Gamma-Delta T lymphocytes in the diagnostic approach of coeliac disease

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**ABSTRACT**

**Goals**  
To validate cut-off values of CD3+TCRγδ+IEL in the (differential) diagnosis of coeliac disease (CD).

**Background**  
CD is characterised by an increase in gamma-delta intra-epithelial lymphocytes (CD3+TCRγδ+IEL).

**Study**  
Percentages were determined by flow cytometric analysis of IELs from small bowel biopsies in 213 CD and 13 potential CD (PCD) patients and in total 112 controls. A cut-off value for percentages of CD3+TCRγδ+IEL to differentiate active CD and controls was obtained from a ROC curve and implemented in controls and PCD patients.

**Results**  
A percentage of ≥14% CD3+TCRγδ+IEL has a high specificity for CD diagnosis and can be of diagnostic help in cases where diagnosis is not straightforward.

**Conclusion**  
A percentage of ≥14% CD3+TCRγδ+IEL has a high specificity for CD diagnosis and can be of diagnostic help in cases where diagnosis is not straightforward.

**INTRODUCTION**

Coeliac disease (CD) is a chronic small intestinal immunemediated 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). 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 intraepithelial lymphocyte (IEL) compartment of CD patients is characterised by an increase in CD3+ T lymphocytes bearing the T cell receptor gamma-delta chain (TcR-γδ) which is both permanent and diet independent. 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+TCRγδ+IEL, a reduced percentage of surface CD3 negative and intracellular CD3 positive lymphocytes (sCD3-iCD3+CD7+ IEL) is described in active CD (ACD). This imbalance in the ratio of TCRγδ+ IEL versus sCD3-iCD3+ IEL was confirmed and shown to be permanent in a paediatric study setting. Although the method was proposed as a new diagnostic criterion, 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 could be of diagnostic use in those cases where diagnosis of CD is not straightforward.

**MATERIAL AND METHODS**

**Patients and data collection**  
Patients visiting Coeliac 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. Different patient subgroups were included for the analysis;
Gammadelta T-cell receptor

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), ECD, PE-Cy7, APC-AF700, APC-H7 and Krome Orange-labeled monoclonal antibodies directed against TCR-γδ, CD3 (surface staining), CD3 (intracellular staining), CD4, CD7, CD8, CD16+56, CD19 and CD45 (Beckman-Coulter or BD Biosciences). 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

1. Patients with proven coeliac disease (n=213). This group was further divided in patients 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)), the presence of intraepithelial lymphocytosis (> 40 intraepithelial lymphocytes per 100 enterocytes) in combination with crypt hyperplasia and at least partial villous atrophy (Marsh IIla-c) and if available, the presence of HLA DQ2 and/or DQ8. The ACD group included therefore only patients with a new diagnosis of CD, without the introduction of a GFD. 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 because of 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. All controls consumed a gluten containing diet at the time of analysis.

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 immunoproliferative 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 was excluded by negative serology and all disease controls consumed a gluten containing diet.

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 described. 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), ECD, PE-Cy7, APC-AF700, APC-H7 and Krome Orange-labeled monoclonal antibodies directed against TCR-γδ, CD3 (surface staining), CD3 (intracellular staining), CD4, CD7, CD8, CD16+56, CD19 and CD45 (Beckman-Coulter or BD Biosciences). 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).
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 nonparametric Mann-Whitney U test. Differences in percentages TCR-γδ+ IELs and sCD3-iCD3+ IELs 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 with the model including only TCRγδ+ IEL densities. Secondly, this model was compared with 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 summarised in Table 1. The median age was 49.3 years (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%).

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 histologic recovery (p=0.35).

Table 1. Baseline characteristics of all groups

<table>
<thead>
<tr>
<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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<tr>
<td>Gender (M/F)</td>
<td>38M / 57F</td>
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<td>30% / 70%</td>
<td>46% / 54%</td>
<td>29% / 71%</td>
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<td>HLA (n) [%]</td>
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<td>- DQ2 heterozygous</td>
<td>50 [53%]</td>
<td>56 [47%]</td>
<td>4 [31%]</td>
<td>32 [36%]</td>
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<td>- DQ2 homozygous</td>
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<td>22 [19%]</td>
<td>5 [38%]</td>
<td>7 [8%]</td>
<td>1 [4%]</td>
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<td>- DQ8 heterozygous</td>
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<td>8 [7%]</td>
<td>1 [8%]</td>
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<td>Marsh 0</td>
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<td>95 [100%]</td>
<td>0</td>
<td>0</td>
<td>21 [100%]</td>
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</table>

ACD: active coeliac disease, GFD: coeliac disease patients on a gluten-free diet, PCD: potential coeliac disease, DC: disease controls
sCD3−iCD3+ IEL

Compared with 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].

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%-Cl: 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
Gammadelta T-cell receptor

significantly in age; controls were relatively younger compared with ACD patients (p<0.001). Correction for this difference in a logistic regression model, yielded a similar AUC, sensitivity and specificity.

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 with those of 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%. Among 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 histologic 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 with 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 histologic abnormalities (i.e., Marsh I) or individuals with positive serology in the absence of histologic abnormalities (i.e., potential coeliac 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. The relative high number 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 useful to determine true CD negativates 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%). For the future, an external and prospective validation cohort would be valuable to confirm our established cut-off value and to confirm its additive value in potential CD.

The first description of an increased CD3+CD4-CD8- population in the intestinal mucosa of patients with active CD dates back to 1986, which was later confirmed as IEL bearing the γ-chain and a δ-chain. 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 independent and was more pertinent when compared to other intestinal disorders (i.e. giardiasis, cow’s milk allergy). The same phenomenon was described in 1991 in patients with potential CD who subsequently developed active CD. Since the introduction of flow cytometry, a more accurate quantification of the gamma-delta subset has become possible. 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 useful 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, a hypothesis which is supported by murine experiments which showed an essential role of
CD3+γδ T-CELL RECEPTOR EXPRESSION IN CELIAC DISEASE

CD3+γδ T lymphocytes are increased and sCD3-γδ T lymphocytes are decreased in ACD compared with 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


