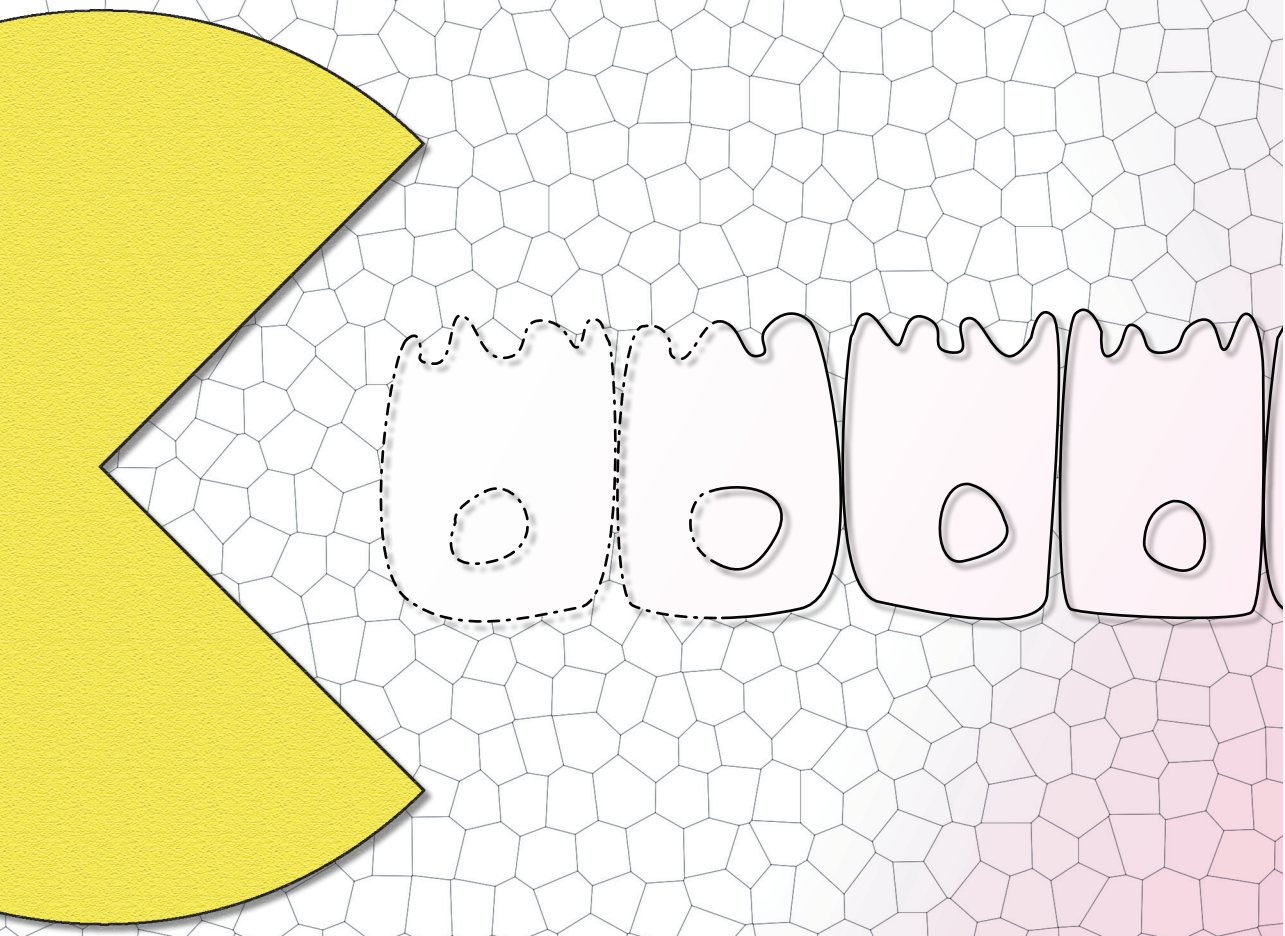
Celiac Disease

How complicated can it get?



Jennifer M-L. Tjon

STELLINGEN BEHORENDE BIJ HET PROEFSCHRIFT
"CELIAC DISEASE: HOW COMPLICATED CAN IT GET?"

1. RCD cellijnen zijn een goed model voor aberrante lymfocyten *in vitro*. *Dit proefschrift.*

2. De naam enteropathie geassocieerd T-cel lymfoom (EATL) dient heroverwogen te worden. *Dit proefschrift.*

3. Aberrante lymfocyten zijn mogelijk minder aberrant dan we denken. *Dit proefschrift.*

4. De expressie van DNAM-1 op aberrante lymfocyten en reguliere T- en NK-cellen is vergelijkbaar, de functie van DNAM-1 verschilt daarentegen. *Dit proefschrift.*

5. Expressie van NK cell markers is niet voorbehouden aan NK cellen en kan dus niet gebruikt worden om onderscheid te maken tussen NK cellen en andere lymfocyten. *O.a Veiga-Fernandes et.al. J Exp Med. 2010 Feb 15;207(2):269-72.*

6. Ingrijpen in de IL-15-afhankelijke anti-apoptotische signaalroute zal het aantal aberrante lymfocyten in refractaire coeliakie type 2 mogelijk reduceren. *Malamut et.al. J Clin Invest. 2010 Jun 1;120(6):2131-43.*

7. Genoomwijde associatie studies kunnen veel genetische risico loci identificeren, voor de identificatie van het causale gen en de functie daarvan zijn functionele studies echter onmisbaar. *Dubois et.al. Nat Genet. 2010 Apr;42(4):295-302.*

8. Betere fenotypische karakterisatie van de TCR-CD3-negatieve CD7-positieve cellen zoals beschreven door Jarry en Lundqvist is nu mogelijk door de opkomst van multicolor flowcytometrie. *Jarry et.al. Eur J Immunol. 1990 May;20(5):1097-103. Lundqvist et.al. Int Immunol. 1995 Sep;7(9):1473-87.*

9. Wetenschappelijk onderzoek naar zeldzame aandoeningen is meestal niet van algemeen maatschappelijk nut, maar kan wel bijdragen aan de verbetering van de kwaliteit van leven van het individu.

10. Het lezen van Engelstalige romans komt het schrijven van een wetenschappelijk artikel ten goede.

11. Don't panic! *Douglas Adams, The hitchhiker's guide to the galaxy.*

JENNIFER TJON, LEIDEN, 30 NOVEMBER 2011

Celiac Disease

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 30 november 2011
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The Nederlandse Coeliakie Vereniging supports scientific research. Also, the NCV organizes contact amongst fellow-sufferers, offers and spreads information, informs and is active in advocacy for persons with coelic disease or dermatitis herpetiformis (www.glutenvrij.nl).

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About the cover: Abberant IEL lyses intestinal epithelial cells.

Contents

SCOPE OF THIS THESIS

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Celiac disease (CD) is a common inflammatory disorder of the small intestine, which is triggered by ingested gluten proteins. Previous studies identified crucial steps in the development of celiac disease and based on this knowledge, we propose a threshold model for the development of celiac disease, which is described in chapter 1. It has also become clear that adult-onset celiac disease has a higher frequency of developing complications: refractory celiac disease (RCD) and enteropathy associated T cell lymphoma (EATL). About a decade ago, RCD was subdivided into RCD type I and RCD type II. This division was based on the respective absence or presence of an aberrant intraepithelial lymphocyte (IEL) population. These aberrant IELs were defined as surface TCR-$CD3^-CD4^-CD8^-CD7^+CD103^+$, intracellular $CD3^+$ cells and were identified as the missing link between regular IELs in uncomplicated celiac disease and lymphoma cells in EATL. The function and cellular origin of the aberrant IELs, however, remained unclear. The aim of this thesis was, therefore, to gain more insight in the phenotypical and functional characteristics of aberrant IELs as this might help to understand the events leading from uncomplicated CD to RCD II and gastrointestinal lymphoma.

Chapter 1 provides an overview of the pathogenesis of uncomplicated CD, including the involvement of the disease predisposing HLA-DQ2 and HLA-DQ8 molecules and their role in the presentation of gluten derived peptides. Based on the available data we propose a threshold model in which the efficiency of gluten presentation to $CD4^+$ T cells determines the likelihood of developing CD and its complications.

Until recently, aberrant IELs were mainly investigated in situ due to lack of model systems. This limited the type and extent of experiments to investigate molecular events linked to the development of RCD II and EATL. In *chapter 2* we describe the isolation and propagation of three cell lines from duodenal biopsies of three individual RCD II patients that display a surface TCR-$CD3^-CD4^-CD8^-CD7^+CD103^+$, intracellular $CD3^+$ phenotype that is characteristic for the aberrant cells found in patients with RCD II. We used these cell lines as a model for aberrant IELs in all the studies described in this thesis. In *chapter 2* we studied the presence and functionality of the individual TCR and CD3 chains.

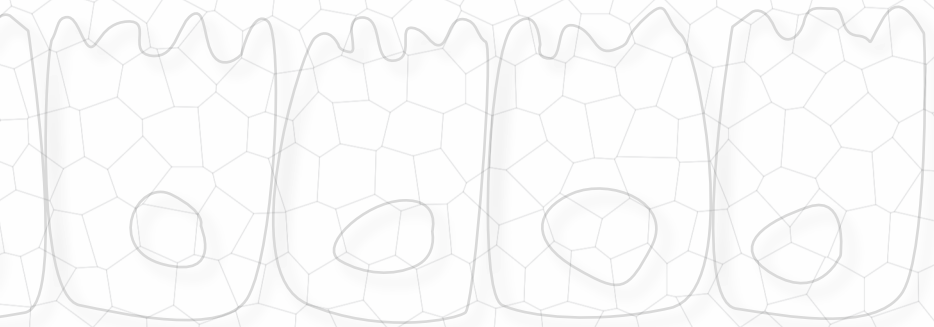
In active celiac disease, regular TCR^+ IELs acquire an NK cell receptor repertoire through which they can lyse epithelial cells. Much less is known about the contribution of aberrant IELs to tissue damage in RCD II and EATL. In *chapter 3* we used the RCD cell lines to investigate the specificity of cytotoxicity of aberrant IELs and the receptors involved.

In *chapter 4* we studied the ability of the RCD cell lines to secrete cytokines after triggering with an array of stimuli. This chapter also suggests a novel role for the activation marker CD30 on (pre)malignant IELs in RCD II and EATL.

The exact cellular origin of aberrant IELs is still unclear. From *chapter 2* we learned that intracellular, all CD3 chains were present, whereas the TCR chains were not always present. Furthermore, the CD3 complex was functional as introduction of exogenous TCR chains resulted in surface TCR-CD3 expression. In *chapter 5* we performed genomic,

transcriptomic and flowcytrometric analysis of the RCD cell lines as a further step to determine the cellular origin of aberrant IELs. We demonstrate that cells with an “aberrant” phenotype are present in the intestinal epithelium of healthy individuals as well. This led us to propose that such cells are part of the normal lymphocyte repertoire and most likely the precursors of the monoclonal aberrant cell populations found in patients with RCD II and EATL. Moreover, these data indicate that aberrant IELs are not derived from mature T cells.

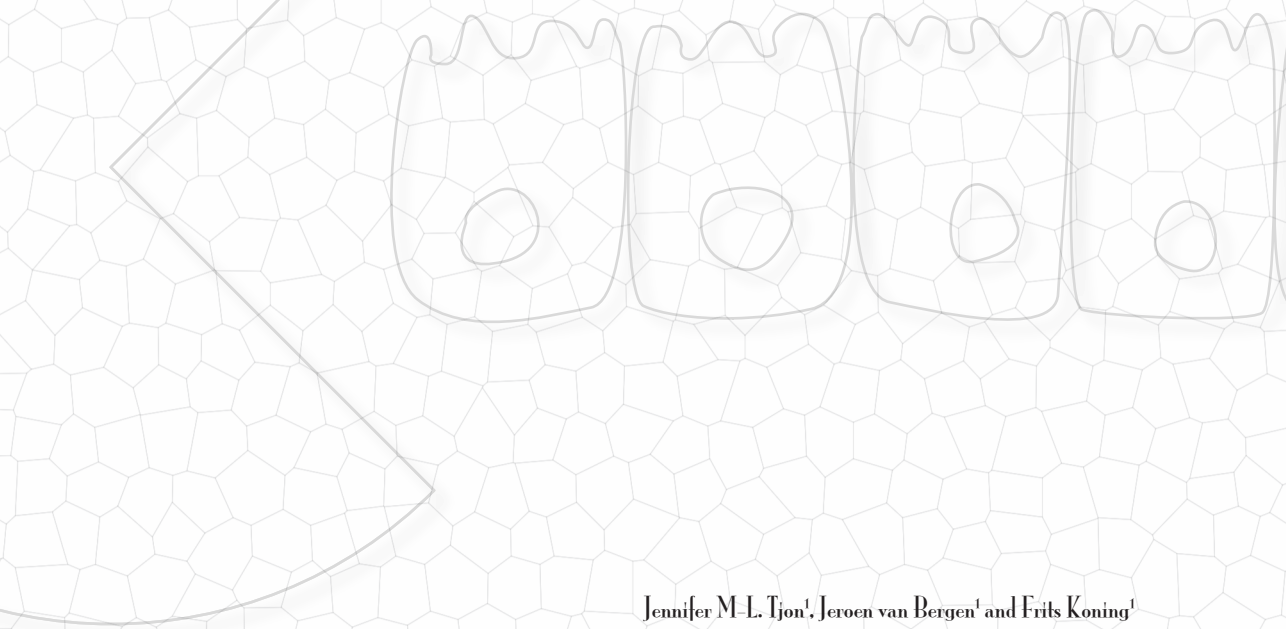
In *chapter 6* the relevance of this thesis and directions for further research and future therapeutic possibilities are discussed.



Chapter 1

General Introduction

Celiac Disease: How Complicated Can It Get?



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Abstract

In the small intestine of celiac disease patients, dietary wheat gluten and similar proteins in barley and rye trigger an inflammatory response. While strict adherence to a gluten-free diet induces full recovery in most patients, a small percentage of patients fail to recover. In a subset of these refractory celiac disease patients an (aberrant) oligoclonal intraepithelial lymphocyte population develops into overt lymphoma. Celiac disease is strongly associated with HLA-DQ2 and/or HLA-DQ8 as both genotypes predispose for disease development. This association can be explained by the fact that gluten peptides can be presented in HLA-DQ2 and HLA-DQ8 molecules on antigen presenting cells. Gluten-specific $CD4^+$ T cells in the lamina propria respond to these peptides and this likely enhances cytotoxicity of intraepithelial lymphocytes against the intestinal epithelium. We propose a threshold model for the development of celiac disease in which the efficiency of gluten presentation to $CD4^+$ T cells determines the likelihood of developing celiac disease and its complications. Key factors that influence the efficiency of gluten presentation include: (1) the level of gluten intake, (2) the enzyme tissue transglutaminase 2 which modifies gluten into high affinity binding peptides for HLA-DQ2 and HLA-DQ8, (3) the HLA-DQ-type as HLA-DQ2 binds a wider range of gluten peptides than HLA-DQ8, (4) the gene dose of HLA-DQ2 and HLA-DQ8 and finally, (5) additional genetic polymorphisms that may influence T cell reactivity. This threshold model might also help to understand the development of refractory celiac disease and lymphoma.

Introduction

With a prevalence of 1% in western populations, celiac disease (CD) is one of the most common inflammatory disorders of the small intestine[1]. CD is often assumed to have its onset in childhood but it has recently been suggested that adults can also develop CD[2]. Clinical manifestations vary according to age group: infants and young children present with diarrhea, abdominal distention and failure to thrive, whereas adults that develop CD not only present with diarrhea but also with silent manifestations such as anemia, osteoporosis or neurological symptoms[1]. Immunohistochemistry of the small intestine of patients shows villous atrophy, crypt hyperplasia and elevated levels of intraepithelial lymphocytes (IELs). The only therapy until now is a gluten-free diet, which will normalize the clinical and histological manifestations and allows the patients to live an otherwise normal life.

A small percentage of adult-onset CD patients develop a primary or secondary resistance to a gluten-free diet (Figure 1). This condition is called refractory celiac disease (RCD) and is characterized by persisting villous atrophy and elevated levels of IELs. Currently, RCD is subdivided into two subtypes: RCD type I (RCD I) and RCD type II (RCD II) that both display clinical and histological resistance to a gluten-free diet[3]. RCD II, however, is associated with the presence of an aberrant IEL population that lacks surface T cell receptor (TCR)-CD3 expression but contains intracellular CD3ε and has clonal TCRγ-gene rearrangements[4]. These aberrant IELs can gain chromosomal abnormalities and develop into surface TCR-CD3$^-$ lymphoma cells[5;6]. RCD II is therefore considered a premalignant condition and roughly 50% of the RCD II patients develop overt lymphoma within 5 years of diagnosis[7;8]. In summary, the majority of CD patients has an uncomplicated disease course and can be treated with a gluten-free diet (Figure 1). RCD II and RCD-associated lymphoma, however, are difficult to treat and have therefore poor 5-year survival rates of <44% and <20% respectively[7].

The development of CD is determined by both environmental and genetic factors. In the 1950s ingestion of wheat products was described to cause malabsorption symptoms in patients[9]. Later on it was established that gluten, the storage proteins in wheat, barley and rye, caused a cell-mediated immune response in the small intestine[10]. In addition to

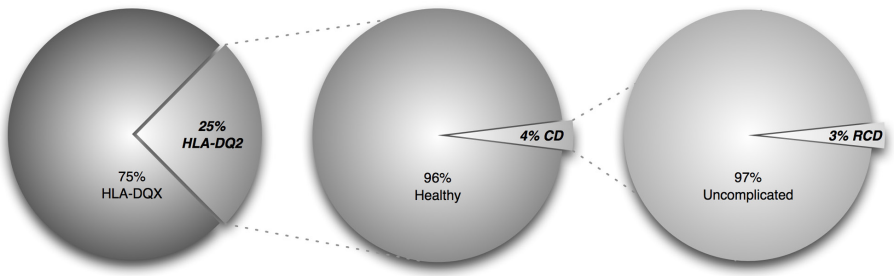

Figure 1. Prevalence of CD and complicated CD in the Caucasian population. Approximately 25% of the general Caucasian population is HLA-DQ2$^+$. From these genetically susceptible individuals only 4% develops CD. In the majority of the CD patients the disease course is uncomplicated. Roughly 3% of the CD patients will not respond to a gluten-free diet and develop RCD. A subset of RCD patients develop RCD II of which approximately 50% develops RCD-associated lymphoma (not shown).

this environmental factor, CD development involves genetic predisposition as the vast majority of the CD patients possess HLA-DQ2 and/or HLA-DQ8[11]. HLA-DQ2 is a genotype that is present in roughly 25% of the European population[12] (Figure 1). Yet only ~4% of all HLA-DQ2$^+$ individuals develop CD (Figure 1)[13]. CD development in HLA-DQ2- and HLA-DQ8-negative individuals is extremely rare[14]. These findings came together with the observation that CD4$^+$ T cell lines from duodenal biopsies of CD patients specifically respond to gluten peptides presented by HLA-DQ2 and/or HLA-DQ8[15;16]. The etiology of RCD is much less clear, but seems to be associated with HLA-DQ2[17].

In short, the complex interplay of multiple genetic and environmental factors will determine the development of CD and its complications. This review describes current knowledge on gluten, HLA-DQ and the immunopathogenesis of CD and its complications. In addition, we present a likely sequence of events in the development of CD and discuss the factors that may influence the risk of CD development.

Gluten: the disease inducing environmental trigger

The visco-elastic properties of gluten are essential for dough formation of wheat flour and give bread its unique texture and taste. Because of its unique properties gluten is widely used in the food industry: not only in products that are readily associated with wheat, like bread, cookies and pasta, but also as a hidden ingredient in sauces, instant soups and even medication. Consequently, the daily gluten intake on a gluten-containing diet in Western-Europe and the United States is high, between 15 and 20 gram per day. The omnipresence of gluten makes adherence to a gluten-free diet challenging for CD patients.

Gluten is a heterogeneous mixture of gliadins and glutenins in wheat or similar proteins in barley and rye. Each wheat variety expresses multiple α-, γ- and ω-gliadins in addition to low and high molecular weight glutenins. Gluten has a very high content of the amino-acids glutamine (30%) and proline (15%). By virtue of its high glutamine content gluten is rich in nitrogen, an essential factor for seed germination. The high proline content renders gluten highly resistant to degradation by gastrointestinal enzymes, making it possible for large immunogenic gluten peptides to reach the mucosal surface[18;19]. As modern wheat varieties contain three complete genomes encoding gliadins and glutenins, up to 100 different gluten proteins may be present in a single wheat variety and many of these are implicated in the pathogenesis of CD.

HLA-DQ: the strongest disease-associated gene locus by far

The strong genetic influence in CD is apparent as the concordance between monozygotic twins is 80%, whereas in dizygotic twins this is only 11%[20], which is approximately the same as the risk for first-degree relatives[21]. The main genetic influence in CD is HLA, which was first indicated by studies describing the predominance of HLA-B8 and HLA-DR3 serotypes in CD patients[22;23]. Later studies established that the strongest association is with HLA-DQ2 (DQA*0501, DQB*0201, termed HLA-DQ2.5 hereafter)[24], which is encoded together with HLA-B8 and HLA-DR3 on the highly conserved ancestral haplotype 8.1[25]. CD is associated to a lesser extent with HLA-DQ8 (DQA*03, DQB*0302)[11]. The strong association between HLA-DQ2.5 and CD is further illustrated by the observation that

individuals homozygous for HLA-DQ2.5 have a five-fold increased risk for development of CD compared to individuals heterozygous for HLA-DQ2.5[26]. Similarly, HLA-DQ 2.5 homozygosity is associated with the development of RCD II and RCD-associated lymphoma, whereas this association is less clear for HLA-DQ2.5 heterozygosity and HLA-DQ8[17]. Another HLA-DQ2 variant exists: HLA-DQ2.2 (DQA*0201, DQB*0202) which has a peptide-binding motif that is almost identical to that of HLA-DQ2.5[27]. Whereas HLA-DQ2.5 predisposes to CD, HLA-DQ2.2 does not. This difference is related to the peptide-binding properties of these HLA-DQ2 variants. The estimated risk effect of HLA-DQ2 and HLA-DQ8 on CD development is estimated to be ~35%[28].

Not all HLA-DQ2.5$^+$ and HLA-DQ8$^+$ individuals develop CD, indicating that these HLA-genotypes are necessary but not sufficient for CD development. Recent large-scale genetic association studies identified many additional genetic loci that all make a small contribution to the risk to develop CD (Box 1 and Table 1). Importantly, most of these genes encode proteins involved in immunity which supports the notion that CD is an immune related disorder and possibly provides clues on the immunopathogenesis of CD.

Locus	Gene candidate	Function	Odds ratio*
6p21	HLA	Antigen presentation	6.23 (5.95-6.52)
3q25-3q26	IL12A	Subunit of IL12, regulates Th1 differentiation	1.36 (1.29-1.44)
3p21	CCR1, CCR2, CCR3 and CCR5	Recruitment of immune cells to the site of inflammation	1.30 (1.23-1.39)
3q28	LPP	Possible role in maintaining cell shape	1.29 (1.25-1.34)
6q23	TNFAIP3	Inhibits NFκB activation and TNF-mediated apoptosis	1.23 (1.17-1.28)
12q24	SH2B3	Adaptor molecule involved in signaling in T cells	1.20 (1.15-1.24)
2q11-2q12	IL18R1 and IL18RAP	Respectively the α- and β-chain of IL18 receptor. IL18 is a proinflammatory cytokine	1.19 (1.14-1.25)
6q25	TAGAP	Role in modulating cytoskeletal changes	1.16 (1.12-1.21)
2p16	REL	Component of NFkB transcription complex	1.15 (1.11-1.20)
2q33	CTLA4	Inhibitory effect on the T cell response	1.14 (1.09-1.19)
	CD28	Stimulatory effect on the T cell response	
	ICOS	Stimulatory effect on the T cell response	
1q31	RGS1	Acts as GTPase activating protein, thereby regulating cell signaling	0.80 (0.76-0.84)
4q27	IL2	Stimulates proliferation of T cells	0.74 (0.70-0.78)
	IL21	Regulates the function of T and NK cells	

Table 1. Loci associated with CD development. *Odds ratios from Dubois et al.[60]

The adaptive immune response: gluten, HLA-DQ and CD4+ T cells

As noted above, CD develops almost exclusively in HLA-DQ2.5+ or HLA-DQ8+ individuals. It is also well established that in CD patients, gluten-derived peptides presented by either HLA-DQ2.5 or HLA-DQ8 induce a CD4+ T cell response. Both HLA-DQ2.5 and HLA-DQ8 prefer to bind peptides with negatively charged amino acids at anchor residues. Gluten peptides, however, are virtually devoid of negative charges and native gluten peptides thus bind poorly to HLA-DQ2.5 or HLA-DQ8. Consequently, CD4+ T cells specific for native gluten peptides are rare. It has become clear that the enzyme tissue transglutaminase 2 (TG2) can modify gluten peptides to fit the requirements for high affinity binding to HLA-DQ2 an HLA-DQ8[29;30]. TG2 can convert non-charged glutamine into negatively charged glutamic acid, a process called deamidation. Because gluten has a high content of glutamine (Q) and proline (P), the sequences QP, QXP and QXXP (in which X can represent any amino acid) are frequently found in gluten peptides. Strikingly, only in the sequence QXP the glutamine is converted, which results in highly selective introduction of negative charges in gluten peptides[31]. This specific deamidation process introduces the negative charges at the positions favored by HLA-DQ2 and HLA-DQ8, thereby expanding the presentable gluten peptide repertoire (Figure 2). As a result, the gluten-specific CD4+ T cell repertoire is substantially expanded which enhances the inflammation and disease development (Figure 2).

TG2 is mostly retained intracellularly in an inactive form and is activated upon its release during tissue damage[32;33]. Therefore, something should trigger tissue damage which initiates TG2 release, allowing the modification of gluten peptides. Whereas CD4+ T cell responses against native gluten peptides are relatively rare, they could represent the first breach in oral tolerance to gluten. The presentation of native gluten peptides by HLA-DQ2 or HLA-DQ8 to CD4+ T cells will lead to the production of IFN-γ (Figure 2). IFN-γ will in turn lead to higher expression of the HLA-DQ molecules and thereby to increased gluten peptide presentation (Figure 2). In the presence of gluten, this could become a self-amplifying loop that could cause limited tissue damage locally. This tissue damage would lead to the release of TG2 that will modify native gluten peptides into high affinity ligands for HLA-DQ2 and/or HLA-DQ8, thereby expanding the gluten-specific CD4+ T

Box 1. Non-HLA genes associated with CD

Candidate gene association study: Candidate genes, selected on the basis of current understanding of CD immunopathology, were tested for association with CD. Genes studied with this approach include, among others, IFN-γ, FAS, TCR, and TG2[55]. No convincing association with CD was found. ***Genetic linkage study:*** This approach is aimed at the identification of chromosomal regions that likely contain disease-causing genes in families with a high prevalence of CD. The genomic region 2q33 showed linkage to CD in multiple populations. This region contains the genes CD28, CTLA4 and ICOS which all control different aspects of the T cell response[55]. Linkage was also found for chromosome 5q31-33[56] and chromosome 19p13.1[57]. Linkage to these regions, however, could not always be replicated in other populations. ***Genome-wide association study (GWAS):*** In recent years it has become possible to perform large-scale case control-based association studies using single nucleotide polymorphisms (SNPs). With this approach it is possible to identify common variants in the genome that predispose to disease. Until now, 10 non-HLA loci associated with CD have been identified and linkage to 2q33 has been confirmed (Table 1)[28;58;59]. Recently, 13 additional true risk variants and 13 suggestive risk variants were identified[60]. Although causality has only been proven for the risk allele SH2B3[61], it is clear that nearly all associated regions contain genes involved in immune response.

cell responses and leading to additional tissue damage: the initiation of a second self-amplifying loop (Figure 2). Alternatively, infections occurring in the gastrointestinal tract would generate a pro-inflammatory milieu that might lead to loss of tolerance to native gluten peptides and generate tissue damage simultaneously and thus initiate deamidation by TG2.

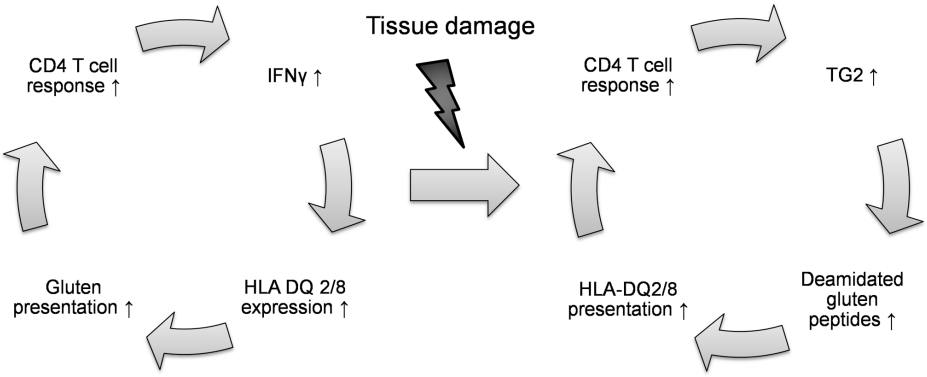

Figure 2. Self amplifying loops in the development of CD. HLA-DQ2$^+$ or HLADQ8$^+$ individuals can present native gluten peptides on their APC to CD4$^+$ T cells. The IFN-γ released in this process can in turn boost HLA-DQ2/8 expression. This low-grade inflammation might eventually lead to tissue damage with TG2 release. TG2 is able to expand the presentable gluten peptide repertoire by deamidation. As a result the CD4$^+$ T cell response is strongly enhanced, leading to more IFN-γ, tissue damage and increased release of TG2. The end result is full-blown CD.

Activated intraepithelial lymphocytes damage the intestinal epithelium

Intraepithelial lymphocytes are localized between intestinal epithelial cells at the basolateral side of the epithelium and are thought to play an important role in immunosurveillance of the epithelium. The IEL population in the small intestine is a mixed population of TCRαβ$^+$ T cells, TCRγδ$^+$ T cells and NK cells, although the vast majority of the IELs are CD8$^+$ TCRαβ$^+$ T cells[34]. Furthermore, most of these TCR$^+$ IELs express a variety of NK cell receptors that is distinct from the NK cell receptors expressed on blood T cells[35]. The NK cell receptors are thought to act mainly as T cell co-stimulators, lowering the threshold for T cell activation in stressful times[36].

In active CD the number of CD8$^+$ TCRαβ$^+$ and TCRγδ$^+$ IELs is markedly increased. It is unclear whether this is a response to changes in the homeostasis of the epithelium or a consequence of the pro-inflammatory milieu created by the CD4$^+$ T cell response in the lamina propria (Figure 3). Similar to IELs of normal controls, IELs from active CD express NK cell receptors. When compared to normal controls, however, IELs from CD patients have acquired a more activating NK cell receptor repertoire[35]. In the healthy small intestine, IELs predominantly express the inhibitory CD94/NKG2A receptor. In contrast,

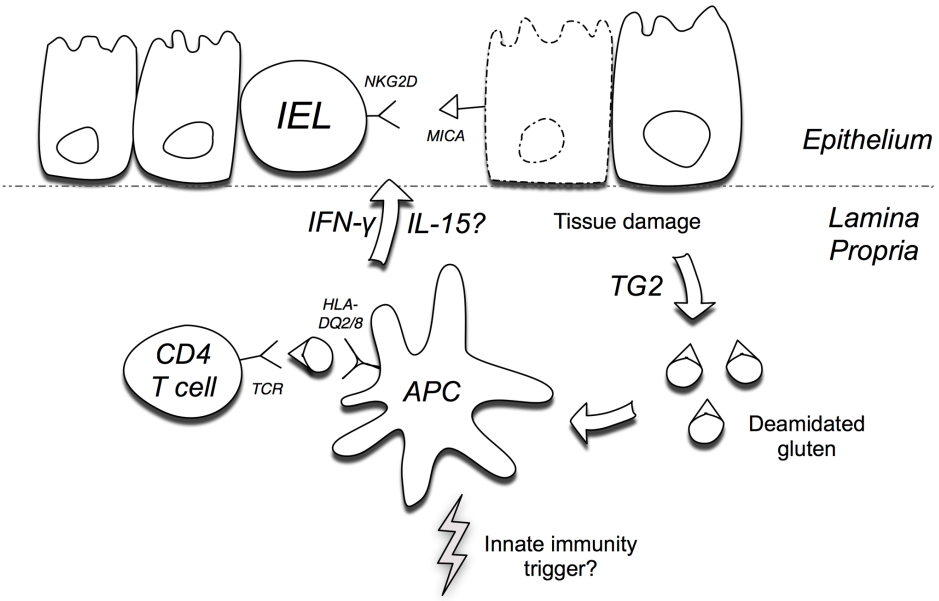

Figure 3. Crosstalk between the CD4⁺ T cell response and IEL cytotoxicity. The CD4⁺ T cell response to gluten may lead to IFN-γ production and potentially to upregulation of IL-15, which in turn boosts IEL-mediated cytotoxicity. Activated IELs lyse the epithelium, which leads to TG2 release and subsequent deamidation of gluten peptides. This may constitute yet another self-amplifying feedback loop, as deamidation of gluten peptides will enhance the CD4⁺ T cell response (see Figure 2).

IELs in CD express high levels of activating receptors like CD94/NKG2C and NKG2D[37;38]. Simultaneously, intestinal epithelial cells in CD upregulate MIC and HLA-E, the ligands for NKG2D and CD94/NKG2C, respectively. Interaction of NKG2D and CD94/NKG2C with their ligands will enhance IFN-γ-production and cytolysis, leading to tissue damage (Figure 3). An important factor in acquiring an activating NK cell receptor repertoire is interleukin 15 (IL-15; Box 2), which has been shown to upregulate both NKG2D and CD94/NKG2C on IELs of active CD patients and boost their ability to lyse enterocytes[37;38]. In addition, IL-15 can alter the NK cell receptor function, leading to NK cell receptor mediated cytotoxicity independent of TCR specificity[37;38]. In conclusion, while gluten-specific CD4⁺ T cells elicit an inflammatory response in the lamina propria, IELs in the epithelium acquire activating NK receptors and the ability to lyse stressed epithelial cells independent of T cell receptor signaling, which likely contributes to the typical tissue damage in CD.

In RCD, the survival, expansion and acquisition of an NK cell-like phenotype by IELs is even more pronounced than in CD, possibly as a result of the presence of larger amounts of IL-15. RCD II patients have an aberrant clonal IEL population that lacks surface TCR-CD3 expression. Studies on aberrant TCR-CD3⁻ IEL lines from RCD II patients showed that, upon stimulation with IL-15, these cell lines express granzyme B and lyse the intestinal epithelial cell line HT29, suggesting a role for aberrant IELs in perpetuating epithelial damage in RCD II[39]. Therefore, IL-15 dependent NK cell-like transformation of IELs may be an essential step in the immunopathology of RCD.

A threshold model for the risk of CD development

The expansion of the presentable gluten peptide repertoire due to the release and activity of TG2 is a critical step in the pathogenesis of full-blown CD. Several lines of evidence support the notion that the level of gluten presentation to T cells critically influences the risk of disease development.

First, HLA-DQ2.5 homozygous individuals have a five-fold higher risk of CD development than HLA-DQ2.5 heterozygous individuals[26]. This gene dose effect directly correlates with the magnitude of the $CD4^+$ T cell response: antigen presenting cells (APC) from HLA-DQ2.5 homozygous individuals induce very strong proliferative T cell responses and IFNγ production, while APC from HLA-DQ2.5/DQX heterozygous individuals induce much weaker responses[40]. These data indicate that the number of HLA-DQ2.5 molecules capable of presenting gluten peptides on the surface of APC will define the magnitude of the $CD4^+$ T cell response.

Second, whereas HLA-DQ2.5 is associated with CD development, the homologous HLA-DQ2.2 is not. Although these two variants have almost identical peptide binding motifs, HLA-DQ2.2 can only bind a subset of the gluten peptides that can bind to HLA-DQ2.5. This difference is explained by the fact that a proline at position 3 in peptides have an adverse effect on peptide binding to HLA-DQ2.2[27]. As gluten epitopes cluster in proline-rich regions[41], many gluten peptides have a proline at position 3 and do not bind to HLA-DQ2.2[40]. Consequently, HLA-DQ2.5 is able to present a much broader repertoire of gluten peptides than HLA-DQ2.2. In addition, HLA-DQ2.5 is better at retaining gluten peptides in its binding groove compared to HLA-DQ2.2[42]. As a result, gluten peptide presentation by HLA-DQ2.5 is protracted compared to presentation by HLA-DQ2.2 which will increase the chance for productive $CD4^+$ T cell stimulation.

Third, CD is associated mainly with HLA-DQ2.5 and to a lesser extent with HLA-DQ8. Although a variety of gluten peptides has been identified that can stimulate HLA-DQ8 restricted T cells from CD patients, one α-gliadin peptide in particular appears to be immunodominant as this peptide invariably induces specific T cell responses in HLA-$DQ8^+$ CD patients[43-46](Kooy et al. unpublished data). In contrast to the HLA-DQ2.5 restricted α-gliadin peptides, the HLA-DQ8 peptide is not derived from a proline-rich region of the α-gliadin protein and therefore likely susceptible to degradation in the gastrointestinal tract. Furthermore, whereas for HLA-DQ2 a single deamidation in a gluten peptide is sufficient to evoke a $CD4^+$ T cell response, for HLA-DQ8 deamidation at two positions is preferred[45], which may limit the generation of strong antigenic gluten peptides. The fact that the immunodominant HLA-DQ8 peptide is more readily degraded

Box 2. IL-15

IL-15 is a cytokine that, just as IL-2, is able to induce T cell proliferation, IFN-γ production and cytotoxicity. Furthermore, IL-15 is known to play an important role in NK cell development and activation[62]. Under normal circumstances, IL-15 expression is strictly regulated at the level of transcription, translation and secretion[63]. In CD this regulation is disrupted, which results in massive upregulation of IL-15 in the epithelium and lamina propria. The abnormal availability of IL-15 results in chronic inflammation by survival, proliferation and activation of IELs[64;65]. Furthermore, IL-15 can exert an inhibitory effect on TGF-β, a negative regulator of the immune response[66]. Recently it has been shown that IL-15 can synergize with IL-21, a cytokine expressed on CD4+ T cells and a stimulator of IFN-γ production and cytolytic activity of CD8+ T cells and NK cells[67;68].

and requires more deamidation steps, limits the availability for antigen presentation and may therefore limit the risk to develop CD.
Fourth, further evidence that the level of gluten presentation is a critical parameter comes from a totally different angle: most CD patients tolerate oat even though it has been shown that the gluten-like molecules in oat can elicit $CD4^+$ T cell responses in CD patients[47;48] There are two striking differences between the relatively safe oat and the disease inducing cereals wheat, barley and rye: (1) while the gluten-like molecules in oat contain only two antigenic sequences, dozens are found in gluten and the gluten-like molecules of barley and rye, (2) the "gluten" content of oat is much lower compared to the other cereals. Consumption of oat thus results in a much lower exposure to antigenic peptides in comparison with the other cereals and this is apparently tolerated as it does not lead to disease in the majority of patients.
Collectively, these data indicate the presence of a threshold to develop CD. Initiation of CD becomes more likely with increased T cell exposure to gluten antigens. This exposure is influenced by the type and amount of HLA-DQ as this determines the efficiency of gluten peptide presentation to $CD4^+$ T cells. For HLA-DQ2.5 homozygous individuals, the threshold to develop CD is most easily exceeded, whereas for HLA-$DQ2.2^+$ and HLA-$DQ8^+$ individuals the threshold is much higher.

CD development: a series of unfortunate events

The past two decades have witnessed the identification of several critical immunological factors in CD, from which a likely sequence of events in the development of this disease can be deduced. It is well known that healthy individuals can have antibodies against native gluten peptides. As an antibody response is controlled by $CD4^+$ T cell help, such individuals most likely have $CD4^+$ T cells specific for native gluten peptides, indicating that the mere presence of such T cells is, in general, not sufficient to exceed the threshold to develop CD (Figure 4). This indicates that in the majority of individuals, tolerogenic and regulatory processes in the intestine keep gluten-specific T cell responses in check. This steady state can be breached by frequent episodes of enteroviral infections as this leads to the secretion of inflammatory cytokines and differentiation of Th1 cells[49], thereby enhancing the response to gluten (Figure 4). Subsequently, the combined effect of low-level gluten reactivity and pathogen induced inflammation could lead to tissue damage and the release of TG2. In turn, the activity of TG2 would generate a large repertoire of deamidated gluten peptides with high affinity for HLA-DQ, thereby boosting the gluten-specific T cell response (Figure 4). At this point in CD development, self-amplifying loops are in action: $CD4^+$ T cell responses against native gluten peptides lead through IFN-γ production to upregulation of HLA and further amplification of the gluten-specific T cell response. TG2 released upon tissue damage expands the presentable gluten peptide repertoire which will ultimately lead to more tissue damage (Figure 2). Due to the massive expansion of the gluten-specific T cell pool, regulatory processes are no longer able to contain the T cell responses, and exposure to gluten suffices to perpetuate inflammation. Eliminating gluten from the diet is the only way to stop this process. At all stages in this scenario the risk to develop CD is influenced by the HLA-DQ genotype as HLA-DQ2.5 homozygous individuals will present more HLA-gluten complexes than HLA-DQ2.5 heterozygous individuals (Figure 4). Simultaneously, an infiltrate of IELs is formed in the epithelium, which is potentially driven by the inflammatory milieu created

by CD4$^+$ T cells in the lamina propria. These IELs upregulate activating NK cell receptors and acquire the ability to lyse enterocytes independent of the TCR.
The development of full-blown CD is most likely the result of an unfortunate series of events which, in isolation, would not lead to disease, but, combined have a detrimental outcome. It is important to note that exposure to gluten, frequent enteroviral infections and occasional TG2 activation likely occur in every individual but usually do not result in CD development, even in HLA-DQ2$^+$ and/or HLA-DQ8$^+$ individuals (Figure 1). It has now become clear that the presence of a higher number of additional non-HLA risk alleles (Table 1) is directly correlated with an increase in the risk to develop CD[50]. This suggests that the influence of non-HLA genes lowers the threshold to develop CD and could skew the balance towards disease development (Figure 4). It is plausible that non-HLA genes also increase the risk to develop complicated CD, although this hypothesis will be difficult to test as this patient population is very small. Thus, even though key pieces of the celiac puzzle have been collected and assembled, the picture is not yet complete.

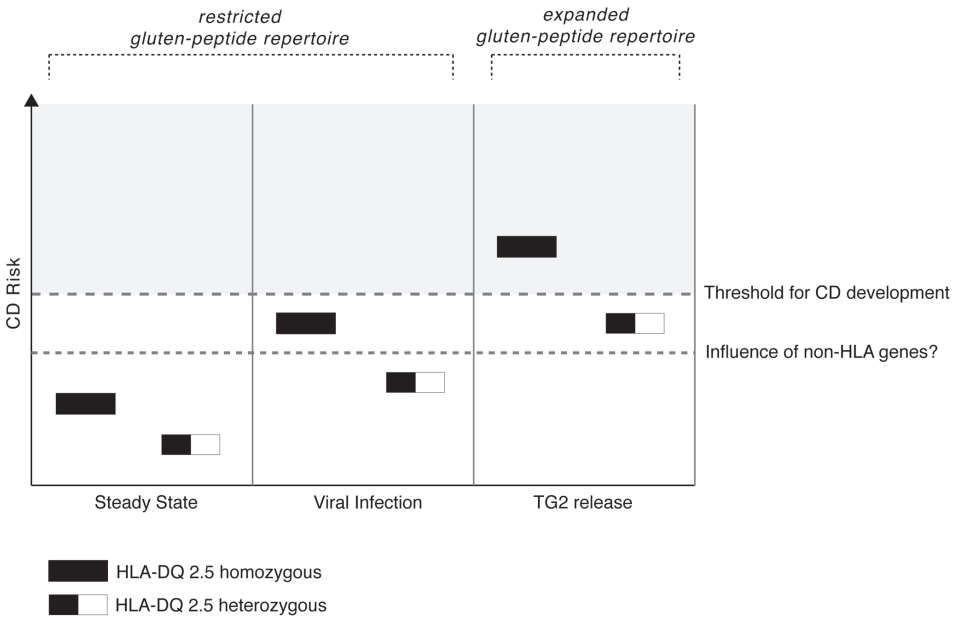

Figure 4. Threshold model for CD development. As HLA-DQ2.5 homozygous individuals can present more gluten peptides on their APC than HLA-DQ2.5 heterozygous individuals, HLA-DQ2.5 homozygotes have a higher risk of CD development a priori. In steady state conditions, presentation of native gluten peptides is unlikely to induce disease. This steady state can be breached by frequent viral infections and low-grade T cell responses to native gluten peptides that lead to release of TG2 upon tissue damage. TG2 activity will expand the presentable gluten peptide repertoire extensively, thereby increasing the risk to develop CD. The recently uncovered non-HLA genes associated with CD likely lower the threshold to develop CD.

Open questions

Although the molecular basis for the involvement of HLA-DQ in CD is now well established, a number of issues remain unclear.

First, it is still controversial how immunogenic gluten peptides from the intestinal lumen reach the lamina propria where they can prime gluten-specific T cells. It has been suggested that gluten peptides can be transported during transient increased intestinal permeability during enteroviral infections[49] or by IgA-mediated retrotranscytosis[51]. Yet, this issue is far from resolved.

Second, a direct effect of gluten on the intestinal mucosa has been attributed to a peptide from α-gliadin, p31-49 (Box 3). It remains unclear, however, whether and how this peptide would exert its activity and contribute to the disease development (Box 3).

Third, TG2 is a crucial factor in expanding the presentable gluten peptide repertoire. In steady state conditions, TG2 is present in an inactive form intracellular and on the cell surface. An intriguing question is therefore, how TG2 is activated and released in CD. We propose that TG2 is released upon tissue damage induced by the initial $CD4^+$ T cell response to native gluten peptides (Figure 2 and 3). Alternatively, a recent study proposed a role for TLR3 ligands released during enteroviral infections that upon ligation with TLR3 could result in TG2 activation[33]. These two possibilities are not mutually exclusive.

Fourth, in active CD, disrupted IL-15 regulation results in massive overexpression of IL-15. It remains unclear what causes this disruption. As on a gluten-free diet the adaptive $CD4^+$ T cell response and IL-15 expression both decrease[39], it is possible that the adaptive $CD4^+$ T cell response has a direct effect on IL-15 expression (Figure 3). Alternatively, innate signals delivered through TLRs may be responsible for elevated IL-15 levels.

Finally, the events leading from uncomplicated CD to RCD II and subsequent lymphoma development are still poorly understood. Lymphoma cells develop from the aberrant IELs in RCD II. One view is that aberrant IELs derive from mature TCR^+ IELs that have undergone oligoclonal expansion and lost surface TCR-CD3 expression due to overstimulation[4]. Alternatively, aberrant IELs could derive from a distinct population of $CD3^-$ $CD7^+$ precursor cells that can develop into T cells and NK cells[52]. In favor of the first hypothesis: although

Box 3. Direct effects of gluten

The role of gluten in the adaptive immune response in CD is well established. In addition, a direct (innate) effect of gluten on the intestinal mucosa has been suggested. One of the first indications for the potential of gluten to elicit a response in the epithelium came from in vivo challenges where administration of gliadin caused villous atrophy and increase of IELs within 2-3 hours after gluten ingestion[69]. This effect was later attributed to the non-immunodominant peptide p31-49 from alpha-gliadin[70]. In vitro studies with p31-49 showed that epithelial alterations were independent of $CD4^+$ T cell activation. Furthermore, p31-49 stimulated IL-15 production in the lamina propria of cultured biopsies from CD patients[71]. The fact that p31-49 could activate the local immune system implied that a receptor for p31-49 should exist. A transcellular transport pathway was proposed where anti-gliadin IgA antibodies were able to bind p31-49. This complex would then bind the transferrin receptor CD71 which would provide protected trafficking across the intestinal epithelium[51]. However, this mechanism would not function in all CD patients as a relatively large fraction of them is IgA deficient[72]. We also assessed the hypothesis that a receptor for p31-49 is present on intestinal epithelial cells. Binding of p31-49 to intestinal epithelial cell lines, however, could not be detected, neither directly nor by either UV-crosslinking or TG2 induced transamidation (Tjon et al. unpublished data). In the absence of a receptor through which p31-43 could exert its activity, the molecular mechanism underlying the biological effects observed with this peptide remains unclear.

aberrant IELs lack surface TCR-CD3 expression, they do express CD3 intracellularly and display TCR-γ gene rearrangements[8]. Furthermore, microarray analysis in one study on TCR-CD3$^+$ IEL lines from CD patients revealed a significant decrease in the transcript levels of TCRα- and TCRβ-chains[38], indicating that IELs may lower TCR expression in CD. We found that aberrant IELs not only express CD3ε intracellularly, but also have intracellular expression of the CD3γ, CD3δ and ζ-chains[53]. In favor of the second hypothesis: the TCR-chains were not always present[53] and TCR-rearrangements were often incomplete (Tjon et. al. unpublished data). Furthermore, the full complement of CD3 chains and incomplete TCR rearrangements have also been observed in NK cell precursors, and even mature NK cells can carry partially rearranged TCRs. A recent study indicated that extrathymic TCR gene rearrangement is an ongoing event in the human small intestine throughout life[54]. This raises the possibility that aberrant IELs derive from cells in an early stage of extrathymic lymphocyte development.

Concluding remarks

Life used to be simple: CD was a rare disease, diagnosed in 1 in a 1000 individuals. Patients were HLA-DQ2$^+$ or HLA-DQ8$^+$ and could be treated effectively with a gluten-free diet. That was about it.

Now we know that CD affects ~1% of the population in Western Europe and the USA, most of which remain undiagnosed. Although good insight has been gained on the immunopathology of CD -inflammation in both lamina propria and epithelium- it remains unclear what triggers the development of CD and why not every patient is equally affected. In addition, with the recognition of RCD and RCD-associated lymphoma that do not respond to a gluten-free diet, CD has become a far more complicated disease.

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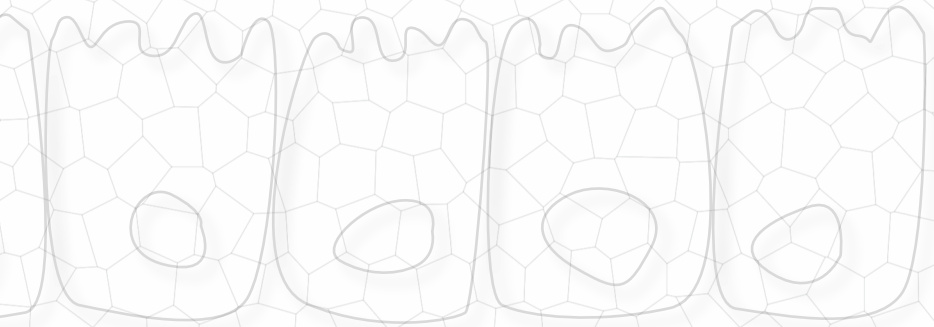

Chapter 2

Defective Synthesis or Association of T Cell Receptor Chains Underlies Loss of Surface T Cell Receptor-CD3 Expression in Enteropathy-associated T Cell Lymphoma

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Abstract

Enteropathy-associated T cell lymphoma, an often fatal complication of celiac disease, can result from expansion of aberrant intraepithelial lymphocytes in refractory celiac disease type II (RCD II). Aberrant intraepithelial lymphocytes and lymphoma cells are intracellularly CD3ε$^+$, but lack expression of the T cell receptor (TCR)-CD3 complex on the cell surface. It is unknown what causes the loss of TCR-CD3 expression. We report the isolation of a cell line from an RCD II patient with the characteristic phenotype of enteropathy-associated T cell lymphoma. We demonstrate that in this cell line the TCR-α and -β chains as well as the CD3γ, CD3δ, CD3ε and ζ chains are present intracellularly and that assembly of the CD3γε, CD3δε and ζζ dimers is normal. However, dimerization of the TCR chains and proper assembly of the TCR-CD3 complex is defective. On introduction of exogenous TCR-β chains, but not of TCR-α chains, assembly and functional cell surface expression of the TCR-CD3 complex was restored. Defective synthesis of both TCR chains was found to underlie loss of TCR expression in similar cell lines isolated from two additional patients. (Pre)malignant transformation in RCD II thus correlates with defective synthesis or defective association of the TCR-chains, resulting in loss of surface TCR-CD3 expression.

Introduction

Celiac disease (CD) is an inflammatory disorder of the small intestine caused by a dysregulated immune response to ingested wheat gluten, which typically leads to villous atrophy and increased numbers of intraepithelial lymphocytes (IELs) in the intestinal mucosa. Whereas most patients recover on a gluten-free diet, a small proportion of patients fails to improve and develops a condition called refractory celiac disease (RCD). RCD is characterized by persisting or recurring villous atrophy with crypt hyperplasia and an increase of IELs despite a gluten-free diet. Two types of RCD are currently recognized: RCD I, without aberrant IELs and RCD II, with aberrant IELs[1;2;3]. The aberrant IELs in RCD II lack CD3, CD4, CD8 and the T cell receptor (TCR) on the surface, but express CD3 intracellularly and display monoclonal TCR-γ gene rearrangement[2;4;5]. Furthermore, it has been shown that interleukin 15 (IL-15) is upregulated in the lamina propria and epithelial cells of RCD patients which induces growth and activation of these clonal IELs[6;7]. Because an expansion of IELs under the influence of IL-15 may eventually give rise to overt enteropathy-associated T cell lymphoma (EATL), the presence of such a clonal IEL population is thought to be a premalignant condition[1;2;6;8]. It is not known what drives lymphoma development and the associated loss of TCR expression in RCD II.

In the present study, we report the isolation of a cell line from a duodenal biopsy of a patient with RCD II. This cell line has the characteristic $CD4^-$, $CD8^-$, intracellular $CD3\varepsilon^+$, surface TCR-$CD3^-$ phenotype of RCD II-associated IELs and proliferates in the presence of IL-15. In addition, these IELs express CD30 on the cell surface, as is typically seen in EATL[9]. We have used this cell line and subunit-specific antibodies to analyze the expression and assembly of the TCR and CD3 subunits. The results indicate that whereas all TCR-CD3 subunits were present intracellularly, proper assembly of the TCRαβ-dimer was defective. Functional cell surface expression of the complex could be restored by the introduction of an exogenous TCR-β chain. A similar analysis of cell lines isolated from two additional RCD II patients indicated defects in the synthesis of the TCR-chains. Defective synthesis or defective association of TCR chains thus causes loss of functional surface TCR-CD3 expression on IELs in RCD II, a process which is likely important in escape from immune regulation and progression into EATL.

Materials and methods

Patient histories

Patient 1 (P1) was typed as HLA-A1/A2, -B8, -Cw7/Cw12, DR3/7, DQ2 and developed celiac disease at the age of 51 years. At age 67, refractory celiac disease type II with aberrant IELs was diagnosed. Until the present study no EATL has developed[10]. Patient 2 (P2) was typed as HLA-A3/32, -B8, -Cw7, DR3, DQ2 and Patient 3 (P3) as HLA-A1, -B8, -Cw7, DR3, DQ2. Both patient 2 and 3 were RCD II patients with aberrant IELs and without EATL.

Cell lines

As controls the following cell lines were used: T cell clone N10[11], a $CD4^+$ gliadin-specific T cell clone isolated from a duodenal biopsy from a CD patient and Jurkat clones deficient for either TCRα ($\alpha^{-/-}$) or TCRβ ($\beta^{-/-}$) expression[12].

Small intestinal biopsy specimens

During upper endoscopy, large spike forceps biopsy specimens (Medi-Globe, Tempe, Arizona) were taken from the second part of the duodenum[13]. Biopsy specimens were taken for direct flow cytometric analysis, TCR gene rearrangement assessment and T cell culture. All biopsy specimens were obtained after given informed consent in accordance with the local ethical guidelines of the VU Medical Center, Amsterdam and the Declaration of Helsinki.

Isolation of intestinal lymphocytes and flow cytometry

IELs were isolated from duodenal biopsies as described by Madrigal et al[14] with minor modifications. Briefly, biopsies were vigorously shaken at 37˚C for 60 min in phosphate buffered saline (PBS) supplemented with 1mM dithiothreitol (Fluka BioChemika, Buchs, Switzerland) and 1mM ethylenediaminetetraacetic acid (Merck, Darmstadt, Germany). The released IELs were washed twice with PBS supplemented with 0,1% BSA (Roche Diagnostics, Mannheim, Germany) and subsequently stained for 30 minutes on ice, with fluorochrome-labeled monoclonal antibodies (MoAbs) directed against CD3, CD4, CD8, CD7, CD45, γδTCR (all from BD Biosciences, San Jose, California) as previously described[15]. Cytoplasmic staining of CD3 was performed after cell permeabilization (Cytofix/CytoPerm Plus™ kit by BD Biosciences). Flow cytometric acquisition was performed using Cellquest software on a fluorescence-activated cell sorter (FACS; FACS-Calibur, BD Biosciences). The data were analyzed using Cellquest software (BD Biosciences). All analyses were performed on lymphocytes, based on $CD45^{bright}$ staining and low sideward scatter.

Assessment of T cell clonality

TCR-γ gene rearrangements were analyzed on two cryopreserved biopsy specimens and on cell line P1 (see "T cell culture"). DNA was extracted from cryosections using proteinase-K digestion and ethanol precipitation of the genomic DNA. TCR-γ gene rearrangements were subsequently analyzed by multiplex polymerase chain reaction (PCR) amplification, using the primers and probes provided by the BIOMED-2 consortium according to their guidelines[16].

T cell culture

Intraepithelial and lamina propria T lymphocytes were isolated from a duodenal biopsy from a RCD II patient. After treatment with 1mM dithiothreitol (2 times for 10 min at room temperature) and 0.75 mM ethylenediaminetetraacetic acid (60 min at 37°C) the biopsy was cultured in Iscove's modified Dulbecco's medium (IMDM; Lonza, Verviers, Belgium) supplemented with 10% normal human serum (NHS), 10 ng/ml recombinant IL-15 (R&D Systems Europe, Abingdon, United Kingdom), gliadin and gliadin treated with tissue transglutaminase. From day five, cells were further expanded in IMDM with 10% NHS containing 10 ng/ml IL-15. Staining of cells from T cell culture was performed with fluorochrome-labeled monoclonal antibodies directed against CD3, CD4, CD8, TCRαβ, TCRγδ, CD103, integrin β7, CD30 (all from BD Biosciences), NKG2D (R&D) and KIR2DL2/KIR2DL3/KIR2DS2 (MoAb GL183, Beckman Coulter). The predominant cell population consisting of $TCR\alpha\beta^-$, $CD3^-$, $CD4^-$, $CD8^-$, $CD30^+$ cells was purified by FACS and cultured in IMDM with 10% NHS containing 10 ng/ml IL-15. Cells were restimulated approximately every 6-7 weeks with 1μg/ml PHA, 10ng/ml IL-15 and $1x10^6$/ml irradiated allogenous PBMC as feeder cells. Stability of the aberrant IEL phenotype of the P1 line was checked with flowcytometry at least once between every two restimulations.

Proliferation assay

Cells from RCD cell line P1 were rested by culturing them in the absence of IL-15 for four days. Cells (10.000 cells per well) were subsequently cultured in triplicate in 96-well plates in the presence or absence of IL-15 and/or IL2 for two to five days at 37°C, after which 0.5 μCi of ^{3}H-thymidine was added to every well. After overnight incubation at 37°C, cells were harvested (Tomtec Harvester, Hamden, CT) and ^{3}H-thymidine incorporation was determined.

Antisera and antibodies

The antisera against TCR-α chain, TCR-β chain, CD3γ chain, CD3δ chain and CD3ε chain were rabbit anti-peptide antisera. As described previously[17;18] the antiserum against the TCR-α chain was raised against a sequence in the extracellular constant region, while antisera against TCR-β chain, CD3γ chain, CD3δ chain and CD3ε chain were raised against peptides corresponding to the carboxy termini of these chains. The anti-ζ-chain monoclonal antibody was obtained from BD pharmingen (San Diego, CA). The anti-CD3 antibody OKT3 used in the stimulation assay was obtained from Orthobiotech (Bridgewater, NJ).

^{35}S Metabolic labeling and cell surface iodination

Metabolic labeling and cell surface iodination were performed as previously described[19]. For ^{35}S metabolic labeling, $10x10^6$ cells were washed thrice in PBS and resuspended in 5 ml methionine- and cysteine-free RPMI (Sigma-Aldrich, Zwijndrecht, The Netherlands) containing 0.5% FCS and 10 ng/ml recombinant human IL-15. 1 mCi of ^{35}S-methionine/cysteine (NEN) was added and cells were incubated overnight at 37°C. After incubation cells were washed in PBS and lysed overnight at 4°C in 1 to 2 ml lysisbuffer containing either 0.5% Nonidet P40 (NP40; Pierce, Rockford, IL) or 1% digitonin (Sigma-Aldrich). For iodination approximately $6x10^6$ cells were washed in PBS and resuspended in 30μl lactoperoxidase solution (2mg/ml, Sigma-Aldrich). 1 mCi ^{125}I Na was added to the cells followed by the addition of 10μl 0.05% H_2O_2/PBS solution. During frequent mixing 0.05%

H_2O_2/PBS solution was added after 5 min (15 µl) and after 15 min (20 µl). After 30 min, free iodine was removed by washing with PBS. Cells were lysed overnight at 4°C in 250-500 µl lysisbuffer containing 1% digitonin.

Immunoprecipitation and SDS-PAGE analysis

^{125}I or ^{35}S lysates were centrifuged at maximum speed for 20 min in an eppendorf centrifuge at 4°C. Lysates were precleared twice, first with 100 µl protein A sepharose beads and 50 µl normal rabbit serum and second with 100 µl protein A sepharose beads only, both under rotation for 1 hour at room temperature. After removal of the beads, 5 µl antiserum (TCR-α, TCR-β, CD3γ, CD3δ, CD3ε antisera, anti-ζ-antibody or normal rabbit serum) was added to 100 µl precleared lysate and rotated for 1 hour at room temperature. Antigen-antibody complexes were isolated with 12.5 µl protein A sepharose beads during 1 hour rotation at room temperature. Beads were washed four times in lysisbuffer and analyzed under either reducing or nonreducing conditions on a one-dimensional 13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After drying of the gels, autoradiography was performed at -80°C using Fuji scientific imaging films (Fuji, Düsseldorf, Germany).

Retroviral transduction

The TCR-α and TCR-β chains isolated from T cell clone N10 (*TCRAV14, TCRBV4*) were cloned into a bicistronic vector as described before[20]. The vector containing the TCR-α chain was combined with the marker green fluorescent protein (GFP), the vector containing the TCR-β chain was combined with truncated nerve growth factor receptor (tNGFR). Both α and β chain constructs were transfected into Phoenix packaging cells[21]. Retroviral supernatant was produced and used to transduce cells from RCD cell lines P1, P2 and P3 with either TCR-α, TCR-β, or both. For transduction, non-tissue-culture-treated 24 well plates (Falcon, BD Biosciences) were treated 2 hours with 25 µg/ml Retronectin (Takara, Otsu, Japan) and blocked 30 min with 2% HSA. After 30 min incubation with the cells, retroviral supernatant was added and cells were placed overnight at 37°C. Presence of the TCR-α chain (GFP) and TCR-β chain (tNGFR) and cell surface expression of the TCR was analyzed with flow cytometry. $TCR\alpha\beta^+$, $NGFR^+$ cells were purified by FACS.

T cell stimulation with anti-CD3

96 well non-tissue-culture-treated plates (Falcon, BD Biosciences) were coated overnight in triplicate with various concentrations of anti-CD3 (OKT3). After coating, plates were blocked with 10% FCS/PBS and washed three times with PBS. Per well 15000 nontransduced P1 cells, P1 cells transduced with the TCR-β chain from T cell clone N10 or cells from T cell clone N10 were added. Plates were incubated at 37°C for 2 days, after which 0.5 µCi of ^3H-thymidine was added. After overnight incubation at 37°C, cells were harvested and ^3H-thymidine incorporation was determined.

Results

The majority of IELs in the small intestine of RCD II patient P1 are aberrant and monoclonal

RCD II is associated with aberrant IELs lacking CD3, CD4, CD8 and the TCR on the cell surface, but expressing CD3 intracellular. To gain insight in the phenotype of the IELs in the small intestine of RCD II patient P1, FACS analysis was performed on IELs directly isolated from a freshly taken duodenal biopsy of patient P1. Analysis of the $CD45^{bright}$ IEL population showed that the majority (71-76%) were, $CD3^-$, $CD4^-$, $CD8^-$, $CD7^+$ but cytoplasmic $CD3^+$, and may thus be defined as aberrant (Figure 1A). Furthermore, PCR analysis demonstrated the presence of monoclonal TCR-γ gene rearrangement in two cryopreserved biopsy specimens of patient P1 (data not shown).

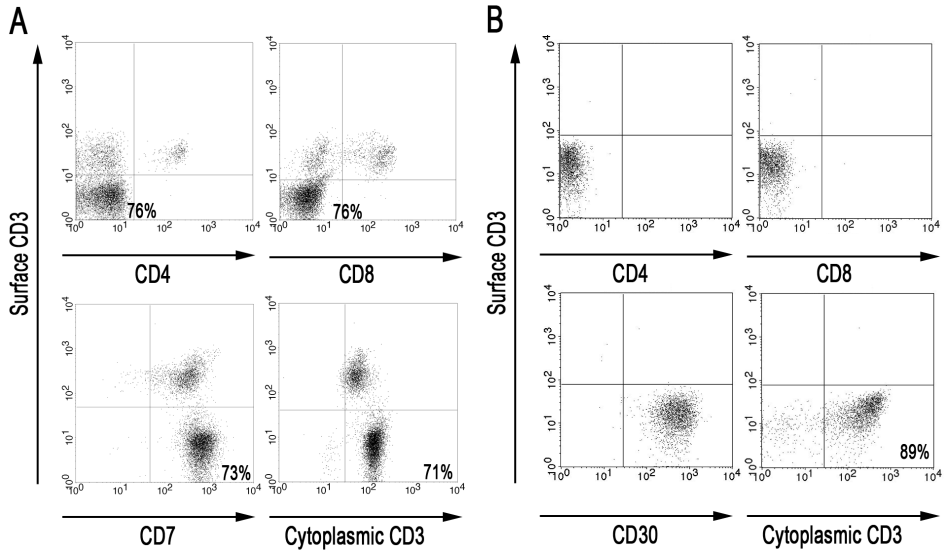

Figure 1. The majority of IELs in the small intestine of RCD II patient P1 are aberrant. (A) FACS analysis of IELs, directly isolated from duodenal biopsies from patient P1. (B) FACS analysis of RCD cell line P1, cultured from duodenal biopsies from patient P1. Analyses were performed on $CD45^{bright}$ cells within a live lymphocyte gate.

RCD cell line P1: a model for aberrant IEL in RCD II and EATL

Culture of a duodenal biopsy from RCD II patient P1 with IL-15 resulted in outgrowth of a cell line in which the predominant population, similar to the freshly isolated IELs, was found to be $CD3^-$, $TCR\alpha\beta^-$, $CD4^-$, $CD8^-$ and cytoplasmic $CD3^+$ (Figure 1B). In addition, these cells were $CD30^+$, which is a characteristic feature of EATL[9] (Figure 1B). Furthermore, the cells displayed the same monoclonal TCR-γ gene rearrangement as the two cryopreserved biopsy specimens of patient P1 (data not shown). The predominant population, hereafter called RCD cell line P1, was purified by FACS and subsequently used as a model for aberrant IELs in RCD II and EATL. As IL-15 is upregulated in the lamina propria and epithelial cells of RCD patients and induces growth and activation of clonal IELs[6;22], the effect of IL-15 on the proliferation of RCD cell line P1 was assessed. Figure 2A shows dose-dependent proliferation of RCD cell line P1. Proliferation in response to high doses of IL-2 was much lower than proliferation in response to IL-15

(Figure 2B). Furthermore, combining IL-15 and IL2 had no additional effect on proliferation compared to IL-15 alone (Figure 2B). The specific response of RCD cell line P1 to IL-15 further supported the notion that this cell line can serve as a model for aberrant IELs in RCD II and EATL.

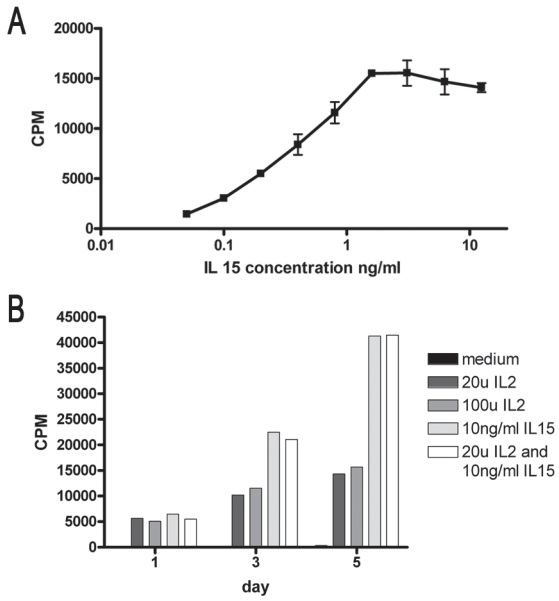

Figure 2. IELs from RCD cell line P1 proliferate in response to IL-15 in a dose-dependent manner. (A) Proliferation in response to various doses of IL-15. (B) Five-day follow up of proliferation in response to IL-2, IL-15 and a combination of both. CPM indicates ^{3}H-thymidine incorporation.

Defective association of the TCR chains underlies the loss of surface TCR-CD3 expression in RCD cell line P1

As a first step to understand why TCR-CD3 surface expression is lost on IELs in RCD II, we investigated the presence or absence of the specific TCR-CD3 chains in RCD cell line P1. For this purpose, cells were labeled with ^{35}S methionine/cysteine and lysed, after which immunoprecipitations with antibodies specific for TCR-α, TCR-β, CD3γ, CD3δ, CD3ε and ζ were performed, followed by SDS-PAGE analysis. For comparison, metabolic labeling and immunoprecipitations were performed on cells from unrelated T cell clone N10 which expresses a normal TCR-CD3 complex on the surface. It is known that in digitonin lysis buffer all components of the TCR-CD3 complex remain complexed while in NP40 lysis buffer the TCR-CD3 complex dissociates into the TCR-αβ heterodimer, ζ-homodimer, a CD3γε heterodimer and a CD3δε heterodimer[17;19]. RCD cell line P1 was found to express all TCR-CD3 subunits intracellularly (Figure 3A). The TCR-β antibody immunoprecipitated two specific bands that appeared to be glycosylation variants of each other as, in agreement with previous observations[23], on deglycosylation with N-glycanase, the upper band merged with the lower band (results not shown). Furthermore, the dimerization of ζ appeared normal as under nonreducing conditions ζζ was seen as a protein band of approximately 30 kD which was reduced to a protein band of approximately 15 kD under reducing conditions (Figure 3A). In addition, several other subunit interactions were observed as indicated by the presence of CD3ε in the CD3γ- and CD3δ-specific immunoprecipitates and vice versa. Under nonreducing conditions, however, there was no evidence for proper formation of a TCR-αβ dimer as only separate TCR-α and -β chains

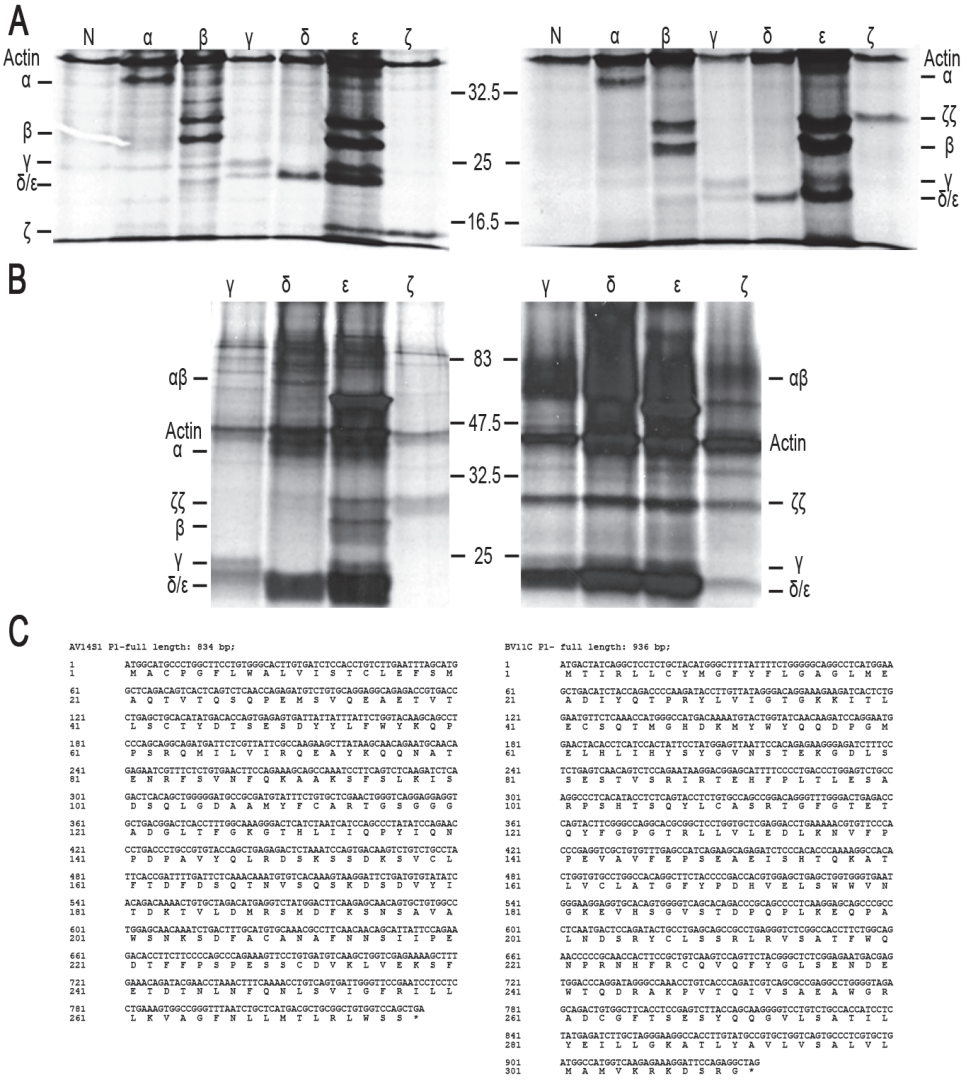

Figure 3. Defective association of the TCR chains underlies the loss of surface TCR-CD3 expression in RCD cell line P1. SDS-PAGE analysis of immunoprecipitates of RCD cell line P1 and T cell clone N10 after ^{35}S metabolic labeling. Antisera used were normal rabbit serum as negative control (N), anti-TCR-α (α), anti-TCR-β (β), anti-CD3γ (γ), anti-CD3δ (δ), anti-CD3ε (ε) and anti-ζ (ζ). (A) Immunoprecipitates obtained from NP40 lysates of RCD cell line P1 were analyzed under reducing (left) and nonreducing (right) conditions. (B) Immunoprecipitates obtained from digitonin lysates of RCD cell line P1 (left) and T cell clone N10 (right) were analyzed under non-reducing conditions. Positions of individual chains and molecular mass markers (kD) are indicated. (C) Sequences of the TCR-α and TCR-β chain of cell line P1 *(TCRAV14S1, TCRBV11c)*.

were observed in the TCR-α- and TCR-β-specific immunoprecipitates. This suggests the lack of formation of a disulfide bridge between the TCR chains (Figure 3A). The lack of proper formation of a TCR-αβ dimer was further substantiated by analysis of CD3- and ζ-specific immunoprecipitations performed on digitonin lysates of RCD cell line P1 and, as a control, surface TCR$^+$ T cell clone N10. In RCD cell line P1 only separate TCR-α and TCR-β chains were observed in association with CD3ε while in T cell clone N10 a TCR-αβ dimer was observed while separate TCR-α and TCR-β chains were absent (Figure 3B). Furthermore, although in all three CD3 subunit-specific immunoprecipitates of T cell clone N10 the ζζ-dimer was present, no evidence for incorporation of the ζζ-dimer into the TCR-CD3 complex was obtained for RCD cell line P1 (Figure 3B). Sequencing of the TCR-α and TCR-β transcripts of RCD cell line P1 revealed that both chains were in frame and encoded full-length TCR-chains (Figure 3C). In conclusion, all TCR-CD3 subunits are present in RCD cell line P1 but proper assembly of the complex is disturbed.

Retroviral introduction of exogenous TCR-β chains in RCD cell line P1 restores TCR-αβ dimer formation and cell surface expression

As the formation of TCR-αβ dimers was found to be disturbed in RCD cell line P1, we next investigated whether the introduction of an exogenous TCR-α and/or TCR-β chain could restore cell surface expression of the TCR-CD3 complex. To this end, a TCR-α and/or TCR-β chain (TCRAV14, TCRBV4) obtained from T cell clone N10, was introduced into RCD cell line P1 by retroviral transduction. For comparison, the exogenous TCR-α or TCR-β chains were also retrovirally transduced into Jurkat clones deficient for TCR-α ($\alpha^{-/-}$) or TCR-β ($\beta^{-/-}$). Cell surface expression of the TCR after transduction was determined with FACS analysis, where GFP-positivity represented proper transduction of the TCR-α chain and NGFR-positivity proper transduction of the TCR-β chain (Figure 4). TCR-CD3

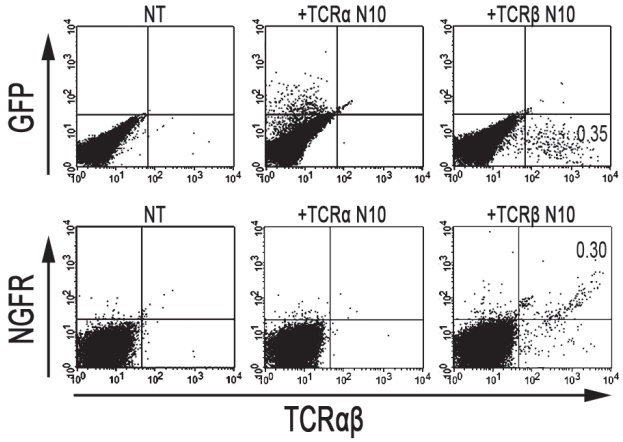

Figure 4. Retroviral introduction of exogenous TCR-β chains in RCD cell line P1 restores surface TCR-αβ expression. FACS analysis after retroviral transduction of cells from RCD cell line P1 with the TCR-α or TCR-β chain from T cell clone N10. The upper panel shows GFP- (TCRα from N10) and TCR-αβ expression on nontransduced P1 cells (NT), P1 cells transduced with the TCR-α chain from T cell clone N10 (+ TCRα N10) and on P1 cells transduced with the TCR-β chain from T cell clone N10 (+ TCRβ N10). The lower panel shows NGFR- (TCRβ from N10) and TCR-αβ expression, also on nontransduced P1 cells (NT), P1 cells transduced with the TCR-α chain from T cell clone N10 (+ TCRα N10) and on P1-cells transduced with the TCR-β chain from T cell clone N10 (+ TCRβ N10).

expression was restored on TCR-α-negative Jurkat cells after transduction with the TCR-α chain (Figure S1). Similarly, TCR-CD3 expression was restored on TCR-β-negative Jurkat cells after transduction with the TCR-β chain (data not shown), indicating that both constructs are functional. Whereas introduction of the TCR-α chain did not restore TCR-CD3 expression on the cell surface of RCD cell line P1, cells transduced with the TCR-β chain did express the TCR-CD3 complex on the cell surface (Figure 4). Similarly, the introduction of two additional TCR-α chains (Figure S1) failed to restore TCR-CD3 expression on the P1 cells while the introduction of another TCR-β chain (data not shown) did restore TCR-CD3 expression. To further substantiate that the introduction of an exogenous TCR-β chain resulted in proper assembly and cell surface expression of a TCR-CD3 complex, TCR-$\alpha\beta^+$, $NGFR^+$ cells were purified by FACS from P1 cells transduced with the TCR-β from N10 (Figure 4). Subsequently, T cell clone N10, RCD cell line P1 and RCD cell line P1 transduced with TCRβ from N10 were either cell surface labeled with ^{125}I, or metabolically labeled with ^{35}S methionine/cysteine. Thereafter, cells were lysed in digitonin buffer to preserve subunit interactions followed by immunoprecipitations and SDS-PAGE analysis. In the metabolically labeled cells the introduction of the TCR-β chain resulted in the formation of a TCR-αβ dimer and proper assembly of a TCR-CD3 complex, including incorporation of the ζζ-dimer into the complex (data not shown). Similarly, a CD3-associated TCR-αβ dimer was observed after cell surface labeling on T cell clone N10 as well as on RCD cell line P1 transduced with the TCR-β chain but not on RCD cell line P1 itself (Figure 5). Proper assembly was further indicated by the presence of a TCR-αβ dimer in the CD3γ, CD3δ and CD3ε immunoprecipitates and by dissociation of the TCR-αβ dimer into its subunits under reducing conditions (Figure 5). Together, these results indicate that impaired dimerization with the endogenous TCR-β chain results in the loss of a functional TCR-CD3 complex on RCD cell line P1.

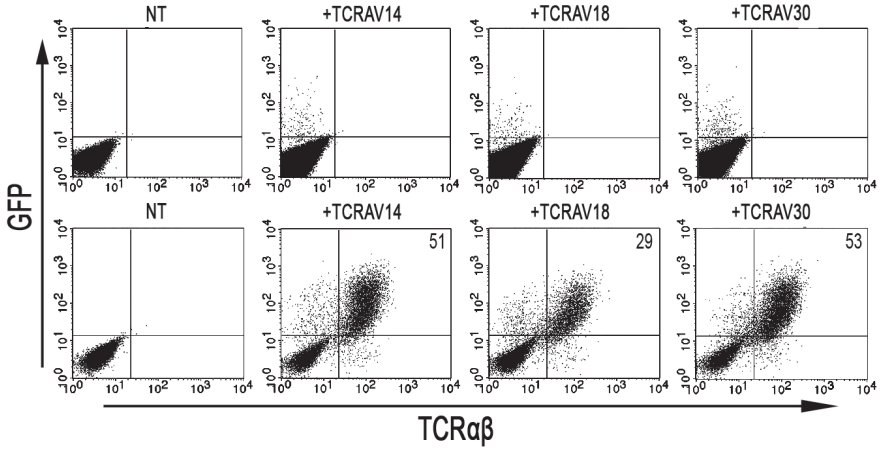

Figure S1. Retroviral introduction of exogenous TCR-α chains does not restore surface TCR-αβ expression on cell line P1. FACS analysis after retroviral transduction of cells from RCD cell line P1 and a Jurkat TCR-α-negative clone with TCRAV14, TCRAV18 and TCRAV30. GFP expression (successful TCR-α introduction) is plotted against surface TCR-αβ expression. (Top panel) nontransduced (NT) P1 cells and P1 cells transduced with TCRAV14, TCRAV18 and TCRAV30. (Bottom panel) nontransduced (NT) TCR-α-negative Jurkat cells and TCR-α-negative Jurkat cells transduced with TCRAV14, TCRAV18 and TCRAV30. Percentages of double positive cells are indicated.

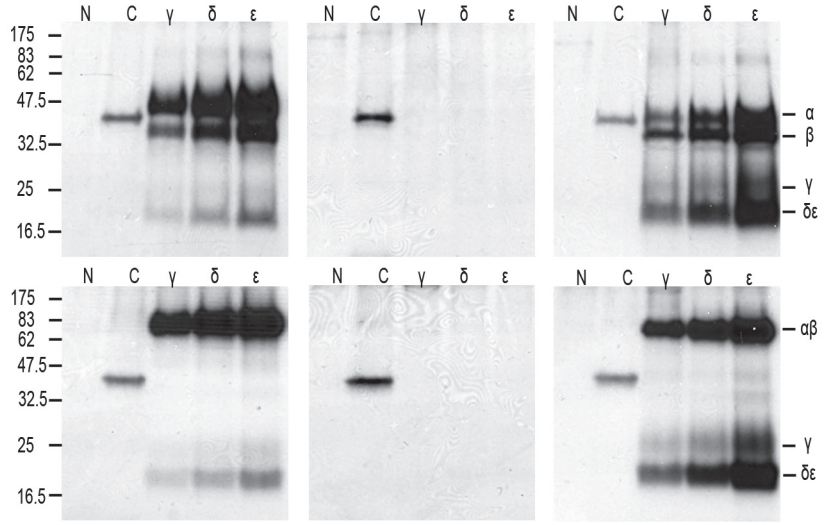

Figure 5. Retroviral introduction of exogenous TCR-β chains in RCD cell line P1 restores TCR-αβ dimer formation and surface expression. SDS-PAGE analysis of immunoprecipitates obtained from digitonin lysates of T cell clone N10 (left), RCD cell line P1 (middle) and RCD cell line P1 transduced with TCR-β from N10 (right) after cell surface iodination. Antisera used were normal rabbit serum as negative control (N), anti-HLA class I (C), anti-CD3γ (γ), anti-CD3δ (δ) and anti-CD3ε (ε). (Top panel) reducing conditions (Bottom panel) nonreducing conditions. Positions of individual chains and molecular mass markers (kD) are indicated.

Restoration of TCR functionality on introduction of an exogenous TCR-β chain in RCD cell line P1

To investigate whether the TCR-CD3 complex expressed on the cell surface after introduction of an exogenous TCR-β chain was functional, we stimulated T cell clone N10, RCD cell line P1 and the RCD cell line P1 transduced with the TCR-β chain from N10 with a plate bound anti-CD3 antibody (OKT3) and determined the proliferative response. As expected, the nontransduced cells did not respond to anti-CD3 stimulation whereas the TCR-β-transduced cells proliferated in response to anti-CD3 stimulation, although to

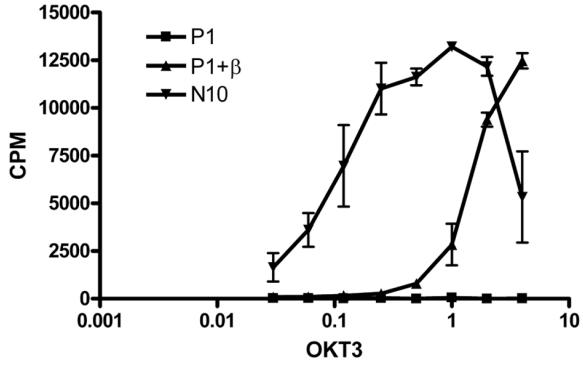

Figure 6. Restoration of TCR functionality upon introduction of an exogenous TCR-β chain in RCD cell line P1. Cells from T cell clone N10, nontransduced P1 cells and P1 cells transduced with the TCR-β chain from T cell clone N10 were stimulated with various amounts of anti-CD3 antibody (OKT3).

a lower extent than control cell line N10 (Figure 6). These results show that functional cell surface expression of a TCR-CD3 complex can be restored by introduction of an exogenous TCR-βchain.

Additional RCD cell lines show impaired TCR-α and TCR-β dimerization as well

In two additional surface TCR-CD3-negative cell lines isolated from duodenal biopsies from RCD type II patients (P2 and P3), the TCR-CD3 complex was studied. Similar to RCD cell line P1, cells from cell line P2 and P3 proliferated specifically in response to IL-15 and ^{35}S metabolic labeling experiments indicated proper assembly of the CD3γε, CD3δε and ζζ-dimers while no evidence for the presence of a TCR-αβ-dimer was obtained (data not shown). Whereas transduction with either the TCR-α or TCR-β chain of T cell clone N10 did not restore cell surface expression of the TCR, simultaneous introduction of both TCR-chains did restore TCR surface expression (Figure 7), confirming that the CD3 complex assembles properly in these cell lines. In contrast to cell line P1, no transcripts coding for either a TCR-α or a TCR-β chain could be detected in cell lines P2 and P3 (results not shown). These results indicate that loss of surface TCR-CD3 expression in RCD II cell line P2 and P3 is due to defects in the synthesis of both TCR-chains.

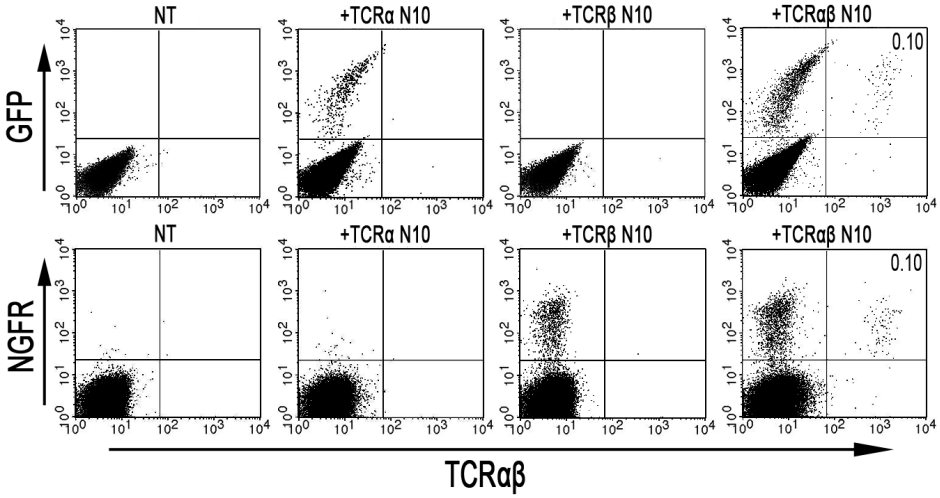

Figure 7. Retroviral introduction of both an exogenous TCR-α and TCR-β chain in RCD cell lines P2 and P3 restores TCR-αβ surface expression. FACS analysis after retroviral transduction of cells from RCD cell line P3 with the TCR-α and/or TCR-β chain from T cell clone N10. (Top panel) GFP- (TCRα from N10) and TCRαβ-expression on nontransduced P3 cells (NT), P3 cells transduced with the TCR-α chain from T cell clone N10 (+ TCRα N10), P3 cells transduced with the TCR-β chain from T cell clone N10 (+ TCR-β N10) and P3-cells with both the TCR-α and TCR-β chain from T cell clone N10 (+ TCR-αβ N10). (Bottom panel) NGFR- (TCR-β from N10) and TCR-αβ expression, also on nontransduced P3 cells (NT), P3 cells transduced with the TCR-α chain from T cell clone N10 (+ TCR-α N10), P3 cells transduced with the TCR-β chain from T cell clone N10 (+ TCR-β N10) and P3 cells with both the TCR-α and TCR-β chain from T cell clone N10 (+ TCR-αβ N10). Percentages of double positive cells are indicated. FACS analysis after retroviral transduction of cells from RCD cell line P2 with the TCR-α and/or TCR-β chain from T cell clone N10 showed similar results.

Discussion

Celiac disease is a common gastrointestinal disorder which afflicts 1 in 200 persons in Europe[24]. 2-5% of celiac disease patients diagnosed as adults, develops a refractory state of CD characterized by persisting villous atrophy and an increase of IELs despite a gluten-free diet [2]. The two types of RCD (RCD I and RCD II) are distinguished by the respective absence or presence of an aberrant IEL population lacking surface TCR-CD3 expression. In all patients with RCD II this abnormal IEL population may be observed, which besides lack of T cell markers such as CD3, CD4, CD8 and TCR-αβ is also associated with clonal TCR-γ gene rearrangement[1;2]. Moreover, the aberrant IEL population is not restricted to the small intestine, but may also be observed in gastric and colonic epithelium[1;25]. The same aberrant IELs and clonal TCR-γ gene rearrangements found in patients with RCD II may be subsequently observed in EATL specimens from these patients, suggesting that RCD II precedes development of EATL[5]. EATL has a very poor 5-year survival rate of 11-20% [2]. Improved understanding of the events leading to RCD II and subsequent EATL development is therefore needed. Until now aberrant IELs have been investigated mainly in situ and this limits the type of experiments that can be performed to investigate molecular events that are linked to malignant transformation[15].

In the present study we report the isolation of three cell lines from small intestinal biopsies of RCD II patients (P1-P3). These cell lines displayed the characteristic intracellular CD3ε^{+}, surface CD3^{-}, CD4^{-}, CD8^{-} and TCR-αβ^{-} phenotype. Moreover, the observed proliferative response of the cell lines to stimulation with IL-15 supports the notion that these cell lines represent a model for aberrant IEL. Strikingly, and in contrast to aberrant IELs in RCD II, the cell lines also expressed CD30 which is typically found on EATL. The latter suggests that while clinically there was no evidence for EATL in these three patients, cells with the characteristic EATL phenotype are already present in the small intestine of these patients and can be propagated *in vitro*. These cell lines offered the unique opportunity to study the cause for loss of surface expression of the TCR-CD3 complex, an event that is typically associated with (pre)malignant transformation. It is well established that in a functional TCR-CD3 complex the TCR-α and TCR-β chain are associated with the CD3γ, CD3δ, CD3ε, and ζ-chains, which enable signal transduction[18;26]. Therefore we hypothesized that loss of TCR-CD3 expression might be due to defects in one of these chains resulting in deficient assembly of the complex. We demonstrate that in RCD II cell line P1 the TCR-α and -β chains as well as the CD3γ, CD3δ, CD3ε and ζ-chains were present intracellularly, that the CD3γε, CD3δε and ζζ-dimers assembled normally, but that dimerization of the TCR-α and -β chains and incorporation of ζζ in the TCR-CD3 complex was defective. Furthermore, we demonstrate that through the introduction of exogenous TCR-β chains, but not of TCR-α chains, the assembly and functional cell surface expression of the TCR-CD3 complex could be restored, indicating that the defect lies with the TCRβ chain. Sequencing of the cDNA encoding the endogenous TCR-α and TCR-β chain from RCD cell line P1 showed both chains to be in frame and according to the consensus sequence (Figure 3C), which correlates with the observed presence of a TCR-β protein in metabolically labeled P1-cells (Figure 3A). In contrast, our results indicate that a lack of expression of both TCR-chains underlies the loss of surface TCR-CD3 expression in cell lines P2 and P3. Therefore, our results indicate that the loss of TCR-CD3 expression in patients with RCD can be mediated by several mechanisms. At present it can not be excluded that cell lines P2 and P3, in which both TCR-chains are lost, represent a more advanced stage of (pre)malignant transformation, whereas the absence of association

despite the presence of wild-type TCR chains in P1, might represent an earlier phase. This possibility and the exact mechanism underlying defective assembly in RCD cell line P1 will be the subject of future investigations. Preliminary experiments indicate that the half-lives of the TCR-α and TCR-β chains of cell line P1 are comparable to those in a T cell clone with normal TCR surface expression. Together, our results indicate that (pre)malignant transformation of IEL in RCD II correlates with abnormal expression or association of the TCR-chains, resulting in defective TCR-CD3 surface expression.

As the loss of TCR-CD3 expression is typically observed in RCD II and EATL, one must assume that this is linked to (pre)malignant transformation[5;27]. An important question, therefore, is what drives the downregulation of the TCR-CD3 complex. Downregulation of TCR expression has been linked to extensive stimulation with antigen presented by antigen presenting cells and serves to prevent apoptosis induction[28;29]. Possibly, the aberrant IELs in RCD II express autoreactive TCR or TCR reactive with (peptides from) stress induced ligands. Down-regulation of the TCR might then be a way to escape from immune-regulatory processes aimed at the elimination of autoreactive cells. Alternatively, aberrant IELs might arise from gluten-specific, HLA-class I-restricted $CD8^+$ IELs[30] which escape from immune regulation by down-regulation of their gluten-specific TCR. The availability of the IEL cell lines established in the present study now allows an in-depth analysis of these possibilities and this will be the topic of future research.

In conclusion, the present study provides the first evidence that loss of TCR-CD3 surface expression on IELs in RCD II is due to defects in the synthesis or assembly of TCR chains providing a first step in understanding the process leading to the development of RCD II and subsequent progression into EATL.

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Authorship

Contribution: J.M.L.T. performed the research, analyzed the data and wrote the paper; W.H.M.V performed experiments and wrote the paper; Y.M.C.K-W, B.H.N, A.R.vd.S, A.T, M.W.J.S and L.H.A.D. performed experiments; C.J.M contributed the duodenal biopsies; M.H.M.H and J.v.B designed the research; F.K designed the research and wrote the paper.
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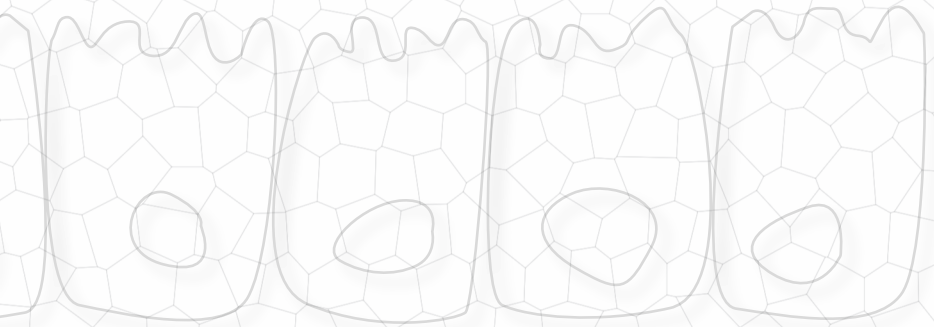

Chapter 3

DNAM-1 Mediates Epithelial Cell-specific Cytotoxicity of Aberrant Intraepithelial Lymphocyte Lines from Refractory Celiac Disease Type II Patients

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Abstract

In refractory celiac disease (RCD) intestinal epithelial damage persists despite a gluten-free diet. Characteristic for RCD type II (RCD II) is the presence of aberrant surface T cell receptor (TCR)-CD3$^-$ intraepithelial lymphocytes (IELs) that can progressively replace normal IELs and can eventually give rise to overt lymphoma. Therefore, RCD II is considered a malignant condition that forms an intermediate stage between celiac disease (CD) and overt lymphoma.

We now demonstrate that surface TCR-CD3$^-$ IEL lines isolated from three RCD II patients preferentially lyse epithelial cell lines. FACS-analysis revealed that DNAM-1 was strongly expressed on the three RCD cell lines, while other activating NK cell receptors were not expressed on all three RCD cell lines. Consistent with this finding, cytotoxicity of the RCD cell lines was mediated mainly by DNAM-1 with only a minor role for other activating NK cell receptors. Furthermore, enterocytes isolated from duodenal biopsies expressed DNAM-1 ligands and were lysed by the RCD cell lines ex vivo.

While DNAM-1 on CD8$^+$ T cells and NK cells is known to mediate lysis of tumor cells, this study provides the first evidence that (pre)malignant cells themselves can acquire the ability to lyse epithelial cells via DNAM-1. This study confirms previous work on epithelial lysis by RCD cell lines and identifies a novel mechanism that potentially contributes to the gluten-independent tissue damage in RCD II and RCD-associated lymphoma.

Introduction

A small proportion of adult-onset celiac disease (CD) patients develops a refractory state with persisting villous atrophy and an increase of intraepithelial lymphocytes (IELs) despite a gluten-free diet[1]. Refractory celiac disease (RCD) can be subdivided into RCD I and RCD II, distinguished by the respective absence or presence of an aberrant IEL population lacking surface expression of the T cell receptor-CD3 complex (sTCR-CD3)[2]. RCD II is now considered an intraepithelial lymphoma that can give rise to high-grade invasive lymphoma. Overt lymphoma likely derives from the aberrant IELs, as they share the sTCR-CD3$^-$ phenotype and display identical monoclonal TCR-γ gene rearrangements[3;4]. The cytokine interleukin-15 (IL-15) which is upregulated in the lamina propria and on epithelial cells of RCD patients[5;6] is thought to be crucial for the expansion and survival of aberrant IELs and lymphoma cells[7]. RCD II and RCD-associated lymphoma have poor 5-year survival rates of 44-58% and <20% respectively[1;8].

In active CD, many CD8$^+$TCR$^+$ IELs have acquired activating NK cell receptors such as NKG2C and NKG2D[9;10]. These receptors co-stimulate TCR-mediated lysis of epithelial cell lines *in vitro*[9;11]. Upon exposure to IL-15, CD8$^+$ IELs can display TCR-independent NKG2D-mediated cytotoxicity against epithelial cell lines[12]. Studies on the cytotoxic capacity of aberrant sTCR-CD3-negative IELs in RCD II indicated that such cells could lyse intestinal epithelial cell line HT29 in a granzyme/perforin dependent fashion[5;11]. Furthermore, lysis was induced by stimulation of NKG2D[11], boosted by IL-15 and could be partially inhibited by an antibody to CD103[5]. Blocking of NKG2D only partially inhibited the lysis of HT29, suggesting a role for additional activating NK cell receptors in the epithelial cytoxicity of aberrant IELs.

In the present study, we set out to identify these additional receptors and further defined the specificity of aberrant IEL cytotoxicity. To this end, we used cell lines isolated from small intestinal biopsies of three RCD II patients (hereafter called RCD cell lines) that display the typical aberrant sTCR-CD3$^-$ phenotype[13].

Materials and methods

Small intestinal biopsy specimens

During upper endoscopy, large spike forceps biopsy specimens (Medi-Globe, Tempe, Arizona) were taken from the second part of the duodenum[14]. Biopsy specimens were used for culture, direct flowcytometric analysis or cytotoxicity assays. 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

RCD cell lines P1, P2 and P3 were isolated from duodenal biopsies of three RCD II patients as previously described[13]. In short, after treatment with 1 mM dithiothreitol (Fluka, Buchs, Switzerland) and 0.75 mM ethylenediaminetetraacetic acid (Merck, Darmstadt Germany), biopsies were cultured in Iscove's modified Dulbecco's medium (IMDM, Lonza, Verviers, Belgium) with 10% normal human serum (NHS) containing 10 ng/ml IL-15 (R&D Systems Europe, Abingdon, United Kingdom). The predominant population of $CD3^-CD4^-CD8^-CD30^+$ cells was purified by FACS, thus giving rise to RCD cell lines P1, P2 and P3. RCD cell lines were propagated in IMDM with 10% NHS containing 10 ng/ml IL-15 and restimulated approximately every 4 to 5 weeks with 1 µg/ml phytohemagglutinin, 10 ng/ml IL-15 and 1 x 10^6/ml irradiated allogenous peripheral blood mononuclear cells as feeder cells. Three $CD8^+$ TCR^+ IEL lines were isolated from duodenal biopsies of three independent CD patients following the method described above and purified by FACS based on CD3-and CD8-positivity. These $CD8^+$ TCR^+ cells, that were homogeneously DNAM-1^+ $NKG2D^+$, were cultured in IMDM with 10% NHS containing 10 ng/ml IL-15 and restimulated every 2 weeks. 15 additional cell lines were isolated from biopsies of RCD II patients following the method for RCD cell lines P1, P2 and P3. These cell lines were not purified by FACS prior to FACS analysis.

Additional control cell lines for P1 and P3 were respectively a $CD8^+$ T cell line and a $CD4^+$ T cell clone isolated from a biopsy of the corresponding patient. Cells were restimulated every 2 weeks and maintained on IMDM with 10% NHS containing 10 ng/ml IL-15 and 20 Cetus units/ml IL-2 (Proleukin, Chiron corporation, Emeryville, CA). Cell lines HT29, Caco2, T84, A549, Daudi, K562, P815 and EBV-BC107 were cultured in IMDM with 10% fetal calf serum (FCS). EBV-BC107 is an EBV B-LCL cell line generated from a healthy blood bank donor[15].

Antibodies

Fluorochrome-conjugated anti-CD3, anti-CD226 (DNAM-1), anti-CD112, anti-CD30 anti-CD94 and anti-perforin were purchased from BD Biosciences (San Jose, CA). Anti-CD155, anti-NKG2D, anti-NKG2C were from R&D Systems Europe. Anti-NKp30, anti-NKp44 and anti-NKp46 were purchased from Beckman Coulter (Fullerton, CA). Anti-NKp80 was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany) and anti-epithelial specific antigen (ESA) from Biomeda (Foster city, CA). For blocking and redirected lysis experiments, anti-CD226 was purchased from BD Biosciences, anti CD30, anti NKG2D and isotype-matched control antibodies from R&D Systems Europe, anti-CD103 and anti-CD155 from Beckman Coulter and anti-CD112 from e-Bioscience (San Diego, CA).

Isolation of cells from duodenal biopsies

Enterocytes and lymphocytes were isolated from two or three duodenal biopsies treated with 200 µg/ml collagenase A (MP Biomedicals LLC, Eschwege, Germany) and 200 µg/ml DNAse II (Roche diagnostics, Almere, Netherlands). After rotating 1 hour at 37°C, cells were filtered through a 70 µm filter (BD Falcon, Erembodegem, Belgium) and washed twice with IMDM with 10% FCS. For subsequent flow cytometric analysis, cells were stained 30 minutes with fluorochrome-conjugated antibodies. For cytotoxicity assays, three duodenal biopsies were treated with 1 mM dithiothreitol (Fluka, Buchs, Switzerland) and 0.75 mM ethylene-diamine-tetra-acetic acid (Merck, Darmstadt Germany); the released cells were then used as target cells. Approximately 80% of the released cells were ESA^+ enterocytes.

Cytotoxicity assay

One million target cells were labeled with 100 µCi ^{51}Cr for 1 hour at 37°C. After extensive washing, labeled target cells were co-incubated with effector cells at effector-target ratios between 50:1 and 1.5:1 for 4 hours at 37°C. For blocking and redirected lysis experiments effector cells were pretreated for 20 minutes at room temperature with 20 µg/ml of the indicated antibodies. Masking ligands on target cells was achieved by the presence of 5 µg/ml monoclonal antibodies in the effector-target mixture during the 4 hour incubation period. Spontaneous chromium release and maximum chromium release by target cells was determined by addition of medium or 1% Triton X100 (Pierce, Rockford, Illinois) respectively. The percentage of specific cytotoxicity was as follows: ([cpm experimental release - cpm spontaneous release]/[cpm maximum release - cpm spontaneous release]) x 100 %.

BLT esterase assay

96-well non-tissue-culture-treated plates (BD Falcon) were coated overnight in duplicate with 5 µg/ml anti-NKG2D, anti-DNAM-1 or both. Subsequently, plates were blocked with 10% FCS/PBS and washed with PBS. 1 x 10^5 cells from cell lines P1, P2 or P3 were added. After 4 hours incubation at 37°C, supernatants were evaluated for esterase secretion using an N-benzyloxycarbonyl lysine thiobenzyl ester (Merck). OD values were obtained at an absorbance of 412 nm on a microplate reader (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Immuno-electron microscopy

Cells were fixed in 2% paraformaldehyde with 0.1% glutaraldehyde and processed for immunogold staining as described elsewhere[16]. Ultrathin cryosections were incubated with granzyme B-specific monoclonal antibody GB11 (Sanquin, Amsterdam, The Netherlands), followed by rabbit-anti-mouse IgG (Dako, Heverlee, Belgium) and 15 nm Protein-A-gold particles. Sections were evaluated with a Philips 410 electron microscope.

Statistical analysis

For cytotoxicity assays and BLT esterase assays, each data point is the mean ± SEM of an experiment performed in duplicate. An unpaired two-tailed *t* test was performed to compare the effect of the antibodies used in this study with their respective controls. A P-value less than 0.05 was considered significant.

Results

RCD cell lines lyse intestinal epithelial cells

RCD cell lines P1, P2 and P3, isolated from three RCD II patients, have the characteristic phenotype of aberrant cells in RCD II: surface $TCR^-CD3^-CD4^-CD8^-CD103^+$, intracellular $CD3^+$ [13]. They grow in an IL-15 dependent fashion[13], which is of interest as IL-15 is known to be over-expressed in the intestine of patients with RCD and lymphoma[5]. Furthermore, they express granzyme B (Supplementary Figure S1) and perforin (not shown), both known to play a key role in lymphocyte-mediated cytotoxicity[17;18]. The RCD cell lines could be isolated repeatedly from successive duodenal biopsies of all three patients, indicating that the isolated cell lines represent a persisting population. Moreover, these cell lines have monoclonal TCR-γ gene rearrangements identical to the aberrant IELs in the patients and therefore closely resemble the aberrant IELs *in vivo*[13].

RCD cell lines P1, P2 and P3 were tested for their ability to lyse an array of target cells: intestinal epithelial cell lines (HT29, Caco2, T84), a non-intestinal epithelial cell line (A549), an erythroleukemia cell line (K562), a Burkitt lymphoma cell line (Daudi) and an EBV-lymphoblastoid cell line (EBV-BC107).

All three RCD cell lines lysed the intestinal epithelial cell lines HT29, Caco2 and T84, but not the non-epithelial cell lines Daudi and EBV-BC107 (Figure 1A-D). RCD cell line P3 was the only cell line that induced lysis of NK cell target K562 (Figure 1A-D), although lysis of K562 was much lower when compared to lysis of the epithelial cell lines (Figure 1D). The non-intestinal epithelial cell line A549 was lysed as well (Figure 1E), indicating that lysis is not confined to intestinal epithelium but can be directed against other types of epithelium. In contrast, three polyclonal $CD8^+TCR^+$ IEL lines derived from small intestinal biopsies of CD patients displayed far less cytotoxicity against HT29 (Figure 1F), but efficiently lysed non-epithelial cell line K562 (Figure 1F), showing that the weak lysis of HT29 was not due to an inherently low cytotoxic capacity. These results indicate that in comparison with $CD8^+$ TCR^+ IEL lines, RCD cell lines are more cytotoxic to epithelial cells. All three RCD cell lines expressed CD103 ($\alpha_E\beta_7$)[13], an integrin present on IELs to retain lymphocytes at the epithelial surface. The only known ligand of CD103 is E-cadherin, which is expressed selectively by epithelial cells[19]. Cytotoxicity of the RCD cell lines against the epithelial cell lines HT29, Caco2, T84 and A549 was indeed partially inhibited by a CD103-specific antibody (Figure 1G, 1H), consistent with a role for the integrin $\alpha_E\beta_7$

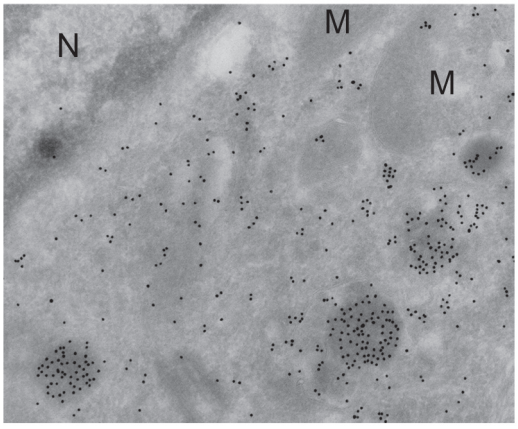

Figure S1. RCD cell lines express granzyme B. Granzyme B was detected using immunogold labeling and immuno-electron microscopy. The result for RCD cell line P1 is shown. "N" represents the nucleus; "M" represents a mitochondrium.

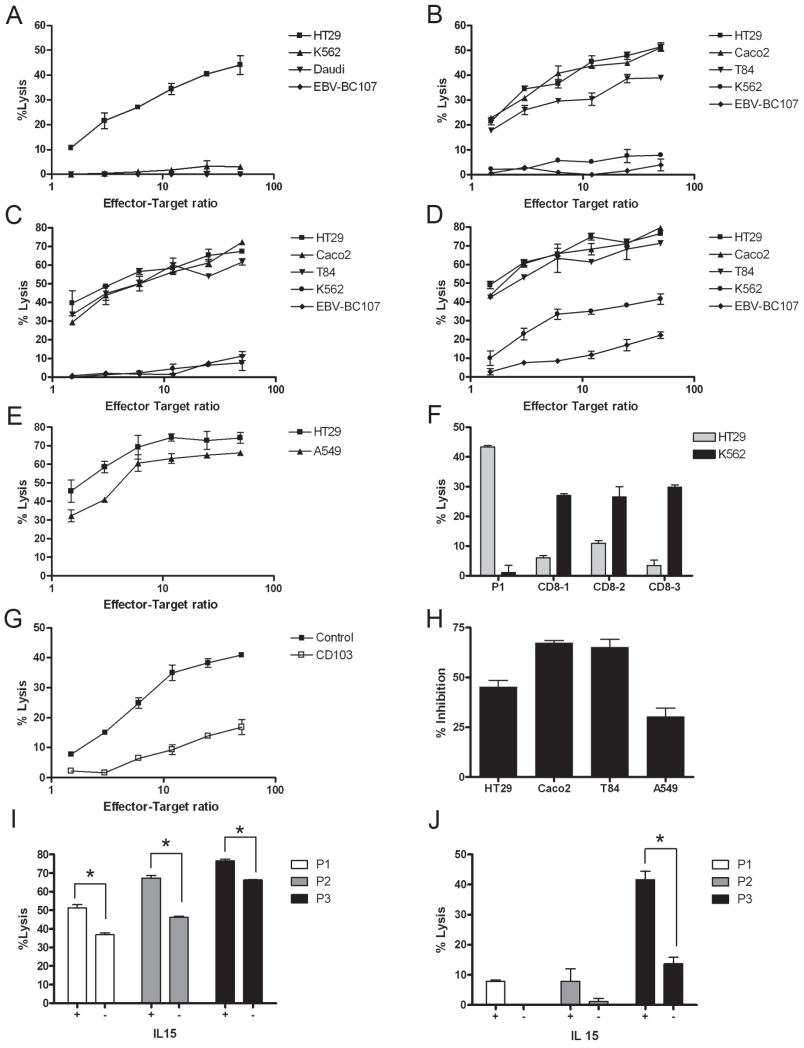

Figure 1. RCD cell lines preferentially lyse epithelial cells. (A) Lysis of HT29, K562, Daudi and EBV-BC107 by RCD cell line P1. Similar results were obtained with P2 and P3, except for lysis of K562 by RCD cell line P3. (B-D) Lysis of HT29, Caco2, T84, K562 and EBV-BC107 by: (B) RCD cell line P1. (C) RCD cell line P2. (D) RCD cell line P3. (E) Lysis of HT29 and A549 by RCD cell line P3. Similar results were obtained with P1 and P2. (F) Lysis of HT29 and K562 by RCD cell line P1 and three independent polyclonal $CD8^+NKG2D^+DNAM\text{-}1^+$ IEL lines in a 50:1 effector-target ratio. (G) Lysis of HT29 by RCD cell line P1 after incubation with an anti-CD103-antibody or with an isotype-matched control antibody. Similar results were obtained with P2 and P3. (H) Percentage of inhibition on the lysis of HT29, Caco2, T84 and A549 by RCD cell line P1 after incubation with an anti-CD103-antibody in a 50:1 effector-target ratio. Similar results were obtained with P2 and P3. (I+J) Cells from RCD cell lines P1, P2 and P3 were cultured for 72 hours in the presence of 10ng/ml IL-15 or in medium alone. After incubation cells were viable and were tested for cytotoxic activity against HT29, Caco2, T84 and K562. *$P < 0.05$. (I) Cytotoxic activity against HT29 in a 50:1 effector-target ratio. Similar results were obtained with the targets Caco2 and T84 (not shown). (J) Cytotoxic activity against K562 in a 50:1 effector-target ratio. All experiments shown are representative of at least three independent experiments. Each data point is the mean ± SEM of an experiment performed in duplicate.

in determining epithelial specificity.
Previous studies indicated that IL-15 potentiates effector functions of sTCR-CD3$^-$ cell lines[5;6]. In our hands, IL-15 indeed boosted the cytotoxicity of the RCD cell lines against intestinal epithelial cells (Figure 1I). However, cells deprived of IL-15 for three days still lysed epithelial target cells, suggesting that cytotoxicity against epithelial cells is an intrinsic property of these cell lines (Figure 1I). In contrast, three-day IL-15 deprivation abrogated lysis of K562 nearly completely (Figure 1J), suggesting that lysis of K562 is more dependent on IL-15 stimulation than the lysis of epithelial cell lines.
Together, these results indicate that RCD cell lines are cytotoxic and preferentially lyse epithelial cells. Furthermore, cytotoxicity against the epithelium was enhanced by IL-15, a cytokine highly upregulated in the epithelium and lamina propria of patients with RCD II and RCD-associated lymphoma[5].

RCD cell lines express DNAM-1 and other activating NK cell receptors

To determine which receptor(s) might mediate epithelial cell-specific cytotoxicity we analyzed the expression of activating receptors by the RCD cell lines. None of the cell lines expressed CD3 (Figure 2), consistent with the phenotype of aberrant IELs but not with that of T cells. In addition, RCD cell lines P1, P2 and >98% of RCD cell line P3 were negative for NK cell marker CD56, supporting the notion that the RCD cell lines were distinct from NK cells. NKG2C and NKG2D, NK cell receptors involved in epithelial cell lysis[9;11], were expressed on RCD cell line P1 and P3, but not P2 (Figure 2). The natural cytotoxicity receptors Nkp30, Nkp44 and NKp46 were only expressed on RCD cell line P3 and none of the RCD cell lines expressed NKp80 (Supplementary Figure S2). In contrast, only DNAM-1, an activating receptor mediating tumor cell lysis by CD8$^+$ T-cells and NK cells[20], was strongly expressed on the cell surface of all three RCD cell lines (Figure 2).

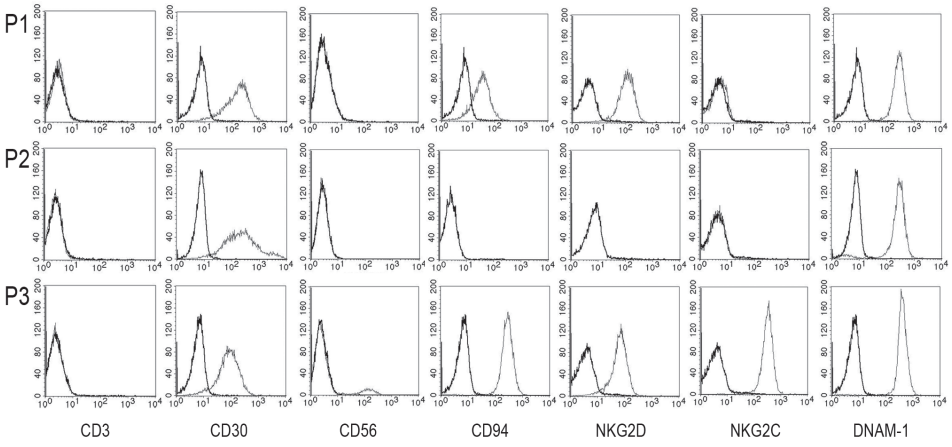

Figure 2. RCD cell lines express DNAM-1. FACS analysis of RCD cell lines P1, P2 and P3. The gray line in the histograms represents staining with the indicated antibody, the bold line the isotype-matched control. Analyses were performed within a live lymphocyte gate. The experiment shown is representative of three independent experiments.

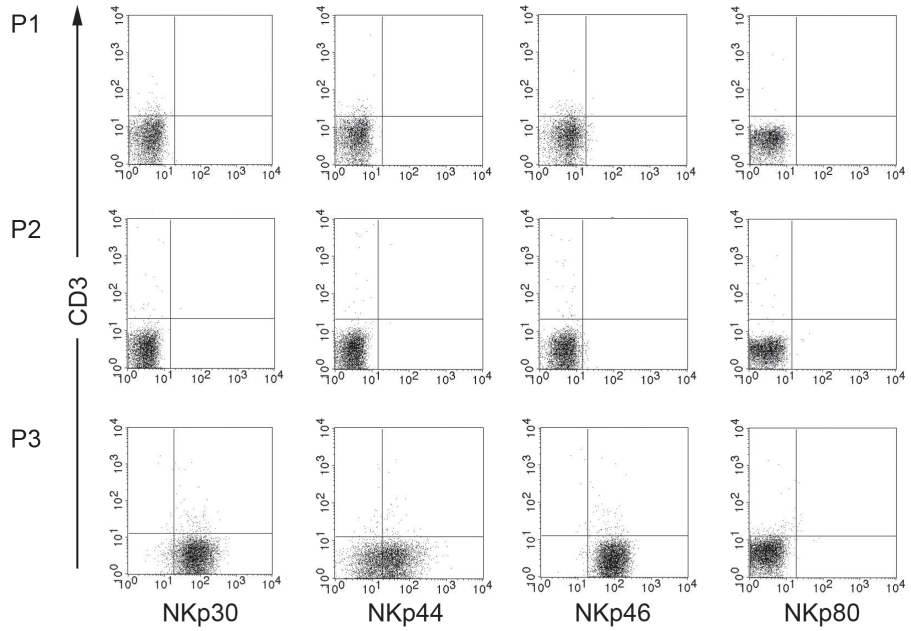

Figure S2. Expression of natural cytotoxicity receptors on RCD cell lines. FACS analysis of RCD cell lines P1, P2 and P3. Analyses were performed within a live lymphocyte gate and quadrants were based on staining with isotype-matched control antibodies (not shown). The experiment was performed twice and peripheral blood NK cells were used as positive control cells (not shown).

DNAM-1 is the main mediator of cytotoxicity of RCD cell lines against intestinal epithelial cells

The observation that all three RCD cell lines expressed DNAM-1 suggested that this receptor might be involved in the killing of intestinal epithelial cells. Indeed, a DNAM-1-specific antibody strongly inhibited lysis of intestinal epithelial cells by all three RCD cell lines (Figure 3A, 3B). In addition, while blocking of NKG2D inhibited lysis slightly in the case of the NKG2D$^+$ RCD cell lines P1 and P3, simultaneous blocking of DNAM-1 and NKG2D abrogated lysis nearly completely (Figure 3A, 3B). As expected, lysis by the NKG2D$^-$ cell line P2 could not be inhibited by an antibody to NKG2D (Figure 3B). In contrast, lysis of K562 by RCD cell line P3 was not inhibited by blocking of DNAM-1 (data not shown). In accordance with previous studies, a CD103-specific antibody was able to partially inhibit lysis of HT29 cells[5] (Figure 1G). When CD103 and DNAM-1 were blocked simultaneously, lysis was prevented completely (Fig. 3C), indicating synergy between DNAM-1 and CD103.

To test whether the DNAM-1 mediated cytotoxicity of RCD cell lines P1, P2 and P3 was independent of other receptor-ligand interactions, a redirected lysis experiment was performed. RCD cell lines P1, P2 and P3, pre-incubated with antibodies specific for DNAM-1, CD103, NKG2D or CD30 or with isotype-matched control antibodies, were co-cultured with Fc-receptor-bearing P815 cells. While neither the CD30-specific antibody nor the control antibody induced lysis of P815 cells, strong lysis was induced by the DNAM-1-specific antibody (Figure 3D). Much weaker effects were observed in the case

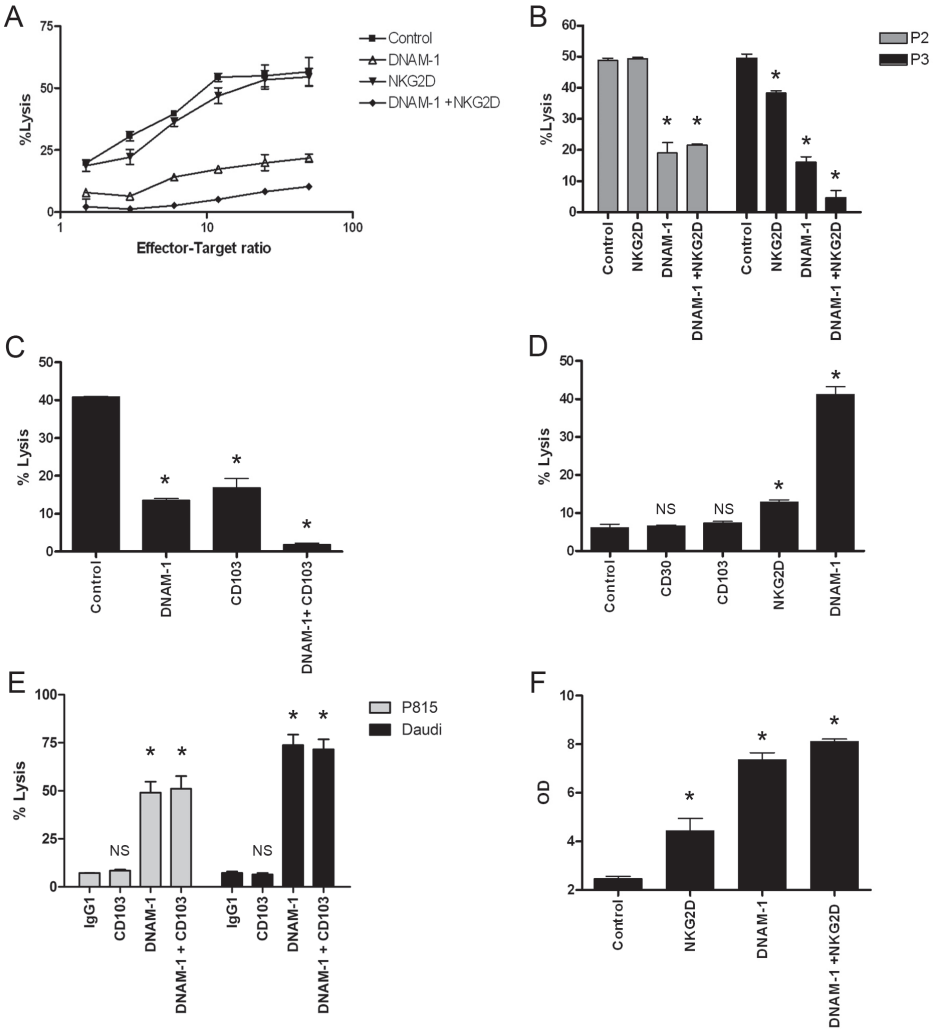

Figure 3. Lysis of epithelial cells by RCD cell lines is mediated mainly by DNAM-1. Cytotoxicity of RCD cell lines P1, P2 and P3 against HT29 in the presence of (combinations of) antibodies directed against DNAM-1, NKG2D, CD103, CD30 or the appropriate isotype control antibodies. Similar results were observed for targets Caco2 and T84. Each data point is the mean ± SEM of an experiment performed in duplicate. *P < 0.05, NS = not significant versus the respective control. (A) Lysis of HT29 by RCD cell line P1. (B) Lysis of HT29 by RCD cell line P2 and P3 in a 25:1 effector-target ratio. (C) Lysis of HT29 by RCD cell line P1 in a 50:1 effector-target ratio. Similar results were observed for RCD cell lines P2 and P3. (D) Redirected lysis of Fc-receptor+ cell line P815 by RCD cell line P3 in a 50:1 effector-target ratio. Similar results were observed for RCD cell line P1 and P2, except for the absence of NKG2D-induced lysis by NKG2D- RCD cell line P2 (E) Redirected lysis of P815 cells or Daudi cells by RCD cell line P3 in a 25:1 effector target ratio. Similar results were obtained with RCD cell line P1 and P2. (F) Esterase release for cell line P3 after incubation with plate-bound antibodies specific for NKG2D and/or DNAM-1, or an isotype-matched control antibody. Optical density (OD) values are shown. Similar results were observed for RCD cell line P1 and P2, except for the absence of NKG2D-induced esterase release by NKG2D- RCD cell line P2. All experiments shown are representative of at least three independent experiments.

of the NKG2D-specific antibody (Figure 3D), and only when using RCD lines expressing NKG2D (Figure 2). The CD103-specific antibody induced lysis of neither P815 cells nor Daudi cells (Figure 3D and 3E). Furthermore, combining the DNAM-1-specific antibody with a CD103-specific antibody showed that the integrin $\alpha_E\beta_7$ had no additional effect on the lysis of both P815 cells and Daudi cells (Figure 3E), confirming the notion that CD103 mediates adhesion to epithelial cells rather than effector cell activation. Consistent with the redirected lysis data, the DNAM-1 specific antibody was able to independently induce esterase release by the RCD cell lines (Figure 3F). Together, these results indicate that DNAM-1 is the dominant receptor triggering lysis of epithelial cells by the RCD cell lines. However, we cannot formally exclude that the DNAM-1-specific antibody was more efficient than the NKG2D-specific antibody in blocking epithelial cell lysis and in inducing redirected lysis.

Consistent with the importance of DNAM-1 in mediating lysis, the intestinal epithelial cell lines HT29, Caco2 and T84 expressed both DNAM-1 ligands CD112 and CD155[21;22] (Figure 4A). Masking of the individual DNAM-1 ligands with CD112- and CD155-specific antibodies had only a marginal effect on lysis of HT29 (Figure 4B), whereas simultaneous blocking of CD112 and CD155 resulted in partial inhibition of lysis (Figure 4B). Blocking with antibodies specific to CD112 and CD155 was never as efficient as blocking with anti-DNAM-1 (Figure 4B). These results indicate that the interaction of DNAM-1 with its ligands CD122 and CD155 indeed leads to lysis of intestinal epithelial cells. We cannot, however, exclude that an additional ligand for DNAM-1 is present on epithelial cells and is involved in the killing of intestinal epithelial cell lines.

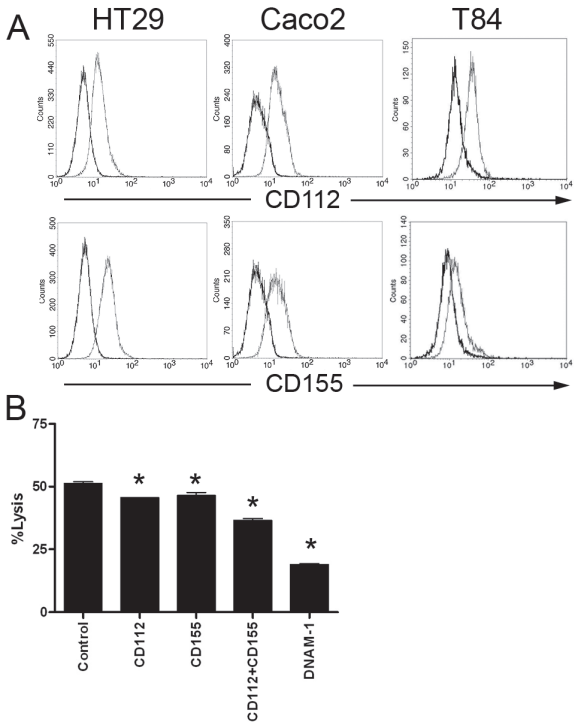

Figure 4. Blocking DNAM-1 ligands CD112 and CD155 on epithelial cell lines inhibits lysis. (A) FACS analysis of CD112 and CD155 on intestinal epithelial cell lines HT29, Caco2 and T84. The gray line in the histograms represents staining with the indicated antibody, the bold line the isotype-matched control. (B) Lysis of HT29 by RCD cell line P2 after pre-treatment with anti-DNAM-1 antibodies or after incubation in the presence of antibodies against CD112, CD155 or a combination of both (effector-target ratio 50:1). Similar results were observed for RCD cell lines P1 and P3. Each data point is the mean ± SEM of an experiment performed in duplicate. All experiments shown are representative of at least three independent experiments. *P < 0.05 versus the isotype control-matched antibody.

In conclusion, DNAM-1 contributes to the cytotoxicity of RCD cell lines against epithelial cells.

Small intestinal enterocytes express DNAM-1 ligands and can be lysed by RCD cell lines ex vivo

To explore the role of DNAM-1 *in vivo*, cells isolated from duodenal biopsies were analyzed by flowcytometry. Both CD112 and CD155 were consistently detected on enterocytes identified by the presence of epithelial specific antigen (ESA), although the expression of CD112 generally appeared to be higher than the expression of CD155 (Figure 5A, 5B). Enterocytes isolated from duodenal biopsies were also used as targets in a cytotoxicity assay. The RCD cell lines lysed enterocytes (Figure 5C), although the degree of cytotoxicity was substantially lower when compared to lysis of intestinal epithelial cell lines. In contrast, control T cell lines isolated from duodenal biopsies from the same patients displayed no cytotoxic activity against enterocytes (Figure 5C). These results indicate that RCD cell lines can destroy DNAM-1 ligand-positive enterocytes in vivo.

In vivo, DNAM-1 is expressed on aberrant IELs in a subset of RCD II patients

To assess the expression of DNAM-1 on aberrant IEL lines from RCD II patients other than the three patients (P1, P2 and P3) studied thus far, cell lines from biopsies of 15 additional RCD II patients were generated. Three of these additional cell lines contained a substantial sTCR-CD3$^-$CD30$^+$ cell population (Figure 6A). These three sTCR-CD3$^-$CD30$^+$

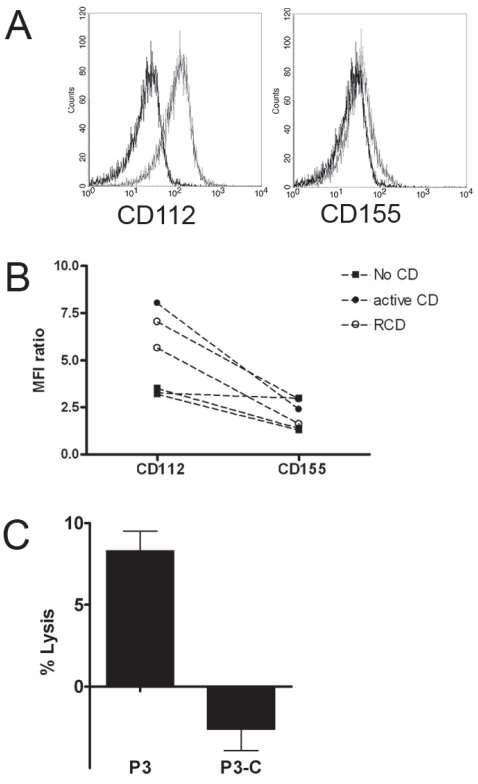

Figure 5. Enterocytes express DNAM-1 ligands and can be lysed by RCD cell lines. (A) FACS analysis of ESA$^+$ enterocytes isolated from a duodenal biopsy. The gray line represents staining with the indicated antibody, the bold line the isotype-matched control. (B) Mean Fluorescent Intensity (MFI) ratios (MFI-antibody of interest/ MFI isotype-matched control) for the expression of CD112 and CD155 on ESA$^+$ enterocytes from 6 independent donors. Closed squares represent individuals without CD, closed circles represent active CD and open circles represent RCD. (C) Cytotoxicity of RCD cell line P3 and control CD4$^+$ T cell clone P3-C, isolated from the same patient, to enterocytes isolated from duodenal biopsies in a 10:1 effector–target ratio. Similar results were obtained for RCD cell line P1 and a control CD8$^+$ T cell line isolated from the same patient. Data are representative of 5 independent experiments.

cell lines homogeneously expressed DNAM-1 (Figure 6B). The other cell lines were largely sTCR-CD3$^+$ and were, therefore, not considered aberrant IEL lines.
Previous studies indicated that DNAM-1 is present on the majority of T cells in peripheral blood[20]. In accordance with this, analysis of non-aberrant sTCR-CD3$^+$ IELs freshly isolated from biopsies of CD patients with active CD and those on a gluten-free diet indicated that these cells can be DNAM-1$^+$ (Figure 7A).

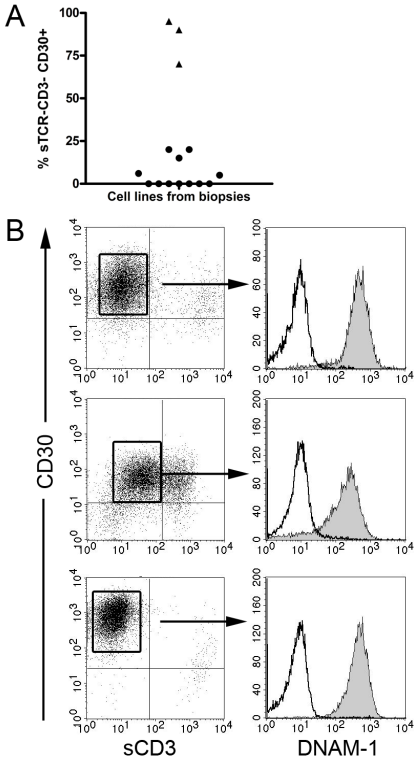

Figure 6. sTCR-CD3$^-$CD30$^+$DNAM$^+$ cell lines could be isolated from only a minority of RCD II biopsy specimens. Cell lines were isolated from small intestinal biopsies of 15 additional RCD II patients. (A) The percentage of sTCR-CD3$^-$CD30$^+$ cells within the total IEL population was determined with flowcytometry. Triangles represent the cell lines studied in figure 6B. (B) DNAM-1 expression on the three cell lines that contained a substantial sTCR-CD3$^-$CD30$^+$ population (triangles figure 6A). Analyses were performed within a live lymphocyte gate and quadrants were set based on staining with isotype-matched controls. Histograms are based on the sTCR-CD3$^-$CD30$^+$ population. The gray filled histogram represents staining with the indicated antibody, the bold line represents staining with the isotype-matched control.

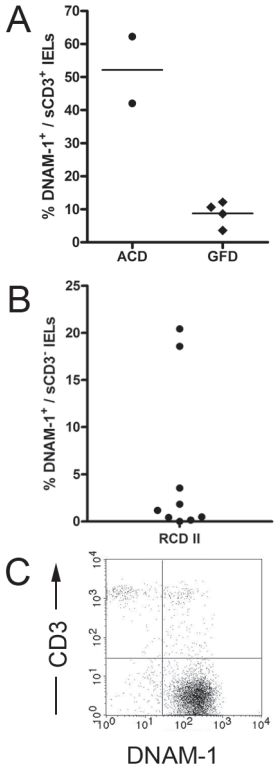

Figure 7. DNAM-1 is expressed on lymphoma cells and on aberrant IELs in a minority of RCD II patients. (A) FACS-analysis of IELs freshly isolated from biopsies of active CD patients (ACD) and CD patients on a gluten-free diet (GFD). The percentage of DNAM-1$^+$ cells within the sCD3$^+$ IEL population is depicted. (B) FACS-analysis of IELs freshly isolated from biopsies of 9 individual RCD II patients. The percentage of aberrant DNAM-1$^+$ cells within the sCD3- IEL population is depicted. (C) FACS analysis of lymphoma cells from ascitic fluid obtained from a patient suffering from invasive RCD-associated lymphoma. Analyses were performed within a live lymphocyte gate on CD45$^+$ cells and quadrants were set based on staining with isotype-matched controls.

In contrast to the aberrant IEL lines *in vitro*, on aberrant sTCR-CD3$^-$ IELs freshly isolated from duodenal biopsies, DNAM-1 was detected in only a minority of RCD II patients (Figure 7B). DNAM-1 was, however, uniformly present on sTCR-CD3$^-$ lymphoma cells directly isolated from ascitic fluid from a patient suffering from invasive RCD-associated lymphoma (Figure 7C). Taken together, DNAM-1 is expressed on aberrant IELs in a subset of RCD II patients and part of a spectrum of receptors that aberrant IELs can deploy to lyse enterocytes.

Discussion

In active CD, epithelial damage is attributed to triggering of activating NK cell receptors on $CD8^+$ IELs[9;11]. This is presumably linked to the adaptive gluten-specific T cell response in the lamina propria as the damage is restored upon withdrawal of gluten in the diet[23]. In contrast, RCD II and the successive state of overt lymphoma are characterized by gluten-independent tissue damage and a monoclonal expansion of aberrant sTCR-$CD3^-$ IELs[24]. It has been shown that such aberrant IELs can be cytotoxic[5;11]. The availability of cell lines isolated from small intestinal biopsies from RCD II patients that exhibit the characteristic phenotype associated with aberrant IELs[13] allowed us to investigate the cytotoxic specificity of such cells and to identify the receptors involved.

The RCD cell lines used in this study closely resemble aberrant IELs as they have the characteristic surface $TCR^-CD3^-CD4^-CD8^-CD103^+$, intracellular $CD3^+$ phenotype and monoclonal TCR-γ gene rearrangements identical to aberrant IELs *in vivo*[13]. In accordance with the phenotype of aberrant IELs *in vivo*, they generally lack expression of NK cell marker CD56, with the exception of a small $CD56^+$ fraction in RCD cell line P3 (Figure 2). In contrast to the majority of aberrant sTCR-$CD3^-$ IELs *in vivo*, the RCD cell lines also express CD30 and DNAM-1, both uniformly expressed on RCD-associated lymphoma cells[25](Figure 7C). In addition, the expression of activating NK cell receptors differed between the three RCD cell lines, with RCD cell line P3 expressing a greater variety of NK cell receptors than RCD cell lines P1 and P2. Strikingly, RCD cell line P2 did not express NKG2D, even though this receptor is present on most cytotoxic IELs[11;26]. Indeed, RCD cell line P1 and P3 (Figure 2) as well as the three $CD8^+$ IEL lines and the additional cell lines described in figure 6 were $NKG2D^+$ (data not shown). As all cell lines tested in this study retained stable expression of surface markers throughout the study, it seems unlikely that RCD cell line P2 lost NKG2D expression during in vitro culture. Lack of sTCR-CD3 expression combined with expression of a subset of NK cell receptors is a feature that the RCD cell lines share with lymphoid tissue inducer cells[27] and with T cells that have undergone reprogramming into NK-like cells due to deletion of the Bcl11b gene[28]. The RCD cell lines, however, expressed neither RORC mRNA nor surface CD127 (data not shown), two characteristic markers for lymphoid tissue inducer cells[27]. Moreover, preliminary data indicate that Bcl11b is expressed by the RCD cell lines. The exact cellular origin of the RCD cell lines thus remains unclear and this will be the subject of further studies.

Our results indicate that the cytotoxicity of the RCD cell lines is directed preferentially at cells of epithelial origin, and that this epithelial cytotoxicity is mediated mainly by DNAM-1. The preference for epithelial cells is explained by the presence of CD103 ($\alpha_E\beta_7$) -an integrin widely expressed on IELs- on the RCD cell lines, as this integrin will interact with its ligand E-cadherin that is selectively expressed on epithelial cells. In contrast to previous studies in which RCD cell lines showed strong cytotoxicity against NK cell target K562[5], in our study only RCD cell line P3 displayed low-level cytotoxicity against K562. This indicates that RCD cell lines differ in their ability to lyse various target cells, which might reflect the differences in NK cell receptor repertoire expressed by these cell lines. While previous studies identified NKG2C and NKG2D as mediators of cytotoxicity directed against intestinal epithelial cells[9;11;12], we now show that DNAM-1 is a third receptor enabling epithelial lysis. It is striking that DNAM-1 can function autonomously on the RCD cell lines while it is known to act as a co-receptor on normal T and NK cells. However, the independent activity of DNAM-1 on the RCD cell lines is analogous to that

of NKG2D on activated IELs in active CD, as NKG2D can act TCR-independently upon exposure to IL-15[12]. All in all, this indicates that there is considerable diversity in RCD cell lines, which may be a reflection of the in vivo diversity of aberrant IELs.

Recently, the inhibitory receptor TIGIT was identified that can be co-expressed with DNAM-1 on T cells and NK cells and competes for binding to the ligands CD155 and CD112[29;30]. TIGIT was shown to have a 100-fold higher affinity for CD155 than DNAM-1[29], suggesting that, when co-expressed, TIGIT has the dominant function and can regulate the function of DNAM-1. Our study, however, indicates a dominant function for DNAM-1 on aberrant IELs in RCD II. This suggests that TIGIT, if present, does not function as a dominant negative regulator on these cells.

While it is well known that DNAM-1 can mediate lysis of tumor cells by $CD8^+$ T cells and NK cells[20;31], this is the first report describing DNAM-1-mediated cytotoxicity by (pre) malignant cells themselves. Various tumor cell types express DNAM-1 ligands CD112 and CD155[15;18;32] and can be lysed by DNAM-1 expressing $CD8^+$ T cells and NK cells. In marked contrast, the cell lines derived from RCD II patients do not express DNAM-1 ligands but instead express the DNAM-1 receptor which triggers specific lysis of intestinal epithelial cells (Supplementary Figure S3). Although lysis of freshly isolated enterocytes by the RCD cell lines was significantly lower compared to lysis of epithelial cell lines, this low degree

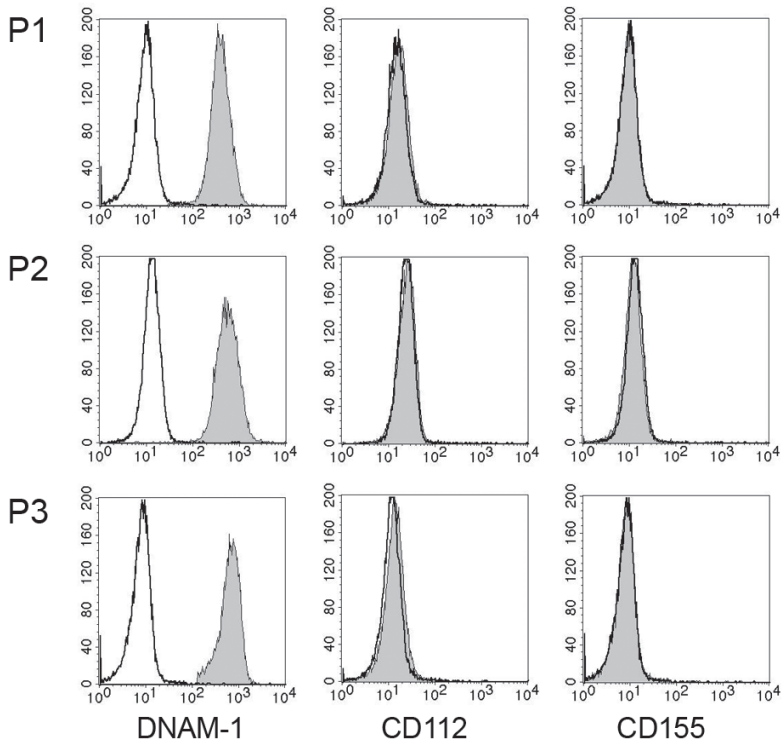

Figure S3. RCD cell lines do not express DNAM-1 ligands. RCD cell lines P1, P2 and P3 were stained with antibodies specific for DNAM-1, CD112 and CD155. The gray filled histogram represents staining with the indicated antibody, the bold line represents staining with the isotype-matched control. Analyses were performed within a live lymphocyte gate.

of lysis could lead to significant damage in vivo as this would be a continuous process in the patient. This might contribute to the gluten-independent intestinal damage in RCD II patients in which such cells are present and in patients suffering from RCD-associated lymphoma. As aberrant IELs in RCD II can disseminate to the entire gastrointestinal tract and RCD-associated lymphoma to extra-intestinal locations like skin, lung and liver[8;33], DNAM-1 mediated cytotoxicity of (pre)malignant cells against epithelium might contribute to tissue damage at various sites in the body.

In vivo, DNAM-1 was expressed on aberrant IELs in 2 out of 9 RCD II patients, and on lymphoma cells isolated from ascitic fluid from a patient suffering from invasive RCD-associated lymphoma. This indicates that DNAM-1 is expressed on aberrant IELs in a subset of RCD II patients. We speculate that these $sCD3^-DNAM\text{-}1^+$ IELs are the precursors of the $sCD3^-DNAM\text{-}1^+$ cell lines that grow out in vitro. However, we cannot exclude that culture conditions have induced DNAM-1 expression of the RCD cell lines.

In conclusion, this study provides the first evidence that in a subset of RCD II patients, aberrant IELs can acquire the ability to lyse epithelial cells via DNAM-1. Together with other activating NK cell receptors, DNAM-1 on aberrant IELs can contribute to the gluten-independent tissue damage in RCD II and RCD-associated lymphoma. This mechanism through which (pre)malignant cells can cause tissue damage might also apply to other tumors of lymphoid origin.

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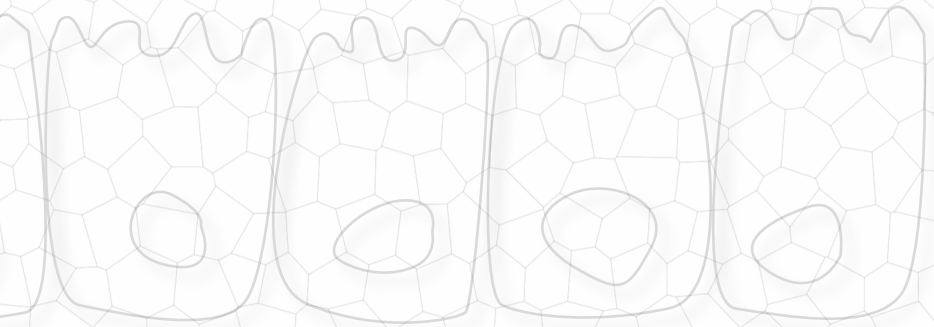
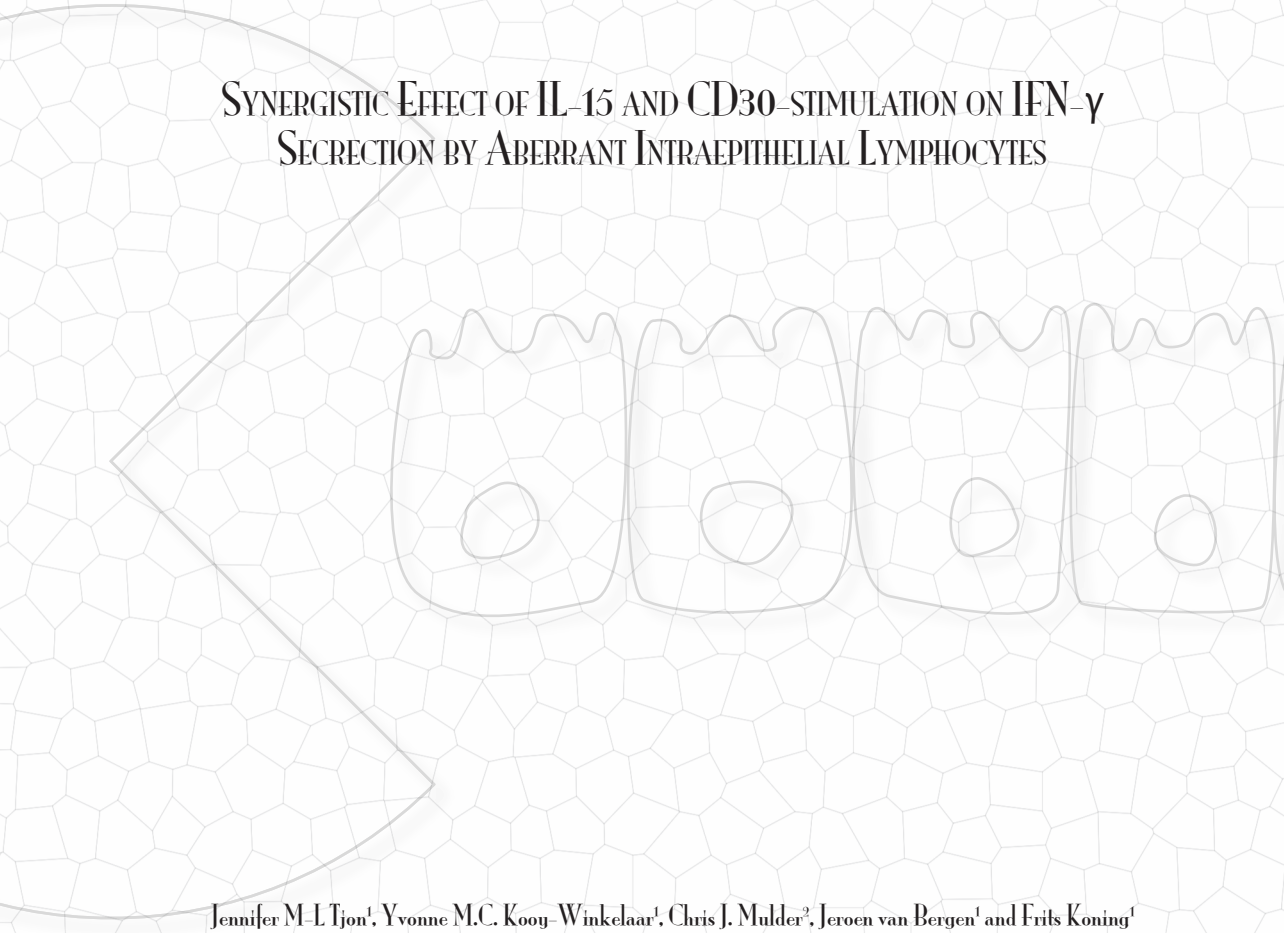


Chapter 4

Synergistic Effect of IL-15 and CD30-stimulation on IFN-γ Secretion by Aberrant Intraepithelial Lymphocytes



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Abstract

Refractory celiac disease type II (RCD II) is characterized by the presence of aberrant intraepithelial lymphocytes (IELs) that lack expression of the T cell receptor (TCR)-CD3 complex. As these aberrant IELs can progressively replace normal IELs and can disseminate to extraintestinal sites, RCD II is now considered an intraepithelial lymphoma that can progress into overt lymphoma. RCD II and overt lymphoma are characterized by persisting inflammation of the small intestine with elevated levels of interleukin (IL)-15 in the epithelium despite a gluten-free diet. In the present study we used aberrant IEL lines isolated from RCD II patients to explore the possible role of aberrant IELs in this ongoing inflammatory response. Analogous to TCR^+ IELs in active CD, the aberrant IEL lines secreted interferon (IFN)-γ upon stimulation with IL-15. With the exception of IL-10 that was secreted by two out of three aberrant IEL lines, other T helper-1 and T helper-2 cytokines were not detected. Furthermore, IFN-γ secretion in response to IL-15 could be boosted by additional cross-linking of some, but not all, NK cell receptors that are involved in aberrant IEL-mediated cytotoxicity.

Strikingly, combined triggering of activation-induced receptor CD30 and stimulation with IL-15 had a synergistic effect on IFN-γ secretion. Previous studies described the presence of CD30 on aberrant IELs in a subset of RCD II patients and on virtually all lymphoma cells. As IL-15 is highly upregulated in both disease stages, combined stimulation might occur in vivo.

This study suggests a novel role for CD30 as a contributor to the persisting inflammatory reaction in RCDII and RCD-associated lymphoma.

Introduction

Celiac disease (CD) is characterized by the presence of gluten-specific $CD4^+$ T cells and an increase of T cell receptor $(TCR)^+$ intraepithelial lymphocytes (IELs) in the mucosa of the small intestine. Exposure to dietary gluten leads to an inflammatory response which results in villous atrophy and crypt cell hyperplasia. An important factor in this inflammatory response is the pro-inflammatory cytokine interferon-gamma (IFN-γ), which is secreted in high amounts by gluten specific $CD4^+$ T cells from the lamina propria[1] and TCR^+ IEL[2]. In contrast, secretion of other T helper-1 (Th1) and/or T helper-2 (Th2) cytokines is less prominent[1-3]. Upon elimination of gluten from the diet, the inflammatory reaction stops and IFN-γ levels decrease[1;2].

Persisting inflammation of the small intestine with elevated numbers of IELs despite a gluten-free diet is a hallmark of refractory celiac disease (RCD)[4]. This ongoing inflammation involves interleukin-15 (IL-15) which is upregulated in the epithelium and lamina propria of active CD and RCD patients[5]. Upon stimulation with IL-15, TCR^+ IELs secrete IFN-γ[6] and display TCR-independent cytotoxicity against the epithelium[7]. Secretion of IFN-γ by TCR^+ IELs might thus have an important role in the propagation of inflammation in RCD. In RCD type II (RCD II) an aberrant IEL population is present which lacks the TCR-CD3 complex on the cell surface but expresses CD3 intracellularly[8]. This aberrant TCR^- IEL population can progress into overt gastrointestinal lymphoma, an often fatal complication of CD. Both RCD II and RCD-associated lymphoma are characterized by persisting inflammation and it seems likely that aberrant TCR^- IELs contribute to this inflammation.

In contrast to TCR^+ IELs, not much is known about cytokine secretion by aberrant TCR^- IELs. In this study, aberrant IEL lines isolated from duodenal biopsies of three RCD II patients[9] were used to study the cytokine profile of aberrant IEL.

Material and methods

Cell lines and culture

RCD cell lines P1, P2 and P3 were isolated from duodenal biopsies of RCD II patients as previously described[9]. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Lonza, Verviers, Belgium) with 10% normal human serum (NHS) containing 10 ng/ml IL-15 (R&D Systems Europe, Abingdon, United Kingdom). RCD cell lines were restimulated approximately every 4 to 5 weeks with 1 µg/ml phytohemagglutinin, 10 ng/ml IL-15 and 106/ml irradiated allogenous peripheral blood mononuclear cells as feeder cells. Prior to stimulation for cytokine release assays, cells were cultured for 72 hours without supplemented IL-15.

Antibodies

Anti-CD3 was from Orthobiothech (Bridgewater, NJ), anti-CD30, anti-NKG2D and isotype-matched control antibodies were from R&D Systems Europe, anti-CD94 was from Beckman Coulter (Fullerton CA) and anti-CD226 (DNAM-1) was from BD Biosciences (San Jose, CA).

Cytokine release assay

96-well non-tissue-culture-treated plates (BD Falcon, Erembodegem, Belgium) were coated overnight in triplicate with 5 µg/ml anti-CD3, anti-CD30, anti-NKG2D, anti-CD94, anti-DNAM-1 or isotype-matched control antibodies. Subsequently, plates were blocked with 10% FCS/PBS and washed with PBS. 20.000 cells from RCD cell line P1, P2 or P3 were added in 100 µl IMDM with 10% NHS or in 100 µl IMDM with 10% NHS supplemented with 10ng/ml IL-15. After 2 days incubation at 37°C, supernatants were harvested for cytokine detection.

Supernatants harvested after stimulation of cells from RCD cell line P1, P2 and P3 were analyzed using the Bio-Plex Pro Human Th1/Th2 assay (Bio-Rad laboratories, Veenendaal, The Netherlands) according to the manufacturer's protocol. The following cytokines were tested: IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, GM-CSF, IFN-γ and TNF-α. Samples were acquired on a Bio-Plex array reader with Bio-Plex software (Bio-Rad laboratories).

After simultaneous stimulation with plate-bound anti-CD30 and IL-15, IFN-γ production was analyzed with the human IFN-γ enzyme-linked immunosorbent assay (ELISA) kit from U-CyTech (Utrecht, The Netherlands) according to the manufacturer's instructions. OD values were obtained at an absorbance of 450 nm on a microplate reader (Bio-Rad Laboratories) and data was analyzed with Microplate Manager software (Bio-Rad Laboratories).

Results

Aberrant IEL lines secrete IFN-γ upon stimulation with soluble IL-15

The three aberrant IEL lines used in this study (RCD cell line P1, P2 and P3) closely resemble aberrant IEL as they lack expression of the TCR, CD3, CD4 and CD8 on the cell surface, but have intracellular expression of CD3[9]. In addition, all three RCD cell lines expressed NK cell receptor DNAM-1 which is involved in epithelial-specific cytotoxocity[10]. Expression of NKG2D and CD94/NKG2C was limited to RCD cell line P1 (NKG2D$^+$) and P3 (NKG2D$^+$ and CD94/NKG2C$^+$)[10]. In contrast to the majority of aberrant IELs in vivo, the RCD cell lines expressed the activation-induced CD30[9;11].

Previous studies indicated that TCR$^+$ IEL lines isolated from patients with active celiac disease secreted mainly IFN-γ upon stimulation with soluble IL-15[6;12]. Therefore, the RCD cell lines and a CD8$^+$ TCR$^+$ cell line derived from patient P1 (P1-CD8) were stimulated with IL-15 to assess the potential secretion of IFN-γ and other Th1 and Th1 cytokines. In analogy to previous studies on TCR$^+$ IEL lines, the RCD cell lines secreted mainly IFN-γ whereas other Th1 and Th2 cytokines were undetectable (Figure 1A). An exception was IL-10, secreted in low amount by RCD cell lines P2 (<25 p/ml) and P3 (<10 pg/ml) (Figure 1A). Strikingly, the control cell line P1-CD8 secreted only low amounts of IL-2 upon IL-15 stimulation (Figure 1A) and therefore displayed a cytokine profile distinct from the RCD cell lines.

To determine which other stimuli could induce IFN-γ secretion by the RCD cell lines, the RCD cell lines were stimulated with plate-bound monoclonal antibodies against CD3, CD30, NKG2D, CD94, DNAM-1, isotype-matched control antibodies or with soluble IL-15. As expected, stimulation with an antibody specific to CD3 did not induce IFN-γ secretion by the CD3$^-$ RCD cell lines, whereas TCR$^+$ cell line P1-CD8 secreted high amounts of IFN-γ

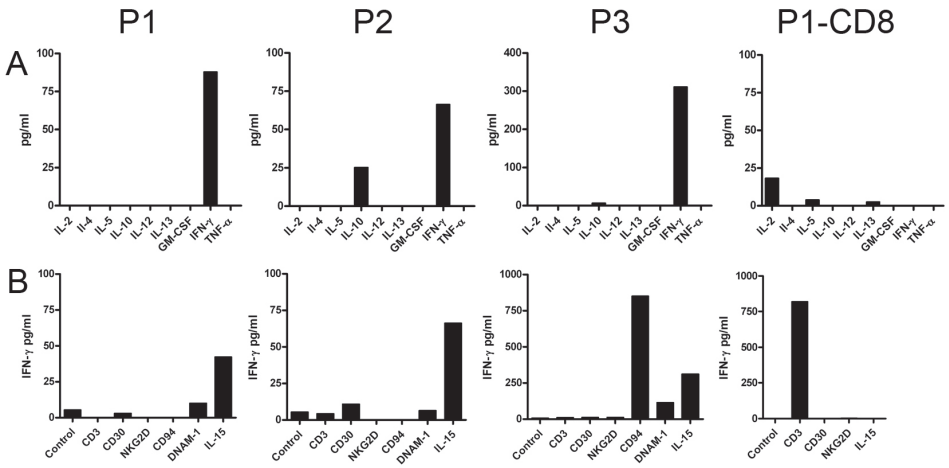

Figure 1. Aberrant IEL lines secrete IFN-γ upon stimulation with soluble IL-15. RCD cell lines P1, P2 and P3 were stimulated with plate-bound monoclonal antibodies against CD3, CD30, NKG2D, CD94, DNAM-1, isotype-matched control antibodies or with soluble IL-15. Control cell line P1-CD8 was stimulated with plate-bound monoclonal antibodies against CD3, CD30, NKG2D, isotype-matched control antibodies or with soluble IL-15. Supernatants were harvested after stimulation and analyzed using the Bio-plex Th1/Th2 assay. (A) Cytokine secretion profile of RCD cell line P1, P2, P3 and P1-CD8 after IL-15 stimulation. (B) IFN-γ secretion after stimulation with the indicated stimuli.

upon anti-CD3 stimulation (Figure 1B). IFN-γ secretion upon anti-CD30 stimulation by the RCD cell lines did not significantly exceed values for the isotype-matched control antibody (Figure 1B). Triggering of DNAM-1 resulted in detectable IFN-γ secretion by RCD cell line P3 but not by RCD cell lines P1 and P2 (Figure 1B). Furthermore, whereas anti-NKG2D stimulation had no effect on NKG2D$^+$ RCD cell lines P1 and P3, anti-CD94 stimulation on CD94/NKG2C$^+$ RCD cell line P3 did result in secretion of high amounts of IFN-γ (Figure 1B).

Together these results indicate that, when stimulated with IL-15, all RCD cell lines secrete the pro-inflammatory cytokine IFN-γ. In addition, two out of three RCD cell lines secrete IL-10. Furthermore, cross-linking of NK cell receptors present on the RCD cell lines mostly did not induce IFN-γ secretion.

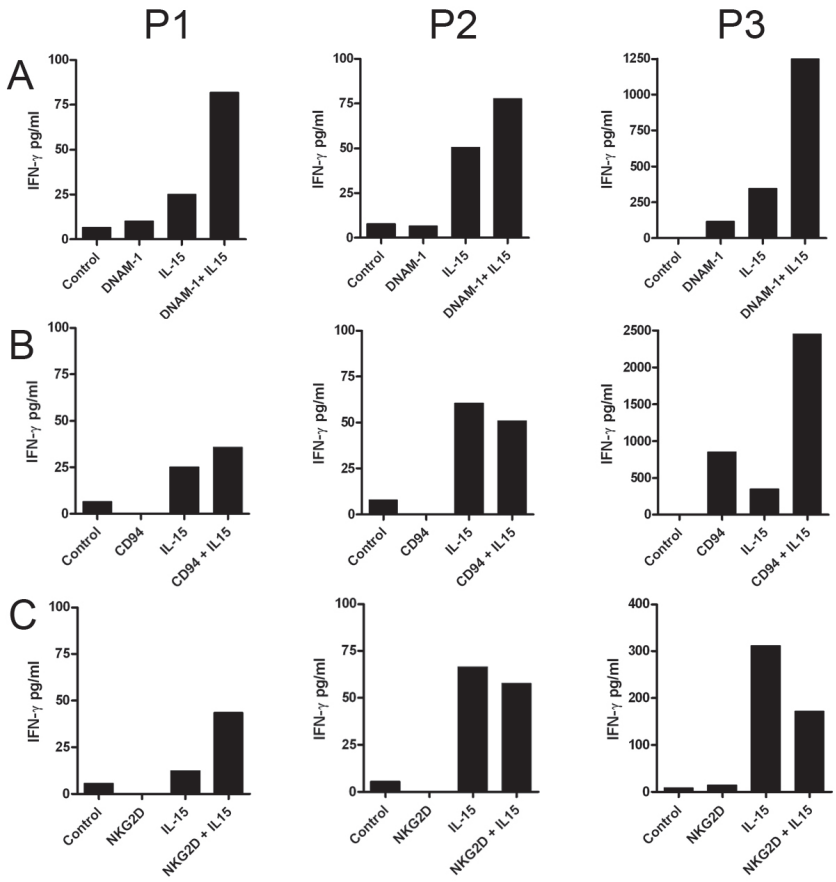

Figure 2. Cross-linking of NK cell receptors on aberrant IEL lines can boost IFN-γ secretion in response to IL-15 stimulation. RCD cell line P1, P2 and P3 were stimulated with: (A) anti-DNAM-1, IL-15 or a combination of anti-DNAM-1 and IL-15. (B) anti-CD94, IL-15 or a combination of anti-CD94 and IL-15. (C) anti-NKG2D, IL-15 or a combination of anti-NKG2D and IL-15. Supernatants were analyzed using the Bio-plex Th1/Th2 assay. Results for IFN-γ secretion are shown.

Cross-linking of NK cell receptors on aberrant IEL lines can boost IFN-γ secretion in response to IL-15 stimulation

Whereas all three RCD cell lines secreted IFN-γ upon stimulation with IL-15, triggering of two out of three NK cell receptors tested induced IFN-γ secretion by RCD cell line P3 but not in RCD cell lines P1 and P2 (Figure 1B). In vivo, stimulation of NK cell receptors and stimulation with IL-15 can occur simultaneously as IL-15 is highly upregulated in RCD. Therefore, the effect of IL-15 stimulation combined with stimulation of NK cell receptors on IFN-γ secretion was studied. The RCD cell lines were stimulated with plate-bound anti-DNAM-1, anti-CD94, anti-NKG2D or isotype-matched control antibodies alone or in combination with soluble IL-15.

Combining anti-DNAM-1 and IL-15 stimulation resulted in increased IFN-γ secretion when compared to stimulation with anti-DNAM-1 or IL-15 alone in all three RCD cell lines (Figure 2A). Similarly, combining anti-CD94 and IL-15 substantially increased secretion of IFN-γ by CD94/NKG2C$^+$ RCD cell line P3, whereas this effect was not found for the CD94/NKG2C$^-$ RCD cell lines P1 and P2 (Figure 2B). In contrast, the effect of combined anti-NKG2D and IL-15 stimulation was less clear as it appeared to increase IFN-γ secretion by NKG2D$^+$ RCD cell line P1, while decreasing IFN-γ secretion by NKG2D$^+$ RCD cell line P3 (Figure 2C).

These data suggest that IFN-γ secretion in response to IL-15 stimulation can be boosted by additional triggering of some, but not all, NK cell receptors.

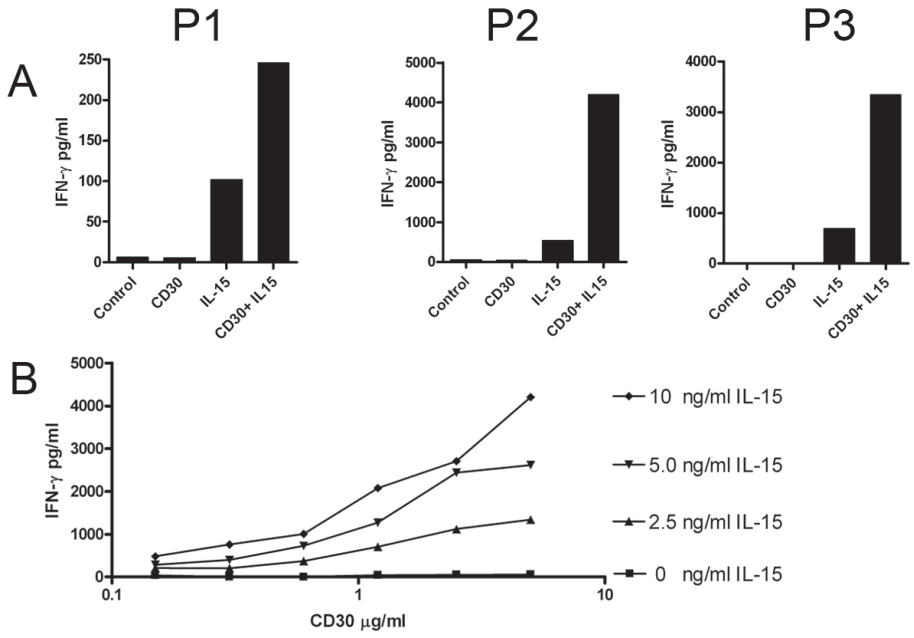

Figure 3. Combined stimulation with soluble IL-15 and stimulation of the CD30 receptor has a synergistic effect on IFN-γ secretion by aberrant IEL lines. (A) P1, P2 and P3 were stimulated with 5 μg/ml anti-CD30 or isotype-matched control antibodies alone or combined with 10ng/ml IL-15. Supernatants harvested after stimulation were analyzed with an IFN-γ ELISA. (B) RCD cell line P2 was stimulated with the indicated amounts of IL-15 combined with 0 to 5 μg/ml anti-CD30. Supernatants were analyzed with an IFN-γ ELISA. Similar results were obtained for RCD cell line P1 and P3.

Combined stimulation with soluble IL-15 and stimulation of the CD30 receptor has a synergistic effect on IFN-γ secretion by aberrant IEL lines

Virtually all RCD-associated lymphoma cells express the marker CD30[13]. Expression of CD30 on the cell surface of aberrant IEL is correlated with a worse prognosis of RCD II and this might indicate the transition into overt lymphoma[11]. The role of CD30 on these cells, however, remains unclear. As all RCD cell lines expressed CD30 we investigated the possible role of CD30 in cytokine secretion. Triggering of the CD30 receptor alone did not result in IFN-γ secretion (Figure 3A). Combining a CD30-specific antibody with IL-15, however, substantially increased IFN-γ secretion when compared to IL-15 stimulation alone (Figure 3A). Titration of the amount of CD30 specific antibody combined with different concentrations of IL-15 indicated that small amounts of anti-CD30 and IL-15 are sufficient to achieve this effect (Figure 3B). These results suggest a role for CD30 as a contributor to the inflammatory response in RCD II and RCD-associated lymphoma.

Discussion

Studies on small intestinal biopsies indicated that the predominant cytokine secreted in active CD is the pro-inflammatory cytokine IFN-γ, whereas other Th1 cytokines were mostly undetectable[1;2]. Although $CD4^+$ lamina propria T cells were shown to produce IFN-γ upon stimulation with gluten1, $CD8^+TCR^+$ IEL were identified as the main producers of IFN-γ[14;15]. RCD is characterized by persisting inflammation and elevated levels of IL-15 in the small intestine despite strict adherence to a gluten-free diet. IL-15 has been shown to induce the production of IFN-γ by $CD8^+TCR^+$ IEL[6;12], suggesting an important role for $CD8^+TCR^+$ IEL in the persisting inflammation that characterizes RCD. In RCD II and RCD-associated lymphoma, the IEL population is largely replaced by a monoclonal aberrant TCR^- IEL population. Therefore, we hypothesized that this aberrant IEL population might contribute to the ongoing inflammation in RCD II and RCD associated lymphoma.

In this study we studied the cytokine profile of aberrant IEL using TCR-$CD3^-$ $CD30^+$ IEL lines isolated from small intestinal biopsies of RCD II patients[9]. Upon stimulation with soluble IL-15, the RCD cell lines secreted IFN- γ, suggesting a role for aberrant IEL in maintenance of inflammation. In addition, RCD cell lines P2 and P3 secreted low levels of the immunoregulatory cytokine IL-10. The role of this cytokine in CD and RCD is unclear as both elevated[2] and decreased[16] levels of IL-10 have been reported for active CD.

Previous studies showed that the activating NK cell receptors NKG2D, CD94/NKG2C and DNAM-1 can induce epithelial cell lysis independent of the TCR, presumably upon stimulation with IL-15[7;10;17]. We observed that stimulation of DNAM-1 or CD94, but not NKG2D, combined with IL-15 stimulation could increase levels of IFN-γ secretion by the RCD cell lines.

The RCD cell lines used in this study express CD30 on the cell surface, a member of the TNF-receptor family that is present on activated T cells and lymphomas[18]. In celiac disease, CD30 has been detected on peripheral blood T cells in active CD[19], on aberrant IELs in a subset of RCD II patients and on RCD-associated lymphoma cells[11]. We show that stimulation of the CD30 receptor alone did not induce secretion of IFN-γ by the RCD cell lines. However, combined triggering of the CD30 receptor and IL-15 stimulation had a synergistic effect on IFN-γ secretion. Expression of its counterpart, CD30 ligand (CD30L) was found on activated T cells, monocytes/macrophages and granulocytes, as well as on resting B cells[20]. In active CD, RCD and RCD-associated lymphoma, IL-15 is upregulated and activated T cells are present that may express CD30L. The interaction of the CD30 with CD30L combined with IL-15 stimulation could, therefore, occur in vivo and might contribute to the inflammatory response via IFN-γ secretion. Determining the expression of CD30L in RCD II and RCD-associated lymphoma and the direct effect of $CD30L^+$ cells on the IFN-γ secretion by aberrant IEL lines will be a topic of further research.

In conclusion, this study indicates a role for aberrant IEL in the propagation of inflammation in RCD II and RCD-associated lymphoma and suggests a novel role for CD30 on (pre)malignant IEL in RCD II and RCD-associated lymphoma.

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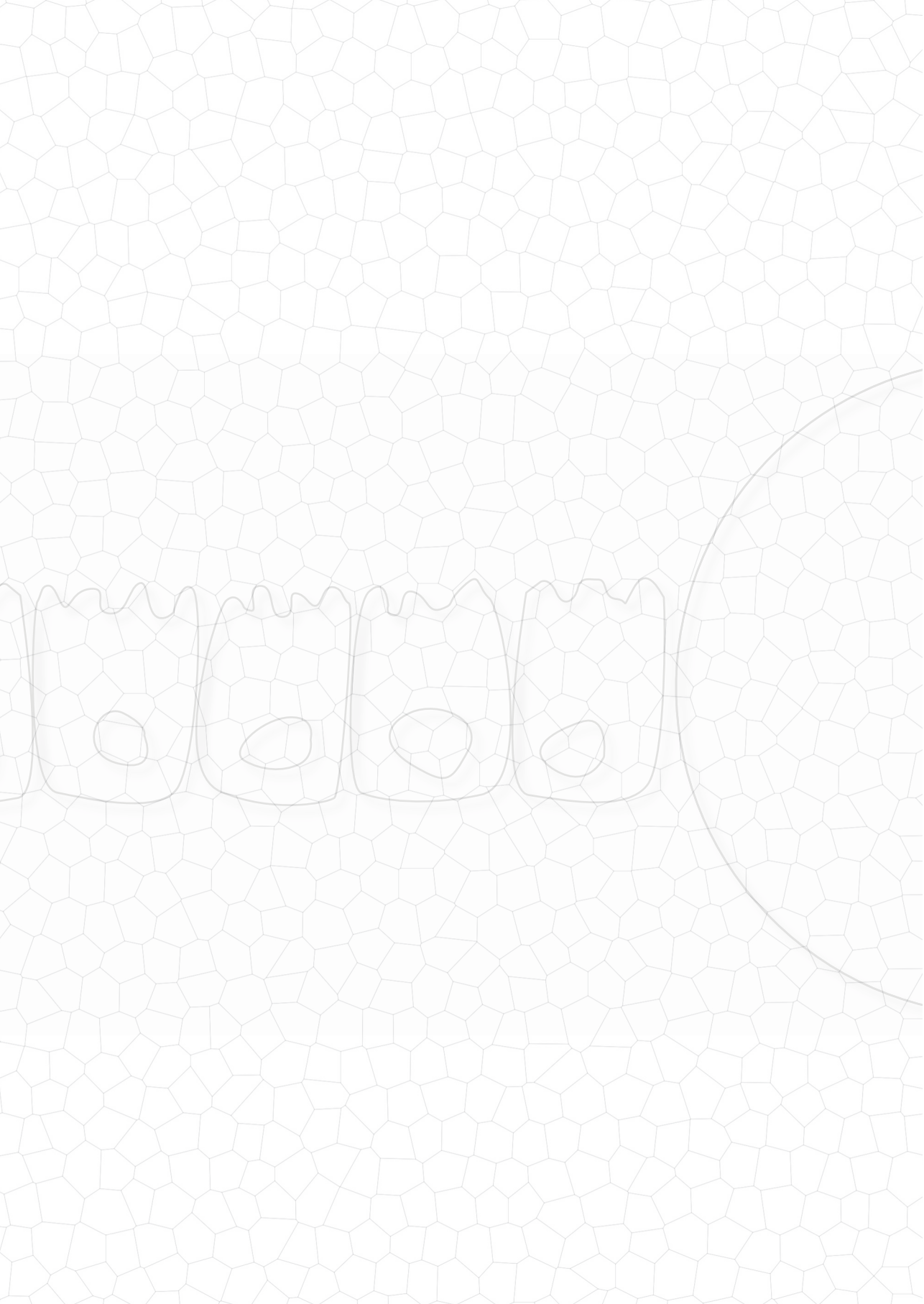

Chapter 5

Characterization of a Unique CD3$^-$CD7$^+$ Intraepithelial Lymphocyte Population: Possible Origin of Aberrant Intraepithelial Lymphocytes in Refractory Celiac Disease Type II

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Abstract

Refractory celiac disease type II (RCD II) is a severe complication of celiac disease (CD) that is characterized by the presence of an aberrant intraepithelial lymphocyte (IEL) population that lacks expression of a surface T cell receptor (TCR)-CD3 complex. These aberrant IEL are considered malignant as they can progressively replace normal IEL and as approximately 50% of the RCD II patients develop high-grade invasive lymphoma. The cellular origin of aberrant IEL is largely unknown. Therefore, we performed genomic, transcriptomic and flowcytrometric analysis of aberrant IEL lines derived from small intestinal biopsies of RCD II patients. Our results indicate a unique phenotype for aberrant IEL: $CD45^{+}CD19^{-}CD14^{-}CD3^{-}CD7^{+}CD56^{-}CD34^{-}CD103^{+}CD127^{-}$. Furthermore, cells with this phenotype can be found in duodenal specimens of healthy children and adults. This study indicates that a unique lymphocyte-subset is present in the small intestine of healthy individuals and non-refractory CD patients that in RCD II patients may undergo malignant transformation into aberrant IEL.

Introduction

Celiac disease (CD) is an enteropathy of the small intestine induced in HLADQ2+ or HLADQ8+ individuals. Active CD is characterized by a CD4+ T cell response to wheat gluten and to similar proteins in barley and rye which typically leads to villous atrophy and crypt hyperplasia[1]. In addition, the number of TCR-αβ+ and TCR-γδ+ 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]. Eliminating gluten from the diet usually restores the intestinal architecture. However, 2-5% of adult-onset CD patients develops refractory celiac disease (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 can be subdivided into two types: RCD type I (RCD I) and RCD type II (RCD II). RCD I presents with < 20% aberrant IEL, whereas in RCD II > 20% of the IEL have an aberrant phenotype[6]. For diagnostic purposes, aberrant IEL are defined as surface T cell receptor (TCR)-CD3-CD4-CD8-CD7+CD103+, intracellular CD3+ cells that commonly display monoclonal TCR-γ gene rearrangements[6;7]. In RCD II, these aberrant IEL replace the normal IEL population and can undergo further malignant transformation into lymphoma cells. In contrast, RCD I patients seldom develop gastrointestinal lymphoma[8]. The clinical outcome of RCD is thus closely linked to the frequency of aberrant IEL, which is also reflected in the 5-year survival rates of 80-96% and 44-58% for RCD I and RCD II respectively[5;8].

The cellular origin of the aberrant TCR- IEL population that expands in RCD II is still unclear. One view is that aberrant IEL originate from mature TCR+ IEL (T-IEL) that have undergone oligoclonal expansion and downregulate the TCR-CD3 complex due to overstimulation[7]. This view is supported by the observation that aberrant IEL have intracellular expression of the CD3γ-, CD3δ-, CD3ε- and ζ-chains and commonly display monoclonal TCR-γ gene rearrangements[7-9]. A possible T-IEL origin is also reflected in the name for RCD-associated lymphoma: enteropathy associated T cell lymphoma (EATL). Intracellular CD3 expression, however, is not exclusively restricted to mature T cells as fetal NK cells can also express all CD3 chains intracellularly[10]. Therefore, an alternative view could be that aberrant IEL derive from the surface TCR-CD3-CD4-CD8-CD7+ IEL population that is present in the fetal and adult intestine[11-14]. This IEL population contains NK cells[15] as well as immature T cells[12;16] and could represent the physiological counterpart of the aberrant surface TCR-CD3- IEL that undergo malignant transformation in RCD II.

In this study we characterized in detail RCD cell lines from three RCD II patients[9] and investigated the phenotype of the surface TCR-CD3-CD7+ IEL population in duodenal biopsy material as a first step to determine the cellular origin of aberrant IEL.

Material and Methods

Duodenal biopsy specimens

During upper endoscopy, large spike forceps biopsy specimens (Medi-Globe, Tempe, Arizona) were taken from the second part of the duodenum. Biopsy specimens were used for cell culture and direct flowcytometric analysis. All biopsy specimens were obtained after given informed consent in accordance with the local ethical guidelines of the VU Medical Center in Amsterdam or the Leiden University Medical Center in Leiden and the declaration of Helsinki.

Fetal intestine

The use of fetal tissue was approved by the Medical Ethical Commission of the Erasmus University Medical Center Rotterdam, and use was contingent on informed consent. Fetal tissues were obtained from elective abortions and gestational age was determined by ultrasonic measurement of the diameter of the skull or the femur. For this study samples from a 19 and a 20 week old human fetus were obtained.

Thymic cell suspensions

Thymic material was obtained as surgical tissue discards from two children without immunological abnormalities (2 and 7 months old) undergoing cardiac surgery at the Erasmus MC Rotterdam, with informed consent from the parents. Thymocytes were isolated as previously described 17 by cutting the thymic lobe and squeezing the pieces through a metal mesh and stored at -80°C until further analysis.

Cell lines and cell culture

RCD cell lines P1, P2, P3 and P4 were isolated from duodenal biopsies of RCD II patients as previously described[9]. In short: biopsy specimens were treated with 1mM dithiothreitol (Fluka, Buchs, Switzerland) and 0.75 mM ethylenediaminetetraacetic acid (Merck, Darmstadt, Germany) after which the biopsy was cultured in Iscove's modified Dulbecco's medium (IMDM, Lonza, Verviers, Belgium) supplemented with 10% normal human serum (NHS) and 10 ng/ml IL-15 (R&D systems Europe, Abingdon, UK). Released cells were propagated on IMDM containing 10% NHS and 10ng/ml IL-15 and restimulated approximately every 4 to 5 weeks with 1 µg/ml phytohemagglutinin, 10 ng/ml IL-15 and 1 x 10^6/ml irradiated allogenous peripheral blood mononuclear cells as feeder cells. Additional 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 RCD cell line P1. For P3 a $CD4^+$ T cell clone (P3-CD4) was isolated from the same biopsy as RCD cell line P3. Cells were restimulated every 2 weeks and maintained on 10% normal human serum (NHS) containing 10 ng/ml IL-15 and 20 Cetus units/ml IL-2 (Proleukin, Chiron corporation, Emeryville, CA). From P2 no additional TCR^+ cell lines were isolated. TCR-$CD3^+$ IEL lines (IEL 1-5) were isolated from 4 CD patients and one patient with Crohn's disease as follows: IEL released directly after DTT and EDTA treatment were cultured separately in IMDM containing 10% NHS supplemented with 10 ng/ml IL-15 and 20 Cetus units/ml IL-2 (Proleukin, Chiron corporation, Emeryville, CA) and restimulated every 2 weeks. The T-IEL lines were a mixed population of $CD4^+$ and $CD8^+$ T cells.

Isolation of cells from duodenal biopsies for flowcytometry

Cells were isolated from two or three duodenal biopsies by treatment with 200 µg/ml collagenase A (MP Biomedicals LLC, Eschwege, Germany) and 200 µg/ml DNAse II (Roche diagnostics, Almere, Netherlands). After rotating 1 hour at 37°C, cells were filtered through a 70 µm filter (BD Falcon, Erembodegem, Belgium) and washed twice with IMDM containing 10% FCS. For isolation of the IEL fraction alone, biopsies were first treated with 1mM dithiothreitol (twice for 10 minutes at room temperature) followed by treatment with 1mM ethylenediaminetetraacetic acid (twice for 1 hour at 37°C). The released IEL in the supernatant were washed twice in PBS containing 0.5% FCS. For subsequent flow cytometric analysis, cells were stained 30 minutes with fluorochrome-conjugated antibodies.

Antibodies and flowcytometry

Fluorochrome-labeled antibodies specific to CD1a, CD2, CD3, CD4, CD5, CD6, CD8α, CD10, CD11a, CD16, CD18, CD25, CD27, CD28, CD30, CD31, CD33, CD34, CD38, CD40, CD44, CD45, CD45RA, CD45RO, CD54, CD62L, CD94, CD103, CD117, CD122, CD152, CD154, CD161, CD226, CD244, integrin β7, KIR3DL1 and NKG2D were purchased from BD Biosciences (San Jose, CA). Fluorochrome-labeled antibodies specific to CD8β, CD127, KIR2DL2/DL3/DS2, KIR2DL1/2DS1, KIR2DS4, KIR3DL1, NKp30, NKp44 and NKp46 were purchased from Beckman Coulter (Fullerton, CA). Fluorochrome-labeled antibodies specific to IL-15Rα, KIR2DL3, KIR2DL4, NKG2A and NKG2C were purchased from R&D systems Europe. Anti-CD19 and anti-CD56 were from Exbio (Vestec, Czech Republic), anti-CD14 was from Immunotools (Friesoythe, Germany) anti-CD7 was from eBioscience (San Diego, CA) and anti-CD52 was from Genzyme, Cambridge. The anti-KIR2DS2/2DL3 antibody was a kind gift from Dr. C. Retiere[18], the anti-KIR3DL2 antibody was a kind gift from Dr. D. Pende[19] and the anti-KIRDL5 antibody was a kind gift from Dr. C. Vilches[20]. For antibody clone names see Table 2. Labeled cells were acquired on an LSR II or FACS-Calibur (both from BD biosciences) and data was analyzed with FACSDIVA software or Cellquest pro software.

PCR-based analysis of TCR-δ TCR-γ and TCR-β gene rearrangements

TCR-δ, TCR-γ and TCR-β gene rearrangements were assessed for RCD cell line P1, P2 and P3 by multiplex PCR as previously described[21]. Resulting PCR products were further evaluated by GeneScan analysis and sequenced to determine gene usage and functionality of the rearrangements.

RNA isolation and microarray hybridization

Total RNA was extracted from cell lines P1, P2, P3, P1-CD4, P1-CD8, P3-CD4 and the 5 T-IEL lines using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) followed by DNAse digestion using the RNase-free DNAse kit (Qiagen). The RNA samples were labeled in duplicate with the Ambion Illumina TotalPrep RNA-amplification kit (Illumina, Son, The Netherlands). cRNA samples were hybridized on 2 Illumina HumanHT-12 V3 Expression BeadChips at the Leiden Genome Technology Center according to the Illumina BeadArray protocol. Samples were randomly distributed among the two BeadChips.

Microarray data analysis

The intensities of the expression results were parsed into the R statistical computing environment using the beadarray package[22]. Gene expression values were $\log_2$ 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 package[23]. The Benjamini and Hochberg method was used to adjust p-values for mulitple-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. The obtained data was then used to do hierarchical clustering of the samples, 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/).

Results

RCD cell lines are distinct from any known lymphocyte class

To investigate the possible cellular origin of aberrant IEL, we used three previously described cell lines isolated from small intestinal biopsies of RCD II patients (P1, P2 and P3) that display the characteristic phenotype of aberrant IEL: surface TCR-CD3-CD4-CD8-CD7+CD103+ intracellular CD3+[9] (Figure 1).

The monoclonal TCR-γ gene rearrangements that are commonly found in aberrant IEL[7] could point to a T cell origin of these cells. Therefore, the presence of TCR gene rearrangements in RCD cell line P1, P2 and P3 was assessed with multiplex PCR analysis[21] and Southern blot analysis.

PCR-based analysis indicated that in RCD cell line P1 TCR-δ gene rearrangements were absent while rearrangement of the TCR-γ gene and TCR-β gene were, respectively, nonfunctional and incomplete (Table 1). Southern blot analysis of the TCR-δ gene- and TCR-β gene rearrangements confirmed these data as bi-allelic loss of the TCR-δ gene and incomplete rearrangement of the TCR-β gene were found (data not shown). Bi-allelic loss of the TCR-δ gene could point to rearrangement of both TCR-α alleles in RCD cell line P1. RCD cell line P2 displayed incomplete TCR-δ gene rearrangement, nonfunctional TCR-γ gene rearrangement and no rearrangement of the TCR-β gene (Table 1.) Southern blot analysis indicated that one allele of the TCR-δ gene was lost while rearrangement of the other was incomplete (data not shown). Furthermore, the TCR-β gene was in germline configuration indicating that no rearrangements of the TCR-β gene were made in RCD cell line P2 (data not shown). In contrast to RCD cell line P1 and P2, no TCR gene rearrangements were found with multiplex PCR for RCD cell line P3 (Table 1) and with Southern blot analysis of this cell line only germ line configuration of the TCR genes was found (data not shown). These data indicate that RCD cell lines can display a diverse

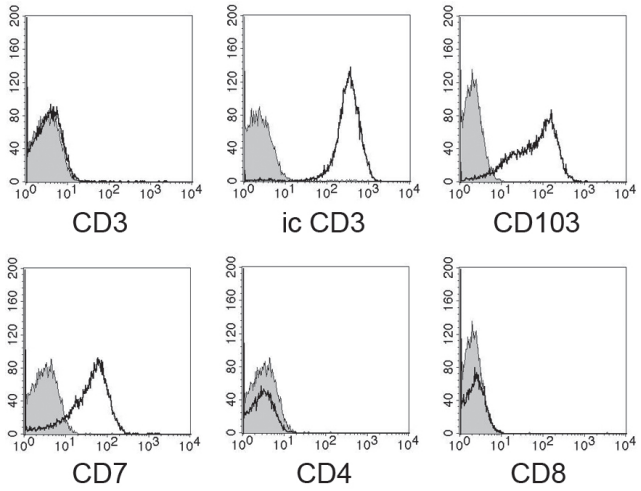

Figure 1. RCD cell lines have an aberrant IEL phenotype. FACS-analysis of RCD cell line P1. The black line in the histogram represents staining with the indicated antibody, whereas the filled histogram represents the accompanying isotype-matched control antibody. Analyses were performed within a live lymphocyte gate based on forward scatter and sideward scatter signals. Essentially identical results were obtained for RCD cell line P2, P3 and P4.

phenotype regarding the TCR gene rearrangements, characterized by incomplete, non-functional TCR gene rearrangements or even total absence of TCR gene rearrangements (Table 1).

To further address the cellular origin of aberrant IEL and to establish a common phenotype for aberrant IEL, expression of a large set of cell surface markers was determined by flowcytometry on RCD cell lines P1, P2, P3 and on RCD cell line P4 which was derived from an additional RCD II patient (Table 2). Although the RCD cell lines did not express the mature NK cell marker CD56 (with the exception of a small $CD56^+$ fraction within RCD cell line P3) (Table 2), they each expressed a unique selection of NK cell receptors (Table 2). Strikingly, all RCD cell lines expressed KIR2DL4 which is typically present on mature NK cells (Table 2). In accordance with previous studies[24-26] these data indicate that RCD cell lines share features with NK cells. All RCD cell lines expressed the pan-leucocyte marker CD45 but lacked expression of B cell marker CD19 and monocyte marker CD14 (Table 2). These cells are, therefore, unlikely to derive from mature B cells or monocytes. Absence of the main lineage markers (CD3, CD19, CD56, and CD14) in combination with expression of the early T/NK cell marker CD7 and intracellular CD3 could indicate an immature lymphocyte phenotype. The RCD cell lines, however, did not express CD34, CD1a or CD117, markers that are associated with T cell and NK cell precursors[27;28]. Furthermore, RCD cell lines P1, P2 and P3 expressed neither intracellular ROR-γ (data not shown) nor the CD127/IL-7 receptor on the cell surface (Table 2). RCD cell line P4 was partly CD127/IL-7 receptor-positive, but intracellular-ROR-γ-negative. The RCD cell lines did therefore also not match the phenotype of lymphoid tissue inducer cells[29].

Together, these results indicate that the RCD cell lines display a phenotype (surface $CD45^+CD19^-CD14^-CD3^-CD7^+CD56^-CD34^-CD103^+CD127^-KIR^+$) which is distinct from any known mature or immature lymphocyte subset.

	TCR-δ	TCR-γ	TCR-β	Conclusion
P1	-	+	+	TCR-β: D-J rearrangement TCR-γ: non-functional V
P2	+	+	-	TCR-δ: incomplete; TCR-γ: 1 out of frame, 1 non-functional V
P3	-	-	-	No rearrangements
TCRαβ$^+$	-	+	+	Rearrangements fitting with TCRαβ phenotype

Table 1. TCR-δ TCR-γ and TCR-β gene rearrangements. TCR-δ TCR-γ and TCR-β gene rearrangements for RCD cell lines P1-P3 and a $CD4^+$ TCRαβ$^+$ clone as positive control.

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	WM59				
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 Rα	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.				

Table 2. FACS analysis of RCD cell line 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.

RCD cell lines express higher levels of NK cell-associated genes when compared with regular TCR$^+$ IEL lines

To define in detail the differences between (pre)malignant aberrant IEL and regular T-IEL, a microarray was performed on RCD cell lines P1-P3 and eight control T cell lines from duodenal biopsies. These controls included (I) five polyclonal T-IEL lines, (II) a CD4$^+$TCR$^+$ cell line from patient P1 and a CD4$^+$TCR$^+$ clone from patient P3 (P1-CD4 and P3-CD4 respectively) and (III) a CD8$^+$TCR$^+$ cell line from patient P1 (P1-CD8).

Unsupervised hierarchical clustering analysis revealed two main braches: one contained the RCD cell lines and while the other contained both the TCR$^+$ cell lines from P1 and P3 and all five T-IEL lines (Figure 2). This indicates that the RCD cell lines are distinct from TCR$^+$ cell lines. Comparison of all TCR$^+$ cell lines with the three RCD cell lines revealed that the RCD cell lines had relatively high expression values of NK cell-associated genes. Most strikingly and in accordance with the FACS analysis (Figure 2, Table 2), the amount of KIR transcripts was roughly 5 to 20-fold higher in RCD cell lines versus controls. Furthermore, the RCD cell lines had higher expression of SH2 domain containing 1B (SH2D1B/EAT-2) that can act as an adaptor molecule for CD244 (2B4) and can elicit an inhibitory effect on

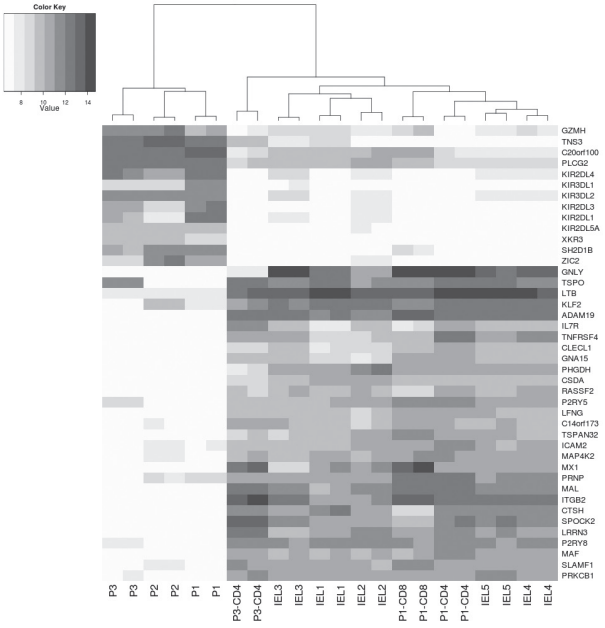

Figure 2. RCD cell lines express higher levels of NK cell-associated genes when compared with regular TCR$^+$ cell lines. Unsupervised hierarchical clustering analysis of the gene expression profiles of RCD cell lines P1, P2 and P3 and TCR+ cell lines P1-CD4, P1-CD8, P3-CD4 and IEL 1-5. The heat map 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 red corresponds to higher log intensity values. KIR allocation was imprecise as the KIR probes anneal to more than 1 KIR-transcript.

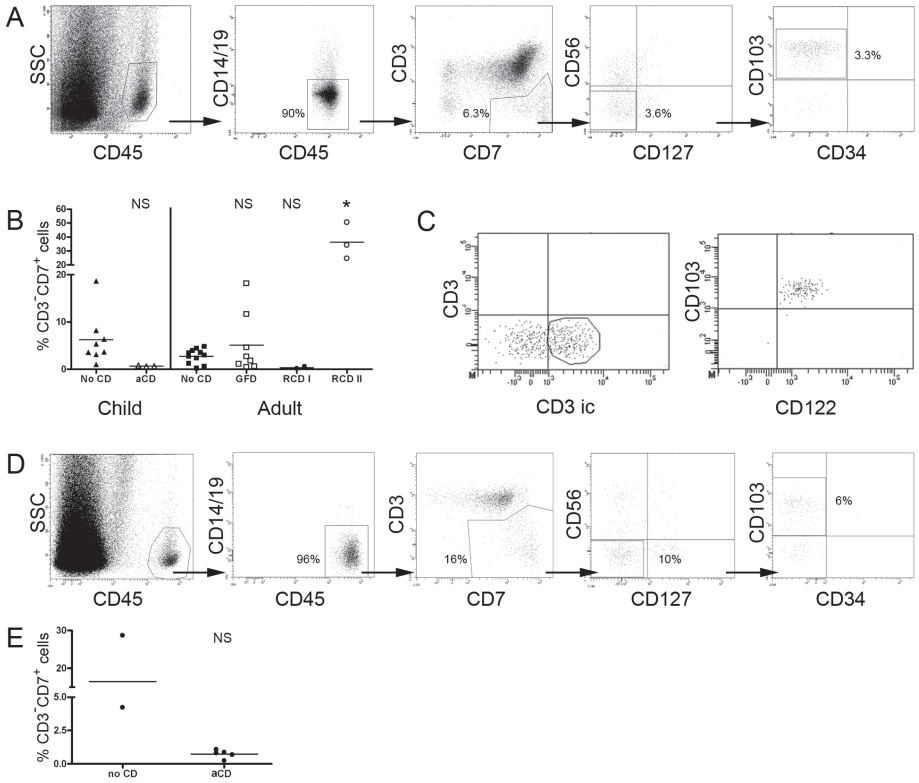

Figure 3. A unique TCR-CD3-CD7+ IEL population resides in the small intestine of healthy adults and children. (A) FACS analysis of cells isolated from small intestinal biopsies from a healthy individual. Each plot represents the cells gated in the previous plot. Percentages are calculated from the total amount of leucocytes within a live lymphocyte gate based on CD45-positivity and low sideward scatter. Quadrants are based on staining with isotype-matched controls. The experiment is a representative for 19 individual donors (B) Percentage of TCR-CD3-CD7+ cells, defined as CD45+CD19-CD14-CD3-CD7+CD56-CD34-CD103+CD127- cells in duodenal biopsies from 8 children without CD (no CD), 3 children with active CD (aCD), 11 adults without CD (no CD), 8 adults with CD on a gluten-free diet (GFD), 2 adults with RCD I and 3 adults with RCD II. Unpaired two-tailed t test was performed on children without CD versus children with active CD and on adults with CD on a gluten-free diet, adults with RCD I or adults with RCD II versus adults without CD. NS = not significant *P < 0.05 (C) FACS analysis of cells isolated from small intestinal biopsies from a healthy individual. Plots are gated on CD45+CD19-CD14-CD3-CD7+CD56-CD34-CD103+CD127- cells using the gating strategy described for Figure 3A. Quadrants are based on staining with isotype-matched controls. The experiment is a representative of 5 experiments. (D) FACS analysis of the IEL population isolated from small intestinal biopsies from a healthy individual. Plots are gated using the gating strategy described for Figure 3A. (E) Percentage of TCR-CD3-CD7+ cells, defined as CD45+CD19-CD14-CD3-CD7+CD56-CD34-CD103+CD127- cells in duodenal biopsies from 2 children without CD (no CD) and 5 children with active CD (aCD). Unpaired two-tailed t test was performed. NS = not significant.

NK cells[30]. Granzyme H (GZMH), that has a role in cytotoxicity, was also higher expressed by the RCD cell lines when compared to the TCR$^+$ cell lines. In contrast, genes associated with T cell mediated immunity such as granulysin (GNLY), lymphotoxin beta (LTB) and tumor necrosis factor receptor 4 (TNSFRSF4) and signaling lymphocytic activation molecule 1 (SLAMF1) had significantly lower expression in the RCD cell lines (Figure 2). These data underscore the notion that RCD cell lines are distinct from mature T cell lines and express several NK cell-associated genes.

A unique TCR-CD3$^-$ IEL population resides in the small intestine of healthy individuals

Characterization of the RCD cell lines indicated a common phenotype for aberrant IEL: CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ (Table 2), distinguishing them from any known lymphocyte type. Flowcytometric analysis of cells from duodenal biopsies from children and adults without CD indicated that CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ cells are also present in the small intestine of healthy individuals (Figure 3A, 3B). These cells comprised 1-5% of the total leucocyte population in both adults and children without CD (Figure 3B). The amount of cells with this phenotype did not differ significantly between healthy children and children with active CD or between healthy adults, adult CD patients on a gluten-free diet and adult RCD I patients (Figure 3B). We cannot exclude, however, that the lack of differences is due to small sample size. As expected, a high percentage of CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ cells were found in the RCD II patients (Figure 3B). In addition, roughly 50% of the CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ population was intracellular CD3$^+$ (Figure 3C). Importantly, we observed that in both children and adults these cells uniformly expressed the IL2/IL-15 receptor β-chain CD122 (Figure 3C), indicating that they could be responsive to IL-15 stimulation. As collagenase can contain trypsin activity, use of this enzyme to isolate cells from duodenal biopsies may have led to loss of surface markers on the isolated cells. Therefore, we also assessed the presence of CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ cells directly in the IEL population without

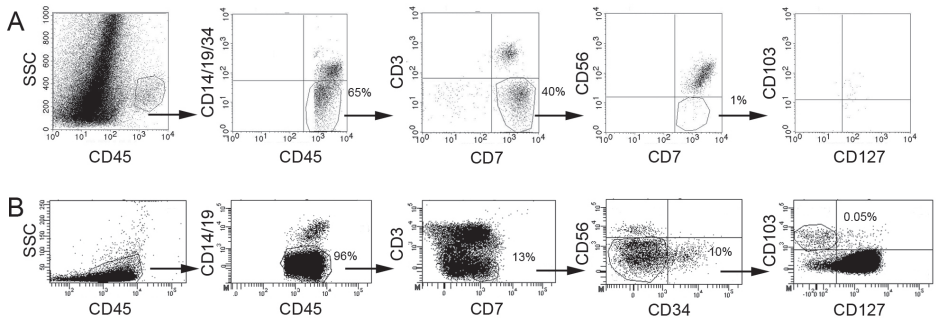

Figure 4. CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ cells are absent from fetal intestine but present in the thymus. Each plot represents the cells gated in the previous plot. Percentages are calculated from the total amount of leucocytes within a live lymphocyte gate based on CD45-positivity and low sideward scatter. (A) FACS-analysis of fetal intestinal material. This experiment is a representative of 2 individual experiments on fetal intestinal material from a 19 and 20 week old fetus. (B) FACS-analysis of a thymic cell suspension. This experiment is a representative of 2 individual experiments on thymic cell suspensions of two children of 2 and 7 months old respectively.

the use of collagenase for isolation. Consistent with the data on the cells isolated with collagenase, the IEL population from healthy children and children with active CD contained CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ cells (Figure 3D, 3E). Preliminary data also confirm the presence of these cells in the IEL population of adults (data not shown).

In two fetal intestinal samples, cells with the CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ phenotype were not detected (Figure 4A). Although TCR-CD3$^-$CD7$^+$ cells were present in the fetal intestine, these were mostly CD56$^+$ NK cells or CD127$^+$ lymphoid tissue inducer cells (Figure 4A). Precursor cells for both T cell and NK cells reside in the human thymus[31;32] and it has been shown that these cells can fully differentiate in secondary lymphoid tissues, including the intestine[16;33;34]. In accordance, FACS-analysis of two thymic cell suspensions indicated that CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ cells constitute approximately 0.05% of the thymocytes (Figure 4B).

Together, these data indicate that a unique TCR-CD3$^-$ CD7$^+$ IEL population that shares the phenotype of aberrant IEL in RCD II is present in the intestine and in the thymus of individuals without celiac disease.

Discussion

RCD II is a severe complication of celiac disease characterized by the presence of an aberrant IEL population and a high frequency in lymphoma development. Roughly 50% of these patients develop overt gastrointestinal lymphoma[8]. It is generally accepted that RCD-associated lymphoma develops from the aberrant TCR-CD3$^-$ IEL population as lymphoma cells share the TCR-CD3$^-$CD4$^-$CD8$^-$CD7$^+$CD103$^+$ phenotype and monoclonal TCR-γ gene rearrangements with the aberrant TCR-CD3$^-$ IEL. The precise cellular origin of these aberrant IEL is still unclear. Identification of the origin of aberrant IEL might aid in the development of modalities for earlier intervention in the development of RCD-associated lymphoma.

In this study we performed an extensive analysis of TCR-CD3$^-$CD7$^+$ cell lines isolated from biopsies of RCD II patients[9] to address the cellular origin of aberrant IEL. Assessment of TCR gene rearrangements indicated that: RCD cell line P1 had clonal, yet non-functional rearrangement of the TCR-γ and TCR-β genes and most likely functional rearrangements of the TCR-α gene, RCD cell line P2 displayed only non-functional rearrangement of the TCR-δ and TCR-γ genes and RCD cell line P3 had no rearrangements of any of the TCR genes. FACS analysis indicated that the RCD cell lines did not express the classical markers for mature T, B and NK cells, but did express a variety of activating receptors, including NCRs and KIRs. Microarray analysis indicated that, when compared with regular T-IEL lines, the RCD cell lines express lower levels of T cell-associated genes, but higher levels of levels NK cell-associated genes. Recently, it has been shown that deletion of the transcription factor Bcl11b, which is expressed in T cells and in some immature NK cells[35], could result in acquirement of NK cell properties in T cell from all developmental stages[36]. This could explain the phenotype of the RCD cell lines in this study, however, microarray analysis showed that Bcl11b was expressed and expression did not differ from the expression in regular T-IEL (data not shown). Together, our data indicate that the RCD cell lines are distinct from mature T-IEL. Furthermore, as there are marked differences between the RCD cell lines, they could represent cells in different stages of T/NK cell development. Both the retroviral transduction experiments in our previous study[9] and analysis of TCR gene rearrangements in this study demonstrate functional rearrangement of the TCR-α gene in RCD cell line P1. Futhermore, Southernblot analysis of the TCR-β gene indicates incomplete rearrangement of the TCR-β gene on one allele and the loss of a germline TCR-β locus on the other allele. This suggests that an in-frame TCR-β could have been present in RCD cell line P1, which is supported by the detection of a full length beta transcript in our previous study[9]. Together, these data suggest that the phenotype of RCD cell line P1 was closest to that of a mature T cell. RCD cell line P2 could represent an immature T cell as TCR rearrangements had been initiated in this cell line. The absence of TCR rearrangements in RCD cell line P3 could indicate that this cell line represents an even earlier stage in T cell or NK cell development. The latter is supported by the observation that RCD cell line P3 expresses more activating NK cell receptors that the other RCD cell lines.

Despite the unique characteristics of each RCD cell line, a shared phenotype could be determined: CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$. In vivo, cells with the CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ phenotype were present in the small intestine of healthy adults and children, but not in the fetal intestine. In addition, this population was identified in thymic cell suspensions, which may indicate that these cells originate in the thymus.

The CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ cell population resembles the previously described TCR-CD3$^-$CD7$^+$ cell population that was shown to contain immature T and NK cells[12;15]. Furthermore, the CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ cell population uniformly expresses CD122, the IL-2/15 receptor β-chain. IL-15, which is highly upregulated in the small intestine of CD patients, might thus drive the expansion of this cell population in CD. Together, our data suggest that CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ cells represent the physiological counterpart of (pre)malignant aberrant IEL. The diverse phenotype of the RCD cell lines studied could be a result of transformation into aberrant IEL at different stages in lymphocyte development within the CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ subset.

In conclusion, this study indicates that the aberrant IEL in RCD II can display diverse phenotypes and may be derived from a unique CD45$^+$CD19$^-$CD14$^-$CD3$^-$CD7$^+$CD56$^-$CD34$^-$CD103$^+$CD127$^-$ IEL population that is present in the small intestine.

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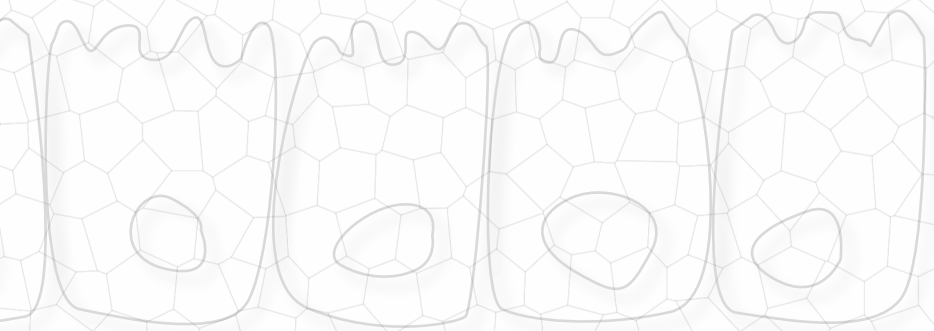

Chapter 6

General Discussion

General Discussion

Although celiac disease (CD) is a relatively benign condition where elimination of gluten from the diet can reverse disease, a small percentage of adult-onset CD patients fail to recover on a gluten-free diet. These refractory celiac disease (RCD) patients have persisting villous atrophy and elevated levels of intraepithelial lymphocytes (IELs) in the small intestine. In a subset of these patients an aberrant monoclonal IEL population is present that lacks expression of the T cell receptor (TCR)-CD3 complex on the cell surface, but expresses CD3 intracellularly. This aberrant IEL population is now regarded as an intraepithelial lymphoma that can develop into overt lymphoma[1]. Therefore, this disease state -RCD type II (RCD II)- is considered an intermediate stage between CD and lymphoma. This thesis reports on novel insights on aberrant IELs that increased our knowledge on these (pre)malignant cells and that might aid in the development of future therapies for RCD II and RCD-associated lymphoma.

RCD cell lines: a model for aberrant IELs in RCD II and RCD-associated lymphoma

About a decade ago, aberrant IELs in RCD II were identified as the missing link between TCR^+ IELs in CD and the TCR^- lymphoma cells of enteropathy associated T cell lymphoma (EATL)[2;3] Although the aberrant IELs did not express the TCR-CD3 complex on the cell surface, CD3 was expressed intracellularly and clonal TCR-γ gene rearrangements were commonly found. One view is, therefore, that aberrant IELs are mature TCR^+ IELs that lost cell surface expression of the TCR-CD3 complex due to overstimulation[4].

Studies on aberrant IELs were mainly performed on IELs *in situ*, which limited the type of experiments that could be performed to investigate molecular events that are linked to malignant transformation. The isolation and propagation of aberrant IEL lines isolated from small intestinal biopsies of RCD II patients (Chapter 2) allowed us to perform more in-depth analysis of aberrant IELs. These RCD cell lines had the characteristic aberrant IEL phenotype: surface $TCR\text{-}CD3^-CD4^-CD8^-CD7^+CD103^+$, intracellular $CD3^+$. In addition, the RCD cell lines displayed monoclonal TCR-γ gene rearrangements identical to the predominant rearrangements found in the aberrant IELs of the patients from which the cell lines were derived. The proliferative response of the RCD cell lines to IL-15, which is highly upregulated in RCD II and RCD-associated lymphoma[5], further supported the notion that RCD cell lines could serve as a model for aberrant IELs (Chapter 2).

Aberrant IELs contribute to the propagation of tissue damage and inflammation in RCD II.

In active CD, the number of TCR^+ IELs is markedly increased. It has become clear that TCR^+ IELs can acquire an activating NK cell receptor repertoire, presumably under the influence of IL-15[6]. These NK cell receptors co-stimulate TCR-mediated lysis of epithelial cells[7]. Upon stimulation with IL-15, which can alter the NK cell receptor function, IELs can even display TCR-independent cytotoxicity[8]. It seems, therefore, likely that these TCR^+ IELs that have acquired an NK cell phenotype play a role in perpetuating tissue damage in RCD.

In addition to a role in tissue damage, TCR$^+$ IELs in active CD contribute to the pro-inflammatory milieu by the secretion of high levels of IFN-γ[9;10]. Analogous to IFN-γ secretion by gluten-specific CD4$^+$ T cells[11], IFN-γ secretion by TCR$^+$ IELs seems to be linked to gluten-intake as TCR$^+$ IELs in active CD patients and CD patients after gluten-challenge secreted higher levels of IFN-γ when compared to healthy controls or CD patients on a gluten-free diet[10]. Furthermore, TCR$^+$ IELs also secreted high amounts of IFN-γ after stimulation with IL-15[12;13]. Strikingly, none of the other T helper 1 (Th1) or T helper 2 (Th2) cytokines seemed to have a role as important as IFN-γ (and to a lesser extent IL-10) in the inflammatory response of active CD.

Much less is known about cytotoxicity and cytokine secretion of the aberrant TCR$^-$ IEL population in RCD II and the subsequent state of lymphoma. The availability of the RCD cell lines as a model for aberrant IELs allowed us to address these issues *in vitro*. In analogy with TCR$^+$ IELs in active CD, the TCR$^-$ RCD cell lines expressed multiple activating NK cell receptors (Chapter 3). In contrast to the TCR$^+$ IELs, however, epithelial-cell specific cytotoxicity was mainly mediated by DNAM-1[14] with only a minor role for other activating NK cell receptors (Chapter 3). We postulated that in a subset of RCD II patients the aberrant IELs acquire the ability to lyse epithelial cells via DNAM-1.

Analogous to TCR$^+$ IELs, the RCD cell lines secreted IFN-γ but none of the other Th1 cytokines upon stimulation with IL-15. This indicated that aberrant IELs can play a role in the propagation of the inflammatory response in RCD II (Chapter 4). Interestingly, simultaneous stimulation with an anti-CD30 antibody and IL-15 had a synergistic effect on IFN-γ secretion (Chapter 4). As CD30 is expressed on aberrant IELs in a subset of RCD II patients[15] and on virtually all RCD-associated lymphoma cells, combined stimulation of the CD30 receptor with IL-15 stimulation could occur *in vivo* and might contribute to the ongoing inflammation in the transition from RCD II into lymphoma.

Together, these results indicate that aberrant IELs could contribute to the ongoing tissue damage and cytokine secretion in RCD II and RCD-associated lymphoma.

Aberrant IELs possibly originate from a unique CD3$^-$CD7$^+$ lymphocyte population

Knowledge on the functional characteristics of aberrant IELs has thus greatly increased. The exact cellular origin of aberrant IELs, however, remains unclear. Following the hypothesis that aberrant IELs used to be TCR$^+$ IELs, we assessed the presence of the TCR-CD3 complex in our RCD cell lines. Intracellulary, all CD3-chains and ζ-chains were present and could form the CD3γε-, CD3δε- and ζζ-dimers (Chapter 2). The TCR-chains, however, were not always present (Chapter 2). Introduction of exogenous TCR-chains, however, restored surface expression and functionality (proliferation and IFN-γ production) in the RCD cell lines (Chapter 2 and unpublished). This indicates that the CD3-complex was functional in the RCD cell lines. Analysis of TCR-gene rearrangements with PCR and Southern blot revealed that clonal TCR-gene rearrangements could be found in the RCD cell lines. These TCR-gene rearrangements were, however, either incomplete, non-functional or out-of frame and can therefore not assemble into a functional TCR. Furthermore, one of the RCD cell lines only displayed germline configuration of the TCR genes (Chapter 5).

The RCD cell lines thus have characteristics of T cells, yet their phenotype does not match that of mature T cells. Phenotypic analysis of the RCD cell lines indicated that aberrant IELs have a unique phenotype: $CD45^{+}CD19^{-}CD14^{-}CD3^{-}CD7^{+}CD56^{-}CD34^{-}CD103^{+}CD127^{-}$ (Chapter 5). This phenotype is distinct from any known lymphocyte class. Furthermore, IELs with this phenotype were present in the small intestine of healthy adults, children and thymic cell suspensions (chapter 5). We hypothesized, therefore, that aberrant IELs do not derive from mature T cells but instead derive from cells in an early stage of extrathymic lymphocyte development.

Directions for further research

The different aspects of aberrant IELs described in this thesis help understand the events leading from uncomplicated CD to RCD II and gastrointestinal lymphoma. Many issues, however, remain to be answered.

First, most patients do well on a gluten-free diet but some individuals develop RCD II and lymphoma. It has been suggested that HLA-DQ2 gene dose is involved in the risk to develop complicated CD as RCD II patients and gastrointestinal lymphoma patients were more often HLA-DQ2.5 homozygous[16]. Genome-wide association studies (GWAS) on uncomplicated CD patients have identified several risk variants on non-HLA-loci for development of CD[17]. The presence of a higher number of additional non-HLA risk alleles is directly correlated with an increase in the risk to develop CD[18]. An interesting issue is whether these genes also influence the risk to develop complicated forms of CD. A GWAS on RCD I, RCDII and RCD-associated lymphoma patients with uncomplicated CD patients and healthy individuals as controls would be a way to uncover the role of non-HLA-genes on the development of complicated CD. Screening of CD patients on potential risk alleles might then lead to earlier intervention. Recent GWAS have indicated that to identify risk variants with small effect sizes the amount of samples should be increased to obtain significant results. As only 2-5% of adult-onset CD patients develop complicated CD, even pooling of sample collections from multiple European countries would not obtain a sufficient sample-size for such a study.

Second, cells with a TCR-CD3-negative phenotype are present in the intestine of healthy adults and children (Chapter 5). These cells could be an immature lymphocyte type that represents the physiological counterpart of the aberrant IELs in RCD II. To test this hypothesis, future studies are needed to determine whether these cells can differentiate into mature T cells and/or NK cells. Hematopoietic precursor cells from fetal liver and postnatal thymus were shown to differentiate into T cells on OP9 cells expressing Notch ligand Delta-like-1(DL1)[19;20]. In analogy, cells with the $CD45^{+}CD19^{-}CD14^{-}CD3^{-}CD7^{+}CD56^{-}CD34^{-}CD103^{+}CD127^{-}$ phenotype could be isolated from the small intestine of a healthy individual and cultured in the presence of stromal cell lines OP9 or OP9-delta-like-1[19]. Such co-culturing could indicate whether the cell population identified in Chapter 5 can indeed differentiate into a mature lymphocyte type. Alternatively, the presence of essential transcription factors Notch1 and GATA3 could indicate in which stage of development these lymphocytes are[21]. Microarray analysis might inform whether these genes are differentially expressed when compared to mature T and NK cells. In addition, in mice the transcription factor E4BP4[22] has been appointed as a critical factor in NK cell development. It remains to be determined if this factor is also important in human NK cell development.

Third, a subset of RCD II patients acquires the ability to lyse epithelial cells via DNAM-1 (Chapter 3). It remains unclear, why DNAM-1 that normally acts as a co-receptor, can now act autonomously. Recently, the inhibitory receptor TIGIT was identified that can be co-expressed with DNAM-1 on T cells and NK cells and competes for binding to the ligands CD155 and CD112[23;24]. TIGIT was shown to have a 100-fold higher affinity for CD155 than DNAM-1 suggesting that, when co-expressed, TIGIT has the dominant function and can regulate the function of DNAM-1[23]. Dysregulation of the balance of expression of TIGIT and/or DNAM-1 might enable DNAM-1 to induce cytotoxicity autonomously. Determining expression of TIGIT and DNAM-1 in different stages of CD and in healthy controls could be a way to address this issue.

Future therapeutic possibilities for RCD II and RCD-associated lymphoma

RCD II and RCD-associated lymphoma are severe complications of CD with 5-year survival rates of 44-58% and <20% respectively[25]. RCD II is very resistant to therapy and transition into lymphoma cannot be prevented. At present, only Cladribine therapy[26] or autologous stem cell therapy[27] might have an effect on reducing the amount of (pre) malignant aberrant IELs.

Blocking of IL-15 has been suggested as a therapy as this cytokine influences many aspects of aberrant IELs including apoptosis[1], proliferation (Chapter 2), cytotoxicity (Chapter 3) and cytokine secretion (Chapter 4). Alternatively, we suggest that blocking of DNAM-1 and CD30 may be interesting as these molecules seem to be linked to malignant transformation of the aberrant IELs into lymphoma cells. Monoclonal antibody therapy against these two receptors might have immediate effects on cytotoxicity and cytokine production, thus limiting tissue destruction and inflammation.

In conclusion, the research described in this thesis improved our understanding of the pathogenesis of complicated CD and gave us suggestions on possibilities for future therapy.

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NEDERLANDSE SAMENVATTING

Nederlandse Samenvatting

Coeliakie

Coeliakie is een chronische ziekte van de dunne darm die wordt veroorzaakt door een ontstekingsreactie tegen gluteneiwitten uit tarwe, rogge en gerst. Coeliakie komt naar schatting voor bij 1 op de 200 mensen en is daarom een van de meest voorkomende voedselintoleranties in de westerse wereld. De symptomen van coeliakie kunnen variëren van ernstige diarree en buikpijn tot vermoeidheidsklachten en bloedarmoede. Er wordt vaak aangenomen dat coeliakie een ziekte is die voornamelijk wordt vastgesteld op kinderleeftijd. De laatste jaren wordt de diagnose coeliakie echter steeds meer bij volwassenen vastgesteld. Het is nog onduidelijk of deze volwassenen al langere tijd coeliakie hadden met lichte symptomen of dat zij de ziekte pas op latere leeftijd ontwikkelen. De diagnose wordt gesteld door een bloedtest waarbij de aanwezigheid van antistoffen tegen het bindweefsel van de dunne darm (anti-endomysium) en tegen het enzym weefseltransglutaminase (anti-tissue transglutaminase) wordt vastgesteld. Als deze antistoffen aanwezig zijn is de kans groot dat er inderdaad sprake is van coeliakie. Omdat de bloedtest niet 100% betrouwbaar is, word er vaak ook een dunne darm onderzoek gedaan waarbij een stukje darmbiopt wordt afgenomen. Dit biopt wordt vervolgens onderzocht op darmschade als gevolg van ontsteking. Tot nu toe is de enige behandeling een levenslang glutenvrij dieet wat ervoor zorgt dat de dunne darm herstelt en de symptomen verdwijnen.

Refractaire coeliakie en lymfoom

De overgrote meerderheid van de coeliakiepatiënten herstelt volledig door het stoppen van eten van gluten-houdende producten. Bij een zeer kleine groep blijven de klachten en de ontsteking van de dunne darm bestaan ondanks het strikt volgen van een glutenvrij dieet. Deze vorm van coeliakie wordt refractaire coeliakie genoemd en is geassocieerd met een sterk verhoogde kans op het ontwikkelen van een lymfoom (lymfklierkanker) van de darm. Patiënten met een lymfoom zijn moeilijk te behandelen en deze complicatie van coeliakie heeft dan ook een zeer lage 5-jaars overlevingskans van minder dan 20%.

Aberrante lymfocyten

Ongeveer 10 jaar geleden werd bekend dat het ontstaan van een lymfoom bij refractaire coeliakiepatiënten samenhing met de aanwezigheid van aberrante lymfocyten in het dunne darmslijmvlies. Lymfocyten zijn immuuncellen die onder te verdelen zijn in T cellen, B cellen en natural killer (NK) cellen. Lymfocyten hebben oppervlaktereceptoren waarmee ze bijvoorbeeld kunnen herkennen of andere cellen geïnfecteerd zijn met een bacterie of virus. Lymfocyten hebben daarom een belangrijke functie in het immuunsysteem. De receptor op T cellen wordt de T cel receptor genoemd.
Aberrante lymfocyten hebben geen T cel receptor op het celoppervlak, maar een onderdeel van de T cel receptor (het CD3-complex) is wel aanwezig binnen in de cel. Mede hierom werd aanvankelijk gedacht dat aberrante lymfocyten T cellen waren die hun

T cel receptor verloren hadden. Lymfoomcellen lijken sterk op de aberrante lymfocyten qua oppervlaktemarkers en refractaire coeliakie patiënten met een hoog percentage aberrante lymfocyten in de darm hebben een grotere kans op het ontwikkelen van een lymfoom. Het is daarom zeer aannemelijk dat lymfoomcellen ontstaan uit aberrante lymfocyten.

Dit proefschrift

Aberrante lymfocyten zijn cruciaal in het ontstaan van een lymfoom van de dunne darm, een complicatie van coeliakie met een zeer slechte overlevingskans. Meer kennis van deze aberrante lymfocyten zou mogelijkheden kunnen bieden om eerder in te grijpen in het ziekteverloop.

***Hoofdstuk 2**: cellijnen als model voor aberrante lymfocyten*
Onderzoek naar aberrante lymfocyten was tot voor kort beperkt omdat het aantal cellen dat uit een darmbiopt verkregen wordt niet toereikend was voor uitgebreid onderzoek. De ontwikkeling van aberrante lymfocytlijnen die buiten het lichaam blijven leven en delen op groeifactoren heeft hier verandering in gebracht. In hoofdstuk 2 wordt beschreven hoe uit dunne darm biopten van 3 refractaire coeliakie patiënten 3 cellijnen werden geïsoleerd. Deze cellijnen ("RCD cell lines" P1, P2 en P3) hebben dezelfde oppervlaktemarkers als de aberrante lymfocyten en ook zijn alle ketens van het CD3 complex in de cel aanwezig. Analyse van het T cell receptor gen toonde aan dat de cellijnen dezelfde genetische kenmerken hadden als de aberrante lymfocyten uit de bijbehorende patiënten. Het feit dat de cellijnen delen op de groeifactor interleukine 15 (IL-15), die zeer hoog tot expressie komt in het darmslijmvlies van refractaire coeliakie patiënten, ondersteunt ook dat onze cellijnen gebruikt kunnen worden als model voor aberrante lymfocyten.

***Hoofdstuk 3**: aberrante lymfocyten dragen bij aan epitheel-specifieke weefselschade door middel van DNAM-1*
Kenmerkend voor refractaire coeliakie is de voortdurende ontstekingsreactie en weefselschade aan de dunne darm ondanks een gluten-vrij dieet. Eerder onderzoek wees uit dat T cellen in de darm van refractaire coeliakie patiënten meer NK cel receptoren op het cel oppervlak hebben. Deze receptoren kunnen antigenen herkennen op het epitheel die tot expressie komen onder invloed van een stress-factor zoals ontsteking. De interactie tussen de NK receptoren en de stress-antigenen resulteert in destructie van de epitheelcel. Deze interactie kan onder invloed van IL-15 onafhankelijk van de T cel receptor en dus ook onafhankelijk van de aanwezigheid van gluten plaatsvinden. In hoofdstuk 3 wordt de mogelijke rol van aberrante lymfocyten beschreven in de voortdurende weefselschade in refractaire coeliakie. Uitgebreide analyse van cellijnen P1, P2 en P3 toonde aan dat deze cellijnen in staat zijn epitheel cellen te lyseren via de DNAM-1 receptor. Omdat DNAM-1 wel aanwezig is op het cel oppervlak van lymfoom cellen maar slechts in een minderheid van de refractaire coeliakie patiënten, gaan we ervan uit dat de expressie van DNAM-1 geassocieerd is met de overgang van refractaire coeliakie naar lymfoom. DNAM-1 is gewoonlijk aanwezig op T en NK cellen en heeft een rol in de immuunreactie tegen tumor cellen.

Het is opmerkelijk dat in een subset van refractaire coeliakiepatiënten de (pre)tumor cellen zelf DNAM-1 gebruiken om epitheelcellen te lyseren.

Hoofdstuk 4*: aberrante lymfocyten scheiden ontstekingsfactoren uit*
Het ontstaan en in stand houden van de ontstekingsreactie in de dunne darm van coeliakiepatiënten wordt voor een belangrijk deel gestuurd door de ontstekingsfactor interferon gamma (IFN-γ). Eerdere studies beschreven dat T cellen in de darm van coeliakiepatiënten grote hoeveelheden IFN-γ uitscheiden in reactie op gluten en/of IL-15. Daarom hebben we onderzocht of aberrante lymfocyten een vergelijkbare functie kunnen hebben in de voortdurende ontstekingsreactie in de darm van refractaire coeliakiepatiënten (Hoofdstuk 4). Evenals normale T cellen kunnen de cellijnen P1, P2 en P3 ook IFN-γ produceren na stimulatie met IL-15. Combineren van de IL-15 stimulatie met stimulatie van de CD30 receptor (CD30 is een oppervlaktemarker die voorkomt op lymfoomcellen) resulteerde in een verhoging van de IFN-γ productie. Omdat in de overgangsfase van refractaire coeliakie naar lymfoom IL-15 en CD30 beiden aanwezig kunnen zijn, is het aannemelijk dat aberrante lymfocyten een rol spelen in het in stand houden van de ontstekingsreactie in de dunne darm.

Hoofdstuk 5*: aberrante lymfocyten zijn lymfocyten van nog onbekende afkomst*
Het gangbare idee is dat aberrante lymfocyten ontstaan uit T cellen. Mede hierom wordt het lymfoom dat kan ontstaan uit deze cellen enteropathie geassocieerd T cel lymfoom (EATL) genoemd. In hoofdstuk 5 wordt echter beschreven dat cellen met het “aberrante” fenotype ook voorkomen in de darm van gezonde volwassenen en kinderen. Deze cellen zouden de normale tegenhanger kunnen zijn van aberrante lymfocyten in refractaire coeliakie. Dit zou betekenen dat aberrante lymfocyten niet ontstaan uit T cellen maar uit een andere lymfocytenpopulatie van nog onbekende origine.

Conclusie

De onderzoeken beschreven in dit proefschrift hebben onze kennis van aberrante lymfocyten in refractaire coeliakie sterk vergroot. De verkregen inzichten zouden in de toekomst wellicht kunnen bijdragen aan de behandeling van refractaire coeliakie patiënten ter voorkoming van het ontstaan van lymfomen.

CURRICULUM VITAE

Curriculum Vitae

Jennifer May-Ling Tjon werd geboren op 18 oktober 1980 te Leiden. In 1998 behaalde zij haar gymnasium diploma aan het Aquino college te Leiden. In hetzelfde jaar begon zij aan de studie Biomedische Wetenschappen aan de Universiteit van Leiden. In 2000 startte zij tevens met de studie Geneeskunde.

Tijdens deze studies werden twee onderzoeksstages uitgevoerd. Bij de afdeling Kindergeneeskunde en Endocrinologie werd onder begeleiding van prof.dr. J.M. Wit en dr. Karperien onderzoek gedaan naar mutaties in het NSD1-gen bij kinderen met Sotos' syndroom. De afstudeerstage naar de rol van serumeiwitten in coeliakie werd uitgevoerd op de afdeling Immunohematologie en Bloedbank onder begeleiding van prof.dr. F. Koning, dr. E.H.A. Dekking en dr. P. Van Veelen. In 2004 werd het doctoraal examen behaald van zowel de studie Biomedische wetenschappen als van de studie Geneeskunde.

Na het doorlopen van de coschappen en het behalen van het artsexamen in 2006 is zij begonnen aan haar promotieonderzoek op de afdeling Immunohematologie en Bloedbank onder begeleiding van prof.dr. F. Koning en dr. J van Bergen, waarvan het resultaat staat beschreven in dit proefschrift.

Sinds januari 2011 is zij werkzaam als assistent in opleiding tot specialist in de interne geneeskunde. Deze opleiding zal doorlopen worden in het Haga ziekenhuis te Den Haag en het LUMC te Leiden.

LIST OF PUBLICATIONS

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**Both authors contributed equally*

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