Part II

Chapter 2

Gamma-Delta T-Lymphocytes might be mandatory in the diagnostic approach of celiac disease


Submitted
Abstract

Introduction: Celiac disease (CD) is characterised by an increase in gamma-delta intra-epithelial lymphocytes (CD3+TCRγδ+ IEL). Our aim is to validate cut-off values of this lymphocyte subset in the (differential) diagnosis of CD.

Methods: Percentages were determined by flow cytometric analysis of IELs from small bowel biopsy specimens in 213 CD patients, 89 controls, 23 disease controls (DC) and 13 potential CD patients (PCD). A cut-off value for percentages of CD3+TCRγδ+ IEL to differentiate between active CD patients and controls was obtained from a receiver operating characteristic (ROC) curve and implemented in DC and PCD patients.

Results: Percentage of intra-epithelial gamma delta T lymphocytes was significantly increased in the majority of CD patients, irrespective of the presence of villous atrophy. A cut-off value of 14% for CD3+TCRγδ+ IEL resulted in 66.3% sensitivity and 96.6% specificity for CD diagnosis (AUC 88.6%).

Conclusion: A percentage of ≥14% gamma-delta intra-epithelial T lymphocytes has a high specificity for CD diagnosis and can be of diagnostic help in those cases where diagnosis is not straightforward.
Introduction

Celiac disease (CD) is a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals. Current diagnostic approach includes serology, compatible HLA-DQ haplotype, and the presence of intraepithelial lymphocytosis in combination with villous atrophy (Marsh IIIa-c)\(^1,2\). Generally the combination of these findings does not pose a diagnostic challenge. Some patients however present with clinical characteristics which raise a high suspicion of CD yet the findings are not sufficient for a definite diagnosis. Such a scenario can for instance be found in seronegative patients with intraepithelial lymphocytosis in the absence of villous atrophy (Marsh I), seropositive patients with normal histology (Marsh 0) or patients who are already on a gluten-free diet (GFD) without an established diagnosis.

It has been shown that the intra-epithelial lymphocyte (IEL) compartment of CD patients is characterised by an increase in CD3+ lymphocytes bearing the T cell receptor gamma-delta chain (TcR-\(\gamma\delta\)) which is both permanent and diet independent\(^3-12\). So far, the role of these lymphocytes in the pathogenesis of CD is not completely understood and cut-off values are unavailable. In addition to a rise in CD3+TCR\(\gamma\delta\)+ IEL, a reduced percentage of surface CD3 negative and intracellular CD3 positive lymphocytes (sCD3-iCD3+CD7+ IEL) is described in active CD (ACD)\(^13\). This imbalance in the ratio of TCR\(\gamma\delta\)+ IEL versus sCD3-iCD3+ IEL was confirmed and shown to be permanent in a paediatric study setting\(^14,15\). While the method was proposed as a new diagnostic criterion\(^16,17\), its implementation in daily practice is so far limited to a few centers.

Here, we analyse these lymphocyte subsets in a large cohort of patients and controls and define the cut-off values which might be mandatory in the future diagnostic approach of CD.

Methods

Patients and data collection

Patients visiting Celiac Center Amsterdam at the VU University medical center and who underwent gastroscopy including flow-cytometry analysis between 2003 and 2014 were included in this study. Patient characteristics are described in Table 1. Different patient subgroups were included for the analysis;
1. Patients with proven celiac disease (n=213). This group was further divided in patient with active CD (n=95) (ACD) and CD in remission due to the introduction of a gluten free diet (n=118) (GFD). ACD is defined as the presence of positive serology (anti-endomysium (EMA) and/or anti-tissue transglutaminase antibodies (TGA)), if available, the presence of HLA DQ2 and/or DQ8 and the presence of intraepithelial lymphocytosis in combination with villous atrophy (Marsh IIIa-c). The GFD group is defined as a history of proven CD according the above mentioned criteria and biopsy proven restoration of villi (Marsh 0-I) in combination with negative serology at the time of the endoscopic evaluation. Follow-up data after the introduction of a gluten free diet were available from thirteen ACD patients.

2. Controls without CD (n=89) (Control). These subjects underwent upper gastrointestinal endoscopy for exclusion of CD due to a variety of symptoms (aphthous stomatitis, gastric reflux disease, nausea and dyspepsia, diarrhoea, abdominal pain and osteopenia) or due to a family history of CD (family screening). All patients lacked circulating anti-endomysium (EMA) and/or anti-tissue transglutaminase antibodies (TGA) and histological abnormalities

3. Patients with potential CD (n=13) (PCD). This group was defined as having positive serology and the presence of HLA DQ2 and/or DQ8 in combination with intraepithelial lymphocytosis without villous atrophy (Marsh 0-II)

4. Patients with enteropathy due to other causes (Disease control group) (n=23)(DC). This additional group of patients all suffered from villous atrophy by any cause other than CD (including malignant immuno-proliferative diseases (n=5), olmesartan use (n=1), collagenous sprue (n=1), autoimmune disease-associated enteropathy (n=6) or villous atrophy of unknown cause (n=10)). In all of them CD serology was negative.

Tissue collection and flow cytometry
During upper gastrointestinal endoscopy multiple large spike forceps biopsies were taken from the second part of the duodenum. Six biopsies were used for immediate immunophenotypical evaluation using flow cytometry. IELs were isolated from the biopsies as previously described18, 19. Briefly, biopsies were vigorously shaken at 37 °C for 60 minutes in PBS supplemented with 1 mM DTT (Fluka Bio-Chemika, Buchs Switzerland) and 1 mM EDTA (Merck, Darmstadt Germany). The released IELs were washed twice with PBS supplemented with 0.1 % BSA (Roche Diagnostics) and stained
for 30 minutes on ice, with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), ECD, PE-Cy7, APC-AF700, APC-H7 and Krome Orange-labeled monoclonal antibodies directed against TCR-γδ, sCD3, iCD3, CD4, CD7, CD8, CD16+56, CD19 and CD45 (all from BD Biosciences, San Jose, CA). A standard 4-color flow cytometer (FACS Calibur, BD Biosciences) or since 2013, a 10-color flow cytometer (Gallios, Beckman-Coulter), was used for analysis. The data were analysed using Cell quest software (BD Biosciences) or Kaluza (Beckman-Coulter). Cells with a strong CD45 expression and low to intermediate forward and sideward scatter were selected, after which both the percentage of IELs expressing TCR-γδ and the percentage of IELs negative for surface CD3 and positive for intracellular CD3 were calculated (Figure 1).

Figure 1. Flow cytometry images of gating strategy for both IEL subsets

Statistical analysis

For the flow cytometric analyses, the percentages of TCR-γδ+ IELs and sCD3-negative-iCD3-positive IELs were described by medians and range for each patient group. Differences in these variables between the groups were tested with the non-parametric Mann-Whitney U test. Differences between time of diagnosis and follow-up were tested with the Wilcoxon signed-rank test. A cut-off value for percentages of TCRγδ+ IEL and
sCD3-iCD3+ IEL to differentiate between ACD patients and controls was obtained from a receiver operating characteristic (ROC) curve. To evaluate whether either one or both IELs are needed to calculate the probability of having CD, likelihood ratio tests were performed to compare two binary logistic regression models. First, the model with both TCRγδ+ IEL and sCD3-iCD3+ IEL densities was compared to the model including only TCRγδ+ IEL densities. Secondly, this model was compared to the model including only sCD3-iCD3+ IEL densities. CD status (yes or no, ascertained by above described variables) was used as a dichotomous dependent variable. Sensitivity and specificity of the combination of both variables were calculated when the model including both turned out to be the best. P-values less than 0.05 were considered statistically significant. All analyses were performed in SPSS 20 (IBM Corp., Armonk, NY USA).

Results
Patients
Overall 338 patients were included. Baseline characteristics are summarized in Table 1. The median age was 49.3 years in this cohort (range: 11.5-81.3). The majority of CD patients (n=213) was HLA-DQ2 heterozygous (n= 106, 49.8%), others being DQ2 homozygous (n= 43, 20.2%), DQ2-DQ8 compound heterozygous (n=10, 4.7%), DQ8 heterozygous (n=10, 4.7%) or DQ8 homozygous (n=3, 1.4%) (data incomplete in n=41, 19.2%).
Table 1. Baseline characteristics of all groups; ACD, GFD, SCD, controls, DC

<table>
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<th></th>
<th>ACD N=95</th>
<th>GFD N=118</th>
<th>PCD N=13</th>
<th>Control N=89</th>
<th>DC N=23</th>
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TCRγδ+ IEL

All CD patients displayed an inverse relationship between age and percentage of TCRγδ+ IEL (spearman’s rho -0.137, P=0.047). As shown in Figure 2a, a significantly higher percentage of TCRγδ+ IEL was found in active CD patients (median 18.5%, range 1.0-58.0%) compared to controls (median 6.0%, range 1.0-15.0%) (p<0.001) and DC (median 2.0% (range 0.5-18.0%) (p<0.001). These increased numbers were found in both patients with villous atrophy as well as in patients on a GFD and normalized antibodies and normalized histology (median 19.0% versus 18.5%, p =0.99). Similarly, these increased TCRγδ+ IEL percentages were also observed in patients with PCD (median 20.0%, range 13.0-66.0%). As shown in Figure 2b, the TCRγδ+ IEL percentage in
ACD did not decrease upon instigation of a GFD (n=13, median follow-up 22.5 months, range 4.1-61.2 months) despite histological recovery (p=0.35).

Figure 2a. Boxplots of CD3+TCRγδ+ IEL in different groups of patients. Densities of TCRγδ+ IEL are expressed as percentage of total IEL.

Figure 2b. Percentage CD3+TCRγδ+ IEL at CD diagnosis compared to follow-up after introduction GFD. Densities of TCRγδ+ IEL are expressed as percentage of total IEL.

**sCD3-iCD3+ IEL**

Compared to TCRγδ+ IEL, percentages of sCD3-iCD3+ IEL show an opposite trend in some but not all subgroups. As shown in **Figure 3a**, a significantly lower percentage of sCD3-iCD3+ IEL was found in ACD patients (median 1.0%, range 0.0-8.0%) compared to both controls (median 8.0%, range 0.0-46.0%) and DC (median 3.0%, range 0.0-86.0%)(both p<0.001). Contrary to TCRγδ+ IEL, these percentages are higher in patients on a gluten-free diet compared to ACD (median 3.0%, range 0.0-18.0%)(p<0.001). In the small group of ACD patients who underwent follow-up after introduction of a gluten-free diet (n=13), sCD3-iCD3+ IEL show a rising trend (from 0.7% at baseline (range 0.2-4.0) to a median of 2.0% during follow-up (range 0.7-22.0%))(p=0.083)[**Figure 3b**]. Percentage of sCD3-iCD3+ IEL was higher in patients with PCD compared to ACD (median in PCD of 2.0%, range 0.3-24.0%)(p=0.045), yet significantly lower than in controls (p=0.041) **[Figure 3a]**.
Figure 3a. Boxplots of sCD3-iCD3+CD7+ IEL in different groups of patients. Densities of sCD3-iCD3+ IEL are expressed as percentage of total IEL.

Figure 3b. Percentage sCD3-iCD3+CD7+ IEL at CD diagnosis compared to follow-up after introduction GFD. Densities of sCD3-iCD3+ IEL are expressed as percentage of total IEL.

Abbreviations: GFD; CD patients in remission (on gluten-free diet), ACD; active celiac disease, DC; disease controls, PCD; potential celiac disease

**TCRγδ+ IEL as a diagnostic tool**

A cut-off value for TCRγδ+ IEL to differentiate between ACD patients and controls was obtained from a receiver operating characteristic (ROC) curve. A cut-off value of ≥14% for CD3+TCRγδ+ IEL resulted in 96.6% specificity and 66.3% sensitivity for a diagnosis of CD, with an area under the curve (AUC) of 88.6% (95% CI: 83.5-93.7%). For sCD3-iCD3+ IEL, a cut-off value of ≤0.5% resulted in 96.6% specificity and 31.2% sensitivity for a diagnosis of CD (AUC: 91.0% (95% CI: 86.5-95.5%). Likelihood ratio tests showed that sCD3-iCD3+ IEL is of added value to TCRγδ+ IEL in predicting CD and vice versa (both p<0.001). That is, both TCRγδ+ IEL and sCD3-iCD3+ IEL combined are superior in the diagnosis of CD than each of the two separately. Combining both dichotomized variables yielded a specificity of 93.2% and a sensitivity of 71.1% in case at least one out of two exceed the cut-off value, and a specificity and sensitivity of 100% and 20.0%, respectively, in case both exceed the cut-off value for diagnosing CD. On baseline, ACD patients and controls differ significantly in age; controls were relatively younger compared to ACD patients (p<0.001). Correcting the calculated cut-off value for age using a logistic regression model and subsequent ROC-curve analysis as mentioned above, results in the same cut-off value with 97.2% specificity and 67.3% sensitivity with an AUC of 93.3%. Due to a
better clinical applicability of a non-logistic approach we choose to use the cut-off value and accessory specificity and sensitivity calculated from the absolute TCRγδ+ percentages and did not correct for age.

The TCRγδ+ cut-off value within the test cohort

The demarcation in IEL subsets between ACD patients and controls is not clear cut. Both IEL subsets show individual variations and a certain degree of overlap was observed between ACD patients and controls. Twenty-eight out of 95 ACD patients (29%) had TCRγδ+ IEL percentages <14% whereas 3 out of 89 controls (3%) had ≥14% TCRγδ+ IEL. The ACD patients with TCRγδ+ IEL percentages <14% were significantly older compared to those with TCRγδ+ IEL percentages ≥14% (p=0.029). The percentage of TCRγδ+ IEL was independent of gender (p=0.92) and HLA-DQ status (HLA-DQ2.5 heterozygosity versus homozygosity: P=0.22) and the incidence of other autoimmune disease was comparable between both groups (p=0.56). Three out of 89 controls (3%) showed TCRγδ+ IEL percentages ≥14%. Of these ‘positive’ controls, one was aged < 18 which could be an explanation for the higher percentage of TCRγδ+ IEL. The other two both showed a family history of CD. Although both patients lacked circulating anti-endomysium (EMA) and/or anti-tissue transglutaminase antibodies (TGA) and histological abnormalities, the high percentages of gamma-delta T lymphocytes might be the first signs of a developing CD.

Implementation of the TCRγδ+ cut-off value

A cut-off of 14% has a specificity of 97% for CD diagnosis. Applying this cut-off value in the PCD patients in our cohort showed that 92% of PCD patients (n=12) display TCRγδ+ percentages ≥14%. With the high specificity of the cut-off value, this makes CD diagnosis in this subgroup very likely and therefore legitimizes the introduction of a gluten-free diet in these PCD patients. Evaluating the added value of the cut-off value in the DC patients in our cohort showed that 96% of DCs patients (n=22) displays TCRγδ+ percentages <14%. One DC patient (with villous atrophy e.c.i.) had a TCRγδ+ percentage of 18%. This patient was HLA DQ2 and/or DQ8 negative, which excludes underlying CD.

Discussion

In the present study we have shown that an imbalance in the ratio of TCRγδ+ IEL vs. sCD3-iCD3+ IEL was present in most active CD patients compared to controls. A cut-off
value of ≥14% for TCRγδ+ IEL has a high specificity for CD. The specificity increased even further when combined with ≤0.5% sCD3-iCD3+ IEL. This could be of diagnostic value in daily practice in secondary or tertiary referral centers, especially in those patients with minimal histological abnormalities (i.e., Marsh I) or individuals with positive serology in the absence of histological abnormalities (i.e., potential celiac disease).

The demarcation in IEL subsets between patients and controls is not clear cut. The question as to why low percentages of TCRγδ+ IEL are found in some CD patients remains unanswered. In ACD, solely age was found to be inversely related to the percentage of TCRγδ+ IEL and no other explanation was found for those patients with low percentages of TCRγδ+ IEL. For example, a low TCRγδ+ IEL percentage was not associated with HLA-DQ haplotype or with the incidence of comorbidity with other autoimmune disease. The relative high percentage of TCRγδ+low CD patients endorses the relatively low sensitivity of this diagnostic tool which makes this cut-off value therefore unsuitable to exclude CD. Therefore the TCRγδ+ cut-off value is mainly usefull to determine true CD negatives with a low percentage of false positives. In other words, ≥14% TCRγδ+ IEL can be used to diagnose CD with a high degree of confidence. This was confirmed when implementing the cut-off value in the subgroup with potential CD which showed ≥14% TCRγδ+ IEL in the majority of patients (92%). We choose to use the calculated cut-off value which was not corrected for age since the clinical usability of a cut-off value would be much higher without the necessity to make logistic corrections. For the future, an external validation cohort would be valuable to confirm our established cut-off value.

The first description of an increased CD3+CD4-CD8- population in the intestinal mucosa of patients with active CD dates back to 198620, which was later confirmed as IEL bearing the γ-chain and a δ-chain13. Since then, several studies have confirmed the presence of high percentages of CD3+TCRγδ+ IEL in the intestinal epithelium of active CD patients, which seemed to be both permanent and diet independent3-15 and was more pertinent when compared to other intestinal disorders (i.e. giardiasis, cow’s milk allergy)21. The same phenomenon was described in 1991 in patients with potential CD22 who subsequently developed active CD15, 23, 24. The presence of gamma-delta T lymphocytes in the intestine cannot be translated to peripheral circulation since this subset with a mucosal phenotype is rare in blood from affected CD patients12. Since the introduction of
flow cytometry, a more accurate quantification of the gamma-delta subset has become possible\textsuperscript{14, 15}. In 2002 a diagnostic algorithm for paediatric CD was proposed including the combined use of a high percentage TCRγδ+ IEL and a low percentage sCD3-iCD3+ IEL\textsuperscript{16}. Its use however has been limited due to the lack of a validated clinical threshold. Here we have defined a clear cut-off value of these IEL subsets that may be mandatory in clinical practice.

Remarkably, although their presence has been suggested for years, the role of intestinal TCRγδ+ subsets in the pathogenesis of CD is not completely understood. It has been suggested that TCR-γδ IEL are involved in mucosal repair\textsuperscript{12}, a hypothesis which is supported by mice experiments which showed an essential role of CD3+TCRγδ+ IEL in promoting epithelial reconstitution following mucosal injury\textsuperscript{25-28}. The observation that TCRγδ-IEL are depleted in complicated, pre-malignant CD, supports the notion that TCRγδ-IEL might play a crucial role in regaining homeostasis in CD and possibly even tumor surveillance\textsuperscript{29}. On the other hand, it cannot be excluded that these TCR-γδ IEL play a pro-inflammatory role in CD. Recently, these regulatory and proinflammatory hypotheses were both confirmed. Distinct subsets TCRγδ-IEL can accumulate during the various stages of CD; in active CD an IL-21 producing effector TCRγδ-IEL subset predominates, in contrast with CD on a GFD where a regulatory TCRγδ-IEL subset under the stimulus of transforming growth factor-β1 predominates\textsuperscript{30}. This might also be the explanation of the persistent high percentage of TCRγδ-IEL despite the absence of the triggering agent in patients on a GFD. In these patients, the regulatory TCR-γδ IEL subset might contribute to recovery from epithelial damage and maintenance of mucosal homeostasis\textsuperscript{30}. Future research will hopefully shed light on the exact roles and stimulants of this lymphocyte subset.

Contrary to TCRγδ+ IEL, the percentage of sCD3-iCD3+ IEL is decreased in CD patients yet this population does not remain stable after the introduction of a gluten-free diet. As was previously shown previously by Camaro et al, this cell population rises after instigation of a GFD\textsuperscript{15}. Additional knowledge of the exact role of this cell subset is required to elucidate their role in the pathogenesis of CD.

For the future, it would be interesting to evaluate patients with common variable immunodeficiency disease (CVID) with villous atrophy since a subset of these patients
might benefit from a GFD although testing for CD (auto)antibodies is impossible in this disease group. It can be hypothesized that CVID patients with a high percentage of CD3+TCRγδ+ IEL might benefit from a GFD. Furthermore, flow cytometric analysis of TCRγδ+ IEL might be helpful in cases of diagnostic doubt on CD in patients compliant to a GFD without the need for a gluten-challenge. Finally, TCRγδ+ IEL could also be of benefit in the 'non-celiac gluten sensitivity' (NCGS) population. It can be hypothesized that patients currently diagnosed as NCGS who are HLA-DQ2 or HLA-DQ8 positive and display a high percentage of TCRγδ+ IEL should be excluded from future clinical trials to prevent bias from potential latent CD. This latter suggestions needs further research within the NCGS population.

**Conclusion**

Gamma-delta T lymphocytes are increased and sCD3-iCD3+ IEL are decreased in ACD compared to controls. In our cohort a percentage of ≥14% gamma-delta T lymphocytes had a high specificity for CD and could therefore be of diagnostic use in those cases where diagnosis is not straightforward.
References


