A functional soluble form of CTLA-4 is present in the serum of celiac patients and correlates with mucosal injury

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Abstract
Celiac disease (CD) is a multifactorial disorder influenced by environmental, genetic and immunological factors. Increasing evidence showed CTLA-4 gene as an important susceptibility locus for autoimmune disorders. A native soluble cytotoxic T-lymphocyte-associated protein-4 (sCTLA-4), lacking of transmembrane sequence, has been described in several autoimmune diseases. We aimed to evaluate the presence of increased sCTLA-4 concentration in the serum of patients with CD and the possible immunoregulatory function. Blood samples were collected from 160 CD patients; sCTLA-4 levels were evaluated by ELISA, western blot and reverse transcription–PCR. The capability of serum sCTLA-4 to modulate T-lymphocyte proliferation in vitro was evaluated by two-way mixed leukocyte reaction assay. We demonstrated high levels of sCTLA-4 in serum of untreated celiac patients. Additionally, we observed that sCTLA-4 concentrations are related to gluten intake and that a correlation between autoantibodies to tissue transglutaminase and sCTLA-4 concentration exists. Moreover, sCTLA-4 levels correlate with the degree of mucosal damage. Conversely, no correlation between sCTLA4 levels and the HLA-related risk was observed. Finally, we show that sCTLA-4 from sera of CD patients displays functional activities. These results strongly suggest a regulation of sCTLA-4 synthesis depending on the presence or absence of dietary gluten and imply a possible immunomodulatory effect on cytotoxic T lymphocyte functions. In gluten-exposed patients, serum sCTLA-4 levels might provide insight about mucosal injury.

Introduction
Celiac disease (CD) is an immune-mediated enteropathy, triggered by the ingestion of wheat, rye and barley gluten proteins in genetically susceptible individuals. The pathological lesion is characterized by flattened small intestinal mucosa with villous atrophy, crypt cell hyperplasia and increased number of intra-epithelial lymphocytes (1) and results in nutrient malabsorption of variable degree. Although formerly thought to be uncommon, greater awareness of its multifaceted clinical presentation and the availability of accurate serologic tests led to the realization that CD is relatively common: large studies in the United States and Europe show the prevalence of the disease to approach 1% (2–6).

CD is a multifactorial disorder that is influenced by both environmental and genetic factors: an autoimmune reaction against the enzyme tissue transglutaminase (TG), which is able to deaminate gliadin, is nearby constantly observed (7).

A significant part of the genetic component involved in CD susceptibility maps to the HLA class II region of the MHC. Virtually all the patients with CD express a HLA class II DQ2 and/or DQ8 haplotype (8). However, such haplotypes,
especially DQ2, are very common in general population in western countries (>30% prevalence for DQ2) (8–11). Evidence has been provided that predisposing HLA haplotypes encode for molecular products allowing an efficient presentation of transglutaminase/gladiin peptides to T cells (12).

The CTLA-4 gene, located on chromosome 2q33, is a likely candidate gene for autoimmune diseases because of its role in controlling the T cell proliferative response. It encodes a T lymphocyte surface molecule whose binding to B7 molecules delivers a negative signal to the T cell and can mediate its apoptosis (13, 14). B7 molecules, namely CD80 and CD86, are expressed on professional antigen-presenting cells, such as mature dendritic cells (including Langherans cells), monocytes and also B lymphocytes (15).

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-deficient mice develop a lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction (13, 14). Association of type-1 diabetes, Graves’ disease and CD with a point mutation in exon 1 of CTLA-4 (position 49A/G) leading to a Thr/Ala substitution in the leader peptide has been described in several different populations (16–22). Moreover, haplotypes in the CTLA-4 region have been proved to be associated with CD in different ethnic groups including Italian (23–26).

A native soluble cytotoxic T-lymphocyte-associated protein-4 (sCTLA-4), deriving from lack of transmembrane sequence, has been described (27). The presence of high concentrations of sCTLA-4 was observed in sera of patients with autoimmune thyroid diseases (27, 28), as well as in patients with other autoimmune diseases, such as type-1 diabetes (29), diffuse cutaneous systemic sclerosis (30), systemic lupus erythematosus (31) and myasthenia gravis (32). In addition, raised plasma levels of sCTLA-4 were observed in patients with allergic asthma (33) and allergy to hymenoptera venom but not in allergic rhinitis (34).

sCTLA-4 may have important immunoregulatory functions (27, 28). The effect of sCTLA-4 binding to CD80/CD86 molecules might depend on the activation state of the cells involved, interfering with T cell co-stimulation and with T cell responses. Thus, sCTLA4 might act indirectly both as inhibitor or as enhancer of the immune response (28).

The functional role of the sCTLA-4 molecule prompted us to evaluate its presence in patients with CD and the regulation of sCTLA-4 synthesis during CD outcome, namely, before and after gluten-free dietary regimen (which results in complete regression of immune-mediated enteropathy). We fore and after gluten-free dietary regimen (which results in complete regression of immune-mediated enteropathy). We observed markedly increased levels of sCTLA-4 in the serum in patients with untreated CD with respect to controls. Moreover, we observed a correlation between serum concentration of sCTLA-4 and IgA antibodies directed against tTG, as well as the degree of villous atrophy in patient with active CD. Furthermore, we show that serum sCTLA-4 is able to bind CD80/CD86 counter receptors and to modulate T lymphocyte proliferation in vitro.

Methods

Patients

Blood samples were collected from 160 CD patients, all of whom provided written informed consent. The research was approved by the Ethics Committee of the School of Medicine, University of Naples ‘Federico II’, Italy, and was in accordance with the principles of the Helsinki II declaration. All were diagnosed from 2002 to 2005. The diagnosis was based on European Society for Paediatric Gastroenterology, Hepatology and Nutrition criteria (35). Modified Marsh classification was used for the characterization of histopathology (T0, normal lymphocyte infiltration and villous atrophy; T1, >30% increased intra-epithelial lymphocyte infiltration, lymphocytic enteritis; T2, T1 with crypt hyperplasia; T3a, T2 with partial villous atrophy; T3b, T2 with subtotal villous atrophy and T3c, T2 with total villous atrophy) (36). Anti-tissue transglutaminase IgA (anti-tTG IgA) was determined by commercial ELISA using microplates coated with recombinant human antigen (Eurospital Diagnostics, Trieste, Italy), as previously described (6). Anti-tTG IgA was above threshold in 71 of 75 CD patients at diagnosis. IgA deficiency was found in two of four anti-tTG IgA-negative subjects (both were positive for tTG IgG autoantibodies). The other two tTG-negative patients were <2 years old and positive for anti-gliadin autoantibodies (evaluated by ELISA).

Seventy-five patients were in active phase of CD (age range 0.8–71). Fifty of them were adult, i.e. >18 years, and the large majority (40/50) was poorly symptomatic and presented mainly anemia or reduced bone mineral density. Eighty-five patients were in remission under gluten-free diet (age range 16–48). From eight patients, serum samples collected at different time points, namely, before and 12 months after gluten-free diet were available. Remission of disease was documented by disappearance of autoantibodies and in a few cases by normalization of histology on duodenal biopsies. The control groups consisted of 45 blood donor volunteers (age range 19–45) and 26 patients with gluten-unrelated enteropathies (infectious enteritis, a specific cholelithiasis, severe food intolerances (age range 11–25) and not afflicted by other autoimmune diseases. Sera were stored frozen until the use and freezing and thawing was avoided.

Genotyping

Genomic DNA was extracted from EDTA + blood samples with a commercially available Kit (Nucleon BACC 2; Amersham Biosciences Europe, Milan, Italy). HLA-DQ typing and HLA-DR typing were performed as previously described (35). All the statistical methods utilized for estimation of control DQA1–DQB1 haplotype frequencies and estimation of the genotypic group risks were previously described and published (35). Thus, based on these previously published data, it was possible to extrapolate a risk of CD according to genotypic group and to classify the CD cohort in five classes. DQ2 carriers could be separated into three groups: with two copies of DQB1*02 (group G1), one copy of DQB1*02 acting in trans with DQA1*05 (group G2) or one copy of DQB1*02 acting in cis with DQA1*05 (group G3). DQ2 non-carriers were divided into two groups: the first with two copies of a DQB1*02. DQ8 or one copy of each (group G4) and the last with only one at-risk allele (group G5). In all populations, the risk is highest for group G1, but the relative risks for the other genotypes vary from one population to another. In the Italian population, the relative risks for
individuals belonging to groups G2, G3, G4 and G5 are 0.68, 0.23, 0.10 and 0.02, respectively (37).

**ELISA**

Specific ELISA kits were used for measuring serum sCTLA-4 levels (Bender Med System, Prodotti Gianni SpA, Milano, Italy), according to the manufacturer’s protocol. Each sample was diluted 1:10 and tested in triplicate. Deviation between triplicates was <10% for any reported value. The lowest sensitivity threshold was 0.1 ng ml⁻¹.

The analytical response was linear approximately between 0.162 and 1.200 of absorbance values (corresponding to 0.1–50 ng ml⁻¹) as assessed by serial dilution test using a strongly positive serum (data not shown). For samples with sCTLA-4 concentration >50 ng ml⁻¹, the ELISA tests were repeated using a greater dilution factor (1:100).

**Western blotting**

Western blotting was used to detect serum sCTLA-4. Proteins were separated by 10–20% gradient PAGE in a discontinuous buffer system on a Mini-Protein system (Bio-Rad, Segrate, Milano, Italy). The separated components were electoblotted onto polyvinylidene fluoride membranes. The blots were washed with 0.15 M NaCl, 0.05 M Tris (Tris-buffered saline), pH 7.5, with 0.3% Tween 20 and reacted with a 1:100 dilution of the anti-sCTLA-4 mAb (clone 14D3, IgG2a, eBiosciences, San Diego, CA, USA) for 1 h at room temperature, washed and then reacted with reporter antibody (HRP-conjugated anti-mouse IgG) for 1 h. The blots were then developed by the use of a commercially available chemiluminescence detection kit (BMB, Indianapolis, IN, USA) according to the manufacturer’s instructions.

**Mixed leukocyte reactions and cell labeling**

Two-way mixed leukocyte reaction (MLR) was used to test the immunoregulatory activity of sCTLA-4. The cells used in these experiments were obtained from a panel of previously HLA-typed laboratory volunteers and were selected to provide two HLA-DR mismatches. PBMCs were isolated by standard Ficoll–Hypaque density gradient centrifugation. Fluorescent labeling of cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) was performed as described previously (38). Briefly, PBMCs were incubated with CFSE in PBS at a final concentration of 2 μM for 3 min, and equal numbers of cells (5 × 10⁵) from two donors were co-cultured in tissue culture medium (RPMI 1640 supplemented with 10% human AB serum, 10 mg ml⁻¹ gentamicin and 50 mM 2-mercaptoethanol). Sera were added at the beginning of the test as indicated. When indicated, cultures contained CTLA-4Ig were utilized as control. Cultures were set up in 1.5 ml volumes in 24-well microculture plates and were incubated for 5 days at 37°C and 5% CO₂ in air. Proliferation (CFSE fluorescence) was assessed by flow cytometry (FACScalibur flow cytometer using CellQuest software, Becton Dickinson) at day 5.

**Depletion of sCTLA-4 protein from sera**

In order to understand the effective role played by serum sCTLA-4 avoiding the effect related to other serum proteins, we performed a MLR assay using sera previously depleted of sCTLA-4. To this end, we incubated the sera from different patients (two positive and two negative after ELISA testing) for 3 h at room temperature on ELISA plates coated with the capture mAb anti-sCTLA-4 (included in the ELISA kit). This procedure was repeated twice before utilizing these sera in a MLR assay. The negativity was assessed by ELISA tests. As a control, we used sera not subject to the depletion procedure.

**mRNA analysis by reverse transcription–PCR**

Total RNA was extracted using RNAse Mini kit (QIAGEN, Milano, Italy) according to the manufacturer’s recommendations. The single-stranded cDNA was synthesized using 1 μg of total RNA by reverse transcription by incubating it at 42°C for 1 h with 20 pmol of oligo(dT) (Roche Molecular Biochemicals, Basel, Switzerland), 500 μM dNTPs (Roche Molecular Biochemicals), 30 U of RNase inhibitor (5 Prime 3 Prime, Boulder, CO, USA) and 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) in a total volume of 20 μl.

PCR reactions were performed with cDNA and primers designed to amplify the entire coding sequence of CTLA-4: 5’-ATGGCTGCGTGGATTTGTCGGGCCAAGG-3’ and 5’-CAATTGGTGGGAAATAAAAAAGGTGAATTG-3’ (predicted size: 672 bp). PCR reaction was as follows: 94°C for 5 min, 30 cycles 94°C for 1 min, 60°C for 1 min and 72°C for 1 min followed by a final extension at 72°C for 5 min. The amplified fragments were separated on 1% agarose gel and visualized by ethidium bromide. As controls, the glyceraldehyde-3-phosphate dehydrogenase primers were used: 5’-ACATC-GTCAAGACCTATGGG-3’ and 5’-GGGTCTACATGGCAACTGGAG-3’.

**Statistical analysis**

Statistical analysis was performed using the Mann–Whitney U-test for comparison of sCTLA-4 levels. The Wilcoxon test was carried out to analyze the differences in sCTLA-4 production before and after gluten-free diet. Spearman regression analysis was used to evaluate the correlation between sCTLA-4 and such as tTG IgA or histological grading. The possible relation between HLA-related genetic risk and sCTLA-4, as well the relation between histological damage and sCTLA-4 and between histological damage and anti-tTG IgA, was evaluated by Newman–Keuls multiple comparison test. A P value of <0.05 was considered statistically significant. All the analyses were performed by using the GraphPad Prism4 software 4.0 (GraphPad Software Inc., CA, USA).

**Results**

sCTLA-4 is present in sera from CD patients and its level is related to autoantibodies, histology and the dietary regimen

We verified the presence of the sCTLA-4 in sera from CD patients by using a sensitive ELISA. In Fig. 1(A), we show the comparison among CD, non-celiac enteropathy patients and normal apparently healthy volunteers. sCTLA-4 levels were markedly raised in the majority of CD patients at diagnosis, significantly higher (P < 0.001) with respect to controls. Only 3 of 45 healthy subjects and 8 of 26 non-celiac enteropathy patients had detectable sCTLA-4. No correlation...
was found between sCTLA-4 levels and age or presence of symptoms.

We confirmed the presence of sCTLA-4 mRNA in PBMCs isolated from CD patients. As shown in Fig. 1(C), an mRNA form coding for sCTLA-4 was evident, suggesting that the molecule we measured in ELISA tests was a result of an alternative splicing.

Next, we spread CD patients in two subgroups: patients with active disease (n = 75) and positive anti-tTG IgA autoantibodies (>9 ng ml⁻¹) and subjects in remission under gluten-free diet (n = 85) and negative tTG autoantibodies (<9 ng ml⁻¹). The results obtained evaluating serum sCTLA-4 concentrations in the two subgroups demonstrated a different ability in producing sCTLA-4. As shown in Fig. 2, serum levels of sCTLA-4 were significantly higher in patients with active CD than in remission (range 0.0–96.4 ng ml⁻¹ and 0.0–48.1 ng ml⁻¹, respectively, P = 0.048). Of interest, a longitudinal study analyzing sera collected from the same patients before and 12 months after starting gluten-free diet showed a clear variation of the amount of sCTLA-4, namely, patients who showed high levels of the soluble spliced form at diagnosis showed nearly undetectable sCTLA-4 after diet. No modification was observed in three CD patients with low sCTLA-4 levels at diagnosis (Table 1) did not show any bands. Moreover, these molecular species were absent in sera resulted negative at ELISA assay (24C and 41C).

Furthermore, a correlation was observed in CD patients between serum sCTLA-4 levels and tTG autoantibody concentrations (Spearman r = 0.2967, P = 0.0002; Fig. 3A). The correlation was significant as well when only patients with active CD were considered (Fig. 3B).

Noteworthy, a significant positive correlation was observed when serum sCTLA-4 levels were compared with histological grading according to modified Marsh classification (Spearman r = 0.5455, P < 0.0001; Fig. 3C).

Serum sCTLA-4 levels do not correlate with HLA-related genetic risk

Using large samples of patients from four European countries, we have recently shown that the genetic risk that individuals will develop CD could be stratified into five classes according to their HLA-DQ genotype (37).

According to these results, we have analyzed the possible correlation among the different classes of risk and the serum sCTLA-4 levels. As shown in Fig. 4, we could not observe any positive statistical differences among the different groups. Thus, we can hypothesize that the ability of different CD patients to produce sCTLA-4 is not related to HLA-DQ genotype.

Serum sCTLA-4 from celiac patients is able to regulate T cell proliferation in vitro

To further examine the role of sera sCTLA-4 on proliferation of T lymphocytes, we performed two-way MLR experiments,
where PBMCs were labeled with CFSE, either in the absence or in the presence of sera containing sCTLA-4. On day 5 after stimulation, cell division was assessed by measurement of CFSE dilution (Fig. 5). Growth of antigen-experienced T cell populations varied depending on the mode of stimulation and was higher for cells in the case of stimulation with plain media. In the presence of sera resulted positive for sCTLA-4 during ELISA analyses (42C and 45C), significantly decreased growth of T lymphocytes was described. Similar results were obtained when CTLA-4Ig was added to the test (as positive control of inhibition of T cell growth). However, in the presence of sera negative for sCTLA-4 (either sera from patient that become negative after the diet regiment, 42C and 45C, and sera from patients negative from the beginning of the study, 27C and 24C), no effect was measurable on T cell proliferation (Fig. 5).

In addition, we performed the same test using sera previously depleted of sCTLA-4. The results showed that these sera (42C and 45C) are ineffective on MLR-induced proliferation (Fig. 5). This suggests a direct role of sCTLA-4 in regulating lymphocytes proliferation and rules out the intervention of putative different serum molecules. Similar results were obtained with sera resulted negative for sCTLA-4 (27C and 24C) also before the depletion procedure (Fig. 5).

Thus, serum sCTLA-4 is able to down-regulate the proliferative ability of T lymphocytes in vitro.

Discussion

CD has been considered as a typical model of disease in which T lymphocytes act as effector of tissue damage (39). The strict genetic association between HLA-DQ2/8 alleles and CD (40) and the studies which have revealed the molecular mechanisms that control post-translational modification of gliadin immunodominant epitopes have strengthen this view (37). Though T cells are the key elements in the induction and progression of CD, their activation must be preceded by an engagement/activation of the innate immune system. The role of resident professional antigen-presenting cells is indeed essential for antigen recognition and T cell activation in CD. This function is operated by tissue myeloid derived cells (usually dendritic cells) which process antigen and deliver the appropriate co-stimulatory signal to T cells.

CTLA-4 is an important negative regulator of T-cell activation and proliferation, interacting with B7 molecules on antigen-presenting cells. It is therefore a plausible candidate as a susceptibility gene in diseases with T-cell mediated pathogenesis. Genetic linkage or association with CD has been consistently reported in a number of studies (21–26). In addition, the CT60 A/G dimorphism of CTLA4 was recently described in patients with both CD and autoimmune thyroid disease (41).

Several papers (26, 39) have described an alternate transcript of the CTLA-4 gene that encodes a protein that lacks a transmembrane region and likely represents a native sCTLA-4. The presence of sCTLA-4 was demonstrated in the sera of several patients with autoimmune thyroid disease, including Graves’ disease and Hashimoto’s thyroiditis (26, 27, 42).

The present study provides the first evidence that serum sCTLA-4 levels are elevated in CD patients. The presence of sCTLA-4 in sera from CD patients was confirmed by western blot tests. Moreover, the detection of the spliced/soluble variant by reverse transcription-PCR in PBMCs from CD patients suggests that the sCTLA-4 measured by ELISA does not result from a cleavage of the full-length form.

Furthermore, we demonstrated, using a two-way MLR system, that sCTLA-4-containing sera are able to

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<th>Time points</th>
<th>Patient sCTLA-4 evaluation (ng ml⁻¹)</th>
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<td></td>
<td>45C 42C 43C 44C 9C 24C 27C 41C</td>
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<tr>
<td>Pre-diet</td>
<td>&gt;50 13.0 8.9 3.0 1.5 &lt;0.1 &lt;0.1 &lt;0.1</td>
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modulate such in vitro immune reaction and that this effect is specifically related to sCTLA-4 molecule. This is consistent with the hypothesis that sCTLA-4 is functionally relevant in CD.

It is apparent that the antigen intake with the dietary regimen plays important roles both in the initiation and development of CD and in inducing sCTLA-4 synthesis/release. In fact in follow-up analyses, all the tested sera that were positive for sCTLA-4 at diagnosis became negative after gluten-free diet. Of course, this aspect needs confirmation/verification on a greater number of cases.

These results may appear as somehow conflicting with those we described in autoimmune thyroid disease, where the levels of sCTLA-4 were not related to disease evolution during time (27). In addition, these findings seem to be conflicting with the hypothesis that the ability to produce sCTLA-4 is ‘constituitive’ and related to genetic features (26, 43).

As a matter of fact, a complex genetic control of sCTLA-4 production, involving not only CTLA-4 gene but also other related polymorphic genes, such as ICOS, has been envisaged (44). We could suppose that the majority of CD patients are able to produce high levels of sCTLA-4 under certain conditions, such as the increase of antigen-specific activated T cells. In the light of such a hypothesis, the difference with respect to thyroid autoimmunity could be easily explained: in autoimmune thyroid diseases, with the improving of clinical manifestations following therapy, the triggering self-antigens are still present for a long time, and thyroid autoantibody positivity tends to persist for years even after thyroid ablation (45). In CD, instead, the autoimmune reaction is driven by an exogenous antigen that totally disappears when dietary gluten is removed.

Our data clearly show that patients with untreated CD display sCTLA4 concentrations significantly higher than controls; however, the values in single patient were markedly variable, ranging from 96.5 ng ml\(^{-1}\) to the detection limit of the ELISA assay. In an attempt to investigate the nature of the possible difference between sCTLA-4 producers and non-producers, we evaluated the possible relationship between serum sCTLA4 (measured under gluten exposure) and HLA-DQ genotypes by grouping the patients according to the presence/combination of different DQ2 and/or DQ8 alleles (37). The results obtained were negative; therefore, it is likely that the differences between sCTLA-4 producers and non-producers are not related to HLA genotype. Of note, the relationship between the ability to produce sCTLA4 and the CTLA4 polymorphisms observed in some autoimmune diseases is not fully clarified (21, 46). Notably, no correlation has been recently found between CT60 (rs3087243), +49 A/G (rs231775) and ~318 (rs5742909) single nucleotide polymorphisms and sCTLA-4 production (47). As above mentioned, a role of other related genes is likely, although no data are available to date.
It is interesting to note from our study that a correlation of anti-tTG IgA with sCTLA-4 concentrations existed throughout the entire CD patient cohort (including both in active phase and in remission). Moreover, a similar positive correlation was evident among the subgroup with active CD. Thus, sCTLA-4 appears to be related to autoantibody production per se, independently from the presence or absence of dietary gluten. As previously discussed, sCTLA-4 could play a critical role in modulating the immune response, especially in the early stage (26, 27, 48, 49). The immunomodulatory effect of sCTLA-4 could be involved in the regulation of B cell activation directly or via T helper function modulation. The above observations suggest that increased production and subsequent secretion of sCTLA-4 into the plasma may start in the very early phase of the disease. Even more importantly, the finding of a significant positive correlation between the amount of serum sCTLA-4 and the grade of gut mucosa damage (graded as Marsh classification) strongly suggests a possible immunomodulatory effect of this soluble molecule on cytotoxic T lymphocyte functions.

From a clinical point of view, this finding may be of special interest in that raised serum sCTLA-4 concentrations might provide insight about possible mucosal damage in patients with suspect of CD.

Actin autoantibodies, which are detectable in a proportion of patients with untreated CD (50), were reported to correlate quite closely with gut mucosal damage. However, their sensitivity and specificity are far lower with respect to tTG, and their additive pathophysiological and clinical meaning with respect to tTG (51) has been recently questioned (52). Thus, additive non-invasive markers of mucosal injury would be welcome.

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**Abbreviations**

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<th>Acronym</th>
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<tr>
<td>anti-tTG IgA</td>
<td>anti-tissue transglutaminase IgA</td>
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<td>CD</td>
<td>Celiac disease</td>
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<td>CFSE</td>
<td>carboxyfluorescein diacetate succinimidyl ester</td>
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<td>CTLA-4</td>
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<td>sCTLA-4</td>
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<td>tTG</td>
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**References**

Soluble CTLA-4 and celiac disease


