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Impaired Control of Effector T cells by Regulatory T Cells: a Clue to Loss of Oral Tolerance and Autoimmunity in Celiac Disease?

Running title: Defective responses to regulatory T cells in celiac disease

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Key words: Celiac disease, Regulatory T cells, IL-15

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ABSTRACT:

Objectives. Regulatory T cells (Tregs) are instrumental for tolerance to self-antigens and dietary proteins. We have previously shown that IL-15, a cytokine overexpressed in the intestine of patients with celiac disease (CD), does not impair the generation of functional Tregs but renders human T cells resistant to Treg suppression. Treg numbers and responses of intestinal and peripheral T lymphocytes to suppression by Tregs were therefore compared in CD patients and controls.

Methods. Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated from duodenal biopsies of CD patients and controls. Concomitantly, CD4+CD25+ T lymphocytes (Tregs) were purified from blood. Responses of IEL and of LPL and PBL to suppression by Tregs were tested by analysing anti-CD3-induced proliferation and IFN- γ production in the presence or not of peripheral Tregs. *Lamina propria* and peripheral CD4+CD25+FOXP3+ T cells were assessed by flow cytometry.

Results. While percentages of CD4+CD25+FOXP3+ LPL were significantly increased in patients with active CD, proliferation and IFN- γ production of intestinal T lymphocytes were significantly less inhibited by autologous or heterologous Tregs in CD patients than in controls ($p < 0.01$). In all tested CD patients, IEL were unable to respond to Tregs. Resistance of LPL and PBL to Tregs suppression was observed in patients with villous atrophy who had serum levels of IL-15 significantly enhanced compared to patients without villous atrophy and to controls.

Conclusion. Our results indicate that effector T lymphocytes from active CD become resistant to suppression by Tregs. This resistance might cause loss of tolerance to gluten but also to self-antigens.

Word count : 261

Study highlights:

WHAT IS CURRENT KNOWLEDGE:

- IL-15 is overexpressed in the intestine of celiac disease patients.
- One important mechanism to achieve and maintain tolerance to dietary proteins relies on the generation and activation of CD4⁺CD25⁺FOXP3⁺ T regulatory cells.
- IL-15 renders *in vitro* conventional lymphocytes resistant to suppressive functions of regulatory T cells.
- In active celiac disease, regulatory T cells are increased in the intestine and can suppress activation of conventional lymphocytes.

WHAT IS NEW HERE:

- Suppression of *lamina propria* T lymphocytes by peripheral autologous and heterologous Tregs is impaired in celiac disease patients and the level of inhibition varies depending on the presence or not of intestinal lesions.
- Intraepithelial lymphocytes of both active and treated celiac disease patients are resistant to the inhibitory effects of peripheral Tregs.

INTRODUCTION

Celiac disease (CD) is a small intestinal enteropathy induced by cereal-derived prolamins (gluten) in genetically susceptible individuals. Activation of *lamina propria* gliadin-specific CD4⁺ T cells is a keystone of CD pathogenesis and results from the selective interplay between prolamins, the triggering environmental factor, and HLA-DQ2/8 molecules, the main genetic risk factor (1). This mechanism, although necessary, is however not sufficient to explain the loss of tolerance to dietary gluten in only a subset of individuals with at risk HLA. Furthermore, humanized mice engineered to express human HLA-DQ8 mount efficient CD4⁺ responses to gluten but do not develop intestinal lesions (2). One important mechanism to achieve and maintain tolerance to dietary proteins relies on the generation and activation of CD4⁺CD25⁺FOXP3⁺ T regulatory cells (Tregs), a subset of lymphocytes initially identified by Sakaguchi in mice for their role against autoimmunity (reviewed in (3)). In humans, patients with mutations affecting the *FOXP3* gene and lacking functional Tregs develop severe autoimmune diseases (4,5) but also dramatic food allergy (6). In mice, antigen feeding induce specific Tregs in gut-associated lymphoid tissues and in *lamina propria* (reviewed in (7)) and the depletion of such cells results in the loss of intestinal and peripheral tolerance to the corresponding food antigen (8). No genetic link of CD with the *FOXP3* gene has been observed (9) and previous studies have suggested that the number of lymphocytes with a Treg phenotype, evaluated in the peripheral blood by flow cytometry (10,11) and in the intestine by immunohistochemistry (12-15) was increased. Recent findings, however, indicate that IL-15, a cytokine overexpressed in the intestine and in the serum of active CD patients (16-18), might, at least in combination with retinoic acid, hamper *in vitro* generation of mouse Tregs in the presence of dendritic cells (19). Contrasting with the later finding, we have observed that IL-15 did not impair but rather promoted the generation of functional human Tregs in an *in vitro* assay without dendritic cells (20). Yet, following pre-incubation in the

presence of IL-15, human peripheral T lymphocytes became resistant to the suppressive functions of Tregs, a resistance that resulted from PI3 kinase activation (20). Altogether, these conflicting data led us to reevaluate by flow cytometry the numbers of Tregs in the peripheral blood and in the *lamina propria* of CD patients and, subsequently, to investigate the responses of intestinal and peripheral T cells of CD patients to Treg immunosuppressive effects.

MATERIAL AND METHODS

Patients and Controls

Twenty-four adult patients (age range, 22–50 years; mean age, 35 years) had uncomplicated CD. The diagnosis was based on detection of villous atrophy and anti-endomysium/transglutaminase antibodies. At the time of study, 15 patients had active CD with partial to total villous atrophy and 9 were on a gluten free diet (GFD). Six of the later patients strictly adhered to GFD (3 months – 7 years; mean, 4 years), had normal villous architecture and were negative for anti-endomysium antibodies, while three were partially compliant to the diet (4 months – 3 years; mean, 2 years), had areas of mild partial villous atrophy on all or some biopsy specimens and were positive for celiac antibodies (IgA anti-endomysium and anti-transglutaminase). All biopsies were obtained during endoscopy performed for diagnosis or follow-up purposes. Twenty-two patients were included as controls (age range, 30–37 years; mean, 41 years). These patients, with no family history of CD or other autoimmune diseases, underwent gastro-duodenal endoscopy for diagnostic purposes and had histologically normal small intestinal samples. Informed consent was obtained from all patients before the study. All experiments were approved by local ethics committee (Institute Pasteur of Tunis).

Lymphocyte Isolation

IELs and LPL were isolated from endoscopic samples as previously described (21, 22). Yield from biopsies was $0.3\text{-}0.8 \times 10^6$ for IEL and $0.5\text{-}1.3 \times 10^6$ for LPL. CD25 negative cells (0.35 to 1.1×10^6) were recovered from LPL after negative selection using magnetic beads (CD25 microbeads, Miltenyi Biotec, Bergisch-Gladbach, Germany). Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Hypaque gradient. For co-culture experiments, CD4+CD25+ (Tregs) and CD4+CD25- T cells were isolated in a two-step procedure using the CD4+CD25+ regulatory T-cell isolation kit (Miltenyi Biotec) according to manufacturer's instructions. Peripheral Tregs were collected with a purity ranging from 63% to 72%.

Flow cytometry analysis

The percentage of *lamina propria* CD4+CD25+FOXP3+ lymphocytes was analyzed by flow cytometry. LPL (2×10^5) were incubated with FITC, PE, PerPC or APC conjugated mAbs to human CD4, CD25, CD45 or with control isotypes (BD Biosciences, Le Pont de Claix, France) for 20 minutes at 4°C. For intracellular FOXP3 detection, cells were fixed, permeabilized using Human FOXP3 Buffer Set (BD Biosciences) and labelled with PE-conjugated anti-human or control isotype (BD Biosciences). Analyses were performed with a BD LSRII flow cytometer using the CELLQuest software (BD Biosciences). As *lamina propria* preparations can be contaminated by some epithelial cells, percentages of CD4+CD25+FOXP3+ lymphocytes were determined in the gate of hematopoietic cells labeled by the pan-anti-CD45 antibody.

Functional analysis

Pre-Print Version

The suppressive functions of peripheral Tregs were tested in co-culture experiments in RPMI 1640 medium supplemented with 10% AB human serum (Sigma, St Louis, MO), 1% sodium pyruvate, 1% non essential aminoacids, 1% HEPES buffer, 5×10^{-5} M/1 β -mercaptoethanol and 40 μ g/ml gentamycin, (Invitrogen, Cergy Pontoise, France). IEL, CD25-LPL and peripheral CD4+CD25- T subsets (10^5 /well) were plated on 96-well plates coated with anti-CD3 antibodies (UCHT1, BD Biosciences) at 2 μ g/ml and added with 1 or 0.25 $\times 10^5$ peripheral Tregs per well in a final volume of 200 μ l for 5 days. In most experiments cocultures were performed in autologous conditions. In two experiments, heterologous Tregs from controls or active CD were co-cultured with IEL, CD25- LPL and peripheral CD4+CD25- T subsets (10^5 /well) from active CD and controls, respectively.

Proliferative responses were assessed by measuring the uptake of [3H] thymidine (Amersham, Saclay, France) 18 hours after adding 0.4 μ Ci/well. IFN- γ production was analyzed on supernatants of cells using Human IFN- γ ELISA Set (BD Biosciences) according to manufacturer's instructions.

Analysis of FOXP3 mRNA expression by real time RT-PCR

Total RNA was extracted from duodenal biopsies using RNeasy Mini Kit (Qiagen) according to the manufacturer's procedure. The extracted RNA was then reverse transcribed using the Murine-Mooloney Leukemia Virus (MMLV) reverse transcriptase and random hexamers (Promega) according to standard procedure. FOXP3 mRNAs were quantified by real time PCR using available gene expressed assay[®] and Taqman PCR Master Mix (Applied Biosystems). Amplification was performed in cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute using an ABI PRISM 7500 sequence detection system. Data were normalized referring to the expression of an endogenous gene RPLP0 (ribosomal Protein, Large, PO) by calculating $2^{-\Delta\text{CT}}$, with ΔCT the difference in threshold cycles for target and reference.

Pre-Print Version

Statistical Analysis

Data obtained from the different groups of patients and healthy controls were compared by the non parametric Mann-Whitney U test. Statistical significance was assigned to a value of $p < 0.05$.

RESULTS AND DISCUSSION

CD4+CD25+FOXP3+ T cells are increased in the *lamina propria* of active CD patients

Real-time PCR analysis of intestinal biopsies demonstrated a significant increase in *FOXP3* mRNA levels in active CD compared to controls ($p < 0.0001$) and patients on a gluten-free diet ($p < 0.01$) (**Figure 1A**). Consistent with this finding, multicolor flow cytometry analysis showed a significantly higher percentage of CD4+CD25+FOXP3+ T lymphocytes among *lamina propria* CD45+ cells isolated from active CD patients than from controls ($p = 0.005$) (**Figure 1, panels B and C**). In contrast, the percentage CD4+CD25+FOXP3+ lymphocytes remained comparable in peripheral blood CD45+ cells from CD and controls ($p > 0.05$) (**Figure 1B**). The increased proportion of *lamina propria* lymphocytes with a Treg phenotype is in keeping with previous reports in CD (12-15) and argues against the hypothesis that a defect in the recruitment or in the *de novo* generation of intestinal Tregs may play a part in the pathogenesis of CD. These results are also consistent with very recent data showing that increased numbers of functional Tregs can be isolated from the *lamina propria* of patients with active CD (23).

Intestinal effector T cells fail to respond to regulatory T cells in CD patients.

The contrast between the intestinal expansion of CD4+CD25+FOXP3 T cells and the rupture of tolerance to dietary gluten in the intestine of active CD suggested that the latter cells may be impaired in their capacity to down-regulate local effector T cell functions or, conversely, that effector T cells may fail to respond to Tregs. To test whether intestinal lymphocytes from patients with CD were responsive or not to the suppressive effects of regulatory lymphocytes, blood samples and duodenal biopsies were obtained from 9 celiac disease patients and 9 controls. Peripheral blood CD4+CD25+ T cells (T regs) were then separated from CD4+CD25- peripheral lymphocytes (PBL) while IEL and LPL were isolated from endoscopic biopsies and CD25+ T cells removed from LPL to obtain a fraction of

CD25-LPL. The suppressive effects of autologous peripheral Tregs were tested on the proliferation and IFN- γ production of IEL, and of CD25- LPL and PBL stimulated with immobilized anti-CD3 antibody. With the idea in mind that the responses to Tregs may be variable within subsets of patients, we defined a “threshold of resistance” when the percentage of inhibition was below the 5th percentile of the control group. In two additional experiments, co-cultures were performed in heterologous conditions.

In all tested controls and CD patients, CD25- PBL and LPL proliferated and produced IFN- γ in response to anti-CD3 stimulation (**Figure 2 and Figure 3**). In controls, these responses were efficiently inhibited by peripheral autologous Tregs (**Figure 4**). In contrast, suppression of proliferation and IFN- γ production was significantly less when LPL from CD patients were co-cultured with autologous peripheral Tregs (**Figure 4**). Moreover, the level of inhibition varied depending on the presence or not of intestinal lesions. Thus, in the 6 CD patients with villous atrophy, no suppression was observed in co-cultures of LPL with autologous T regs while suppression was detected in the three CD patients on GFD who had recovered a normal villous architecture (**Figure 4**). Interestingly, inhibition of proliferation and IFN- γ production of CD25- PBL by autologous Tregs was also impaired in active CD patients. In addition, in one CD patient who did not adhere strictly to GFD and remained with partial villous atrophy, Tregs were unable to inhibit IFN- γ production by peripheral CD25-PBL despite efficient inhibition of their proliferation (**Figure 4**).

IEL proliferation and more particularly cytokine production in response to coated anti-CD3 antibody varied between individuals, a variability previously ascribed to the lack of accessory signals in this *in vitro* assay (24) or to the release of immunosuppressive cytokines by epithelial cells (25). Yet, no significant difference was observed between groups of patients and controls ($p > 0.05$). (**Figure 4 and data not shown**). In controls, the sensitivity of IEL to the inhibitory effect of autologous Tregs was variable and overall less than that of

PBL and LPL. Yet a striking finding was that, in all tested CD patients, IEL were completely resistant to the inhibitory effects of Tregs and the difference was highly significant when compared to controls (**p = 0.0005**). Altogether, these results indicated that, although the numbers of CD4+CD25+FOXP3+ T cells were increased in the intestine of active CD patients, these cells could not efficiently down-regulate effector T cell functions in the intestine but also in the periphery.

The fact that the suppressor effect of Tregs on LPL and PBL was comparable in patients on GFD with normal histology and in controls suggested that the defect was not primary but secondary to the inflammatory process. Moreover, that Tregs from patients on GFD and with normal histology could suppress LPL and PBL but not IEL, rather pointed to an abnormal response of effector cells. It was, however, difficult to ascertain that the defect lied at the level of effector T cells, since immuno-suppression had been tested in autologous conditions. To confirm this hypothesis, co-cultures were performed in heterologous conditions. As shown in figure 5, Tregs from active CD patients could efficiently down-regulate proliferation and IFN- γ production of PBL, LPL and IEL from controls. In contrast, Tregs from controls were unable to suppress effector T cells from active CD patients (**Figure 5**) while they could efficiently suppress autologous PBL (**data not shown**). Altogether these results are in keeping with a recent article that shows that Tregs isolated from the *lamina propria* of patients with active CD are functional (23) and confirm that effector cells from patients with active CD fail to respond to Tregs either autologous or heterologous. We and others have previously shown that IL-15, a cytokine overproduced in CD patients, prevents the response of effector T cells to the suppressive effects of Tregs (20, 26). The lack of inhibitory effect of Tregs in CD may thus depend on the production of IL-15. In favor of this hypothesis, we observed that the serum levels of IL-15 were significantly increased in CD patients (4.9 pg/ml; 0.8 to 22.4 pg/ml) compared to controls (0.49 pg/ml; 0 to 2.79 pg/ml)

(**p<0.001**) (18). IL-15 is known to reverse the anergic state of Tregs and to allow their proliferation in response to anti-CD3 stimulation (27). The presence of circulating IL-15 may thus explain the small but significant proliferative response to anti-CD3 stimulation of peripheral Tregs from CD patients but not from controls (**Figures 2 and 5 and data not shown**). Similarly, a small secretion of IFN- γ by the latter cells was observed (**Figures 3 and 5 and data not shown**). This finding is reminiscent of the recent demonstration by McClymont et al. that a subset of human Tregs from patients with type I diabetes produced IFN- γ while preserving their suppressive capacity (28). It will be interesting to define whether IL-15 participates in the appearance of this T cell subset since we have previously found that IL-15 was particularly increased in the serum of CD patients with type I diabetes (18). As already described (18), serum concentrations of IL-15 depended on disease activity (**Figure 6**) and were correlated with levels of anti-transglutaminase antibodies (**data not shown**). The fact that serum levels of IL-15 varied between patients may explain why two previous studies using co-culture of autologous peripheral Tregs and CD25-CD4⁺ T cells reported conflicting results when testing the immunosuppressive effect of peripheral T regs on autologous conventional T cells (10, 11). It is also noticeable that CD8⁺ T cells are much more sensitive to the effect of IL-15 and become thereby more easily unresponsive to the effect of Tregs after pre-incubation in IL-15 (18). IEL contain both CD8⁺ TcR $\alpha\beta$ ⁺ cells and TCR $\gamma\delta$ ⁺ cells also responsive to IL-15. The high sensitivity of IEL to IL-15 together with the fact that IL-15 can remain increased in the epithelium of CD patients on GFD (16), might explain the lack of response of IEL to Treg suppression even in treated CD patients whose CD25- LPL and peripheral T cells responded to the inhibitory effect of autologous peripheral Tregs.

In conclusion, we observe that peripheral Tregs from active CD display normal suppressive functions despite partial loss of their anergic state, a possible consequence of their *in vivo* exposure to IL-15. More strikingly, we demonstrate that intestinal but also peripheral

T lymphocytes become resistant to the suppressive effects of Tregs during active CD and that the defective response persists in IEL even after gluten eviction. Together with our previous studies showing that, due to IL-15 overexpression, intestinal T lymphocytes become unresponsive to the immunoregulatory cytokine TGF- β (29), these data provide a rationale for the loss of intestinal tolerance to gluten (30). Our demonstration of the inability of regulatory T cells of CD patients to control the expansion of lymphocytes using a polyclonal stimulus (anti-CD3 antibody) further suggests that such defect may underlie the loss of tolerance towards different antigens and the frequent occurrence of autoimmunity in CD (31, 32). The persistent resistance of IEL to Treg suppression in treated CD might also explain why the latter cells remain increased and abnormally activated despite GFD (33). Together with the anti-apoptotic effect of IL-15 (16, 34), the lack of effective retro-control of IEL activation may also, on the long term, promote the onset of IEL-derived lymphomas, a situation recapitulated in IL-15 transgenic mice which develop multi-organ lymphocytic infiltrates, aggressive autoimmune skin lesions and, ultimately, CD8⁺/NK T cell lymphoma or leukemia (35). Restoring the response of effector T cells to the regulatory effects of Tregs is likely central to prevent the onset of CD autoimmune complications. The normalization of LPL and PBL responses after GFD may explain why GFD can prevent the later complications. It will therefore be interesting to investigate whether unresponsiveness to Tregs persists or resumes despite GFD in patients developing refractory celiac disease.

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Specific author contribution: Nadia Belhadj Hmida performed suppression assays and flow cytometry analyses and contributed to data analysis. Mélika Ben Ahmed performed quantitative RT-PCR analyses, supervised suppression assays and contributed to study design, data analysis and drafting of the article. Amel Moussa, Majd Ben Rejeb and Yosra Said helped in patient's recruitment and performed duodenal biopsies for celiac disease patients and controls. Nadia Kourda performed histological analyses for celiac disease patients and controls. Bertrand Meresse contributed to flow cytometry analysis. Maha Abdeladhim helped for suppression assays. Hechmi Louzir contributed to drafting of the article. Nadine Cerf-Bensussan contributed to study design, data analysis and drafting of the article

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FIGURE LEGENDS

Figure 1

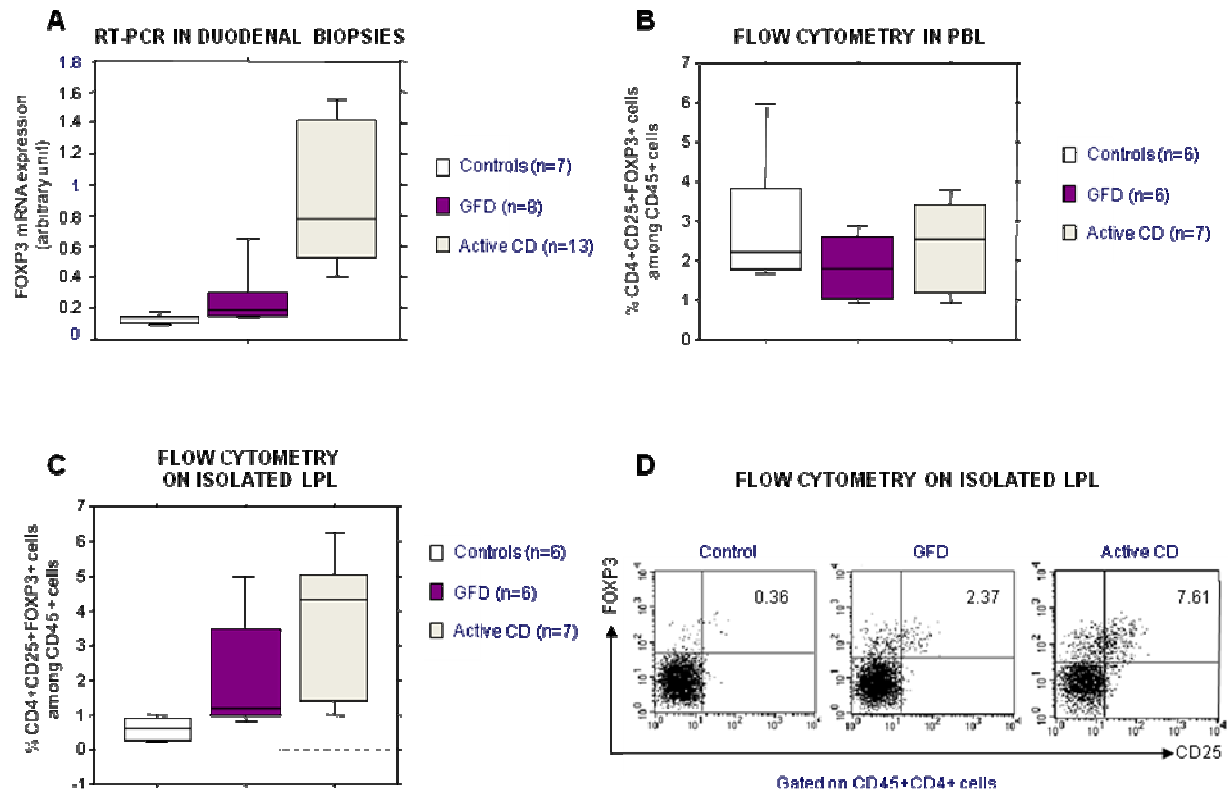


Figure 1: Intestinal CD4+CD25+FOXP3 T lymphocytes are increased in active CD.

(A) Expression of FOXP3 mRNA in duodenal biopsies from 8 active CD patients, 8 CD patients on gluten free diet (GFD) and 7 healthy controls was analyzed by quantitative real-time RT-PCR. Data were normalized referring to expression of ribosomal protein, large, PO (RPLPO). Results are expressed in arbitrary units as $2^{-\Delta\text{CT}}$, with ΔCT the difference in threshold cycles for target and reference. (B, C, D) Peripheral and *lamina propria* lymphocytes were isolated from 7 active CD patients, 6 CD patients on gluten free diet (GFD) and 6 healthy controls. The percentage of CD4+CD25+FOXP3+ lymphocytes among CD45+ peripheral (B) and intestinal (C) cells was assessed by flow cytometry. While percentages of CD4+CD25+FOXP3+ lymphocytes were comparable in peripheral blood of CD and controls ($p > 0.05$), the percentage of CD4+CD25+FOXP3+ lymphocytes was significantly higher in

lamina propria of active CD or CD on GFD than of controls (p=0.005 and p=0.03, respectively). (D) Double staining of CD25 and FOXP3 in *lamina propria* CD4+ cells is shown in one representative donor from each study group. Percentages of CD25+FOXP3+ cells among CD45+CD4+ cells are indicated.

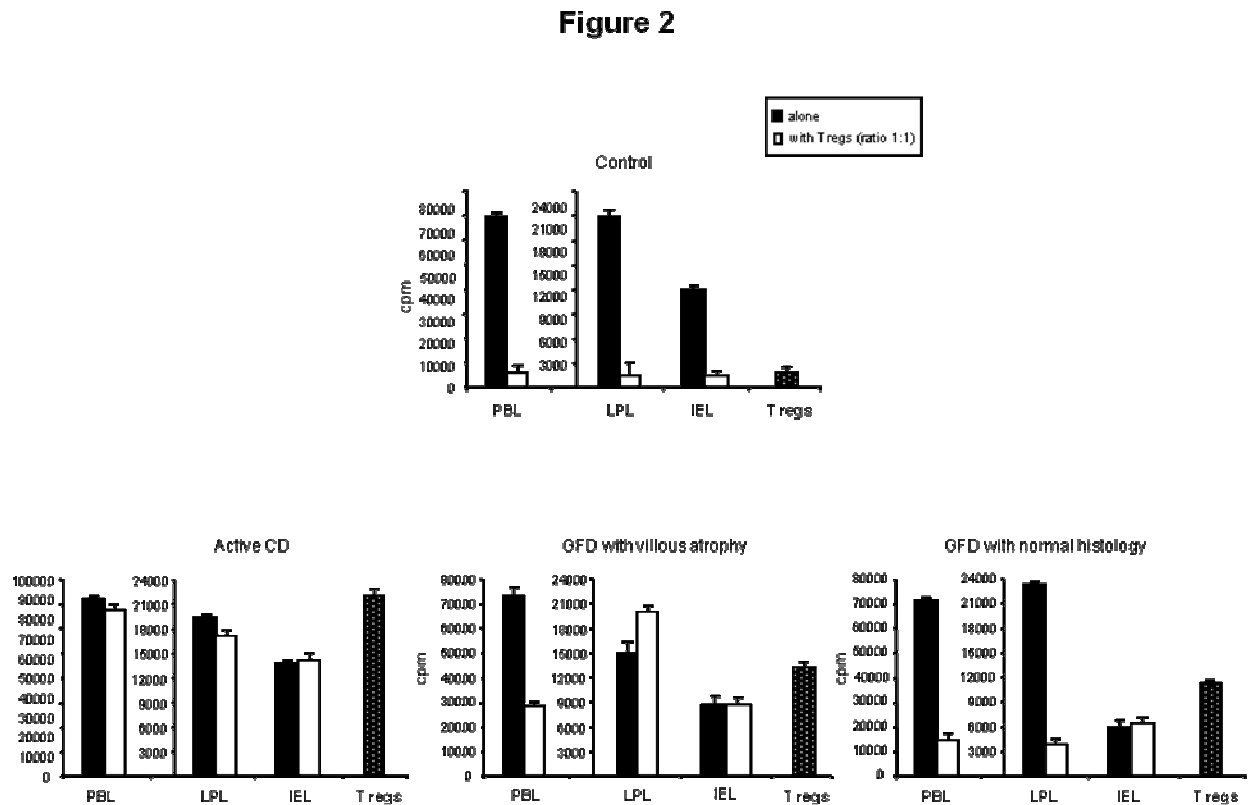


Figure 2: Proliferation of peripheral and intestinal lymphocytes of CD patients is not efficiently inhibited by autologous peripheral T regs.

Purified intraepithelial lymphocytes (IEL), CD25- *lamina propria* lymphocytes (LPL), CD4+CD25- peripheral T lymphocytes (PBL) from CD patients and controls were stimulated with immobilized anti-CD3 antibody (2 µg/ml) in the presence or not of autologous CD4+CD25+ peripheral T lymphocytes (Tregs) a ratio 1:1 and 1:4 of regulatory to responsive T cells. To test whether Tregs were or not anergic, purified Tregs were activated alone in the presence of immobilized anti-CD3 (last black dotted histogram of each panel labeled T regs).

Proliferative responses were assessed by measuring the uptake of [3H] thymidine. Results of co-cultures at ratio 1:1 from one representative individual out of 3 active CD patients, 3 CD patients on GFD with villous atrophy, 3 CD patients on GFD without villous atrophy and 9 controls are shown. Standard deviations from triplicates are represented.

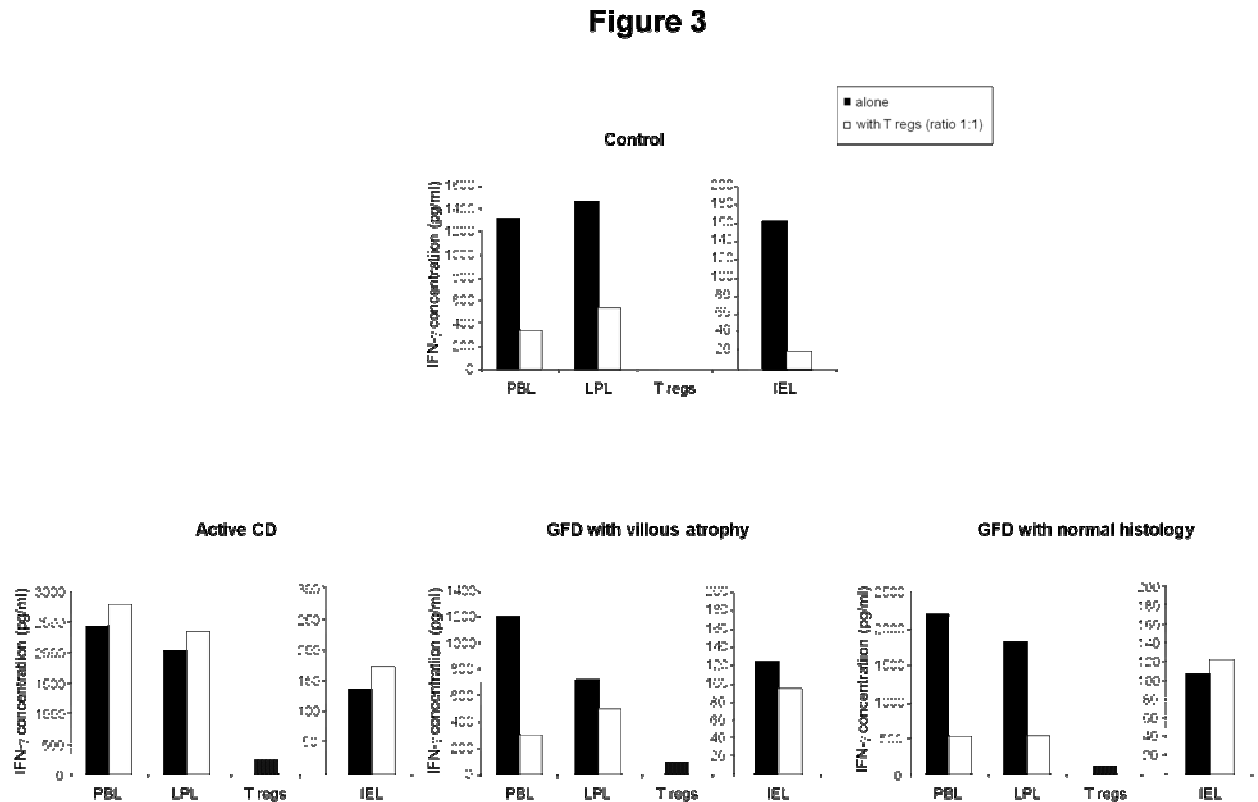


Figure 3: Interferon gamma secretion by peripheral and intestinal lymphocytes of CD patients is not efficiently inhibited by autologous peripheral T regs.

Purified intraepithelial lymphocytes (IEL), CD25- lamina propria lymphocytes (LPL), CD4+CD25- peripheral T lymphocytes (PBL) from CD patients and controls were stimulated as described in Figure 2. Supernatants from triplicate cultures were pooled and IFN- γ production was analyzed by ELISA. Results of co-cultures at ratio 1:1 from one representative individual out of 3 active CD patients, 3 CD patients on GFD with villous atrophy, 3 CD patients on GFD without villous atrophy and 9 controls are shown.

Figure 4

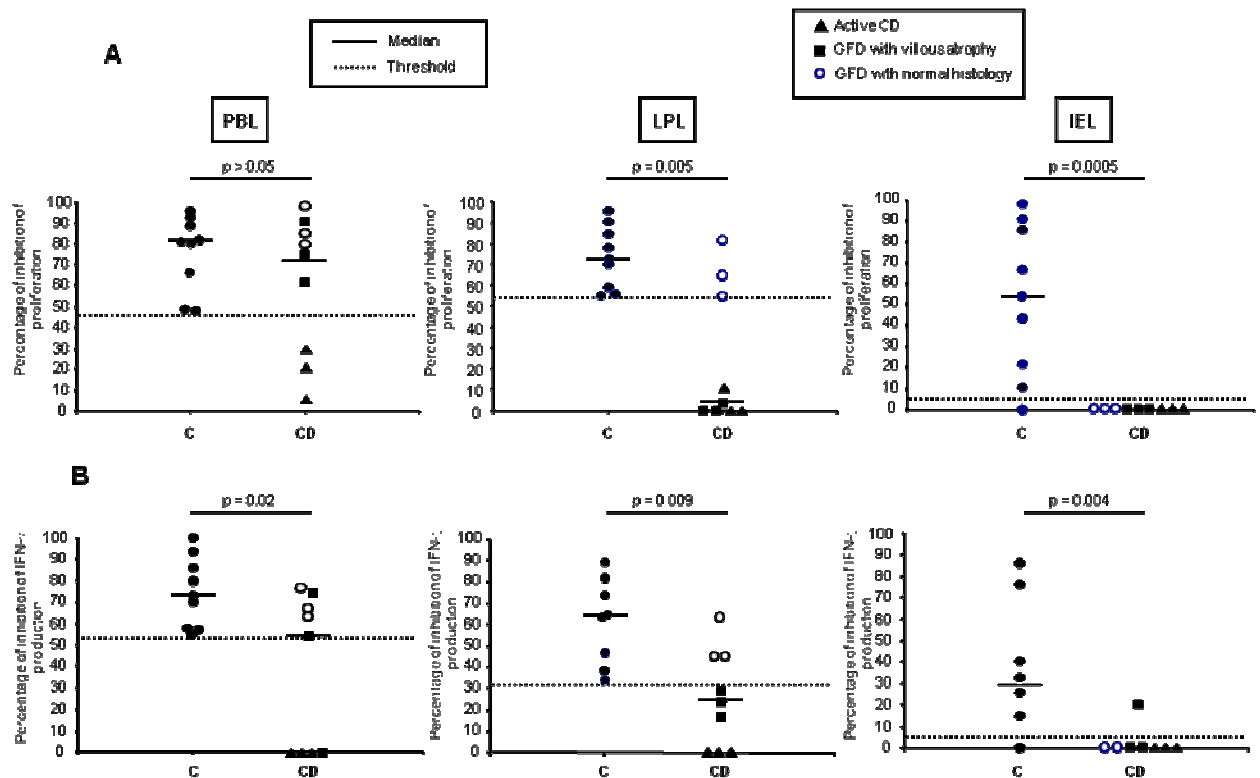


Figure 4: Global comparison of the responses of peripheral and intestinal lymphocytes to the suppressive effects of autologous Tregs in CD patients and control individuals.

Inhibition of proliferation (A) and IFN- γ production (B) of IEL, CD25- lamina propria lymphocytes (LPL), CD4+CD25- peripheral T lymphocytes (PBL) by autologous CD4+CD25+ peripheral T cells was tested as in Figures 2 and 3. Histograms summarize data from co-cultures at ratio effector to Treg 1:1 in 9 controls and 9 CD patients depending on their diet and intestinal histology. In each histogram, the horizontal bar indicates the

percentage of inhibition corresponding to the 5th percentile of the controls used to define a “threshold of resistance”.

Figure 5

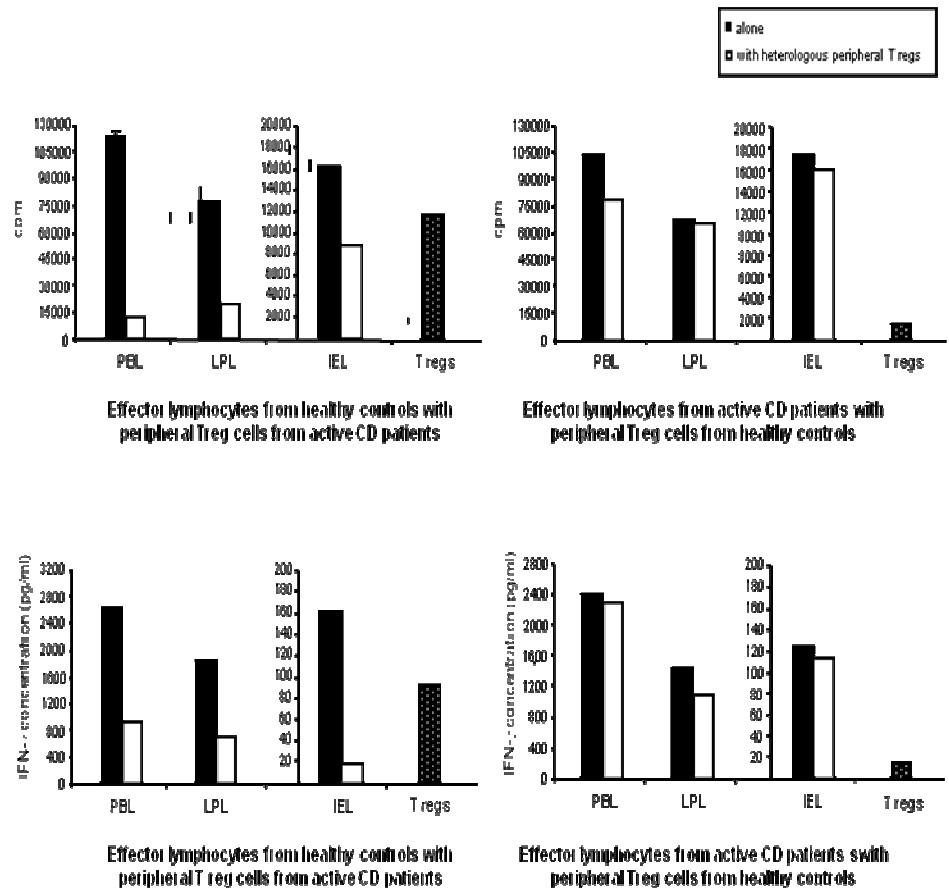


Figure 5: Lack of suppression of effector lymphocytes from active CD patients by heterologous regulatory T cells from controls.

IEL, CD25⁻ LPL and peripheral CD4⁺CD25⁻ T subsets from controls or from active CD patients were plated on 96-well plates coated with anti-CD3 antibodies at 2 μg/ml and cultured with heterologous peripheral Tregs from active CD patients or from controls respectively at a ratio 1:1 in for 5 days. (A) Proliferative responses were assessed by measuring the uptake of [3H] thymidine. Standard deviations from triplicates are represented. (B) Supernatants from triplicate cultures were pooled and IFN-γ production was analyzed by ELISA. Results of one representative out of two independent experiments are shown.

Figure 6

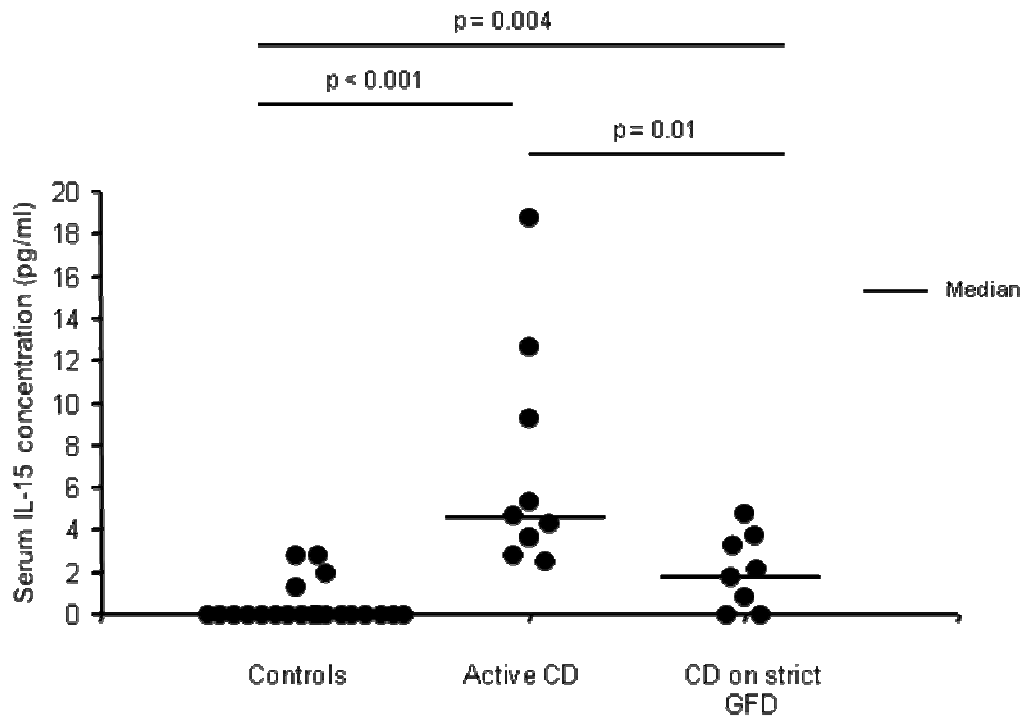


Figure 6: Increase in IL-15 serum levels in active CD.

IL-15 serum levels were compared by ELISA in 20 controls, 10 patients with active celiac disease (CD) and 8 patients with CD on gluten free diet (GFD).