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Expression pattern of Th17 signalling pathway and mucosal inflammation in celiac disease

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Abstract

Objective: We aimed to investigate the mucosal activation of a broad range of genes associated with the Th17 signalling pathway in children at different stages of CD; including children with increased risk for CD, children with untreated and gluten-free diet (GFD)-treated CD.

Material & methods: Small intestinal biopsies were taken from children with untreated and GFD-treated CD, transglutaminase antibody (TGA) positive children with potential CD and reference children. Real-time PCR-arrays were used to study the gene expression pattern of Th17 related genes and qPCR was used to study the IL-17A expression.

Results: The mucosal expression of CD8A was elevated at all stages of CD. Children with untreated CD had diminished levels of IL-17RE, IL-23R, RORc, STAT6, CCL22, NFATC2, IL-18, CD4, CD247 and matrix metalloproteinase(MMP)9, but elevated levels of MMP3, IL-17, IFN- γ and CD8A, compared to references. The majority of the aforementioned genes, being differentially expressed in untreated CD, displayed similar expression in GFD-treated children and references. Children with untreated and GFD-treated CD had elevated expression of IFN- γ , but reduced expression of CD247. Interestingly, children with potential CD displayed reduced FOXP3, IL-21 and IL-17A levels.

Conclusion: Mucosal up-regulation of Th17 immunity occurs at the late stage of disease and is down-regulated with dietary treatment indicating that IL-17 immunity is not a fundamental feature of CD as Th1 immunity, which is not fully down-regulated by GFD.

Key words: Arrays, celiac disease, children, FOXP3, gene expression, gluten free diet, IL-17, mucosa, Th17.

Introduction

Celiac disease (CD) is a common autoimmune disorder which develops in genetically susceptible individuals, positive for HLA-DQ2/DQ8, as a result of intolerance to dietary gluten. CD has often been considered as a classical malabsorption state characterized by severe mucosal villous atrophy with crypt hyperplasia together with immune cell infiltration and inflammation in the small intestinal mucosa. Nowadays, also mild inflammation with partial villous atrophy is becoming a more common phenotype of CD at presentation. Individuals with elevated CD associated transglutaminase autoantibodies without villous atrophy, who are considered to have increased risk of CD and so called potential CD, are also a part of the broad spectrum of CD. Approximately one third of the subjects with potential CD developed villous atrophy and acute CD within a few years if they continued on a gluten containing diet [1]. So far the only available treatment of CD is a lifelong strict gluten-free diet (GFD), which causes clinical improvement and normalization of the villous atrophy together with improvement of the intestinal inflammation.

It is known that CD is characterized by mucosal up-regulation of the interferon (IFN)- γ pathway [2-4]. Gluten reactive T-cells have been shown to secrete IFN- γ , emphasizing the role of T-helper (Th)1 associated immune responses in the pathogenesis [5]. We recently reported that children with untreated CD displayed mucosal up-regulation of the IFN- γ pathway, which remained elevated even one year after GFD treatment. This suggests that activation of the Th1 response seems to be more fundamentally associated with CD, being present already in individuals with potential CD and also remaining in GFD-treated CD patients with normal villous structure [6].

Recently elevated interleukin (IL)-17 responses were reported after exposure to wheat gliadin in acute CD but not in potential CD indicating association of up-regulated IL-17 pathway with villous atrophy [7-9]. However, T-cell clones reactive with deamidated gliadin peptide did not show IL-17

secretion [5] which suggests that activation of IL-17 may not be induced directly by dietary gluten, but rather develops at later stage of mucosal inflammation. IL-17 has been speculated to act as a counter-regulatory molecule limiting the intestinal inflammation (induced by Th1 cells) [8]. We have reported that IL-17 induced anti-apoptotic bcl-2 responses in an intestinal epithelial cell line [9]. Another regulatory factor, FOXP3, has been reported to be up-regulated also in acute CD. In summary, the complex network of mucosal immune activation in CD is poorly understood and it seems that changing pattern of Th1/Th17/Treg activation is seen at different stages of CD.

To understand the role of IL-17 immunity in CD, we analysed the intestinal expression profile of Th17 related genes in reference children, children with transglutaminase antibody (TGA) positivity (potential CD), and in children with untreated CD and GFD-treated CD.

Methods

Study subjects

Small intestinal biopsy samples were obtained from 33 children (mean age: 6.9 years, range 1-18 years, 21 girls and 12 boys) who underwent small intestinal biopsy sampling at the Paediatric Clinic, University Hospital Linköping or at the Paediatric Clinic in Norrköping or Motala for suspicion of or confirmation of CD (Table 1). The study population comprises four groups: nine children with untreated CD displaying a morphological picture of CD (untreated CD); eight CD children who have followed a strict exclusion diet for one year with a normalized mucosa (GFD treated CD); eight children with potential CD, *i.e.* TGA-positive children having a normal mucosa and following gluten contain diet (potential CD); and eight TGA negative children displaying a morphologically normal mucosa (references).

RNA preparation

Cryopreserved biopsies were used for RNA isolation, according to standardized methodology at our laboratory as described previously [6]. Total RNA was isolated from frozen biopsies, DNase treated and quality checked using Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturers' guidelines, as described before [6].

Th17 signalling pathway PCR array

For the real-time PCR-array analyses, 1.4µg of total RNA was reversely transcribed into cDNA (15 min at 42°C and 5 min at 95°C), mixed into a PCR-cocktail (together with DNA polymerase, SYBR®Green dye, ROX reference dye and double-distilled H₂O) and loaded on the Human Th17 for Autoimmunity & Inflammation RT² Profiler™ PCR Array (PAHS-073, SABiosciences, Frederick, Maryland, USA). Subsequently, PCR amplification was performed (10 min at 95°C, 40

cycles of 15 s at 95°C, 1 min at 60°C), according to the manufacturer's guidelines, as described earlier [6].

Only genes with threshold cycle (Ct)-values <35 were considered to be detectable, according to the manufacturer's guidelines. The Ct-values of the genes were normalized with the average Ct-value of all five housekeeping genes (HKG) on the array: Beta-2-microglobulin (B2M), Hypoxanthine phosphoribosyltransferase 1 (HPRT1), Ribosomal protein L13a (RPL13A), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin (ACTB). The Δ Ct and the median fold change ($2^{-\text{MEDIAN } \Delta\Delta\text{Ct}}$) were calculated for the genes on the array.

Quantitative RT-PCR

The mucosal expression of IL-17 was undetectable with the PCR-arrays and was therefore analysed with conventional quantitative PCR (qPCR), which is a more sensitive method for IL-17 detection. For qPCR (qPCR) of IL-17A, total RNA was reversely transcribed into cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA). qPCR of IL-17A (cat. no. Hs00174383_m1) was then carried out using TaqMan Gene Expression Assay (Applied Biosystems), as described earlier [9]. 18s rRNA was used as endogenous control (Hs99999901_s1) and an exogenous cDNA pool calibrator as an interassay standard to which normalized samples were compared. The relative expression level of IL-17A was quantified using the comparative $\Delta\Delta$ Ct method, where $2^{-\Delta\Delta\text{Ct}}$ represents the relative amount of the gene of interest in the sample, according to the manufacturer's guidelines, as described earlier [9]. For presentations the relative amounts ($2^{-\Delta\Delta\text{Ct}}$) of IL-17 was multiplied by a factor 1000 and expressed as relative units. To enable statistical analyses if the C_t value not reached a quantitative level, an artificial value corresponding to half of the lowest quantitative value in relative units was given to the sample.

Statistical analysis

The data analysis was performed with SPAW Statistics 17.0 for Windows (SPSS Inc., Chigaco, IL, USA) and GraphPad prism software (San Diego, CA). Mann-Whitney U test was used for comparisons between the groups and p-values <0.05 were considered as statistically significant.

For the PCR-arrays a cut off criterion for the gene expression fold change ($2^{-\text{MEDIAN } \Delta\Delta C_t}$) between the samples was used. A fold change of 1.4 was considered to be an enhancement, whereas a fold change of -1.4 was considered to be a reduction of the studied genes. The difference in the gene expression between the groups was considered significant if it fulfilled the following two criteria: a fold change of (± 1.4) together with a p-value of <0.05 between the groups, as described before [6]. The statistical analyses were not corrected for multiple comparisons.

The expression profiles of genes passing the double cut-off criterion were visualised with Self Organizing Map (SOM)-clusters formed by GeneCluster 2.0 (Whitehead Institute, Center for Genome Research, Massachusetts Institute of Technology, Cambridge, MA), using Tamayo's algorithm [10]. The expression data was pre-processed by normalization (mean: 0, variance: 1) before it was clustered according to Tamayo et al [10] as described before [6].

In order to disclose multivariate responses Principal Component Analysis (PCA) of the HKG-normalised Real time-PCR-array data was employed, using GeneEx (MultiD Analyses/TATAA Biocenter, Gothenburg, Sweden). The data was pre-processed by calculating the relative expression of the genes, log2-transformation and mean-centering. Genes passing the double cut-off criteria; the fold change cut-off (± 1.4) together with a significant difference in gene expression between the study groups (Mann-Whitney U test (p<0.05) between children with untreated-CD and/or GFD-treated-CD and/or children without CD (references + potential CD) were visualised with PCA.

Ethical Considerations

The study was approved by the Regional Ethics Committee for Human Research at the University Hospital of Linköping, Sweden, and written informed consent was obtained from parents and the children who were old enough to agree to participation.

Results

Detectable PCR-array products (<35 cycles) were obtained from the majority (n=72) of the 84 genes associated with the Th17 signalling pathway (Supplementary Table 1). The expression of 61 genes in total differed (with a fold change value higher than ± 1.4) among the groups of children studied (Figure 1). The expression level; Δ -Ct-value, of 23 genes was statistically different between the groups (Supplementary Table 2). The Ct-values of IL-17A in the samples were all below 35 cycles, which is the cut off level for detection in the PCR arrays. The expression of IL-17A was analyzed with conventional qPCR, due to its higher sensitivity [9]. The IL-17A levels of the samples analyzed with qPCR displayed moderate mRNA expression (median Ct-value: 31.6; range 28.1-40 cycles).

Cluster analysis

Unsupervised cluster analysis (SOM-clustering) of the 23 genes passing the double cut-off criterion displayed 4 distinct clusters; C0-C3, including 5, 3, 5 and 10 genes respectively (Figure 2).

Mucosal gene expression in potential CD

Children with potential CD had elevated CD8A gene expression level, but reduced IL-21, FOXP3, CSF2, TRAF6, MMP9 and CLEC7A expression when compared to the references (Figure 2-3, Supplementary Table 2). Children with potential CD had reduced expression of FOXP3 and CLEC7A compared to untreated CD and GFD treated CD, respectively (Figure 2-3, Supplementary Table 2). The expression of CD4, IL-17RE, RORc, NFATC2 and IL-18 was higher in potential CD in comparison to untreated CD (Figure 2, 4, Supplementary Table 2). Interestingly, the qPCR analyses of IL-17 showed that children with potential CD expressed lower levels of IL-17A than the reference children, but also compared to children with untreated and GFD-treated CD (Figure 3).

Mucosal gene expression in untreated and GFD-treated CD

Children with untreated CD had elevated expression of IFN- γ , IL-17A, MMP3, CD3D, CD3G and CD8A, but reduced expression of IL-17RE, IL-23R, RORc, NFATC2, CD4, CD247, IL-18, CCL22, STAT6, MMP9 and TRAF6 when compared to the reference children (Figure 2-5, Supplementary Table 2). In GFD-treated CD, the expression of IL-17A, MMP3, IL-17RE, IL-23R, RORc, NFATC2, CD4, IL-18, and STAT6 was similar as the references and therefore seemed to be normalised by GFD-treatment (Figure 2, 4-5, Supplementary Table 2). Similarly as children with untreated CD, the GFD-treated children displayed enhanced IFN- γ , CD3D, CD3G and CD8A expression, but diminished CD247 expression when compared to the references. Furthermore, the GFD-treated children expressed lower levels of IL-21 than the reference group (Figure 2-3, Supplementary Table 2).

The expression of TIRAP, IL-6R and IL-7R was lower in untreated CD in comparison to GFD-treated CD (Figure 2, Supplementary Table 2). qPCR analyses showed that IL-17A transcripts were elevated in children with untreated CD as compared to GFD-treated CD children (Figure 3).

Principal component analysis (PCA)

Principal component analysis (PCA) of the 23 differentially expressed genes; CCL22, CD247, CD3D, CD3G, CD4, CD8A, CLEC7A, CSF2, FOXP3, IFN- γ , IL-17RE, IL-18, IL-21, IL-23R, IL-6R, IL-7R, MMP3, MMP9, NFATC2, RORc, STAT6, TIRAP and TRAF6, revealed separation of the groups; children with untreated CD, GFD-treated CD, potential CD and reference children without CD (Figure 6).

Discussion

During recent years the role of IL-17 immunity in CD has received a lot of interest due to the key function of Th17 immunity in mucosal defence. Here, we used real-time PCR arrays, an applicable tool for gene expression studies [11] [6] to analyse the mucosal immune activation stage of a broad range of Th17 related genes in small intestinal biopsies from children at different stages of CD *i.e.* untreated, potential and GFD-treated CD.

We found that children with untreated CD and villous atrophy expressed elevated levels of IFN- γ , IL-17A, MMP3 and CD8A but diminished levels of IL-17RE, RORc, NFATC2, CCL22, CD4, CD247, IL-18 and STAT6 as compared to the reference children. The elevated expression of CD8A was observed in all stages of CD; *i.e.* in potential, untreated and GFD-treated CD. This indicates that activation of the cytotoxic T-cells (Tc) is a basic feature in CD related mucosal inflammation and not only triggered by gliadin because it is still seen after one year on GFD. As expected, high levels of mucosal IFN- γ expression were seen in children with GFD-treated CD when compared to the references indicating that the dysregulated IFN- γ response is not normalized by a strict GFD-treatment for one year. Thus, elevated mucosal IFN- γ and CD8A in patients on GFD suggests that Th1 activation is the underlying mucosal immune aberrancy in CD and not totally down-regulated by dietary treatment. These results also suggest that in the etio-pathogenesis of CD an unknown factor which not is gliadin is playing a role. The dysregulated Th2 response in untreated CD, characterised by reduced CCL22, IL-18 and STAT6 levels, is likely a reflection of the increased Th1 response since these changes were normalized by the GFD-treatment. This is in agreement with previous studies [6, 12].

Our results also show that the mucosal IL-17A response was elevated at the late stage of CD when villous atrophy has developed. Mucosal IL-17A displayed elevated expression in children with untreated CD when compared to GFD treated children and also when compared to children with potential CD. This is in agreement with previous studies [8, 9, 12, 13]. In contrast to the elevated IL-17A level in untreated CD, the expression of RORc, IL-17RE and IL-23R was diminished. RORc is a transcription factor for IL-17 activation, IL-23R is expressed on Th17 cells and IL-23 is important for the maintenance of IL-17 secretion. It seems that there are simultaneous attempts to regulate the increased mucosal IL-17 immunity in CD. Alternatively, IL-17 transcripts are not up-regulated in the “classical” Th17 cell, but in T-cells which show plasticity. It was recently reported that gliadin-specific mucosal Th17 cells of patients with CD are different from Th17 cells of healthy individuals [14]. Th17 cells in CD show plasticity; they secrete pro-inflammatory cytokines such as IL-17, IFN- γ , IL-21, but also mucosa-protective IL-22 and regulatory TGF- β [14]. The role of IL-17 expressing cells may thus be dualistic in CD mucosa. It has been suggested that IL-17 up-regulation in mucosa is actually related to the mechanisms of protection [8]. In a previous study we showed that IL-17 induced anti-apoptotic rather than apoptotic responses in an epithelial cell line [9]. IL-17 in mucosal anti-microbial defence has been shown to contribute to the gut barrier function and up-regulation of IL-17 diminishes the dissemination of pathogens from the intestinal lumen [15]. Furthermore, the small intestine seems to be responsible for the control of the response by eliminating Th17 cells and by induction of a phenotype change in Th17 cells, which acquire suppressive phenotype characteristics and participate in the regulation of immune responses in small intestine [16].

On the other hand commensal bacteria can induce mucosal IL-17 response [17-20], and it is possible that changes in microbiota could be responsible for the up-regulation of IL-17 when villous atrophy develops. Alterations of intestinal microbiota are seen in active CD [21-25], but the

importance of microbes as triggers of mucosal damage has not been proven. The innate immunity associated markers TIRAP and TRAF6, which are involved in TLR signalling pathways, were also diminished in children with untreated CD compared to the GFD-treated CD children and the references. The expression of both markers was similar in the references as in children following a strict GFD for one year. These findings might reflect the alterations in the microbiota in untreated CD, which previously has been reported in CD [25-29].

Interestingly, IL-17A gene expression together with FOXP3 and IL-21 expression were markedly reduced in children with potential CD when compared to all other groups of children. Diminished IL-21 levels have earlier been reported in potential CD as compared to patients with untreated CD and controls [30]. Accordingly, the activation of mucosal Th1 immunity alone, as seen in potential CD and treated CD, may not lead to the villous atrophy, but additional immunological changes are needed. In addition to IL-17, the expression of MMPs was up-regulated at the time of villous atrophy.

Our results show that MMP3 was enhanced in untreated CD with villous atrophy when compared to the references, which is in agreement with previous studies reporting elevated MMP3 expression, both at mRNA and protein level, in children with untreated CD [31, 32]. We show here that MMP3 levels in the GFD-treated children did not differ from those in the reference group. GFD seems to induce a normalization of the MMP3 expression concurrently with the normalization of the intestinal mucosa. The simultaneous up-regulation of MMP3 and IL-17 seen in our study is of interest, since it was reported that IL-17 may induce secretion of MMP3 in vitro [33]. Also IFN- γ has been shown to up-regulate MMPs [34]. MMPs are known to be involved in tissue remodelling, but may also cause degradation of the extracellular matrix and basement membranes and thereby promote influx of immune cells to the intestinal epithelium and contribute to the intestinal

inflammation. It is likely that MMP3 are involved in the pathogenesis of CD of epithelial destruction at the time of villous atrophy in CD [35].

The reduced expression of CD247, CD4, and NFATC2 which was seen in CD children with villous atrophy may indicate that the T-cell activation and signalling is disturbed in untreated CD. CD247 is an amplification module in the TCR/CD3 signalling cascade, but also a master regulator and sensor of innate and adaptive immune responses. A defective expression/function of CD247 was suggested to be associated with pro-inflammatory conditions, autoimmune diseases and refractory CD [36-40]. Cells expressing low levels of CD247 produce IFN- γ , but they do also possess enhanced migratory capacity and the ability to enrich in inflamed tissues [37]. It is however unknown if the down-regulation of CD247, which often occur concurrently with Th1 responses, is induced by or a consequence of the inflammatory response. Down-regulation of CD247 most likely represents a beneficial mechanism attenuating hyper-activated immune responses in acute inflammatory conditions [41]. NFATC2 is a multi-faceted activation marker, important for cytokine production by peripheral T-cells, which also has been suggested to regulate mucosal T-cell activity and to be important in innate mucosal immune responses [42]. Treatment with a GFD seems to normalise the defective T-cell activation and signalling, since the expression of the aforementioned genes were similar in GFD-treated CD and references. Thus, the diminished expression of these genes involved in T-cell signalling and activation most likely represents a normal mechanism by which the immune system regains homeostasis/balance during inflammatory conditions.

Multivariate data analysis techniques such as PCA and cluster analysis are nowadays more common used for analysis and visualization of expression data. Here, four distinct clusters were formed when SOM-clustering was used to display the expression pattern of the 23 differentially expressed genes in children at different stages of CD. In our study, the PCA separated the groups of children with

untreated CD from children with GFD-treated CD, potential CD and reference children without CD. The 23 genes included in the PCA comprise genes directly involved in IL-17 pathway, such as IL-17 RE, IL-23R, IL-21, RORc and IL-6R, and also other genes related to T-cell activation and regulation, such as IFN- γ , CD247, CD3D, CD3G, CD4, CD8A, NFATC2, STAT6, FOXP3 and IL-7R, but also genes involved in innate immunity, such as CLEC7A, CCL22, CSF2, IL-18, TIRAP, TRAF6 and MMP3. It should be noted that we did not corrected the statistical analyses for multiple comparisons, and thus the results should be interpreted with caution.

In summary, we show that the primary immune-pathogenesis of CD is characterized by the mucosal activation of IFN- γ and CD8, which is present in potential CD and also in treated CD despite of the mucosal healing after elimination of dietary gluten. This may indicate that the fundamental cause of gliadin intolerance is a trigger of this kind of cytotoxic T-cell immunity often induced by intracellular pathogens. Although the role of IL-17 and MMPs is not fully understood in the pathogenesis of CD, their up-regulation at the time of villous atrophy suggests that IL-17 and MMPs could be explored as useful biomarkers of active CD in the follow-up of individuals with potential CD.

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Disclosure

The authors have no conflict of interest to declare.

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Table 1. Detailed information about the study population.

CD-status	Age/Gender	TGA	Marsh grade	Comment
Ref (TGA-)	M/1.5	Neg (<4)	Marsh 0	-
Ref (TGA-)	F/1	Neg (<4)	Marsh 0	IgA deficiency
Ref (TGA-)	F/2	Neg (<4)	Marsh 0	(Mb Down)
Ref (TGA-)	M/1	Neg (<4)	Marsh 0	-
Ref (TGA-)	F/1.5	Neg (<4)	Marsh 0	-
Ref (TGA-)	M/1	Neg (<4)	Marsh 0	-
Ref (TGA-)	F/10	Neg (<4)	Marsh 0	Heredity
Ref (TGA-)	F/1	Neg (<4)	Marsh 1	Heredity
Pot CD (TGA+)	F/11	9	Marsh 0	Pot-CD, Heredity
Pot CD (TGA+)	F/10.5	27	Marsh 0	Pot-CD
Pot CD (TGA+)	F/9.5	6	Marsh 0	Pot-CD, Heredity
Pot CD (TGA+)	M/3	10	Marsh 0	Pot-CD
Pot CD (TGA+)	F/11	18	Marsh 0	Pot-CD
Pot CD (TGA+)	M/10	6	Marsh 0	Pot-CD
Pot CD (TGA+)	F/9.5	9	Marsh 0	Pot-CD, New biopsy 1 year later: CD.
Pot CD (TGA+)	M/8	6	Marsh 2	Pot-CD, New Biopsy 1 year later: CD.
CD	M/13	10	Marsh 1	Heredity
CD	F/18	6	Marsh 2	-
CD	F/5.5	>100	Marsh 3b	-
CD	M/9.5	97	Marsh 3b	-
CD	M/13	56	Marsh 3b	-
CD	F/2	Neg (<4)	Marsh 3b	-
CD	F/2.5	>100	Marsh 3b	-
CD	F/4	>100	Marsh 3b	-
CD*	F/2	100	Marsh 3c	-
GFD-CD	F/17.5	NA	Marsh 0	-
GFD-CD	F/2.5	Neg (<4)	Marsh 0	-
GFD-CD	F/12.5	NA	Marsh 0	-
GFD-CD	M/14	4	Marsh 0	Heredity
GFD-CD	M/3	NA	Marsh 1	-
GFD-CD	F/13	NA	Marsh 1	-
GFD-CD	F/5	Neg (<4)	Marsh 1	-
GFD-CD	M/6	Neg (<4)	Marsh 1	Heredity

CD=celiac disease, GFD=gluten-free diet, TGA=transglutaminase antibodies, NA= not analysed, Pot-CD=Potential CD.

Figure Legends

Figure 1. Fold change values ($2^{-\Delta\Delta C_t}$) of Th17 associated gene expression levels in biopsies from children with potential CD (pot-CD), untreated CD (CD) and GFD-treated CD (GFD-CD) and references (TGA-neg Ref). Cut off: $-1.4 > x > 1.4$.

Figure 2. Gene expression profiles in biopsies from the study groups; TGA negative references, potential CD, untreated CD and GFD-treated CD. The expression patterns of the 23 genes passing the double cut-off criteria (a fold change >1.4 together with $p < 0.05$) are visualized in a self-organizing map (SOM)-cluster. The y-axis represents a relative scale with normalized gene expression. Lines connecting the dots indicate the mean expression profiles of the genes and the two outer lines indicate the standard deviation.

Figure 3. Th17 associated gene expression in small intestinal biopsies from children with potential CD (pot CD), untreated CD (CD), gluten-free diet (GFD)-treated CD (GFD-CD) and references.

The results from the real-time PCR analyses of CD8A (A), IL-21 (B), FOXP3 (C), MMP9 (D) are presented as ΔC_t -values, whereas the results from the qPCR analyses of IL-17A (E) is displayed as relative units. P-values <0.05 were considered significant and p-values <0.10 were considered as trends.

Figure 4. Th17 associated gene expression in small intestinal biopsies from children with potential CD (pot CD), untreated CD (CD), gluten-free diet (GFD)-treated CD (GFD-CD) and references. The results from the real-time PCR analyses of IL-17RE (A), RORc (B), CD4 (C), NFATC2 (D) and IL-18 (E) are presented as ΔC_t -values. P-values <0.05 were considered significant and p-values <0.10 were considered as trends.

Figure 5. Th17 associated gene expression in small intestinal biopsies from children with potential CD (pot CD), untreated CD (CD), gluten-free diet (GFD)-treated CD (GFD-CD) and references. The results from the real-time PCR analyses of IFN- γ (A), MMP3 (B), IL-23R (C), CD247 (D) and STAT6 (E) are presented as Δ Ct-values. P-values <0.05 were considered significant and p-values <0.10 were considered as trends.

Figure 6. Principal component analysis (PCA) visualization (score plot) of gene expression data from intestinal biopsies, based on 23 of the genes (CCL22, CD247, CD3D, CD3G, CD4, CD8A, CLEC7A, CSF2, FOXP3, IFN- γ , IL-6R, IL-7R, IL-17RE, IL-18, IL-21, IL-23R, MMP3, MMP9, NFATC2, RORC, STAT6, TIRAP, TRAF6) displaying differential expression in untreated celiac disease (CD) (black), gluten-free diet (GFD)-treated CD (blue), children with potential CD (red) and reference children without CD (green).

Supplementary material

Supplementary Table 1. The 84 genes studied in biopsies from the study subjects.

	All individuals (groups)		TGA- Ref	Pot CD	CD	GFD-CD
Gene abbreviation	Response ratio: Pos/Total	Analysed statistically	Response ratio: Pos/Total	Response ratio: Pos/Total	Response ratio: Pos/Total	Response ratio: Pos/Total
CACYBP	32/33	Yes	8/8	8/8	9/9	7/8
CCL1	2/33	No	1/8	1/8	0/9	0/8
CCL2	33/33	Yes	8/8	8/8	9/9	8/8
CCL20	33/33	Yes	8/8	8/8	9/9	8/8
CCL22	33/33	Yes	8/8	8/8	9/9	8/8
CCL7	3/33	No	1/8	1/8	1/9	0/8
CD247	33/33	Yes	8/8	8/8	9/9	8/8
CD28	10/33	No	4/8	3/8	2/9	1/8
CD34	33/33	Yes	8/8	8/8	9/9	8/8
CD3D	33/33	Yes	8/8	8/8	9/9	8/8
CD3E	33/33	Yes	8/8	8/8	9/9	8/8
CD3G	33/33	Yes	8/8	8/8	9/9	8/8
CD4	33/33	Yes	8/8	8/8	9/9	8/8
CD40LG	33/33	Yes	8/8	8/8	9/9	8/8
CD8A	33/33	Yes	8/8	8/8	9/9	8/8
CEBPB	33/33	Yes	8/8	8/8	9/9	8/8
CLEC7A	33/33	Yes	8/8	8/8	9/9	8/8
CSF2	29/33	Yes	8/8	5/8	8/9	8/8
CSF3	7/33	No	2/8	2/8	3/9	0/8
CX3CL1	32/33	Yes	8/8	7/8	9/9	8/8
CXCL1	33/33	Yes	8/8	8/8	9/9	8/8
CXCL12	33/33	Yes	8/8	8/8	9/9	8/8
CXCL2	32/33	Yes	8/8	8/8	9/9	7/8
CXCL5	21/33	Yes	5/8	5/8	6/9	5/8
CXCL6	33/33	Yes	8/8	8/8	9/9	8/8
EDG1	33/33	Yes	8/8	8/8	9/9	8/8
FOXP3	31/33	Yes	8/8	7/8	9/9	7/8
GATA3	32/33	Yes	8/8	8/8	9/9	7/8
ICAM1	33/33	Yes	8/8	8/8	9/9	8/8
ICOS	32/33	Yes	8/8	8/8	9/9	7/8
IFNG	33/33	Yes	8/8	8/8	9/9	8/8
IL10	33/33	Yes	8/8	8/8	9/9	8/8
IL12B	13/33	No	2/8	2/8	3/9	6/8

IL12RB1	33/33	Yes	8/8	8/8	9/9	8/8
IL12RB2	33/33	Yes	8/8	8/8	9/9	8/8
IL13	19/33	Yes	4/8	5/8	5/9	5/8
IL15	33/33	Yes	8/8	8/8	9/9	8/8
IL17A	0/33	No	0/8	0/8	0/9	0/8
IL17C	32/33	Yes	8/8	8/8	9/9	7/8
IL17D	13/33	No	4/8	1/8	1/9	7/8
IL17F	27/33	Yes	8/8	5/8	9/9	5/8
IL17RB	33/33	Yes	8/8	8/8	9/9	8/8
IL17RC	33/33	Yes	8/8	8/8	9/9	8/8
IL17RD	31/33	Yes	8/8	7/8	9/9	7/8
IL17RE	33/33	Yes	8/8	8/8	9/9	8/8
IL18	33/33	Yes	8/8	8/8	9/9	8/8
IL1B	33/33	Yes	8/8	8/8	9/9	8/8
IL2	31/33	Yes	8/8	7/8	8/9	8/8
IL21	32/33	Yes	8/8	7/8	9/9	8/8
IL22	13/33	No	4/8	2/8	1/9	5/8
IL23A	33/33	Yes	8/8	8/8	9/9	8/8
IL23R	26/33	Yes	8/8	6/8	5/9	7/8
IL25	0/33	No	0/8	0/8	0/9	0/8
IL27	0/33	No	0/8	0/8	0/9	0/8
IL3	0/33	No	0/8	0/8	0/9	0/8
IL4	6/33	No	2/8	0/8	2/9	2/8
IL5	33/33	Yes	8/8	8/8	9/9	8/8
IL6	25/33	Yes	7/8	4/8	9/9	5/8
IL6R	33/33	Yes	8/8	8/8	9/9	8/8
IL7R	33/33	Yes	8/8	8/8	9/9	8/8
IL8	33/33	Yes	8/8	8/8	9/9	8/8
ISG20	33/33	Yes	8/8	8/8	9/9	8/8
JAK1	33/33	Yes	8/8	8/8	9/9	8/8
JAK2	33/33	Yes	8/8	8/8	9/9	8/8
MMP13	33/33	Yes	8/8	8/8	9/9	8/8
MMP3	33/33	Yes	8/8	8/8	9/9	8/8
MMP9	33/33	Yes	8/8	8/8	9/9	8/8
NFATC2	33/33	Yes	8/8	8/8	9/9	8/8
NFKB1	33/33	Yes	8/8	8/8	9/9	8/8
RORC	33/33	Yes	8/8	8/8	9/9	8/8
SOCS1	33/33	Yes	8/8	8/8	9/9	8/8
SOCS3	33/33	Yes	8/8	8/8	9/9	8/8

STAT3	33/33	Yes	8/8	8/8	9/9	8/8
STAT4	33/33	Yes	8/8	8/8	9/9	8/8
STAT5A	33/33	Yes	8/8	8/8	9/9	8/8
STAT6	33/33	Yes	8/8	8/8	9/9	8/8
SYK	33/33	Yes	8/8	8/8	9/9	8/8
TBX21	33/33	Yes	8/8	8/8	9/9	8/8
TGFB1	33/33	Yes	8/8	8/8	9/9	8/8
TIRAP	33/33	Yes	8/8	8/8	9/9	8/8
TLR4	33/33	Yes	8/8	8/8	9/9	8/8
TNF	33/33	Yes	8/8	8/8	9/9	8/8
TRAF6	33/33	Yes	8/8	8/8	9/9	8/8
YY1	33/33	Yes	8/8	8/8	9/9	8/8

Supplementary Table 2. Th17 associated gene expression levels in small intestinal biopsies from children with potential celiac disease (CD) (pot-CD), untreated CD (CD), gluten-free diet (GFD) treated CD (GFD-CD) and references (Ref). Mann-Whitney U test (M-W) was used for comparison of the gene expression levels (ΔCt values) between the groups, p-values <0.05 were considered significant and p-values <0.10 were considered as trends. Genes marked in bold displays differentially expressed genes between the groups; statistically (based on ΔCt -values) and with fold change values ($2^{\Delta\text{median } \Delta\Delta\text{Ct}}$) passing the cut off levels at: $-1.4 \geq x \geq 1.4$.

	<i>Ref (TGA-)</i>	<i>Pot-CD</i>	<i>CD</i>	<i>GFD- CD</i>	<i>Pot-CD vs Ref (TGA-)</i>	<i>CD vs Ref (TGA-)</i>	<i>GFD- CD vs Ref (TGA-)</i>	<i>CD vs Pot-CD</i>	<i>GFD- CD vs Pot-CD</i>	<i>GFD- CD vs CD</i>
Gene symbol	Median ΔCt	Median ΔCt	Median ΔCt	Median ΔCt	M-W	M-W	M-W	M-W	M-W	M-W
CACYBP	3.1	3.0	2.4	3.3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CCL2	5.3	5.8	4.6	5.1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CCL20	5.1	6.4	6.3	5.5	0.06	n.s.	n.s.	n.s.	n.s.	n.s.
CCL22	7.0	7.8	7.9	7.4	n.s.	0.02	n.s.	n.s.	n.s.	n.s.
CD247	4.6	4.8	6.1	5.1	n.s.	0.04	0.05	n.s.	n.s.	n.s.
CD34	6.1	6.0	6.1	6.2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CD3D	3.5	2.8	2.5	1.9	n.s.	0.03	0.02	n.s.	n.s.	n.s.
CD3E	5.0	4.8	5.2	4.6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CD3G	5.4	4.4	4.5	3.6	0.07	0.01	0.05	n.s.	n.s.	n.s.
CD4	7.7	8.3	9.0	7.9	n.s.	0.007	n.s.	0.04	n.s.	0.03
CD40LG	8.4	9.4	10.3	9.0	n.s.	0.06	n.s.	n.s.	n.s.	n.s.
CD8A	8.6	6.6	6.9	6.2	0.04	0.02	0.02	n.s.	n.s.	n.s.
CEBPB	6.3	6.8	6.3	6.4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CLEC7A	9.5	10.6	9.8	9.3	0.05	n.s.	n.s.	n.s.	0.03	0.07
CSF2	11.7	13.1	12.7	12.0	0.05	0.10	n.s.	n.s.	n.s.	n.s.
CX3CL1	9.8	9.8	9.9	9.6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CXCL1	5.8	5.7	5.8	5.5	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CXCL12	5.9	5.8	6.4	5.3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CXCL2	10.2	11.7	10.2	10.2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CXCL5	13.5	13.5	12.7	12.8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CXCL6	7.2	6.7	6.9	6.3	n.s.	n.s.	n.s.	n.s.	n.s.	0.10
EDG1	6.0	6.1	6.3	6.1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
FOXP3	12.4	13.4	12.2	12.9	0.006	n.s.	n.s.	0.007	0.08	n.s.
GATA3	12.2	12.0	12.4	12.3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ICAM1	5.4	5.8	5.2	5.4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ICOS	10.6	11.8	11.3	11.0	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IFNG	8.2	8.0	5.4	6.2	n.s.	0.001	0.04	n.s.	n.s.	n.s.
IL10	9.4	10.1	9.4	10.3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL12RB1	7.6	7.6	7.7	6.9	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL12RB2	10.0	9.5	9.2	10.6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL13	13.9	13.1	13.2	13.7	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL15	4.9	5.3	4.8	4.6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL17C	12.0	12.1	11.5	12.1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL17F	12.4	13.7	12.5	13.6	0.06	n.s.	n.s.	n.s.	n.s.	n.s.
IL17RB	5.4	4.9	5.1	5.2	n.s.	0.10	n.s.	n.s.	n.s.	n.s.
IL17RC	6.5	5.7	6.7	5.6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL17RD	10.8	10.1	11.3	11.1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL17RE	4.4	4.1	5.0	3.9	0.10	0.05	0.09	0.007	n.s.	0.004
IL18	3.9	3.8	4.8	3.7	n.s.	0.001	n.s.	0.001	n.s.	0.008

IL1B	8.5	8.8	9.1	8.6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL2	10.0	9.6	11.4	10.1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL21	10.1	12.8	11.3	12.3	0.009	n.s.	0.01	0.08	n.s.	n.s.
IL23A	9.1	9.4	9.2	9.6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL23R	11.7	13.5	13.1	12.3	0.09	0.003	n.s.	n.s.	n.s.	0.02
IL5	11.6	11.2	11.5	10.8	n.s.	n.s.	0.09	n.s.	n.s.	n.s.
IL6	11.5	13.7	11.4	12.6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL6R	5.6	5.8	6.2	5.1	n.s.	n.s.	n.s.	n.s.	0.09	0.03
IL7R	8.8	8.3	9.6	8.2	n.s.	n.s.	n.s.	n.s.	n.s.	0.04
IL8	7.7	8.0	7.2	8.5	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ISG20	2.2	2.2	2.3	2.1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
JAK1	2.3	2.1	2.4	2.4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
JAK2	5.5	5.8	4.9	5.2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
MMP13	10.9	11.4	9.6	10.7	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
MMP3	6.9	6.7	5.5	6.7	n.s.	0.02	n.s.	0.07	n.s.	n.s.
MMP9	6.9	8.6	8.5	7.7	0.05	0.03	n.s.	n.s.	n.s.	n.s.
NFATC2	9.2	8.5	10.2	9.1	n.s.	0.04	n.s.	0.003	n.s.	0.05
NFKB1	3.3	3.1	3.0	3.3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
RORC	6.6	6.4	7.5	6.0	n.s.	0.002	n.s.	0.004	n.s.	0.002
SOCS1	6.9	6.4	7.0	7.1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SOCS3	7.2	7.3	6.4	6.8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
STAT3	2.7	2.9	2.7	2.7	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
STAT4	6.4	6.0	6.9	5.9	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
STAT5A	6.0	6.0	6.6	6.4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
STAT6	2.5	2.7	3.5	2.6	n.s.	0.006	n.s.	0.07	n.s.	0.005
SYK	6.8	6.6	7.4	6.0	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TBX21	8.6	8.1	8.9	8.1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TGFB1	5.0	4.8	5.5	4.8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TIRAP	8.4	8.2	8.7	7.8	n.s.	n.s.	n.s.	0.10	n.s.	0.02
TLR4	7.3	7.9	7.6	7.5	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TNF	6.7	7.2	7.3	7.0	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TRAF6	5.3	5.8	5.9	5.5	0.002	0.001	n.s.	n.s.	n.s.	0.10
YY1	3.7	3.7	3.4	3.5	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.