLA-DQ8 was revealed when it was demonstrated that CD4⁺ T cells in the lamina propria (LP) can recognize gluten presented in a HLA-DQ2/8-restricted fashion ^{86, 87, 88-93}. Such gluten-specific T cells have a pro-inflammatory phenotype characterized by the secretion of IFN γ ⁹⁴ and IL-21 ⁹⁵. Moreover, generally such CD4⁺ T cells are found in small intestinal biopsies of patients but not in healthy controls and they are therefore generally held responsible for the intestinal inflammation of CD lesions due to gluten consumption.

Gluten is unusually rich in the amino acids proline and glutamine, and the abundance of proline makes it relatively resistant to digestion by gastrointestinal enzymes, as compared to other food-derived proteins ⁹⁶. After partial digestion immunostimulatory gluten fragments can reach the intestine and trigger a cascade of immune reactions in the small intestinal LP in CD patients, which

has been extensively characterized in the last years. The local tissue-repair enzyme tTG can modify such gluten fragments by deamidation of particular glutamine residues into a negatively charged glutamic acid. This increases the binding affinity of gluten peptides to HLA-DQ2 and HLA-DQ8, thus amplifying the gluten-specific immune response^{97,98}. Various immunodominant epitopes have been characterized for HLA-DQ2 and HLA-DQ8 and placed CD4⁺ T cells as key players in the gluten-mediated immune response^{86, 87, 99, 100}.

The inflammatory reaction to gluten in the lamina propria has been shown to be absolutely necessary for the development of celiac disease, but might not be sufficient, as additional factors such as the activation and expansion of IEL most likely contribute to tissue destruction. In order to study the pathogenesis of CD one relies on limited patient material, as currently no mouse model is available that shows all key features of CD: the adaptive response to gluten, the innate response in the epithelium and LP and the autoimmunity component by the generation of tTG antibodies (reviewed in¹⁰¹). In fact, mice engineered to express human HLA-DQ8 or HLA-DQ2 and a gluten-specific TCR, mainly produced the anti-inflammatory cytokines IL-10 and TGFβ upon gluten challenge^{102, 103}, indicating that multiple factors are required in order to break the strong oral tolerance towards gluten. In another mice model, oral tolerance to gluten could be abrogated by the administration of both IL-15 and retinoic acid (a vitamin A metabolite)¹⁰⁴, implicating a great improvement in the development of murine CD models. If there is a causal relationship of retinoic acid intake and CD is currently unknown and the factors leading to the break of oral tolerance against gluten in CD patients remain to be elucidated.

REFRACTORY CELIAC DISEASE

Most celiac patients show remission from CD symptoms when following a gluten-free diet (GFD). Nevertheless a small percentage of patients do not respond to a GFD initially or relapse despite a strict GFD and are therefore diagnosed with refractory celiac disease (RCD). Refractory celiac disease is considered a rare disorder, with an estimate prevalence of 2-5 % in adult onset CD patients, which are generally above the age of 50⁷⁴.

The diagnosis of RCD is based on exclusion criteria, where other pathological intestinal diseases such as autoimmune enteropathy, common variable immunodeficiency and tropical sprue are ruled out, as these diseases can mimic (R)CD symptoms¹⁰⁵. According to the phenotype, composition and clonality of IEL, RCD can further be subdivided in type I and type II, with substantial differences in clinical outcome. RCDI presents with oligoclonal, phenotypically normal IEL, composed mainly of T cells (T-IEL). RCDII on the other hand shows a massive expansion of clonal aberrant IEL, lacking surface markers characteristic for T cells such as CD4, CD8, CD3 and the TCR, but expressing CD3 intracellularly (ic)¹⁰⁶⁻¹⁰⁸. The diagnosis of RCDI and RCDII is therefore performed on the basis of the existence or absence of aberrant cells by histology or

flow cytometry. To detect aberrant IEL, intestinal samples are stained for CD3ε in combination with T cell receptor or CD8 by immunohistochemistry ¹⁰⁹ or- more accurately- IEL are isolated from biopsies and analyzed by flow cytometry ¹¹⁰. Clonal aberrant IEL are further analyzed by PCR and the presence of clonal rearrangements confirms the diagnosis of RCDII ¹⁰⁶. As in CD, RCD shows a strong association with HLA-DQ2 and HLA-DQ2 homozygosity is particularly frequent in RCDII patients ¹¹¹.

No standardized therapeutic strategy has been defined for the treatment of RCDI and RCDII, except for parental nutrition in cases of severe malnutrition. Whereas RCDI can be treated effectively with immunosuppressant drugs ¹¹², moderate success rate have been described for the treatment of RCDII with various chemotherapeutic drugs ^{108, 113} or autologous stem cell transplantation ¹¹⁴.

Consequently, about 50% of RCDII patients progress and can develop an overt enteropathy-associated lymphoma (EATL), thereby defining RCDII as a serious premalignant condition ^{107, 108, 115}. This is further illustrated by chromosomal aberrations found in aberrant IEL, which are particularly frequent within the chromosome 1q22-44 region ¹¹⁶. Upon transformation aberrant IEL can disseminate to other tissues such as the LP, blood and other organs ^{108, 117}. As the 5-year survival rate in patients with RCDI is 80-96 % but only 44-58 % for patients with RCDII, this clearly demonstrates the necessity to further differentiate the diagnosis of RCDI and II to predict the clinical outcome ^{108, 112, 115}.

INTRAEPITHELIAL LYMPHOCYTES IN CELIAC AND REFRACTORY CELIAC DISEASE

A classical feature of active CD is the expansion of IEL in the small intestine (IEL lymphocytosis), which is also used as a diagnostic criterion for CD. Histological studies on healthy biopsy material and CD lesions have set an IEL count for the upper limit of normal at 25 IEL/100 enterocytes, whereas >30 IEL/100 enterocytes is considered pathological ¹¹⁸. The distribution pattern of IEL also changes in CD, from a crypt-enriched pattern under physiological conditions, to a more scattered, continuous distribution mainly caused by elevated numbers of IEL in the tip of the villi ²² (Figure 2). IEL lymphocytosis is a consequence of a significant increase of TCRγδ T-IEL, as well as TCRαβ T-IEL, whereas TCRαβ T-IEL counts decrease on a GFD to physiological levels-in contrast to persistently high numbers of TCRγδ T-IEL ¹¹⁹⁻¹²¹ (Figure 2). The GFD therefore leads to a moderate decrease in IEL counts, but already 12 hours after oral or rectal gluten challenge elevated IEL numbers can be detected, demonstrating a rapid epithelial response to gluten which precedes the onset of epithelial damage ^{76, 122}. IEL lymphocytosis is not specific to CD as it is also found in gastric infections with *h. pylori*, bacterial overgrowth in the small intestine ¹²³, tropical sprue

²³ and can be caused by the use of nonsteroidal anti-inflammatory drugs ^{23,124}, but not in other gastroinflammatory diseases such as Crohn's disease or autoimmune enteropathy. Like in CD the latter two diseases share a marked infiltration of lymphocytes in the LP, but do not reveal such a characteristic IEL expansion ²³. Whether the increase of IEL in intestinal inflammation, such as in CD is caused by an expansion of local IEL, an influx of (mature or semi-mature) lymphocytes from extra-intestinal sites or by lymphocytes generated by local development from immature IEL is not known.

However, several studies have demonstrated that in CD IEL can be highly cytotoxic ^{125,126} and can kill epithelial cells via Fas, granzyme and perforin-mediated pathways ^{127,128} and via the NK cell receptor NKG2D which binds to its ligand MICA on stressed enterocytes ^{31,32,70} (Figure 2). These studies also demonstrated a prominent role for the inflammatory cytokine IL-15 in NKG2D up-regulation, therefore contributing to cytotoxicity. IL-15 plays an important role in the pathogenesis of CD ¹²⁹, as it has been shown to be up-regulated both in the LP and the epithelium of CD patients, in comparison to controls ^{128,130}.

Besides T-IEL, the epithelium also contains an ill-defined CD3⁺ population which harbors functional NK cells ^{68,69} and is decreased in CD patients in comparison to controls ^{69,72,131-133}. Studies carried out on this enigmatic CD3⁺ population further indicate that it is heterogeneous ^{69,72,131-133}, most likely harboring various IEL subsets, amongst others also NK cells. Therefore the exact phenotype of the different CD3⁺ IEL subsets is not determined, neither is the function of these CD3⁺ cells under physiological and pathological conditions, nor the mechanisms leading to its reduction in CD. As aberrant IEL in RCDII patients show a similar phenotype of CD3⁺CD4⁺CD8⁺TCR⁺ but intracellular CD3⁺, it was proposed that CD3⁺ IEL could harbor the physiological precursors for aberrant IEL in RCDII ¹³³. On the contrary it was also postulated that aberrant IEL could be derived from derailed T-IEL, which have down-regulated their TCR due to overstimulation ¹⁰⁶.

As previously mentioned, in RCDII more than 80% of IEL can display an aberrant phenotype, which distinguishes RCDII from RCDI where no to <20% of IEL have such a CD3⁺ phenotype ^{106,110}. In RCDI patients IEL show the same characteristic phenotypes as active or treated CD, with increased or physiological levels of TCR $\gamma\delta$ T-IEL ¹⁰⁸. In RCDII aberrant IEL lack the T-IEL markers CD3, CD4, CD8 and TCR, but express the common IEL markers CD45, CD7 and CD103 ^{106-108,134}. Furthermore, aberrant IEL as T-IEL in CD express NKG2D ³², but lack the NK cell marker CD56 and CD16 ¹⁰⁶ and show clonal TCR rearrangement, which are mostly TCR γ -but also TCR δ or β rearrangements have been detected. Importantly, TCR rearrangements in aberrant IEL are almost always non-functional, out-of-frame or even completely absent ^{106,108,135,136}. Similar to T-IEL in CD, aberrant IEL show strong cytotoxicity towards epithelial cells and aberrant IEL cell lines (derived from RCDII biopsies) can lyse epithelial cells via NKG2D ³² and via DNAM ¹³⁷ (Figure 2). As described for CD, IL-15 was

also shown to be up-regulated in the LP and the epithelium of RCDII patients, compared to controls¹²⁸. Different cell types such as enterocytes can present IL-15 via the IL-15R α to adjacent cells (such as IEL) which respond via the IL-2/15R β (CD122). This mode of activation is called trans-presentation and can take place in the intestinal epithelium (reviewed in¹³⁸). Aberrant IEL in RCDII patients have been shown to express CD122¹²⁸ and to be very sensitive to IL-15 by responding to low concentrations of IL-15^{128, 134, 139}. IL-15 can further induce IFN γ expression and cytotoxicity³² and the anti-apoptotic pathway of Jak3, Stat5 and Bcl-xl in aberrant IEL¹³⁹. IL-15 therefore plays a crucial role in the accumulation of aberrant IEL in RCDII. As IL-15 strongly induces proliferation and exerts anti-apoptotic effects on aberrant IEL, it has been proposed that RCDII and EATL patients should be treated with IL-15-specific antibodies, neutralizing the detrimental effects of IL-15 on aberrant IEL¹³⁹.

Whether or not elevated IL-15 is sufficient in inducing expansion and transformation of aberrant IEL, or in other words if blocking of IL-15 is sufficient in preventing the expansion or induction of cell death in an already enlarged aberrant IEL pool is not known. In fact, the expansion of aberrant IEL could be the result of multiple factors. It is furthermore important to determine the origin of aberrant IEL in order to understand how aberrant IEL develop and what makes them susceptible to outgrowth and transformation.

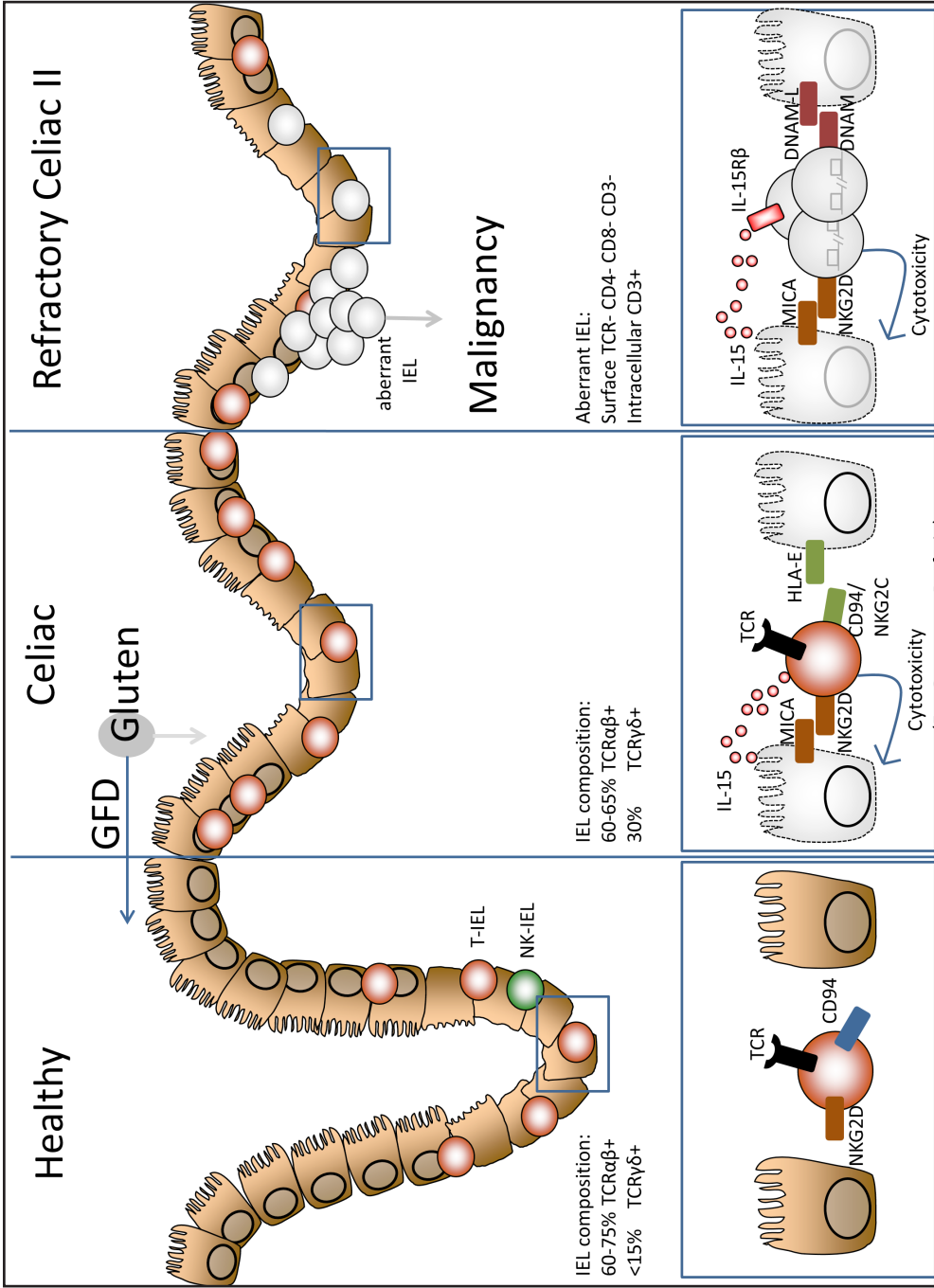


FIGURE 2. INTRAEPITHELIAL LYMPHOCYTES IN HEALTH AND CELIAC DISEASE

The cartoon depicts the key features of the epithelium and the role of intraepithelial lymphocytes (IEL) under physiological, celiac (CD) and refractory celiac (RCD) conditions. The frame enlarges the interaction of IEL with enterocytes and depicts the current understanding of receptor-ligand interactions resulting in cytotoxicity towards epithelial cells. IEL are mainly situated in the crypts and made up of mainly T cells (T-IEL) and some NK cells (NK-IEL). Under physiological conditions T-IEL consist mainly of $CD8^+TCR\alpha\beta^+$ and $<15\% TCR\gamma\delta^+$, whereas the latter increase to around 30% in celiac disease patients (CD). In CD patients, gluten ingestion leads to villous atrophy thus decreasing the distance between the tip of the villi and the crypts. Moreover, the distribution pattern of IEL changes, as IEL are scattered throughout the whole mucosa and can therefore also be found at the (remaining) tips of the villi. The treatment for CD is a life-long gluten-free diet (GFD) which in most patients leads to a complete mucosal repair. Nevertheless, a few percent of patients develop refractory celiac disease (RCD), with no remission despite a GFD. Whereas RCD type I mimics CD pathology, RCDII is characterized by the expansion of so called aberrant IEL (surface $TCRCD4^+CD8^+CD3^-$ intracellular $CD3^+$), which present with non-functional TCR rearrangements. These aberrant IEL are considered pre-malignant and can transform into a malignant enteropathy-associated T cell lymphoma (EATL). IEL can actively promote tissue damage which leads to the macroscopic mucosal changes characteristic for CD and RCD. Under physiological conditions epithelial cells lack the expression of CD94- and NKG2D-ligands, whereas in CD the ligands for NKG2D and CD94 (MICA and HLA-E) are upregulated, resulting in enhanced epithelial cell lysis. The pro-inflammatory cytokine IL-15, which can be produced by enterocytes, further enhances epithelial lysis via NKG2D upregulation on IEL. This cytotoxicity is mediated by Fas, Granzyme or Perforin-mediated pathways. Aberrant IEL are also highly cytotoxic and can lyse epithelial cells via NKG2D- or via DNAM-mediated pathways. As in CD, IL-15 also plays an important role in the pathogenesis of RCDII, as IL-15 is upregulated in RCDII lesions and aberrant IEL strongly respond to IL-15 via the IL-15R β , leading to uncontrolled expansion of such cells. IEL- intraepithelial lymphocytes; GFD- gluten free diet.

SCOPE OF THE THESIS:

Over the last few decades several studies have shed light on the complex network of immune reactions triggered by gluten in the small intestine of celiac disease patients and the cytotoxic mechanisms of T-IEL in CD. In recent years it has also been demonstrated how aberrant IEL in RCDII patients can participate in tissue damage. Nevertheless, several features of RCD are still poorly understood. How does RCD develop and why do some patients develop the severe condition of RCDII and EATL? Our studies have focused on two main questions:

WHAT DRIVES THE EXPANSION OF ABERRANT IEL IN RCDII PATIENTS?

From studies previously carried out by the group of Cerf-Bensussan et al. we have learned that *in vitro* IL-15 can drive the expansion of aberrant IEL through proliferation and anti-apoptotic effects. We hypothesized that the expansion could be also influenced by other factors present in the small intestine.

WHAT IS THE ORIGIN OF ABERRANT IEL?

In order to understand the pathological mechanisms leading to the massive expansion of aberrant IEL and ultimately the transformation into an overt lymphoma, one has to understand the origin of the disease. If there is a physiological cell population that could be the counterpart of aberrant IEL, what can we learn about their phenotype and physiological function? This information could be used to draw conclusions about the mechanisms on how aberrant IEL develop, expand and ultimately transform into a lymphoma. This knowledge is essential to develop more effective therapeutic approaches.

THIS CHAPTER (CHAPTER 1) provided an overview of key players and mechanisms in immunity, especially in mucosal immunology of the small intestine. The structure, cell types and networks of immune cells that are unique to the small intestine in particular have been described. Subsequently the current knowledge of celiac and refractory celiac disease was summarized, with an emphasis on intraepithelial lymphocytes in health and under celiac and refractory celiac conditions.

IN CHAPTER 2 we investigate which other factors can drive the expansion of aberrant IEL, besides the well characterized IL-15. We therefore made use of aberrant IEL lines, generated from RCDII patients and study the interaction of these aberrant IEL lines with possible bystander intestinal immune cells, focusing on proliferation-induction and anti-apoptotic effects.

WHAT IS THE ORIGIN OF ABERRANT IEL?

It was postulated that aberrant IEL could either be derived from a derailed T-IEL population, which down-regulates CD3 and the TCR, or that it originates from a physiological IEL subsets within the existing CD3⁺ population.

IN CHAPTER 3 we tested the latter hypothesis by comparing aberrant IEL lines and T-IEL lines, using transcriptomic and detailed phenotypic analysis to initially define a distinct common phenotype of aberrant IEL. This information was then used to identify their possible physiological counterpart *in situ* in non-RCDII individuals.

The comparison of aberrant IEL lines with T-IEL lines in CHAPTER 3, generated a broad knowledge of the phenotype of aberrant IEL, but also about the existence of a heterogeneous physiological innate IEL population, which harbors the putative precursor for aberrant IEL. This led to several questions: What is the phenotype, relationship and function of this innate IEL population? What are the parallels with a similar thymic population?

IN CHAPTER 4 we further characterize this heterogeneous small intestinal innate IEL compartment in depth with multicolor flow cytometry and studied its plasticity in different patient cohorts. As we have learned in CHAPTER 3 aberrant IEL lines and their putative physiological precursor cells reflect features of T and NK cells, we now investigated the hypothesis that these cells function as immature IEL which could mature *in situ*. We therefore test the ability and the requirements of these cells to develop into mature NK and T cells, comparing the developmental capacity of IEL derived from non-CD, CD and RCDII patients *in vitro*.

IN CHAPTER 5 the thymic counterpart of the small intestinal cell population is studied, which is described in CHAPTER 3 and 4. We now investigate the phenotype and developmental capacity of the thymic cell population with parallels to the well-described CD34⁺ T/NK cell precursor population in the human thymus.

IN CHAPTER 6 the implications of our studies described in Chapter 2-5 for our understanding of CD and RCDII pathogenesis in particular are discussed. Furthermore, we discuss the relevance of these studies in the context of IEL development under physiological and pathological conditions and suggest directions for future research.

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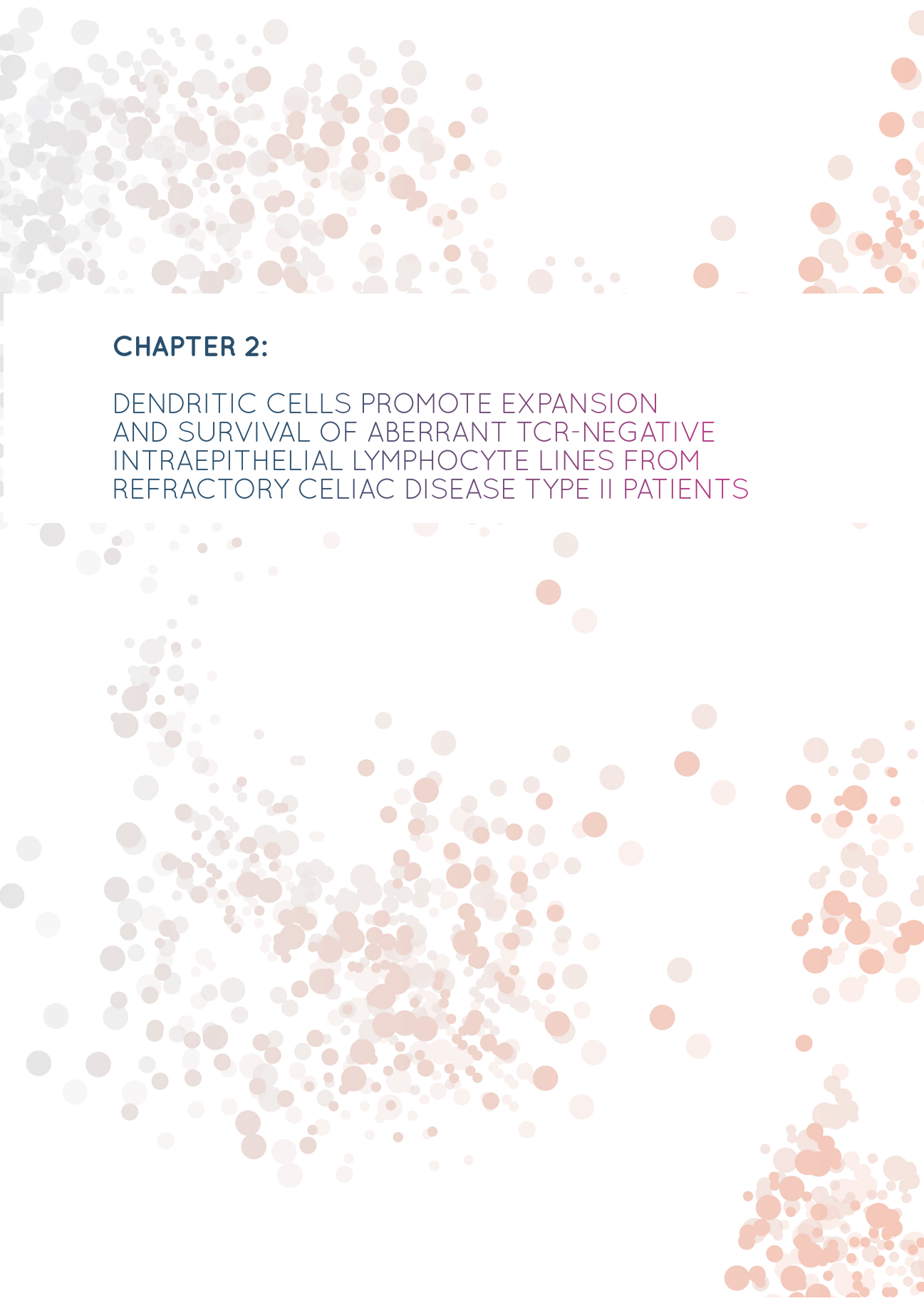
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CHAPTER 2:

DENDRITIC CELLS PROMOTE EXPANSION
AND SURVIVAL OF ABERRANT TCR-NEGATIVE
INTRAEPITHELIAL LYMPHOCYTE LINES FROM
REFRACTORY CELIAC DISEASE TYPE II PATIENTS

CHAPTER 2

2

DENDRITIC CELLS PROMOTE EXPANSION AND SURVIVAL OF ABERRANT TCR-NEGATIVE INTRAEPITHELIAL LYMPHOCYTE LINES FROM REFRACTORY CELIAC DISEASE TYPE II PATIENTS

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ABSTRACT

Celiac disease (CD) patients who fail to respond to a gluten-free diet suffer from refractory celiac disease (RCD). A marked expansion of intraepithelial lymphocytes (IEL) lacking surface TCR/CD3 expression characterizes the RCD subtype II. In up to 50% of RCDII patients these so-called aberrant IEL (a-IEL) develop into lymphoma and can disseminate into other tissues. Elevated levels of Interleukin-15 (IL-15) in the intestine of CD and RCD patients likely contribute to the expansion of a-IEL. Here, we investigated if interactions with other cells might also influence a-IEL expansion. Similar to IL-15, cells from the monocyte lineage, particularly mature dendritic cells (DCs), promoted proliferation, prevented apoptosis and induced IFN γ secretion of a-IEL derived from RCDII biopsies (RCDII cell lines), which in turn induced CXCL10. In contrast to IL-15, mature DCs did not induce proliferation of regular TCR+IEL lines, generated from CD biopsies and IL-15-blocking antibodies did not inhibit DC-induced proliferation of RCDII cell lines. Furthermore, proliferation was dependent on cell-cell contact, but independent of the HLA-genotype of the stimulating cells. Our results suggest that contact with DC, either in the epithelium or upon dissemination, contributes to uncontrolled expansion of a-IEL in RCDII, independent of HLA-genotype and IL-15.

ABBREVIATIONS

CD- Coeliac Disease, RCD- Refractory Coeliac Disease, EATL- Enteropathy associated T cell Lymphoma, IEL- Intraepithelial Lymphocytes, T-IEL- TCR+/CD3+ Intraepithelial Lymphocytes, a-IEL- aberrant IEL

INTRODUCTION

Celiac disease (CD) is an enteropathy of the small intestine, caused by a strong immune response to dietary gluten that occurs in HLA-DQ2⁺ and/or HLA-DQ8⁺ individuals. Histological findings include villous atrophy, crypt hyperplasia ¹, increased numbers of TCR- $\alpha\beta$ ⁺ and TCR- $\gamma\delta$ ⁺ intraepithelial lymphocytes (T-IEL) ^{2,3} and elevated interleukin-15 (IL-15) levels in the epithelium and lamina propria ⁴. Clinical symptoms include anemia, diarrhea and failure to thrive. CD is treated with a gluten-free diet, which usually leads to remission and amelioration of histological and serological findings. However, 2-5% of adult-onset CD patients develop refractory CD (RCD) with persisting epithelial damage and increased numbers of IEL ^{5,6}, despite adherence to a gluten-free diet.

RCD can be further subdivided into RCD type I (RCDI) and RCD type II (RCDII), based on the quantity and phenotype of IEL in duodenal biopsies. RCDII patients have >20% surface T-cell receptor-negative-also called aberrant- IEL (a-IEL) of the total IEL population ⁷. In approximately 50% of RCDII patients these a-IEL gradually replace the normal IEL population and undergo malignant transformation ⁸. These patients develop a severe life threatening condition, called enteropathy-associated T cell lymphoma (EATL) with a 2 year survival of less than 30% ^{5,9}. a-IEL in RCDII contain clonal TCR rearrangements ^{6,8,10} and display a unique phenotype: they express no lineage markers (CD3, CD14, CD19, CD56) (Lin⁻) nor CD34 or CD127, but are positive for the T/NK cell marker CD7 and express intracellular CD3 (icCD3). a-IEL also express multiple NK cell markers and therefore share features with T and NK cells ^{7,8,10,11}.

While in non-celiac adults less than 10 % of IEL possess this ‘aberrant’ phenotype ¹¹, in RCDII patients a-IEL can make up more than 80% of the IEL population. The combination of elevated IL-15 levels in the duodenal epithelium of RCD patients and greater IL-15 sensitivity of a-IEL compared to other IEL is likely to contribute to the selective expansion and survival of aberrant cells in RCDII ^{4,12,13}. The fact that cells with this ‘aberrant’ phenotype from non-celiac duodenal biopsies express the IL-2/15R β and become positive for the proliferation marker Ki67 upon incubation with IL-15 ¹¹ suggests that the precursors of a-IEL in RCDII patients are also IL-15 sensitive.

Aberrant IEL in situ are located in the intraepithelial compartment and are therefore likely to closely interact with epithelial cells. In addition, contact with dendritic cells (DCs) could be made via DC protrusions extending into the epithelium ¹⁴⁻¹⁶ and contact to DCs and other types of blood-borne cells could take place upon dissemination of a-IEL. We therefore investigated proliferation, apoptosis and cytokine production of a-IEL derived from RCDII biopsies (RCDII cell lines) after co-

culture with intestinal epithelial cell lines and several blood-borne cell types, including DCs.

MATERIALS AND METHODS

Small intestinal biopsy specimen

During upper endoscopy, large spike forceps biopsy specimens (Medi- Globe, Tempe, AZ) were taken from the second part of the duodenum. All biopsy specimens were obtained after given informed consent in accordance with the local ethical guidelines of the VU Medical Center in Amsterdam and the Declaration of Helsinki.

Cell lines and cell culture

RCDII lines P1 and P2 were isolated from duodenal biopsies of RCDII patients and maintained as described [13]. TCR+IEL (T-IEL) lines were isolated from 4 CD patients, as described for RCDII lines. PBMC were isolated from buffy coats using standard ficoll gradient (PBMC layer). After ficoll separation the PBMC layer was removed, remaining cells were separately collected from the layer above the erythrocytes and termed neutrophils if CD16 was expressed or eosinophils if CD16 expression was not detected. Monocytes were isolated from fresh PBMC using CD14 beads (Miltenyi, Bergisch Gladbach, Germany) using LS MACS columns and the standard separation protocol (Miltenyi). Dendritic cells (DC) were generated by culture of these monocytes in RPMI/10% FCS medium supplemented with 1000 U/ml GM-CSF (R&D Systems, Abingdon, UK), 500 U/ml IL-4 (R&D Systems, Abingdon, UK) and macrophages were generated by culture in RPMI/10% FCS medium supplemented with 50 U/ml GM-CSF. On day 7, DC and/or macrophages were matured by incubation of 100 ng/ml LPS (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 10% FCS/ IMDM medium for 48h. HT-29 cells were cultured in IMDM/10% FCS medium.

Cytotoxicity assay

One million target cells were labeled with 100 μ Ci [^{51} Cr] for 1 h at 37°C. After extensive washing, labeled target cells were incubated with the RCDII lines as effector cells at an effector: target (E:T) ratio between 50:1 and 1.5:1 for 4 h at 37°C. Spontaneous chromium release and maximum chromium release by target cells was determined by addition of medium or 1% Triton X-100 (Pierce, Rockford, IL), respectively. The percentage of specific cytotoxicity was as follows: $\frac{[\text{cpm experimental release} - \text{cpm spontaneous release}]}{[\text{cpm maximum release} - \text{cpm spontaneous release}]} \times 100\%$

Proliferation assays

Responder cells were rested by culturing them in the absence of IL-15 for 4 days. 20 000 responder cells per well were subsequently cultured in triplicate in 96-well plates in the presence or absence of 10 ng/ml IL-15 or with irradiated (3000 Rad) stimulator cells for 3 days at 37°C, after which 0.5 μCi ^3H -thymidine was added to every well. Stimulator cells were 1×10^5 /well PBMC, 2×10^4 /well DC/macrophages/monocytes, 2×10^4 /well HT29 and responder cells were 2×10^4 /well RCDII lines or T-IEL lines, unless stated otherwise. For the transwell experiments the inserts were discarded after 3 days co-culture and 0.5 μCi ^3H -thymidine was added directly to the 96 well plate (HTS Transwell-96 system, 0,4 μm pore size, Corning, MA, USA). ^3H -thymidine incorporation was determined during the final 17h of the incubation.

Flowcytometry and antibodies

After co-culture, cells were harvested, washed in 0,5% FCS/PBS and stained with 7-AAD and Annexin PE using the apoptosis detection kit according to the BD Biosciences protocol (BD Biosciences, San Jose, CA). In short, 7-AAD and Annexin PE incubation was done for 15 min at room temperature (RT) in the supplied binding buffer and the labeled cells were acquired within 1h on a LSR II or FACS-Calibur (both from BD Biosciences). PBMC subsets were stained with CD14-PeCy7, CD19-PE, CD56-FITC, CD16-FITC (all BD Biosciences, San Jose, CA) and CD3-AlexaF750 (Invitrogen, NY, USA) and sorted with FACS Aria III (BD Biosciences). For proliferation assays 10 μg /ml IL-15 antibodies MAB 647 and 2 μg /ml MAB247 (both R&D systems Europe, Abingdon, UK) were used.

Cytokine production

IFN γ and CXCL10 concentrations were measured using the Bio-Plex assay (BioRad, Hercules, CA, USA) according to the manufacturer's protocol (www.biorad.com).

Data analysis

Flow cytometry results were analyzed with FACS DIVA 6.1.2 software. Data from cytotoxicity, proliferation and cytokine measurements were analyzed and computed in 4-GraphPad.

RESULTS

MONOCYTES INDUCE PROLIFERATION BUT NOT CYTOTOXICITY OF RCDII CELL LINES

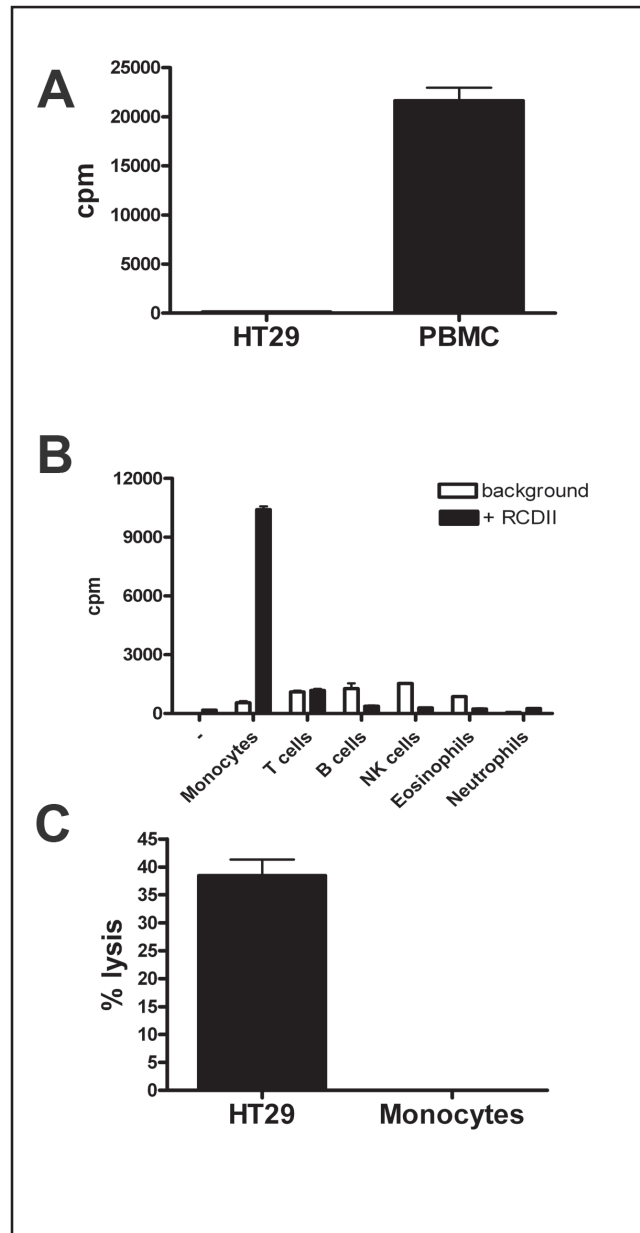
In vivo, a-IEL are in direct contact with epithelial cells and likely also with blood-borne cells, either locally or upon dissemination. *In vitro*, a-IEL derived from RCDII biopsies (RCDII cell lines) efficiently lyse epithelial cell lines via the NK cell receptor DNAM-1¹⁷, but it was not known whether the interaction between RCDII cell lines and other cell types could contribute to proliferation. To test this, we co-cultured RCDII lines with PBMC and the epithelial cell line HT-29. We observed that RCDII cell lines proliferated upon co-culture with PBMC, but not when co-cultured with the intestinal epithelial cell line HT-29 (Figure 1A). To investigate which cell type among PBMC induced RCDII line proliferation, we purified PBMC subpopulations by flow cytometry and co-cultured these with the RCDII lines. Monocytes (CD3⁻CD14⁺) induced proliferation of RCDII lines, but B cells (CD3⁻CD19⁺), NK cells (CD3⁻CD56⁺), T cells (CD3⁺), neutrophils (CD3⁻CD56⁻CD16⁺) and eosinophils (CD3⁻CD56⁻CD16⁻) did not (Figure 1B). In contrast, RCDII lines lysed HT-29 but not monocytes (Figure 1C). Thus, monocytes induced proliferation but not cytotoxicity of RCDII lines, while the opposite was true for intestinal epithelial cells.

MATURE MONOCYTE-DERIVED DENDRITIC CELLS ARE SUPERIOR TO MONOCYTES IN INDUCING PROLIFERATION OF RCDII CELL LINES

Monocytes are recruited from peripheral blood to tissues, where they differentiate into macrophages and dendritic cells (DC). Macrophages and DC are found in the intestinal lamina propria and DC protrusions can be found in the epithelium¹⁴⁻¹⁶. We therefore investigated the induction of proliferation by macrophages and DC differentiated *in vitro* from purified peripheral blood monocytes. While macrophages, monocytes, immature DC and LPS-matured DC induced proliferation of RCDII lines, the strongest responses were found with mature DC (Figure 2A). In contrast, T-IEL lines derived from CD patients did not proliferate when co-cultured with DC (Figure 2B). The interaction between RCDII lines and DC did not alter the expression of the maturation markers CD1a, HLA-DR, CD86, CD80, CD83 and DC-SIGN on DC (data not shown). Thus, RCDII lines proliferate upon interaction with cells of the monocyte lineage, particularly mature DC.

FIGURE 1. MONOCYTE-DERIVED CELLS INDUCED PROLIFERATION, BUT NOT CYTOTOXICITY OF RCDII LINES.

(A) Proliferation was measured after 3 days of co-culture with PBMC or HT-29 and RCDII cell lines. On the y-axis the ³H-incorporation (CPM) is depicted. **(B)** Proliferation (cpm) was measured after 3 days of co-culture with RCDII lines and monocytes, T cells, B cells, NK cells, eosinophils or neutrophils. Black bars indicate the proliferation when co-cultured with the RCDII line, white bars represent the background proliferation of the corresponding PBMC population. **(C)** Monocytes were sorted from fresh healthy PBMC by CD14⁺ MACS sort and used in a cytotoxicity assay. An effector:target ratio of 50:1 is depicted. On the y-axis the percentage of lysis is shown. Results are depicted for RCDII line P2. Similar results were obtained with RCDII line P1 for all tests. Graphs are representative of at least two independent experiments.



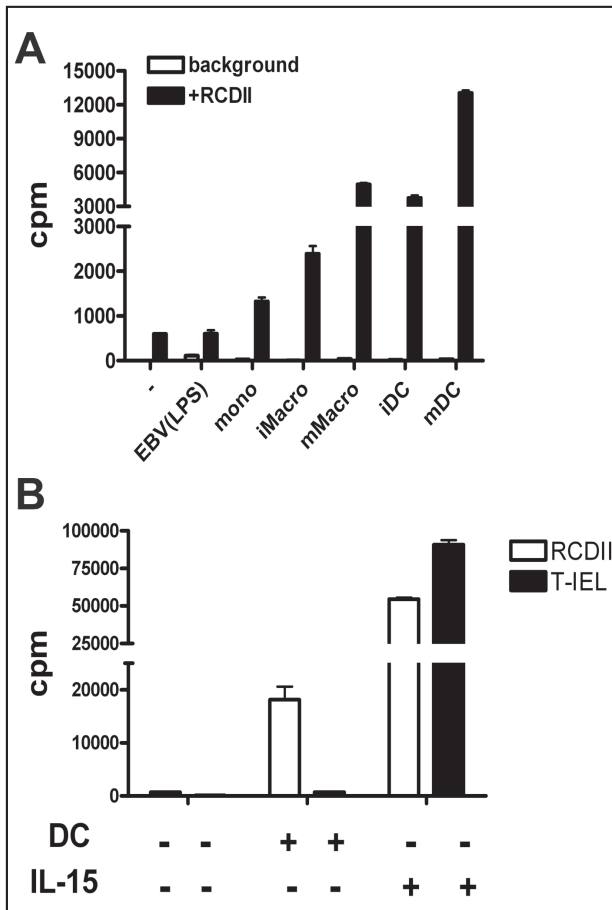


FIGURE 2. DENDRITIC CELLS INDUCED STRONGEST PROLIFERATION OF RCDII LINES WHEREAS T-IEL LINES SHOWED NO RESPONSE. (A) MACS sorted CD14⁺ monocytes (mono), monocyte-derived in vitro cultured macrophages (macro), immature dendritic cells (iDC) or 48h LPS-matured DC (mDC) were co-cultured with RCDII cell line P2 and proliferation (cpm) was measured after 3 days. Control 1 represents the mean background proliferation of monocyte-derived cells, control 2 the background proliferation of the RCDII line with an LPS-treated EBV line. Results were representative of 3 experiments. On the y-axis the ³H-incorporation (CPM) is depicted. **(B)** Proliferation (cpm) was measured after 3 days of co-culture with DC and a RCDII line P2 (white bars), or with DC and a representative T-IEL line (black bars). A plus or minus indicates whether DC or IL-15 as a control was added. The result is representative of 2 experiments. The T-IEL line used in this experiment is representative for four T-IEL lines tested, RCDII lines P1 and P2 showed similar results for all experiments depicted here.

PROLIFERATION OF RCDII LINES WAS INDEPENDENT OF IL-15 OR HLA GENOTYPE, BUT DEPENDENT ON CELL-CELL CONTACT

Since monocytes, macrophages and DC are known to produce Interleukin-15 (IL-15)¹⁸, we tested whether the proliferative effect seen in co-culture experiments was due to the IL-15 production and/or presentation by cells of the monocyte lineage. Blocking of IL-15 with a combination of IL-15 antibodies showed no effect on proliferation in monocyte RCDII line co-cultures, while they effectively blocked the response to soluble IL-15 (Figure 3A). These results indicate that it is unlikely that IL-15 is the factor responsible for the monocyte- and DC-induced proliferation of RCDII lines.

Activation of T and NK cells is modulated by receptors binding to Human Leucocyte Antigens (HLA). Since many of these receptors bind only to a selected set of HLA allele products, we investigated whether the proliferation of RCDII lines was affected by the HLA genotype of the stimulating cells. To address this, we used a panel of PBMC with divergent HLA-genotypes as a convenient source of monocytes. PBMC from 10 donors selected for divergence in HLA allotypes induced similar proliferation of RCDII lines (Figure 3B). What's more, RCDII line P1 proliferated in response to autologous PBMC (Figure 3C). As observed for DC (Figure 2B), a control T-IEL line showed no response to PBMC. The HLA genotype of the stimulator cells therefore did not detectably influence the proliferative response of RCDII lines.

To address whether cell-cell contact was required for the induction of proliferation by DC, a transwell experiment was performed. In the absence of cell-cell contact, the proliferation of RCDII lines was severely reduced but did not disappear completely (Figure 3D). Therefore, cell-cell contact played a major role in the induction of proliferation, but soluble factors also contributed.

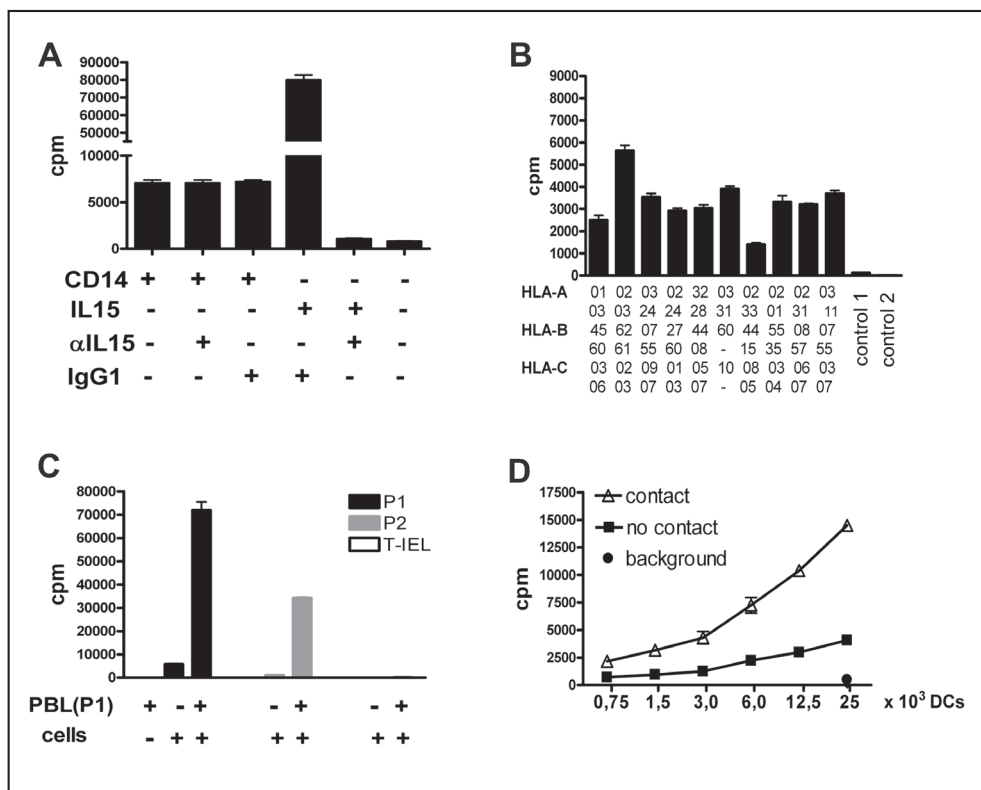


FIGURE 3. PROLIFERATION OF RCDII LINES WAS INDEPENDENT OF IL-15 OR HLA GENOTYPE, BUT DEPENDENT ON CELL-CELL CONTACT. Proliferation was measured by ³H incorporation (CPM) after 3 days of co-culture. **(A)** Co-culture of RCDII line P2 with monocytes (CD14), 10 ng/ml IL-15, IL-15 antibodies MAB647 (10 µg/ml) and MAB247 (2 µg/ml) (αIL-15) or 12 µg/ml of the isotype control (IgG1). (n=2) **(B)** Co-culture of RCDII line P2 and PBMC of divergent HLA-typed donors. The HLA-type is depicted for HLA-A, B and C on the x-axis to reflect the random HLA-type of the donors. Control 1 is the background of the representative RCDII line, control 2 stands for the representative background proliferation of PBMC used in the experiment. **(C)** Co-culture of RCDII line from patient 1 (P1) (black bar) and patient 2 (P2) (gray bar) or of a representative T-IEL (white bar) with PBMC from patient 1 (PBL(P1)). The legend on the x-axis indicates if autologous PBMC from patient 1 (PBL(P1)), or the corresponding cell lines P1,P2 or T-IEL (cells) were added to the co-culture experiment. IL-15, taken along as a positive control is not depicted here (162000 cpm for P1, 63000 cpm for P2 and 80000 cpm for T-IEL). **(D)** A transwell experiment was combined with a proliferation (cpm) assay. Cell-cell contact is depicted by the open triangle symbol, no cell-cell contact by the black square symbol. The round symbol represents the mean background

proliferation of the RCDII line P2 and the DC. On the x-axis the amount of DC added to each well is depicted as $\times 10^3$ cells. Results are representative of two independent experiments. RCDII line P1 and P2 showed similar results in all tests.

DENDRITIC CELLS INHIBIT APOPTOSIS OF RCDII LINES

IL-15 is known to induce proliferation and inhibit apoptosis of RCDII lines and other lymphoid cell types such as T-IEL^{4,12}. Since DC induced proliferation of RCDII lines, we also investigated whether apoptosis was inhibited. After 6 days of incubation in the absence of DC, the majority of RCDII lines underwent early (AnnexinV+/7-AAD-) and late (AnnexinV+/7-AAD+) stage apoptosis (Figure 4A). In the presence of DC the percentage of apoptotic cells was drastically reduced (Figure 4B). In line with previous observations¹², IL-15 also drastically reduced apoptosis of RCDII lines. These data demonstrated that, similar to IL-15, DC prevented apoptosis of RCDII lines.

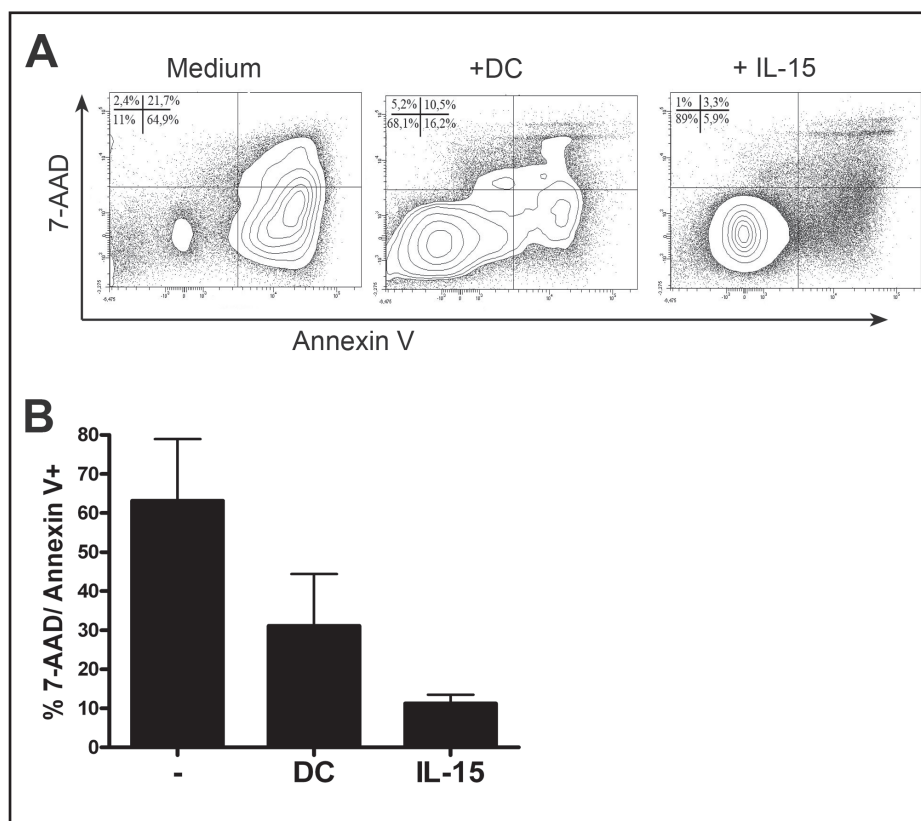


FIGURE 4. ANTI-APOPTOTIC EFFECTS OF DC ON RCDII LINES. Apoptosis induction was measured by 7-AAD and Annexin V staining by flow cytometry. **(A)** After 6 days incubation of RCDII line P2 with medium alone (Medium), after DC co-culture (+DC) or after 10 ng/ml IL-15 (+IL-15) cells were analyzed by flow cytometry. The percentages of Annexin V⁺ and 7-AAD⁺ cells are depicted in the graph. **(B)** Summarizing the data from co-culture experiments of 4, 6 and 7 days for RCDII line P2, as percentages of Annexin V⁺ and 7-AAD⁺ RCDII cells with medium alone (-), after DC co-culture (DC) and after IL-15 (10 ng/ml) incubation (IL-15). RCDII line P1 and P2 showed similar results.

DENDRITIC CELL-RCDII LINE CO-CULTURES PRODUCE IFN γ AND CXCL10

Previous studies showed that IL-15 triggered interferon- γ (IFN γ) production by RCDII lines ⁴. We therefore measured IFN γ secretion after co-culture of RCDII lines with DC or IL-15. In co-culture experiments with DC higher concentrations of IFN γ were measured in the supernatants compared to IL-15 incubation (Figure 5A). A control T-IEL line generated from a CD patient showed no IFN γ response to either stimulus, but did proliferate in response to IL-15 (Figure 5A). IFN γ is known to induce the secretion of CXCL10 from monocytes, macrophages and DC ^{19,20}. Indeed, supernatants of DC and RCDII co-cultures contained high concentrations of CXCL10 (Figure 5B), which was mainly detected when cells had direct cell-cell contact (Figure 5C) and was not secreted after IL-15 incubation (Figure 5B). In contrast, co-culture of DC with a T-IEL line resulted in very little CXCL10 secretion, consistent with the absence of IFN γ in such co-cultures (Figure 5A and B). In short, IFN γ is produced by RCDII lines upon co-culture with DC, which induces CXCL10 secretion by DC, especially after direct cell-cell contact.

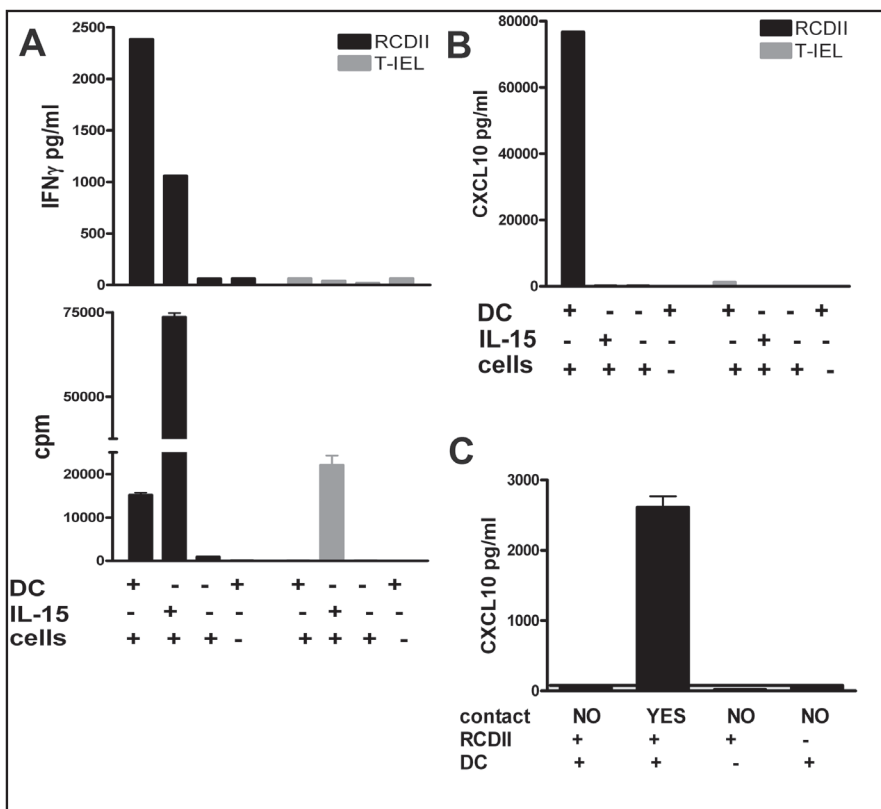


FIGURE 5. IFN γ WAS DETECTED IN THE SUPERNATANTS OF DC-RCDII CO-CULTURES AND INDUCED THE SECRETION OF THE IFN γ -DEPENDENT CHEMOKINE CXCL10. Supernatants of co-culture experiments were analyzed after 3 days by Bioplex Human Cytokine assay. **(A)** In the upper graph the concentration of IFN γ in the supernatants of DC co-culture is depicted on the x-axis in pg/ml. Proliferation of cells was measured by ^3H -incorporation (CPM) from the same experiment as depicted in the lower panel. Black bars indicate DC co-culture with RCDII line P2, gray bars with a representative T-IEL cell line. A plus or a minus in the legend on the x-axis indicates if DC, 10 ng/ml IL-15 or the corresponding cell line (cells) was added to the co-culture experiment. **(B)** The concentration of CXCL10 (in pg/ml) in supernatants of DC co-culture with RCDII line P2 (black bar) or T-IEL control lines (gray bar) is shown. The legend on the x-axis indicates whether DC, 10 ng/ml IL-15 or the corresponding cell lines (cells) was added. **(C)** The concentration of CXCL10 (in pg/ml) in supernatants of transwell experiments with DC and RCDII line P2 is depicted. The legend on the x-axis indicates if cell-cell contact was permitted (YES) or not (NO) and if the RCDII line or DC were added (+) or not (-). Similar results were obtained for both RCDII lines P1 and P2 and are representative of at least two independent experiments.

DISCUSSION

Interleukin-15 is generally believed to contribute to the outgrowth of a-IEL seen in RCDII patients, and its proliferation-inducing and anti-apoptotic effects on RCDII lines are well documented ^{4, 12}. Here we demonstrate that not only IL-15 but also monocytes, macrophages and especially dendritic cells (DC), prominent cell types of the innate immune system, can induce proliferation and inhibit apoptosis of RCDII lines. This effect on proliferation was specific to RCDII lines as regular T-IEL lines did not proliferate when co-cultured with monocyte-derived cells.

Although we were unable to identify the receptor-ligand interactions between DC and RCDII lines triggering proliferation, several important candidates could be excluded. First, since proliferation was drastically reduced in the absence of cell-cell contact, soluble mediators are unlikely to play a major role. Second, since RCDII lines (like a-IEL, their *in vivo* counterparts in RCDII) do not express TCR/CD3 complexes on the cell surface, TCR-dependent recognition of HLA-class I or II cannot be responsible for proliferation induced by DC. Third, co-culture experiments with a diverse panel of HLA-typed PBMC- including autologous PBMC- demonstrated that the HLA-genotype of the stimulator cells did not influence the observed proliferative response. This argues against HLA-allotype-specific recognition by NK cell receptors such as Killer-cell Immunoglobulin-like Receptors (KIR), which are expressed by the RCDII lines ¹¹. Finally, IL-15-specific antibodies did not inhibit the induction of proliferation by DC, while they did block the response induced by soluble IL-15. In conclusion, recognition is most likely mediated by receptors on the RCDII lines that bind to non-polymorphic surface-expressed ligands on DC.

Contact between cells of the monocyte lineage and a-IEL can occur either in the epithelium in RCD patients or after dissemination into the lamina propria ²¹, the blood and other intestinal tissues ^{6, 22} reflecting ongoing malignant transformation and development into an enteropathy-associated T cell lymphoma. DC normally reside in the lamina propria, but can extend protrusions into the epithelial layer ^{14, 15} and thereby could contact a-IEL. Raki et al. reported increased numbers of dendritic cell subsets and macrophages in the lamina propria and in the basolateral membrane of epithelial cells in lesions of active celiac patients, reflecting the ongoing inflammatory responses of the untreated disease ²³. While this has not been investigated in RCD patients, DC cell numbers may be similarly increased in these patients due to the ongoing severe inflammation in the intestine.

Our results indicate that IFN γ produced by RCDII lines leads to the secretion of CXCL10 by DC. *In vivo*, this would attract CXCL10-sensitive macrophages and DC to the lesion ²⁴, thus creating a self-augmenting inflammatory process. It has been described that CXCL10 (Chirido et al., personal

2

communication) and its receptor CXCR3²⁵ are more abundantly expressed in the mucosa of CD patients than in non-CD individuals, suggesting that this chemokine-receptor interaction is involved in ongoing inflammation in active celiac disease. Thus far, no data are available for refractory celiac disease. Nevertheless, high expression levels of CXCL10 have also been found in the mucosa of IBD patients^{26,27} and increased serum levels of CXCL10 have further been described in several autoimmune diseases and could be of value as a biomarker for inflammation²⁸⁻³⁰. Thus, contact-dependent production of IFN γ and subsequently CXCL10 as a result of DC-lymphocyte interactions could help sustain persistent inflammation in refractory celiac disease and other intestinal and autoimmune diseases.

In conclusion, contact between a-IEL and cells of the monocyte lineage can protect the a-IEL from apoptosis and induce proliferation and IFN γ production. Since both IL-15 and DC quantities are increased in duodenal lesions^{4,12}, and since a-IEL are more sensitive to these stimuli than regular T-IEL, together they may contribute to the uncontrolled expansion of a-IEL in RCDII and support the development of an invasive lymphoma.

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F.S. designed the study, did experiments, analyzed data and wrote the manuscript; J.M.L.T. generated cell lines described in this paper; J.v.B. designed the study, analyzed data, wrote the manuscript; F.K. designed the study, analyzed data, wrote the manuscript. We thank C. Mulder for providing biopsies and Y.Kooy-Winkelaar for technical assistance.

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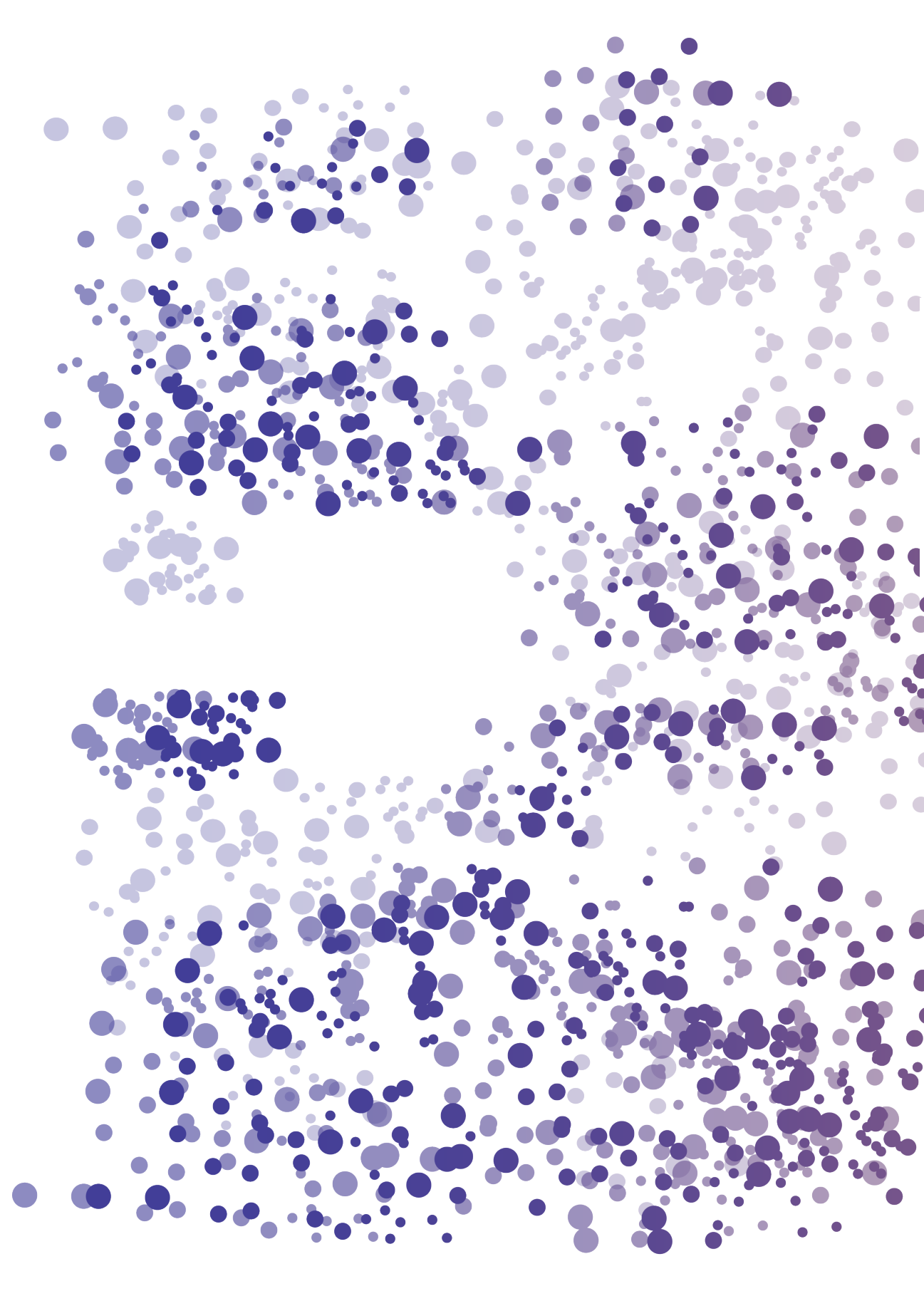
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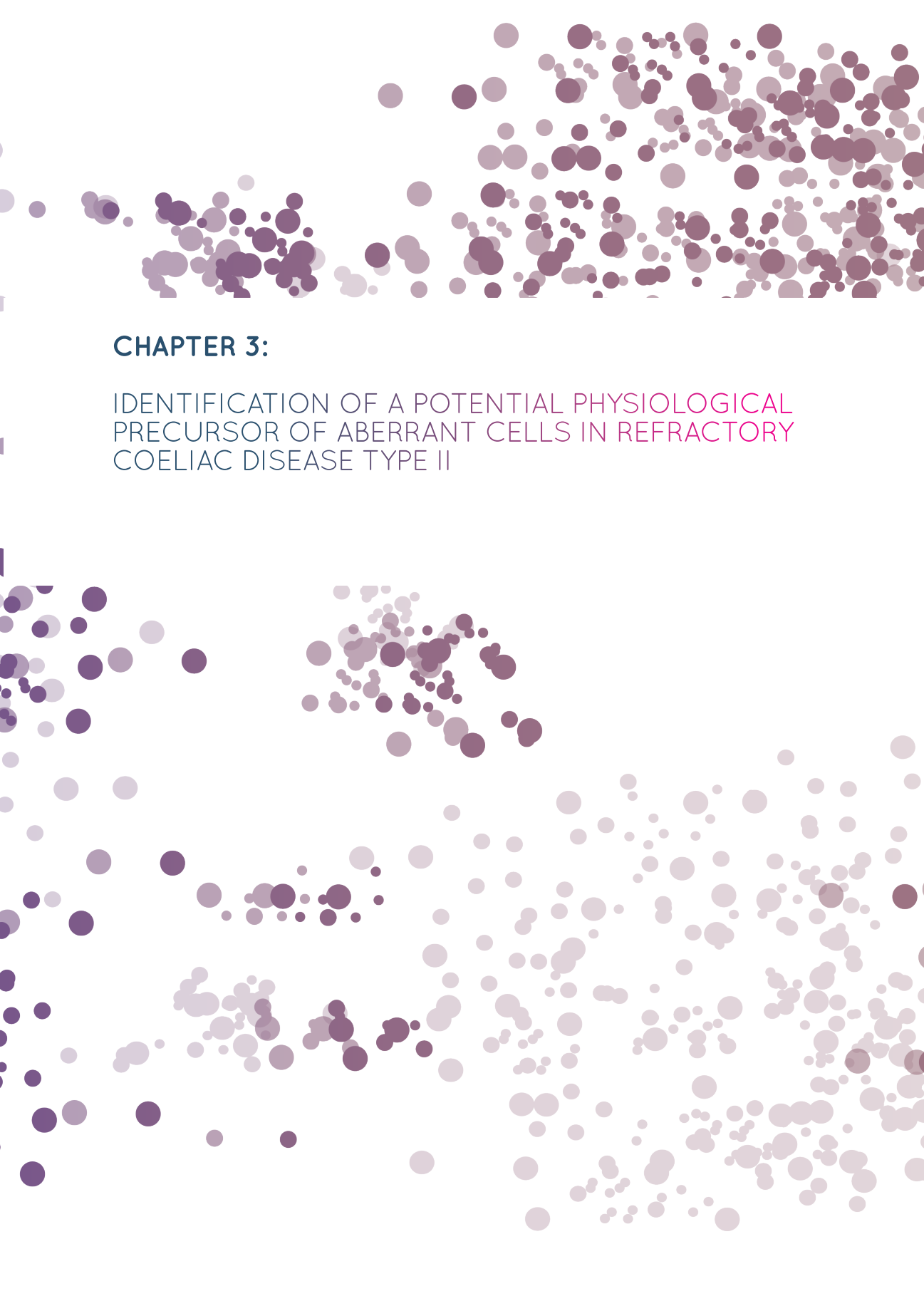
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CHAPTER 3:

IDENTIFICATION OF A POTENTIAL PHYSIOLOGICAL
PRECURSOR OF ABERRANT CELLS IN REFRACTORY
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IDENTIFICATION OF A POTENTIAL PHYSIOLOGICAL PRECURSOR OF ABERRANT CELLS IN REFRACTORY COELIAC DISEASE TYPE II

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ABSTRACT

Refractory coeliac disease type II (RCDII) is a severe complication of coeliac disease (CD) characterized by aberrant intraepithelial lymphocytes (IEL) of unknown origin that display an atypical CD3⁺CD7⁺icCD3⁺ phenotype. In approximately 40% of RCDII patients these lymphocytes develop into an invasive lymphoma. In the current study we aimed to identify the physiological counterpart of these cells.

RCDII cell lines were compared to TCR⁺ IEL (T-IEL) lines by microarray analysis, RT-qPCR and flow cytometry. This information was used to identify cells with an RCDII-associated phenotype in duodenal biopsies from non-refractory individuals by multicolour flowcytometry.

RCDII lines were transcriptionally distinct from T-IEL lines and expressed higher levels of multiple NK cell receptors. In addition to the CD3⁺CD7⁺icCD3⁺ phenotype, the RCDII lines were distinguishable from other lymphocyte subsets by the absence of CD56, CD127 and CD34. Cells matching this surface lineage-negative (Lin⁻) CD7⁺CD127⁻CD34⁻ phenotype expressed a functional IL-15 receptor and constituted a significant proportion of IEL in duodenal specimens of non-coeliacs, particularly children, and were also found in the thymus. In non-coeliacs, the Lin⁻CD7⁺CD127⁻CD34⁻ subset was one of four subsets within the CD3⁺CD7⁺icCD3⁺ population that could be distinguished on the basis of differential expression of CD56 and/or CD127.

Our studies indicate that the CD3⁺CD7⁺icCD3⁺ population is heterogeneous and reveal the existence of a Lin⁻ subset that is distinct from T, B, NK and Lymphoid tissue-inducer cells. We speculate that this IL-15 responsive population represents the physiological counterpart of aberrant cells expanded in RCDII and transformed in RCDII-associated lymphoma.

ABBREVIATIONS

CD- Coeliac Disease, RCD- Refractory Coeliac Disease, EATL- Enteropathy associated T cell Lymphoma, IEL- Intraepithelial Lymphocytes, T-IEL- TCR⁺/CD3⁺ Intraepithelial Lymphocytes, Lin-Lineage, ic- intracellular, KIR- Killer-cell Immunoglobulin-like Receptors, LTi- Lymphoid Tissue inducer Cell

INTRODUCTION

Coeliac disease (CD) is an enteropathy of the small intestine induced in HLA-DQ2⁺ and/or HLA-DQ8⁺ individuals by a polyclonal CD4⁺ T cell response to gluten, leading to villous atrophy and crypt hyperplasia ¹. In addition, the number of TCR- $\alpha\beta$ ⁺ and TCR- $\gamma\delta$ ⁺ intraepithelial lymphocytes (IEL) is strongly increased ² and interleukin 15 (IL-15) is highly upregulated in the epithelium and in the lamina propria ^{3,4}. Elimination of gluten from the diet usually restores the intestinal architecture, accompanied by a reduction in TCR- $\alpha\beta$ ⁺ IEL but not TCR- $\gamma\delta$ ⁺ IEL. However, 2-5% of adult-onset CD patients develops refractory CD (RCD) with persisting epithelial damage and increased numbers of IEL ⁵, despite strict adherence to a gluten-free diet.

Based on immunophenotyping of the IEL population, RCD is subdivided into two types: RCD type I (RCDI) and RCD type II (RCDII). RCDI presents with < 20% aberrant IEL, whereas in RCDII > 20% of the IEL have an aberrant phenotype ⁶. For diagnostic purposes, aberrant IEL are defined as surface TCR-CD3-CD4-CD8-CD7⁺ but intracellular CD3⁺ cells that commonly display monoclonal TCR- γ gene rearrangements ^{6,7}. In RCDII, the aberrant IEL gradually replace the normal IEL population and can undergo malignant transformation into lymphoma ⁸.

The cellular origin of the aberrant CD3⁺CD7⁺icCD3⁺ population that expands in RCDII is still unclear. One view is that aberrant IEL originate from mature TCR⁺CD3⁺ IEL (T-IEL) that downregulate the TCR-CD3 complex due to overstimulation ⁷. This view is supported by the observation that aberrant IEL have intracellular expression of the CD3 γ -, CD3 δ -, CD3 ϵ - and ζ -chains and commonly display monoclonal TCR- γ gene rearrangements ^{7,9}. Intracellular CD3 expression, however, is not exclusive to mature T cells as pre-T cells and some NK cells also express all CD3 chains intracellularly ^{10,11}. Therefore, an alternative view is that aberrant IEL derive from a surface CD3⁺CD7⁺ IEL population that is already present in the healthy small intestine. By immunohistochemical analysis ¹² and 3 colour flow cytometry ¹³ the CD3⁺CD7⁺ IEL population was studied in coeliac and non-coeliac patients, demonstrating a reduction of this population in coeliac compared to non-coeliac patients. Later studies focused on the composition and characterization of this IEL population in the intestine of non-coeliac individuals ^{12,14-18}. The CD3⁺CD7⁺ IEL population proved to contain NK cells ¹⁷ and immature T cells ^{14-16,18}. Due to limitations of the techniques applied and the small amount of cells available from biopsy material an accurate identification and quantification of rare (sub)populations within the IEL population is challenging. In the present study we therefore combined microarray analysis, real time quantitative PCR and multicolour flow cytometry to extensively characterize aberrant RCDII cell lines from RCDII patients. Subsequently, we have used this information to design a multicolour staining protocol to investigate the presence and phenotype of RCDII-like cells in duodenal biopsy material from

coeliacs and non-coeliacs as a first step to determine the cellular origin of aberrant IEL.

MATERIALS AND METHODS

Human specimens

Duodenal biopsies were obtained as previously described⁹. Fetal tissues were obtained from elective abortions and gestational age was determined by ultrasonic measurement of the diameter of the skull or the femur. Thymic material was obtained as surgical tissue discards from four children without immunological abnormalities (2, 5, 7 and 8 months old) undergoing cardiac surgery. Thymocytes were isolated as described¹⁹ and stored at -80°C until further analysis. All specimens were obtained after informed consent, medical ethical commission approval and in accordance with the local ethical guidelines of the VU Medical Center in Amsterdam (adult duodenal biopsies), the Leiden University Medical Center (paediatric duodenal biopsies) or the Erasmus MC in Rotterdam (fetal intestine, thymuses) and in accordance with the declaration of Helsinki.

Cell lines and cell culture

RCDII cell lines P1, P2, P3 and P4 were isolated from duodenal biopsies of RCDII patients and maintained as described⁹. Control cell lines for P1 were a CD4⁺ T cell line (P1-CD4) and a CD8⁺ T cell line (P1-CD8) isolated from the same biopsy as RCDII cell line P1. A CD4⁺ T cell clone (P3-CD4) and a CD8⁺ T cell line (P1-CD8) was isolated from the same biopsy as RCDII cell line P1 or P3, respectively. T-IEL lines (CD4⁺ and CD8⁺ T: IEL1-5) were isolated from 4 CD patients and one patient with Crohn's disease, as described for RCDII lines. IEL1-CD4 was derived from IEL1, IEL5-CD8 from IEL5. NK cells were negatively selected from PBMC by depletion using MACS beads (Miltenyi, Bergisch Gladbach, Germany). RCDII lines and T cells were restimulated with mixed irradiated allogeneic PBMC, 1 µg/ml phytohemagglutinin, 20 Cetus u/ml IL-2 (Proleukin, Chiron corporation, Emeryville, CA) and 10 ng/ml IL-15 (R&D systems Europe, Abingdon, UK) approximately every 4 or 2 weeks, respectively and maintained on 10% normal human serum (NHS) containing 10 ng/ml IL-15 and 20 Cetus units/ml IL-2. After isolation, NK cells were cultured for 2 weeks in AMV-I Medium containing 10% NHS and IL-15 (10 ng/ml).

Isolation of cells from duodenal biopsies specimen for flowcytometry

Cells were isolated from two or three duodenal biopsies by treatment with 1mM DTT (Fluka, Buchs, Switzerland) for 20 minutes followed by the addition of 1mM EDTA (Merck, Darmstadt,

Germany) under rotation for 2 hours at 37°C. Since DTT incubation decreases CD103 expression²⁰ all CD45⁺ cells were considered IEL. The IEL were then washed with PBS containing 0,5% FCS and fixed for 10 min at 4°C with PBS containing 0,1% paraformaldehyde and 0,5% FCS. After washing the cells were kept at 4°C until flow cytometric analysis.

Antibodies and flowcytometry

Fluorochrome-labeled antibodies specific for CD1a, CD2, CD3, CD4, CD5, CD6, CD7, CD8 α , CD11a, CD16, CD18, CD25, CD27, CD28, CD30, CD31, CD33, CD34, CD38, CD40, CD44, CD45RA, CD45RO, CD54, CD56, CD62L, CD94, CD103, CD117, CD122, CD152, CD154, CD161, CD226, integrin β 7, KIR3DL1 and NKG2D were purchased from BD Biosciences (San Jose, CA). Fluorochrome-labeled antibodies specific for CD8 β , KIR2DL2/DL3/DS2, KIR2DL1/2DS1, KIR2DS4, KIR3DL1, CD244, NKp30, NKp44 and NKp46 were purchased from Beckman Coulter (Fullerton, CA). Fluorochrome-labeled antibodies specific for IL-15R α , KIR2DL3, KIR2DL4, NKG2A and NKG2C were purchased from R&D systems (Abingdon, UK). Anti-CD19 was from Exbio (Vestec, Czech Republic), anti-CD14 from Immunotools (Friesoythe, Germany), anti-CD7, CD127, CD45 and Ki-67 were from eBioscience (San Diego, CA) and anti-CD52 was from Genzyme, Cambridge. The anti-KIR2DS2/2DL3 antibody was a kind gift from Dr. C. Retiere²¹, the anti-KIR3DL2 antibody from Dr. D. Pende²² and the anti-KIRDL5 antibody from Dr.C.Vilches²³. For antibody clone names see Table 2. For surface staining, cells were incubated for 30 minutes with fluorochrome-conjugated antibodies. For intracellular CD3- or Ki-67-staining, the cells were subsequently lysed with BD lysing solution for 10 min at room temperature (RT) then washed thoroughly and treated with permeabilising solution 2 (BD) for 10 min at RT, after which the cells were incubated with anti-CD3 or anti-Ki-67 for an additional 30 minutes and washed again. Antibody-stained cells were acquired on an LSR II or FACS-Calibur (both from BD biosciences) and the results were analyzed with FACSDIVA 6.1.2 or FlowJo 7.6.3 software. Relative mean fluorescence intensity (MFI) was calculated as follows: (MFI staining antibody)/(MFI isotype control). Percentages obtained from FACS analysis were computed in 4-GraphPad.

RNA isolation and preparation

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) followed by DNase digestion using the RNase-free DNase kit (Qiagen, Venlo, the Netherlands). The RNA samples were labeled in duplicate with the Ambion Illumina TotalPrep RNA-amplification kit (Illumina, Son, The Netherlands) for the microarray study.

Microarray hybridization and Microarray data analysis

Biotinylated cRNA samples were hybridized on 2 Illumina HumanHT-12 V3 Expression BeadChips according to the Illumina BeadArray protocol. Samples were randomly distributed among the two BeadChips. The intensities of the expression results were parsed into the R statistical computing environment using the beadarray package²⁴. Gene expression values were log2 transformed and normalized using the quantile method across all samples. Samples were grouped into lymphoma and control for a two-sample comparative study, fold changes and adjusted p-values were calculated using the limma package²⁵. The Benjamini and Hochberg method was used to adjust p-values for multiple-testing errors. The cut-off criteria for differential expression was set at a relative difference of > 7 fold change and adjusted P-value < 0.001. Unsupervised hierarchical clustering of the samples was done, using the complete clustering method with the euclidean distance metric. The result was plotted using the heatmap.2 function from the R plots package. Gene ontology clusters were determined with PANTHER (<http://www.pantherdb.org/>).

Real-time quantitative PCR (RT-qPCR)

cDNA was synthesized using superscript reverse transcriptase III (Invitrogen, USA), following the manufacturer's instructions. Gene-specific PCR primers were designed using the Roche web-based universal probefinder software tool, version 2.45 (www.roche-applied-science.com). RT-qPCR was performed in a Step One Plus Real Time PCR System (Applied Biosystems, USA). The reactions were set up in a Microamp 96 well plate (Applied Biosystems, USA), using the forward and reverse primers (each at 400 nM) in Fast Start Universal SYBR Green Master Mix solution (Roche, Germany). The thermal profile for all reactions was 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. All RT-qPCR measurements were done in triplicate. The Δ CT values were determined using GAPDH as the reference gene.

Statistics

Statistical analysis was conducted using two-tailed unpaired Student's t-test. A P value < 0,05 was considered significant.

RESULTS

MOLECULAR ANALYSIS REVEALS HIGHER EXPRESSION LEVELS OF NK CELL-ASSOCIATED GENES ON RCDII LINES COMPARED TO TCR⁺ IEL LINES

To define the differences between aberrant IEL and regular T-IEL in detail, aberrant cell lines from RCDII patients (RCDII lines) and control T-IEL cell lines were subjected to microarray analysis.

The RCDII cell lines were isolated from small intestinal biopsies of RCDII patients (P1, P2 and P3) and displayed the characteristic phenotype of aberrant IEL: surface TCR, CD3⁺, CD4⁺, CD8⁺, CD7⁺, CD103⁺, intracellular (ic) CD3⁺⁹. The controls included (I) five polyclonal CD103⁺ CD4⁺ and CD8⁺ TCRαβ⁺ T-IEL lines (T-IEL), (II) a CD4⁺TCR⁺ cell line from patient P1 (P1-CD4), (III) a CD4⁺TCR⁺ clone from patient P3 (P3-CD4) and (IV) a CD8⁺TCR⁺ IEL line from patient P1 (P1-CD8).

Unsupervised hierarchical clustering analysis revealed two main branches: one contained the RCDII lines while the other contained all control TCR⁺ cell lines, including those from patients P1 and P3 (Figure 1A). Therefore, the RCDII lines were transcriptionally distinct from TCR⁺ cell lines, yet similar to each other. The RCDII lines had relatively high expression values of NK cell-associated genes. The amount of Killer-cell Immunoglobulin-like Receptors (KIR) transcripts was roughly 5- to 20-fold higher in RCDII lines versus controls. Furthermore, the RCDII lines showed higher expression levels of PLCG2 (phospholipase C γ2), which plays a pivotal role in secretion of cytotoxic granules in NK cells²⁶. The RCDII lines also had higher expression levels of SH2 domain containing 1B (SH2D1B/EAT-2) which can act as an adapter molecule for the cell surface receptors CD84, CD244 and CD150 (SLAMF1)²⁷. All RCDII cell lines expressed CD244 (Table 1), a receptor expressed by NK cells and effector/memory T cells²⁸, while SLAMF1 expression was downregulated in the RCDII cell lines.

In addition, several other genes that are associated with T cell mediated immunity according to the PANTHER classification were downregulated in the RCDII lines compared to the T cell lines, in particular granulysin (GNLY), lymphotoxin β (LTB) and tumor necrosis factor receptor 4 (TNFSF4) (Figure 1A, B). The differential expression of 7 genes (TNS3, SLAMF1, FoxD1, SH2D1B, PLCG2, LAT, LTB) was confirmed by RT-qPCR (Figure 1B and data not shown). An additional 12 surface markers (CD154, CD278, CD6, CD44, CD7, CD244, CD82, CD160, CD127, CD18, CD5, CD158d) were analyzed by flow cytometry (Figure 2 and data not shown) and also corroborated the microarray results. Furthermore, analysis of TCR rearrangements revealed that none of the RCDII lines possessed functionally rearranged TCRα/β or TCRγ/δ loci²⁹. Together, these data showed that RCDII lines were distinct from mature T cell lines and expressed several NK cell-associated genes.

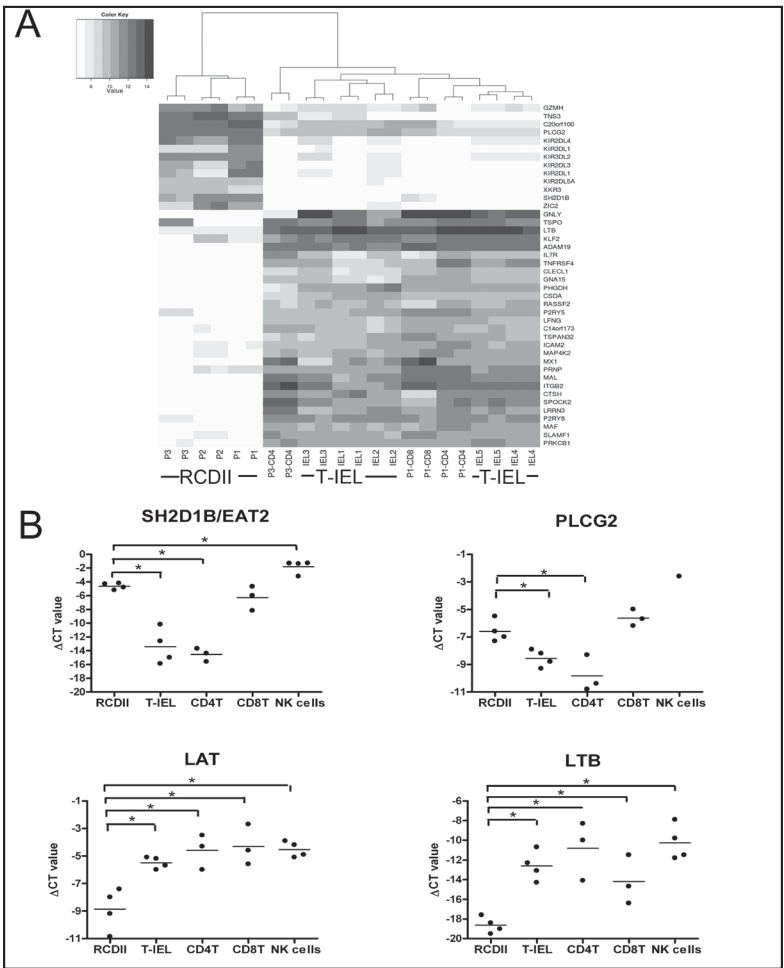


FIGURE 1. RCDII LINES EXPRESS HIGHER LEVELS OF NK CELL-ASSOCIATED GENES WHEN COMPARED WITH REGULAR TCR⁺ CELL LINES. **(A)** Unsupervised hierarchical clustering analysis of the gene expression profiles of RCDII lines P1, P2 and P3 and TCR⁺ cell lines P1-CD4, P1-CD8, P3-CD4 and IEL 1-5. The heat map depicts the log intensity values of differentially expressed genes with a false discovery rate (FDR) of $P < 0.01$ and a fold change difference of > 7 . Darker grey corresponds to higher log intensity values. KIR allocation was imprecise as KIR probes anneal to transcripts of multiple KIR genes. **(B)** Quantification of differential gene expression by RT-qPCR. Relative expression of SH2D1B, PLCG2, LAT and LTB in RCDII lines (P1-P4), T-IEL lines (IEL1-4) as well as CD4⁺ T cell lines (CD4T: P1-CD4, P3-CD4, IEL1-CD4), CD8⁺ T cell lines (CD8T: P1-CD8, P3-CD8, IEL5-CD8) and NK cell lines from 4 independent donors (NK cells). The Δ CT value is depicted on the y-axis.

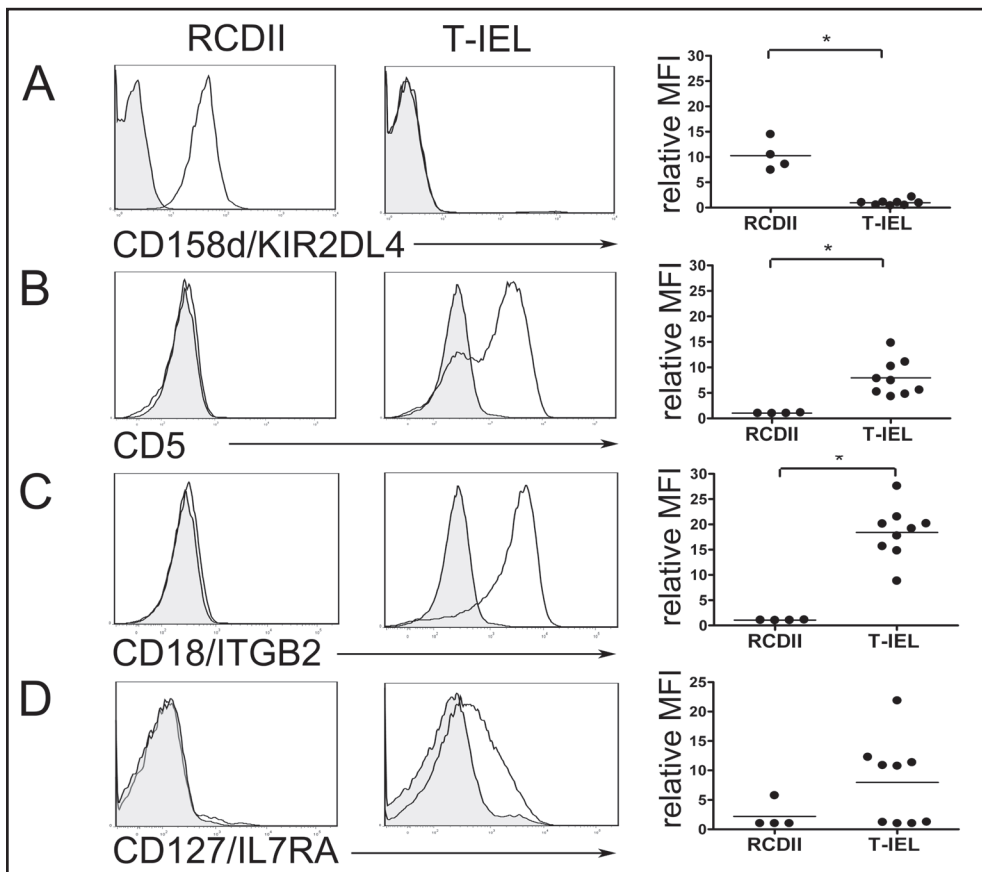


FIGURE 2. CONFIRMATION OF DIFFERENTIALLY EXPRESSED GENES FROM FIGURE 1 BY FLOW CYTOMETRY ANALYSIS. RCDII lines (P1-P4) and T-IEL (IEL1-5, IEL1-CD4, P1-CD8, P3-CD8, IEL5-CD8) were analyzed for expression of (A) CD158d (KIR2DL4), (B) CD5, (C) CD18 (ITGB2) and (D) CD127 (IL7RA) by flowcytometry. On the left, a histogram of a representative staining for one RCDII and one T-IEL line (grey filling= isotype, no filling= antibody) is shown. The scatter plots on the right depict the relative mean fluorescence intensities (relative MFI = MFI antibody/MFI isotype) of all RCDII and T-IEL lines. * $P < 0,05$

RCDII LINES DISPLAY A PREVIOUSLY UNDESCRIBED LYMPHOCYTE PHENOTYPE

To confirm and extend the results of the microarray analysis, we determined the expression of a large set of cell surface markers on RCDII lines P1, P2, P3⁹ and on RCDII line P4 by flow cytometry (Table 1). RCDII line P4 (supplementary Figure 1) was derived from an additional RCDII patient. Like T-IEL, all RCDII lines expressed the pan-leucocyte marker CD45 but lacked expression of B cell marker CD19 and monocyte marker CD14 (Table 1). Although the RCDII lines did not express the NK cell marker CD56 (with the exception of a small CD56⁺ fraction within RCDII line P3, see Table 1), they each expressed a unique selection of NK cell receptors (Table 1). Consistent with the microarray results (Figure 1A), all RCDII lines but not the control T cell lines expressed KIR2DL4/CD158d (Figure 2A), which has thus far been detected on mature NK cells and memory T cells only. In addition, these cells expressed the IL-2/15R β chain (CD122), normally found on NK cells and memory T cells (Table 1). These results confirm and extend previous studies³⁰⁻³² and indicate that RCDII lines share features with both T and NK cells.

The absence of the main lineage (Lin) markers (CD3, CD14, CD19, CD56) in combination with intracellular CD3 would be consistent with an immature lymphocyte phenotype. However, the RCDII lines did not express CD34, CD1a or CD117 (Table 1), markers associated with T cell and NK cell precursors^{33,34}. Furthermore, RCDII lines P1, P2, P3 expressed no ROR-C transcripts (data not shown), nor the CD127/IL-7 receptor on the cell surface (Table 1, Figure 2D). RCDII line P4 was partly CD127 (IL-7R α)-positive, but ROR-C negative (data not shown). The RCDII lines did therefore also not match the phenotype of LT_i cells, a Lin⁻CD127⁺ROR- γ ⁺ cell population present in the small intestine^{35,36}.

Together, these results demonstrate that the RCDII lines share features with both T and NK cells and display a previously undescribed IEL phenotype: Lin⁻CD7⁺CD127⁺CD34⁻.

Cell Type	Marker	Antibody clone name	P1	P2	P3	P4
Leucocyte	CD11a	G43-25B				
Leucocyte	CD18	L130				
Leucocyte	CD54	LB-2				
Leucocyte	CD62L	SK11-EMPTY				
Leucocyte	CD31	VM59				
Leucocyte	CD52	Campath 1-H				
Leucocyte	CD45	2D1				
Monocyte	CD14	MEM-15				
Monocyte	CD33	HIM3-4				
B (pre-B)	CD10	ALB2				
B	CD40	5C3				
B	CD19	LT19				
T/B	CD6	MT605				
T/B	CD5	L17F12				
T/B/NK	CD38	HB7				
T/B/NK	CD30	BerH8				
T/B/NK	CD45 RA	L48				
T	CD3	S4.1				
T/NK	CD3 ic	SK7				
T	CD4	SK3				
T/NK	CD8- α	SK1				
T	CD8- β	2ST8.5H7				
T	CD45 RO	UCHL1				
T	CD152	BN13				
T	CD154	TRAP1				
T	CD44	G44-26				
T	CD28	CD28.2				
T/LTI	CD127	ebioRDR5				40%.
T/NK	CD2	S5.2				
T/NK	CD25	M-A251				
T/NK	CD27	M-T271				
T/NK	CD7	6B7				
T/NK	Integrin beta-7	F1B504				
IEL T/NK	CD103	Ber-ACT8				
NK/T	CD122	TU27				
NK/T	IL15 R α	151303.				
NK/T	CD56	MEM-188			2%.	
NK/T	CD161	DX12				
NK/T	CD16	GO22				
NK/T	CD226	DX11				
NK/T	KIR2DL1/S1	EB6				
NK/T	KIR2DL2/L3/S2	GL183				
NK/T	KIR2DL4	181703.				
NK/T	KIR2DL5	UP-R1				
NK/T	KIR2DS2/L3	1F12				
NK/T	KIR2DS4	FES172				
NK/T	KIR3DL1	DX9				
NK/T	KIR3DL2	Q66				
NK/T	CD94	HP-3D9				
NK/T	NKG2A	131411.				
NK/T	NKG2C	134591.				
NK/T	NKG2D	149810.				
NK/T	CD244 (2B4)	C1.7				
NK/T	NKp30	210845.				
NK/T	NKp44	253415.				
NK/T	NKp46	195314.				
NK/T	NKp80	4A4.D10				
pre T/NK	CD1a	HI149				
pre T/NK	CD117	104D2				
lymphoid precursor	CD34	8G12				

TABLE 1. FLOWCYTOMETRY ANALYSIS OF RCDII CELL LINES P1-P4. White: no expression of the marker; Black: uniform expression of the marker; Gray: partial expression of the marker, percentage positive cells is indicated. CD3ic = intracellular CD3

LIN⁺CD7⁺CD127⁺CD34⁺ IEL ARE ALSO FOUND IN NON-COELIAC INDIVIDUALS

Next we investigated if cells with a Lin⁺CD7⁺CD127⁺CD34⁺ phenotype were also present in non-coeliacs. Multicolour flow cytometric analysis of IEL from duodenal biopsies from children and adults without CD showed that cells with this phenotype were indeed present (Figure 3A). These cells constituted on average 4% (range 0-8%, n=10) of the CD45⁺SSC^{low} population in adults without CD and were more abundant (average 13%, range 4-32%, n=10) in children without CD (Figure 3B). In pediatric CD patients, this subset formed on average 0.7% (range 0-2%, n=11) of the CD45⁺SSC^{low} population while this was on average 1.8 % (range 0-10%, n=16) in adult CD patients. Thus, Lin⁺CD7⁺CD127⁺CD34⁺ IEL are present in the duodenum of non-coeliac individuals as well as in CD patients.

In contrast to the duodenum, few Lin⁺CD7⁺CD127⁺CD34⁺ IEL were found in the ileum (average 0.3%, n=2; data not shown) and colon (average 0.1%, n=3; data not shown). Furthermore, Lin⁺CD7⁺CD127⁺CD34⁺ IEL were not detected in two fetal intestinal samples (data not shown). Most CD3⁺CD7⁺ cells in the fetal intestinal samples were CD56⁺ or CD127⁺ cells (data not shown). In human thymic cell suspensions, Lin⁺CD7⁺CD127⁺CD34⁺ cells expressing CD103 constituted on average 0.1% (range 0.0-0.3%, n=4) of CD45⁺SSC^{low} thymocytes (Figure 3C). These findings indicate that the postnatal duodenum is the preferential intestinal site for Lin⁺CD7⁺CD127⁺CD34⁺ IEL, and suggest that this population may be thymus-derived.

The classical definition of aberrant IEL in RCDII includes the presence of intracellular CD3ε. Indeed, the large majority (average 88%, range 66-99% n=4) of Lin⁺CD7⁺CD127⁺CD34⁺ IEL in RCDII duodenal biopsies expressed CD3 intracellularly (Figure 3 D, E), while this was on average 51% (range 8-91 n=11) in coeliac and 31% (range 27-37 n=9) in non-coeliac individuals (Figure 3 D, E). On average 23% (range 11-37%, n=3) of Lin⁺CD7⁺CD127⁺CD34⁺ IEL in the thymus showed intracellular CD3 expression (Figure 3 D, E).

In conclusion, a sizeable fraction of the Lin⁺CD7⁺CD127⁺CD34⁺ cells in the duodenum and in the thymus expressed intracellular CD3ε.

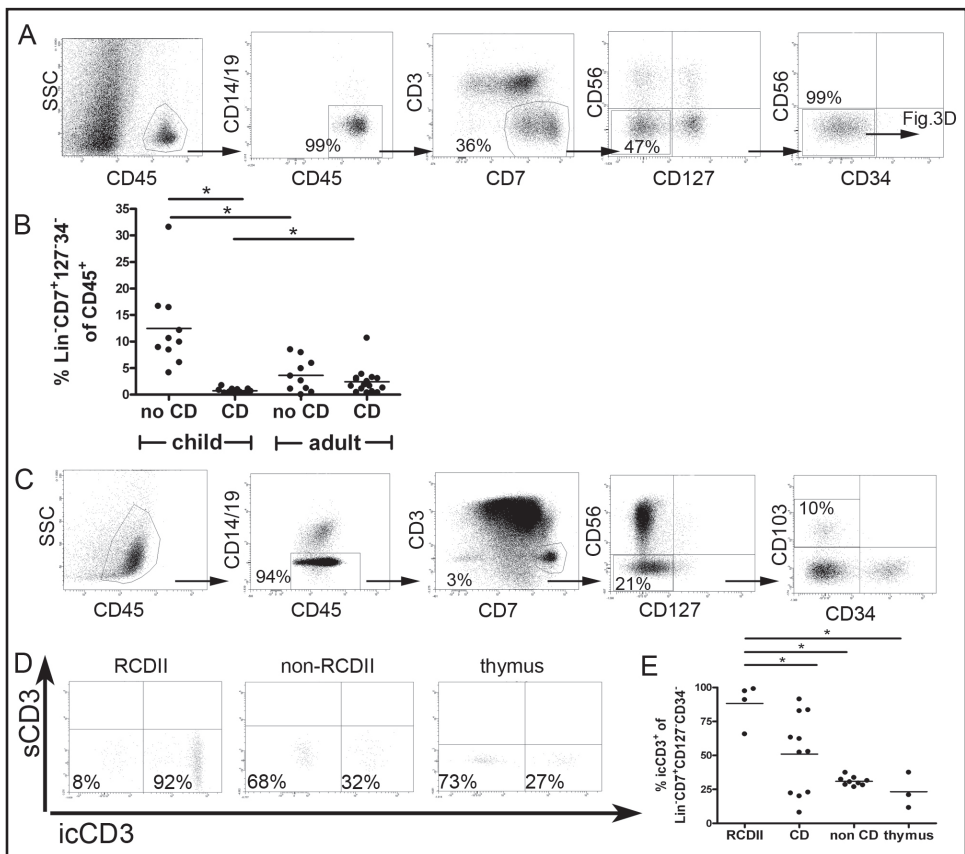


FIGURE 3. LIN⁻CD7⁺CD127⁻CD34⁻ IEL: A PHYSIOLOGICAL LYMPHOCYTE POPULATION IN NON-COELIAC AND COELIAC INDIVIDUALS. **(A)** FACS analysis of cells isolated from the epithelium of duodenal biopsies from a non-coeliac child. Each plot shows the cells gated in the previous plot with the calculated percentages from the previous gate. The experiment shown is qua gating and populations representative of non-coeliac adults (n=10) and children (n=10). **(B)** Fraction of LIN⁻CD7⁺CD127⁻CD34⁻ IEL in duodenal biopsies expressed as a percentage of the CD45⁺SSC^{low} population. This graph summarizes data from 11 children with CD (CD), 10 non-coeliac children (no CD), 16 adults with CD (CD) and 10 adults without CD (no CD). **(C)** FACS analysis of a healthy thymus (8 months old child) using the same gating strategy and calculations as described in Fig. 3A. Plots are representative of 4 donors. **(D)** Representative FACS plot depicts intracellular CD3 staining of LIN⁻CD7⁺CD127⁻CD34⁻ IEL in an RCDII patient, a non-RCD individual, and a thymus sample. **(E)** Percentages of intracellular (ic) CD3⁺ cells within LIN⁻CD7⁺CD127⁻CD34⁻ IEL in RCDII patients (n=4), CD (n=11), non-CD (n=9) individuals and thymus (n=3). *P < 0,05 (unpaired Student's t test)

LIN⁻CD7⁺CD127⁻CD34⁻ FORM A SUBPOPULATION OF CD3⁻CD7⁺icCD3⁺ CELLS

As the diagnosis of RCDII and EATL is made on the basis of the identification of a CD3⁻CD7⁺icCD3⁺ cell population either by histological or flow cytometric means^{6-8,37}, we inverted our gating strategy to determine the specific phenotype of the CD3⁻CD7⁺icCD3⁺ population.

Using this strategy we observed that in non-RCD patients (CD patients and non-coeliac individuals) this duodenal CD3⁻CD7⁺icCD3⁺ cell population was composed of four distinct populations: CD56⁺CD127⁻, CD56⁺CD127⁺, CD56⁻CD127⁻ and CD56⁻CD127⁺ (Figure 4A). In RCDII patients (n=4) only two populations: CD56⁻CD127⁻ and CD56⁻CD127⁺ cells were observed (Figure 4B). In the thymus the CD3⁻CD7⁺icCD3⁺ cells predominantly expressed CD56⁺ or were CD56⁻CD127⁻ (Figure 4C).

These findings revealed that the duodenal CD3⁻CD7⁺icCD3⁺ IEL population is heterogeneous and that a subset of aberrant IEL in RCDII patients biopsies express the IL-7R (CD127).

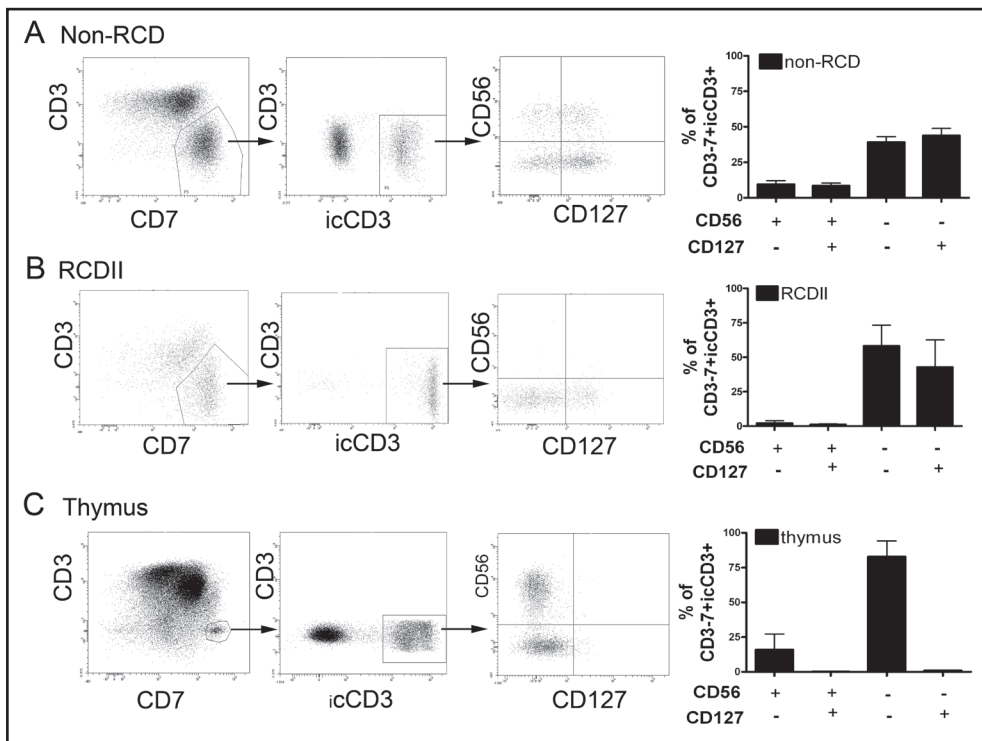


FIGURE 4. CD3⁻CD7⁺icCD3⁺ CELLS FORM A HETEROGENOUS POPULATION.

FACS analysis of CD45⁺SSC^{low}CD14⁻/CD19⁻ intraepithelial cells isolated from duodenal biopsies of (A) non-RCD individuals (no CD: n=9, CD: n=11), (B) RCDII patients (n=4) and of (C) from healthy thymuses (n=3). Representative dot plots are shown on the left; each plot depicts the cells gated in the previous plot. The graphs on the right summarize the results: the average (+/- SD) size of each of four subsets of CD3⁻CD7⁺icCD3⁺ cells is shown, based on the presence or absence of CD127 and CD56.

LIN⁺CD7⁺CD127⁺CD34⁺ IEL EXPRESS THE IL-2/15 RECEPTOR β -CHAIN AND PROLIFERATE IN RESPONSE TO IL-15

Expression of the cytokine IL-15 is elevated in the duodenum of CD and RCD patients³. As IL-15 is critical for the survival and expansion of RCDII cell lines^{3,38} we examined the expression of the IL-2/15 receptor β -chain (CD122) on IEL subsets from non-coeliac (n=5) coeliac (n=2) and RCDII (n=1) duodenal biopsies (Figure 5A, B). Lin⁺CD7⁺CD127⁺CD34⁺ IEL expressed CD122 (Figure 5A). In fact, CD122 expression levels on these cells were higher than on NK cells, which are known to be IL-15 responsive. CD122 expression levels on Lin⁺CD7⁺CD127⁺CD34⁺ cells on the other hand were lower than on NK cells and no CD122 was detected on T cells (Figure 5B). CD122 expression by PBMC from the same patients, on the other hand, showed a different pattern as Lin⁺CD7⁺CD127⁺CD34⁺ cells in blood had a lower CD122 expression than NK cells, while T cells, like in biopsies, were negative for CD122 expression (Figure 5C).

As Lin⁺CD7⁺CD127⁺CD34⁺ IEL expressed the IL-2/15 receptor β -chain (CD122) in the absence of the IL-2 receptor α -chain (CD25, data not shown), they expressed a receptor for IL-15 but not a high-affinity IL-2 receptor. To test whether these cells were indeed IL-15 responsive, IEL released from paediatric non-coeliac biopsies were incubated with IL-15 for three days. In the presence of IL-15, approximately 25% of Lin⁺CD7⁺CD127⁺CD34⁺ IEL were positive for the nuclear proliferation marker Ki-67, compared to approximately 5% in the absence of IL-15 (Figure 6). A similar picture was observed for the NK cells among the IEL (Figure 6), which also expressed CD122 (Figure 5). In contrast, no increase in Ki-67⁺ cell numbers was detectable for Lin⁺CD7⁺CD127⁺CD34⁺ cells and CD3⁺T cells (Figure 6), which did not express CD122 (Figure 5).

Together, these results indicate that duodenal Lin⁺CD7⁺CD127⁺CD34⁺ IEL proliferate in response to IL-15 via the IL-2/15 receptor β -chain.

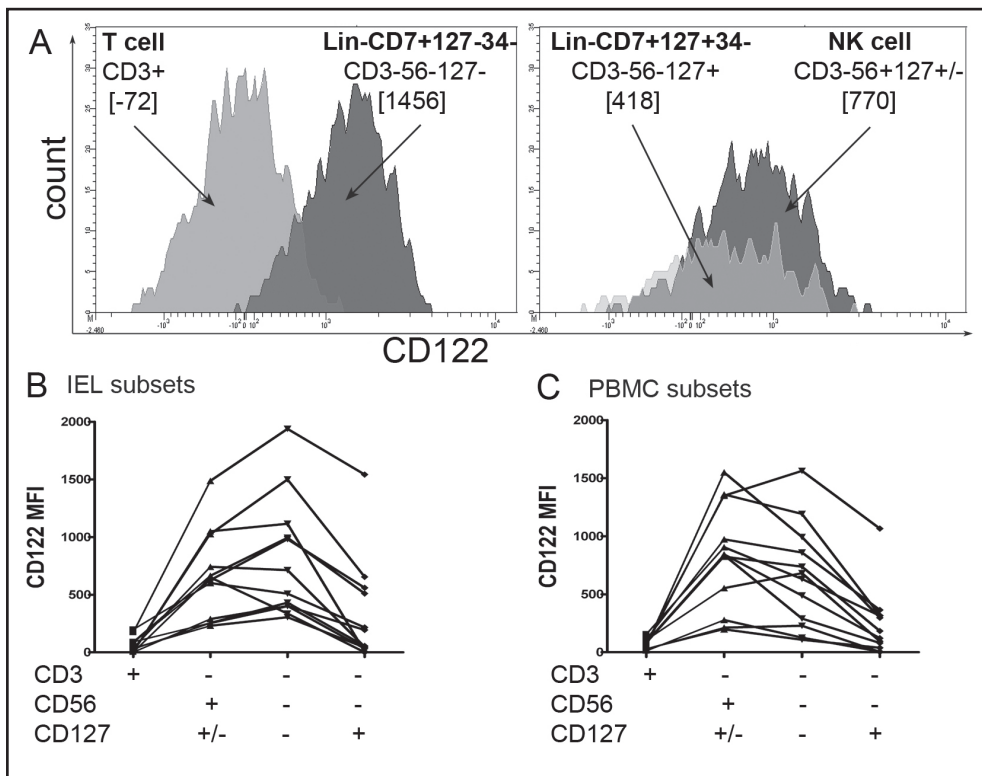


FIGURE 5. CD122 (IL2/IL-15R β) EXPRESSION ON T, LIN⁻CD7⁺CD127⁻CD34⁻ IEL, LIN⁻CD7⁺CD127⁺CD34⁻ AND NK CELLS. (A) Representative experiment of the CD122 expression of IEL subsets of a non-coeliac child. The mean fluorescence intensity (MFI) of surface CD122 expression of T cells (CD3⁺), Lin⁻CD7⁺CD127⁻CD34⁻ IEL, Lin⁻CD7⁺CD127⁺CD34⁻ IEL and NK cells (CD3⁻CD56⁺CD127^{+/-}) is depicted between square brackets. The CD122 expression is shown on the x-axis and the cell count on the y-axis. All IEL depicted here are CD45⁺SSC^{low}CD14⁻CD19⁻CD7⁺CD34⁻. **(B)** CD122 MFI expression of IEL subsets. Expression of CD3, 56 and CD127 is used to differentiate the sub populations as explained for (A) within CD45⁺SSC^{low}CD14⁻CD19⁻CD7⁺CD34⁻ cells from biopsies. **(C)** CD122 MFI expression of PBMC subsets. Expression of CD3, 56 and CD127 is used to differentiate the sub populations within CD45⁺SSC^{low}CD14⁻CD19⁻CD7⁺CD34⁻ cells in PBMC from the same donors as in (B). Figure (B) and (C) summarize data from 8 donors (no-CD n=5, CD n=2, RCDII n=1). Lines connect data from individual donors.

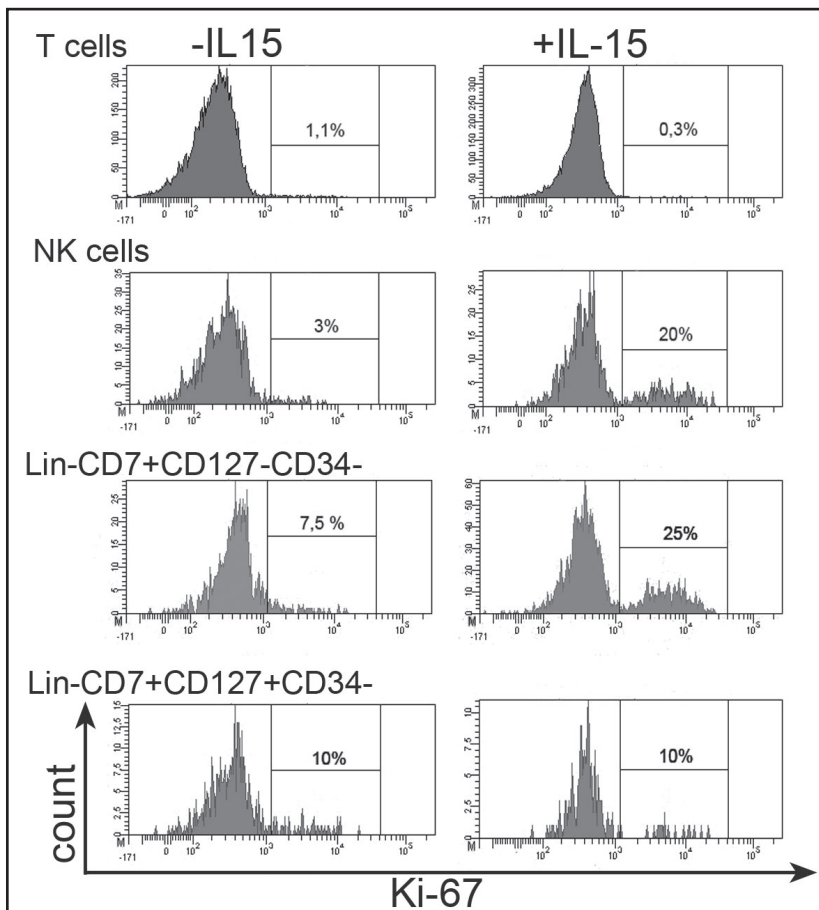


FIGURE 6. LIN⁺CD7⁺CD127⁻CD34⁻ IEL PROLIFERATE IN RESPONSE TO IL-15. IEL released from a non-celiac paediatric biopsy were incubated for three days with 10ng/ml IL-15 and subsequently analyzed by flow cytometry. Intracellular Ki-67 staining upon incubation in the absence (left) or presence (right) of IL-15 is shown for T cells (CD3⁺), NK cells (CD3⁺CD56⁺CD127⁺), Lin⁺CD7⁺CD127⁻CD34⁻ cells and Lin⁺CD7⁺CD127⁺CD34⁻ cells among IEL, gated as in Fig.5. One experiment is shown, representative of two.

DISCUSSION

In this study we performed an in-depth analysis of aberrant RCDII lines isolated from biopsies of RCDII patients to uncover the cellular origin of these aberrant cells. Compared to TCR⁺CD3⁺ IEL, these RCDII lines expressed more NK-cell associated transcripts, including KIR. The RCDII lines lacked surface expression of lineage markers, as well as the expression of CD127 and CD34 (Lin⁺CD7⁺CD127⁺CD34⁺ IEL), but highly expressed the IL-15/2R β chain (CD122). Cells with this phenotype were also found in duodenal biopsies of non-coeliacs and in the healthy thymus, but were less frequent in ileal and colonic epithelium and undetectable in the fetal intestine.

Characteristic for aberrant cells in RCDII, Lin⁺CD7⁺CD127⁺CD34⁺ IEL do not match any previously described physiological (non-pathological) IEL subset. In the duodenum of non-coeliac individuals, particularly children, these cells constituted up to 30% of CD45⁺SSC^{low} cells. Strikingly, these cells were 18-fold more frequent in non-coeliac children compared to children with CD. This difference in numbers between coeliac and non-coeliac individuals mirrors previous studies regarding the CD3⁺CD7⁺ population^{12,13}. Even after correction for the 2-4 fold increase in total IEL numbers in active celiac disease^{39,42}, absolute numbers of Lin⁺CD7⁺CD127⁺CD34⁺ IEL remain reduced in CD compared to non-CD children.

Lin⁺CD7⁺CD127⁺CD34⁺ cells that also expressed the gut-homing receptor CD103⁴³ were also present in the human thymus. This may point to a thymic origin of these cells in the gastrointestinal epithelium. The low frequency of these cells in the ileum and colon compared to the duodenum suggests that the latter would be a preferential site for homing of such thymic emigrants. Strikingly, Lin⁺CD7⁺CD127⁺CD34⁺ IEL were not detected in the fetal intestine. Therefore, if these cells in the duodenum are indeed of thymic origin, this suggests migration from the thymus later in life. Together, the data fit a scenario in which the frequencies of these intestinal cells peak when the thymus is highly active, i.e. during the first postnatal decade.

Data from this study support and extend further studies focusing on the intestinal CD3⁺CD7⁺ cell population, which included immature T cells and NK cells^{12-18,44}. Using multicolour flow cytometry we could further define the different subpopulations in this heterogeneous CD3⁺CD7⁺ cell population. Additional markers such as iCD3, CD56, CD127 and CD34 proved that Lin⁺CD7⁺CD127⁺CD34⁺ IEL are distinct from T cells, NK, LTi and early CD34⁺ precursor cells. It is tempting to speculate that the Lin⁺CD7⁺CD127⁺CD34⁺ IEL represent intermediate NK/T-lymphocyte precursors that undergo extrathymic differentiation, as was shown for other intestinal IEL subsets^{18,45-47}.

In fetal thymic organ cultures, a minority of the CD3⁺CD7⁺ cells developed into T cells¹⁴. In line

with a pre-T cell phenotype, RCDII cell lines P1, P2 and P4 (but not P3) contained partial TCR rearrangements²⁹. Lin⁺CD7⁺CD127⁺CD34⁻ IEL may therefore be identical to the immature T cells contained within the CD3⁺CD7⁺ cell population. On the other hand, the RCDII lines as well as duodenal Lin⁺CD7⁺CD127⁺CD34⁻ IEL *in vivo* expressed the IL-2/15 receptor β -chain (CD122) which could be detected on CD3⁺CD7⁺ cells in the epithelium¹³, but is normally found on NK but not T cell precursors³³. Thus it will be important to test the ability of Lin⁺CD7⁺CD127⁺CD34⁻ IEL to differentiate into T or NK cells in *in vitro* differentiation systems. This will be the subject of further investigation.

The presence of copious amounts of IL-15 in RCD is likely to contribute to the expansion and survival of (aberrant) IEL^{3,9,38}. In CD it induces NK- programming of TCR $\alpha\beta$ ⁺CD8⁺ IEL^{30,31,48,49}. Lin⁺CD7⁺CD127⁺CD34⁻ IEL, regardless of the disease status, expressed the IL-2/15 receptor β -chain in the absence of the IL-2 receptor α -chain. In line with this finding, in non-CD children Lin⁺CD7⁺CD127⁺CD34⁻ IEL proliferated in response to IL-15, providing a potential link between the upregulation of IL-15 and the outgrowth of cells with a Lin⁺CD7⁺CD127⁺CD34⁻ phenotype.

Microarray analysis showed that, compared to regular T-IEL lines, the RCDII lines expressed lower levels of several T cell-associated genes, but higher levels of NK cell-associated genes. Notably, the lines each expressed a unique combination of KIR, normally expressed after the appearance of NKp46 (present on P3 only) and CD56 (present on a small fraction of P3 only) in NK cell development or well after the completion of TCR rearrangements in T cells. It is therefore conceivable that IL-15, a critical cytokine in NK cell development, induces not only the expansion and survival, but also the acquisition of NK cell receptors on aberrant IEL in RCDII.

Quantification of the CD3⁺CD7⁺icCD3⁺ population provides the basis for diagnosis of RCDII^{6-8,37}. Our results demonstrate that in non-coeliac individuals, as well as in CD patients, the CD3⁺CD7⁺icCD3⁺ cells are heterogeneous and composed of four subsets based on the expression of CD127 and CD56, one of which displays the Lin⁺CD7⁺CD127⁺CD34⁻ IEL phenotype. In RCDII patients, CD56⁺CD127⁻ and CD56⁻CD127⁺ subsets were observed, which fits the observation that lymphomas arising in RCDII patients are usually CD56⁻⁵⁰. This diverse phenotype of CD3⁺CD7⁺icCD3⁺ cells *in vivo*, however, was mirrored in the RCDII cell lines, as a sizeable fraction of P4 expressed CD127 and a minor fraction of P3 expressed CD56. Proliferation and a (pre)malignant transformation of aberrant IEL in RCDII and EATL could therefore take place at different time points of cellular development, potentially ranging from pre-T/NK cells to nearly mature T cells with near-complete TCR rearrangements.

In conclusion, our studies indicate that 'aberrant' IEL in RCDII may be derived from a preexistent Lin⁺CD7⁺CD127⁺CD34⁻ IEL population present in the duodenum of non-coeliac and coeliac individuals.

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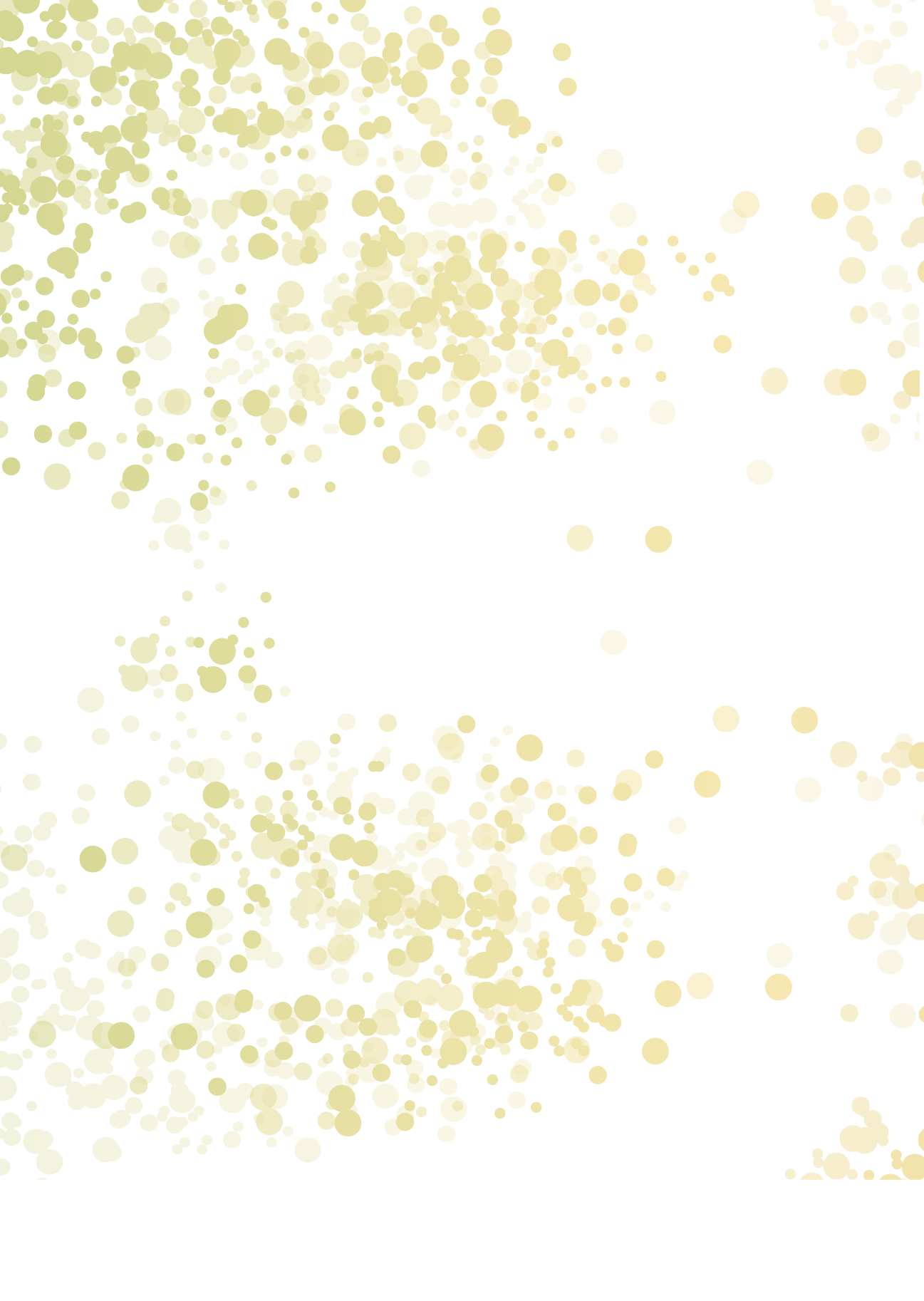
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CHAPTER 4:

CELIAC AND REFRACTORY CELIAC DISEASE
ALTER THE COMPOSITION OF THE INNATE
INTRAEPITHELIAL LYMPHOCYTE COMPARTMENT AND
ITS DIFFERENTIATION POTENTIAL

CHAPTER 4

CELIAC AND REFRACTORY CELIAC DISEASE ALTER THE COMPOSITION OF THE INNATE INTRAEPITHELIAL LYMPHOCYTE COMPARTMENT AND ITS DIFFERENTIATION POTENTIAL

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ABSTRACT

Active celiac disease (CD) is characterized by an oligoclonal expansion of intraepithelial T cells (T-IEL), whereas refractory celiac disease type II (RCDII), a severe complication of CD, is defined by the clonal expansion of Lineage-negative (Lin⁻) IEL that can transform into overt lymphoma. Previously we demonstrated that CD3-negative IEL could be divided into 4 phenotypically distinct subsets based on the expression of CD56 and CD127. To gain insight into their function and relationship we here determined the phenotype, composition and differentiation potential of this innate IEL compartment in non-celiac controls as well as in patients with CD and RCDII.

Unsupervised hierarchical clustering analysis of the expression of 14 NK and T cell surface markers demonstrated that the innate IEL subsets cluster together and separate from T-IEL. Moreover, co-expression of the IL-7R, IL-21R and IL-15R α was a hallmark of CD127⁺ innate IEL while CD127⁻ innate IEL expressed the IL-2/15R β . Age and disease dependent alterations in both the T-IEL and innate IEL compartments were observed, in particular a significant increase in the proportion of Lin⁺CD127⁻ and Lin⁺CD127⁺ IEL expressing intracellular CD3 ϵ and CD3 γ in patients with CD. *In vitro*, in the presence of IL-15, Lin⁺CD127⁻ IEL from controls and patients with CD, but not from RCDII patients, could differentiate into NK and T cells, the latter largely dependent on notch-signaling.

Together our data reveal complexity and plasticity of the innate IEL compartment that is influenced by age and disease and provide evidence that innate IEL have the ability to give rise to NK and T cells. This developmental plasticity may allow rapid responses to local cues in health and disease.

ABBREVIATIONS

IEL- Intraepithelial Lymphocytes, Lin- Lineage, ILC- innate lymphoid cells, PBL- Peripheral Blood Lymphocytes, PBMC- Peripheral Blood Mononuclear Cells, RAG- Recombination Activating Gene, TdT- Terminal Deoxynucleotidyl Transferase

INTRODUCTION

In the intestine, a single-layer epithelium provides a barrier against potentially harmful entities and mediates the uptake of nutrients. Intraepithelial lymphocytes (IEL) scattered throughout the epithelium form one of the largest lymphocyte populations in the body and are thought to be essential for maintaining the integrity of this barrier ¹.

IEL consist mainly of TCR $\alpha\beta$ and TCR $\gamma\delta$ T cells (T-IEL). Frequently disregarded, a CD3-negative innate IEL compartment exists, which was first described by Spencer et al. ^{2,3} and Jarry et al. ⁴. Later studies revealed that this innate IEL compartment harbors cells expressing a variety of NK cell receptors ⁵⁻⁹, functional NK cells ⁹ and NK cell-like type 1 Innate Lymphoid Cells (ILC1) ¹⁰. However, the composition, function and origin of this innate IEL compartment is still poorly understood. While it has been described that human innate IEL contain T cell precursors, their exact phenotype or developmental requirements have not been established ^{11,12}. As yet there is no evidence for intraepithelial NK cell development.

Previous studies reported that ILC subsets are increased in the lamina propria ^{13,14}, as well as in the epithelium of IBD patients ¹⁰ while in celiac disease (CD) patients a decrease in the CD3⁺ innate IEL population ^{3,7-9,15}, as well as a decline in NK cell numbers is found ¹⁶. A severe complication of CD is refractory celiac disease (RCD), defined by a lack of remission despite a gluten free diet. RCD type II patients present with a massively expanded innate IEL population. This innate IEL population, characterized by the presence of intracellular CD3 ϵ , was termed aberrant IEL ¹⁷.

We recently showed that cells matching this phenotype were also present in healthy individuals and proposed that these cells were the physiological counterparts of aberrant IEL in RCDII ¹⁸. Moreover, we observed that four innate IEL populations could be distinguished based in the expression of CD56 and CD127, indicating substantial heterogeneity within the innate IEL compartment.

We now have conducted an extensive phenotypic analysis of innate IEL and compared them with T-IEL and their counterparts in blood. Furthermore we investigated the influence of aging and disease on the composition of the T-IEL and innate IEL compartment. Finally, we investigated if CD3⁺ IEL could be precursors to mature T-IEL and /or NK cells. Our results indicate that innate IEL are heterogeneous and that the composition of the innate IEL and T-IEL compartments is influenced by both age and disease. What's more, our results provide evidence that innate IEL harbor precursors to functional NK and T-IEL, which may provide plasticity in response to

environmental cues.

MATERIALS AND METHODS

Human specimens

Fetal intestine from elective abortions and duodenal biopsies were obtained as described previously^{18,52}. Informed consent was given for all human specimens after medical ethical commission approval and in accordance with the local ethical guidelines of the VUmc (duodeni) and the LUMC (duodeni, fetal intestines) and in accordance with the declaration of Helsinki.

Cell isolation

IEL were isolated from fetal intestinal material and duodenal biopsies as described¹⁸. Briefly, the material was treated with 1 mM DTT (Fluka, Buchs, Switzerland)/HBSS (Life Technologies, Bleiswijk, NL) and subsequently 1 mM EDTA (Merck, Darmstadt, Germany)/HBSS. IEL from fetal intestines cells were further purified using a percoll (GE Healthcare, Hoevelaken, NL) gradient. Isolated cells were either kept on ice in PBS/0.5 % FCS, fixed for 10 min at 4°C with PBS containing 0.1% paraformaldehyde and 0.5% FCS, or viably frozen and stored at -80°C.

Flow cytometry

Fluorochrome-labelled antibodies for CD3ε, CD4, CD5, CD7, CD16, CD25, CD45, CD56, CD103, CD117, CD122, CD14, CD19, CD34, KIR3DL1 (DX9), IL-21R, IFNγ and TCRγδ were from BD Biosciences (San Jose, CA, USA). KIR2DL2/DL3/DS2 (GL183), KIR2DL1/2DS1 (EB6), CD244, and NKp46 were from Beckman Coulter (Fullerton, CA, USA). IL-15Rα, TCRαβ, Granzyme B and goat-anti hamster (secondary antibody for HMT3-2) were from Biolegend (San Diego, CA, USA). Anti-CD127, CD45, CD3ζ and CD160 were from eBioscience (San Diego, CA, USA) and CD8 from Invitrogen (Bleiswijk, The Netherlands). CD3γ [HMT3-2] was from Millipore (Billerica, MA, USA) and TCRδ [TCS-1] from ThermoScientific (Etten-Leur, The Netherlands). Cells were incubated for 30 minutes with fluorochrome-conjugated antibodies for surface staining, and subsequent intracellular staining was done using the permabilisation kit from eBioscience. Cells were acquired on a LSRII or on a FACSAriaIII (BD Biosciences) for cell sorting. T cell contamination for sorts of Lin⁺IEL was calculated as 0%, CD56^{Low} cell contamination 2,5% (n=3, reanalysis of 500 sorted cells/well). Results were analyzed using FlowJo V10 software, and 4-GraphPad. Unsupervised hierarchical clustering of flow cytometric measurements was done using the complete clustering method with the Euclidean distance measurement. The result was plotted in a heatmap using the

heatmap.2 function from the R gplots package.

Differentiation assays and cell culture

OP9-DL1 cells 53 were irradiated (1500 RAD) and plated at 5000 cells/well in 96-well plates. Lineage-negative (Lin⁻) cells were sorted from intestinal IEL as CD45⁺CD14⁻CD19⁻CD3⁻CD7⁺CD56⁻CD127⁻CD4⁻ and plated at 1 or 100 cells/well onto OP9-DL1. These co-cultures were maintained in Iscove's modified Dulbecco's medium (IMDM; LonzaVerviers, Verviers, Belgium) containing 10 % pooled normal human serum (NHS), 5 ng/ml human IL-7 (Peprotech, Rocky Hill, NJ, USA), 10 ng/ml human SCF, 10 ng/ml Flt3L and 10 ng/ml IL-15 (all R&D systems Europe, Abingdon, UK) for two weeks. Lin⁻IEL from duodenal biopsies were plated at 1 cell/well and feeder-cell mixture was added after two weeks of OP9-DL1 culture. This feeder-cell mixture contained 100.000 mixed irradiated allogeneic PBMC, 1 µg/ml phytohemagglutinin, 20 Cetus u/ml IL-2 (Proleukin, Chiron corporation, Emeryville, CA) and 10 ng/ml IL-15. Clones were maintained on IMDM/10% NHS containing 10 ng/ml IL-15 and 20 Cetus units/ml IL-2. In all cases, the phenotypes of the resulting cell populations were determined by flow cytometry. NK cells were obtained from PBMC by negative selection using MACS beads (Miltenyi, Bergisch Gladbach, Germany) and cultured for 2 weeks in AMV-I Medium containing 10% NHS/IL-15. K562 and Daudi cells were cultured in IMDM/8 % FCS and split at regular intervals.

Cytotoxicity assay

K562 and Daudi were labeled with 100 µCi [⁵¹Cr] for 1 h at 37°C and washed. Target cells were incubated with a Lin⁻IEL-derived NK cell clone or blood-derived polyclonal NK cells at an effector: target (E:T) ratio of 25:1 for 4 h at 37°C. Spontaneous and maximum ⁵¹Cr-release was assessed by the addition of medium or 1% Triton X-100 (Pierce, Rockford, IL), respectively. The percentage of specific lysis was determined by: (cpm experimental release- cpm spontaneous release)/(cpm maximum release- cpm spontaneous release) x 100 %.

Functional cell activation assays

Non tissue cultured plates (BD Microtest Plates) were coated over night with anti-CD3 and anti-CD28 (both at 2,5 µg/ml) and IgG1 (5 µg/ml) all from Biolegend in PBS, washed with PBS and incubated with 10% NHS/ IMDM Medium for 1h subsequently. Alternatively, cells were activated with PMA (20ng/ml) and Ionomycin (1µg/ml) both from SigmaAldrich (St. Luis, MO, USA), IL-15 and IL-12 (both 10ng/ml from R&D systems Europe, Abingdon, UK) for 1h and Brefeldin A (10 µ/ml) was added in the culture for an additional 3h after which cells were harvested, stained and

analyzed by flow cytometry.

Statistical Analysis

Differences in percentages of IEL subsets were first calculated for an overall statistical significance with a Kruskal-Wallis test, and then using the Mann-Whitney U test with the Bonferroni Correction for multiple testing.

ACKNOWLEDGMENTS

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RESULTS

INNATE IEL FORM A HETEROGENEOUS POPULATION WHICH IS DISTINCT FROM T-IEL

In a previous study we identified multiple innate IEL subsets that could be distinguished on the basis of CD127 and CD56 expression: conventional CD56⁺CD127⁻ NK cells (cNK), CD56⁺CD127⁺ NK cells, Lin⁻CD127⁻ IEL and Lin⁻CD127⁺ IEL (Figure 1A and ¹⁸). In the current study, we determined the phenotypes of these innate IEL subsets by flow cytometry in a large number of duodenal samples. We used a panel of 14 T and NK cell surface markers known to be expressed at various stages of NK and T cell development: CD160, IL-21R, CD18, NKp80, CD94, CD16, KIRs, CD122, CD117, LAT, CD5, NKp46, IL-15Ra, CD244. For comparison, PBL samples were stained using the same antibody mixtures. Representative FACS-histograms of three cell surface markers are shown in Figure 1B. An overview of all data obtained is shown in supplementary Figure 1. While all IEL subsets expressed CD160, the IL-21R was expressed by a small subset of T-IEL and CD127⁺ innate IEL but not by CD127⁻ innate IEL (Figure 1B). Similarly, the integrin CD18 showed a distinct pattern of expression, as a fraction of T-IEL, cNK and CD127⁺ NK were CD18 positive, while both Lin⁻IEL subsets lacked CD18 expression (Figure 1B).

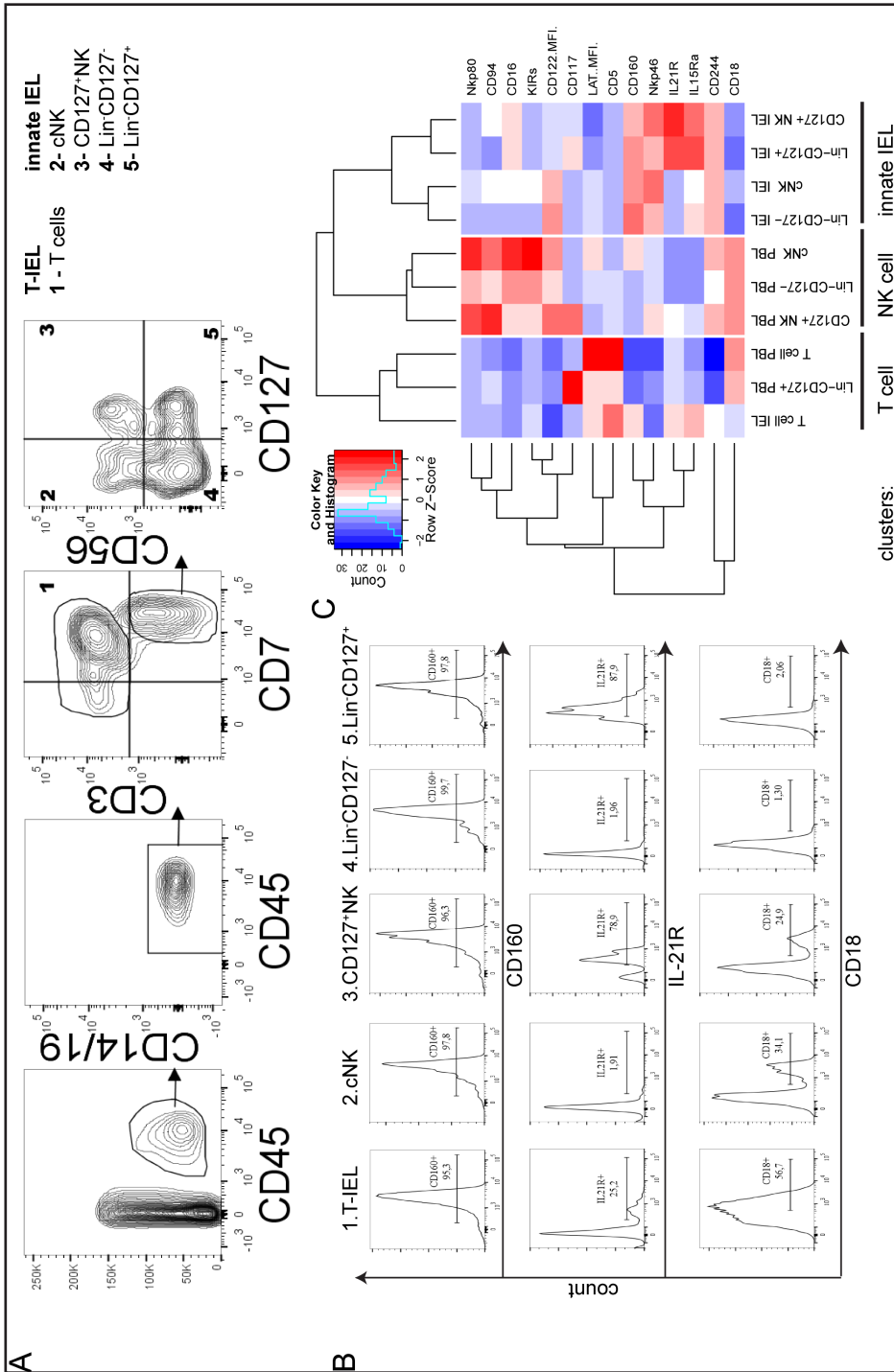
Unsupervised hierarchical clustering analysis (Figure 1C) of the entire set of flow cytometric data (supplementary Figure 1) using IEL and PBL samples revealed three distinct clusters of phenotypically similar cells: 1) a T cell cluster, 2) an NK cell cluster and 3) an innate IEL cluster with the four innate IEL subpopulations (Figure 1C). As the T-IEL clustered with T cells from PBL and

the NK cells from PBL clustered together, this indicates that the innate IEL populations are distinct from conventional NK cells and T cells including T-IEL.

Within the innate IEL the CD127⁺ and CD127⁻ subsets clustered together and are distinguished by the co-expression of the IL-7R, the IL-21R and the IL-15R α by CD127⁺ but not CD127⁻ cells and the much more prominent expression of IL-2/15R β (CD122) by CD127⁻ cells compared with CD127⁺ cells (Figure 1C). Strikingly, the unsupervised clustering groups the cNK IEL with the Lin⁻CD127⁻ IEL and the CD127⁺NK IEL with the Lin⁻CD127⁺ IEL so that neither the innate NK subpopulations nor the innate Lin⁻ subpopulations cluster together.

Taken together, these data confirmed the notion that the innate IEL compartment consists of 4 phenotypically distinct subsets and indicates that while the CD127⁺ subsets could respond to IL-7 and IL-21, the CD127⁻ subsets would be more sensitive to IL-15.

FIGURE 1. INNATE IEL ARE FORMED BY DISTINCT SUBSETS THAT ARE DISSIMILAR TO T-IEL. **(A)** IEL were isolated from duodenal biopsies, stained with antibodies to CD45/CD14/CD19/CD3/CD7/CD56/CD127 and analyzed by flow cytometry. A representative plot for the gating strategy for T cells (T-IEL) (1) innate IEL including conventional NK (cNK) (2) CD127⁺NK (3) Lineage-negative (Lin⁻) CD127⁻ (4) and Lin⁻CD127⁺ (5) cells is depicted. Each plot shows the cells gated in the previous plot. **(B)** IEL were isolated from duodenal biopsies and stained as described in (A) and analyzed by flow cytometry. The percentage of CD160, IL-21R and CD18 expression for IEL within the different subsets is depicted in the histograms and the corresponding marker on the y-axis. **(C)** Pediatric and adult duodenal IEL and PBL from peripheral blood were stained with a standard panel of antibodies (against CD45/CD14/CD19/CD3/CD7/CD56/CD127), combined with antibodies detecting Nkp80, CD94, CD16, KIRs (a cocktail recognizing KIR2DL1/KIR2DL2/KIR2DL3/KIR2DS1/KIR2DS2/KIR3DL1), CD122, CD117, LAT, CD5, CD160, Nkp46, IL21R, IL15R α , CD244 and CD18 and analyzed by flow cytometry. T cells, conventional NK (cNK), CD127⁺NK, Lineage-negative CD127⁻ (Lin⁻CD127⁻) and Lin⁻CD127⁺ cells from IEL or PBL were gated as described in (A). A heatmap was generated from the data by unsupervised hierarchical clustering. The heatmap depicts the z score generated by the average percentages of cells expressing the above-mentioned markers or the average mean fluorescence intensity (MFI) of the marker (see color key). N= $\geq$ 3 per marker (see Supplementary Figure 1).



AGE- AND CELIAC DISEASE-ASSOCIATED CHANGES IN INNATE IEL

As the entire innate IEL population is decreased in celiac disease patients compared with non-celiac controls,^{3, 7-9, 15, 16} and children show higher frequencies of Lin⁺CD127⁻ IEL than adults¹⁸, we considered the possibility that the composition of the innate IEL compartment is influenced by disease and/or ageing.

We therefore determined the relative abundances of the IEL subpopulations in the fetal intestine, duodenal biopsies of healthy children and adults, children and adults suffering from CD, and adults with complicated celiac disease (RCDII). T-IEL were the least abundant in fetal IEL and most prominent in adults with an intermediate percentage in children (Figure 2A). Conversely, both cNK and CD127⁺NK populations were most abundant in the fetal IEL and significantly lower in IEL of both children and adults. As reported earlier¹⁸ Lin⁺CD127⁻ IEL were significantly higher in children compared to adults. Similarly, cNK IEL were higher in children compared to adults.

In CD, both in children and in adults, the percentage of T-IEL were increased, in agreement with previous reports^{3, 19, 20}. Conversely, the percentages of the innate IEL populations were lower, particularly in children in which all four subsets were significantly affected, while in adults only the CD127⁺ subsets were lowered (Figure 2A). In striking contrast, in RCDII patients both the percentages of Lin⁺CD127⁻ and Lin⁺CD127⁺ IEL were significantly increased, accompanied by a decrease in the percentage of T-IEL when compared with adults with CD (Figure 2A). The latter observation confirms the diagnosis of RCDII on the basis of the percentage of CD3-negative aberrant IEL²¹.

Previously, it was not recognized that the CD3-negative aberrant IEL population could contain both CD127⁻ and CD127⁺ cells. Analysis of these subpopulations in the cohort of 10 patients included in the present study indicates that the relative contribution of these subsets is patient dependent: while all patients harbored a sizable, but variable proportion of CD127⁻ and CD127⁺ cells, in three patients only the CD127⁻ subset was detected (Supplementary Figure 2). As these subsets can be distinguished on the basis of the expression of distinct cytokine receptors, the local cytokine milieu in the intestine may determine the selective outgrowth of one of these innate IEL subsets.

Premalignant aberrant Lin⁺CD127⁻ IEL expanded in RCDII patients¹⁸ express intracellular CD3 (icCD3). We therefore analyzed the intracellular expression of the ζ -chain, CD3 ϵ and CD3 γ expression in all four innate IEL populations and T-IEL and PBL as a positive control. As expected

T-IEL from both controls and patients with CD and in PBL expressed both CD3 ϵ and CD3 γ (Figure 2B, C) and the ζ -chain (not shown). In patients and non-CD controls all four innate IEL subsets expressed the ζ -chain (data not shown). In controls the large majority of cells in all four IEL subpopulations lacked expression of both icCD3 ϵ and icCD3 γ while the remainder were either icCD3 ϵ single positive or icCD3 $\epsilon\gamma$ double positive. In contrast, in CD patients the majority of both Lin⁻CD127⁺ and Lin⁻CD127⁻ IEL populations expressed both icCD3 ϵ and icCD3 γ , while the percentage of icCD3 $\epsilon\gamma$ positive cNK and CD127⁺ NK IEL was decreased (Figure 2B,C). In PBL high frequencies of icCD3 $\epsilon\gamma$ ⁺ cells were only detected in Lin⁻CD127⁺ cells (Figure 2C). Furthermore, Lin⁻CD127⁻ IEL and Lin⁻CD127⁺ IEL in RCDII patients showed enormous levels of icCD3 ϵ expression, which was on average 3x higher than T-IEL (Figure 2D).

Thus, the composition of both the T-IEL and innate IEL compartment is influenced by age and disease and in CD there is a significant increase in the intracellular expression of CD3 $\epsilon\gamma$ within both the Lin⁻CD127⁻ and Lin⁻CD127⁺ IEL subsets. Together this points to plasticity of the IEL compartment.

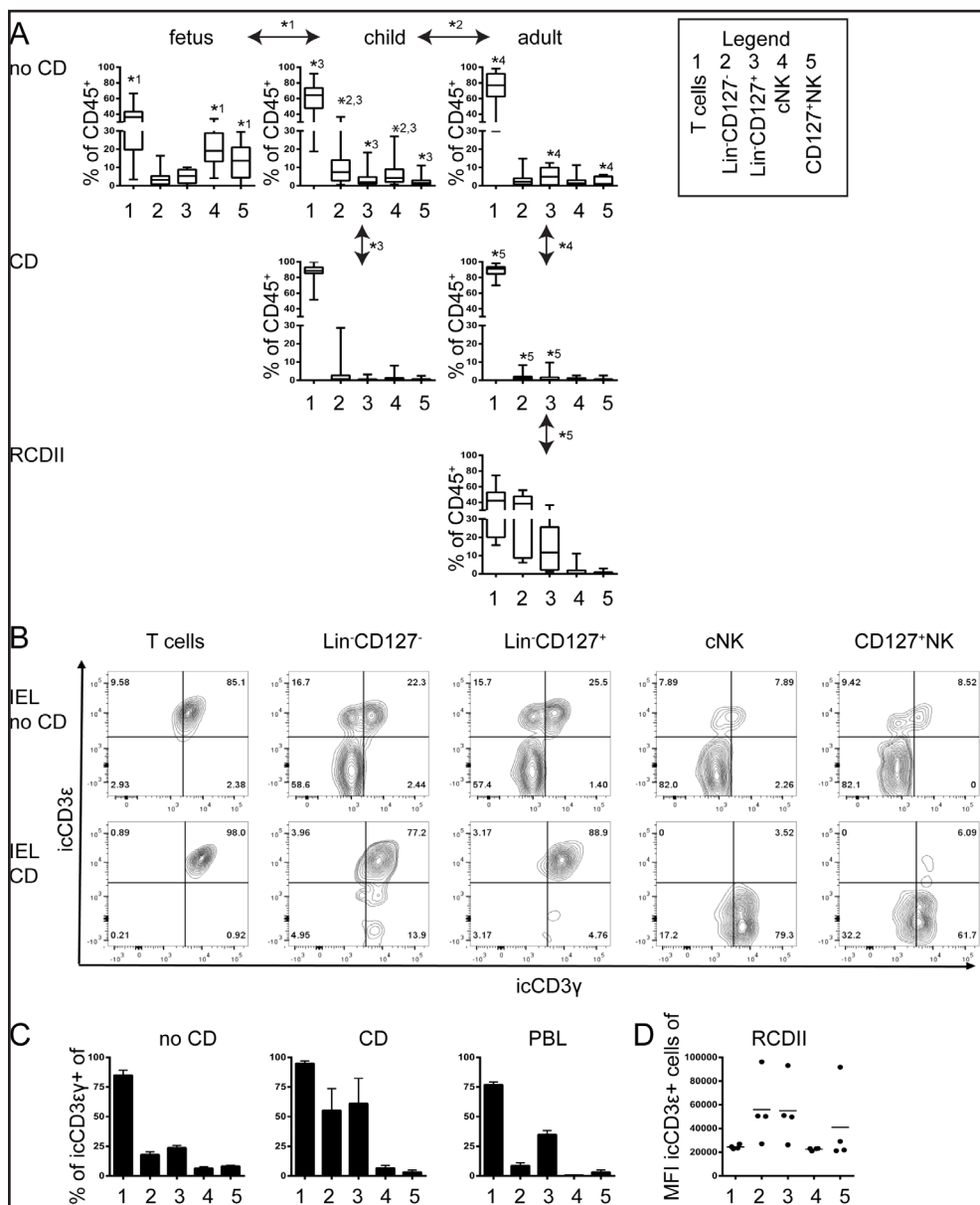


FIGURE 2. PLASTICITY OF THE INNATE IEL COMPARTMENT: SHAPED BY AGE AND DISEASE. (A) IEL were isolated from duodenal biopsies from children and adults with celiac disease (CD) or without CD (no CD) and refractory celiac disease type II (RCDII). IEL were stained with antibodies to CD45/CD14/CD19/CD3/CD7/CD56/CD127 and analyzed by flow cytometry. The percentages of T cells (1), Lineage-negative CD127 (Lin⁻CD127⁻) (2), Lin⁺CD127⁺ IEL (3), conventional NK (cNK) (4) and CD127⁺NK (5), gated as in Fig1A is depicted calculated from all

CD45⁺ cells measured. A star indicates statistical significance as calculated by the Mann-Whitney U test with Bonferroni Correction. *1 compares percentages of IEL subsets from: the fetus with children (no CD), *2 children with adults (both no CD), *3 children without CD (no CD) with children with CD (CD), *4 adults no CD versus adults CD and *5 adults CD with RCDII. N=17 fetus, n=51 children no CD, n=10 adults no CD, n=28 children CD, n=30 adults CD, n= 10 RCDII. **(B)** IEL were isolated from pediatric biopsies from celiac patients (IEL CD) and non CD patients (IEL no CD) and stained for surface CD45/CD14/CD19/CD3/CD7/CD56/CD127 as well as intracellular CD3ε (icCD3ε) and CD3γ (icCD3γ). Representative dot plots of the intracellular CD3ε and CD3γ staining of T cells, Lineage-negative CD127⁻ (Lin⁻CD127⁻) and Lin⁻CD127⁺ IEL, conventional NK (cNK) and CD127⁺NK cells, gated as in Fig1A are shown. **(C)** IEL isolated from biopsies of CD, no CD and PBL were stained and analyzed as described in (A). The bar graphs depict the percentages (mean ± SD) of intracellular (ic) CD3ε and CD3γ (icCD3γ⁺) cells (y-axis) within the subpopulations (x-axis). N=3 for IEL CD and no CD, N=6 for PBL. **(D)** IEL isolated from RCDII patients were stained as in (A) and for icCD3ε. The mean fluorescence intensity (MFI) of the icCD3ε⁺ cells (x-axis) is depicted for the different IEL subsets (y-axis). N=4

LIN⁻CD127⁻ IEL FROM NON-CD CONTROLS AND CD PATIENTS, BUT NOT FROM RCDII PATIENTS, CONTAIN NK AND NOTCH-DEPENDENT T CELL PRECURSORS

As both Lin⁻ IEL subsets contained cells that expressed multiple CD3 chains intracellularly, we investigated if these subsets might contain precursors to mature T and/or NK cells. For this purpose we determined the in vitro differentiation potential of cells isolated from controls, CD and RCDII patients in the OP9 stroma-based co-culture system²². As duodenal biopsy material yielded only small numbers of Lin⁻IEL (usually up to 100 cells), we decided to focus on the Lin⁻CD127⁻ IEL subset as in the majority of biopsies this subset was the largest innate subset and we have previously shown that they are responsive to IL-15¹⁸, a key cytokine in CD and RCDII pathogenesis. For all experiments we isolated Lin⁻CD127⁻ IEL by flow cytometric sorting.

First, we investigated if IL-15 was required for the outgrowth of Lin⁻CD127⁻ IEL in stromal co-cultures. For this purpose Lin⁻CD127⁻ IEL were isolated and co-cultured with OP9-DL1 in the absence or presence of IL-15. After two weeks, significant numbers of CD45⁺ cells were detected only in co-cultures to which IL-15 had been added (Figure 3A) showing, that IL-15 was required for the survival and expansion of Lin⁻CD127⁻ IEL in this culture system.

As notch ligands are expressed in the intestinal epithelium^{23,24} and notch receptor triggering has been shown to drive icCD3 expression²⁵ and T cell development in developing lymphocytes, we next investigated the differentiation potential of Lin⁻CD127⁻ IEL on stroma cells lacking (OP9) or expressing the notch ligand DLL1 (OP9DL1)²⁶. For this purpose we used Lin⁻CD127⁻ IEL isolated from CD biopsies. While Lin⁻CD127⁻ IEL co-cultured with OP9 developed mainly into cNK cells, co-cultures with OP9DL1 resulted in the outgrowth of both cNK and T cells (Figure 3B, left two panels). The differential outgrowth of T cells on notch-ligand DLL1 indicated, that Lin⁻CD127⁻ IEL contained notch-dependent T cell precursor cells. Therefore this outgrowth was unlikely due to contaminating mature T cells.

Subsequently, we determined the differentiation capacity of Lin⁻CD127⁻ IEL isolated from biopsies of controls and patients with RCDII. Similar to Lin⁻CD127⁻ isolated from CD patients, co-cultures of OP9DL1 with Lin⁻CD127⁻ IEL from controls yielded cNK and T cells, but the outgrowth of cNK cells dominated (Figure 3B, middle panels). In contrast, Lin⁻CD127⁻ IEL isolated from RCDII biopsies did not differentiate into NK or T-IEL in OP9DL1 co-cultures (Figure 3B, right panel). As T-IEL were still present in RCDII patients, yet no T-IEL outgrowth was detected from RCDII Lin⁻CD127⁻ IEL on OP9DL1, this further underlies the validity of our previous studies.

Next we determined the cell surface phenotype of the cells generated in the co-cultures. An overview of the results is shown in Figure 3C and examples for the phenotype of cells generated from a patient with CD and a patient with RCD are shown in Figure 3D. T cells from non-CD and CD patients consisted mainly of CD8⁺TCRαβ⁺ cells and some TCRγδ⁺ cells. NK cells showed a variable level of CD8 expression, but lacked CD127 and were therefore considered cNK cells (Figure 3C,D). On the contrary, Lin⁻CD127⁻ IEL from RCDII biopsies retained their original phenotype after culture (Figure 3D).

Thus, Lin⁻CD127⁻ IEL from controls and CD patients could give rise to cNK and T cells, where increased T cell differentiation was detected in co-cultures with cells from CD patients, compared to non-CD patients, which may relate to the increased icCD3εγ expression in Lin⁻CD127⁻ IEL from CD patients (Figure 2). In contrast Lin⁻CD127⁻ IEL from RCDII patients did not differentiate into either cNK or T cells, which may be linked to the malignant transformation of these cells.

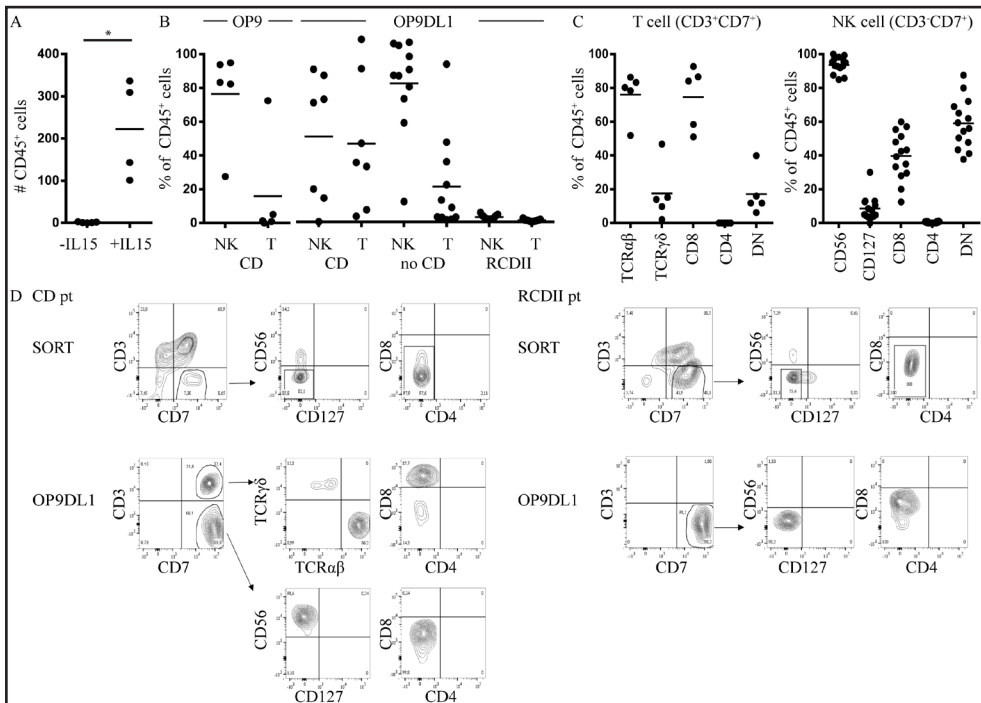


FIGURE 3. LIN⁺CD127⁺IEL FROM NON-CD CONTROLS AND CD PATIENTS, BUT NOT FROM RCDII PATIENTS, CONTAIN NK AND NOTCH-DEPENDENT T CELL PRECURSORS. (A) IEL isolated from adult biopsies (adult Lin⁺CD127⁺IEL) were sorted (gated as in Figure 1) at 100 cells/well on OP9-DL1. After 14 days of co-culture in the presence of Flt3L/IL-7/SCF with (+IL-15) or without IL-15 (-IL-15), cells were harvested and analyzed by flow cytometry. The amount of CD45⁺ (y-axis) cells after co-culture is depicted. **(B)** Lin⁺CD127⁺IEL from biopsies of CD patients (CD), non-CD controls (no CD) and from RCDII biopsies were sorted at 100 cells/well on OP9 or OP9DL1 and cultured in the presence of Flt3L/IL-7/SCF and IL-15. After two weeks, the cultures were analysed by flowcytometry, and the percentage of NK (CD3⁺CD7⁺CD56⁺) and T cells (CD3⁺CD7⁺) was calculated as a percentage of CD45⁺ cells. Each data point represents the result of a single culture from a total of 7 non-CD controls, 4 CD patients and 7 RCDII patients. While for non-CD and CD patients Lin⁺CD127⁺IEL were sorted directly from biopsies, for the RCDII patients Lin⁺CD127⁺IEL were sorted directly from biopsies (n=1), sorted from short-term cultures of biopsies in IL-15-containing medium (n=2), or plated from cultured RCDII lines, which were homogeneously Lin⁺CD127⁺ (n=4). **(C)** These graph gives an overview of the phenotype of T and NK cells generated as described in (B). The percentages of cells staining positive for TCRαβ, TCRγδ, CD4, CD8 or CD4⁺CD8⁺ (DN= double negative) is depicted for T cells (CD3⁺CD7⁺) and NK cells (CD3⁺CD7⁺).

(D) Representative FACS plots of Lin⁺CD127⁺ IEL at the time of sort (sort) and after 14 days of culture on OP9DL1 (with FLT3L, SCF, IL-7, IL-15). This graph shows a representative example of Lin⁺CD127⁺ IEL isolated from a CD patient and a RCDII patient (both after IEL isolation from biopsies, staining and sorting as described in Figure 1A). Cells were stained for CD3, CD7, CD56, CD127, CD4, CD8, and additionally TCRαβ and TCRγδ if applicable.

CLONAL LIN⁺CD127⁺ IEL GENERATED FUNCTIONAL TCRαβ, TCRγδ AND NK CELLS

Subsequently, we investigated whether single Lin⁺CD127⁺ IEL isolated from duodenal biopsies could give rise to T and/or NK cells. However, single Lin⁺CD127⁺ IEL seeded on OP9DL1 did not yield sufficiently large cell numbers for phenotypic analysis (data not shown). We therefore cultured single cells on OP9DL1 cells for 14 days for differentiation, after which a 'feeder mix' consisting of irradiated allogeneic PBL was added for expansion of the differentiated cells. After an additional 10-20 days, three types of cells arose from single duodenal Lin⁺CD127⁺ IEL: Vδ1⁺TCRγδ T cells, CD8⁺TCRαβ T cells and NK cells (Figure 4A).

To investigate if the generated T and NK cell clones were functional, we stimulated these clones with plate-bound antibodies directed against CD3 and CD28 or with a mixture of PMA/Ionomycin/IL-15 and IL-12- designed to achieve optimal activation of the clones- and tested for the induction of IFNγ and Granzyme B production. In the T cell clones, anti-CD3/CD28 triggering induced moderate IFNγ production in the T cell clones and the PMA/Ionomycin/IL15/IL2 mix strongly upregulated both IFNγ production and Granzyme B (Figure 4B). In the NK cell clone, anti-CD3/CD28 triggering had no effect and the PMA/Ionomycin/IL15/IL2 mix induced a strong IFNγ response, but had no effect on the already very high levels of Granzyme B expression prior to stimulation (Figure 4B). In addition, this NK cell clone lysed classical NK cell targets K562 and Daudi, albeit to a lesser extent than peripheral-blood-derived NK cells (Figure 4C).

In summary, the generated T cell clone was sensitive to TCR crosslinking and able to produce IFNγ and Granzyme B upon stimulation, while the NK cell clone expressed high levels of Granzyme B, was capable of lysing classical NK cell targets and produced IFNγ upon stimulation.

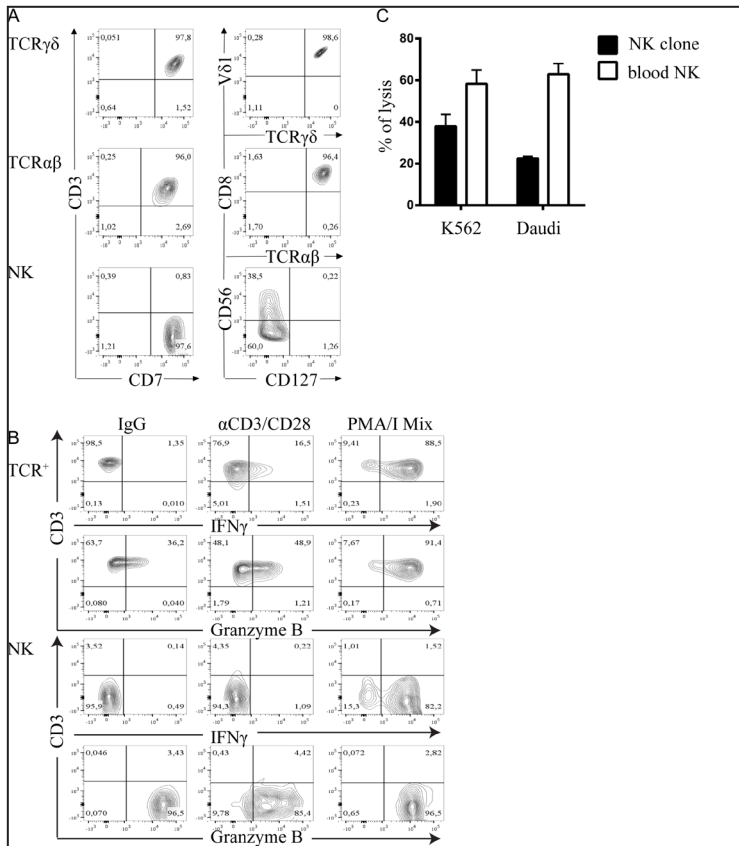


FIGURE 4. LIN⁺CD127⁺ IEL DERIVED CLONES SHOW FUNCTIONAL PROPERTIES OF T OR NK CELLS. **(A)** Lin⁺CD127⁺ IEL were sorted from pediatric duodenal biopsies and plated at 1 cell/well on OP9DL1 in the presence of Flt3L/IL-7/SCF and IL-15. After 14 days of co-culture, cultures were expanded with mixed irradiated allogeneic PBMC, IL-2/IL-15 for another 10-20 days. Dot plots show the phenotype of representative single-cell cultures (TCRγδ, TCRαβ and NK clone), harvested after expansion. N=4 independent experiments. **(B)** A Lin⁺CD127⁺ IEL-derived T and NK cell clone were activated with coated anti-CD3 and anti-CD28 (both 2.5 μg/ml) or IgG1 (5 μg/ml) for 4h or by a cocktail of PMA/Ionomycin/IL-12 and IL-15 in the presence of Brefeldin A (10 μg/ml) for the last 3h. Cells were harvested, stained and analysed by flow cytometry for intracellular production of IFNγ or Granzyme B. **(C)** Lin⁺CD127⁺ IEL-derived NK cell clone (see Figure 4A) and control peripheral blood NK cells, both expanded in the presence of IL-15, were used in a cell-mediated lysis (CML) assay, using ⁵¹Cr-labelled K562 and Daudi cells as target cells at an effector:target ratio of 25:1. The percentage of specific lysis was calculated based on ⁵¹Cr-release after 4 hours.

DISCUSSION

While the existence of a CD3⁺ IEL population in the small intestine is well established, the exact composition and function of this lymphocyte subset has remained largely unknown. Here, we phenotypically characterized four CD3⁺ innate IEL subsets and demonstrated that these are all clearly distinct from each other. In agreement with our previous results, two distinct Lin⁺ IEL populations and two NK-like IEL populations could be identified based on the differential expression of CD127 and CD56. Moreover, co-expression of the IL-7R (CD127), the IL-21R and the IL-15R α distinguished two of these subsets from the two other subsets that typically express the IL-15R β , indicating that changes in the local cytokine milieu may have profound effects on the composition of the innate IEL compartment.

Furthermore, we observed that the composition and intracellular expression of CD3 chains was influenced by age and disease. In particular, while the relative proportion of the innate IEL subsets was decreased in CD, the intracellular expression of CD3 chains was markedly increased in both Lin⁺ IEL subsets. In contrast, both Lin⁺ IEL subsets were found expanded in RCDII. Finally, we provide evidence that the Lin⁺CD127⁺ IEL subset in controls and patients with CD contained precursors to cNK and T cells as co-culture of Lin⁺CD127⁺ IEL on the stromal cell line OP9DL1 led to the outgrowth of both cNK and T cells in an IL-15 dependent fashion.

Besides IL-15, the expression of the notch-ligand DLL1 promoted T cell development. Importantly, notch-ligands such as DLL1 are expressed in the intestine^{23,24}, where they provide differentiation signals to intestinal stem cells in the crypts^{27,28} and as such could also play a role in lymphocyte differentiation in the intestinal epithelium. Lin⁺CD127⁺ IEL derived from RCDII patients on the other hand failed to develop into T or NK cells, which is likely linked to the premalignant nature of these cells.

Intestinal Lin⁺ T cell precursors have been identified in mice and were found mainly in cryptopatches in the lamina propria²⁹. These cells express CD34, CD117 and CD127 (IL-7R α) and gave rise to TCR $\alpha\beta$ and TCR $\gamma\delta$ T cells^{30,31}. T cell precursors with a Lin⁺ IEL phenotype were also detected in the mouse epithelium; these express iCD3 ϵ but no to little RAG-1, which may explain the restricted TCR repertoire found in non-conventional T cells^{32,33}. Extensive analysis of Lin⁺ subsets suggested a stepwise differentiation process of maturation, which include intermediary T cell precursors with a phenotype matching that of the human Lin⁺CD127⁺ IEL described in our studies: iCD3 $\epsilon^{+/-}$ CD8 $\alpha^{+/-}$, CD117⁺, IL7-R α /CD127⁺, CD25⁺.

Evidence for the existence of T cell precursors in the human intestine is scant. In line with our current data, MacDonald and colleagues reported that human intestinal CD3⁺ cells contain T cell precursors. In murine reaggregate thymic organ cultures and in transplanted SCID mice, this population gave rise to mature T cells^{11,12}. The exact innate subset and developmental requirements of these T cell precursors, however, was not determined.

In fact, TCR rearrangements^{11,34} and preTα^{12,34-36} were detected in intestinal IEL and CD3⁺ cells. Furthermore, RAG-1 expression has been demonstrated in CD3⁺ IEL^{6,34-36}, as well as the expression of TdT³⁶, but to a variable degree³⁷. In line with the latter observation, we did not detect significant intracellular TdT protein expression in duodenal biopsies while such expression was evident in CD34⁺ cells in the thymus (data not shown). As it is now clear that innate IEL contain different subpopulations (ref.¹⁸ and this study), which depending on the age and disease status of the donors were present in variable numbers, this may explain the conflicting results in previous studies. Our current results thus extend those previous observations as they provide further evidence for the existence of T cell precursors in the Lin⁺ IEL compartment in the duodenal epithelium.

Furthermore, our data indicated that the majority of Lin⁺ IEL developed into cNK cells in the *in vitro* culture system used. In humans, NK cell precursors have so far been described in secondary lymphoid tissue as lymph nodes³⁸, tonsils³⁹, liver⁴⁰, in the uterus⁴¹, the thymus⁴² and as CD117⁺ cells the lamina propria of the intestine⁴³, but not in the intestinal epithelium.

A confounding factor could be the outgrowth of contaminating NK and/or T cells in the *in vitro* differentiation system. Several observations argue against this possibility. First, we used a strict gating strategy throughout our studies, which showed no T cell contamination (0+/-0%) and very few CD56^{low} cells were detected after the sort (2,5+/-3%). Second, culture of Lin⁺CD127⁺ IEL from RCDII patients did not lead to T cell outgrowth, even though T cells were present before the FACS sort. Third, bulk sorting of intestinal Lin⁺CD127⁺ IEL followed by culture with 'feeder mix' – optimal culture conditions for mature T and NK cells – did not yield any outgrowth of T or NK cells in four independent experiments (data not shown). Finally, the observation that notch-signaling augmented T cell differentiation points to the fact that T cells arising from Lin⁺CD127⁺ IEL were derived from notch-dependent pre-T cells and thus not from contaminating mature T cells.

It is well known that T-IEL numbers, both TCRαβ⁺ and TCRγδ⁺ are elevated in patients with CD compared to controls^{3,19}. We observed that Lin⁺CD127⁺ IEL isolated from CD patients displayed increased expression of intracellular CD3ε and CD3γ and yielded higher T cell numbers compared to Lin⁺CD127⁺ IEL from non-celiac controls. Moreover, we found outgrowth of both TCRαβ⁺ and

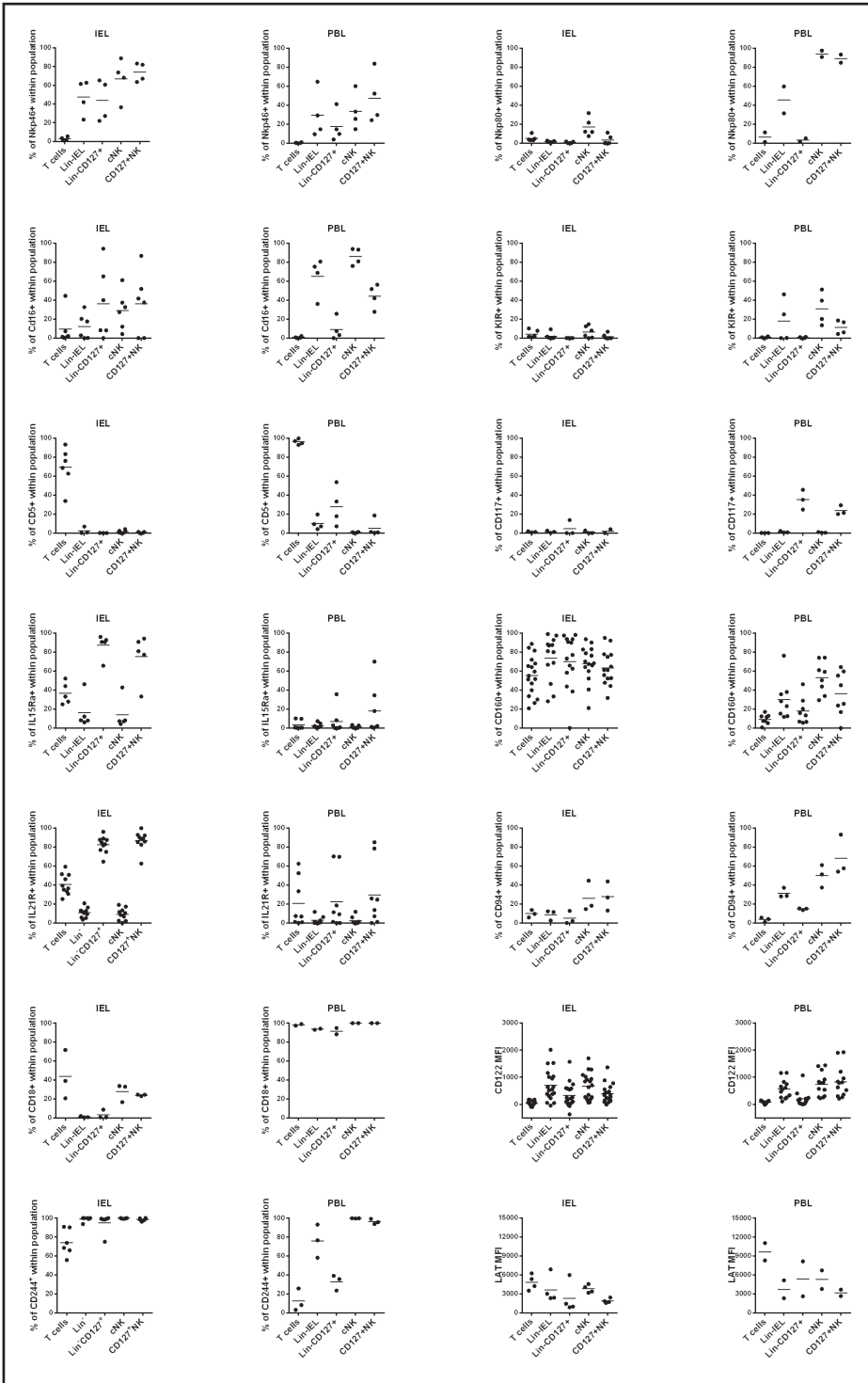
TCR $\gamma\delta^+$ cells in bulk cultures as well as at the clonal level. Thus, it is tempting to speculate that in CD Lin $^-$ CD127 $^-$ IEL are more prone to give rise to T cells due to local alterations, like changes in the cytokine milieu induced by the gluten-specific T cell response. For example, the well-known upregulation of IL-15 in CD ^{44} would impact on the IL-15 β^+ Lin $^-$ CD127 $^-$ IEL^{18,44,45} while IL-21 produced by gluten-specific T cells in the lamina propria⁴⁶ could drive proliferation and/or differentiation of the IL-21R $^+$ Lin $^-$ CD127 $^-$ IEL. RCDII on the other hand presents with a massive expansion of Lin $^-$ CD127 $^-$ IEL that express extraordinarily high levels of intracellular CD3 chains, which by itself is known to block T cell differentiation⁴⁷ and this may explain our observation that Lin $^-$ IEL from RCDII patients did not develop into cNK or T cells in the OP9DL1 based differentiation system.

Interleukin-15 plays an important role in CD and RCDII as it is upregulated in the intestinal epithelium and has potent proliferative and anti-apoptotic effects^{44,45,48,49}. In particular, patients with RCDII have a massive increase in the Lin $^-$ IEL numbers¹⁷ and in approximately 50 % of these patients, malignant transformation leads to enteropathy associated T cell lymphoma, a usually fatal complication⁵⁰. Cell lines isolated from such patients are IL-15 responsive and treatment with anti-IL-15 has therefore been suggested to be a potential treatment option. Our current data, however, indicate that next to an expansion of Lin $^-$ CD127 $^-$ IEL, Lin $^-$ CD127 $^+$ IEL are also frequently expanded in RCDII. As the former express the IL-15R $\beta\gamma$ while the latter express the IL-7R, IL-21R and IL-15R α , but considerably less IL-15R β , treatment with anti-IL-15 by itself is unlikely to be sufficient. Intriguingly, the simultaneous presence of IL-15R α Lin $^-$ CD127 $^+$ IEL and IL-15R β Lin $^-$ CD127 $^-$ IEL in the intestinal epithelium suggests that the former could present IL-15 in trans to the latter.

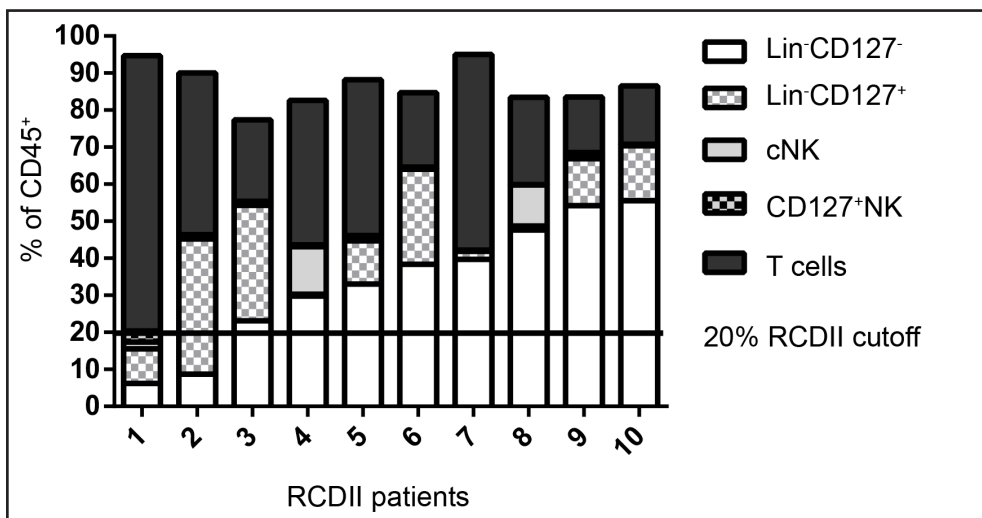
Recently, ILC have aroused broad interest, as these cells constitute minor cell populations in the intestine, but exert major roles in the healthy intestine and in inflammatory intestinal diseases⁵¹. Three types of innate lymphoid cells, ILC1, ILC2 and ILC3, have been described in recent years, each with distinct functional features. ILC2 and ILC3 express CD127/IL-7R α and ILC1 are either CD127 $^+$ ¹³ or CD127 $^-$ ¹⁰. Most ILC subsets reside in the lamina propria but evidence for the existence of intraepithelial ILC1 subset was recently obtained¹⁰. In fact, this ILC1 population could be identical to the intraepithelial cNK subset described in our studies, as both populations are CD56 $^+$ CD127 $^-$. Also, intraepithelial ILC1 express granzyme-B, like the cNK cells derived from Lin $^-$ CD127 $^-$ IEL (data not shown). Moreover, ILC1 cells share other features with Lin $^-$ CD127 $^-$ IEL, such as the expression of CD160, a functional IL-15 receptor and NKp44 (data not shown). Thus, further work will be needed to clarify the relationship between these ILC and ILC-like subsets.

In the colonic lamina propria of patients with IBD, the closely related ILC1^{10,13} and ILC3¹⁴ populations are expanded, as well as ILC1 in the ileal epithelium¹⁰, while innate IEL are reduced in the duodenal epithelium of celiac disease patients. This suggests that these innate cell types, depending on their localization, play distinct roles in disease pathogenesis.

In conclusion we provide evidence for the complexity and plasticity of the innate intraepithelial cell compartment. This may allow rapid and tailored responses to changes in the environment.



SUPPLEMENTARY FIGURE 1 (RELATED TO FIGURE 1). Flow cytometry data for the heatmap of Fig1C. Graphs depict the percentages of cells expressing Nkp80, CD94, CD16, KIRs (a cocktail recognizing KIR2DL1/KIR2DL2/KIR2DL3/KIR2DS1/KIR2DS2/KIR3DL1), CD122, CD117, LAT, CD5, CD160, Nkp46, IL21R, IL15R α , CD244 and CD18. This data was used to generate the heatmap in Figure 1C. The percentage of T cells, conventional NK (cNK), CD127⁺ NK, Lineage-negative (Lin⁻) and Lin⁻CD127⁺ expressing the marker of interest (y-axis) in blood (n=3-10) and duodenal IEL (n=3-8) samples is shown.



SUPPLEMENTARY FIGURE 2. COMPOSITION OF IEL SUBSETS IN RCDII PATIENTS. IEL were isolated from duodenal biopsies from adults with refractory celiac disease II (RCDII). IEL were stained with antibodies to CD45/CD14/CD19/CD3/CD7/CD56/CD127 and analyzed by flow cytometry. The percentages of Lineage-negative CD127⁻ (Lin⁻CD127⁻), Lin⁻CD127⁺ IEL, conventional NK (cNK) and CD127⁺NK and T cells, gated as in Fig.1A is depicted calculated from all CD45⁺ cells measured. The numbers on the y-axis indicate the patients analyzed.

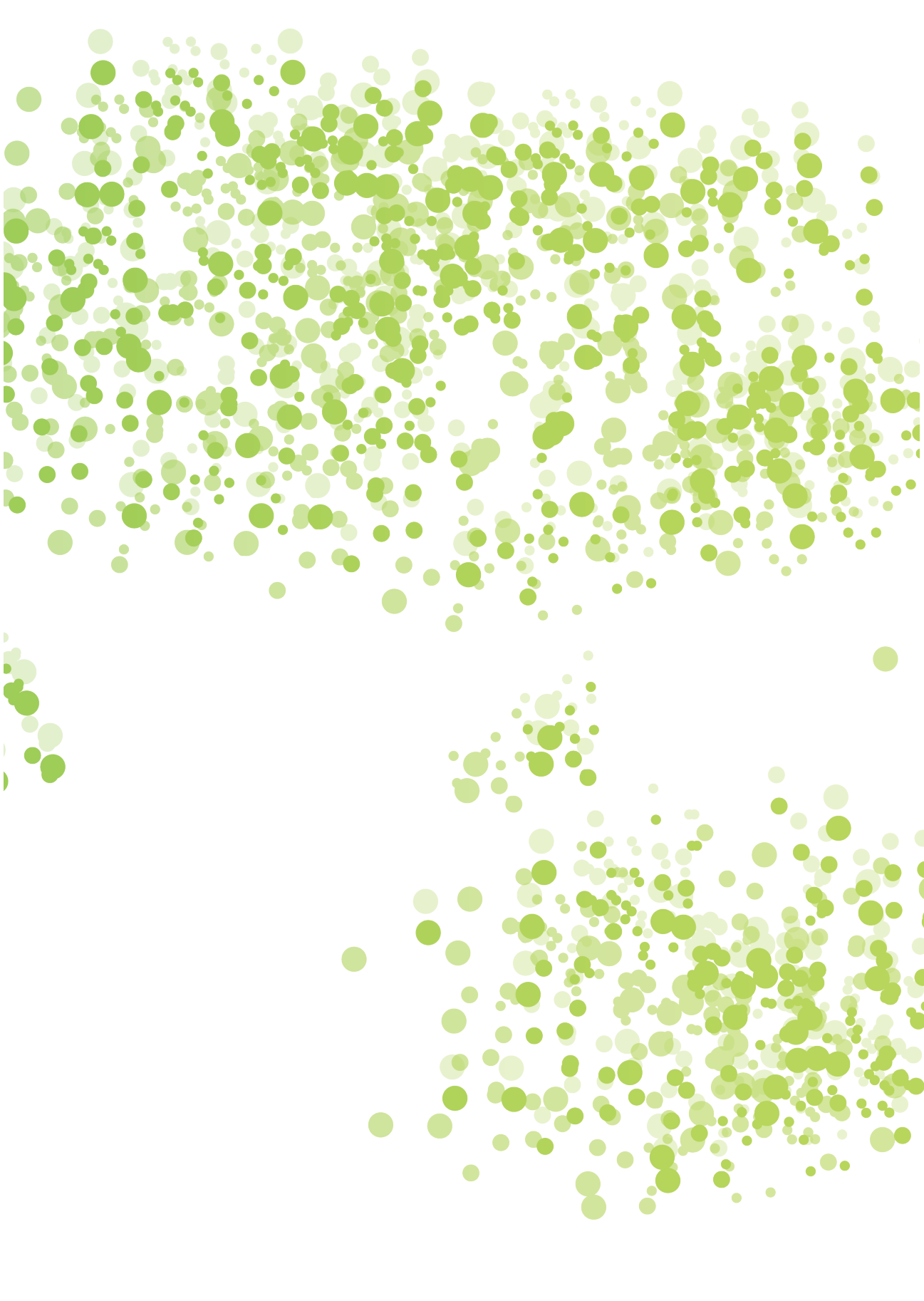
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CHAPTER 5:

HUMAN CD34⁺CD103⁺LINEAGE⁻CD127⁻ THYMOCYTES
HARBOR BIPOENTIAL T/NK CELL PRECURSORS

CHAPTER 5

HUMAN CD34⁺CD103⁺LINEAGE⁻CD127⁻ THYMOCYTES HARBOR BIPOTENTIAL T/NK CELL PRECURSORS

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ABSTRACT

The thymic environment primarily supports T cell maturation, but thymic NK cells and NK cell precursors have also been identified. We recently identified a CD34⁺CD103⁺Lin⁻CD127⁻ population in the small intestinal epithelium that contained NK and T cell precursors.

Given the presence of NK/T cell precursors and CD103⁺ cells in the thymus, we here examined the existence, phenotype and NK/T cell differentiation potential of thymic CD34⁺CD103⁺Lin⁻CD127⁻ (now referred to as thymic CD103⁺Lin⁻CD127⁻) cells.

Thymic CD103⁺Lin⁻CD127⁻ cells were phenotypically similar to thymic NK cells: CD5⁺, Nkp46⁺, KIR⁺. In contrast to mature NK cells, however, the cells lacked CD16 and CD56. Furthermore, thymic CD103⁺Lin⁻CD127⁻ cells were devoid of markers commonly used to identify T cell precursor stages such as CD3, CD4, CD8, CD34 and CD1a, but a significant proportion of cells expressed CD3e intracellularly.

Upon culture with OP9DL1, thymic CD103⁺Lin⁻CD127⁻ cells primarily developed into mature NK cells in an IL-15-dependent fashion. However, in clonogenic assays single CD103⁺Lin⁻CD127⁻ cells gave rise to mixed populations of NK cells, immature and mature T cells and undifferentiated Lin⁻ cells.

In conclusion, this study shows that human CD103⁺Lin⁻CD127⁻ thymocytes resemble thymic NK cells and contain bi-potential NK/T cell precursors.

ABBREVIATIONS

IEL- Intraepithelial Lymphocytes, Lin- Lineage, DN- Double-negative, DP- Double-positive, PBMC- Peripheral Blood Mononuclear Cells, TdT- Terminal Deoxynucleotidyl Transferase

INTRODUCTION

T and NK cells share a common progenitor that derives from hematopoietic stem cells in the bone marrow. These progenitors can differentiate into NK cells locally, but they can also seed the thymus to differentiate into T cells. In the thymus, the precursor cells are initially Lineage-negative (Lin⁻): they lack markers for all mature lineages such as T, B, myeloid and NK cells. The earliest T cell progenitors are Lin⁻, CD34⁺, CD1a⁻ and CD4⁻CD8⁻ (double-negative or DN). Subsequently, these DN thymocytes mature via an immature single-positive (ISP CD4⁺) and a double-positive (DP) stage into CD4⁺ or CD8⁺ SP T cells that exit the thymus. Rearrangement of the TCR loci takes place from the earliest DN stage (DN1) to the DP CD3⁺ stage and culminates in the expression of TCR/CD3 complexes on the surface of DP CD3⁺ thymocytes. With this progression along the T cell path, CD34 is lost together with the potential to differentiate into NK cells. The latest stage at which thymocytes developing into T cells can still become NK cells is the second DN stage (DN2), when the cells are still CD34⁺.

A subset of DN and CD8 SP thymocytes expresses CD103 (αE), which together with the integrin β7 forms a receptor for E-cadherin, expressed on thymic epithelial cells ¹. The function of these CD103⁺ thymocytes is currently unclear.

We recently described a CD103⁺ and CD34⁻ Lineage-negative (Lin⁻) cell population in the epithelium of the small intestine. Despite the lack of CD34, this population still had the capacity to develop into T and NK cells in in vitro differentiation assays. In the current study, we characterize a similar population in the thymus, and show that these CD34⁻CD103⁺Lin⁻CD127⁻ thymocytes (now referred to as thymic CD103⁺Lin⁻CD127⁻) harbor bipotential T/NK cell precursors.

MATERIALS AND METHODS

Human specimens

Thymus was obtained as surgical tissue discards from children undergoing cardiac surgery. Informed consent was given for all human specimens after medical ethical commission approval and in accordance with the local ethical guidelines of the ErasmusMC and in accordance with the declaration of Helsinki.

Cell culture

Thymocytes were isolated as described ¹⁶. OP9 or OP9-DL1 cells were irradiated (1500 RAD) and plated at 5000 cells/well in 96-well plates. Lineage-negative (Lin⁻) cells were sorted from CD3-depleted (anti-CD3 MACS beads, Miltenyi, Bergisch Gladbach, Germany) thymocytes as CD34⁻CD103⁺Lin⁻CD127⁻ [CD45⁺CD14⁻CD19⁻CD3⁻CD7⁺CD56⁻CD127⁻CD103⁺CD34⁻] or as CD34⁺CD1a⁻ [CD45⁺CD14⁻CD19⁻CD3⁻CD34⁺CD1a⁻] and plated at 1, 100 or 1000 cells/well onto OP9 or OP9-DL1. These co-cultures were maintained in Iscove's modified Dulbecco's medium (IMDM; LonzaVerviers, Verviers, Belgium) containing 10 % pooled normal human serum (NHS), 5 ng/ml human IL-7 (Peprotech, Rocky Hill, NJ, USA), 10 ng/mL human SCF, 10 ng/ml Flt3L and if stated 10 ng/ml IL-15 (all R&D systems Europe, Abingdon, UK) for up two weeks. Clonal CD103⁺Lin⁻CD127⁻ thymic cells were sorted at 1cell/well on 100.000 mixed irradiated allogeneic PBMC, 1 µg/ml phytohemagglutinin, 20 Cetus u/ml IL-2 (Proleukin, Chiron corporation, Emeryville, CA) and 10 ng/ml IL-15 ('feeder mix'). Clones were maintained on IMDM/10% NHS containing 10 ng/ml IL-15 and 20 Cetus units/ml IL-2 and restimulated with 'feeder mix' when appropriate.

Flow cytometry

Fluorochrome-labelled antibodies for CD3ε, CD4, CD5, CD7, CD16, CD45, CD56, CD103, CD117, CD122, CD14, CD19, CD34, KIR3DL1 (DX9) and TCRγδ were from BD Biosciences (San Jose, CA, USA). KIR2DL2/DL3/DS2 (GL183), KIR2DL1/2DS1 (EB6), CD244, and NKp46 were from Beckman Coulter (Fullerton, CA, USA). TCRαβ was from Biolegend (San Diego, CA, USA). Anti-CD127, CD45 and CD160 were from eBioscience (San Diego, CA, USA) and CD8 from Invitrogen (Bleiswijk, The Netherlands). Cells were incubated for 30 minutes with fluorochrome-conjugated antibodies for surface staining, and subsequent intracellular staining was done using the permabilisation kit from eBioscience. Cells were acquired on a LSRII or on a FACSAriaII (BD Biosciences) for cell sorting. Results were analyzed using FlowJo V10 software, and 4-GraphPad. Unsupervised hierarchical clustering of flow cytometry measurements was done using the complete clustering method with the Euclidean distance measurement. The result was plotted in a heatmap using the heatmap.2 function from the R gplots package.

Acknowledgments

We thank E. de Haas and G. de Roo for flow cytometric cell sorting.

RESULTS

THE HUMAN THYMUS CONTAINS CD103⁺LIN⁻CD127⁻ CELLS THAT RESEMBLE THYMIC cNK CELLS

We recently identified a Lin⁻CD127⁻ IEL population in the human intestinal epithelium with NK and T cell differentiation potential: [CD45⁺SSC^{low}CD14⁻CD19⁻CD3⁻CD7⁺CD4⁻CD8⁻CD56⁻CD127⁻CD34⁻CD103⁺]². Given the reported presence of CD103⁺ DN cells in the thymus¹, we considered the possibility that cells with this Lin⁻CD127⁻ IEL phenotype were also present in the thymus.

Indeed, flow cytometric analysis of human thymocytes (Figure 1A) revealed a distinct population of CD103⁺ cells within Lin⁻CD127⁻ thymocytes (referred to as CD103⁺Lin⁻CD127⁻ thymocytes from now on). Like Lin⁻CD127⁻ IEL, these CD103⁺Lin⁻CD127⁻ thymocytes lacked surface expression of CD4, CD8, CD34 and CD1a, and a sizeable fraction expressed intracellular CD3ε (Figure 1A).

Using an expanded set of surface markers, we compared the phenotype of CD103⁺Lin⁻CD127⁻ thymocytes to Lin⁻CD127⁻ IEL and other lymphocyte subsets within the intestinal epithelium, the blood and the thymus. Unsupervised hierarchical clustering analysis of the results showed that CD103⁺Lin⁻CD127⁻ thymocytes most resembled conventional thymic CD3⁻CD56⁺ NK cells (cNK, Figure 1B). Similar to thymic cNK cells, CD103⁺Lin⁻CD127⁻ thymocytes expressed NKp46, CD244 and killer cell immunoglobulin-like receptors (KIRs). However, CD103⁺Lin⁻CD127⁻ thymocytes were, by definition, CD56⁻. Also they less frequently expressed CD160 and CD16 and expressed lower levels of CD122 (IL-2/15Rβ) than thymic cNK cells.

These experiments revealed several notable differences between CD103⁺Lin⁻CD127⁻ thymocytes and Lin⁻CD127⁻ IEL. While CD103⁺Lin⁻CD127⁻ thymocytes expressed CD160, CD122, and CD16 at lower levels than Lin⁻CD127⁻ IEL, they did express KIRs. Finally, CD103⁺Lin⁻CD127⁻ thymocytes did not express intracellular TdT (Figure 1C), expressed during rearrangement of the TCR locus. In summary, the human thymus contains CD103⁺Lin⁻CD127⁻ cells that are similar to, but distinct from thymic cNK cells and Lin⁻CD127⁻ IEL.



(B) Thymocytes, duodenal intraepithelial lymphocytes (IEL) and PBL from peripheral blood were stained with a standard panel of antibodies against CD45/CD14/CD19/CD3/CD7/CD56/CD127, gated as in (A) and combined with antibodies detecting CD160, Nkp46, CD122, CD16 KIR (a cocktail recognizing KIR2DL1/ KIR2DL2/ KIR2DL3/ KIR2DS1/ KIR2DS2/ KIR3DL1), CD244 and analyzed by flow cytometry. A heatmap was generated from the flow cytometric data by unsupervised hierarchical clustering. The heatmap depicts the z score generated by the average percentages of cells expressing the above-mentioned markers or the average mean fluorescence intensity (MFI) of the marker per subset (see color key). Analyzed cell populations were: T cells; conventional NK (cNK); Lin⁺CD127⁺ and Lin⁺CD127⁻ cells from thymocytes, IEL and PBL; CD127⁺NK

from IEL and PBL. Thymic Lin⁻CD127⁻ were additionally stained for CD103 (Lin⁻CD127⁻ thymus). **(C)** Thymocytes were stained, analyzed and gated as described in (A). A histogram depicts the TdT expression of CD103⁺Lin⁻CD127⁻ and CD34⁺ thymocytes. The percentage of TdT⁺ positive cells is indicated in the histogram. The cell count is depicted on the y-axis.

IL-15-DEPENDENT DIFFERENTIATION OF CD103⁺LIN⁻CD127⁻ THYMOCYTES INTO NK CELLS

Our previous results indicated that Lin⁻CD127⁻ IEL contain precursors to NK and T cells (Chapter 4). To test the ability of thymic CD103⁺Lin⁻CD127⁻ cells to develop into NK or T cells, we made use of OP9DL1-based stromal developmental assays, optimized for T cell differentiation³. Because thymic CD103⁺Lin⁻CD127⁻ cells showed similarities to NK cells, including the expression of CD122 (IL-2/15Rβ), we first tested the effect of IL-15, a critical factor in NK cell differentiation⁴⁻⁶.

In the absence of IL-15, FACS-sorted thymic CD103⁺Lin⁻CD127⁻ cells cultured on OP9DL1 in the presence of SCF, IL-7 and Flt3L did not expand within two weeks (Figure 2A). In the presence of IL-15 however, cultures starting from 100 and 1000 thymic CD103⁺Lin⁻CD127⁻ cells yielded thousands of CD45⁺ cells within the same time frame. Under these conditions, thymic CD103⁺Lin⁻CD127⁻ yielded comparable numbers of CD45⁺ cells as thymic CD34⁺CD1a⁻ cells, the earliest T/NK cell progenitors in the human thymus⁶ (Figure 2A).

Upon co-culture with OP9DL1 in the presence of IL-15, CD103⁺Lin⁻CD127⁻ thymocytes developed into CD3⁺CD56⁺ cNK cells (Figure 2B). In line with this, many of these NK cells expressed intermediate or high levels of CD8α. In contrast, under identical culture conditions CD34⁺CD1a⁻ thymocytes yielded a mixture of Lin⁻, NK and T cells (Figure 2B and data not shown). Lin⁻ cells derived from CD34⁺CD1a⁻ thymocytes had often acquired CD4 and occasionally CD8α (Figure 2B), suggesting that they had differentiated into CD4⁺ ISP.

Thus, under conditions that allowed differentiation into the NK as well as the T cell lineage, CD103⁺Lin⁻CD127⁻ thymocytes developed exclusively into NK cells.

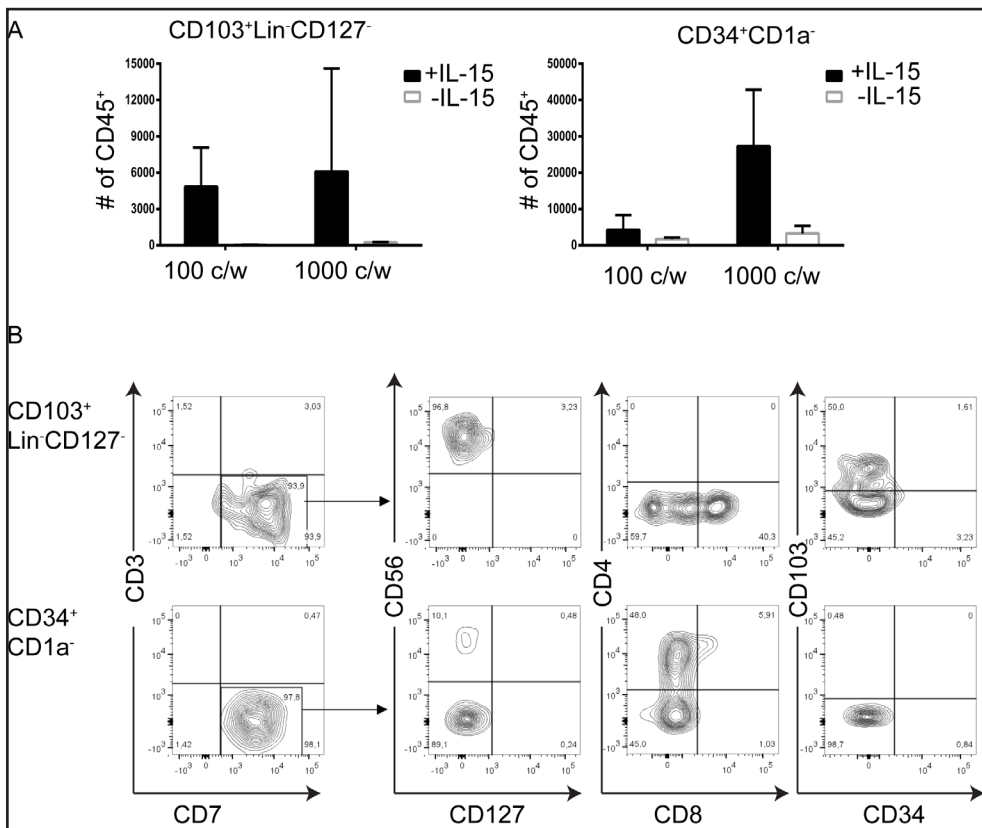


FIGURE 2. CD103⁺LIN⁻CD127⁻ THYMOCYTES ARE IL-15-DEPENDENT AND DEVELOP INTO NK CELLS ON OP9DL1. (A) Human thymocytes were stained with antibodies to CD45, CD14, CD19, CD3, CD7, CD56, CD127, CD103, CD4, CD8, CD34, CD1a and analyzed by flow cytometry. CD34⁺CD103⁺LIN⁻CD127⁻ (CD4⁺CD8⁻) and CD34⁺CD1a⁻ thymocytes were sorted at 100 cells/well or 1000 cells/well on OP9DL1. Sorted cells were co-cultured with FLT3L, SCF, IL-7 with or without IL-15 (-IL-15), harvested and analyzed by flow cytometry. The graph depicts the amount of CD45⁺ (# of CD45⁺) after 1 and 2 weeks of OP9DL1 co-culture with IL-15 (+IL-15) or without IL-15 (-IL-15). **(B)** A representative FACS plot depicting the phenotype of CD103⁺LIN⁻CD127⁻ and CD34⁺CD1a⁻ cells, obtained after a one week culture of 1000 FACS-sorted cells on OP9DL1 in the presence of FLT3L, SCF, IL-7 and IL-15 after 1 week of co-culture. Gates were set on CD45⁺ cells. The phenotype of CD3⁺CD7⁺ cells is shown in the right panel with respect to CD56, CD127, CD4, CD8, CD103 and CD34 expression.

THYMIC CD103⁺LIN⁻CD127⁻ CELLS CONTAIN BIPOTENTIAL NK/T CELL PRECURSORS

In an effort to obtain thymic CD103⁺LIN⁻CD127⁻ clones, we cultured single CD103⁺LIN⁻CD127⁻ thymocytes with irradiated mixed allogeneic feeder cells, phytohemagglutinin (PHA), IL-2 and IL-15. After expansion, most of these cultures contained a mixture of cells that had retained the CD3⁺CD56⁻ phenotype, CD3⁺CD56⁺ NK cells and CD3⁺CD56⁻ T cells (Figure 3A). A minority of cultures contained CD3⁺CD56⁻ and CD3⁺CD56⁺ cells or CD3⁺CD56⁺ cells only (Figure 3C). While a fraction of CD3⁺CD56⁺ NK cells, like peripheral blood NK cells, expressed CD8 α , the CD3⁺ T cells arising from single CD103⁺LIN⁻CD127⁻ thymocytes had not fully differentiated (Figure 3B). Most T cells expressed neither CD4 nor CD8, and only a small minority expressed CD4.

What's more, approximately half expressed the TCR $\alpha\beta$, but the other half of the CD3⁺ cells was TCR-negative. This suggested that these CD3⁺TCR⁻ cells expressed a pre-TCR α /TCR β complex.

As single CD103⁺LIN⁻CD127⁻ thymocytes yielded mixed cultures of Lin⁻ cells, NK cells and immature and mature T cells, these results formally demonstrated that CD103⁺LIN⁻CD127⁻ thymocytes contain bipotential NK/T cell precursors.

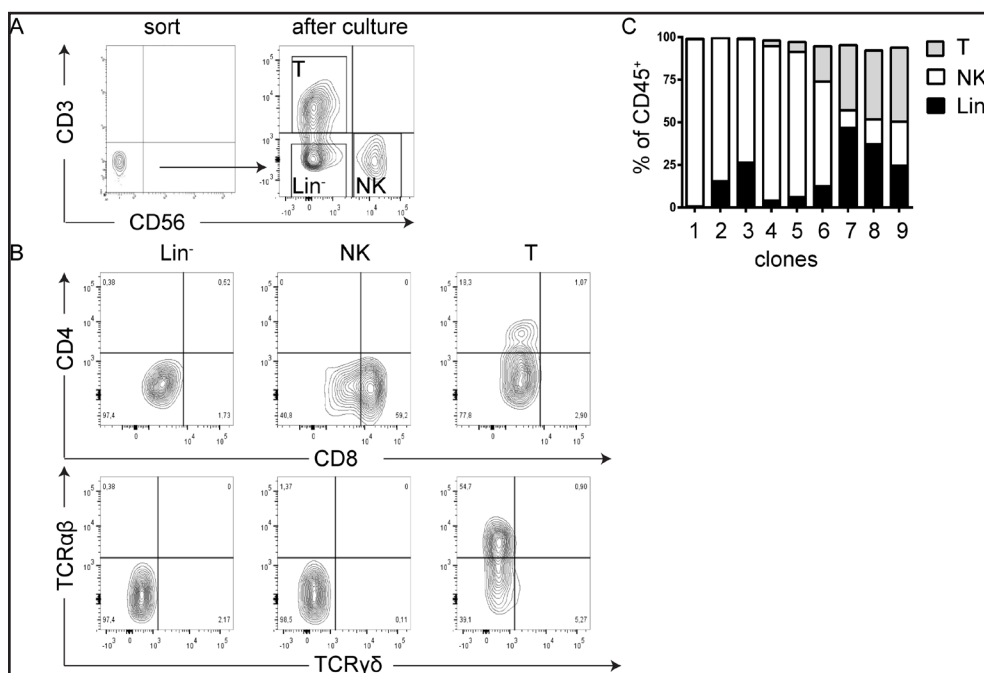


FIGURE 3. CD103⁺LIN⁻CD127⁻ THYMOCYTES HARBOR BI-POTENTIAL NK/T CELL PRECURSORS.

Human thymocytes were stained and analyzed as described in Fig.1A and CD34⁺CD103⁺LIN⁻CD127⁻ (CD4⁺CD8⁺) thymocytes were sorted at 1 cell/well on mixed irradiated allogeneic PBMC and cultured in the presence of 10 ng/ml IL-15 and 20 U/ml IL-2. After 3-4 weeks, the resulting cultures were analyzed by flow cytometry. Gates were set on CD45⁺ cells. **(A)** The graph depicts the FACS sort gate used to isolate CD103⁺LIN⁻CD127⁻ thymocytes (sort) and the expression of CD3 and CD56 on one of the cultures resulting from the expansion of a single CD103⁺LIN⁻CD127⁻ thymocyte (after culture). **(B)** Analysis of the expression of CD4, CD8, TCRαβ and TCRγδ by the CD3⁺CD56⁻ (Lin⁻), CD3⁺CD56⁺ (NK) and CD3⁺CD56⁺ (T) cells present in the culture depicted in Figure 3A. **(C)** Overview over the percentages of CD3⁺CD56⁻ (Lin⁻), CD3⁺CD56⁺ (NK) and CD3⁺CD56⁺ (T) cells present in cultures arising from single cell cultures of CD103⁺LIN⁻CD127⁻ thymocytes.

DISCUSSION

This study shows that human CD103⁺Lin⁻CD127⁻ thymocytes resemble thymic NK cells and contain bipotential NK/T cell precursors. This finding is surprising for several reasons.

First, CD103⁺Lin⁻CD127⁻ thymocytes did not express CD34, expressed on developing lymphocytes with the potential to develop into NK as well as T cells. Although CD3⁺CD4⁺CD8⁺CD34^{bright} thymocytes are capable of differentiating into NK and T cells, CD3⁺CD4⁺CD8⁺CD34^{dim} thymocytes have already lost the ability to differentiate into T cells ⁶. Second, using a single-cell culture protocol similar to ours, a previous study identified CD3⁺CD4⁺CD8⁺CD56⁺CD5⁺CD34⁻ thymocytes, encompassing CD103⁺Lin⁻CD127⁻ cells, as committed NK cell precursors without T cell potential ⁷. Third, KIR expression frequencies on CD103⁺Lin⁻CD127⁻ thymocytes were comparable to those on thymic conventional NK cells (cNK). Since acquisition of KIR is a late event in NK cell differentiation ⁸, it is likely that a subset of CD103⁺Lin⁻CD127⁻ thymocytes was already committed to the NK cell lineage. Finally, although T cell differentiation is usually notch-dependent ^{6,7,9,10}, in the case of CD103⁺Lin⁻CD127⁻ cells T cell development did not require notch-signaling, as it occurred using irradiated PBMC, but not in the presence of OP9 cells expressing the notch-ligand DL1.

In conclusion, our results indicate that CD103⁺Lin⁻CD127⁻ thymocytes are an unconventional thymocyte subset that contains – most likely KIR-negative- cells with the ability to differentiate into NK cells and into T cells in a notch-independent fashion.

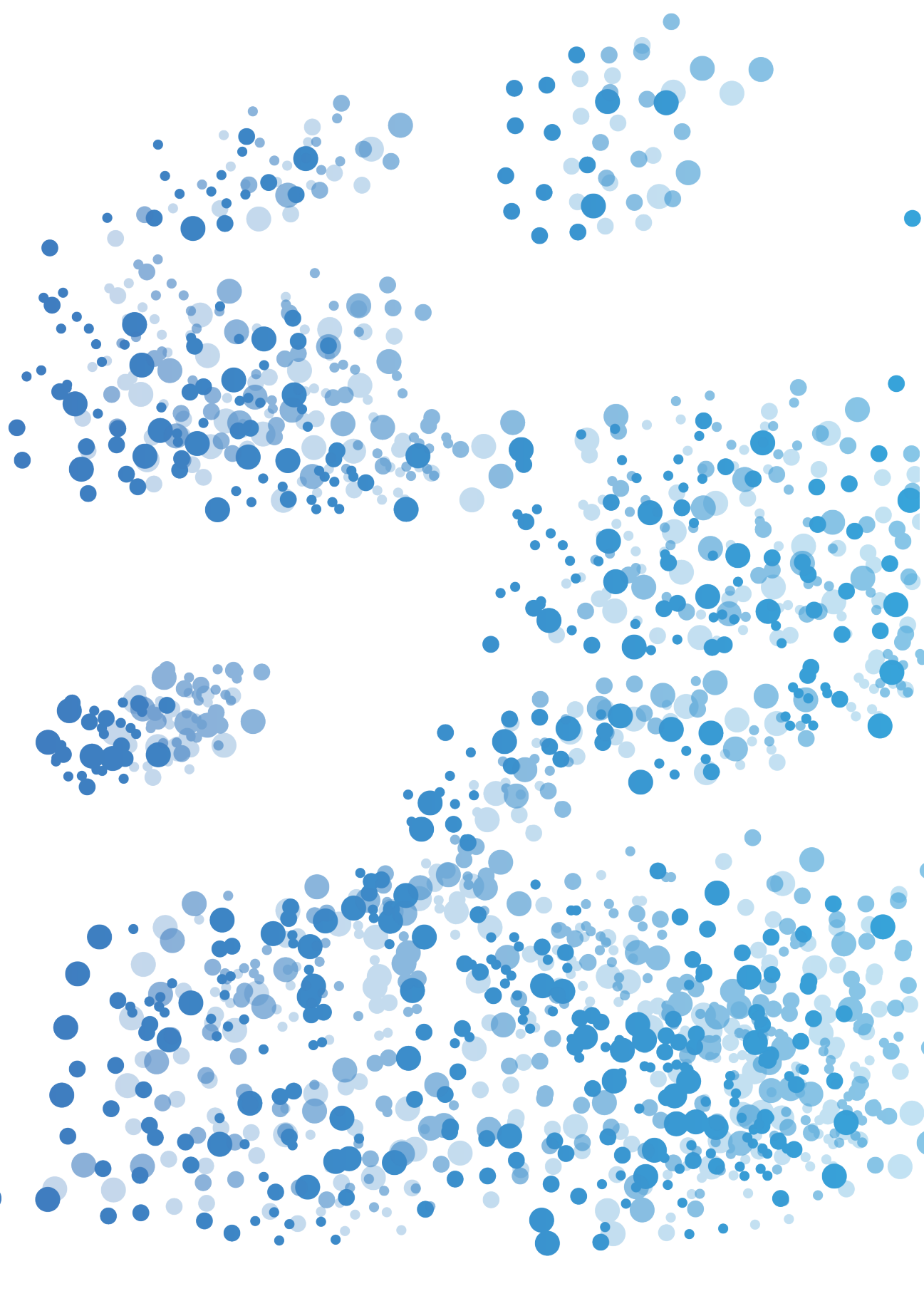
Given the above, it is challenging to place the CD103⁺Lin⁻CD127⁻ thymocytes in previously described NK and T cell developmental pathways. An unusual feature of these cells is the fact that many express intracellular (ic) CD3ε. As icCD3ε can be induced by notch-signaling ¹¹ the presence of icCD3ε may reflect ongoing notch-signaling in the thymic environment or represent a relic of previous T cell lineage differentiation events ¹². As notch-signaling was not required for T cell development of CD103⁺Lin⁻CD127⁻ thymocytes, these cells most likely had already undergone TCR rearrangement prior to expansion. This would place these cells somewhere between the CD34⁺ DN3 and CD34⁺ DN4 stage of thymic T cell differentiation, when the cells are going through the final steps of the TCR rearrangement process. However, in that case one would have to postulate that CD103⁺DN3/DN4, unlike regular DN3/DN4 cells, still have the capacity to develop into NK cells. This is not inconceivable, as thymic NK cells express icCD3ε ^{13,14} and TCR transcripts ¹⁵, suggesting that they can derive from later stages of T cell development.

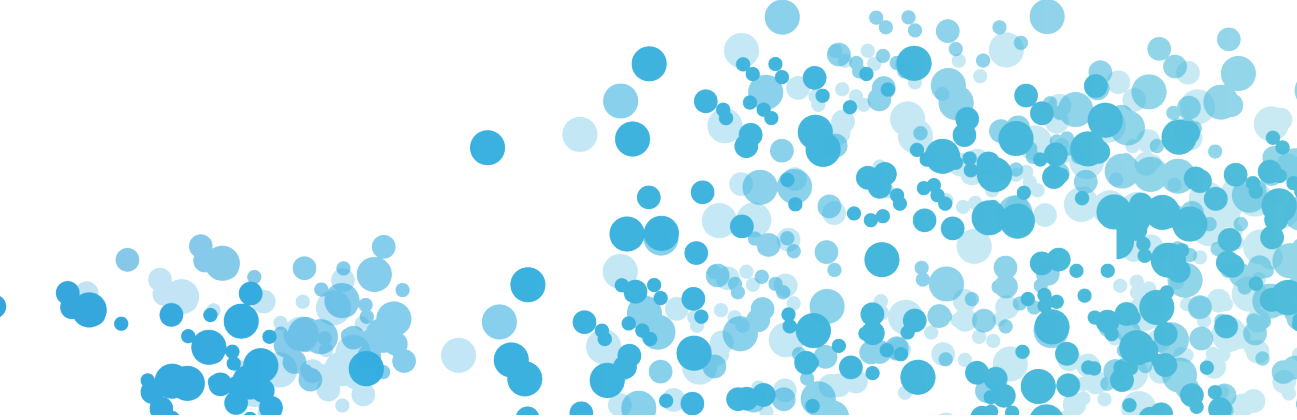
Thus, it will be important to test the TCR rearrangement status of CD103⁺Lin⁻CD127⁻ thymocytes.

In conclusion, our studies identified a novel thymocyte population that does not easily fit in the known thymocyte developmental pathways, but that does have the ability to give rise to NK and T cells.

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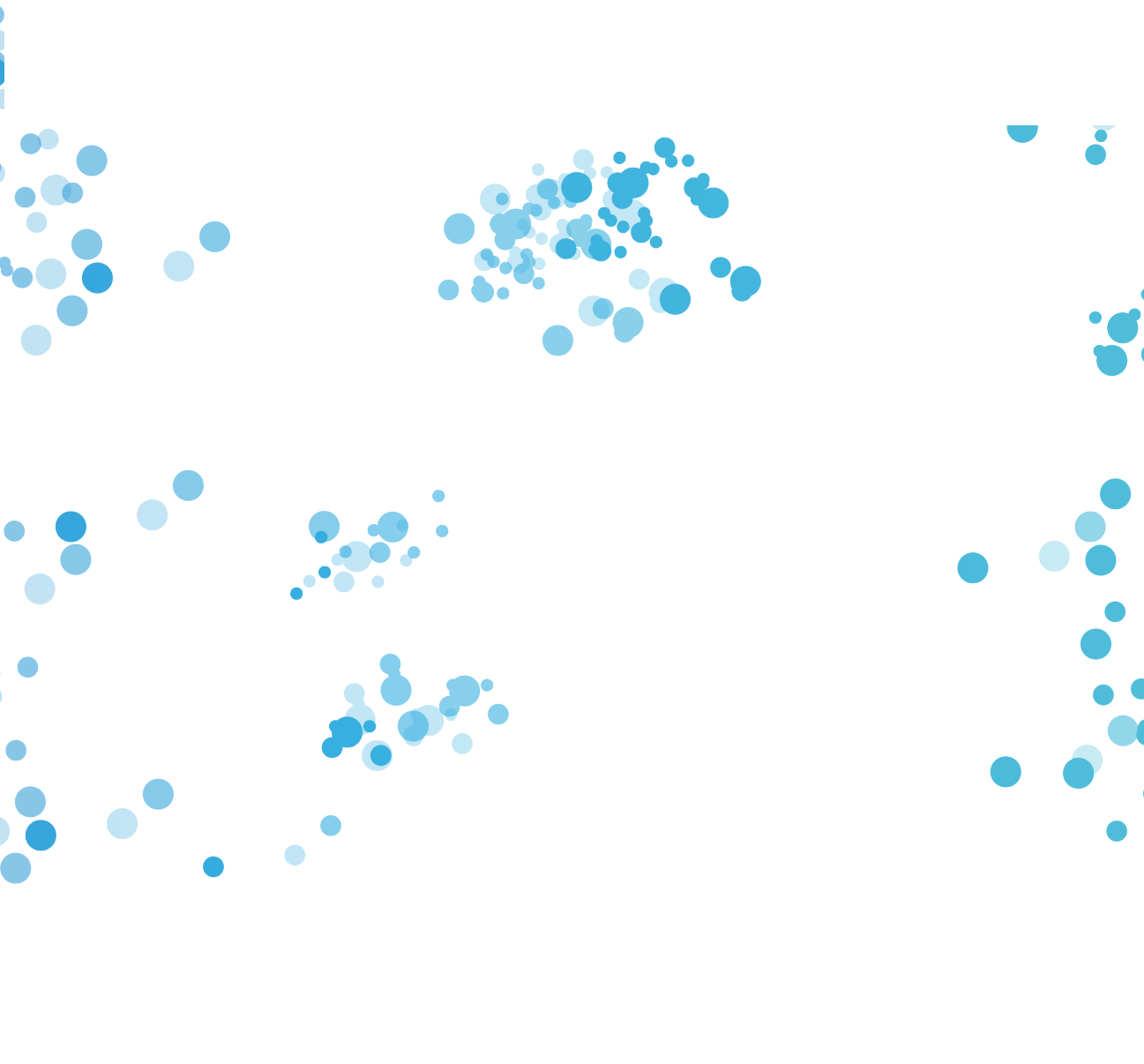
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CHAPTER 6:

GENERAL DISCUSSION



CHAPTER 6

GENERAL DISCUSSION

WHAT IS ALREADY KNOWN ABOUT RCD AND CD3⁺ IEL?

In recent years, the pathological mechanisms leading to small intestinal damage in CD have been elucidated, in particular the nature of the pro-inflammatory gluten-specific T cell response in the lamina propria (LP) and the receptor-ligand interactions leading to cytotoxicity of intraepithelial lymphocytes (IEL) towards enterocytes in the epithelium. However, refractory celiac disease (RCD), where gluten removal does not lead to remission of celiac symptoms, is incompletely understood. Aberrant IEL, which massively expand in the epithelium of RCDII patients, have been demonstrated to participate actively in cytotoxicity towards the epithelium^{1,2} and IL-15 has been shown to be a major factor that drives the expansion of such cells³. It is currently unknown whether IL-15 is solely responsible for the outgrowth of these cells in RCDII. Moreover, the origin of aberrant IEL has also remained elusive, but it was suggested that they could originate from CD3⁺ IEL⁴. Several studies focusing on CD3⁺ IEL have indicated that this population is heterogeneous and contains functional NK cells⁵, ILC1⁶, as well as IEL with unknown functions, which express numerous NK cell receptors^{5,7-9}. While it is clear that T cell development predominantly takes place in the thymus¹⁰, evidence for T cell differentiation in the intestine has been presented^{11,12}. Such extrathymic, intestinal T cell development, however, is subject of a long standing debate and intestinal NK cell development has only been described to occur in the lamina propria¹³.

WHAT IS ALREADY KNOWN?

- IL-15 induces the expansion and survival (anti-apoptotic effects) of aberrant IEL;
- Aberrant IEL were postulated to be either derived from mature T-IEL or from an ill-defined CD3⁺ IEL population;
- CD3⁺ IEL are heterogeneous, express a variety of NK cell receptors and consist of functional NK cells and ILC1;
- CD3⁺ IEL harbor T cell precursors, the phenotype and developmental requirements of which have not been elucidated;
- NK cell precursors have been described as CD117⁺ cells in the intestinal LP.

HOW DID OUR STUDIES CHANGE THE CURRENT UNDERSTANDING OF RCD AND IEL?

DC-INDUCED EXPANSION OF ABERRANT IEL LINES FROM RCDII PATIENTS

In **Chapter 2** we outline our studies on the effect of blood-borne cell types on aberrant IEL lines from RCDII patients, which demonstrated that dendritic cells (DC), prominent cell types of the intestinal innate immune system, could induce proliferation and had anti-apoptotic effects. As in recent years it has been demonstrated that IL-15 can induce proliferation^{3,14} and has anti-apoptotic effects on aberrant IEL¹⁵, our results thus indicate that multiple factors can contribute to the expansion of aberrant IEL in RCD.

In active CD lesions, increased numbers of macrophages and DC have been demonstrated, which is likely the result of the ongoing inflammation¹⁶. Until now no studies for RCD patients have been conducted, but it is likely that due to the severe intestinal inflammation in these patients increased numbers of cells of the monocytic lineage can also be expected. Hence, increased frequencies of monocytic cells, such as DC, could contribute to the expansion and subsequent dissemination of aberrant IEL into the underlying LP, the site where most DC reside.

As our studies made use of cell lines derived from RCDII patients, we can only hypothesize that such proliferative effects indeed happen *in situ*. It remains to be investigated to what extent DC can contribute to the expansion of aberrant IEL, why aberrant IEL are particularly sensitive to DC-induced proliferation and if all DC subsets are equally stimulatory for aberrant IEL. Moreover, the sensitivity towards DC-induced proliferation could reflect the stage of malignancy, as not all aberrant IEL lines were equally responsive to stimulation with DCs. Nevertheless, regular T-IEL lines showed no proliferative effect in DC co-cultures. It therefore needs to be investigated, if the response to DC correlates with malignant transformation, or the localization of aberrant IEL *in vivo*. Ultimately, this knowledge could be used to develop novel biomarkers for disease and/or novel therapeutic approaches.

PLASTICITY OF THE INNATE IEL COMPARTMENT: ABERRANT IEL LIKELY ORIGINATE FROM LIN⁺CD127⁻ IEL

The appearance and expansion of aberrant IEL is a characteristic feature which distinguishes RCDI from RCDII. RCD is a severe complication of CD and possibly due to long-term gluten exposure in patients that are not properly diagnosed with CD¹⁷. Evidence for an involvement of the gluten-specific T cell response in the development of RCD is the fact that these patients are often HLA-DQ2 homozygous¹⁸ which leads to stronger gluten-specific T cell responses¹⁹. Aberrant IEL in RCDII patients are considered pre-malignant, as these cells show the same phenotype and monoclonal TCR rearrangements as the malignant cells detected in the enteropathy-associated T cell lymphoma (EATL)²⁰. In order to understand the mechanisms driving the expansion and malignant transformation of these aberrant IEL, knowledge on their origin is essential. It has been hypothesized that aberrant IEL either originate from mature T cells²¹ or from a physiological CD3⁻ population⁴.

In **Chapter 3** we present evidence that aberrant IEL in RCDII can be characterized as Lineage-negative (Lin⁻) CD127⁻ IEL (Lin⁻CD127⁻ IEL), that these cells were indeed present in non-RCDII individuals and that Lin⁻CD127⁻ IEL shared important features with their aberrant IEL counterparts. Though the use of multicolor flow cytometry and the explicit phenotypical characterization of aberrant IEL as Lin⁻CD127⁻ IEL, it now became feasible to refine the common phenotype of aberrant IEL described by several groups^{3,14,15,20-25} and to investigate its physiological counterpart in depth.

First described in EATL patients by Spencer et al.⁴, the CD3⁻ IEL population has been further investigated (as CD3⁻CD7⁺) in the following years. Since this IEL population lacks receptors for adaptive lymphocytes, such as CD3/TCR (or B cell markers) we termed this population “*innate IEL*”. Previous studies have analyzed the expression of various NK and T cell markers, as well as adhesion molecules and markers of early progenitor cells on this innate IEL population (Table 1). Whereas the majority of innate IEL expressed the IL-2/15R β (CD122)^{7,8}, the markers CD56⁷,⁸, CD16⁷⁻⁹ and CD94^{7,8} were only expressed by some innate IEL. Killer-cell immunoglobulin-like receptor (KIR) expression was not detected^{7,8} and innate IEL did not stain for the T cell marker CD5^{7,9}, but were partially icCD3 ϵ ⁺⁵. Our studies validated and further defined the expression pattern of these markers on innate IEL subsets. As suggested by previous studies, innate IEL reflected a phenotype reminiscent of immature lymphocytes, therefore early progenitor markers such as TdT, CD34 and c-kit (CD117) were tested, but not detected⁵. In line with the latter study, we also did not detect any TdT and CD34 expression on innate IEL, although CD117

expression was detected on a subset of Lin⁺CD127⁺ IEL (**Chapter 4**). Together these studies clearly demonstrated that the innate IEL population is heterogeneous and harbors functional NK cells⁵, but also harbors other (immature) cell types. In fact, on the basis of the expression or absence of the NK cell receptor CD56 and the IL-7R (CD127), we were able to define four subpopulations within CD3⁺CD7⁺ innate IEL. These include 1.) conventional NK cells (cNK) or ILC1 (CD56⁺CD127⁺) -in this thesis referred to as cNK-, 2.) CD127⁺ NK (CD56⁺CD127⁺), 3.) Lin⁺CD127⁻ (CD56⁺CD127⁻) and 4.) Lin⁺CD127⁺ (CD56⁻CD127⁺) IEL (Figure 1). Our results further support the finding that T-IEL expressed a distinct phenotype as that of innate IEL (Table 1, **Chapter 4**).

Moreover, our studies revealed that Lin⁺CD127⁻ IEL were present in high frequencies in young children without CD, while in CD patients the frequencies of these cells were significantly decreased in comparison to non-CD controls, supporting previous findings which demonstrated that in CD the innate IEL population is reduced in comparison to controls^{4, 5, 7, 8, 26, 27}. In contrast, we observed that in RCDII patients Lin⁺CD127⁻ IEL and Lin⁺CD127⁺ IEL are massively increased (**Chapter 4**). In Chapter 4 we conclude that both disease and age influence the composition of the distinct innate IEL subsets (Figure 1). Our results therefore extend early findings by MacDonald et al., in which the authors noted a steady increase in T-IEL and changes in CD3⁺ IEL with age^{11, 12, 28, 29}.

As Lin⁺CD127⁻ IEL lacked lineage markers (CD14/CD19/CD3/CD56/CD34) and the IL-7R (CD127), Lin⁺CD127⁻ IEL were neither of monocyte, B, T or NK cell lineage, nor innate lymphoid cells type 2 or 3 (ILC2, ILC3), as these ILC are defined as Lin⁺CD127⁺. ILC are innate-like lymphoid cells that have attracted great attention in the last years. Even though these cells compose minor cell populations at mucosal sites, they are notably important in the immune response to bacteria and viruses, as well as for tissue remodeling^{30, 31}. Intestinal ILC were mainly described to reside in the LP. We postulated that Lin⁺CD127⁻ IEL were distinct from their ILC counterparts, due to the lack of CD127 expression (LP- ILC1/ILC2/ILC3 are CD127⁺) and the absence of CD56 expression, which is expressed by intraepithelial ILC1⁶ (Table 2). In fact, as intraepithelial ILC1 show large phenotypic overlap with cNK-described in our studies- these cell types could be identical. So far ILC1 were the only ILC population described to reside in the epithelium⁶. How Lin⁺CD127⁻ IEL and the additional innate IEL subsets described here are positioned in relation to the newly described ILC family requires further investigation. For this detailed studies defining the expression of cytokine receptors and cytokine production, along with an analysis of transcription factors will be crucial^{30, 31} (Figure 2, Table 2).

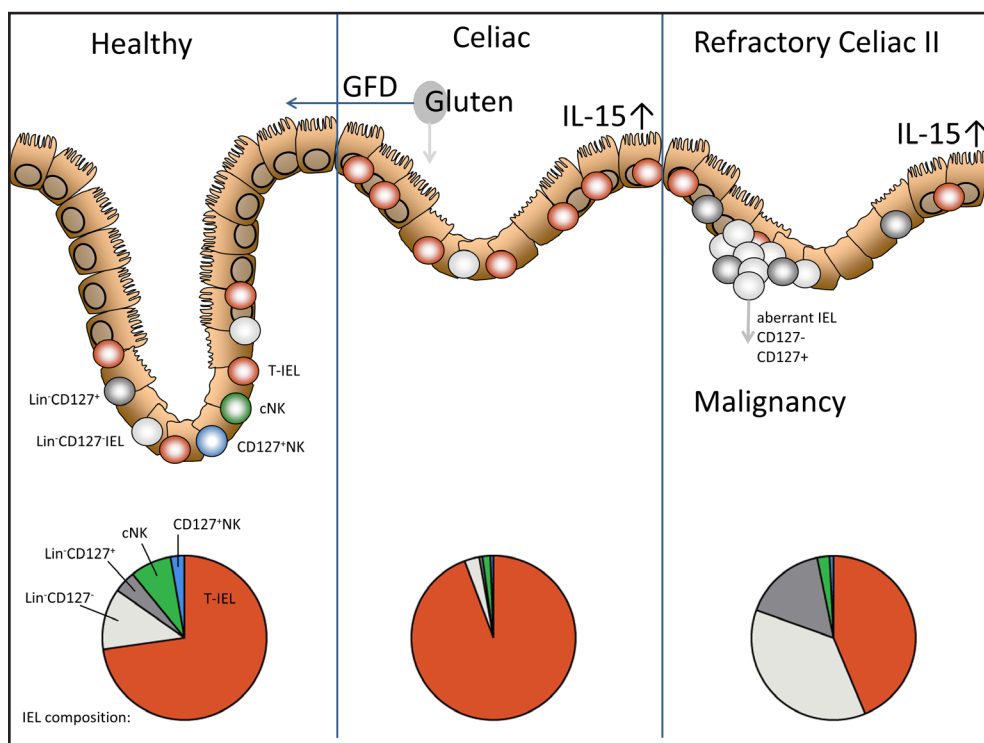


FIGURE 1. HETEROGENEITY OF THE IEL COMPARTMENT. Intraepithelial lymphocytes (IEL) are composed of CD3⁺ T cells (T-IEL) and a diverse innate IEL population (CD3⁻ IEL), which includes conventional NK cells (cNK), CD127⁺ NK cells, Lineage-negative (Lin⁻) CD127⁻ and Lin⁻CD127⁺ IEL. The composition changes rigorously during celiac disease and refractory celiac disease type II. The pie chart below indicates the mean % of the IEL subsets from all CD45⁺ lymphocytes; the colors of the chart correspond to the colors used to discriminate the different IEL subsets in the comic above. From left to right: non-CD children, CD adults, RCDII adults

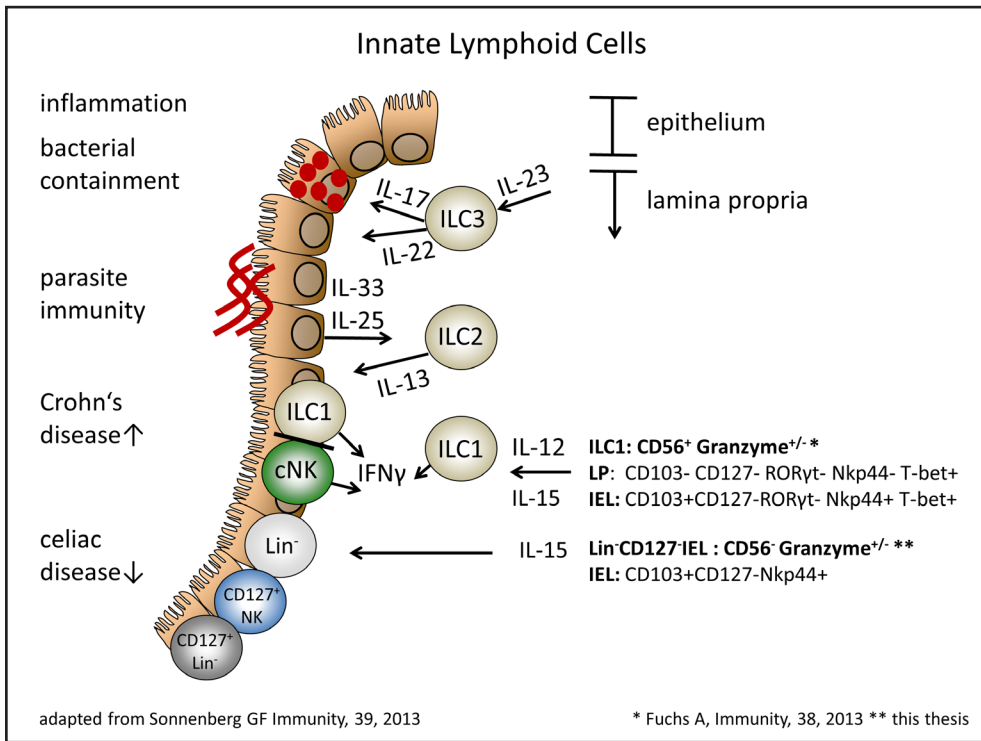


FIGURE 2. INNATE LYMPHOID CELLS AND LIN⁻CD127⁻IEL: A COMPARISON.

This figure is adapted from Sonnenberg et al.³¹ and describes the main findings for ILC subsets, their localization in the intestine and the important cytokines for ILC physiology. Note that ILC1 were the only ILC subset described to reside in the epithelium so far⁶. Their phenotype is elucidated on the right hand side of the graph in comparison to our findings on Lin⁻CD127⁻IEL, which are depicted as the white cell in this cartoon (Lin⁻). Due to the functional and phenotypical overlap of cNK (as described in this thesis) and ILC1, described by Fuchs et al.⁶, these cell types could be identical and are therefore depicted together.

Multicolor flow cytometry allows simultaneous analysis of multiple markers on small subsets of cells in suspension. However, this approach has the drawback that only a limited number of markers can be simultaneously analyzed. The choice of markers to be analyzed therefore can influence the type of relationship which is revealed by hierarchical clustering (**Chapter 4**). Furthermore, cells might up- or downregulate certain markers, depending on stimuli *in situ* or *in vitro*. This could be the case for cytokine receptors such as CD122, IL-15R α or CD127 or for NK cell receptors such as CD56. In fact, some Lin⁺CD127⁺ IEL lines, which were generated from 4 RCDII patients (P1-P4) (described in **Chapter 3**), showed intermittent plasticity during cell culture. The cell line P4, for example, temporarily up- or down-regulated CD127 expression. It was also notable that cell line P3 was infrequently tested positive for CD56 while negative at other moments. Upon repeated sorting of CD127⁺ or CD56⁺ cells from these cell lines, the marker-negative sorted cells showed the same functional characteristics as their marker-positive counterparts and reacquired CD127 or CD56 expression in culture (data not shown). Hence, some plasticity was observed in long term culture for these two cell lines, while the other two cell lines showed a stable Lin⁺CD127⁺ IEL phenotype. In light of this, one has to be careful to *ex vivo* distinguish independent cell subsets, as *in situ* cells might in fact change their phenotype depending on the local milieu. The studies described in **Chapter 3 and 4**, which provided evidence that Lin⁺CD127⁺ IEL represent aberrant IEL-as their overlapping phenotype and IL-15 responsiveness suggests- therefore need to be interpreted carefully. More work is needed in order to precisely define the capacity to up- or down-regulate certain receptors for innate IEL subsets, hence the kinship of distinct innate IEL subsets needs to be investigated.

Deep profiling of the IEL subsets, described in this thesis, from different patient cohorts and tissues through microarray or RNA sequencing would result in an improved and unbiased comparison of these cell subsets. This could generate a broad knowledge of their phenotypical and possible functional relationship. Of particular interest would be the expression of transcription factors, cytokines and cytokine receptors under steady-state (directly isolated *ex vivo*) and after incubation with certain cytokines, relevant for CD disease etiology (IL-15 or IL-21).

In conclusion, our studies have refined and further characterized the heterogeneous innate IEL population. By investigating the phenotype of four different innate IEL subpopulations, we could extrapolate the similarities and dissimilarities of the IEL subsets (**Chapter 4**). Furthermore, our studies clearly demonstrated that the innate IEL population is diverse and harbors a Lin⁺CD127⁺ IEL population from which aberrant IEL could originate (**Chapter 3**). The IEL compartment therefore has to be considered a diverse, complex lymphocyte compartment with various subsets-each with distinct functions.

marker	CD3+		CD3-			REF
CD8	85,0%	89,0%	+/-	10,0% CD8aa	10,0%	1,2,3,5
CD5	100%		-	-		1,2
	gd:CD5-					
CD2	96,9%	72,0%	55,1%	25,2%	55,0%	1,2,3,4
CD69	97,0%	97,0%	97,0%	+	97,0%	1,3,5
CD38	91,0%		98,0%	+		1,3
CD122	9,2%		91,5%	92,0%		1,3
CD56	25,0%		44,7%	44%		1,3
CD16	4,3%	0,5%	12,6%	-	12,0%	1,2,3
CD94	31,3%	30,0%	16,2%	24,0%		1,3
CD28	25,5%	25,0%	0,5%	-		1,5
CTLA-4	5,0%		-			5
CD25	3,6%		-	-		1,3
HLA-II	high	39,0%	low	2,0%		1,5
CD71	-	-	-	-	-	1,3,5
KIR3DL1	-		-			1
KIR2DL1	-		-			3
CD44	+	+ 95,0%	-	-	-	1,3,5
CD161	70,0%		98,0%			3
Perforin	5,0%	5,0%	75,0%	75,0%		3,5
CD94L		6,0%		5,0%		5
CD94	+	97,0%	+	95,0%		3,5
CD18	43,7%		0,0%			3
ICAM-I	33,2%	34,0%	85,6%	86,0%		3,5
VLA-4	33,1%		41,1%			3
VCAM-I	14,6%		-			3
CD1	14,5%	5,0%	26,0%	<1%		4,5
icCD3ε	100,0%		10-25%			5
CD117	2,0%		-			5
CD10	3,0%		-			5
TdT	-		-			5
CD34	-		-			5
CD80	-		-			5
CD86	4,0%		10,0%			5
CD31	57,0%		-			5
TNFR1	5,0%		<1%			5
TNFR2	61,0%		31,0%			5
CD30	-		-			5
CD70	-		-			5

TABLE 1. THE PHENOTYPE OF CD3⁺ AND CD3⁻ IEL. References (REF): 1 Eiras et al. Cytometry 1998 ⁷ 2 Jarry et al. Eur J Immunol 1990 ⁹ 3 Eiras et al. Scand J Immunol 2000 ⁸ 4 Lundqvist et al. Int Immunol 1995 ³² 5 Leon et al. Gastroenterology 2003 ⁵

named	ILC1	ILC3	ILC1	ILC3	Lin ⁺ CD127 ⁻	Lin ⁺ CD127 ⁺	cNK	CD127 ⁺ NK
REF	1		2		thesis			
CD4	ND	ND	-	-	-	-	-	-
CD25	ND	ND	low	low	-	-	-	-
CD56	+	+	-	+/-	-	-	+	+
CD117	ND	ND	-	+	-	+	-	-
CD127	-	+	+	+	-	+	-	+
Nkp44	+	+	-	+	+	+	+	+
Nkp46	+	+	-	+/-	+/-	+/-	+	+
CCR6	-	+	+	ND	ND	ND	ND	ND
CD16	ND	ND	-	ND	-	+/-	(+/-)	+/-
CD94	+	-	-	-	-	-	(+/-)	(+/-)
Granz. B	+	+/-	-	-	+/-	+/-	+/-	+/-
CD160	+	-	ND	ND	+	+	+	+
CD122	+	-	ND	ND	+	-	+	-
Tbet	+	-	+/-	-	ND	ND	ND	ND
RORyt	-	+	-	ND	ND	ND	ND	ND
CD244	+	-	ND	ND	+/-	+/-	+/-	+/-
CD103	+				+	+	+	+
site	IEL	LP	LP	LP	IEL	IEL	IEL	IEL
disease	CrD↑		CrD↑		CD↓	CD↓	CD↓	CD↓

TABLE 2. LIN⁺CD127⁻ IEL IN COMPARISON WITH ILC1 AND ILC3 SUBSETS.

References (REF): 1 Fuchs et al. 2013 ⁶ 2 Bernink et al. 2013 ³³, CrD- Crohn's disease, CD- Celiac Disease

DEVELOPMENT OF LIN⁺CD127⁻ IEL IN THE INTESTINE AND THE THYMUS

As Lin⁺CD127⁻ IEL resembled immature cells, we hypothesized that these cells could represent NK and/or T-IEL precursors. In **Chapter 4** we present evidence that Lin⁺CD127⁻ IEL from celiacs and non-celiacs can indeed differentiate into cNK and T-cells, while Lin⁺CD127⁻ IEL derived from RCDII patients did not. In **Chapter 5** we investigate the developmental potential of the thymic counterparts of Lin⁺CD127⁻ IEL, and observed that these harbor bipotential T/NK cell precursors.

Human NK cells are thought to develop mainly in the bone marrow, and NK cell development in secondary lymphoid tissue has so far been described for lymph nodes ³⁴, tonsils ³⁵, the liver ³⁶, the uterus ³⁷, the thymus ¹⁰ and the LP of the intestine ¹³. Our studies demonstrated that Lin⁺CD127⁻ IEL primarily developed into cNK cells *in vitro*, which is the first evidence that NK cell precursors can reside in the epithelium of the small intestine (**Chapter 4**).

T cells primarily develop in the thymus from immature CD34⁺ progenitor cells which egress the bone marrow and seed the thymus for maturation into TCRαβ or TCRγδ T cells. Human T and NK cells share a common progenitor, which in the thymus still expresses CD34 ¹⁰. During maturation cells lose CD34 expression and develop either into NK or T cells. Nevertheless, our studies (**Chapter 5**) show that single thymic CD103⁺Lin⁺CD127⁻ cells, which lack CD34, could still give rise to NK and T cells and are therefore bipotential precursor cells. The intestinal counterpart on the other hand did not have this bipotential capacity and could only give rise to either NK or T cells.

It remains to be investigated whether thymic CD103⁺Lin⁻CD127⁻ cells could indeed egress from the thymus and home to the intestine, as suspected by the expression of the epithelial homing marker CD103^{38,39}. Recent thymic emigrants have been reported to express CD103⁴⁰ which indicates that thymic CD103⁺Lin⁻CD127⁻ cells could indeed exit the thymus. Nevertheless, CD103 expression could also reflect their thymic intraepithelial localization, similar to intraepithelial lymphocytes in the intestine⁴¹.

Studies with humanized mice and/or athymic mice could elucidate if intestinal Lin⁻CD127⁻ IEL are indeed derived from the thymus. Vital cell labeling such as with BrdU and injection of thymic Lin⁻CD127⁻ IEL into humanized mice could demonstrate if these thymic cells indeed home to the intestine and can give rise to T and NK cells *in situ*.

Whether T cell development indeed can take place in the intestine is the subject of long standing debate. In mice, evidence both in favor and against intestinal T cell development has been provided. In studies with RAG2 transgenic mice, where fluorescence indicates recent and past RAG transcripts, no RAG2-expressing lymphocytes were found in the intestine⁴². Nevertheless, while athymic mice have severely reduced numbers of T cell in the intestine, residual T cells are still present^{43,44}. Hence, intestinal T cell development cannot be excluded, but is thought to have a minor contribution to the T cell pool in mice⁴⁵. Studies on human intestinal T cell development are scarce and no direct evidence for *in situ* T cell development has been presented. MacDonald and colleagues were the first to demonstrate that CD3⁺ T cell precursors reside in the human intestine and they provided evidence that these cells might be precursors for T cells^{11,12}. Moreover, studies have indicated that CD3⁺ cells can rearrange TCR genes^{11,46}, express pre-T α ⁴⁷ or enzymes which are essential for TCR rearrangements, such as recombination activating gene 1 (RAG-1)^{32,46,47}, as well as terminal deoxynucleotidyl transferase (TdT)⁴⁸ (Table 3). In contrast, other authors claim that TdT is virtually undetectable⁴⁹. Due to the small numbers of cells that can be isolated from intestinal biopsies, we were not able to detect RAG transcripts Lin⁻ IEL and in agreement with the previous study no TdT-positive cells were found within the IEL compartment (**Chapter 4**).

	Author	Type	compartment	Intestine	donor
RAG1+	Lundqvist 1995	CD3-	IEL	Small	adults
	Bas 2003	CD3-	IEL/LP	small	adults
	Bas 2009	CD3-	IEL	small	children
	Williams 2004	biopsy	all	small	children 1-2yrs
PreTα+	Howie 1998	cells	IEL/LP	fetal	fetus
	Bas 2003	cells	IEL/LP	small	adults
	Bas 2009	CD3-	IEL	small	children
TCR rearrangement+	Howie 1998	cells	IEL	fetal	fetus
	Williams 2004	cells	IEL/LP	small	children 1-2yrs
	Gunther 2005	CD3-	LP	fetal	fetus
	Williams 2004	biopsy	all	small	children 1-2yrs
TdT+	Williams 2004	biopsy	all	small	children 1-2yrs
TdT-	Taplin 1996	cells	IEL	small	adults

TABLE 3. EVIDENCE FOR HUMAN INTESTINAL T CELL DEVELOPMENT.

References: Lundqvist et al. 1995 ³², Bas et al. 2003 ⁴⁶, Bas et al. 2009 ⁴⁷, Howie et al. 1998 ¹², Williams et al. 2004 ⁴⁸, Gunther et al. 2005 ¹¹, Taplin et al. 1996 ⁴⁹, Leon et al. 2003 ⁵

These data demonstrate conflicting evidence concerning RAG and TdT expression in the human small intestine. This evident discrepancy could be explained by the detection limit of the technique applied, as the frequency of the CD3⁺ population varies, depending on the age and disease condition of the individual (**Chapter 4**). A significant reduction of innate IEL in CD could explain why less RAG expression was found in CD patients compared to controls ⁴⁷. Besides this, most studies have analyzed complete biopsy material, the entire IEL/LP, or CD3⁺ population (Table 3). Thus, as it is likely that only a small subset of cells expresses RAG and/or TdT and that this expression is also tightly regulated in the intestine, the detection is problematic. The use of sensitive techniques to analyze RAG and TdT expression and TCR rearrangements in single or a few cells is therefore needed to confirm our findings for Lin⁺CD127⁺ IEL and thymic CD103⁺Lin⁺CD127⁺ cells.

Strikingly, while Lin⁺CD127⁺ IEL numbers were reduced in CD patients, the frequency of icCD3⁺ expression within Lin⁺CD127⁺ IEL was strongly increased and this may relate to the elevated levels of T-IEL in patients with CD. Moreover, this is in accordance with the elevated T-IEL frequencies generated in culture from Lin⁺CD127⁺ IEL from CD patients. In contrast, differentiation of Lin⁺CD127⁺ IEL into T or NK cells was impaired in patients with RCDII, likely due to the pre-malignant state of the Lin⁺CD127⁺ IEL (**Chapter 4**).

Thus, our results indicate that IEL maturation can take place in the intestine and that local clues, such as inflammation, can alter the differentiation and expansion potential of innate IEL subsets. Depending on the triggers *in situ* the IEL compartment could therefore swiftly adapt to the environmental needs. Selective expansion has already been described for human ILC subsets,

which under inflammatory conditions were found in higher frequencies in the intestinal LP or epithelium^{6,33,50,51}. In fact, IL-15, but also other common γ chain cytokines such as IL-21 or IL-7 could influence IEL plasticity.

T-cell differentiation from Lin⁺CD127⁺ IEL was augmented by the expression of the notch-ligand DLL1, which is present in the small intestine⁵². The role of notch signaling has been extensively described for T cell development in the thymus⁵³ and for intestinal stem cell differentiation⁵⁴. Thus it is likely that notch-ligands, such as DLL1 could also influence the lineage decisions of immature IEL precursor cells *in situ*. Depending on the localization of DLL1 and the IEL precursor cells, the strength of the signal and the interplay of other confounding factors (such as common γ chain cytokines) could influence IEL maturation, an issue that deserves further attention. In fact, DeSmedt and colleagues have demonstrated that notch-signaling can induce icCD3 expression in developing NK cells⁵⁵. As higher frequencies of icCD3⁺ cells are found in CD patients and even further elevation is seen in RCDII patients, intestinal notch-signaling could thus play a role in the induction of CD3 expression in CD and RCD. It is not known whether icCD3⁺ and icCD3⁻ innate IEL subsets possess distinct functions or represent different cell types. In fact, the functional role of intracellular CD3 expression in innate IEL is obscure. In humans, icCD3⁺ NK cells are present in the fetal liver⁵⁶ and we could also detect them in the fetal intestine (data not shown), but they form only a minor population in blood. It has been suggested that icCD3 expression could be a remnant of organ-specific ligand-receptor interactions, such as notch signaling⁵⁷. Also, it has been proposed that in the intestine icCD3 expression might ensure survival or initiate TCR rearrangements, possibly even independently from the pre-TCR⁵⁸. Hence, it can be hypothesized that icCD3 expression could discriminate between distinct developmental stages in innate IEL. If icCD3 expression is an indication of maturation towards the T cell lineage, icCD3⁺ could indeed be pre-T-IEL while icCD3⁻ IEL could be immature or pre-NK cells (Figure 3).

Due to technical restraints, we could not investigate this hypothesis. One possibility to study progeny-descendant cell relationships is the use of barcoding. Such experiments have been designed to tag individual cells with unique barcodes that pass on their specific barcode to their progeny, thus allowing tracing of cell differentiation at a single cell level^{59,60}. Alternatively, studies in mice would allow cell manipulations and fate mapping experiments. Such fate mapping techniques could be designed to trace CD3 expression in the precursor as well as the progeny (icCD3⁺ non-T) cells due to the stable expression of fluorescent proteins. These experiments could therefore address the developmental and functional differences of icCD3⁺ and icCD3⁻ innate IEL subsets.

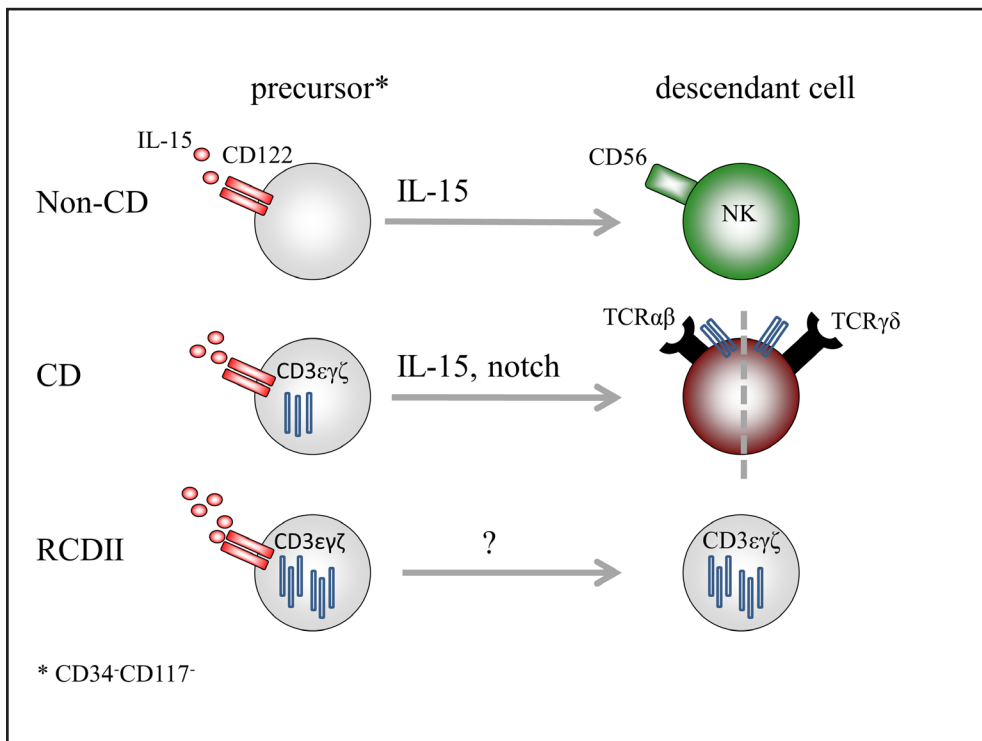


FIGURE 3. HYPOTHESIS OF THE DIFFERENTIATION OF LIN⁻CD127⁻ IEL INTO NK AND T CELLS BASED ON OUR STUDIES. Lin⁻CD127⁻ IEL can differentiate into NK cells or TCRαβ or TCRγδ T-IEL. Based on our findings that icCD3 expression is higher in Lin⁻CD127⁻ IEL in celiac disease (CD) patients than non-CD controls and T cell differentiation from Lin⁻CD127⁻ IEL was found to be elevated, we assume that the icCD3⁺ cells constitute pre-T cells and the icCD3⁻ cells pre-NK cells. No further T or NK cell differentiation was detected from Lin⁻CD127⁻ IEL in RCDII patients. This suggests that in RCDII-through as yet unidentified triggers- Lin⁻CD127⁻ IEL show a developmental block. CD122- IL-15Rβ

Further evidence from mouse models suggested that overexpression of CD3ε in immature thymocytes by itself could act as an proto-oncogene, resulting in a developmental block in T cell differentiation⁶¹ and lymphoma development⁶². While the exact mechanism how CD3ε can act as an oncogene is unclear, it is striking that in aberrant IEL of RCDII patients extraordinary high levels of icCD3 expression are found, well above the level present in conventional T cells. In this respect it is important to note that disturbed notch-signaling is involved in more than half of all T-ALL lymphoma subsets⁶³. Thus it is possible that disrupted notch-signaling could also play a role in the upregulation of icCD3 chains, expansion and transformation of aberrant IEL in RCDII patients.

Our studies on primary intestinal cells pointed out technological limitations. The cell amounts which can be isolated from biopsies or the thymus are small, and sorting by flow cytometry yields the danger of impurity of the sorted cells. Therefore we have addressed the issue of cell contamination in various experiments (**Chapter 4**) and concluded that the chance of mature T cell contamination was negligible, while the chance of NK cell contamination was very low. Nevertheless, we could not exclude contamination completely, as flow cytometric sorting is not 100% pure, and purity could vary between each analysis. In conclusion, flow cytometry has proven to be a sensitive method to analyze rare subsets of cells and isolate these for further *in vitro* assays. Nevertheless, barcoding experiments or studies in mouse models are essential to investigate the underlying mechanisms and to confirm our data on human primary cells.

The *in vitro* studies described in Chapter 4 on the developmental potential of Lin⁺CD127⁺ IEL were performed with the mouse stromal cell lines OP9 and OP9DL1, as a convenient tool for the generation of T cells from precursor cells⁶⁴. Despite the presence of essential components for T cell differentiation, this cell culture system has some drawbacks. First, no equivalent human cell line exists and no positive/negative selection takes place in OP9/DL1 stromal cultures, as these cultures lack the complexity of thymic stromal niches. As OP9DL1 only expresses HLA-I, only CD8⁺ cells can be generated⁶⁴, in line with our observation that the T-cells generated from Lin⁺CD127⁺ IEL consistently expressed CD8 (**Chapter 4**). Furthermore, we did not detect any intermediate stages of T cell development (such as double-positive T cells) when culturing Lin⁺CD127⁺ IEL on OP9DL1. This could be due to the relatively long time span of 2 weeks before analysis. Hence, at the time of analysis T cell maturation could have already been completed. Due to the low growth potential of the cells, we were not able to check the phenotype of the co-cultured cells at earlier timepoints.

In summary, we provided evidence that Lin⁺CD127⁺ IEL, the putative precursor population for aberrant IEL in RCDII patients, can generate NK and mature TCR $\alpha\beta$ and TCR $\gamma\delta$ T-IEL (in the presence of IL-15), whereas T-cell generation was augmented by notch-signaling. This maturation was influenced by disease state, as T-IEL differentiation frequencies were higher in CD patients and no further development was observed in cells from RCDII patients. This could imply a developmental block in Lin⁺CD127⁺ IEL in RCDII, reflecting the pre-malignant nature of these cells. Which triggers influence the (block of) differentiation, expansion and transformation of these cells, still needs to be elucidated (Figure 3).

WHAT ARE THE NEW FINDINGS?

In this thesis we investigated if, next to IL-15, other factors contribute to the expansion of aberrant IEL in RCDII, by exploring the effects that potential intestinal bystander cells could have on aberrant IEL lines. Furthermore, we investigated the origin of aberrant IEL. In order to do so, we defined distinct subsets of the CD3⁺ IEL population and investigated their phenotype, plasticity and the developmental capacity of cells from the intestine and the thymus. The main findings are:

- Dendritic cells induced expansion and anti-apoptotic effects in RCDII cell lines (**Chapter 2**);
- Aberrant IEL are likely derived from a subset of innate CD3⁺ IEL, characterized as Lineage-negative (Lin⁻) CD127⁻ IEL (**Chapter 3**);
- The innate IEL population was heterogeneous, contained four subsets, which could be distinguished on the basis of CD127 and CD56 expression (**Chapter 3**); each with a distinct cytokine receptor profile: CD127⁻ IEL expressed IL-15R β , CD127⁺ IEL the IL-15R α and IL-21R (**Chapter 4**);
- Lin⁻CD127⁻ IEL were dependent on IL-15: they expressed IL-15R β , responded to IL-15 by proliferation (**Chapter 3**) and required IL-15 for outgrowth and development (**Chapter 4**);
- The innate IEL populations demonstrated plasticity, which was shaped by age, and disease state, both in celiac and refractory celiac disease (**Chapter 4**);
- Lin⁻CD127⁻ IEL harbored NK and notch-dependent T cell precursors: cells isolated from non-CD controls mainly generated NK cells, while higher frequencies of T cells were detected in cultures of CD patients. In contrast, Lin⁻CD127⁻ IEL from RCDII patients had no differentiation potential (**Chapter 4**);
- A similar CD34⁺CD103⁺Lin⁻CD127⁻ population was found in the thymus (**Chapter 3**), but in contrast to the intestine this contained bipotential NK/T precursors (**Chapter 5**)

DIRECTIONS FOR FUTURE RESEARCH

Our studies shed light on the diversity and plasticity of CD3⁺ innate IEL subsets and challenged our understanding of the composition of the IEL compartment. They further demonstrated how knowledge of a premalignant cell population in a rare disease, such as RCDII, can lead to the identification of a novel physiological cell population. It brought up the concept, that IEL maturation can be influenced by disease. Nevertheless, several questions still need to be answered:

PLASTICITY: WHAT IS THE EXPRESSION PROFILE OF INNATE IEL SUBSETS IN HEALTH AND DISEASE?

To gain further insight into the physiology and relationship of the different innate IEL populations in healthy versus diseased tissues, unbiased approaches such as microarray or RNA sequencing should be performed. Preferably at the single cell level. It is challenging to generate sufficient amounts of RNA for such an analysis, nevertheless it should be technically feasible to perform⁶⁵. These high throughput technologies could yield crucial information with regard to the expression of transcription factors (TF), cytokines and cytokine receptor in these innate IEL subsets; and how such innate IEL respond to specific stimulation, for example to cytokines. Furthermore, it could indicate which markers can be used to further differentiate these subpopulations and which alterations are linked to the development of intestinal diseases like CD, RCD and inflammatory bowel disease (IBD). Alternatively, a comprehensive analysis could also be performed with the use of a Cytometer coupled to a Time-of-flight analysis (CyTOF). In the near future a CyTOF is expected to allow simultaneous analysis of up to 100 markers simultaneously^{66, 67}. Thus, the following issues could be addressed:

- What is the expression profile of innate IEL subsets in health and disease?
Parallel sorting of T-IEL, innate IEL subsets from healthy, CD and RCDII biopsies (or other gastrointestinal diseases) and subsequent microarray or RNA sequencing analysis (focusing on TF, cytokine responsiveness)
- How do cytokines alter the expression profile of the different subsets?
Parallel sorting of IEL subsets after incubation with cytokines (such as IL-15, IL-21, IL-7) and subsequent microarray, RNA sequencing or CyTOF analysis (focusing on TF, phosphorylated proteins, cytokine production, proliferation)

DIFFERENTIATION: HOW ARE LINEAGE DECISIONS OF LIN⁺CD127⁺ IEL AND INNATE IEL REGULATED?

We have provided evidence that Lin⁺CD127⁺ IEL can differentiate into T and NK cells. This needs to be further investigated and validated. Moreover, it needs to be determined which factors influence lineage decisions. Two important candidates which could play a role and have been discussed are IL-15 and notch-ligands, such as DLL1. Which cell types express IL-15 and notch-ligands (these could be enterocytes, goblet cells, DC) and where are IL-15 and DLL1 (co-)localized? Is there a differential expression, when comparing CD/RCDII with non-CD controls? Hence, these questions could be studied as follows:

- What is the lineage relationship of Lin⁺CD127⁺ IEL with T and NK cells?
IEL could be transduced with a barcode library and transduced Lin⁺CD127⁺ IEL could be subsequently sorted onto OP9DL1. After differentiation T and NK cells can be sorted and the barcodes in both populations are analyzed by a microarray platform. According to the barcodes recovered the lineage relationship can be calculated.
- Which factors influence lineage decisions of Lin⁺CD127⁺ IEL?
 - To study the interaction of lymphocytes with IL-15 and notch-ligands, such as DLL1, histology on small intestinal biopsies could shed light on the presence and localization of DLL1 and IL-15 in different patient cohorts;
 - Fate mapping experiments in mice with stable expression of fluorescent CD3 proteins could discriminate icCD3⁺ innate IEL subsets to investigate the role of CD3 chains in T/ NK cell differentiation;
 - The addition of different soluble factors, such as cytokines (IL-7, IL-21) or supernatants of activated gluten-specific T cells to Lin⁺CD127⁺ IEL onto OP9DL1 could evaluate if such factors can influence lineage decisions;
 - Co-culture of Lin⁺CD127⁺ IEL on human intestinal epithelial cultures (e.g. organoids) could indicate if (and which) intestinal cells can also provide differentiation signals for T/ NK cell development

TUMORGENESIS: WHICH ONCOGENES CAUSE MALIGNANCIES OF LIN⁻CD127⁻ IEL IN EATL?

As discussed above, the overexpression of icCD3ε could potentially lead to transformation. It needs to be investigated how (i) notch-signaling can induce icCD3 expression⁵⁵ and (ii) how overexpression of CD3ε leads to tumorigenesis⁶². This could be elucidated as follows:

- Which factors can act as an oncogene driving the transformation of aberrant IEL into EATL?
 - DNA sequencing could yield information on whether certain mutations, SNP, or chromosomal translocations such as those previously described for Chromosome 1q⁽⁶⁸⁾ and data not shown) can be detected. Implications of these mutations could be tested *in vitro*;
 - Overexpression of CD3 chains by retroviral transduction of primary cells (IEL, thymocytes) could determine if and how high copy numbers of CD3 chains induce uncontrolled cell growth and differentiation arrest in immature cells.

WHAT ARE THE IMPLICATIONS FOR FUTURE THERAPIES?

Our studies thus demonstrated that the small intestinal epithelium not only harbors T-IEL, but also a diverse innate IEL compartment, which exhibited plasticity and differentiation potential. What's more, our studies highlighted the idea that CD can profoundly influence not only the composition, but also the developmental capacities of IEL. The failure to further differentiate seems to be an important hallmark of the premalignant nature of Lin⁻CD127⁻ IEL in RCDII patients and future studies need to investigate which factors can influence maturation, expansion and transformation of IEL subsets. This knowledge may ultimately lead to the development of diagnostic tests, aiming at detecting derailed IEL maturation before the development of a full blown lymphoma and classifying CD patients at risk of developing RCDII or RCDII patients at risk to develop EATL. If we understand the different maturation stages of innate IEL in the healthy gut, in CD and in RCDII, this knowledge could help us understand which triggers lead to expansion and transformation. This information could then be used to further predict clinical outcome in RCDII or responsiveness to the treatment of the lymphoma. Such a 'scaling' of aberrant IEL maturity has already been suggested to predict clinical outcome in RCDII²⁴ and calls for further investigation.

Our studies have also shown, that RCDII patients harbor expanded Lin⁻CD127⁻ and Lin⁻CD127⁺ IEL populations, both likely responding to different cytokines. Treatment with anti-IL-15 therefore is probably not sufficient in these patients, as this would risk the selective expansion of Lin⁻CD127⁺ IEL. Alternatively, RCDII could be treated with inhibitors of downstream signaling molecules

of the common γ chain receptors (including IL-15 and IL-7), such as Jak3 or STAT5 inhibitors. Increased Jak3 and STAT5 phosphorylation has been demonstrated in IEL from RCDII patients, compared to controls ¹⁵.

Ultimately, by understanding the distinct developmental stages and their implications for cell survival and function, specialized treatment and intervention strategies could be developed to prevent malignancy. Knowledge of the confounding factors leading to transformation of Lin⁺IEL in RCDII will generate cues for an optimized treatment of EATL. It is now becoming clear that EATL likely represents an immature or semi-mature innate IEL lymphoma, rather than a mature T cell lymphoma.

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NEDERLANDSE SAMENVATTING

COELIAKIE EN REFRACTAIRE COELIAKIE

Coeliakie is een darmziekte die veroorzaakt wordt door gluten: eiwitten uit tarwe, rogge en gerst. Deze gluten eiwitten worden door immuuncellen als lichaamsvreemd herkend en dit heeft een ontsteking in de darm tot gevolg. Deze herkenning van gluten gebeurt alleen bij mensen die een genetische dispositie hebben: ze zijn HLA-DQ2 of HLA-DQ8 positief. HLA-DQ2 en -DQ8 binden fragmenten van gluten die door afbraak ontstaan en deze complexen kunnen immuuncellen activeren.

Coeliakie patiënten mogen hun leven lang geen gluten eten omdat elke keer dat zij dat doen de immuuncellen geactiveerd worden. Dit heeft een ontsteking, dus schade in de darm als gevolg. Een glutenvrij dieet leidt bij de meeste coeliakiepatiënten tot herstel van de darmschade en het verdwijnen van de klachten. Echter bij een klein aantal coeliakie patiënten werkt het glutenvrij dieet niet zodat, ondanks het strikt volgen van een glutenvrij dieet, de darm nog steeds ontstoken is. Deze patiënten worden refractaire coeliakie (RC) patiënten genoemd. Tot nu toe weet men niet waarom deze patiënten niet beter worden hoewel ze een glutenvrij dieet volgen.

HET IMMUUNSYSTEEM VAN DE DUNNE DARM

Omdat veel RC patiënten afwijkende immuuncellen in hun dunne darm hebben, zijn deze immuuncellen in dit proefschrift nauwkeurig onderzocht. De darm bevat het grootste aantal immuuncellen van het lichaam. Deze zijn ervoor verantwoordelijk om aan de ene kant tegen bacteriën en virussen te vechten maar aan de andere kant tegelijkertijd ook tolerant tegen voedingstoffen te zijn. De immuuncellen die in de darmwand zitten (zogenaamde intra-epitheliale lymfocyten) zijn meestal T-cellen, die direct contact met 'buiten' hebben. Daarom bezitten deze belangrijke functies om de darm te beschermen. Bij coeliakie is het aantal intraepitheliale lymfocyten sterk verhoogd en deze kunnen epitheliale cellen vernietigen en zo aan het ziekte proces bijdragen.

Maar het is ook bekend dat er niet alleen maar T-cellen in de darmwand zitten. Hoewel de exacte identiteit en functie(s) van deze andere cellen nog grotendeels onbekend is, is het wel duidelijk dat de aantallen van juist deze cellen bij refractaire coeliakie sterk verhoogd is. Omdat zij sterk afwijken van de 'gewone' darmcellen worden zij 'aberrant' genoemd. Deze afwijkende cellen kunnen zich aanzienlijk snel vermenigvuldigen. Vaak ontspreiden zij en ontwikkelen zich tot een lymfoom (kanker). Tot nu toe is deze lymfoom heel slecht te behandelen. Verder is niet bekend waar deze afwijkende cellen vandaan komen.

DIT PROEFSCHRIFT

In dit proefschrift hebben wij onderzocht wat de oorsprong van deze ‘afwijkende’ cellen in RC patiënten is en hoe zij zich ontwikkelen. Hiervoor hebben wij darmcellen (intra-epitheliale lymfocyten) van refractaire coeliakie patiënten vergelijken met cellen uit de darm van ‘gewone’ coeliakie patiënten, en met cellen van patiënten die geen coeliakie of RC hebben (controle groep). Wij hebben onderzocht wat deze aberrante cellen nodig hebben om te delen en hebben deze cellen uitgebreid gekarakteriseerd om te kunnen bepalen hoe afwijkend deze cellen in vergelijking met ‘gewone’ cellen uit de controle groep echt zijn.

HOOFTSTUK 1: INLEIDING

Hoofdstuk 1 geeft een algemene inleiding over het immuunsysteem, deze gaat met name over het immuunsysteem in de darm. Er wordt samengevat wat er tot nu toe bekend is over intra-epitheliale lymfocyten bij gezonde individuen, coeliakie en refractaire coeliakie patiënten.

HOOFDSTUK 2: DENDRITISCHE CELLEN INDUCEREN DELING VAN ABERRANTE CELLEN

Onze studies met aberrante cellen uit de darm van RC patiënten laten zien, dat deze cellen gaan delen als zij contact hebben met andere immuuncellen, namelijk dendritische cellen. Deze reactie is specifiek voor aberrante cellen, omdat ‘gewone’ darmcellen niet gaan delen bij contact met dendritische cellen. Eerdere studies hebben aangetoond dat er veel dendritische cellen in een ontstoken darm zijn. Contact van aberrante cellen met dendritische cellen zou dus (onder andere) vermoedelijk tot expansie van aberrante cellen leiden- een kenmerk van RC.

HOOFDSTUK 3: ‘ABERRANTE’ CELLEN ZIJN EEN NATURLIJKE CELPOPULATIE

In hoofdstuk 3 gaan we in op de vraag waar aberrante cellen vandaan komen. Een hypothese postuleert dat aberrante cellen ontspoorde rijpe T-cellen zijn, een andere dat ze al aanwezig zijn in een gezond individu, maar in een zo kleine hoeveelheid dat ze daarom nog niet ontdekt zijn. Om te onderzoeken of deze cellen inderdaad aanwezig zijn, hebben wij eerst een uitgebreide karakterisering van deze cellen uit darmbiopsen van refractaire coeliakie patiënten uitgevoerd. Nadat wij wisten hoe deze cellen er precies uitzagen, hebben wij gekeken of er soortgelijke cellen in darmbiopsen van ‘gewone’ coeliakie patiënten en de controle groep te vinden zijn. Wij hebben ontdekt dat zulke cellen juist veel voorkomen bij kinderen zonder coeliakie, en minder bij patiënten met coeliakie. Onze conclusie was dus dat ‘aberrante’ cellen niet specifiek voor

refractaire coeliakie zijn, maar een normale fysiologische celpopulatie zijn. In refractaire coeliakie kunnen deze 'natuurlijke' cellen zich vermeerderen en uiteindelijk ontspreiden en zo tot een lymfoom leiden. Omdat deze cellen kenmerken van zowel T-cellen als ook van NK-cellen (natural killer cells) hebben, hebben wij aangenomen dat zij onrijpe cellen zouden kunnen zijn, die zich nog niet tot rijpe T- of NK-cellen hebben ontwikkeld.

HOOFDSTUK 4: HET LYMPHOCYTEN COMPARTIMENT IN DE DARM IS DIVERS

Studies over cellen in de darmwand waren tot nu toe voornamelijk gericht op één celtype, namelijk de T-cellen. Omdat wij uit onze studies in hoofdstuk 3 weten dat er ook andere cellen zijn (die kenmerken van zowel T- als NK-cellen bevatten) wilden wij weten hoeveel en welke verschillende celtypen er überhaupt in de darmwand zitten.

In hoofdstuk 4 laten wij zien dat het intra-epitheliale lymfocyten compartiment heel erg divers is en dat dit er bij coeliakie en RC patiënten anders uit ziet dan bij de controle groep. Wij vonden dat er naast T-cellen ook nog vier verschillende subtypes van (niet T-)cellen in de darmwand zitten, die wij innate lymfocyten noemen. Dit zijn de door ons beschreven cellen uit hoofdstuk 3, NK-cellen en cellen die verder nauwelijks bekend zijn en deel uitmaken van de innate lymfocyten in de dunne darm. Verder zagen wij leeftijdsafhankelijke veranderingen in dit compartiment in individuen uit de controle groep. Dit betekent dat darmcellen sterk beïnvloed worden door zowel leeftijd van het individu, als ook door ziekte, zoals coeliakie.

ONRIJPE CELLEN KUNNEN NOG VERDER ONTWIKKELEN

De meeste immuuncellen in de darm zijn rijpe cellen (rijpe T-cellen), die zich niet meer verder kunnen ontwikkelen. Over potentiële onrijpe of voorlopercellen is minde bekend. In hoofdstuk 3 hebben wij cellen beschreven die kenmerken van zowel T- als ook NK-cellen hebben en dus onrijp zouden kunnen zijn. Zulke onrijpe cellen zijn in de mens nauwelijks beschreven en tot nu toe slechts bij muizen aangetoond. Om te onderzoeken of de door ons beschreven cellen inderdaad onrijp zijn, hebben wij deze cellen uit de darm geïsoleerd en in kweek (in vitro) getest of zij zich verder kunnen ontwikkelen (hoofdstuk 4).

Een van de bevindingen in dit hoofdstuk is dat deze cellen uit de controle groep (geen coeliakie of RC patiënten) zich voornamelijk tot NK-cellen differentiëren. Bij coeliakie patiënten ontwikkelen zij zich meer tot T-cellen en bij RC patiënten kunnen deze cellen zich niet meer verder ontwikkelen. Uit deze studies hebben wij geconcludeerd dat zulke cellen inderdaad onrijpe cellen zijn. Refractaire coeliakie zou dus als gevolg van een ontwikkelingsstop kunnen ontstaan. Welke factoren deze ontwikkeling kunnen hinderen en of de huidige bestaande therapie voor onrijpe

cellen zou kunnen toegepast worden, is het doel van toekomstig onderzoek.

HOOFDSTUK 5: VERGELIJK VAN ONRIJPE CELLEN UIT DE THYMUS MET DE DARM

In hoofdstuk 5 beschrijven wij een vergelijkbare celpopulatie in de menselijke thymus die in tegenstelling tot de darm tegelijkertijd T- en NK-cel kan worden (in de darm worden deze cellen óf T-cel óf NK-cel). Deze celpopulatie in de thymus is dus nog onrijper omdat zij nog alle twee de kanten uit kan gaan.

HOOFDSTUK 6: WAT WIJ GELEERD HEBBEN/KUNNEN LEREN OVER INNATE LYMFOCYTEN EN HUN ONTWIKKELING

In hoofdstuk 6 worden onze studies over de darm- en thymuscellen samengevat en onze resultaten vergeleken met de resultaten van andere studies. Tot nu toe is er nog weinig bekend over innate lymfocyten in de darm. Door onze studies weten wij nu dat dit compartiment divers is en hoe wij het kunnen onderscheiden. Dat biedt belangrijk perspectief voor vervolgonderzoek naar de functie van deze cellen in het darmepitheel. Ook hebben wij laten zien dat het hier mogelijk voorlopercellen betreft. Dit laatste is een controversieel onderwerp omdat het onduidelijk is of er (in de mens) überhaupt ontwikkeling van onrijpe cellen in de darm kan plaats vinden.

Vervolgstudies zijn dus noodzakelijk om meer inzicht in de functie van deze innate lymphocyten in het darmepitheel te verkrijgen. Als wij meer over de cellen in de darmwand weten – zowel T cellen als innate lymfocyten- waar ze vandaan komen, tot welke cellen ze kunnen ontwikkelen en door welke factoren dit beïnvloed wordt- dan kan dit er toe bijdragen dat we darmziektes beter kunnen behandelen. Het uiteindelijke doel is om een effectieve therapie te ontwikkelen voor patiënten met refractaire coeliakie. Vervolgonderzoek zou er ook aan kunnen bijdragen dat we deze ziekte vroeg op kunnen sporen zodat eerder ingegrepen kan worden.

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Manuscript in preparation

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CURRICULUM VITAE

Frederike Schmitz was born on december 23, 1982 in Göttingen, Germany. She participated in an exchange program in 1999/2000 to study in Lemoore, California, United States and graduated from Lemoore High School. After returning to Germany, she finished secondary school (Ernst-Moritz Arndt Gymnasium) in Bonn, Germany and obtained her 'abitur' degree in 2002. After graduation, Frederike studied Biomedical Chemistry in Mainz, Germany for one year, and subsequently changed to Nutrition Science in Jena, Germany, from which she obtained her masters (dipoma) degree in 2008. During her studies, Frederike completed two external internships, at the Allergy-Centrum Charité, University Hospital of Berlin, in the field of allergy and nutrition and at the Walter and Eliza Hall Institute of Medical Research (WEHI) in Melbourne, Australia, investigating toxicity of oats in patients with celiac disease. The latter internship arose her interest in celiac disease and she started her PhD studies on intraepithelial lymphocytes in celiac disease with Frits Koning in Leiden, the Netherlands. The work during her PhD period is subject of this thesis. After her PhD Frederike will work as a post-doctoral researcher in the lab of David Artis in Philadelphia, USA.

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