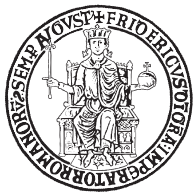

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PhD Program
"Human Reproduction, Development and Growth"

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PhD Thesis

Biological effect of P31-43

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to my sweet daughters
Francesca and Elena

INDEX

BACKGROUND AND AIM OF THE PROJECT

Celiac Disease: definition and epidemiology

Celiac disease (CD) is an intolerance to wheat gliadin and prolamine present also in barley and rye. The intake of these cereals in the diet determines, in the small intestine, a cellular and humoral immune response in people genetically predisposed[1].

Diagnosis of celiac disease was based, in the past, mainly on the clinic manifestations and the prevalence of the disease, which was considered rare, around 1:1000, with large differences in incidence in different geographical areas. Thanks to recent studies based on serological tests (EMA and tTG2 antibodies) it was found that celiac disease has a prevalence of around 1:100 [2,3], even in those European countries such as Denmark and the Netherlands, where the estimation of the disease was known to be very low, or in the United States, where it was believed that the disease almost did not exist [4]. This reversal of the situation can be explained by the "iceberg model", originally introduced by R. Logan in 1991 [5], in which the visible part of the iceberg corresponds to the cases of celiac disease diagnosed because clinically evident, while the submerged part is represented by the cases not diagnosed because asymptomatic or "atypical". Furthermore, a delayed introduction of gluten in the diet, instead of preventing the development of celiac disease, as it was thought at the beginning, did nothing but increase the "atypical" onset of disease [6]. Likely factors such as age of introduction of gluten in the diet and its quantity may influence the clinical presentation of celiac disease [7,8].

Celiac genetic background

The existence of a strong genetic component in CD is determined by a prevalence of about 10% of the disease among first-degree relatives of celiac patients [9] in addition to the high correlation, 75-80%, found between monozygotic twins [10]. Currently the only certain association is with the HLA genes that map on chromosome 6. The " immunogenic peptide" of the gliadin are indeed processed and presented to T lymphocytes associated with molecules of major histocompatibility complex class II (MHCII). The

deamidation by the tissue- transglutaminases type 2 (tTG2) converts glutamine residues in glutamic acid residues, giving increased affinity of gluten peptides for HLA molecules, which are positively charged, and thus increasing their immunogenic power [11]. Inhibition studies with anti-HLA antibodies have shown that T lymphocytes derived from celiac mucosa recognize gliadin peptides only when presented by DQ molecules [12]. Genetic studies highlight that about 90% of celiacs have a identical HLA, the DQ2 heterodimer, encoded by DQa1* 0501 and DQb1 * 0201 genes [13]. In many patients negative for this haplotype there is an association with two other class II alleles, the DQa1 * 0301 and DQb1 * 0302, encoding the DQ8 [1]. The presence of DQ2 and / or DQ8 alleles is therefore highly sensitive to the CD. HLA typing, can exclude the disease, presenting a high negative predictive value [13]. We can not say the same about the positive predictive value or about the specificity of these alleles for the disease, in fact, not all individuals that have the DQ2 and / or DQ8 indeed develop the disease, if we consider that these alleles are present in approximately 20 -25% of the general population [14].

Clinical features and diagnosis

The clinical manifestation of celiac disease can range from a"classical" presentation, characterized by typical gastrointestinal symptoms, to a total absence of symptoms ("silent" CD), passing through many clinical conditions, some dominated by extraintestinal events (" atypical " CD), further characterized by a state of real emergency (crisis celiac). Currently, cases that present with the classic malabsorption syndrome characterized by chronic diarrhea, reduced growth, anorexia, apathy and irritability [15] are less frequent and among these few are in celiac crisis or with ipoprotidemia and edema for the severe malabsorption syndrome. Patients with less typical clinical manifestations are increasing, symptoms such as abdominal pain, meteorism and dyspepsia can be at the onset of CD. More frequently extraintestinal symptoms ranging from abnormal haematological events to the manifestation of the central nervous system [16] and other organs and systems are the only clinical manifestation of CD.

In relation to this strong clinical polymorphism, it is clear that the diagnosis of celiac disease should be based on other factors, first of all is the search in the serum of anti-endomysial (EMA) [17,18] and anti-tissue-type transglutaminases 2 (tTG2) antibodies. The latter in particular are highly sensitive and speci-

fic for celiac disease [19,20] and in close relation with susceptibility HLA genes [21].

However, the presence of one of these types of antibodies it is not enough to be confident with the diagnosis. The criteria set by the European Society of Gastroenterology, Hepatology and Pediatric Nutrition (ESPGHAN) for the diagnosis of celiac disease are two[22]:

- histology of the intestinal mucosa compatible with the damage from gluten (villi atrophy and crypts hypertrophy);
- remission of symptoms or negative serology antibodies after gluten-free diet.

Therapy

Treatment of celiac patients is based mainly on exclusion of gluten from the diet (gluten free diet "-GFD) [23]. GFD is as a diet in which are excluded all products containing wheat, barley and rye. Even small amounts of these cereals may be harmful to the celiac. Oat toxicity is still debated, but it seems that this cereal is not harmful in most coeliacs [23]. Of fundamental importance is the role of an early diagnosis since the more prolonged exposure to gluten correlates with greater risk to develop serious complications of CD or autoimmune diseases [8].

Pathogenesis: dual action of gliadin

T-mediated effects of gliadin: the role of P57-68 in adaptive immune response

The term "gluten" is referred in general to the protein mass of elastic consistency that remains after removing the starch by mixing with water the wheat flour. Gliadins are the main proteins conteined in gluten. They are monomeric proteins of low nutritional value, constituted in high proportion by only two amino acids, proline (Pro) to 20% and Glutamine (GLN) 38% that is why these proteins are named "prolamine"[24]. Recent studies indicate that all four electrophoretic fractions of gliadin (α , β , γ ,ω) differentiated according to their N-terminal sequence, are able to release peptides capable of triggering the immune reaction at the base of CD, when are digested by intestinal enzymes [25]. Among the gliadin pep-

tides that have more immunogenic power are recognized the fragment 33Mer, of which the peptide P57-68 appears to be one of the dominant epitopes recognized by the celiac subject [26]. When genetically susceptible individuals are exposed to gliadin peptides, there is the activation of specific T lymphocytes, as demonstrated by the activation marker CD25 [27]. An immune cellular response is established, represented by the migration in lamina propria of a linfomonocitary infiltrate with an high prevalence of CD4 + cells and the migration of intraepithelial CD8 + T lymphocytes. This reaction is supported by a subpopulation of CD4 + T lymphocytes (type Th1) which produce cytokines such as IFN γ, TNFα and IL2 [28] and is belived to be mainly responsible for the maintenance of tissue damage, with the characteristic picture of villous atrophy and crypts hyperplasia. Of great importance is also a type of humoral immune response, represented by the secretion, at mucosal level, of type IgA, IgM and IgG immunoglobulin, and is supported by a subpopulation of T lymphocytes CD4 + (type Th2) which produce cytokines such as IL4, IL5 and IL10 [28]. Recently it was also shown a biological effect of anti-transglutaminases; particularly in isolated cells in culture, they can induce proliferation [29] and may interfere with differentiation [30]. In conclusion, although it is still not defined the role of these antibodies in tissue damage, they assume, as we have seen a crucial role for diagnosis of the disease [17,18,19,20].

Not T-mediated (or "toxic") effects of gliadin: the role of P31-43 in innate immune response

The "immunological" alteration of CD intestinal mucosa are able to explain much of the pathogenesis of CD and of intestinal lesions that are observed. But they appear to present strong limitations since leaves unresolved what may be considered one of the pivotal questions of the pathogenesis of CD, as well as any other type of food intolerance: why the celiac not develop oral tolerance against gliadin? Oral tolerance is the tendency to develop immune tolerance to antigens encountered for the first time orally and is well known that among the main mechanisms for the development of this phenomenon is the presentation of antigen to T cell clone in absence of adjuvant substances. When a clone is stimulated by T cells presenting the antigen in the absence of adjuvant substances, it tends to go to anergy rather than clonal expansion, resulting in peripheral tolerance to that specific antigen [31].

And it is partly in an attempt to understand how gliadin is able to deflect these mechanisms of oral tolerance, which, in the pathogenesis of CD, the so-called hypothesis of gliadin toxicity, could then find an area of interest [32]. It appears that upstream of T-mediated reaction at the base of the disease, there are a number of pathological interactions between toxic nature of the gliadin and intestinal structures which include various effects, also leading to the activation of innate immunity [13,24], able to interfere with the establishment of oral tolerance to gliadin [32].

The existence of toxic interaction between the gliadin and intestine was already assumed in the 80s when it was noted that the gliadin peptides were able to bind to glycoproteins of the brush-border [33] and that caused the agglutination of K562 cells [34]. But it was only with the identification of P31-43 of gliadin that has toxic effects in vitro [35] and in vivo [36], that the hypothesis of gliadin toxicity began to assume an increasing importance. This peptide does not show immunogens activity on T cells, but is capable of preventing the recovery of patients with atrophic intestinal mucosa [37] and activate the mechanisms of innate immunity in the mucosa from celiac subjects [13,37].

Innate Immunity

Activation of innate immunity is a necessary condition for the induction of an immune adaptive response, it seems to be the cornerstone in the pathogenesis of CD. Therefore is logical to assume that any factor capable of inducing innate immunity may increase, in DQ2 or/and DQ8 subjects, the risk of breaking the oral tolerance to gliadin with subsequent development of celiac enteropathy.

Epithelial proliferation

Beside induction of innate immunity, another key element in the pathogenesis of CD is, without doubt, the intestinal crypts proliferation. In previous studies conducted in our laboratory we have observed some biological actions of P31-43 which seem to mimic the actions of epidermal growth factor EGF [37]; they could therefore play a fundamental role in the phenomenon of crypts proliferation . We have observed the ability of P31-43 to induce changes in the cytoskeleton of actin in cultured Caco2 cells, phenomena

similar to those induced by EGF. We have shown an increase in proliferative activity in NIH3T3 cells, that can be prevented by anti-EGFR antibody. Further studies have indicated also that the EGF-like action of P31-43 is not expressed through a ligand activity, but rather through the ability of the peptide to cause a slowdown of the endocytosis and delay of the endocytic maturation causing the decay of the downstream EGFR signal and amplify the actions induced by endogenous EGF. Similar effects were also observed in cultures from intestinal celiac patient treated with P31-43.

Celiac disease and innate immunity: the emerging role of Interleukin 15

Interleukin 15 (IL15) has a pivotal role in innate immunity mechanisms [38,39]. This stems from the significant actions that this cytokine is able to elicit on many cells of many compartment of immunity. It has pleiotropic actions including antiapoptotic effects, induction of proliferation and activation on NK cells, neutrophils, eosinophils, mastocyte , monocytes / macrophages and dendritic cells [39]. Similar effects are induced by this cytokine also on typical cells of the adaptive responses, such as T lymphocytes (with an action IL2-like) and B lymphocytes [40]. It has been shown that many not immune cells respond to IL15 inhibiting apoptosis and increasing proliferation. Among these include fibroblasts, osteoclasts, endothelial cells, adipocytes, myocytes, glia and neuronal cells, keratinocytes and epithelial cells of various types [39]. Of great interest is the observation of Reinecker et al [41] that intestinal epithelial cells are able to express this cytokine as well as respond to it by increasing the proliferation. A possible relationship between activation of innate immunity and induction of proliferation may be hypnotized for IL15 activity.

IL15 is constitutively expressed in many cell types (macrophages / monocytes, dendritic cells, keratinocytes and epithelial cells of various types, fibroblasts, nerve cells) [39]. Two isoforms of IL15 are described derived from an alternative "splicing" of mRNA, they differ on the length of the signal peptide capable of directing the protein along the secretory way of the cell [39,40] The isoform with the long signal peptide (LSP), is obtained from an mRNA containing 8 exons and is found along the secretory way of the cell (ER, Golgi and plasma membrane); the isoform with short signal peptide (SSP), is obtained from an mRNA of higher molecular weight, which has an extra exon, the 4a, which is inserted in such a way as to interrupt the sequence of the signal peptide. This isoform is localized in the cytoplasm and is not found

along the secretory way of the cell. In any case, it was found that in physiological conditions this cytokine is not secreted but remains anchored to the cell membrane, carrying out his action of "signaling" through a juxtacrine mechanism [42,43]. An increase in the amount of cytokine in the serum was found only in certain autoimmune diseases such as rheumatoid arthritis, chronic inflammatory bowel disease, systemic lupus, type I diabetes mellitus, vasculitis [39].

The regulation of IL15 expression on the membrane may be in the cell at three main levels[39]:

1. At the level of transcription: through a mechanism of alternative "splicing" the cell may express two different isoforms of IL15, only one LSP is sent to the cell

membrane and is functionally active.

2. At the level of translation of mRNA to protein.

3. At the level of intracellular trafficking of recycling vesicles carrying the cytokine.

IL15 receptor consists of three chains: two of these form βγc complex, constitutively expressed, which is capable of binding to the low affinity IL15 and

IL2 (thus explaining the redundancy of the two cytokines as concerning the effects on T cells and NK cells) and is sufficient to trasduct the signal phosphorylating downstream effectors of the via JAK / STAT. The chain alpha, inducible, is able to binds with the complex βγc to increased activity; it bind to high affinity only IL15, becoming responsible for the specific activity of this cytokine compared to IL2 [39].

P31-43 and Interleukin 15

Based on the previous report about the ability of P31-43 to delay the maturation of endosomes [37], it is feasible to assume that a generic block of the endocytosis, besides being able to amplify the EGF signal, could more generally extend the downstream signal of several other receptors. Several authors have shown an IL15 overexpression in the lamina propria and in intestinal epithelium of celiac patients, which manifests itself mainly at the cell surface [42,43]. Ciccocioppo et al [43] have observed an active role by the enterocytes of celiac patients in the secretion of the cytokine. It was also demonstrated that the gliadin and P31-49 are capable of increasing in vitro the expression of MIC-A on the surface of epithelial cells of celiac patients in active phase, an effect correlated with IL15 activity [44]. Finally, of considerable impor-

tance is the observation of Maiuri et al [45] that IL15 blocking antibodies can prevent epithelial apoptosis induced by P31-43, which for the first time draws attention to the possibility that some of effects of P31-43 may be mediated by IL15.

My PhD program has been focused to the study some of these aspects in order to clarify "Relationship between proliferative effects and activation of innate immunity induced by gliadin".

This thesis reports the result I obtained during my PhD course in "Human, Reproduction, Development and Growth" (XXIV cycle) from 2008 to 2011.
During the past 3 years, my research has been focused in the study of the following lines of research:

- Gliadin peptide P31-43 localises to endocytic vesicles and interferes with their maturation.
- Gliadin-mediated proliferation and innate immune activation in celiac disease are due to alterations in vesicular trafficking.
- IL-15 interferes with functionally suppressive Foxp3+ regulatory T cells expanded in the celiac small intestine.

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CHAPTER 1
GLIADIN PEPTIDE P31-43 LOCALISES TO ENDOCYTIC VESICLES AND INTERFERES WITH THEIR MATURATION

Project

Celiac disease (CD) is characterised by a derangement of both the adaptive and the innate immune response to gliadin. Some gliadin peptides that are deamidated by tissue transglutaminase (e.g., A-gliadin P57-68) bind to HLA DQ2 and/or DQ8 molecules [1] and induce an adaptive Th1 proinflammatory response. In the case of the innate immune response, [2] A-gliadin P31-43, which is not recognised by T cells, [3,4] induces IL15 production, which in turn is thought to cause expansion of intra¬epithelial lymphocytes (IEL) in CD and epithelial apoptosis. [5-6-7] Furthermore, IL15 has been implicated in the increased expression of NKG2D on lymphocytes. The interaction between the major histocompatibility complex (MHC) class I chain-related gene A (MICA), and NKG2D is at least in part responsible for IEL-induced enterocyte apoptosis and villous atrophy. [8-9]

Many biological activities have been associated with gliadin peptides in several cell types [10-11-12-13-14] including reorganisation of actin and increased permeability in the intestinal epithelium. [15-16] Other effects are specific to celiac tissues. In untreated celiac patients, P31-43 prevented the restitution of enterocyte height, which normally occurs after 24-48 h of culturing mucosal explants with medium alone. [17] P31-43 damaging activity has been demonstrated in organ culture of treated celiac biopsies, [18] and in in vivo feeding studies. [19] Similar results have been obtained in vivo on small intestinal and oral mucosa with the A-gliadin peptide 31-49. [20-21]

It has yet to be established to what extent these properties relate to the ability of these A-gliadin peptides to activate innate immunity mechanisms. Virtually nothing is known about the mechanisms underlying the biological properties of P31-43 or about the metabolic pathways involved in the activation of innate immunity in CD. Similarly, it is not known why celiac patients are particularly sensitive to these biological activities.

We recently investigated the molecular basis of the non-T cell-mediated properties of the gliadin peptides most likely to play an important role in the very early phases of CD, and we found that P31-43 causes

actin alterations and cell proliferation, both of which depend on activation of the epidermal growth factor receptor (EGFR), in several cell types, and in the organ culture of celiac mucosa. [22-23] In this system P31-43 interferes with EGFR decay and prolongs EGFR activation. We also showed that P31-43 increases IL15 on the cell surface, by interfering with its trafficking (MV Barone, submitted). These data suggest that enhancement of EGFR and IL15 signalling may be important biological contributors to the pathogenesis of CD. Here we demonstrate that both P31-43 and P57-68 enter CaCo 2 cells and interact with endocytic compartment, but only P31-43 interferes with the endocytic pathway by delaying maturation of early endosomes to late endosomes. We also show that the P31-43 sequence is similar to hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which is a key protein of endocytic maturation. [24] P31-43 is localised at the vesicles membranes and interferes with the correct localisation of Hrs to endocytic vesicles thus delaying the maturation of early endosomes to late endosomes. Consequently the activation of EGFR and other receptors is expanded with multiple effects on various metabolic pathways and cellular functions.

These data have been published on PloS ONE, for the manuscripts see below.

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PLoS one

Gliadin Peptide P31-43 Localises to Endocytic Vesicles and Interferes with Their Maturation

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Abstract

Background: Celiac Disease (CD) is both a frequent disease (1:100) and an interesting model of a disease induced by food. It consists in an immunogenic reaction to wheat gluten and glutenins that has been found to arise in a specific genetic background; however, this reaction is still only partially understood. Activation of innate immunity by gliadin peptides is an important component of the early events of the disease. In particular the so-called "toxic" A-gliadin peptide P31-43 induces several pleiotropic effects including Epidermal Growth Factor Receptor (EGFR)-dependent actin remodelling and proliferation in cultured cell lines and in enterocytes from CD patients. These effects are mediated by delayed EGFR degradation and prolonged EGFR activation in endocytic vesicles. In the present study we investigated the effects of gliadin peptides on the trafficking and maturation of endocytic vesicles.

Methods/Principal Findings: Both P31-43 and the control P57-68 peptide labelled with fluorochromes were found to enter CaCo-2 cells and interact with the endocytic compartment in pulse and chase, time-lapse, experiments. P31-43 was localised to vesicles carrying early endocytic markers at time points when P57-68-carrying vesicles mature into late endosomes. In time-lapse experiments the trafficking of P31-43-labelled vesicles was delayed, regardless of the cargo they were carrying. Furthermore in celiac enterocytes, from cultured duodenal biopsies, P31-43 trafficking is delayed in early endocytic vesicles. A sequence similarity search revealed that P31-43 is strikingly similar to Hrs, a key molecule regulating endocytic maturation. A-gliadin peptide P31-43 interfered with Hrs correct localisation to early endosomes as revealed by western blot and immunofluorescence microscopy.

Conclusions: P31-43 and P57-68 enter cells by endocytosis. Only P31-43 localises at the endocytic membranes and delays vesicle trafficking by interfering with Hrs-mediated maturation to late endosomes in cells and intestinal biopsies. Consequently, in P31-43-treated cells, Receptor Tyrosin Kinase (RTK) activation is extended. This finding may explain the role played by gliadin peptides in inducing proliferation and other effects in enterocytes from CD biopsies.

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Introduction

Celiac disease (CD) is characterised by a derangement of both the adaptive and the innate immune response to gliadin. Some gliadin peptides that are deamidated by tissue transglutaminase (e.g., A-gliadin P57-68) bind to HLA DQ2 and/or DQ8 molecules [1] and induce an adaptive Th1 proinflammatory response. In the case of the innate immune response, [2] A-gliadin P31-43, which is not recognised by T cells, [3,4] induces IL15 production, which in turn is thought to cause expansion of intra epithelial lymphocytes (IEL) in CD and epithelial apoptosis. [5–6–7] Furthermore, IL15 has been implicated in the increased expression of NKG2D on lymphocytes. The interaction between the major histocompatibility complex (MHC) class I chain-related gene A (MICA), and NKG2D is at least in part responsible for IEL-induced enterocyte apoptosis and villous atrophy. [8–9]

Many biological activities have been associated with gliadin peptides in several cell types [10–11–12–13–14] including reorganisation of actin and increased permeability in the intestinal epithelium. [15–16] Other effects are specific to celiac tissues. In untreated celiac patients, P31-43 prevented the restitution of enterocyte height, which normally occurs after 24–48 h of culturing mucosal explants with medium alone. [17] P31-43 damaging activity has been demonstrated in organ culture of treated celiac biopsies, [18] and in *in vivo* feeding studies. [19] Similar results have been obtained *in vivo* on small intestinal and oral mucosa with the A-gliadin peptide 31–49. [20–21]

It has yet to be established to what extent these properties relate to the ability of these A-gliadin peptides to activate innate immunity mechanisms. Virtually nothing is known about the mechanisms underlying the biological properties of P31-43 or about the metabolic pathways involved in the activation of innate

immunity in CD. Similarly, it is not known why celiac patients are particularly sensitive to these biological activities.

We recently investigated the molecular basis of the non-T cell-mediated properties of the gliadin peptides most likely to play an important role in the very early phases of CD, and we found that P31-43 causes actin alterations and cell proliferation, both of which depend on activation of the epidermal growth factor receptor (EGFR), in several cell types, and in the organ culture of celiac mucosa. [22–23] In this system P31-43 interferes with EGFR decay and prolongs EGFR activation. We also showed that P31-43 increases IL15 on the cell surface, by interfering with its trafficking (MV Barone, unpublished data). These data suggest that enhancement of EGFR and IL15 signalling may be important biological contributors to the pathogenesis of CD.

Here we demonstrate that both P31-43 and P57-68 enter CaCo 2 cells and interact with endocytic compartment, but only P31-43 interferes with the endocytic pathway by delaying maturation of early endosomes to late endosomes. We also show that the P31-43 sequence is similar to hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which is a key protein of endocytic maturation. [24] P31-43 is localised at the vesicles membranes and interferes with the correct localisation of Hrs to endocytic vesicles thus delaying the maturation of early endosomes to late endosomes. Consequently the activation of EGFR and other receptors is expanded with multiple effects on various metabolic pathways and cellular functions.

Materials and Methods

Cell culture, materials and transfections

xCaCo-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, San Giuliano Milanese, Italy), 10% foetal calf serum (FCS) (GIBCO, San Giuliano Milanese, Italy), 100 units/ml penicillin-streptomycin (GIBCO, San Giuliano Milanese, Italy), and 1 mM glutamine. Lipopolysaccharide-(LPS) free synthetic peptides [22] (Inbios, Naples, Italy, >95% pure, MALDI-TOFF analysis, as expected) were obtained by Ultrasart-D20 filtration (Sartorius AG, Gottingen, Germany). Levels of LPS were undetectable (<0.20 EU/mg as assessed with a commercial kit: QCL-1000 kit, Cambrex Corporation, NJ). The P31-43 sequence is LGQQQPFPPQQPY; and the P57-68 sequence is QLQPFPQPQLPY. The labelled peptides were produced as the unlabelled peptides. Solutions were used in the following concentrations: P31-43-lissamine (liss), P31-43 CY3 and P57-68-liss at 20 micrograms/ml: unlabeled peptides were used as previously reported, [22] at 70 micrograms/ml; EGF at 100 nanograms/ml; EGF-Alexa-488 at 20 nanograms/ml (Molecular Probes, San Giuliano Milanese, Italy); Dextran-Alexa488 (MW 10000) (Molecular Probes, San Giuliano Milanese, Italy) at 0.5 milligrams/ml; goat polyclonal antibody against EEA1 (C-15) at 2 micrograms/ml (Santa Cruz, DBA, Milan, Italy); mouse monoclonal antibody against LAMP2 (H4B4) at 2 micrograms/ml (Santa Cruz, DBA, Milan, Italy); secondary antibodies anti goat-Alexa-488 conjugated (Molecular Probes) for EEA1 staining at a ratio of 1:100; and anti mouse-Alexa 488 conjugated (Molecular Probes) for LAMP2 staining at a ratio of 1:100. Rab5-EGFP and Rab7 EGFP were kindly provided by Prof. M. Zerial (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) and Hrs-EGFP was kindly provided by Prof. P.P. Di Fiore (Fondazione Istituto FIRC di Oncologia Molecolare, Milan, Italy).

Transfections and BrdU incorporation

We used the lipofectamine kit (Invitrogen, San Giuliano Milanese, Italy) according to the manufacturer's instructions to transfect all plasmids (Rab5-EGFP, Rab7-EGFP, Hrs-EGFP and Hrs-Ha). Briefly, CaCo-2 cells seeded on coverslips for 48 h were transfected with the plasmids for 16 h. The next day, transfected cells were pulsed and chased as described below or stained for Bromodeoxyuridine (BrdU) with a monoclonal antibody (Sigma-Aldrich, Milan, Italy) and total nuclei were identified by Hoechst staining. BrdU incorporation was performed as described elsewhere [22], briefly, CaCo-2 cells seeded on coverslips were transfected with Hrs-EGFP and serum starved in DMEM 0.1% foetal calf serum, antibiotics and glutamine for 30–48 h followed by 24 h treatment with gliadin peptides and/or growth factors. BrdU (100 mM, Boehringer) was added for one hour before fixing the samples. The cells fixed with paraformaldehyde and permeabilised with triton, were stained for BrdU as described [22] and observed at the miscroscope (Axiophot microscope, Carl Zeiss MicroImaging, Inc.). Greater than 100 HRS-EGFP positive cells in several fields were evaluated for BrdU incorporation in each sample. The number of HRS-EGFP/BrdU positive cells was expressed as a proportion of the total Hoechst positive nuclei.

Pulse and chase experiments

In pulse and chase experiments, transfected and untransfected cells were pulsed for 30 minutes at 37°C with a mixture of labelled and unlabeled peptides to avoid an excess of fluorochromes in the medium. Overall 20 micrograms/ml of P31-43-liss, P31-43-CY3 and P57-68-liss and 50 micrograms/ml of unlabeled peptides were used to reach the working concentration of 70 micrograms/ml. [22] The mixtures of labelled and unlabeled peptides were called P31-43-liss, P31-43-CY3 and P57-68-liss. After a 30-minutes treatment with the peptides mixtures (pulse), cells were washed five times with complete medium to eliminate fluorochrome excess. Unlabeled peptides (70 micrograms/ml) were added to the cells and incubation was continued for 3 h at 37°C (chase). Transfected coverslips were briefly fixed (5 minutes) with paraformaldehyde 3% (Sigma-Aldrich) at room temperature, then mounted. Untransfected coverslips treated with labelled peptides were washed and stained for EEA1 and LAMP2.

EEA1 and Lamp staining

CaCo-2 cells seeded on glass coverslips were stained for 1 h at room temperature with anti-EEA1 or -LAMP2 antibody after fixation with 3% paraformaldehyde for 5 min at room temperature and mild permeabilisation with 0.2% Triton (Biorad, Milan, Italy) for 3 min at room temperature. Secondary antibodies Alexa-488 conjugated (Invitrogen) anti-goat for EEA1 and anti-mouse for LAMP2 were added to the coverslips for 1 h at room temperature. Control panels for EEA1 staining with P31-43-liss and non specific anti-goat antibody together with secondary antibodies Alexa-488 conjugated anti-goat, did not show any cross-excitation of fluorochromes (Figure S1). Similar results were obtained for Lamp2 and P56-68-liss (not shown). The coverslips were then mounted on glass slides and observed by confocal microscope (LSM 510 Zeiss). In total 40 to 50 cells were observed in each sample. Images were generated with the same confocal microscope. Co-localisation analysis was performed with AIS Zeiss software. Magnification of the micrographs was the same for all the figures shown (63× objective) unless stated differently in the legends.

Time-lapse experiments

Cells were seeded on glass-bottom dishes (3 cm in diameter obtained from Falcon Becton Dickinson Labware; La Pont de Claix, France) to allow live observation, and they were kept in a specially designed incubator (OXO-lab, Naples, Italy) that controls temperature and CO2. After treatment with gliadin peptides, cells were

observed by confocal microscopy for 10 min, during which sets of frames were acquired at 30-seconds intervals. The image stack was analysed with the help of a program that allows to record the tracks followed by individual particles [25]. Using this methodology the subsequent positions of each vesicle was identified by the observer, who, with the help of a pointer on the computer screen, stored the coordinates in a text file. The program calculated the distance and direction for each time step; the values shown are the average speed of each vesicle during the observation period. The list of coordinates was also used to draw the paths covered by the vesicles, which were superimposed onto the confocal images.

Co-localisation analysis

Samples were examined with a Zeiss LSM 510 laser scanning confocal microscope. We used Argon/2 (458, 477, 488, 514 nanometers) and HeNe1 (543 nanometers) excitation lasers, which were switched on separately to reduce cross-talk of the two fluorochromes. The green and the red emissions were separated by a dichroic splitter (FT 560) and filtered (515-to 540-nm band-pass filter for green and >610-nm long pass filter for red emission). A threshold was applied to the images to exclude about 99% of the signal found in control images. The weighted co-localisation coefficient represents the sum of intensity of co-localising pixels in channels 1 and 2 as compared to the overall sum of pixel intensities above threshold. This value could be 0 (no co-localisation) or 1 (all pixels co-localise). Bright pixels contribute more than faint pixels. The co-localisation coefficient represents the weighted co-localisation coefficients of Ch1 (red) with respect to Ch2 (green) for each experiment. [26–27] The image collection and exposure times were identical for the two peptides.

Data bank analysis

Swissprot, Trembl and InterPro data banks were searched for sequences matching peptide P31-43 and P57-68 by using Blast and FastA. Sequence alignment was performed by using ClustalW and visualized by PrettyPlot from the EMBOSS suite.

Immunoblotting and subcellular fractionation

Near-confluent Caco2 cells in a 90-mm dish were incubated with EGF and P31-43 at various times, after homogenization

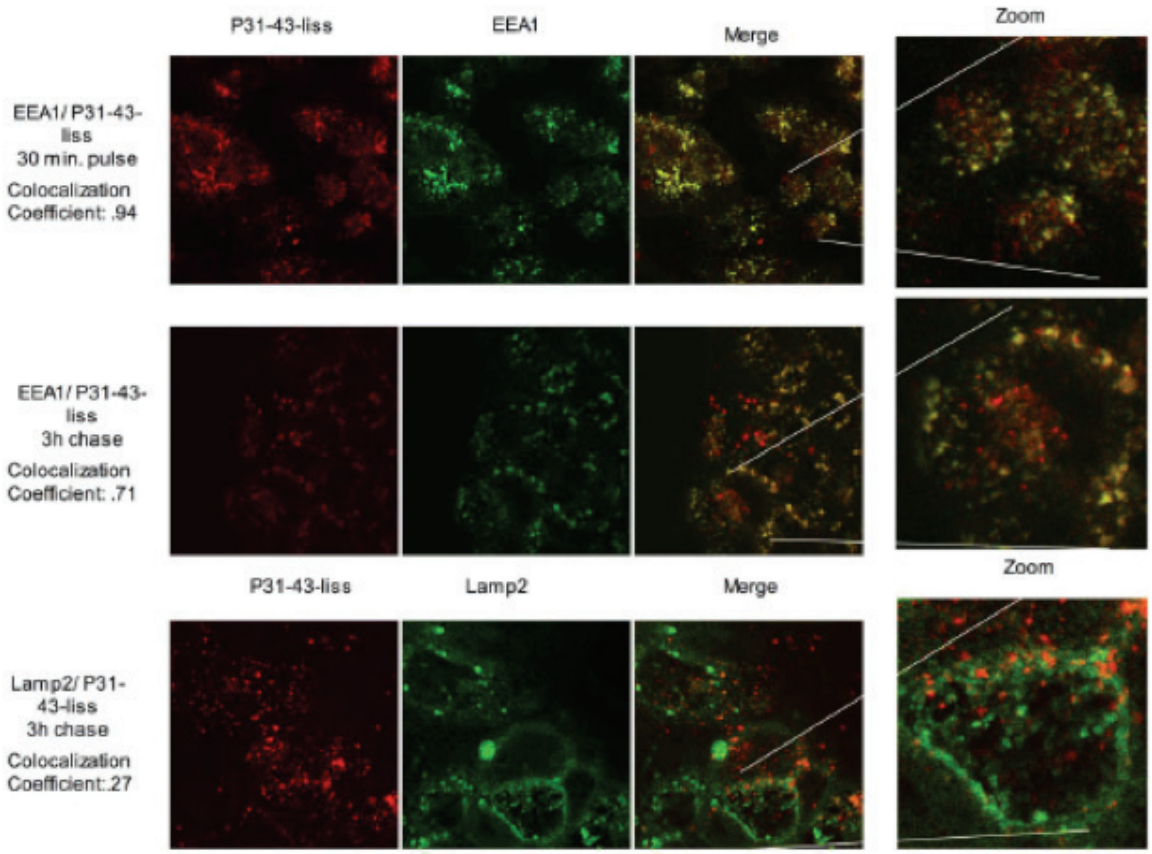

Figure 1. Vesicles interacting with P31-43-liss are early endocytic vesicles after a 3 h chase. In CaCo-2 cells, both after 30 minutes of pulse and 3 hours of chase with P31-43-liss (red), the peptide interacted with vesicles that are positive for EEA1 (green staining, top and middle panel). P31-43-liss did not co- localise with LAMP2-positive vesicles (green staining, bottom panel) after 3 hours of chase. A merge of the red and green panels of the EEA1 or LAMP2 and P31-43-liss or P57-68-liss staining is shown, the yellow/orange colour indicates co- localisation. The zoom panels represent a digital 4 x enlargement of the region highlighted by white lines in the merge panels. The co- localisation coefficient was calculated as reported in the "Methods" section. The results are representative of four independent experiments..
doi:10.1371/journal.pone.0012246.g001

(10 mM Tris-HCl [pH 7.4], 1 mM EDTA, and the mixture of phosphatase and protease inhibitors) the nuclear fraction was eliminated by centrifugation. The soluble cytosolic and the membrane fraction were obtained by ultracentrifugation. Electrophoresis and immunoblotting were performed as described elsewhere. [22] Briefly the proteins of the soluble cytosolic and the membrane fractions were separated by SDS-PAGE and incubated with anti-Hrs mouse monoclonal antibody (Alexis, Vinci-Biochem, Florence, Italy) or anti-EGFR rabbit polyclonal antibody (Cell Signaling Celbio, Milan Italy) or anti-tubulin mouse monoclonal antibody (SIGMA-Aldrich, Milan, Italy). Densitometric analysis was performed as before. [22]

Organ culture studies

All biopsies were treated as previously described. [22] To examine the entry of P31-43-CY3 into cells, we cultured three intestinal biopsies from untreated celiac patients in the active phase of the disease and three from control subjects affected by gastro-oesophageal reflux for 3 h with P31-43-CY3 (20 micrograms/ml) and with unlabelled P31-43 (50 micrograms/ml). The samples were then washed, and chased for 24 h. Three samples from CD patients and three from controls were harvested after a 3-h pulse; three other samples from patients and controls were harvested after 24 h of chase. All samples were prepared for cryosectioning. Air-dried, 5 microns sections were stained for EEA1 and analysed with a confocal microscope. Anti EEA1 antibody was applied to the sections for 1 hour at room temperature and secondary antibody anti goat Alexa-488 conjugated was applied to the sections for 1 h at room temperature in a dark chamber. The protocol of the study was approved by the Ethical Committee of the University "Federico II", Naples, Italy (Etical approval code: C.E. n. 230/05).

Results

Different vesicle subpopulations carry P31-43 or P57-68 peptides

Peptides P31-43 and P57-68 enter the cells and interact with vesicular compartment. We used markers of the endocytic pathway to identify the vesicular compartment that interacts with P31-43-liss and P57-68-liss.

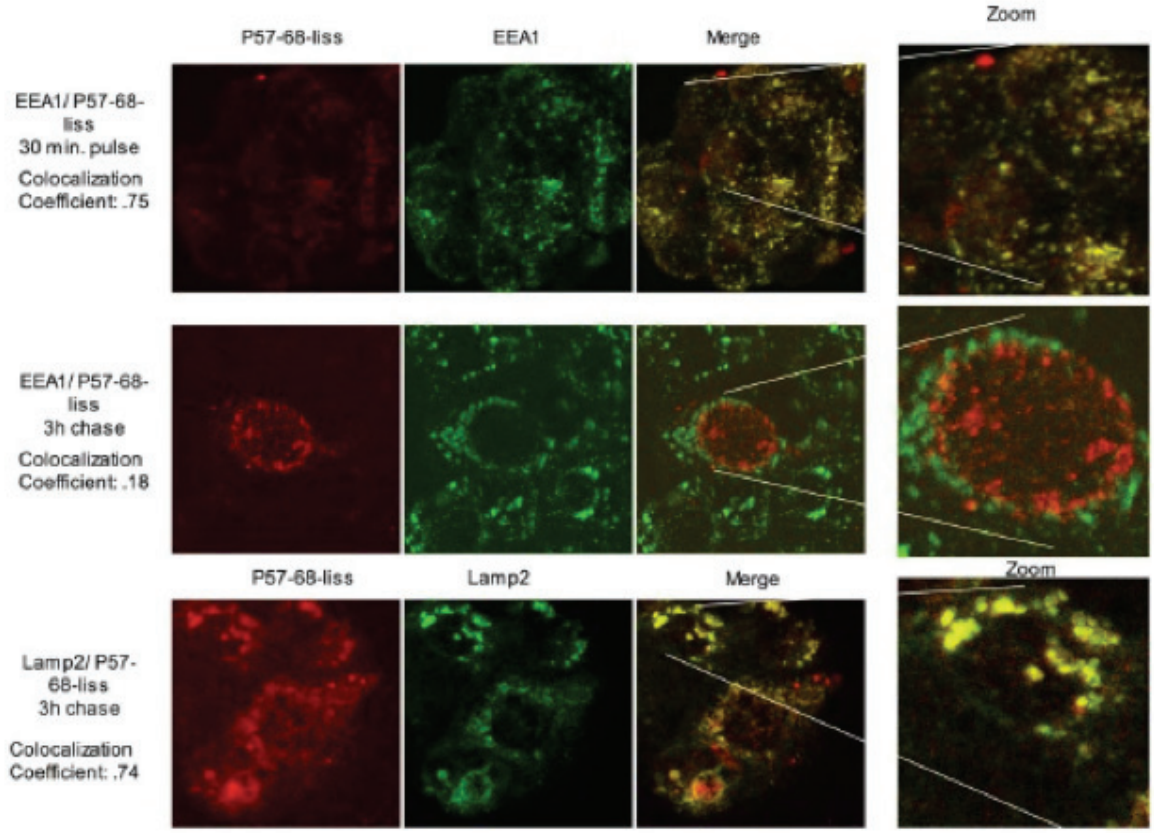

Figure 2. Vesicles interacting with P57-68-liss are late endocytic vesicles after a 3 h chase. At 30 minutes pulse, P57-68-liss co- localised with EEA1-positive vesicles (top panel); however after 3 hours of chase P57-68-liss no longer co- localised with EEA1-positive vesicles (middle panel). After 3 hours of chase, P57-68-liss co- localised with LAMP2-positive vesicles (bottom panel). Merge of the red and green panels of the EEA1 or LAMP2 and P31-43-liss or P57-68-liss staining is shown, yellow/orange colour indicates co- localisation. The zoom panels represent a digital 4× enlargement of the region highlighted by white lines in the merge panels. The co- localisation coefficient was calculated as reported under "Methods". The results are representative of four independent experiments.
doi:10.1371/journal.pone.0012246.g002

We used EEA1 (Figure 1) and Rab5-EGFP (not shown) as markers of early endocytosis. CaCo-2 cells treated with labelled peptides for 30 minutes (pulse) and 3 hours (chase) were stained with anti-EEA1 antibodies to identify early endocytic vesicles. Both peptides co-localised with EEA1-positive vesicles at 30 minutes (Figure 1 and 2), but only P31-43-liss-carrying vesicles were EEA1-positive after 3 hours of chase. Similar results were obtained with CaCo-2 cells overexpressing Rab 5-EGFP protein (not shown). Normal endocytic maturation requires the progression from early vesicles to late vesicles. We used the late endocytic markers LAMP2 (Figure 1) and Rab7-EGFP (not shown) to investigate the progression of endocytosis. CaCo-2 cells treated with labelled peptides for a 30-minutes pulse and a 3-hours chase were stained with anti-LAMP2 antibodies to identify late endocytic vesicles. Neither P31-43-liss nor P57-68-liss peptides co-localised with LAMP2-positive vesicles at 30 minutes (not shown). Only P57-68-liss-carrying vesicles were LAMP2-positive after 3 hours of chase (Figure 1 and 2). Taken together, these observations indicate that P31-43-containing vesicles, but not P57-68-containing vesicles are impaired in their maturation from early to late endosomes.

P31-43-liss-carrying vesicles move more slowly than P57-68-liss-carrying vesicles

To test the hypothesis that P31-43 could interfere with vesicle movements, we examined living cells labelled with the two peptides. The paths followed by individual vesicles were recorded after treatment with P31-43-liss and P57-68-liss peptides (Movie S1 and Movie S2). The movement of peptide-carrying vesicles was analysed for 10 minutes immediately after a 30-minutes pulse and after a 3-hours chase (Figure 3); data are reported as the average of three independent experiments. P57-68 vesicles had longer trajectories in pulse (0.44±0.009 microns/10 minutes) and chase (0.51±0.095 microns/10 minutes) experiments. P31-43-liss-interacting vesicles moved much less under both conditions (0.28±0.035 microns/10 minutes in pulse and 0.29±0.021 microns/10 minutes in chase experiments). Directionality of the endosome movements seems not to be affected by P31-43.

P31-43-induced delay of endocytic veside dynamics is unrelated to their cargo

To determine whether P31-43 is selectively directed to a specific population of slower vesicles or is able to delay endocytosis of vesicles where it is directed, we loaded CaCo-2 cells with Alexa-488 labelled dextran, a compound that is readily endocytosed in the cells, and EGF-Alexa, which normally enters the cells bound to the EGFR. CaCo-2 cells were pulsed for 30 minutes and chased for 3 hours with dextran-Alexa-488 alone or combined with P31-43-liss or P57-68 liss and then observed for 10 minutes in time-lapse microscopy. In experiments with dextran-Alexa-488 and P31-43-liss, most vesicles carry both fluorochromes, and are slower than vesicles carrying dextran alone or combined with P57-68 (Figure 4A), suggesting that the peptide, rather than the contents of the endocytic vesicle, is responsible for slower vesicle movement and delayed endocytosis.

We similarly investigated whether P31-43, which is known to interfere with EGF-carrying vesicles, [22] also affects the dynamics of EGF-Alexa-488-containing vesicles. Time-lapse analysis showed that P31-43-liss, unlike P57-68, delays EGF-Alexa-488-carrying vesicles (Figure. 4B). The fact that the peptide can delay endocytic vesicles carrying dextran or EGF strongly supports the hypothesis that the peptide is able to delay early endocytotic vesicles,

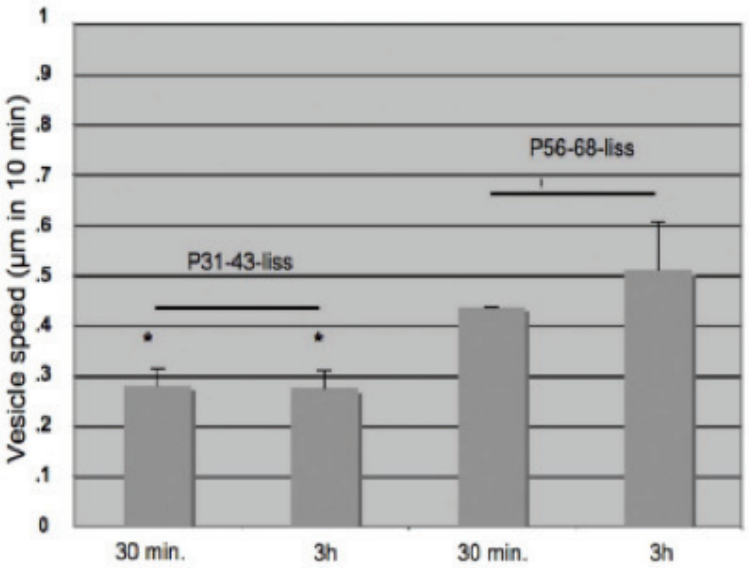

Figure 3. P31-43-liss-carrying vesicles are slower than P57-68-liss-carrying vesicles. Statistical analysis of three experiments performed resulted as follows: live CaCo2 cells pulsed for 30 minutes and chased for 3 hours with lissamine-labelled peptides were used for time-lapse experiments in which we acquired images every 30 seconds for 10 minutes at the indicated times. The image stacks were assembled to produce a video of vesicles dynamics. In each experiment, the position of at least 25 vesicles per cell was recorded and reconstructed to mark the trajectories of each vesicle during the observation time. The speed of vesicles was calculated by averaging the trajectories produced in 10 minutes at the indicated times. Bars represent mean and standard deviation. Asterisks indicate $P<0.05$ (Student's t-test). P31-43 carrying vesicles both at 30 minutes of pulse and 3 hours of chase are statistically significantly slower than P57-68 carrying vesicles.
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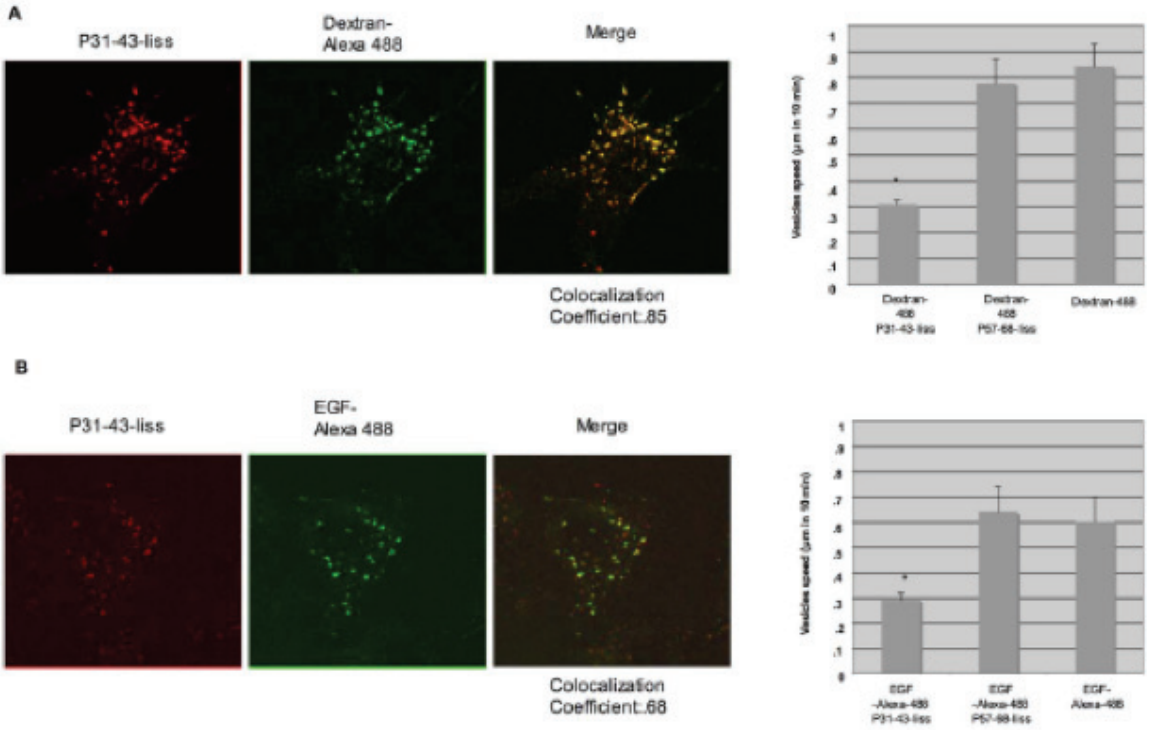

Figure 4. P31-43 delays endocytosis regardless of vesicles cargo. A) Dextran-Alexa-488-carrying vesicles moved faster than dextran-Alexa-488- and P31-43-liss-carrying vesicles. Live CaCo2 cells were pulsed for 30 minutes with dextran-Alexa-488 with and without P31-43-liss or P57-68-liss then chased for 3 hours in time lapse experiments. Only the 30 minutes pulse experiments are shown. The histogram shows the statistical analysis of three experiments. We calculated the speed of vesicles by averaging the trajectories produced in 10 minutes as in Figure 2. Bars represent mean and standard deviation. P31-43-liss and Dextran-Alexa-488 co-localised in the vesicular compartment. B) Live CaCo2 were pulsed for 30 minutes and chased for 3 hours (not shown) with EGF-Alexa-488 conjugated with and without P31-43-liss or P57-68-liss. Only the 30 minutes pulse experiments are shown. The histogram on the right side shows the statistical analysis of three experiments. We calculated the speed of vesicles by averaging the trajectories produced in 10 minutes as in Figure 2. Bars represent mean and standard deviation. P31-43-liss co- localises with EGF-Alexa 488 in the vesicular compartment. Asterisk indicates $P<0.05$ (student's t-test). The results show that P31-43 carrying vesicles are slower regardless of the cargo they are carrying.
doi:10.1371/journal.pone.0012246.g004

regardless of the contents of the vesicles. The effects exerted by P31-43 on the endocytic pathway, in the case of EGFR, as we have shown previously, [22] result in an extension of the activation period of this receptor, which accounts for the stimulation of EGFR dependent pathways. [22] This mechanism is likely to be responsible also for activation of other receptors sharing the same endocytic pathway.

P31-43 shares sequence similarity with Hrs, a key molecule in the maturation of endocytic vesicles

We carried out a FASTA search on the SWISSPROT database using gliadin peptide 31–43 as query sequence to look for endogenous proteins with a similar primary structure. The search returned, for both P31-43 and 31–49, a match with amino acids 719–731 of human Hrs, a protein with no known relationship to gliadin. The degree of similarity is high, with a better score and e-value than many matches with gliadin family proteins. Figure 5A shows the alignment of P31-43 with human Hrs and with Hrs from mouse, rat and *Drosophila melanogaster*. Of the 13 residues in the peptide, 7 are identical and 2 similar to the corresponding residues of human Hrs, the only major difference being the peptide N-terminal leucine instead of the consensus proline. The similarity area maps within a proline/glutamine-rich domain of Hrs and is conserved better than the surrounding area among Hrs orthologs. This finding is interesting because Hrs is localised both in the cytosol and in endocytic vesicles and is involved in the transport of EGFR, PDGFR and other receptor-containing early endosomes to lysosomes. [24–28] We carried out the same data bank search using the gliadin peptide P57-68, and failed to find any relevant similarity with Hrs or other human proteins.

P31-43 is localised at the level of the membranes in endocytic vesicles

We next investigated the sub-cellular localisation of gliadin peptides P31-43 and P57-68. P31-43 has been described by electron microscopy to be localized at the level of endocytic membranes. [29] When cells are transfected with Hrs, enlarged early endosomes are formed due to increased fusion; [30] furthermore detection of membrane vesicles by confocal immunofluorescence microscopy is facilitated. We then overexpressed Hrs-EGFP in CaCo2 cells for 48 h to obtain larger endosomes. P31-43 or P57-68 were added for 15 minutes and then the experiment was stopped. In Figure 5B one of these experiments is shown. As expected large endosomes can be seen with Hrs-EGFP

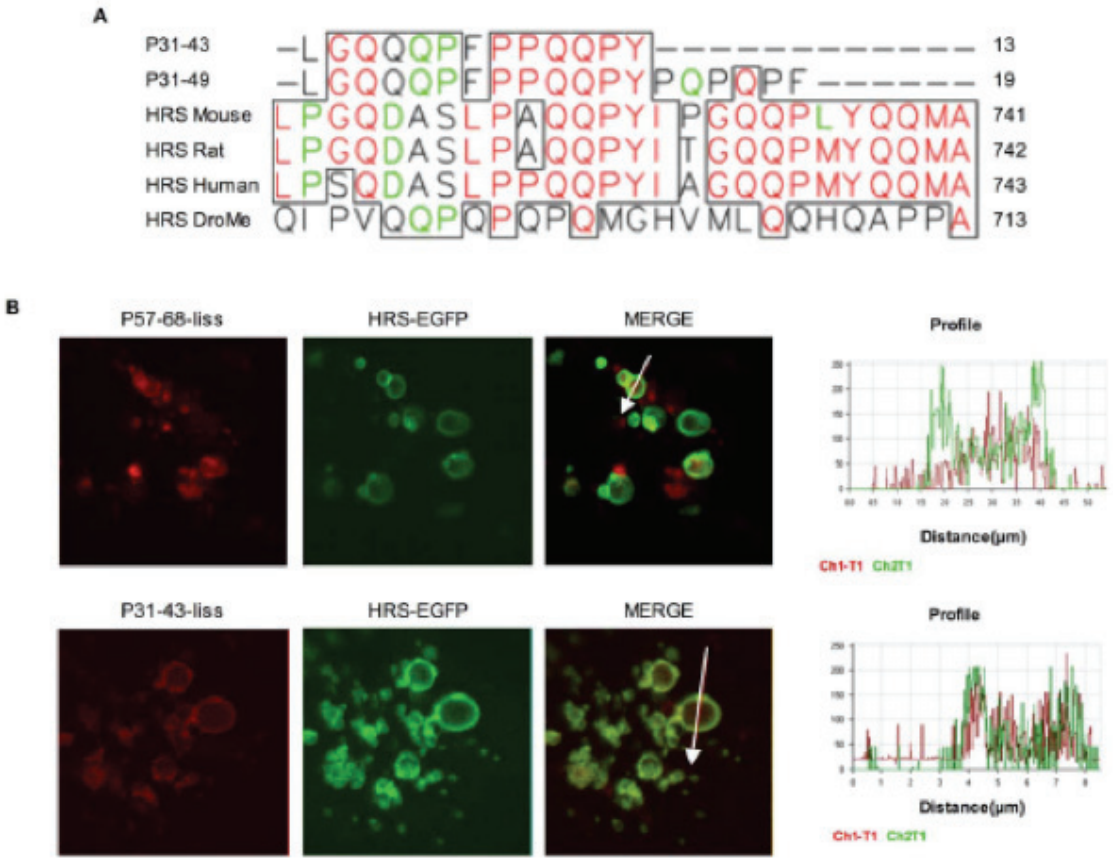

Figure 5. P31-43 is similar to Hrs and is localised at the membrane vesicles. A) Multiple alignments of gliadin peptides P31-43 and P31-49 are shown, with Hrs from mouse, rat, human and *Drosophila*. Numbers on the right side of the figure represent the terminal amino acid position of each sequence shown. Of the 13 residues in P31-43, 7 are identical (red) and 2 similar (green) to the corresponding residues of human Hrs. B) Enlarged vesicles visible by multiple digital enlargement (8 x) of the cytosol of a single cell. Hrs-EGFP was transfected for 48 hours in CaCo2 cells. P31-43 or P57-68 was added for 15 minutes. On the right side is the profile of the red and green channels along the white arrows that run across a single vesicle in the figure. The figure is representative of four similar independent experiments. Enlargement of the endocytic vesicles allow the observation of the vesicle membrane. In a short time (15 minutes) only P31-43 but not P57-68 co- localises with Hrs-EGFP at the vesicle membrane.
doi:10.1371/journal.pone.0012246.g005

present on the vesicles membranes. Surprisingly P31-43-liss, but not P57-68 co-localises with Hrs on the vesicles membranes, indicating that the two peptides may have different ways of entering the same endocytic vesicles.

P31-43, but not P57-68, competes with Hrs localisation in endocytic vesicles

Because there is a close sequence similarity between P31-43 and Hrs within a region that is important for the localisation of Hrs to the vesicle membrane, we evaluated whether the peptide could interfere with Hrs localisation to the vesicles. To this aim, we treated CaCo-2 cells with P31-43 or EGF, and then separated cytosolic and membrane bound proteins by ultracentrifugation to quantify the amount of endogenous Hrs present in each compartment. [30] P31-43 resulted in an increase in the amount of Hrs in the cytosolic fraction, which reached a maximum 3 h after treatment (Figure. 6A,B), whereas the amount of Hrs was decreased in the membrane fraction. This effect was unrelated to the enhanced EGF pathway because EGF treatment alone did not affect Hrs concentration in either compartment, in agreement with previous results. [30] The Hrs concentration was normalised to a control protein stained on the same blot, namely, tubulin in the cytosolic fraction and EGFR in the membrane fraction. [30] We used tubulin and EGFR because they selectively localise to the cytosolic and membrane fractions and their concentration is not affected by P31-43 treatment.

The results of confocal analysis are in agreement with the results of the western blot experiment. In fact, 24 h after transfection of the Hrs-EGFP fusion protein into CaCo-2 cells, fluorescence was mostly associated with vesicles, whereas 3 h treatment with P31-43 resulted in diffused cytoplasmic staining (Figure 6C). Hrs remained associated to vesicles in the control peptide P57-68 (Figure 6C).

Hrs overexpression prevents entry into the cell cycle induced by P31-43

Evidence suggests that gliadin peptides affect the cell cycle by delaying receptor inactivation. [22] Should this effect be due to competition of the gliadin peptide P31-43 with Hrs, large over

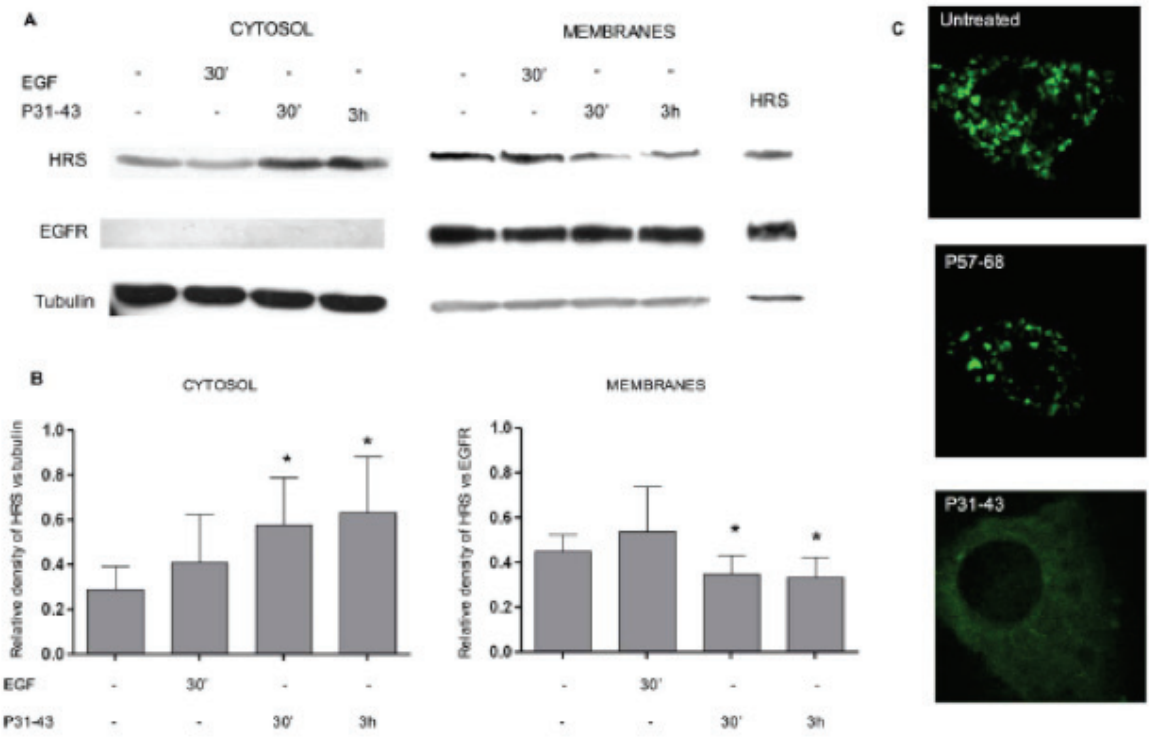

Figure 6. P31-43 competes with Hrs localisation to the endocytic vesicles. A) Western blot analysis of endogenous Hrs after separation by ultracentrifugation of cytosolic and membrane proteins. EGFR was used as a control for membrane fraction and tubulin as a control for cytosol. The last line on the right is a lysate of CaCo-2 cells transfected with Hrs as a size control for the protein. The results are representative of three independent experiments. B) Densitometric analysis. The Hrs concentration was normalised to a control protein, namely tubulin in the cytosolic fraction and EGFR in the membrane fraction. Mean and SD of three independent experiments is shown. Asterisk indicates $P<0.05$ (student's t-test). After P31-43 treatment, Hrs increases in the cytosolic fraction and decreases in the membrane fraction in comparison to the not treated sample in a statistically significant way. C) Confocal analysis of CaCo-2 cells transfected for 24 h with Hrs-EGFP, not treated and treated with P57-68 and P31-43 for the last 3 h of transfection. The results are representative of four independent experiments.
doi:10.1371/journal.pone.0012246.g006

expression of HRS cDNA would result in reversal of the effect. We therefore evaluated whether Hrs can compete with the effects induced by P31-43 on the cell cycle. As shown in Figure 7, CaCo-2 cells over expressing Hrs were stimulated to proliferate by adding EGF, P57-68 or P31-43. Under these conditions, EGF stimulation greatly increased BrdU incorporation in Hrs-transfected and untransfected CaCo-2 cells (Hrs = 66.25%±9.53%; No Hrs = 68.99%±7). As expected from a previous study, [22] P31-43 induced proliferation of non-Hrs-expressing CaCo-2 cells, with a BrdU incorporation of 51%±2% that decreased to 22.8%±8.4% in Hrs-expressing CaCo-2 cells. P57-68 did not induce proliferation. These data indicate that Hrs can compete with the effects exerted by P31-43 on cell proliferation.

In cultured small intestine samples from biopsies, P31-43 enters the enterocytes and interacts with early endocytic vesicles

We investigated whether P31-43 enters the cell and traced its localisation in intestinal biopsies from CD patients. For this set of experiments, we labelled P31-43 with CY3, a fluorochrome that is excited at 553 nanometers and emits at 575 nanometers. Intestinal biopsies were obtained from patients with CD and control subjects, kept in culture, and pulsed for 3 h with P31-43-CY3 (Figure 8A and B). In all biopsies, after 3 h pulse, peptide P31-43-CY3 was seen in epithelial cells, both in the crypts and in the villi (not shown) where it interacted with vesicles at the apical portion of the cells. Staining with anti EEA1 antibodies shows that the vesicles interacting with P31-43-CY3 are early endocytic vesicles. The overlay panels in Figure 8A and B show co- localisation of P31-43-CY3 and EEA1 in controls subjects and in patients with CD in the active phase of the disease. After 24 h chase, P31-43-CY3 was seen only in cells from CD patients. In this case the labelled peptide also interacted with the vesicular compartment and co-localised with EEA1 (Figure 8B). In normal controls, P31-43-CY3 entered the cells and interacted with EEA1-positive vesicles after a 3 h pulse, but the peptide was no longer seen after a 24-hours chase (Figure 8A). This indicates that, in healthy controls, this peptide is readily processed by the vesicular compartment. In the celiac environment, in which the peptide interacts with the vesicular compartment, P31-43 is delayed in early endocytic vesicles also at 24-hours chase.

Discussion

In this paper we demonstrate that A gliadin peptides P31-43 and P57-68 enter CaCo2 cells. P31-43 localises on the endocytic membranes and delays vesicle trafficking by interfering with Hrs-

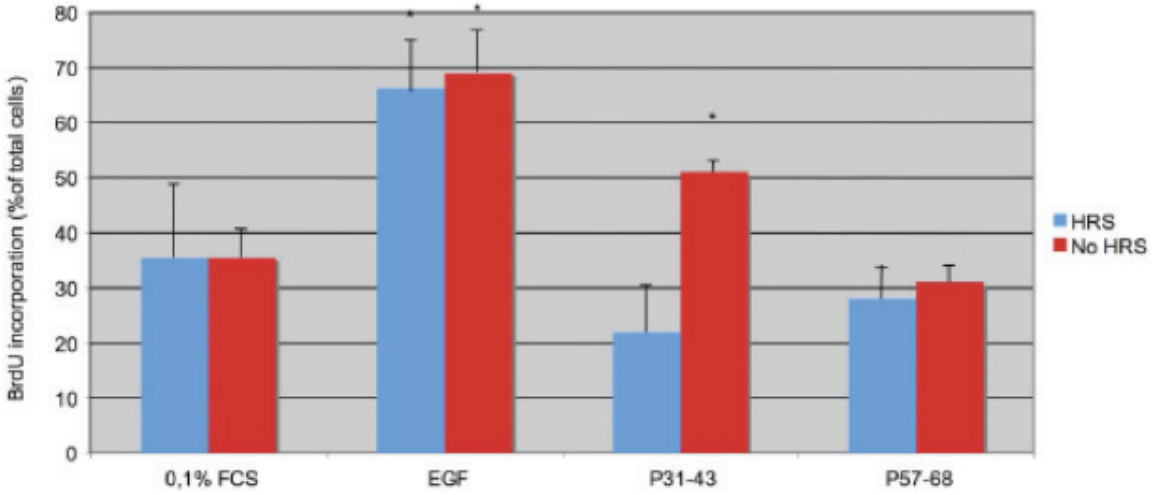

Figure 7. Hrs competes with the effects of P31-43 on the G0 >S transition. Bromodeoxyuridine (BrdU) incorporation of CaCo-2 cells transfected or not with Hrs-EGFP cDNA and treated as indicated 24 h after transfection. The bars represent the fraction of BrdU incorporating cells as a percent of total cells and are the mean ± SD of three independent experiments. Asterisks indicate P<0.05 (Student's t-test) with respect to the 0,1% FCS. These results indicate that Hrs compete with the effects exerted by P31-43 on cell proliferation.
doi:10.1371/journal.pone.0012246.g007

mediated maturation of early endosomes in cells and enterocytes. Consequently, EGFR and possibly other receptors activation is extended with multiple effects on various metabolic pathways and cellular functions.

Although little is known about the processing of gliadin peptides, there is evidence that they enter enterocytes. [31–32–33]. Recently two papers [29–34] have described entrance and localization of P31-43 and P57-68 gliadin peptides, one localising P31-43 to the level of early endocytic vesicles using electromicroscopy, (which is consistent with our findings), and the other localising it to the level of the late vesicles using light microscopy of biotinylated peptides. Zimmer et al have shown, that P31-43, which is found in early vesicles, is not presented to stimulate gluten sensitive T-cells, in contrast P57-68 is found in late vesicles and can be presented in this manner. [29] The results of our experiments show that both P31-43 and P57-68 enter CaCo-2 cells and interact with the vesicular compartment. Their entrance is an active process that requires a temperature of 37C and Ca++ in the media. Methyl-Beta-Cyclodextrin, an inhibitor of endocytosis, prevents the entrance of both peptides indicating that they enter the cells by endocytosis. [35–36]

We mapped the distribution of P31-43 and P57-68 along the endocytic pathway using markers of early endosomes (EEA1; RAB5-EGFP) and late endosomes (LAMP 2; RAB7-EGFP). P57-68 could progress from the early, EEA1 positive, endocytic compartment to the late, LAMP2 positive, compartment after a 3 h chase. P31-43 instead interacted both at 30 minutes and 3 hours with the early endocytic compartment. Vesicular dynamic correlates with proper maturation of early endocytic vesicles [30] and can be altered by proline/glutamine rich proteins such as Huntingtin. [37] We therefore, investigated the motility of vesicles carrying P31-43-liss and P57-68-liss. Live observation of cells treated with fluorescent peptides (time lapse) indicated that the P31-43-carrying vesicles are slower than those carrying P57-68 at both 30 minutes and 3 hours. Taken together, these results suggest that P31-43 remains in the early endocytic vesicles, thereby delaying maturation of these vesicles into late endosomes by affecting endocytic motility. Moreover P31-43, but not P57-68 was able to delay endocytic vesicles containing EGF-Alexa [22] and dextran indicating that P31-43 interferes with vesicular dynamics no matter what cargo they are carrying. Consequently EGFR and other receptors can stay longer activated. There is in fact compelling evidence that endocytic membrane trafficking regulates signalling by extra cellular ligands. [38]

The delay of decay of the EGF receptor may have different consequences in different cell types because it affects several pathways and different functions (cell reproduction and survival, permeability, motility, endocytosis etc.) (Figure 9). [39–40]. We previously showed that gliadin peptides, and in particular P31-43, induce actin rearrangements and cell proliferation in various cell types, thereby mimicking the effect of EGF. Peptide 31–43 induces phosphorylation of EGFR and of the downstream effector signalling molecule ERK [22] which indicates activation of the EGFR pathway. Enhancement of the EGF pathway by gliadin and P31-43 is due to delayed inactivation of EGFR. [22].

It is likely that endocytic delay could also affect the innate immune response and cytokine metabolism. We have shown (MV Barone, unpublished data) that in CaCo2 cells gliadin peptide P31-43 can enhance the recycling endocytic compartment. As a consequence of this process, more transferrin receptor and IL15 accumulates on the cell surface. Recently, the recycling transferrin receptor has been implicated in the pathogenesis of CD. In fact, transferrin receptors are increased in celiac intestine and also function as IgA receptors that retrotranscytose P31-49 linked to IgA. [41] Taken together these data suggest that an important pathogenetic event in CD is the interference of gliadin peptide P31-43 with the endocytic compartment.

A data bank search using P31-43 as the query sequence, revealed strong sequence similarity with a region of Hrs, which is an important regulator of endocytic trafficking. Hrs is the main coordinator of endocytosis and signalling. It is part of a large complex, located to early endocytic vesicles and the multivesicular body, that is involved in the ubiquitination of proteins destined to lysosomes. It can be phosphorylated in cells treated with growth

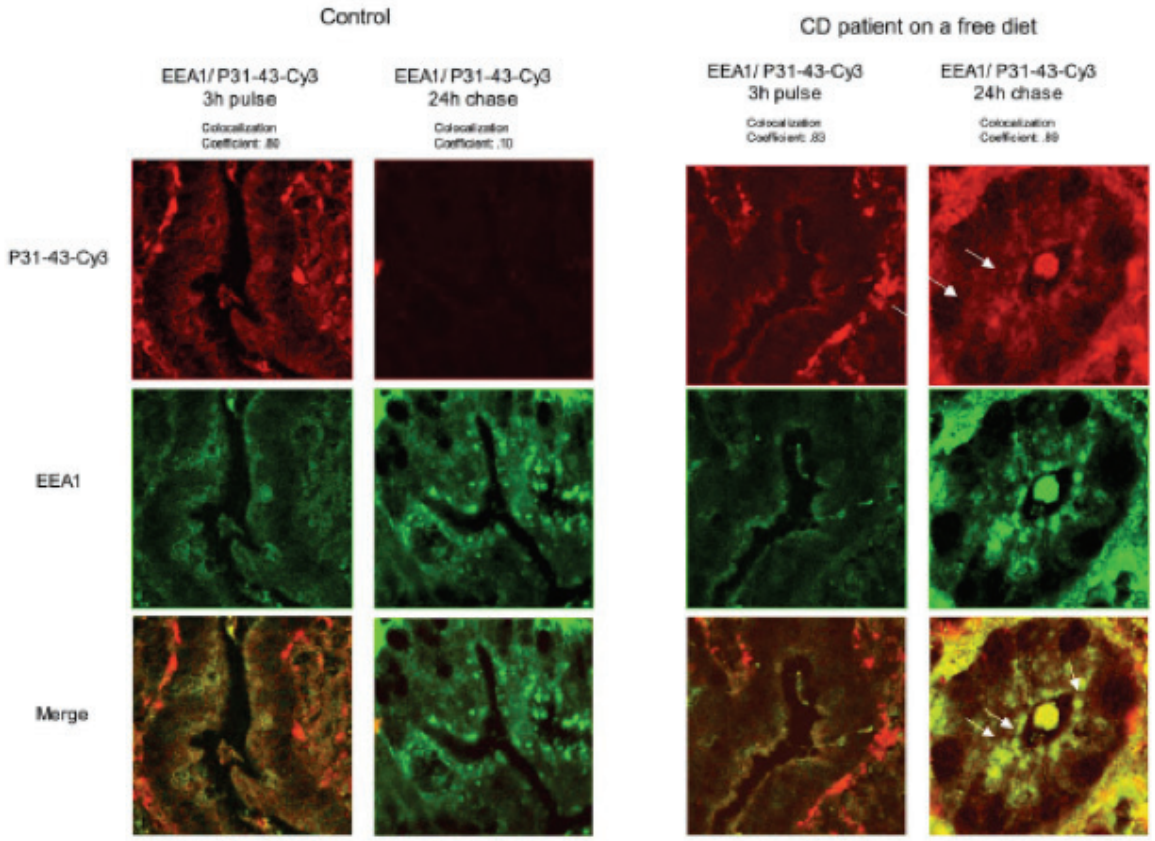

Figure 8. After a 24-h chase, gliadin peptide P31-43-CY3 is still present in epithelial cells of crypts of celiac disease patients, but not of controls. Intestinal biopsies from control subjects (A) and celiac disease patients on a gluten-containing diet (B) were cultivated with CY3-labelled P31-43 for 3 h and then chased for 24 h as indicated. Thin sections of the cultivated biopsies were then stained with anti-EEA1 antibodies. In control sections (A), P31-43-CY3 was visible only after 3-h pulse, but not after a 24 h chase. In the section of a celiac disease patient, P31-43-CY3 was present at both 3 h and 24 h in the epithelial cells of crypts. Overlay panels show that in cultivated biopsies from celiac patients at any time and in controls only at 3 h pulse, the P31-43 peptide co- localised with EEA1. White arrows indicates localisation of peptide in the early endocytic vesicles. Representative results from 3 independent experiments are shown.
doi:10.1371/journal.pone.0012246.g008

factors and cytokines [30] and is itself ubiquitinated. These post-translational modifications are needed for efficient sorting by Hrs of ubiquitinated membrane proteins to the degradation pathway. In cells where Hrs has been silenced, mutated or dislocated from the endosomes, EGFR and other receptor tyrosine kinases stay activated longer [42] and are recycled back to the cell surface. [43]

The sequence similarity between gliadin peptide P31-43 and Hrs involves a small area of the proline/glutamine-rich domain of the latter. Although gliadin is a well known proline/glutamine-rich protein, the homology of P31-43 with this Hrs domain is specific because the rest of the gliadin proline/glutamine-rich sequence does not share the same degree of similarity with Hrs. Moreover, P57-68, another gliadin peptide with a similar amino acid composition, does not produce the same effects in cells. This Hrs domain, at its COOH end, contains the clathrin-binding domain that brings clathrin to clathrin coated vesicles, [27] and is one of the domains needed to localise Hrs to the vesicle membranes. [44–45–46]

We have demonstrated in Hrs-EGFP transfected CaCo2 cells that P31-43, but not P57-68 co-localises with Hrs on the membrane of endocytic vesicles after 15 minutes of treatment, suggesting that the two peptides may have a different route to enter endocytic vesicles. Up to now no receptor has been found for P31-43 uptake. (Barone et al. unpublished results). Vilasi et al. [47] have proposed an alternative possibility investigating the interaction of the gliadin peptides with a very simple model of lipids micellae. They showed that P31-43 but not P57-68 can directly interact with the micellae, a good indication that it is possible for P31-43 to travel through the membranes and possibly reach the HRS molecules on the surface of the vesicles. We next evaluated whether P31-43 could interfere with Hrs localisation to the endocytic vesicles.

Western blot analysis of proteins extracted from the cell cytosol and membranes, together with immunofluorescence, showed that P31-43 treatment for 3 h, moved HRS from the vesicles to the cytosol. Furthermore, if P31-43 interferes with Hrs localization, it follows that a large excess of Hrs should prevent the proliferative activity of the gliadin peptide on cells. In fact, over expression of Hrs-EGFP prevented the effect of P31-43 on CaCo-2 proliferation. Taken together these results suggest that P31-43 interferes with Hrs-mediated maturation of early endosomes.

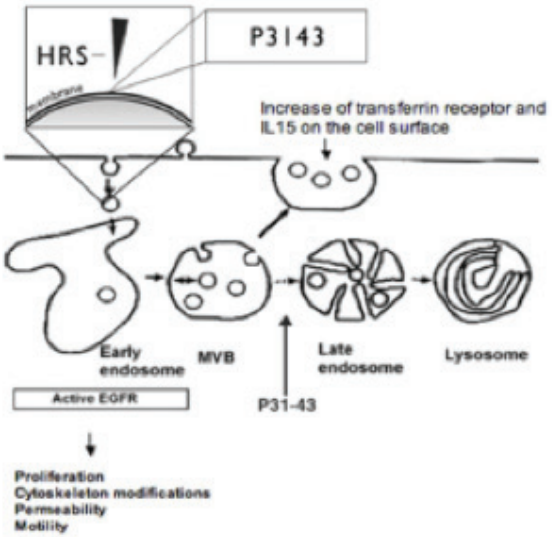

Figure 9. Overview of the effects of P31-43 on the endocytic pathway. Endocytosis has many effects on signalling: in fact, signalling pathways and endocytic pathways are regulated in a reciprocal manner. It is now widely accepted that the "Endocytic Matrix" is a master organiser of signalling, governing the resolution of signals in space and time. Consequently endocytosis affects several cell functions that range from proliferation to cell motility [50]. Growing evidences [22, 33, 34 and the present paper], point to an effect of certain gliadin peptides (i.e. P31-43) on the endocytic compartment. By interfering with Hrs localisation to the endocytic membranes, P31-43 induces two important effects: a) it delays endocytic maturation, and b) it alters the recycling pathway. By delaying the maturation of endocytic vesicles P31-43 reduces EGFR and other RTK degradation and prolongs their activation which in turn results in increased proliferation, actin modification and other biological effects. The alteration of the recycling pathway is able to direct more transferrin receptor and likely other recycling receptors such as IL15 to the membranes.
doi:10.1371/journal.pone.0012246.g009

We also examined P31-43 trafficking in cultured intestinal biopsies from CD patients and controls using pulse and chase experiments. We show that P31-43 enters the enterocytes of cultured intestinal biopsies and localise, after a 3 h pulse, in early endocytic vesicles of enterocytes of intestinal biopsies from normal control subjects and non-treated celiac patients. However, after a 24-h chase, the peptide was still in the early endosomes of celiac enterocytes, but not in those of controls. This suggests that celiac patients are particularly susceptible to the effect of P31-43. We previously reported that endocytosis of EGF is delayed in enterocytes of atrophic celiac mucosa cultured *in vitro* with P31-43. [22] In the same context, P31-43 increased proliferation of crypts enterocytes – an effect that was prevented by EGFR inhibitors. [22]. Similar to the effects we observed in CaCo-2 cells, P31-43 probably delayed maturation of early endocytic vesicles also in cultured biopsies. This process prolongs EGFR activation and culminates in increased EGFR-dependent proliferation of crypt enterocytes as we have previously shown. [22] These observations suggest that the EGF pathway plays a central role in initiating and maintaining the high proliferation rates observed in the crypts of celiac patients. [48–49] This finding explains at least in part the role played by gliadin in remodelling of the celiac mucosa.

From a general point of view it is interesting to note that peptides from a very common alimentary protein, the gliadin, can have several metabolic effects due to the interference with important cellular functions, such as those regulated by the endocytic pathway. It remains to be established why P31-43 has a peculiar effect on the celiac intestinal mucosa. Celiac patients may have an alteration of the endocytic pathway (or some other related metabolic pathway) that renders cells more sensitive to the effect of P31-43 on endocytic maturation.

Supporting Information

Figure S1 Control panel for EEA1 staining did not show any cross-excitation of fluorocromes: CaCo-2 cells, after 30 minutes pulse with P31-43-liss (red), were fixed permibilised and stained with an isotype matched primary antibody and anti-goat secondary antibodies Alexa-488 conjugated (green). The control shows that there isn't any cross-excitation of fluorocromes between the Alexa-488 conjugated secondary antibody and the lissamine linked to the peptide. Merge of the red and green panels is shown. The results are representative of 4 independent experiments.
Found at: doi:10.1371/journal.pone.0012246.s001 (2.37 MB TIF)

Movie S1 In Materials and Methods of the text.
Found at: doi:10.1371/journal.pone.0012246.s002 (1.88 MB AVI)

Movie S2 In Materials and Methods of the text.
Found at: doi:10.1371/journal.pone.0012246.s003 (2.42 MB AVI)

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Author Contributions

Conceived and designed the experiments: MVB SA. Performed the experiments: MVB MN MM RT MTSR. Analyzed the data: MVB MN GP MM RT MTSR. Contributed reagents/materials/analysis tools: GP SA. Wrote the paper: MVB SA.

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CONCLUSIVE REMARKS

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The delay of decay of the EGF receptor may have different consequences in different cell types because it affects several pathways and different functions (cell reproduction and survival, permeability, motility, endocytosis etc.). [35-36]. We previously showed that gliadin peptides, and in particular P31-43, induce actin rearrangements and cell proliferation in various cell types, thereby mimicking the effect of EGF. Peptide 31-43 induces phosphorylation of EGFR and of the downstream effector signalling molecule ERK [22] which indicates activation of the EGFR pathway. Enhancement of the EGF pathway by gliadin and P31-43 is due to delayed inactivation of EGFR. [22]

It is likely that endocytic delay could also affect the innate immune response and cytokine metabolism. We have shown (MV Barone, unpublished data) that in CaCo2 cells gliadin peptide P31-43 can enhance the recycling endocytic compartment. As a consequence of this process, more transferrin receptor and IL15 accumulates on the cell surface. Recently, the recycling transferrin receptor has been implicated in the pathogenesis of CD. In fact, transferrin receptors are increased in celiac intestine and also function as IgA receptors that retrotranscytose P31-49 linked to IgA. [37] Taken together these data suggest that an important pathogenetic event in CD is the interference of gliadin peptide P31-43 with the endocytic compartment.

A data bank search using P31-43 as the query sequence, revealed strong sequence similarity with a region of Hrs, which is an important regulator of endocytic trafficking. Hrs is the main coordinator of endocytosis and signalling. It is part of a large complex, located to early endocytic vesicles and the multivesicular body, that is involved in the ubiquitination of proteins destined to lysosomes. It can be phosphorylated in cells treated with growth factors and cytokines [32] and is itself ubiquitinated. These post-translational modifications are needed for efficient sorting by Hrs of ubiquitinated membrane proteins to the degradation pathway. In cells where Hrs has been silenced, mutated or dislocated from the endosomes, EGFR and other receptor tyrosine kinases stay activated longer [38] and are recycled back to the cell surface. [39]

The sequence similarity between gliadin peptide P31-43 and Hrs involves a small area of the proline/

glutamine-rich domain of the latter. Although gliadin is a well known proline/glutamine-rich protein, the homology of P31-43 with this Hrs domain is specific because the rest of the gliadin proline/glutamine-rich sequence does not share the same degree of similarity with Hrs. Moreover, P57-68, another gliadin peptide with a similar amino acid composition, does not produce the same effects in cells. This Hrs domain, at its COOH end, contains the clathrin-binding domain that brings clathrin to clathrin ¬coated vesicles, [40] and is one of the domains needed to localise Hrs to the vesicle membranes. [41-42-43]

We have demonstrated in Hrs-EGFP transfected CaCo2 cells that P31-43, but not P57-68 co-localises with Hrs on the membrane of endocytic vesicles after 15 minutes of treatment, suggesting that the two peptides may have a different route to enter endocytic vesicles. Up to now no receptor has been found for P31-43 uptake. (Barone et al. unpublished results). Vilasi et al. [44] have proposed an alternative possibility investigating the interaction of the gliadin peptides with a very simple model of lipids micellae. They showed that P31-43 but not P57-68 can directly interact with the micellae, a good indication that it is possible for P31-43 to travel through the membranes and possibly reach the HRS molecules on the surface of the vesicles. We next evaluated whether P31-43 could interfere with Hrs localisation to the endocytic vesicles.

Western blot analysis of proteins extracted from the cell cytosol and membranes, together with immunofluorescence, showed that P31-43 treatment for 3 h, moved HRS from the vesicles to the cytosol. Furthermore, if P31-43 interferes with Hrs localization, it follows that a large excess of Hrs should prevent the proliferative activity of the gliadin peptide on cells. In fact, over¬expression of Hrs-EGFP prevented the effect of P31-43 on CaCo-2 proliferation. Taken together these results suggest that P31-43 interferes with Hrs-mediated maturation of early endosomes.

We also examined P31-43 trafficking in cultured intestinal biopsies from CD patients and controls using pulse and chase experiments. We show that P31-43 enters the enterocytes of cultured intestinal biopsies and localise, after a 3 h pulse, in early endocytic vesicles of enterocytes of intestinal biopsies from normal control subjects and non-treated celiac patients. However, after a 24-h chase, the peptide was still in the early endosomes of celiac enterocytes, but not in those of controls. This suggests that celiac patients are particularly susceptible to the effect of P31-43. We previously reported that endocytosis of EGF is delayed in enterocytes of atrophic celiac mucosa cultured in vitro with P31-43. [22] In the same context, P31-

43 increased proliferation of crypts enterocytes – an effect that was prevented by EGFR inhibitors. [22]. Similar to the effects we observed in CaCo-2 cells, P31-43 probably delayed maturation of early endocytic vesicles also in cultured biopsies. This process prolongs EGFR activation and culminates in increased EGFR-dependent proliferation of crypt enterocytes as we have previously shown. [22] These observations suggest that the EGF pathway plays a central role in initiating and maintaining the high proliferation rates observed in the crypts of celiac patients. [45-46] This finding explains at least in part the role played by gliadin in remodelling of the celiac mucosa.

From a general point of view it is interesting to note that peptides from a very common alimentary protein, the gliadin, can have several metabolic effects due to the interference with important cellular functions, such as those regulated by the endocytic pathway. It remains to be established why P31-43 has a peculiar effect on the celiac intestinal mucosa. Celiac patients may have an alteration of the endocytic pathway (or some other related metabolic pathway) that renders cells more sensitive to the effect of P31-43 on endocytic maturation.

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CHAPTER 2

Gliadin-mediated proliferation and innate immune activation in celiac disease are due to alterations in vesicular trafficking

Project

Celiac disease (CD) is characterised by derangement of both the adaptive and the innate immune responses to gliadin. Some gliadin peptides that are deamidated by tissue transglutaminase (e.g., A-gliadin P57-68) bind to HLA DQ2 and/or DQ8 molecules and induce an adaptive Th1 pro-inflammatory response. [1] There is also evidence that gliadin contains other peptides (i.e., P31-43) able to initiate a response involving innate immune.[2,3]

Damage to the intestinal mucosa in CD is mediated both by inflammation due to the adaptive and innate immune responses (with IL-15 as a major mediator of the innate immune response) and by proliferation of crypt enterocytes as an early alteration of CD mucosa causing crypt hyperplasia.[4-6] The celiac intestine is characterised, in fact, by an inversion of the differentiation/proliferation program of the tissue with a reduction in the differentiated compartment, up to complete villi atrophy, and an increase of the proliferative compartment, with crypt hyperplasia.[7,8]

We previously investigated the early events of celiac disease and in particular the interaction between gliadin peptides and intestinal epithelial cells. We found that the so-called gliadin toxic peptide (P31-43) delays endocytic vesicle maturation and consequently reduces epidermal growth factor receptor (EGFR) degradation and prolongs EGFR activation, which in turn results in increased cell proliferation and actin modifications in celiac crypt enterocytes and in various cells lines.[9] P31-43 enters CaCo2 cells and intestinal enterocytes, interacts with early endocytic vesicles,[10,11] reduces their motility and delays their maturation to late endosomes. [10] Taken together, this information points toward an effect of certain gliadin peptides, i.e., P31-43, on endocytic function and indicates epidermal growth factor (EGF) signalling as one of the major pathways in the celiac intestine.

The pro-inflammatory cytokine IL-15 is a major mediator of innate immune in CD. In fact, IL-15 is higher in the lamina propria and the intestinal epithelium of untreated celiac patients as compared with treated

patients and controls.[3,12,13] It induces differentiation of dendritic cells[14] and is also secreted by the intestinal epithelium.[15] Moreover, IL-15 affects the proliferation, localisation and function of intraepithelial lymphocytes (IELs) in the intestinal mucosa of CD patients.[16-19]

Gliadin peptides 31-43 and 31-49 are not recognized by T cells and induce an innate immune response in the celiac mucosa.[2] P31-43-induced activation of various markers of the innate immune response is inhibited by neutralising anti-IL-15 antibodies.[2] IL-15 mediates P31-43-induced expression of the stress molecule MIC-A in enterocytes[3] and reproduces most of the epithelial modifications caused by gliadin in CD patients, including IEL migration.[12-14] IL-15 also exerts pleiotropic activity that ultimately results in immunoregulatory cross-talk between cells of the innate and adaptive branches of the immune response.[20] Moreover, IL-15 can induce proliferation in intestinal epithelial cells21.

IL-15 expression is tightly regulated at both the transcriptional and post-transcriptional levels.[22-24] Although IL-15 transcripts are widely expressed, the IL-15 protein is seldom detected in the supernatants of cells that display mRNA for this interleukin.[22,24] IL-15 has been found in the Golgi complex and in transferrin-carrying endocytic vesicles.[25,26] Trafficking of the IL-15/IL-15R alpha complex in the endocytic pathway plays a central role in the regulation of IL-15 expression at the post-transcriptional level. IL-15 is chaperoned through the secretory pathway by complexing with IL-15 R alpha, as this complex forms in the Golgi and is transported to the membrane where it recycles and is trans-presented to neighbouring cells.[27-31] Interestingly, in the intestine, IL-15 is present on the surface of enterocytes, which suggests that cell-to-cell contact could play a role in IEL regulation.[13]

The aims of this study were to determine if the proliferative activity of P31-43 on celiac enterocytes and cells is not only EGFR-dependent but also mediated by IL-15. We also investigated whether P31-43 increases IL-15 in an intestinal epithelial cell line (CaCo2 cells) and the molecular and cellular bases of this phenomenon in relation to the derangement of the vesicular function induced by P31-43.

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PLoS one

Gliadin-Mediated Proliferation and Innate Immune Activation in Celiac Disease Are Due to Alterations in Vesicular Trafficking

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Abstract

Background and Objectives: Damage to intestinal mucosa in celiac disease (CD) is mediated both by inflammation due to adaptive and innate immune responses, with IL-15 as a major mediator of the innate immune response, and by proliferation of crypt enterocytes as an early alteration of CD mucosa causing crypts hyperplasia. We have previously shown that gliadin peptide P31-43 induces proliferation of cell lines and celiac enterocytes by delaying degradation of the active epidermal growth factor receptor (EGFR) due to delayed maturation of endocytic vesicles. IL-15 is increased in the intestine of patients affected by CD and has pleiotropic activity that ultimately results in immunoregulatory cross-talk between cells belonging to the innate and adaptive branches of the immune response. Aims of this study were to investigate the role of P31-43 in the induction of cellular proliferation and innate immune activation.

Methods/Principal Findings: Cell proliferation was evaluated by bromodeoxyuridine (BrdU) incorporation both in CaCo-2 cells and in biopsies from active CD cases and controls. We used real-time PCR to evaluate IL-15 mRNA levels and FACS as well as ELISA and Western Blot (WB) analysis to measure protein levels and distribution in CaCo-2 cells. Gliadin and P31-43 induce a proliferation of both CaCo-2 cells and CD crypt enterocytes that is dependent on both EGFR and IL-15 activity. In CaCo-2 cells, P31-43 increased IL-15 levels on the cell surface by altering intracellular trafficking. The increased IL-15 protein was bound to IL15 receptor (IL-15R) alpha, did not require new protein synthesis and functioned as a growth factor.

Conclusion: In this study, we have shown that P31-43 induces both increase of the *trans*-presented IL-15/IL5R alpha complex on cell surfaces by altering the trafficking of the vesicular compartments as well as proliferation of crypt enterocytes with consequent remodelling of CD mucosa due to a cooperation of IL-15 and EGFR.

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Introduction

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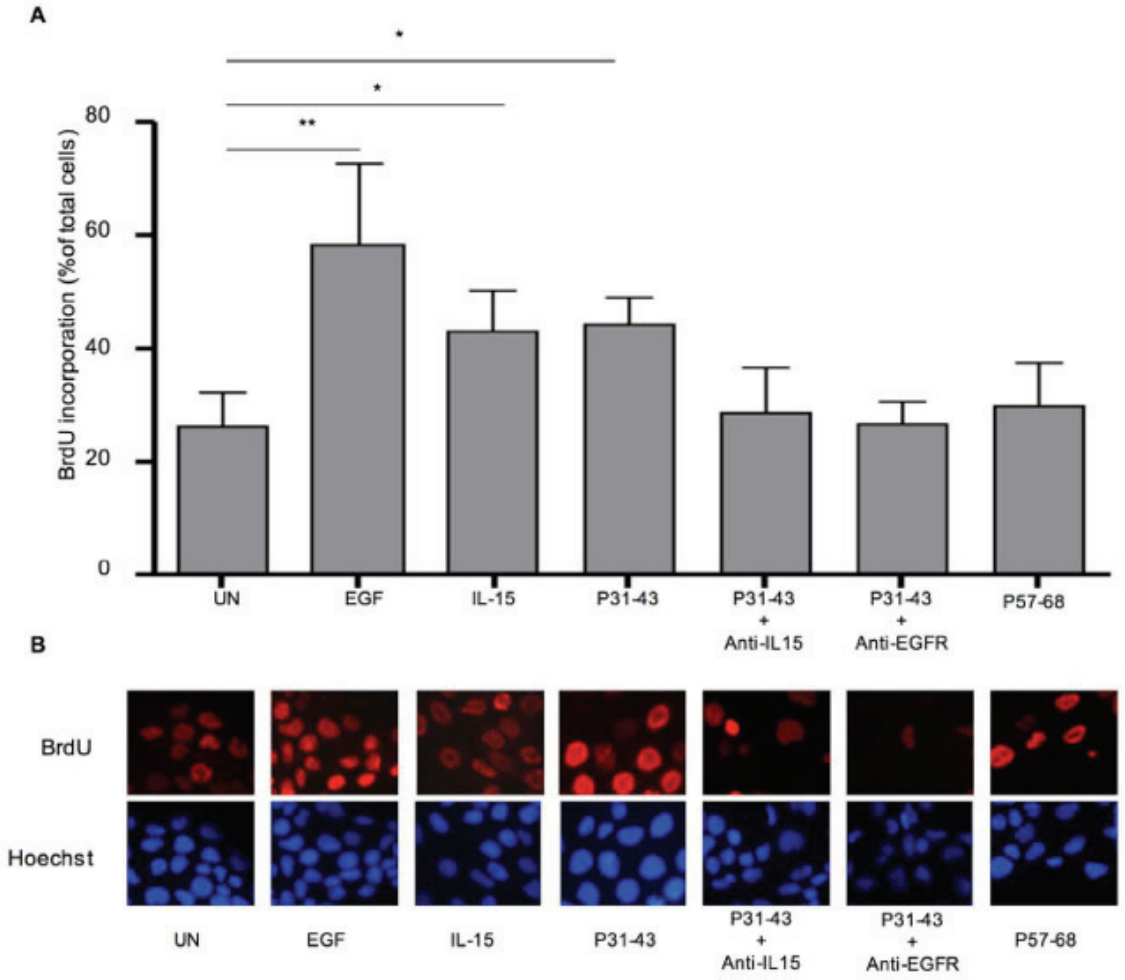

Figure 1. P31-43-induced EGFR- and IL-15-dependent proliferation in CaCo-2 cells. (A) Quantification of BrdU incorporation of CaCo-2 cells incubated overnight with medium alone, or treated as indicated. Columns represent the mean and bars represent the standard deviation of five independent experiments. More than 300 nuclei were counted for each experiment in several optical fields and the number of BrdU-positive cells was expressed as a proportion of the total nuclei. * = $p<0.05$ ** $p<0.01$ (Student's t-test). (B) Immunofluorescence staining of BrdU incorporation of CaCo-2 cells treated as indicated. Hoechst stains of total nuclei. Single representative optical fields (63x objective).
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pathway by complexing with IL-15 R alpha, as this complex forms in the Golgi and is transported to the membrane where it recycles and is *trans*-presented to neighbouring cells [27–31]. Interestingly, in the intestine, IL-15 is present on the surface of enterocytes, which suggests that cell-to-cell contact could play a role in IEL regulation [13].

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Materials and Methods

Cell culture, materials and transfections

CaCo-2 cells were grown for 5–6 days in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, San Giuliano Milanese, Italy), 10% fetal calf serum (GIBCO), 100 units/ml penicillin-streptomycin (GIBCO), and 1 mM glutamine (GIBCO) with medium changed every two days.

Synthetic peptides were obtained from Inbios srl (Naples, Italy) and they were >95% pure as evaluated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Lipopolysaccharide (LPS)-free peptides were obtained by Ultrasart-D20 filtration (Sartorius AG, Gottingen, Germany)[9].

The levels of LPS in these peptides were below the detection threshold, i.e., <0.20 EU/mg as assessed with the QCL-1000 kit (Cambrex Corporation, NJ). The P31-43 sequence was LGQQQPFPPQQPY and the P57-68 sequence was QLQPFPQPQLPY. Dose/response experiments indicated that the best concentration of peptides for experiments involving bromodeoxyuridine (BrdU) incorporation and IL-15 expression on the cell surface (Fig. S1) was 100 μg/ml [9].

IL-15 PE-conjugated monoclonal antibody (clone: 34559; isotype: IgG1) was purchased from R&D Systems (Minneapolis, MN, USA). Rat isotype-matched PE-labelled control IgG1s were purchased from Pharmingen (San Diego, CA, USA). Recombinant human IL-15 (R&D Systems, Minneapolis, MN, USA) was used at a concentration of 10 ng/ml for FACS analysis and the blocking monoclonal anti-human IL-15 antibody (R&D Systems, Minneapolis, MN, USA) at 5 μg/ml in all experiments. We used the goat, anti-human IL-15R alpha (R&D Systems, Minneapolis, MN, USA), rabbit anti-human IL-15 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-alpha tubulin (Sigma-Aldrich, Milan, Italy) and rabbit anti-EGFR (Cell Signaling Celbio, Milan, Italy) antibodies for western blotting. BrdU was detected with a

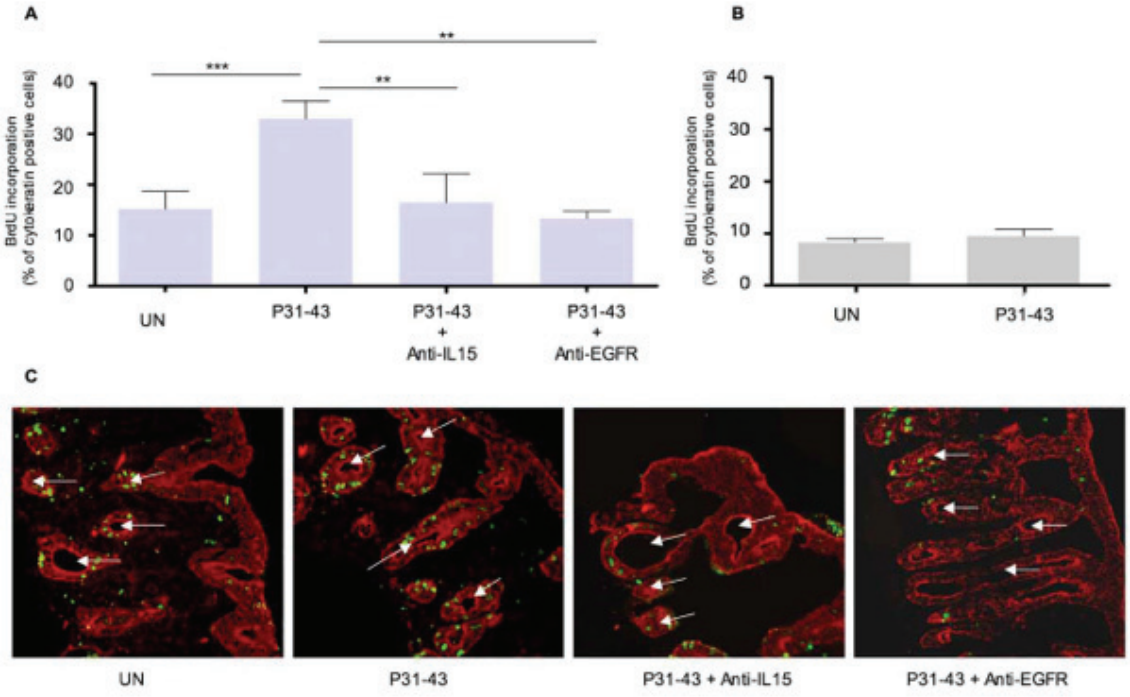

Figure 2. P31-43-induced proliferation of crypt enterocytes in celiac disease (CD) biopsies in the active phase of the disease depends on EGFR and IL-15 functions. (A) Quantification of BrdU incorporation of crypt enterocytes of intestinal biopsies from CD patients incubated with P31-43, with and without blocking antibodies anti-IL-15 and anti-EGFR. More than 300 cytokeratin-positive cells were counted in several fields in each sample and the number of BrdU-positive cells was expressed as a proportion of the total cytokeratin-positive cells. Mean and standard deviation of five independent experiments (Student's *t* test). ** = $p<0.01$; *** = $p<0.001$ (B) Quantification of BrdU incorporation of crypt enterocytes of intestinal biopsies from controls incubated with P31-43. More than 300 cytokeratin-positive cells were counted in several fields in each sample and the number of BrdU-positive cells was expressed as a proportion of the total cytokeratin-positive cells. Mean and standard deviation of three independent experiments (C) Immunofluorescence of crypts of duodenal biopsies from patients with active CD stained for cytokeratin to identify epithelial cells [red] and for BrdU [green]. Representative single optical field (40x objective). Lumen of the crypt is highlighted by white arrows. For methods, see supplementary material.
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monoclonal antibody (GE Healthcare, Bickinghamshire, UK) and an anti-mouse-Alexa-488 conjugated secondary antibody (Molecular probes, San Giuliano Milanese, Italy). Nuclei were stained with Hoechst (Sigma-Aldrich, Milan, Italy). BrdU incorporation experiments to evaluate cell proliferation were carried out as described elsewhere.[9] Blocking antibodies EGFR (528) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and IL-15 (R&D Systems, Minneapolis, MN, USA) were used at concentrations of 2 μg/ml and 5 μg/ml, respectively, for the BrdU assay.

Transfection of siIL-15R alpha was carried out following the manufacturer's instructions (QIAGEN) with HIPerFect Transfection Reagent. Briefly, CaCo-2 cells were incubated in standard growth conditions and 500 ng of IL-15R alpha siRNA were diluted in 100 μl of culture medium without serum to give a final siRNA concentration of 10 nM. Twenty microliters of HIPerFect Transfection reagent were added to the siRNA mix by vortexing. The transfection mix was added drop-wise onto the cells which were incubated for 72 h. Cells were than processed for WB or FACS analysis. Transfection of IL-15-EGFP was carried out as described before [25].

Over night (O/N) treatment is intended as 16 h treatment.

IL-15 analysis

CaCo-2 cells were stimulated at 37°C with P31-43, P57-68, cycloheximide (Sigma-Aldrich, Milan, Italy) or with medium alone. After incubation, cells were removed from the dish by scraping on ice and plated in 96-well V-bottom plates (Costar Celbio, Milan, Italy). Cells were plated at a density of 1×10^5 cells/well and were washed with PBS and analysed for surface or intracellular cytokine expression. Membrane cytokines were identified by labelling cells with PE-conjugated anti-IL-15 mAb for 30 min at 4°C. In the experiments to detect intracellular cytokines, 10 μg/ml brefeldin A (Sigma-Aldrich, Milan, Italy) was added to the incubation media for 3 h. Intracellular cytokines were identified as previously reported [32]. Finally, cells were read with a cytometer. Cycloheximide was used at a final concentration of 2 mM [33]. Dose-response curve was done for P31-43 stimulation to find optimal P31-43 concentration (Figure S1).

Some of the cells stimulated with P31-43 or medium alone were treated with acid buffer (2 mM glycine and 150 mM NaCl) for 10 min at 4°C and then labelled with PE-conjugated anti-IL-15 mAb [28]. Flow cytometry was carried out with a FACSCalibur system (BD Bioscience, San Diego, CA, USA) and the results were analysed with CellQuestPro software (BD Bioscience, San Diego, CA, USA).

Immunoprecipitation

Lysates were prepared as described previously and protein concentration was measured with a Bio-Rad protein assay kit (Hercules, CA, USA) [9]. Equal amounts of cell lysates (2 mg protein/mL) were used for immunoprecipitation. IL-15R

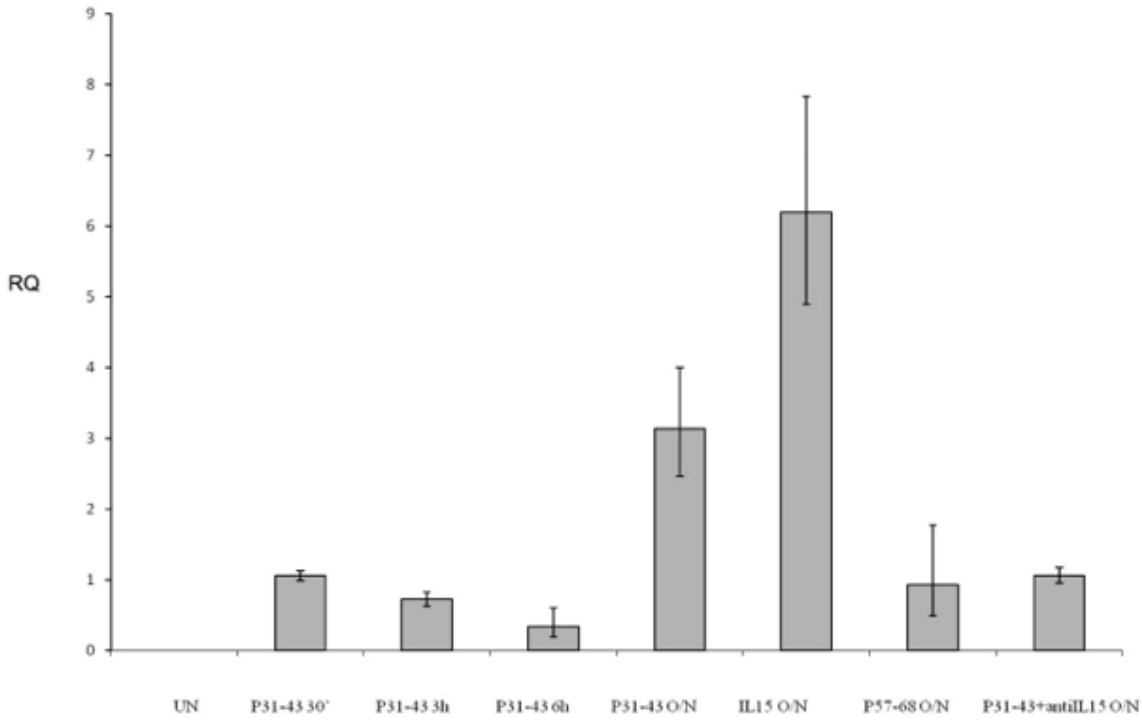

Figure 3. Overnight treatment with gliadin peptide P31-43, but not P57-68, increased levels of IL-15 mRNA in CaCo-2 cells. Quantitative PCR analysis shows an increase of IL-15 mRNA after O/N treatment of CaCo-2 cells with P31-43 but not after 30 min, 3 h and 6 h. This increase can be prevented by IL-15 blocking antibodies. RQ = relative quantity of IL-15 mRNA. Columns represent means, and bars are standard deviations of a representative experiment done in triplicate. Four separate experiments show similar results. UN = untreated. For methods, see supplementary material.
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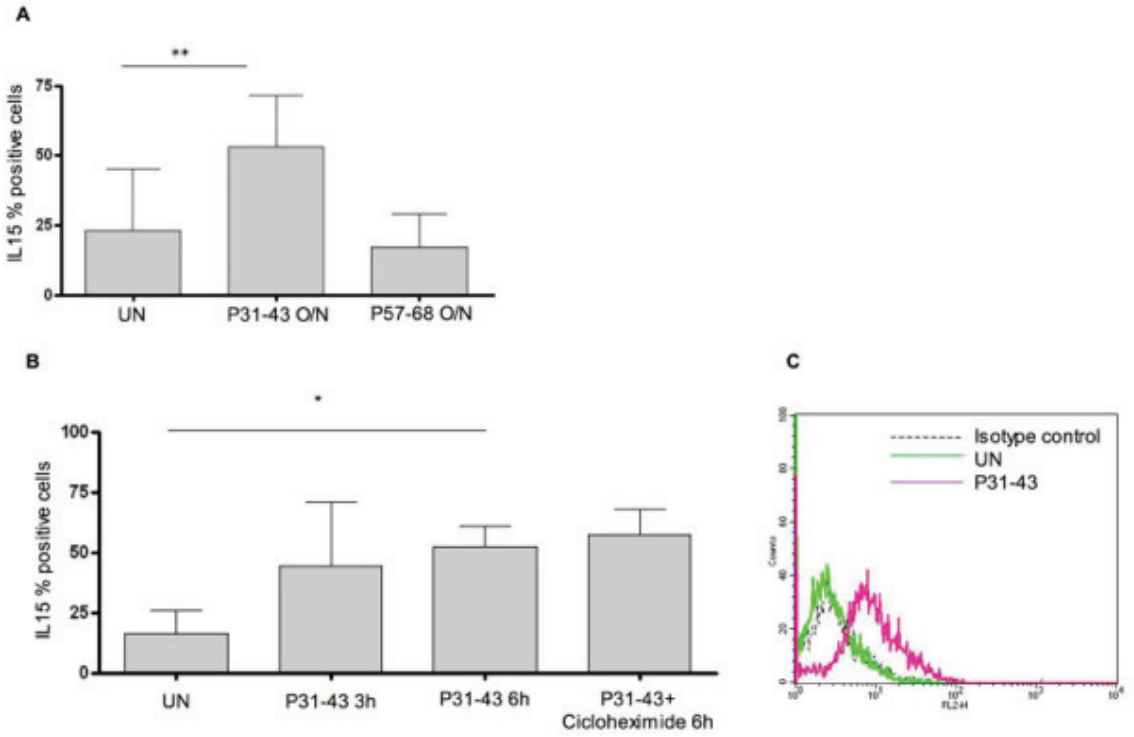

Figure 4. Gliadin peptide P31-43 increased IL-15/IL-15R alpha complex on the cell surface in CaCo-2 cells. (A) P31-43 increased IL-15 on the cell surface in CaCo-2 cells. FACS analysis of IL-15 on the cell surface after overnight (O/N), or 3 h or 6 h of treatment (B) with P31-43. Columns represent means and bars are the standard deviations of ten independent experiments for panel A and three independent experiments for panel B; * = $p<0.05$ (Student's *t*-test), ** = $p<0.01$ (Student's *t*-test) (C) Histogram of one representative experiment of CaCo-2 cells treated O/N with P31-43. Black dotted curve corresponds to negative control (isotype-matched Ab), the green open curve depicts specific IL-15 staining after medium treatment and pink open curve is specific IL-15 staining after O/N culture with P31-43.
doi:10.1371/journal.pone.0017039.g004

alpha was immunoprecipitated using the anti-IL-15R alpha goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were immunoblotted with specific antibodies.

Western blot

Briefly, CaCo-2 cells were starved overnight in DMEM containing 0.1% FBS and then stimulated with P31-43 for various intervals at 37°C. Cells were washed twice and resuspended in lysis buffer. Cell lysates were analysed by SDS-PAGE and transferred to nitrocellulose membranes (Whatman Gmbh, Dassel, Germany). The membranes were blocked with 5% non-fat dry milk and probed with anti-IL-15, anti-IL-15R alpha, anti-tubulin and anti-EGFR. Bands were visualised with the ECL system (GE Healthcare, Amersham, Buckinghamshire, UK). Band intensity was evaluated by integrating all the pixels of the band without the background, calculated as the average of the pixels surrounding the band [9].

Organ culture studies

Biopsy fragments from the duodenum were obtained from five untreated patients with active CD and three controls (affected by gastroesophageal reflux) for organ culture studies. The protocol of the study was approved by the Ethical Committee of the University "Federico II", Naples, Italy (ethical approval: C.E. n. 230/05). Informed written consent was obtained from all patients. The biopsy fragments were cultivated as reported elsewhere (for details, see Text S1) [9,34].

Statistical analyses

GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graphic representation. Statistical analysis of differences was performed with Student's *t*-test. A p value <0.05 was considered statistically significant.

Results

P31-43-induced proliferation depends on EGFR and IL-15 functions in CaCo-2 cells and in enterocytes of cultured biopsies from patients with active celiac disease

We previously demonstrated that P31-43 induces proliferation of fibroblasts (NIH 3T3 cell line) and of crypt enterocytes from cultured biopsies of CD patients with active disease but not from controls. This proliferation is mediated in an EGFR-dependent manner [9].

We have now investigated whether P31-43 induces proliferation of an intestinal cell line such as CaCo-2 cells and whether this effect, as well as the P31-43 induced proliferation of celiac crypt enterocytes, is mediated not only by EGFR activation but also by IL-15 function. As shown in Fig. 1, not only EGF and IL-15 but

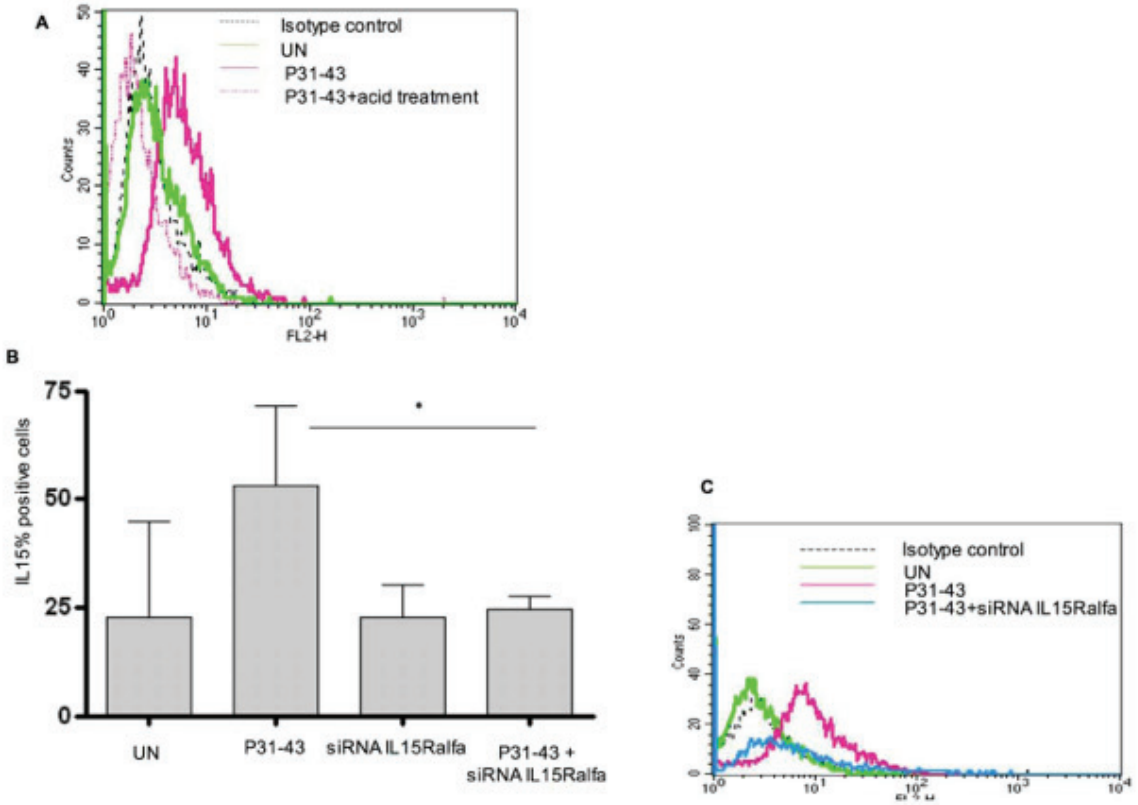

Figure 5. Acid treatment and sill15 mRNA reduce the increase of IL-15 expression on CaCo-2 cell surfaces induced by P31-43. (A) FACS analysis of IL-15 on the cell surface after overnight (O/N) treatment with P31-43. Histogram of one representative experiment of CaCo-2 cells treated O/N with P31-43 and P31-43 plus acid treatment. Black dotted curve corresponds to negative control (isotype-matched Ab), the green open curve depicts specific mAb staining after medium treatment, pink open curve is specific mAb staining after O/N with P31-43 and the dotted pink open curve represents specific mAb staining after O/N with P31-43 plus acid treatment. Data are representative of one of five independent experiments. (B) siRNA IL-15R alpha reduces P31-43 mediated increase of IL-15 on CaCo-2 cell surfaces. FACS analysis of IL-15 on Caco-2 cell surfaces. Statistical analysis of ten independent experiments for UN (untreated) and P31-43 O/N treated cells and of four experiments for cells treated with siRNA IL-15R alpha. Columns represent means and bars are standard deviations. * = $p<0.05$ (Student's t-test). (C) Histogram of one representative experiment of CaCo-2 cells treated O/N with P31-43 and P31-43 plus SiRNA IL-15R alpha. The dotted black curve corresponds to the negative control (isotype-matched Ab), the green open curve depicts specific IL-15 staining after medium treatment, the pink open curve depicts specific IL-15 staining after treatment with P31-43 and the blue open curve represents specific IL-15 staining after O/N treatment with P31-43 in the presence of siRNA IL-15R alpha.
doi:10.1371/journal.pone.0017039.g005

also P31-43 induces proliferation of CaCo-2 cells, measured as the percentage of cells that incorporate BrdU. Treatment with P31-43 increased proliferation of CaCo-2 cells from 26.40%±5.7% in the untreated sample to 44.33%±4.5%. This proliferation is dependent on IL-15 and EGFR functions. In fact, both IL-15 and EGFR blocking antibodies reduced the percentage of proliferating cells to 28.57%±7.8% with IL-15-blocking antibodies and 26.67%±4% with EGFR-blocking antibodies. Similar results were obtained when CaCo-2 cells were treated with peptic-tryptic digest of gliadin (PTG, not shown). Peptide P57-78 had no effect on CaCo-2 cell BrdU incorporation.

We next investigated whether, in biopsies from CD patients in the active state of the disease, P31-43-induced proliferation of enterocytes required IL-15 function. As expected, P31-43 induced a statistically significant increase in BrdU incorporation in crypt enterocytes from CD patients (Fig. 2A and C) [9]. Prevention of P31-43-induced proliferation was accomplished not only with the use of anti-EGFR blocking antibody (Fig. 2A and C), but also with IL-15-blocking antibody (Fig. 2A and C) [9]. In fact, after treatment with IL-15 blocking antibody, the percentage of BrdU-positive cells decreased from 33%±3.4% in the P31-43 treated sample to 16.5%±5.6%. Similar results were obtained when biopsies from active CD patients were treated with PTG (not shown). In control patients, neither P31-43 (Fig. 2 B) nor PTG (not shown) induced any proliferation [9].

Altogether, these data indicate that gliadin peptide-induced proliferation of CaCo-2 cells and of CD enterocytes is mediated by both IL-15 and EGFR activities.

Effect of gliadin peptide P31-43 on transcriptional regulation of IL-15

We treated CaCo-2 cells with P31-43 for 30 min, 3 h, 6 h or O/N to determine whether the peptide affected IL-15 mRNA levels. Quantitative PCR analysis showed an increase in IL-15

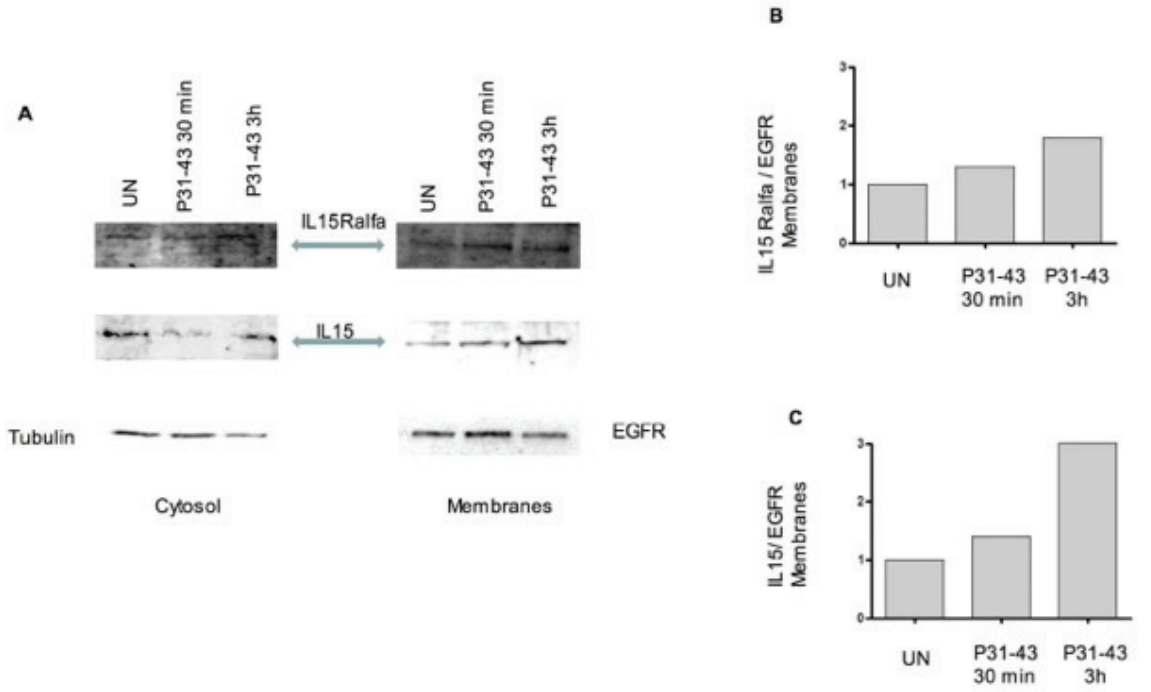

Figure 6. Both IL-15 and IL-15R-alpha expression increase in the isolated membrane fraction after stimulation with P31-43 for 30 min and 3 h. A) Western blot analysis of membrane proteins separated from total cell lysates shows an increase in membrane protein fractions of IL-15 and IL-15R alpha after P31-43 treatment. B and C) densitometric analysis of the Western blot experiment shown in a. EGFR was used to normalise membrane protein measurements. Increments (i) of IL-15 and IL-15R alpha were calculated as follows: iIL-15 = (IL-15 treated [t]/IL-15 untreated [un])/(EGFR Treated [T]/EGFR Untreated [UN]). iIL-15R = (IL-15R [t]/Il-15R [un]/(EGFR [T]/EGFR [UN]). The blots shown are representative of three similar independent experiments.
doi:10.1371/journal.pone.0017039.g006

mRNA only after O/N treatment with P31-43, the control peptide P57-68 was not able to increase IL15 mRNA at the same levels (Fig. 3). Intriguingly, this increase in IL-15 mRNA is IL-15-dependent as it can be prevented by IL-15 blocking antibodies. This finding suggested that P31-43 acts on pre-existing IL-15 protein to further increase IL-15 mRNA accumulation in CaCo-2 cells. Indeed, exogenous IL-15 induced an even greater increase of IL-15 mRNA than did P31-43. (Methods are described in Text S2)

P31-43 increased IL-15 protein expression on the surface of CaCo-2 cells, it did not do so in the cytoplasm or in the cell supernatant

To investigate whether P31-43 affects the expression of IL-15 protein, we evaluated (by FACS analysis) the intracellular and surface pools of IL-15 in CaCo-2 cells before and after exposure to P31-43. Overnight treatment with P31-43 did not affect the intracellular pool of IL-15 (Figure S2) and neither did shorter treatment times (not shown). We next evaluated whether P31-43 affects the extra-cellular release of IL-15 by CaCo-2 cells. After overnight incubation with P31-43, there was no statistically significant increase in IL-15 in the supernatant as measured by ELISA assay (Figure S3). However, the percentage of IL-15-positive cells on the surface increased from 22.92%±22.24% to 53.20%±18.26% after overnight treatment (Fig. 4A). This increase is specific for P31-43 because the control peptide P57-68 did not affect the percentage of cells expressing IL-15 on the surface (from 22.92%±22.24% to 17.09%±11.98%). IL-15 on the cell surface appeared to increase in expression after only 3 h of incubation with P31-43. This increase became statistically significant after 6 h of incubation, when it was comparable to that observed after overnight treatment (Fig. 4B). These findings indicate that either P31-43 increases the production of the IL-15 protein or mobilizes, from a pre-existing protein pool, IL-15 on the surface of the cells. We next analysed whether protein synthesis blockade induced by cycloheximide treatment was able to interfere with P31-43-induced increase of IL-15 on cell surfaces. Cycloheximide treatment failed to prevent the P31-43-mediated expression of IL-15 on the cell surface (57.42%+/−10.52% vs. 52.40+/−8.35, in the absence of cycloheximide) (Fig. 4B), suggesting that protein synthesis is not required for the P31-43 effect and that IL-15 is mobilized from an existing intracellular pool to the cell surface.

Cell surface IL-15 is linked to IL-15R alpha

Duitman et al. demonstrated that membrane-associated IL-15 is directed to the cell surface in complex with IL-15R alpha, which serves as a chaperone for its ligand [30]. We therefore investigated whether cell surface IL-15, which is increased by P31-43, is also attached to the receptor in CaCo-2 cells (Fig. 5–7). PCR analysis confirmed the presence of IL-15R alpha mRNA in CaCo-2 cells (not shown).

In addition, acid treatment known to release IL-15 from the ligand/receptor complex, reduced IL-15 on the surface of P31-43-treated CaCo-2 cells, suggesting that membrane-bound IL-15 is

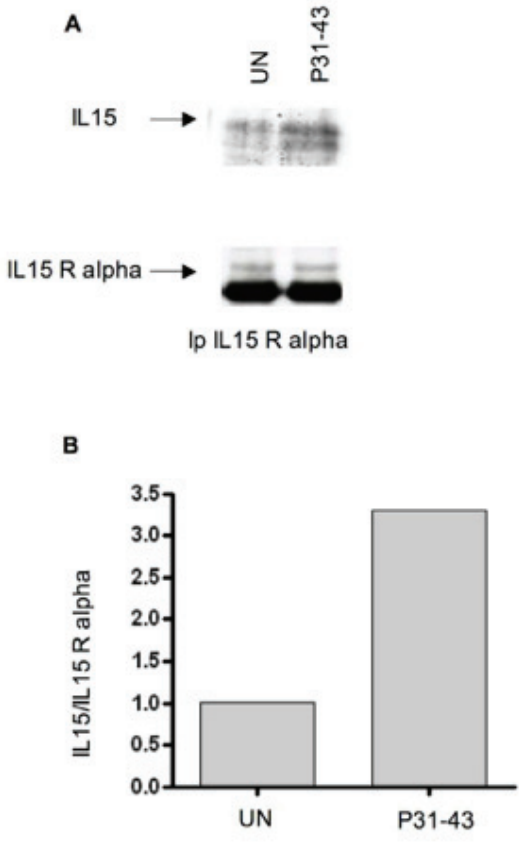

Figure 7. P31-43 treatment increases, on the cell membrane, IL-15/IL-15R alpha association. A) Demonstration of an IL-15/IL-15R alpha complex in an isolated membrane fraction. WB analysis of membrane proteins immunoprecipitated with IL-15R alpha antibodies. IL-15 and IL-15R alpha are visualized in the upper and lower gels with their respective antibodies. UN = untreated. B) Densitometric analysis of the Western blot experiment shown in (A). Fold increase of upper band of IL-15 (black arrow) was calculated respective to the IL-15R alpha band. The increment of IL-15 association (A) was calculated as follows: aIL-15 = (IL-15 [t]/IL-15 [un])/(IL-15R alpha [T]/IL-15R alpha [UN]). The blot shown is representative of three independent experiments.
doi:10.1371/journal.pone.0017039.g007

also linked to its receptor in this system (Fig. 5 A, B) [29]. To further confirm this hypothesis, we silenced IL-15R alpha by transfecting a specific siRNA, which reduced IL-15R alpha protein expression by almost 50% as shown in Figure S4. In Fig. 5 C,D the P31-43-induced increase of IL-15 on the cell surface is significantly inhibited (from 55%+/−18% to 24%+/−2.8%) in the presence of siIL-15R alpha. As expected, the increase of IL-15 on the cell membranes is mirrored by an increase in IL-15R alpha at the same site. In fact, both FACS analysis of the cells (not shown) and western blot analysis of proteins isolated from membrane fraction show an increase in IL-15 and IL-15R alpha after 30 min and after 3 h of treatment with P31-43 (Fig. 6). Finally, the existence of an IL-15/IL-15R alpha complex was demonstrated by analysis of proteins immunoprecipitated by anti-IL-15R alpha antibody from isolated cell membranes [29]. This analysis showed both immunoprecipitated IL-15 ligand and receptor by western blotting using specific antibodies (Fig. 7). Moreover, treatment with P31-43 increased the association of IL-15 and IL-15R alpha on the cell membrane by more than three-fold as compared to untreated cells when analysed by densitometry.

P31-43-induced surface IL-15 is biologically active

Most of the biological activity of IL-15 is believed to be mediated by the membrane-attached form of the protein [27,28]. We therefore evaluated the functional activity of IL-15 on the CaCo-2 cell surface by co-culturing irradiated CaCo-2 cells, treated or not with P31-43, with CTLL2, a cell line responsive to the mitogenic effects of both IL-15 and IL-2 [35]. As shown in Fig. 8, the proliferation rate of CTLL2 cells, evaluated as ^{3}H-thymidine incorporation, increased from 24,945 cpm±13,792 of the untreated sample, to 36,431 cpm±13,265 after P31-43 treatment of CaCo-2 cells. As expected, P57-68 treatment of CaCo-2 cells was not able to induce proliferation of CTLL2 (11,952+/−6,108). Furthermore, CTLL2 cells did not proliferate in response to direct treatment with P31-43 alone (not shown). The increase of ^{3}H-thymidine incorporation was dependent on IL-15 because IL-15 blocking antibody treatment prevented CTLL2 proliferation induced by CaCo-2 cells treated with P31-43 (21,129+/−12,648). This finding indicates that IL-15 increased on the cell surface after P31-43 treatment can function as a growth factor. (Methods are described in Text S3)

P31-43 alters trafficking of IL-15-containing recycling vesicles and increases recycling markers on CaCo-2 cell surfaces

IL-15 has been found in the Golgi complex and in transferrin-carrying endocytic vesicles [25,26]. We previously demonstrated that P31-43 alters the vesicular trafficking [9]. Therefore, we evaluated whether P31-43 affects the recycling pathway by carrying more IL-15 to the cell surface. IL15-EGFP localises to a recycling vesicular compartment when it is transfected in CaCo-2 cells [25]. After treatment with P31-43, IL-15EGFP-containing vesicles accumulated in the cytosol as shown in Fig. 9 A,B. The fluorescence intensity of the P31-43-treated cells exhibited a statistically significant increase from 54±2.9 to 79.3±4.7 after P31-43 treatment. To identify the IL-15EGFP-containing vesicular compartment, we treated CaCo-2 cells transfected with IL-15-EGFP with the recycling marker transferrin-Tex-Red for 90 min. [36]. As shown in Fig. 9A and B, treatment with P31-43 increased the number of transferring-containing vesicles, indicating that P31-43 can alter the trafficking of the recycling vesicles (fluorescence intensity/cell increased from 52.25±6.8 to 73.3±5.6 after P31-43 treatment). Treatment with P57-68 had no effect on the number of transferring-carrying vesicles. Furthermore, IL-15-EGFP co-localised with transferrin-Tex red in the same vesicular compartment before and after P31-43 treatment. To confirm P31-43 induced alterations of the recycling vesicular compartment, we investigated the levels of recycling marker transferrin receptor on the cell surface by FACS analysis before and after overnight treatment with P31-43 or P57-68. As shown in Fig. 10, the percentage of cells displaying the transferrin receptor on their surfaces significantly increased (from 18%±7% to 34.4%±13%) after P31-43 treatment while P57-68 treatment had no effect on the cell surface levels of transferrin receptor (from 18%±7% to 13%±4.7%). Therefore, P31-43 increased the expression of recycling vesicle markers on the cell surface, suggesting that the increase of IL-15 on the cell surface may relate to re-distribution of IL-15 from an intracellular vesicular compartment to the cell membrane. (Methods are described in Text S4.)

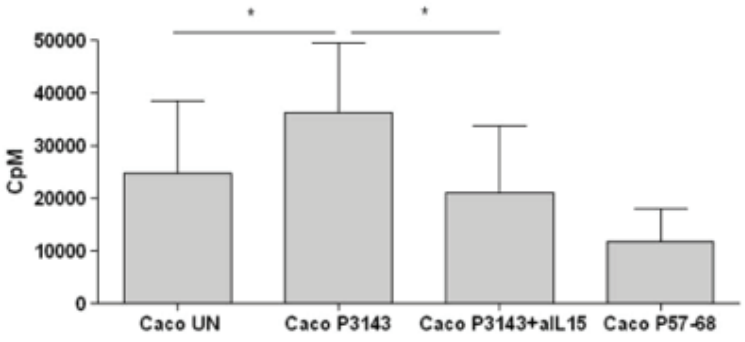

Figure 8. The complex IL-15/IL-15R alpha induced by P31-43 on the surface of CaCo-2 cells functions as a growth factor for CTLL2 cells. ^{3}H-thymidine incorporation by CTLL2 cells induced to proliferate by CaCo-2 cells untreated or treated with P31-43 or P31-43 and anti-IL-15 or P57-68 was measured. CaCo-2 and CTLL2 cells were co-cultivated overnight. Data are expressed as ^{3}H-TdR (CpM 1×10^{6} cells). Columns represent the mean, and bars represent the standard deviation of five independent experiments. *$p<0.05$ (Student's t-test). For methods, see supplementary material.
doi:10.1371/journal.pone.0017039.g008

Discussion

In this paper, we demonstrate that P31-43-induced cell proliferation both in crypt enterocytes and in CaCo-2 cells is not only dependent on EGFR but also on IL-15. P31-43 increased CaCo-2 cell surface expression of IL-15, the major mediator of innate immunity in CD, by altering the endocytic trafficking of the IL-15/IL-15R alpha complex. Thus, gliadin effects on proliferation and innate immunity activation are mediated by cooperation between growth factors (EGFR) and innate immunity mediators (IL-15) due to alterations in vesicular trafficking. It is now well accepted that endocytosis has many effects on signalling; in fact, signalling pathways and endocytic pathways are regulated in a reciprocal manner. It is also widely accepted that the "Endocytic Matrix" is a master organiser of signalling, governing the resolution of signals in space and time. Consequently, endocytosis affects several cell functions that range from proliferation to cell motility [37].

We first investigated the role of IL-15 in P31-43-induced cell proliferation. In previously published reports P31-43 has been found to be delayed in early endocytic vesicles both in crypt enterocytes of CD atrophic mucosa and in cell lines [9–11]. It has also been found that P31-43 can interfere with the correct localisation, on the vesicles surface, of the major coordinator of vesicle dynamics and maturation, namely the Hepatocyte growth factor Regulated tyrosine kinase Substrate (HRS) [10]. As a consequence maturation of the early endocytic compartment is delayed and the activation of EGFR and other receptors is prolonged, which results in several different biological events including cell proliferation [9–10].

In fact, the increase of proliferation of celiac crypt enterocytes induced by P31-43 was EGFR-dependent, as proliferation increase could be prevented by inhibitors of this pathway [9]. In this study, we show that IL-15, EGF and P31-43 or PTG can induce proliferation of an intestinal cell line such as CaCo-2. Moreover, we show that P31-43-induced proliferation is dependent on IL-15 and EGFR function. In fact, blockage of either the EGFR or IL-15 signalling pathways prevented P31-43-induced proliferation. These observations can be reproduced in intestinal biopsies from CD patients cultured for 24 hours. In this system, we show that PTG and peptide P31-43-induced crypt enterocyte proliferation is dependent not only on EGFR activation but also on IL-15 activity.

The present data and previously published reports [9] point to cooperation between a cytokine (IL-15) and a growth factor (EGF) to induce cellular proliferation. A complex between IL-15R alpha and EGFR is in fact present in CaCo-2 cells and is increased by P31-43 treatment (unpublished results). Such cooperation in signal transduction is not new. In fact, IL-15 and EGFR share the downstream effectors ERK and STAT [38]. IL-15 also interacts with the tyrosine kinase receptor AXL to prevent apoptosis in fibroblasts [39].

We next investigated whether gliadin-induced inflammation in CD is also affected by P31-43 alterations of the endocytic compartment. EGFR itself has a leading role in the regulation of the inflammation and can mediate innate immune responses in airway epithelium in respiratory diseases [40]. On the other hand, IL-15 is recognised as a major mediator of innate immunity in CD. In fact, it is not only increased in CD mucosa [3,12,13], but it is also necessary for the proliferation, localisation and function of intraepithelial lymphocytes (IELs) in the intestinal mucosa of CD patients [16–19]. Moreover, increased IL-15 activity mediates, to a large extent, the immune response induced by P31-43 in CD [2].

Therefore, we chose IL-15 activity as an indicator of the inflammation triggered by P31-43 and CaCo-2 cells as a model to study the capacity of P31-43 to increase IL-15 activity in an effort to understand the molecular mechanisms underlying this phenomenon.

IL-15 expression is tightly regulated at both transcriptional and post-transcriptional level [22–24].

Real-time PCR analysis showed that IL-15 mRNA increased in an IL-15-dependent manner only after prolonged incubation of CaCo-2 cells with P31-43, which suggests that the effects of gliadin on IL-15 mRNA could be secondary to other earlier effects. P31-43 increased IL-15 expression on the cell surface but not in the cytoplasm or at the level of protein secretion. The protein increase on the cell surface occurred earlier than the increase of IL-15 mRNA levels and independently from new protein biosynthesis, indicating that P31-43 first affected IL-15 protein distribution and then mRNA levels. Previous observations indicated that intracellular IL-15 localises to recycling vesicles that contain Transferrin Receptor and to the Golgi complex [20,25]. Therefore, we investigated the effect of P31-43 on the early/endosomal vesicle recycling pathway. P31-43 treatment increased the number of vesicles carrying both IL-15-EGFP and Transferrin-Tex-Red.

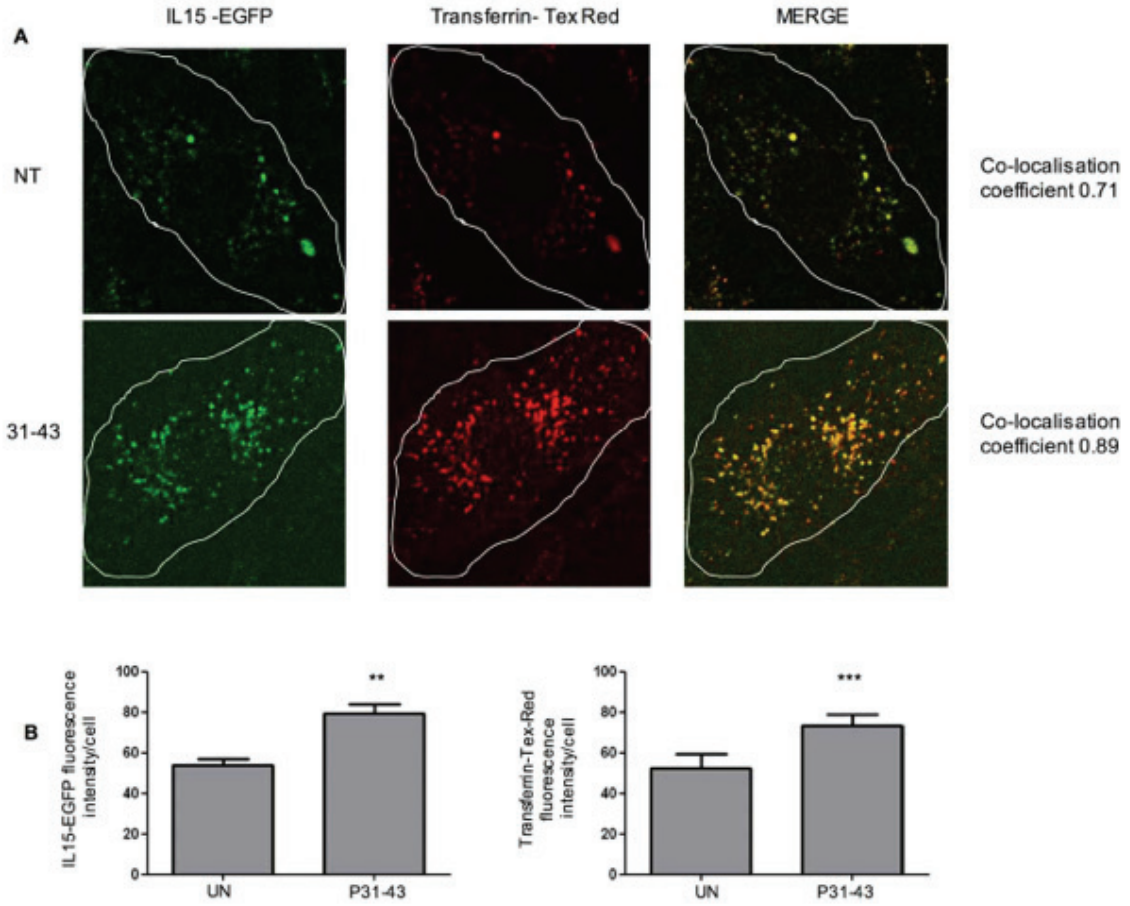

Figure 9. P31-43 alters trafficking of IL-15-containing recycling vesicles and increases recycling markers expressed on CaCo-2 cell surfaces. (A) IL-15-EGFP and Transferrin-Tex Red accumulate and co-localise after P31-43 treatment in a recycling vesicular compartment. IL-15-EGFP was transfected into CaCo-2 cells and observed by microscope after treatment with Transferrin-Tex Red and P31-43. White lines show the area of a single cell. (63x objective and 2x zoom). IL-15-EGFP (green) co-localises with Transferrin-Tex-Red (red) positive vesicles. Merge of the red and green panels is shown with yellow/orange colour indicating co-localisation. The co-localisation coefficient was calculated as reported under "Methods". The results are representative of three independent experiments. For methods, see supplementary material. (B) Statistical analysis of fluorescence intensity/cell. For treated and untreated samples, three independent experiments were done, measuring fluorescence intensity of 10 cells in random fields in each experiment. ** = $p<0.01$, *** = $p<0.001$ (Student *t*-test). For methods, see supplementary material.
doi:10.1371/journal.pone.0017039.g009

Probably due to the accumulation of early endocytic vesicles induced by P31-43 [9–10] and to the delay of the maturation of this compartment to lysosomes [10,11]. This could explain the increase of fluorescence for IL15 in absence of increased IL15 protein synthesis. Moreover, FACS analysis showed that P31-43 increases a typical marker of recycling vesicles, such as the Trasferrin Receptor, on the cell surface. These data are consistent with the hypothesis that P31-43-induced alteration of the endocytic pathway may be responsible for the increase of IL-15 expression on the cell surface.

It has recently been demonstrated that IL-15 is transported to the cell surface as a complex with its receptor, IL-15R alpha, which functions as a chaperone for the ligand, through the Golgi apparatus. This complex represents the *trans* form of IL-15 and enables the trafficking of this cytokine through the secretory and recycling pathways [30]. Here, we have shown that both IL-15 and IL-15R alpha increase in isolated membrane fractions of CaCo-2 cells after stimulation with P31-43. In addition, we showed, by immunoprecipitation, the presence of an IL-15/IL-15R alpha complex in the membrane fraction of CaCo-2 cells increased by P31-43 treatment. Finally, acid treatment and siRNA anti-IL-15R alpha reduce the amount of IL-15 present on the cell surface. Taken together, these data demonstrate that cell surface IL-15 is linked to the receptor.

The IL-15/IL-15R alpha complex present on the surface of CaCo-2 cells after P31-43 treatment is a functional growth factor for IL-15-sensitive CTLL2 cells. The membrane-bound, *trans*-presented IL-15 performs a number of IL-15 primary functions [27,28]. In non-immune cells, *trans*-presented IL-15 protects fibroblasts and epithelial cells from apoptosis and induces their proliferation [21]. It is also capable of inducing angiogenesis, of mediating anabolic effects in muscle cells, and of stimulating the lipolysis of adipocytes and the survival of neuronal cells [27]. IL-15 induces these effects by activating intracellular pathways directly by cell-to-cell contact [28].

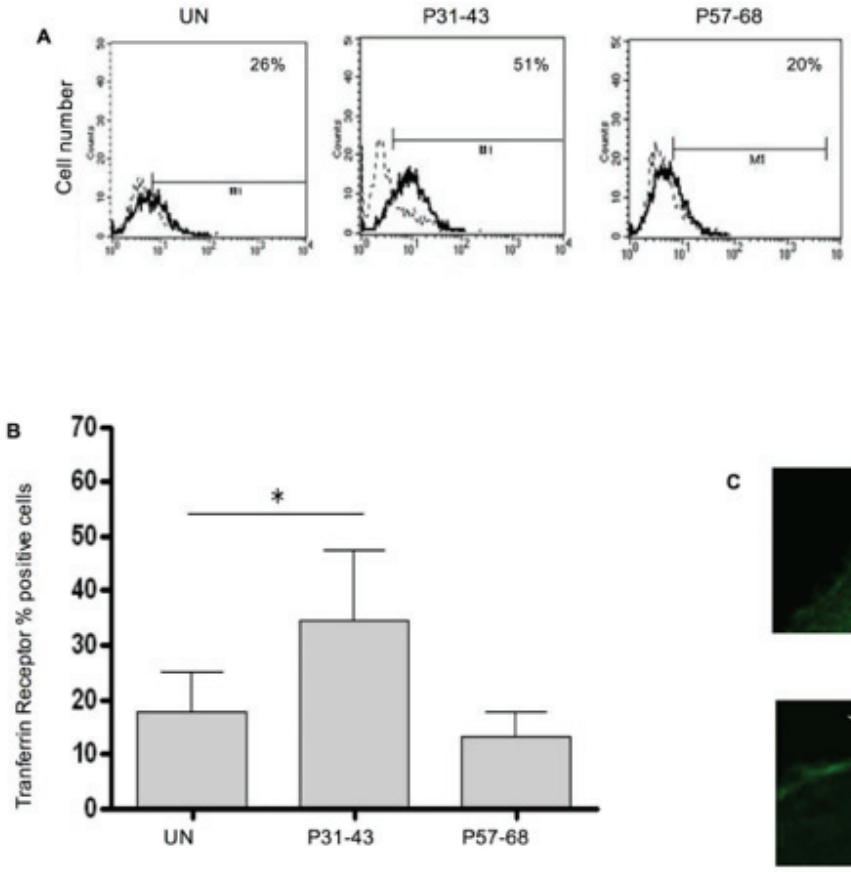

Figure 10. P31-43 increases expression of recycling marker transferrin receptor on the cell surface. A) FACS analysis of transferrin receptor, one experiment is shown. B) Statistical analysis of CaCo-2 cells percentage expressing the recycling marker, Transferrin Receptor, on the cell surface after P31-43 or P57-68 O/N treatment. Columns represent means and bars are the standard deviations of ten independent experiments. *$p<0.05$ (Student's *t* test). C) Confocal images of transferrin receptor expression on CaCo-2 cell surfaces. White arrows point to cell surface. 63x objective.
doi:10.1371/journal.pone.0017039.g010

In conclusion, we have shown that P31-43 induces at least two main effects by altering the trafficking of cell vesicular compartments. This leads to overexpression of the *trans*-presented IL-15/IL5R alpha complex, an activator of innate immunity, and, due to cooperation of IL-15 and EGFR, the proliferation of crypt enterocytes with consequent remodelling of the CD mucosa.

These observations are relevant to our understanding of the early events occurring in the celiac mucosa exposed to gliadin because the increase of IL-15 and IL-15R alpha is a major event in the initial phases of CD [3,12,13,41]. Our observation that in the celiac intestine IL-15 plays a major role in the gliadin-induced proliferation of epithelial cells, one of the hallmarks of CD, reinforces the importance of our results obtained in CaCo-2 cells and CD biopsies, which may increase understanding of the pathogenesis of CD. Why the celiac mucosa seems to be particularly sensitive to the effects of some gliadin peptides, such as peptide P31-43, remains to be elucidated. Preliminary data suggest that in CD cells, the endocytic compartment is morphologically and functionally altered. We hypothesize that in CD mucosa, an alteration of the vesicular compartment renders the tissue more sensitive to the effects of gliadin.

Supporting Information

Figure S1 Dose-response effect of P31-43 treatment on IL-15 expression on CaCo-2 cell surfaces FACS analysis of IL-15 on Caco-2 cells surfaces after O/N treatment with varying concentrations of P31-43 peptide. UN = untreated. Columns indicate percentage of positive cells (mean and standard deviation of three independent experiments). *$p<0.05$ (Student's t-test). Optimised concentration of P31-43 for IL-15 expression on cell surface was 100 μg/ml.
(TIF)

Figure S2 Overnight treatment with gliadin peptide P31-43 does not increase intracellular IL-15 expression. FACS analysis of IL-15 in the cytoplasm of CaCo-2 cells. Columns indicate percentage of positive cells (mean and standard deviation of four independent experiments).
(TIF)

Figure S3 Overnight treatment with gliadin peptide P31-43 does not increase secreted IL-15. ELISA assay of IL-15 in medium of cultured CaCo-2 cells. Columns indicate pg/ml (mean and standard deviation of three independent experiments).
(TIF)

Figure S4 siRNA IL-15R alpha reduces IL-15R alpha protein expression. (A) CaCo-2 cells were transfected with IL-15R alpha siRNA, lysed and immunoblotted for IL-15R alpha expression. β-Tubulin was used as an internal control. (B) Densitometric analysis of IL-15R alpha expression compared to alpha-tubulin expression. The decrease (d) of IL-15R alpha was calculated as follows: dIL-15R = (IL-15R [t]/IL-15R [un])/(Tubulin [T]/Tubulin [UT]). Shown is one representative experiment out of three independent experiments.
(TIF)

Text S1 Organ Culture Study.
(RTF)

Text S2 RNA Extraction and Real-Time PCR.
(RTF)

Text S3 CTLL2 Proliferation Assays.
(RTF)

Text S4 Transferrin and Transferrin Receptor Analysis.
(RTF)

Author Contributions

Conceived and designed the experiments: MVB MN DZ MM. Performed the experiments: MVB MN DZ MM SS GL MTSR CG. Analyzed the data: MVB RT SA SF. Contributed reagents/materials/analysis tools: EM FM RA. Wrote the manuscript: MVB SA.

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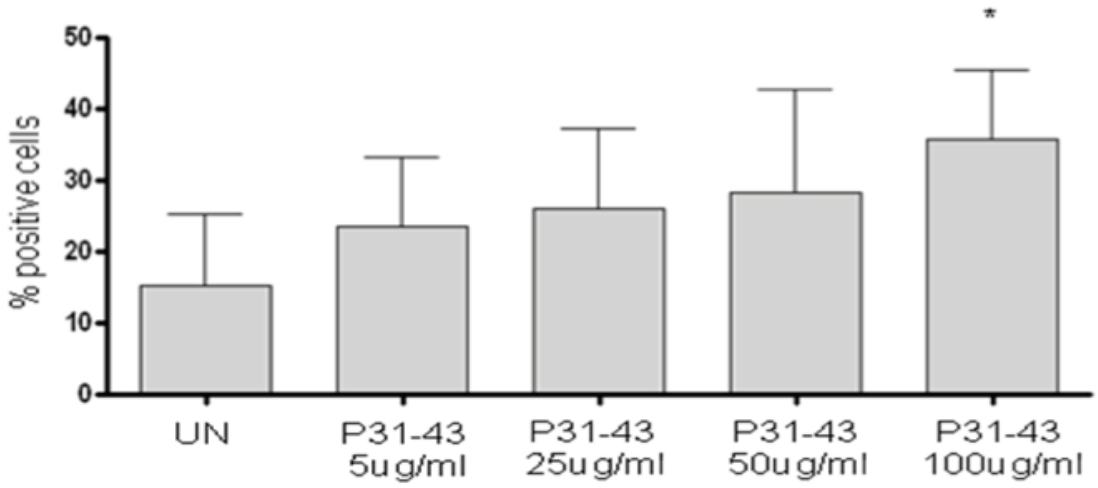
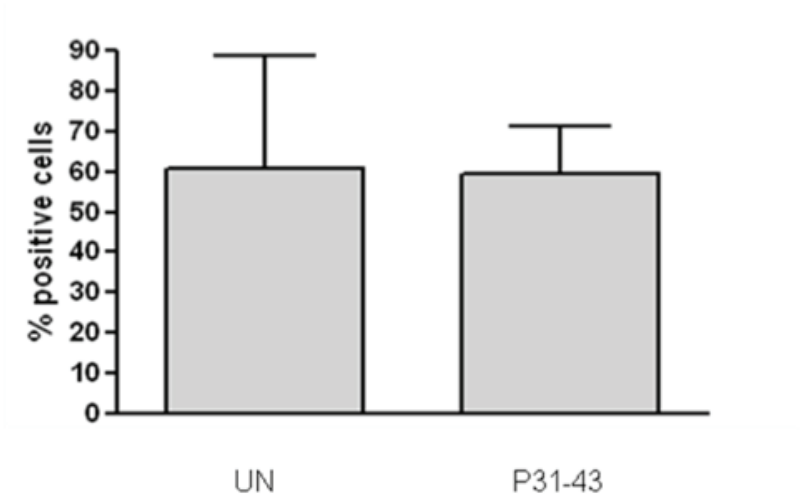
APPENDIX I: SUPPLEMENTARY FIGURE



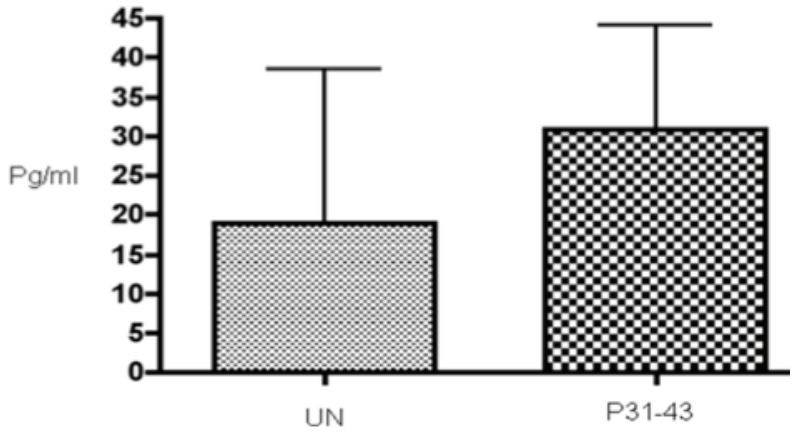
Supplementary Figure 1

Dose-response effect of P31-43 treatment on IL-15 expression on CaCo-2 cell surfaces. FACS analysis of IL-15 on Caco-2 cells surfaces after O/N treatment with varying concentrations of P31-43 peptide. UN=untreated. Columns indicate percentage of positive cells (mean and standard deviation of three independent experiments). *$p < 0.05$ (Student's t-test). Optimised concentration of P31-43 for IL-15 expression on cell surface was 100 µg/ml.



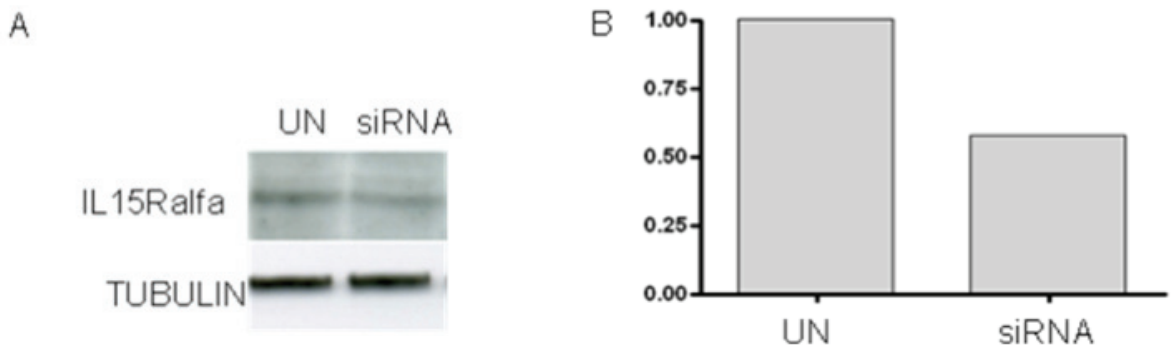
Supplementary Figure 2

Overnight treatment with gliadin peptide P31-43 does not increase intracellular IL-15 expression. FACS analysis of IL-15 in the cytoplasm of CaCo-2 cells. Columns indicate percentage of positive cells (mean and standard deviation of four independent experiments).



Supplementary Figure 3

Overnight treatment with gliadin peptide P31-43 does not increase secreted IL-15. ELISA assay of IL-15 in medium of cultured CaCo-2 cells. Columns indicate pg/ml (mean and standard deviation of three independent experiments).



Supplementary Figure 4

siRNA IL-15R alpha reduces IL-15R alpha protein expression. (A) CaCo-2 cells were transfected with IL-15R alpha siRNA, lysed and immunoblotted for IL-15R alpha expression. β-Tubulin was used as an internal control. (B) Densitometric analysis of IL-15R alpha expression compared to alpha-tubulin expression. The decrease (d) of IL-15R alpha was calculated as follows: dIL-15R = (IL-15R [t]/ IL-15R [un])/(Tubulin [T]/ Tubulin [UT]). Shown is one representative experiment out of three independent experiments.

APPENDIX II: SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL 1

Organ Culture Study

The intestinal samples were cultured for 24 h with medium alone or with P31-43 (100 µg/ml) or with peptic-tryptic gliadin peptides (PTG) (0.5 mg/ml) with or without blocking anti-IL-15 antibody (50 ng/ml) or blocking anti-EGFR antibody (2 mg/ml). All medium cultures were enriched with BrdU 10 µM (Sigma-Aldrich, Milan, Italy). Specimens were harvested, snap-frozen in liquid nitrogen, embedded in OCT and stored at –80 ° C until required.

We used double immunofluorescence to evaluate crypt proliferation in 5 µm cryoStat sections from cultured biopsies. After a short (3 min) treatment with 1.5 N HCl, the sections were incubated with mouse monoclonal anti-BrdU 1:150 (GE Healthcare Amersham, Buckinghamshire, UK) for 1 h, followed by 30 min with secondary Alexa488-labelled anti-mouse IgG 1:150 (Invitrogen, San Giuliano Milanese, Italy) to identify BrdU-positive cells. After several washes in PBS, specimens were fixed with 3% paraformaldehyde (Sigma-Aldrich, Milan, Italy) for 5 min and incubated for 1 h with polyclonal rabbit anti-cow cytokeratin 1:50 (Dako, Glostrup Denmark) to stain epithelial cells. Slides were then covered for 30 min with Alexa633-labelled goat anti-rabbit immunoglobulins 1:200 (Invitrogen, San Giuliano Milanese, Italy), contrasted with Hoechst staining (Sigma-Aldrich, Milan, Italy) and then mounted in Mowiol4-88. All incubations were carried out at room temperature in a dark humid chamber. The number of BrdU-positive cells divided by the total number of cytokeratin-positive cells gave the percentage of BrdU-positive cells.

SUPPLEMENTARY MATERIAL 2

RNA Extraction and Real-Time PCR

cDNAs were generated from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The resulting cDNA samples were subjected to a 10-cycle PCR amplification protocol followed by real-time PCR using TaqMan® PreAmp Master Mix Kit Protocol (Applied

Biosystems, PN 4366127). Each TaqMan Gene Expression Assay consisted of two sequence-specific PCR primers and a TaqMan assay-FAM dye-labelled MGB probe. Eighty ng of total cDNA (as total input RNA) was used for each replicate assay. Three replicates were run for each sample in a 96-well plate format. The endogenous control gene used was beta-2-microglobulin (B2M). Assays were run with 2× Universal PCR Master Mix without UNG (uracil-N-glycosylase) on Applied Biosystems 7300 Real-Time PCR System using universal cycling conditions (10 min at 95 °C; 15 sec at 95 °C, 1 min 60 °C, 40 cycles).

SUPPLEMENTARY MATERIAL 3

CTLL2 Proliferation Assays

Cytotoxic T-cell line 2 (CTLL2) cells were analysed for proliferation in response to CaCo-2 cells treated with gliadin peptides. CTLL 2 cells were plated at a density of 0.3 x 105 in 96-well round bottom plates together with 0.6 x 105 (ratio 1:2) gamma-irradiated CaCo-2 cells, which had been previously pulsed overnight with medium alone, P31-43 (100 µg/ml), P31-43 and blocking anti-IL-15 (5 µg/ml) or P57-68 in 200 µl of complete medium.

After 24 h of incubation, the cells were pulsed for 16 h with 1 µCi/well 3H-thymidine (Amersham-Pharmacia, Uppsala, Sweden). Radioactivity was assessed with a β counter (1600 TP, Hewlett Packard California, San Francisco, USA).

SUPPLEMENTARY MATERIAL 4

Transferrin and Transferrin Receptor Analysis

Texas Red-conjugated biferric-Transferrin (Molecular Probes) was used for pulse and chase experiments to highlight transferrin-positive vesicles and investigate colocalisation with IL-15-EGFP (42). CaCo 2 cells were seeded on coverslips for 48 h and then transfected with IL-15-EGFP. After 48 h of transfection, they were pulsed for 15 min with Texas Red-conjugated biferric-transferrin (pulse phase). Coverslips were than washed and reincubated in growing media for 90 min (chase phase) in the presence of P31-43. The cells were than fixed and observed via microscopy after mounting (Zeiss LSM 510).

For colocalisation analysis, the samples were examined with a Zeiss LSM 510 laser scanning confocal microscope. We used Argon/2 (458, 477, 488, 514 nm) and HeNe1 (543 nm) excitation lasers, which were switched ¬on separately to reduce cross-talk of the two fluorochromes. The green and red emissions were separated by a dichroic splitter (FT 560) and filtered (515-to 540-nm band-pass filter for green and > 610-nm long pass filter for red emission). A threshold was applied to exclude approximately 99% of the signal found in control images. The weighted co-localisation coefficient represents the sum of intensities of the co-localising pixels in channels 1 and 2 as compared to the overall sum of pixel intensities above the threshold. This value could be 0 (no co-localisation) or 1 (all pixels co-localise). Bright pixels contributed more than faint pixels. The co-localisation coefficient represents the weighted co-localisation coefficients of Ch1 (red) with respect to Ch2(green) for each experiment.[43]

Transferrin receptor expression on CaCo2 cells was analysed by FACS analysis and immuno-fluorescence. For Facs analysis, CaCo-2 cells were plated in tissue culture dishes (35 x 10 mm) in 1.5 ml DMEM and 0.1% fetal calf serum and stimulated overnight at 37°C with 100 µg/ml P31-43, 100 µg/ml P57-68 or medium alone. After 24 h, cells were scraped from the dishes at 4°C and transferred to a 96-well V-bottom plates (Costar Celbio, Milan, Italy). Flow cytometry analysis was performed as follows: after stimulation, 3-5x104 cells were washed with PBS and labelled with unconjugated anti-Transferrin Receptor (Calbiochem, clone T56/14) mouse monoclonal antibody. Cells were incubated with the primary antibodies for 30 min at 4°C. After two washes with PBS, the cells were labelled with anti-mouse PE-conjugated secondary polyclonal antibody (Dako, Denmark, Polyclonal Rabbit anti-Mouse Immunoglobulins/PE) for 20 min at 4°C. After washing, the labelled cells were analysed on a FACSCalibur flow cytometer using CellQuestPro software (BD Bioscience, San Diego, California).

Transferrin receptor was stained on CaCo2 cells with anti-Transferrin Receptor (Calbiochem, clone T56/14) mouse monoclonal antibody followed by secondary anti-mouse-Alexa-488 conjugated (Molecular probes, San Giuliano Milanese, Italy). The cells were seeded on coverslips and treated at 4°C, to block endocytosis, with anti-transferrin primary antibody for 45 min followed by staining for 45 min with secondary anti-mouse-Alexa-488. The cells were than fixed for 5 min with paraformaldehyde, mounted and observ.

CHAPTER 3
IL-15 INTERFERES WITH SUPPRESSIVE ACTIVITY OF INTESTINAL REGULATORY T CELLS EXPANDED IN CELIAC DISEASE

Introduction

Celiac disease (CD) is a chronic disorder caused by the ingestion of the gluten prolamines of wheat, rye, and barley in genetically predisposed individuals [1]. Although the pathogenesis of CD is not fully understood, it has been clearly shown that in the CD mucosa gluten peptides deamidated by tissue transglutaminase trigger CD4+ T cells to produce large amounts of interferon gamma IFN [2-3]. This mucosal inflammatory response leads to a profound remodeling of the intestinal mucosa, up to complete villous atrophy. However, the spectrum of histological changes is quite wide and there are CD patients, indicated as potential CD, who present the genetic and immunological features of CD, but whose small-bowel mucosa is architecturally normal [4, 5].

Beside the Th1 response, it has been highlighted the fundamental role of other pro-inflammatory cytokines, such as IL15 [6]. More recently also other cytokines, such as IL21, bridging innate and adaptive immunity, have been found to play an important role [6]. In these studies an important contribution to the comprehension of the mechanisms leading to disease has come from in vitro studies based on ex vivo organ cultures of intestinal biopsies taken from CD patients on a gluten-free diet (GFD) [6].

CD can be seen as the result of a break of tolerance where the regulation of the mucosal immune response to dietary gliadin might be altered. Several Tregs subsets are involved in immune tolerance [7]. These subsets include natural Treg cells expressing the forkhead box P3 (Foxp3) transcription factor able to maintain tolerance to self components and antigen-induced Foxp3+ cells able to contain the activity of Th1 and Th17 cells [8]. Tr1 cells which down-regulate naive and memory T cell responses upon local secretion of IL-10 and TGF–β [9], and TGFβ-producing Treg cells (Th3) [10] are other important subsets with regulatory properties. Many factors may interfere with Treg cells function. For CD it is relevant to know

that IL15, largely expressed in the CD mucosa, interferes with immune regulation, acting on TGF-β1 activity, thus contributing to the loss of intestinal homeostasis and promoting chronic inflammation [11]. Nevertheless, concomitantly with this pro-inflammatory response, high amount of the anti-inflammatory cytokines IL-10 and IL-4 are also produced in the untreated CD intestinal mucosa [12]. This apparent paradoxical milieu of both pro-inflammatory and suppressive cytokines strongly suggests that regulatory mechanisms might operate to counterbalance the gliadin-triggered, abnormal immune activation in untreated mucosa [13]. Our recent studies have revealed that the treatment with IL-10 of small intestinal mucosa from CD patients in remission prevents the massive immune activation induced by gliadin challenge [14]. Moreover, we have observed that celiac intestinal mucosa harbors a subset of Treg, Tr1 cells, that through the release of both IL-10 and TGF-β, inhibit the pathogenic response to in vitro gliadin challenge [15]. Although Tr1 cells, identified in CD, have some similar properties to Treg cells, they do not express Foxp3 [16]. This suggests that they are functionally distinct and may represent another level of regulation of the inflammatory response. Several studies have found that the number of Foxp3+ T cells are significantly increased in the small intestinal mucosa with active CD [17-19]. Futhermore, whereas the functional activity of circulating CD4+CD25+ T cells from CD patients has been recently investigated [20-22], the suppressive capacity relative to their intestinal counterparts has never been reported.

The aim of our study was to investigate the presence of Foxp3 cells in the celiac small intestinal mucosa and their correlation with disease state by combined immunochemistry (IHC) and flow cytometry (FACS) ex vivo analysis. Furthermore, we used an in vitro organ culture to investigate the induction of Foxp3 by gluten. Finally, we evaluated the functional capacity of intestinal Treg cells from celiac patients and the effects that IL-15 exerts on their suppressive function.

These data have been published on The American Journal of Gastroenterology, for the manuscripts see below.

IL-15 Interferes With Suppressive Activity of Intestinal Regulatory T Cells Expanded in Celiac Disease

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OBJECTIVES: **Celiac disease (CD) is a condition in which the regulation of the mucosal immune response to dietary gliadin might be altered. The transcription factor forkhead box P3 (Foxp3) has been identified as a marker of a subset of regulatory T cells (Treg). In this study, we have investigated the presence and the suppressive function of Treg cells in the celiac small intestinal mucosa, their correlation with the disease state, and the inducibility by gliadin in an organ culture system; moreover, we tried to define whether interleukin 15 (IL-15), overexpressed in CD, could influence the regulatory activity of such cells.**

METHODS: **The expression of Foxp3, CD3, CD4, and CD8 were analyzed by immunohistochemistry and flow cytometry in duodenal biopsies taken from patients with untreated CD, treated CD, and from non-CD controls, as well as *in vitro* cultured biopsy samples from treated CD patients, upon challenge with gliadin. Furthermore, we analyzed the suppressive function of CD4+CD25+ T cells, isolated from untreated CD biopsy samples, on autologous responder CD4+CD25− T cells, in the presence of a polyclonal stimulus, with or without IL-15.**

RESULTS: **Higher density of CD4+CD25+Foxp3+ T cells was seen in duodenal biopsy samples from active CD patients in comparison with treated CD and non-CD controls. In coculture, CD4+CD25+ T cells were functionally suppressive, but their activity was impaired by IL-15. Cells from CD subjects showed increased sensitivity to the IL-15 action, likely due to enhanced expression of IL-15 receptor. Finally, we demonstrated an expansion of Foxp3 in treated CD mucosa following *in vitro* challenge with gliadin.**

CONCLUSIONS: **These data suggest that CD4+CD25+Foxp3+ T cells are induced *in situ* by gliadin. However, their suppressor capacity might be impaired *in vivo* by IL-15; this phenomenon contributes to maintain and expand the local inflammatory response in CD.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

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INTRODUCTION

Celiac disease (CD) is a chronic disorder caused by the ingestion of the gluten prolamines of wheat, rye, and barley in genetically predisposed individuals (1). Although the pathogenesis of CD is not fully understood, it has been clearly shown that in the CD mucosa, gluten peptides deamidated by tissue transglutaminase trigger CD4+ T cells to produce large amounts of interferon gamma (IFN-γ) (2,3). This mucosal inflammatory response leads to a profound remodeling of the intestinal mucosa, up to complete villous atrophy. However, the spectrum of histological changes is quite wide and there are CD patients, indicated as potential CD, who present the genetic and immunological features of CD, but whose small-bowel mucosa is architecturally normal (4,5).

In addition to the Th1 response, it has highlighted the fundamental role of other pro-inflammatory cytokines, such as interleukin 15 (IL-15) (6). More recently, also other cytokines, such as IL-21, bridging innate, and adaptive immunity, have been found to have an important role (6). In these studies, an important contribution to the comprehension of the mechanisms

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leading to disease has come from *in vitro* studies based on *ex vivo* organ cultures of intestinal biopsies taken from CD patients on a gluten-free diet (6).

CD can be seen as the result of a break of tolerance in which the regulation of the mucosal immune response to dietary gliadin might be altered. Several Tregs subsets are involved in immune tolerance (7). These subsets include natural Treg cells expressing the forkhead box P3 (Foxp3) transcription factor that is able to maintain tolerance to self components, and antigen-induced Foxp3+ cells that are able to contain the activity of Th1 and Th17 cells (8). Tr1 cells that downregulate naive and memory T-cell responses upon local secretion of IL-10 and transforming growth factor-β (TGF-β) (9), and TGFβ-producing Treg cells (Th3) (10) are other important subsets that possess regulatory properties. Many factors may interfere with the function of Treg cells. For CD, it is relevant to know that IL-15, largely expressed in the CD mucosa, interferes with immune regulation, functioning on TGF-β1 activity, thus contributing to the loss of intestinal homeostasis and promoting chronic inflammation (11). Nevertheless, concomitantly with this pro-inflammatory response, high amount of the anti-inflammatory cytokines IL-10 and IL-4 are also produced in the untreated CD intestinal mucosa (12). This apparent paradoxical milieu of both pro-inflammatory and suppressive cytokines strongly suggests that regulatory mechanisms might operate to counterbalance the gliadin-triggered, abnormal immune activation in untreated mucosa (13). Our recent studies have revealed that the treatment with IL-10 of small intestinal mucosa from CD patients in remission prevents the massive immune activation induced by gliadin challenge (14). Moreover, we have observed that celiac intestinal mucosa harbors a subset of Treg cells, Tr1 cells, which, through the release of both IL-10 and TGF-β, inhibit the pathogenic response to *in vitro* gliadin challenge (15). Although Tr1 cells, identified in CD, have some properties similar to that of Treg cells, they do not express Foxp3 (16). This suggests that they are functionally distinct and may represent another level of regulation of the inflammatory response. Several studies have found that the number of Foxp3+ T cells is significantly increased in the small intestinal mucosa with active CD (17–19). Futhermore, although the functional activity of circulating CD4+CD25+ T cells from CD patients has been recently investigated (20–22), the suppressive capacity relative to their intestinal counterparts has never been reported.

The aim of our study was to investigate the presence of Foxp3 cells in the celiac small intestinal mucosa and their correlation with the disease state by combined immunohistochemistry and flow cytometry (FACS) *ex vivo* analysis. Furthermore, we used an *in vitro* organ culture to investigate the induction of Foxp3 by gluten. Finally, we evaluated the functional capacity of intestinal Treg cells from celiac patients, and the effects that IL-15 exerts on their suppressive function.

METHODS

Patients and controls

Biopsy specimens were obtained from the distal duodenum of 25 untreated CD patients (seven male and eighteen female patients; median age, 29 years; range 17–54). They were snap-frozen in liquid nitrogen or immediately processed. Diagnosis was based on typical mucosal lesions with crypt cell hyperplasia and total villous atrophy. All untreated CD patients were positive for serum anti-endomysial antibodies. Duodenal biopsy samples were also obtained from 15 treated CD patients (seven male and eight female patients; median age 35 years; range 21–51), who were in clinical and histological remission, and negative for anti-endomysial antibodies. Finally, 10 non-celiac individuals (six male and four female patients; median age 41 years; range 25–57) with normal intestinal mucosa and negative serology for anti-endomysial were recruited. Functional studies were performed on both intestinal CD4+CD25+ T cells, isolated from biopsy samples of nine untreated CD patients, and on peripheral blood CD4+CD25+ T cells, purified from five untreated CD patients and four non-CD controls. The study received approval from a local ethics committee (Hospital Moscati, Avellino, Italy).

Immunohistochemistry

Acetone-fixed sections (5 μm), from biopsy samples that were not cultured or after culture, were individually incubated for 1 h with mouse monoclonal antibodies (see **Supplementary Table 1** online), followed by incubation (20 min) in the dark with rabbit anti-mouse tetramethyl-rhodamine isothiocyanate-conjugated (Vector 1:200) antibodies. Costaining experiments were set up by mixing rat anti-human Foxp3 with mouse anti-CD3, -CD8, or -CD4, and incubating the cryosections individually for 1 h with each single mixture. After incubation with primary antibodies, the cryosections were incubated with a mixture of goat anti-rat tetramethyl-rhodamine isothiocyanate-conjugated antibody and horse anti-mouse fluorescein isothiocyanate-conjugated antibody for 30 min. Finally, all the sections were counterstained with ToPro-3 (Molecular Probes, Leiden, The Netherlands), mounted in phosphate-buffered saline:glycerol (1:1), and imaged with a Leica SP confocal microscope (Heidelberg, Germany). Non-immune mouse immunoglobulins were used as isotype controls of primary antibodies. The density of cells expressing Foxp3 in the lamina propria was evaluated within a total area of 1 mm^2 of lamina propria. Slides were analyzed by two observers who were blinded.

Organ culture

The mucosal specimens were cultured as described elsewhere (22). Briefly, biopsy samples taken from 10 patients with inactive CD and from six control patients were placed on iron grids, with the mucosal face upwards in the central well of an organ culture dish. Cultures were prepared with or without the addition of 1 mg/ml of peptic–tryptic digest (Frazer III fraction) of gliadin (PT-gliadin; Sigma, St Louis, MO), and were placed in a tight container with 95% O_2/5% CO_2 at 37 °C, at 1 bar. After 24 h of culture, the tissue was embedded in Optimal Cutting Temperature and snap-frozen in liquid nitrogen.

Ex vivo cytofluorimetric analysis

Mucosal explants from nine healthy donors, nine CD patients and four gluten-free diet, either following 24 h of gliadin chal-

lenge or freshly processed, were digested with collagenase-A. Immediately after the digestion, the cells were passed through a cell strainer (40μm Nylon, Becton Dickinson (BD), Bedford, MA) and isolated from mucosal explants. A total of 5–10×10^6 cells obtained from fresh biopsy samples were washed in phosphate-buffered saline and labelled with a pre-titrated optimal dilution of each fluorochrome-conjugated mouse monoclonal antibodies against surface antigens (see **Supplementary Table 1** online). Appropriate isotype-matched control antibodies (BD) were included in all experiments. Intracellular cytokine production was detected by using a triple-staining technique and by FACS analysis. Briefly, after washing twice with phosphate-buffered saline, cells were fixed and permeabilized with Cytofix/cytoperm (BD), according to the manufacturer's instructions, followed by staining with fluorescein isothiocyanate (FITC)-anti-Foxp3 antibody (PCH101; eBioscience, San Diego, CA) in permeabilization buffer. Cells were then analyzed by a flow cytometer (FACSCalibur and CellQuest Software, BD), using a live gate set around viable lymphocytes based on their forward-scatter/side-scatter characteristics.

IL-15 receptor-α surface expression (IL-15Rα) was evaluated by incubating peripheral blood mononuclear cells (PBMCs) with optimal concentration of phycoerythrin-conjugated mouse mAb against CD4 (BD Pharmingen, San Jose, CA), phycoerythrin-Cy5-conjugated mAb against CD25 (BD Pharmingen), anti-IL-15Rα goat IgG (R&D System, Minneapolis, MN, USA and Alexa Fluo 488, R&D System), and donkey anti-goat as a secondary mAb (R&D System). Cells were also incubated with the respective mouse isotype controls. After two washings, the cells were fixed with 2% paraformaldehyde and analyzed by flow cytometry.

Purification of T cell subsets

Autologous PBMCs isolated by heparinized blood obtained from patients with active CD and healthy donors were purified by density-gradient centrifugation (Ficoll, MP Biomedicals, LLC, Solon, OH). However, freshly isolated mucosal cells were processed from intestinal mucosal explants after collagenase digestion. CD4+CD25+ T cells and CD4+CD25− T cells were separated using the Dynabeads Regulatory CD4+ CD25+ T cell kit (Dynal-Biothec, AS, Oslo, Norway). In the first step, CD4+ cells were separated by negative selection, using the Antibody Mix Human CD4 (Dynal-Biothec, AS, Oslo, Norway). In the second step, Depletion MyOne Dynabeads (Dynal-Biothec, AS, Oslo, Norway) was added to remove the non-CD4 cells. In the third step, Dynabeads CD25 (Dynal-Biothec, AS, Oslo, Norway) was added to CD4+ T cells to capture the CD4+CD25+ T cells and the remaining fraction was used as CD4+CD25− T cells. In the last step, Dynabeads CD25 was removed from the cells. All purification steps were performed according to the manufacturer's instructions and collected cells were found to be >95% pure by flow cytometry.

Suppression assay

The CD4+CD25+ (Treg) and CD4+CD25− responder T cells (Tresp) were cultured, respectively, at different ratios in the suppression experiments (1:1, 1:0.5, 1:0.25, 1:0.125, 1:0). Cells were cultured (1–2×10^4 cells/well) in U-bottom 96-well plates with RPMI medium supplemented with 2-mM L-glutamine, 100-U/ml penicillin, 100-μg streptomycin, and 10% fetal bovine serum. Cells were stimulated for 3 days in the presence of Treg suppression Inspector (Miltenyi Biotec, Bergisch Gladbach, Germany) that consists of Anti-Biotin MACSi Bead Particles that are pre-loaded with biotinylated CD2, CD3, and CD28 antibodies. As additional control, Tresp cells were cultured alone with and without the Treg suppression Inspector (Miltenyi Biotec) and Treg cells were cultured alone with Treg suppression Inspector (Miltenyi Biotec). Furthermore, the cocultured Tresp/Treg cells were stimulated with 10-ng/ml of IL-15 (R&D System). On the last day, ^{3}H-thymidine (1uCi/well; Amersham-Pharmacia Biotec, Uppsala, Sweden) was added to the cultures and incubated for 16h. Radioactivity was assessed with a β-counter (1600 TP, Hewlet Packard California, San Francisco, CA, USA). Percentage proliferation was determined as (cpm incorporated in the coculture)/(cpm of responder population alone)×100%. Percentage of inhibition was calculated as (1 – number of cells in coculture that divide/number of responder cells that divide)×100.

Cytokine assay

At the end of the cell culture, supernatants were collected and analyzed for the content of IFN-γ by enzyme-linked immunosorbent assay. Briefly, 96-well plates (Immunoplate MaxiSorp, Nunc, Merelbeke, Belgium) were coated by incubating with anti-human IFN-γ monoclonal antibody (Endogen) overnight at 4 °C. After washing, wells were blocked by incubation with phosphate-buffered saline

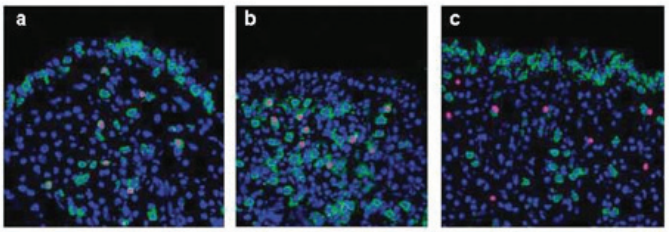

Figure 1. Phenotyping of forkhead box P3 (Foxp3+) cells on jejunal biopsy samples from untreated celiac disease (CD). Immunofluorescence costaining experiments revealed that all the Foxp3+ cells were CD3+ (**a**), CD4+ (**b**), and CD8− (**c**). Pink indicates the Foxp3 molecule staining, green indicates the CD3, CD4 and CD8 molecule staining, and blue indicates the nuclei counterstaining by using ToPro-3. Original magnification: ×40.

solution containing 2% bovine serum albumin. Supernatants were added and incubated for 1 h at room temperature. After two washes, the wells were incubated with biotinylated anti-human IFN-γ monoclonal antibody (Endogen) for 30 min at room temperature. Finally, the wells were incubated with streptavidin-horseradish peroxidase (BD Pharmingen) and then was added 100 µl of TMB (3,3′,5,5′-tetramethylbenzidine; Sigma), a chromogenic substrate for horseradish peroxidase. Absorbances were read on an enzyme-linked immunosorbent assay reader at 450 nm.

Statistical analysis

Data are presented as mean values±s.d.; paired two-tailed Student's *t*-test was used to calculate *P*-values within the same individuals and unpaired two-tailed Student's-*t* test was used to calculate *P*-values between study groups. *P*-values < 0.05 were considered statistically significant.

RESULTS

Increased expression of Foxp3 T cells in untreated CD mucosa

To localize the anatomical site of cells expressing Foxp3, we performed immunohistochemical analysis of untreated CD, treated CD, and non-CD control duodenal sections, using a mouse anti-human Foxp3 antibody. In untreated CD, the number of cells/mm^2 of lamina propria expressing the transcription factor Foxp3+ was significantly higher (mean±s.d.: 91±24) in comparison with treated CD (8±2, $P < 0.001$) and non-CD controls (6±3, $P < 0.001$; see **Supplementary Figure 1** online upper panel online). Foxp3 cells exhibit a nuclear localization in positive cells (see **Supplementary Figure 1** online lower panel). Those cells were not found in the epithelium layer, but only in the lamina propria, particularly in the subepithelial compartment (see **Supplementary Figure 1** online lower panel). No significant differences were noted in the number of Foxp3+ cells in biopsy samples of treated CD in comparison with

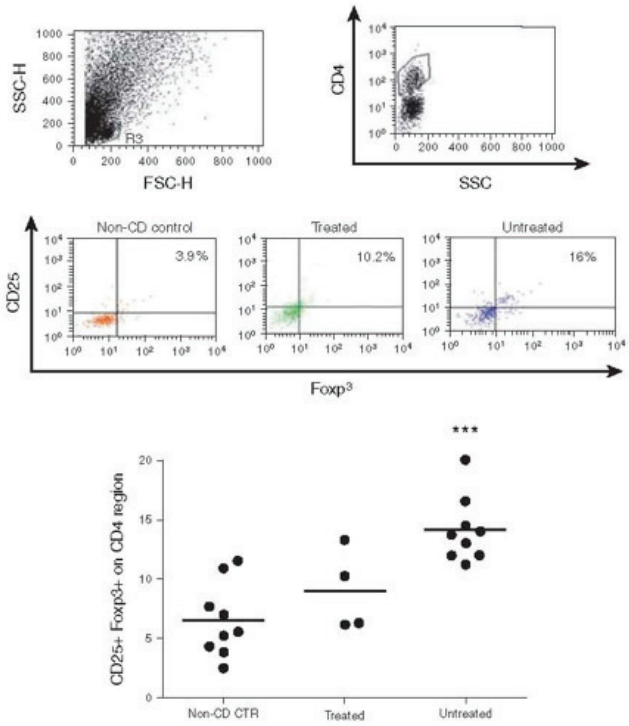

Figure 2. Increased CD4+CD25+forkhead box P3 (Foxp3+) intestinal T cells in untreated celiac disease (CD) patients as compared with treated-CD and non-CD control. Freshly isolated human intestinal cells were collected, stained, and analyzed by flow cytometry, as described in Methods, and assessed by a FACSCalibur (BD). Upper panel, representative dot plot from one experiment from non-CD control, treated-CD, and untreated-CD intestinal cells each is shown. Lymphocytes gated on forward- and side-scatter properties to exclude dead and/or granular cells, representative gates of CD4 populations are shown. Dot plot of Foxp3+CD25+ are gated on CD4+cells. Foxp3+ staining was performed after cell permeabilization. Lower panel, the mean percentages of the intestinal whole Foxp3$^+$CD25$^+$ T cells in total CD4$^+$ cells isolated from non-CD control patients (n=9), untreated patients (n=9), and treated patients (n=4) was presented in each scatter plot. Horizontal bars represent mean values. ***$P < 0.0001$ comparing the non-CD control vs. untreated-CD patients.

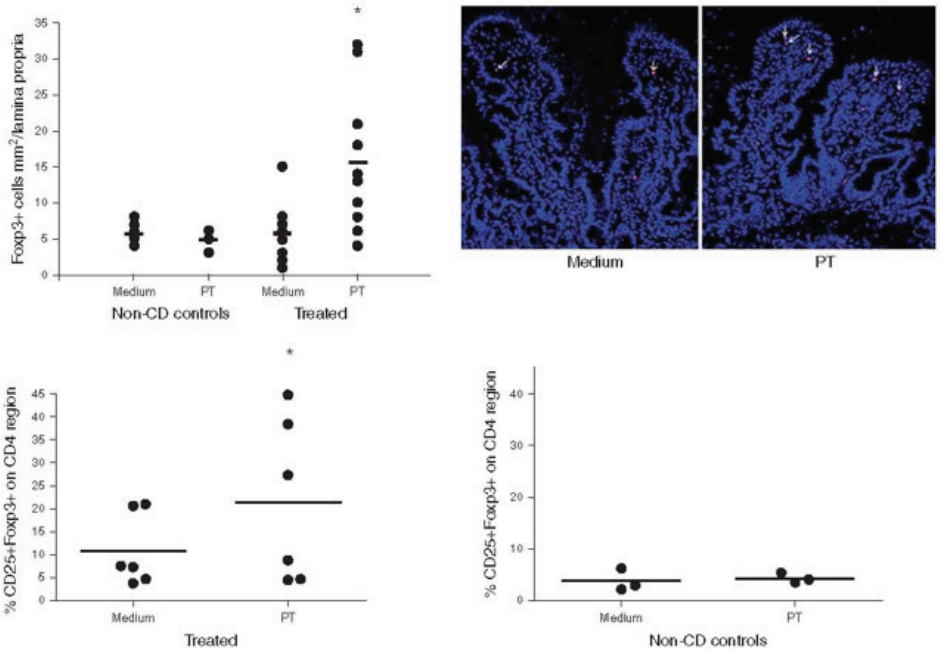

Figure 3. PT-gliadin challenge increased Foxp3+ cells in the jejunal biopsy specimens from treated-celiac disease (CD) patients. Upper left panel, number of forkhead box P3 (Foxp3) cells analyzed by immunohistochemistry, in mucosal explants from non-CD controls and treated CD cultured *in vitro* with medium alone or with PT-gliadin. Foxp3+ cells were counted per square millimeter of lamina propria. Dashes indicate the mean values. Statistical significance was evaluated by comparing responses to PT-gliadin with responses to medium alone for each group of subjects. *$P<0.05$. Upper right panel, Foxp3+ cells in the jejunal mucosa from a celiac patient cultured *in vitro* with medium only or with a PT-gliadin. In the later, increased numbers of immunostained cells are evident particularly in the subepithelial region (arrows). Original magnification: x20. Lower panel, CD4+CD25+Foxp3+ cells, analyzed on FACSCalibur, in intestinal biopsies from treated CD patients (left panel) and non-CD controls (right panel) cultured for 24h in the presence of only medium or PT-gliadin. Data represent the mean of percentages of CD25+Foxp3+ cells in the CD4+population. Each scatter plot is representative of mean of three or six independent experiments, respectively. Statistical significance was evaluated for lower panel as indicated in the upper left panel. *$P<0.05$.

biopsy samples of non-CD controls (see **Supplementary Figure 1** online upper panel). We next explored the phenotype of Foxp3+ cells, with antibodies to CD3, CD4, and CD8, by costaining experiments. Herein, we demonstrated that all the intestinal Foxp3+ cells, in untreated CD, treated CD, and non-CD control duodenal sections, expressed a CD3+CD4+ double-positive phenotype (**Figure 1a, b**), whereas CD8+ T cells expressing Foxp3 were not found (**Figure 1c**).

FACS confirmed the findings obtained by immunohistochemistry. Specifically, we analyzed the frequency of Foxp3+CD25+ cells in a CD4+ population. As shown in **Figure 2**, the percentage of Foxp3+CD25+CD4+ cells was significantly higher in untreated celiac patients (mean±s.d.: 14.1±2.7%) compared with treated CD patients (8.9±3.4%, $P<0,01$) and non-CD controls (6.8±2.4%, $P<0,001$; **Figure 2**). No significant differences were noted in the percentage of Foxp3+CD25+CD4+ cells in treated CD in comparison with non-CD controls (**Figure 2**).

Foxp3+ cell expansion in treated-CD biopsy samples cultured with gliadin

An *in vitro* gliadin-challenge system was used, which reproduces many features of the mucosal immune response that occurs in the established celiac lesion (23). In the lamina propria of treated-CD biopsy specimens cultured in the presence of PT gliadin, the number of cells/mm² expressing Foxp3 (16±9) was significantly higher ($P<0.01$) than in those cultured in medium alone (6±4; **Figure 3**, upper left panel). By contrast, no statistically significant differences were noted in the number of Foxp3+ cells, when biopsy samples obtained from non-CD controls were cultured in the presence of PT-gliadin (5±1), compared with those cultured in medium alone (5±2; **Figure 3**, upper left panel). As seen in uncultured biopsy samples taken from untreated CD, Foxp3+ cells in PT-gliadin-challenged mucosa were not found in the epithelium layer, but were found only in the lamina propria, mainly localized beneath the epithelium (**Figure 3**, upper right panel).

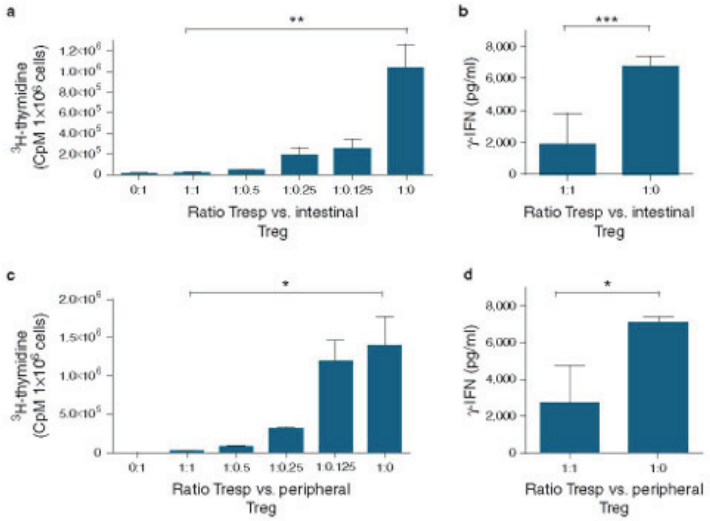

Figure 4. In coculture, CD4+CD25+ cells isolated from intestinal or peripheral blood of untreated-celiac disease (CD) patients significantly suppressed the proliferation and interferon gamma (IFN-γ) production of responder T cells (Tresp). Proliferative responses were measured by [^{3}H]thymidine uptake. IFN-γ secretion was evaluated in supernatants of cocultures by an enzyme-linked immunosorbent assay test. Upper panel, *in vitro* study evaluating the suppressive capacity of CD4+CD25+ T cells, purified from intestinal biopsy of seven active CD patients, on the proliferation (**a**) and IFN-γ production (**b**) by Tresp cells. Results are from at least three independent experiments. **$P<0.005$ and ***$P<0.001$ comparing the 1:0 vs. 1:1 ratio). Lower panel, *in vitro* study evaluating the suppressive capacity of CD4+CD25+ T cells, purified from peripheral blood of four active CD patients, on the proliferation (**c**) and IFN-γ production (**d**) by Tresp cells. Results are from at least three independent experiments. *$P<0.05$ comparing the 1:0 vs. 1:1 ratio.

The FACS analysis of the frequency of Foxp3+CD25+ cells before and after challenge with PT-gliadin in CD4+ population, confirmed the immunohistochemical data. As shown in **Figure 3**, lower left panel, there was a significantly higher ($P<0,01$) frequency of Foxp3+CD25+ T cells in treated-CD biopsy samples cultured with PT-gliadin (21.4±17.9%) than in those cultured in medium alone (10.8±7.9%). No significant differences were noted in the percentage of Foxp3+CD25+CD4+ cells in biopsy samples of non-CD controls cultured with PT-gliadin (4.2±0.87%), in comparison with biopsy samples cultured with medium alone (3.7±2.1%; **Figure 3**, lower right panel).

Intestinal CD4+CD25+ cells in CD are regulatory T cells

The high Foxp3 expression by CD4+CD25+ cells from the mucosal explants of active CD raised the possibility that these cells may be Treg cells. Therefore, we co-cultured intestinal CD4+CD25+ T cells from untreated CD patients with CD4+CD25− peripheral Tresp at a ratio of 1:1, in the presence of a polyclonal stimulus. After 3 days, CD4+CD25+ T cells showed a hypoproliferative response (anergy), whereas Tresp cells proliferated vigorously (**Figure 4a**). In coculture, intestinal CD4+CD25+ T cells significantly suppressed the proliferation of Tresp cells (**Figure 4a**) ($P<0.005$) in a cell dose-dependent manner, and induced a significant decrease of IFN-γ production ($P<0,001$; **Figure 4b**). Moreover, in line with previous reports (21), we have shown that the peripheral blood CD4+CD25+ T cells of untreated CD patients were Treg cells, being capable of suppressing a significantly autologous responder CD4+CD25− stimulated by anti-CD3/anti-CD28 in a cell dose-dependent manner, in both terms of proliferation ($P<0.05$; **Figure 4c**) and IFN-γ secretion ($P<0.05$) (**Figure 4d**).

Ability of IL-15 to overcome Treg-mediated immunosuppression in CD patients

As our data show increased number of Foxp3+ cells and a satisfactory suppressive activity to explain the strong inflammatory response in untreated CD patients, we hypothesized that IL-15 hyperexpressed in the untreated CD mucosa could impair such suppression. Therefore, we assessed whether IL-15 could alter Treg-mediated immunosuppression. Freshly isolated Treg cells from intestinal or peripheral blood of untreated CD patients were cocultured, with Tresp at a ratio of 1:1, in a medium containing anti-CD3/CD28, in the presence or absence of IL-15 (10 ng/ml). In CD patients, IL-15 was effective in counteracting both intestinal and peripheral blood Treg cell-mediated suppression of anti-CD3-activated Tresp cells, partially in terms of proliferation (**Figure 5a, b**, respectively) and completely in terms of IFN-γ production (**Figure 5c**).

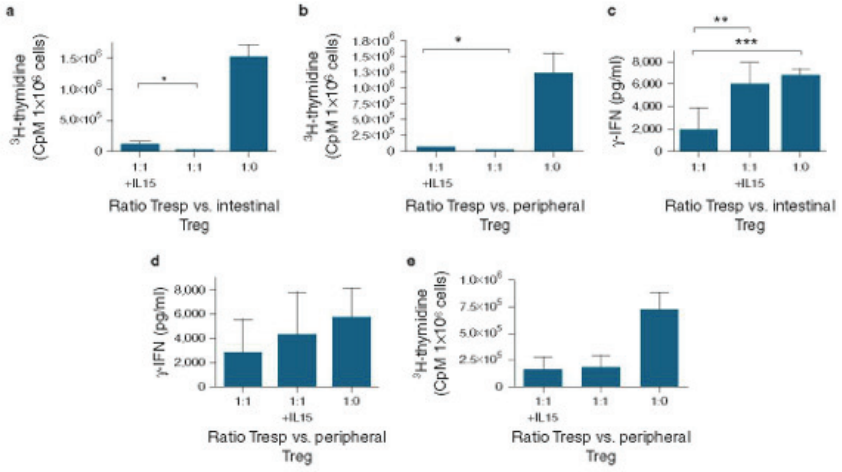

Figure 5. Interleukin 15 (IL-15) impairs the suppressive activity of regulatory T cells (Treg). (**a, b**) Effect of IL-15 on suppressive capacity of Treg cells isolated, respectively, from intestinal biopsy and peripheral blood of five active celiac disease (CD) patients assessed on proliferation. (**c**) Effect of IL-15 on suppressive capacity of Treg cells isolated from peripheral blood of five active CD patients assessed on IFN-γ production. Proliferative responses were measured by [^{3}H]thymidine uptake. Interferon gamma (IFN-γ) secretion was evaluated in supernatants of cocultures by an enzyme-linked immunosorbent assay test. (**d** and **e**) Effect of IL-15 on suppressive capacity of Treg cells isolated from peripheral blood of four non-CD controls assessed, respectively, on IFN-γ production and proliferation. Results are from four independent experiments. The Treg and responder T cells (Tresp) were cocultured at 1:1 ratio and stimulated with IL-15 (10 ng/ml). $^*P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$, when compared with the condition in presence of IL-15.

To test whether the inhibitive effect of IL-15 on Treg cells was unique to CD patients, we performed analysis on non-CD controls. We cocultured only peripheral blood CD4 + CD25 + T cells with Tresp cells, with or without IL-15, as CD4 + CD25 + T cells isolated from the intestinal biopsy samples of non-CD control patients were not enough for functional studies. Our data demonstrated that the phenomenon was nonspecific for CD patients as in non-CD controls IL-15 partially prevented the inhibition of IFN-γ secretion (**Figure 5d**). However, it did not overcome the Treg cell-mediated block of Tresp cell proliferation (**Figure 5e**).

Finally, we wondered whether the greater sensitivity to IL-15 seen in CD patients could be explained by an increased expression of its receptor. Cell-surface IL-15Rα was then monitored by FACS in peripheral blood Treg and Tresp cells of both CD patients and non-CD controls. We found that IL-15Rα was highly expressed on Treg cells in both CD patients and non-CD controls (**Figure 6**), whereas it was moderately detectable in Tresp cells (**Figure 6**). However, a direct comparison of Treg cells from CD patients and non-CD controls revealed that the surface density of IL-15Rα was significantly higher ($P<0.05$) in Treg cells from CD patients (mean±s.d.: 261±150 and 33±28, respectively; **Figure 6**).

DISCUSSION

In this study, we found, by two complementary methods—immunohistochemistry and FACS *ex vivo* analysis—an increased number of Foxp3 + cells in the intestinal mucosa of patients with active CD compared with both treated CD and non-CD controls. These cells were not found in the epithelium layer, but were mainly localized in the subepithelial layer of the lamina propria. The data obtained in biopsies from active celiac patients are in agreement with recent observations (17–19), indicating an increased density of Foxp3 + cells by immunohistochemistry. In our study, we have confirmed these results also by FACS *ex vivo* analysis.

In general, these data suggest that Foxp3 expression is linked to the Th1 -driven mucosal immune response to gliadin. In fact, the expansion of this subset proportional to the intensity of local inflammation, could have a role in the negative feedback loop of T-cell activation. In support of this hypothesis, we found in three CD patients with a partially healed mucosal tissue, an increased number of Foxp3 + cells when compared with the normal mucosa of both treated CD and non-CD controls, but lower with respect to the density found in the mucosa of untreated CD (data not shown). Moreover, the data that we are collecting on duodenal biopsies from "potential" CD patients (patients with positive CD serology, and low local inflammation) point in the same direction. Thus, the increased density of Foxp3 + cells seems to be correlated with the histological lesion, suggesting that the immune system is actively trying to downregulate ongoing inflammation either through the rapid redistribution of Treg cells from the circulation to the inflamed site, or through the local proliferation of these regulatory cells.

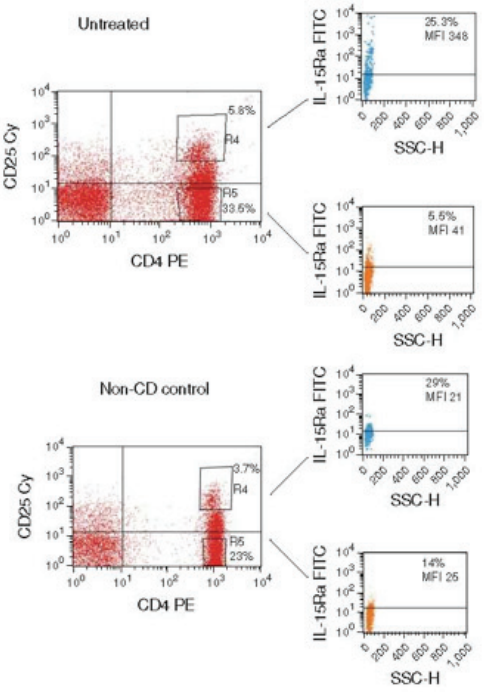

Figure 6. Increased expression of interleukin-15 receptor alpha surface expression (IL-15Rα) on CD4+CD25+ T cells from active celiac disease (CD) patients. Freshly isolated peripheral blood mononuclear cells (PBMCs) cells from active celiac patients and non-CD controls stained for CD4, CD25, and IL-15Rα, and analyzed by flow cytometry (FACS). Representative dot plot from one experiment from active CD patients and non-CD control was shown. CD4+CD25+ and CD4+CD25− T cells were analyzed for IL-15Rα expression and mean fluorescence intensity (MFI) of IL-15Rα was indicated. The dot plot shows a higher intensity of IL-15Rα on CD4+CD25+ peripheral blood T cells from active CD patient than non-CD controls ($P<0.05$). Numbers indicate the MFI of at least three experiments.

In humans, the correlation between Foxp3 expression and suppressive capacity is not as clear as in the murine system. In fact, recently it has been shown that expression of Foxp3 does not exclusively occur in CD4+CD25+ Treg cells, as in humans it can also be transiently induced in activated CD4+CD25− T effector cells, which do not express Foxp3 in the resting state (24,25). Therefore, the statement that CD patients are characterized by accumulation of suppressive cells in the intestinal mucosa must be considered carefully, in the absence of a functional suppressive assay. Although the functional activity of CD4+CD25+ T cells isolated from peripheral blood of untreated-CD patients was recently investigated (20–22), the suppressive capacity of such T cells in the intestinal mucosa of CD patients has never been reported. Towards this aim, and to ascertain whether the observed Treg cells are indeed suppressive, we isolated CD4+CD25+ cells from biopsy samples of active CD and tested their suppressive capacity in an *in vitro* coculture assay. Our data show that intestinal CD4+CD25+ T cells of CD patients are able to exert their regulatory effects *in vitro* in terms of inhibition of proliferation and IFN-γ secretion of Tresp cells. Moreover, in line with recent report (21), we confirm the suppressive activity of peripheral blood CD4+CD25+ T cells of CD patients. Therefore, our current results suggest that intestinal and peripheral blood Treg cells of untreated CD patients are not functionally deficient and could be able to control the ongoing immune response to gluten and the consequent inflammation.

On the contrary, despite the increased frequency and suppressive activity *in vitro*, Treg cells fail to control the development of the inflammation in the small intestinal mucosa with active CD. It is possible that the suppressor capacity of these cells may be abrogated *in vivo* or it is insufficient to counterbalance the strong proinflammatory response. Recently, it has been shown that IL-15 not only has a pleiotropic role at the interface between innate and adaptive immunity in CD, but also exerts effects interfering with anti-inflammatory pathways that are normally activated in the small intestinal mucosa by the cytokine TGF-β1 (11). The massive increase of the proinflammatory cytokine IL-15 in CD led us to investigate whether IL-15 might interfere with the suppressive activity of intestinal Treg cells. In active CD patients, we have shown that IL-15 impairs the functions of Treg cells making Tresp cells refractory to the regulatory effects of CD4+CD25+ T cells, in terms of proliferation and production of IFN-γ. This phenomenon was nonspecific for CD patients, as in non-CD controls the addition of IL-15 to cocultures of Treg/Tresp cells prevented the inhibition of IFN-γ secretion. Nevertheless, this effect was less marked than in CD. The greater sensitivity to IL-15 of CD patients is likely to be due to their increased expression of IL-15 receptor. Recently, it has been observed that the mRNA expression of IL-15 receptor-α was increased in duodenal biopsy samples of untreated CD patients as compared with controls (26). How IL-15 can impair the suppressive activity of Treg cells in *vitro* remains to be defined. Previous data indicated that, in active CD, IL-15 was involved in the local downregulation of TGF-β signaling (11), which is required to maintain the regulatory function of Treg cells (27). Studies are now in progress to address in our system whether and how IL-15 might interfere with the regulatory function of TGF-β.

Aside from evidence that natural Foxp3+ Treg cells arise and mature in the thymus, there is mounting evidence that Foxp3+ Treg cells can develop extrathymically under certain conditions. As a consequence of this expansion, Treg cells cause downmodulation of inflammation associated with pathogen-specific immune responses. Recently, it was observed that small intestine lamina propria dendritic cells promote *de novo* generation of Foxp3 Treg cells through retinoic acid, which is a vitamin A metabolite that is highly expressed in gut associated lymphoid tissue (28). Together, these data demonstrate that the intestinal immune system has

evolved a self-contained strategy to promote Treg cell induction. The *in vitro* gliadin challenge system reproduces many features of the mucosal immune response, which occur in the established celiac lesion (29,30). In such a system, we provide evidence that in CD intestinal mucosa, Foxp3 + Treg cell can be expanded locally during gliadin-specific stimulation as a likely attempt to curtail the mucosal immune response. In fact, in the lamina propria of celiac biopsy samples cultured in the presence of a PT-gliadin, but not in those from controls, the number of cells expressing Foxp3 were significantly higher, particularly in the subepithelial compartment, than in samples cultured in medium alone. The FACS analysis of the frequency of Foxp3 + CD25 + cells before and after challenge with PT-gliadin in CD4 + population confirmed the immunohistochemical data.

In conclusion, we have shown that in CD-untreated intestinal mucosa, the expanded CD4 + CD25 + Foxp3 + T cells are regulatory cells. We proved that they are induced *in situ* by gliadin. However, they can be impaired *in vivo* in their suppressor capacity by IL-15. Their sensitivity to the IL-15 action is likely due to enhanced expression of IL-15 receptor.

On the basis of these results and on the finding that IL-15 is overexpressed in intestinal mucosa of patients with active CD, we suggest that, in target tissues, the function of Treg may be substantially limited by these cytokines, and that therapies that aim at neutralizing such cytokines may not only decrease bystander T-cell activation but also reconstitute the suppressor function of regulatory T cells.

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CONFLICT OF INTEREST

Guarantors of the article: Delia Zanzi, MS and Giuseppe Mazzarella, MS.

Specific author contributions: Delia Zanzi was involved in study design, data analysis, and supervised the flow cytometric analyses. Rosita Stefanile contributed to study design and immunohistochemical analyses. Sara Santagata performed flow cytometric analyses. Gaetano Iaquinto and Nicola Giardullo helped in patients' recruitment. Laura Iaffaldano performed flow cytometric analyses. Giuliana Lania and Ilaria Vigliano carried out purification of T Cell subsets and performed suppression assay. Aufiero Rotondi Vera performed organ culture. Katia Ferrara performed the enzyme-linked immunosorbent assay test. Salvatore Auricchio and Riccardo Troncone contributed to study design, data analysis, and critical revision. Giuseppe Mazzarella contributed to study design and data analysis, and supervised immunohistochemical analyses. Riccardo Troncone and Giuseppe Mazzarella were also involved in drafting of the article.

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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Coeliac disease (CD) is a condition characterized by a dysregulated mucosal immune response to gliadin.
- ✓ Increased number of Foxp3+ T cells are present in the small-intestine biopsy samples from patients with active CD.
- ✓ Suppressor capacity of circulating CD4+CD25+ T cells from CD patients are reported, but no functional data are available for their intestinal counterparts.

WHAT IS NEW HERE

- ✓ Intestinal CD4+CD25+T cells from CD patients are functionally suppressive.
- ✓ IL-15 impairs the regulatory activity of both intestinal and peripheral blood Treg cells of CD patients. Their sensitivity to IL-15 is partly explained by the increased expression of IL-15Rα.
- ✓ CD4+CD25+Foxp3+ T cells are induced *in situ* by gliadin.

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CONCLUSIVE REMARKS

In this study we found, by two complementary methods, immunohistochemistry and flow cytometry ex vivo analysis, an increased number of Foxp3+ cells in the intestinal mucosa of patients with active CD compared with both treated CD and non-CD controls. These cells were not found in the epithelium layer, but were mainly localized in the subepithelial layer of the lamina propria. The data obtained in biopsies from active celiac patients are in agreement with recent observations [17-19] indicating an increased density of Foxp3+ cells by immunohistochemistry. In our study, we have confirmed this results also by flow cytometry ex vivo analysis.

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In humans the correlation between Foxp3 expression and suppressive capacity is not as clear as in the murine system. In fact, recently it has been shown that expression of Foxp3 does not exclusively occur in CD4+CD25+ Treg since in humans it can also be transiently induced in activated CD4+CD25- T effector cells, which do not express Foxp3 in the resting state [24, 25]. Therefore, the statement that CD patients are characterized by accumulation of suppressive cells in the intestinal mucosa must be considered

carefully, in the absence of a functional suppressive assay. While the functional activity of CD4+CD25+ T cells isolated from peripheral blood of untreated CD patients was recently investigated [20-22], the suppressive capacity of such T cells in the intestinal mucosa of CD patients has never been reported. To this aim and to ascertain if the observed Treg cells are indeed suppressive, we isolated cells CD4+CD25+ cells from biopsies of active CD and tested their suppressive capacity in an in vitro coculture assays. Our data show that intestinal CD4+CD25+ T cells of CD patients are able to exert their regulatory effects in vitro in terms of inhibition of proliferation and IFN-γ secretion of Tresp cells. Moreover, in line with recent report [21], we confirm the suppressive activity of peripheral blood CD4+CD25+ T cells of CD patients. Therefore, our current results suggest that intestinal and peripheral blood Treg cells of untreated CD patients are not functionally deficient and could be able to control the ongoing immune response to gluten and the consequent inflammation.

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Based on these results and on the finding that IL-15 is over-expressed in intestinal mucosa of patients with active CD, we suggest that in target tissues the function of regulatory T cells may be substantially limited by these cytokines and that therapies that aim at neutralizing such cytokines may not only decrease bystander T cell activation but also reconstitute the suppressor function of regulatory T cells.

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CHAPTER 4
ENDOCYTIC TRAFFICKING IS CONSTITUTIVELY ALTERED IN CELIAC DISEASE

Background and aims

Celiac disease (CD) occurs frequently, and is caused by ingestion of prolamins from cereals in subjects with a genetic predisposition. The small intestinal damage depends on an intestinal stress/ innate immune response to certain gliadin peptides (e.g., A-gliadin P31-43) in association with an adaptive immune response to other gliadin peptides (e.g., A-gliadin P57-68). P31-43 has an effect on the maturation and function of early endocytic vesicles and consequently on epithelial growth factor receptor (EGFR) signaling and CD enterocyte proliferation. The reason that the stress/innate immune and proliferative responses to certain gliadin peptides are disruptive in CD and not in control intestine is so far unknown.

The aim of this work is to demonstrate that, in CD cells, a constitutive alteration of the endocytic compartment exists that may represent a predisposing condition to the damaging effects of gliadin in CD patients.

Methods

Immunofluorescence and pulse-chase experiments were used to study endocytic morphology and function in CD fibroblasts and intestinal biopsies. Western blot (WB) analysis, immunoprecipitation, immunostaining and quantitative PCR were also used.

Results.

We found morphological and functional alterations of the endocytic compartment in fibroblasts and enterocytes from biopsies of CD patients. These changes included an increase in the number of early endosomes, delayed EGF endocytic trafficking, an increase in total phosphorylated proteins including EGFR and

the downstream signaling molecule ERK (extracellular signal regulated kinase) and increased EGF mRNA and enterocyte proliferation.

Conclusions

The same pathway with which gliadin peptide P31-43 can interfere is constitutively altered in CD cells. This observation potentially explains the specificity of the damaging effects of certain gliadin peptides on CD intestine.

Keywords

Endocytosis; Celiac Disease; Enterocytes Signaling; EGFR; ERK.

INTRODUCTION

Celiac disease (CD) is characterized by derangement of adaptive and innate immune responses to wheat gliadins. Some gliadin peptides that are deamidated by tissue transglutaminase (e.g., A-gliadin P57-68) bind to HLA DQ2 and/or DQ8 molecules and induce an adaptive Th1 pro-inflammatory response.1 Other gliadin peptides (e.g., P31-43) are able to initiate both a stress [2,3] and an innate immune response. [4,5]

In CD, damage to the intestinal mucosa is mediated by inflammation due to both the adaptive and the innate immune responses (IL-15 is a major mediator of the innate immune response) and by proliferation of crypt enterocytes, which causes crypt hyperplasia and mucosal remodeling, both of which are hallmarks of CD mucosa. [6,7,8] In the celiac intestine, there is an inversion of the differentiation/proliferation program of the tissue. This inversion involves a reduction in the differentiated compartment that can result in complete villus atrophy and an increase in the proliferative compartment, with resultant crypt hyperplasia. [9,10]

Recent observations [11,12,13,14] from our laboratory and by others point to an effect of certain gliadin peptides (e.g. P31-43) on the maturation and function of early endocytic vesicles in cell lines and in intestinal biopsies. Endocytosis has many effects on signaling; in fact, signaling pathways and endocytic pathways are regulated in a reciprocal manner. Consequently, endocytosis affects several cell functions ranging from proliferation to cell motility. [15]

We have previously investigated the interaction between gliadin peptides and intestinal epithelial cells in CaCo2 cells and in biopsies from CD patients. We found that by interfering with the localization to the endocytic membranes of Hrs (hepatocyte growth factor-regulated kinase), a key molecule in the maturation of early endocytic vesicles, P31-43 induces two important effects: a) it delays endocytic maturation, and b) it alters the recycling pathway. By delaying the maturation of endocytic vesicles, P31-43 reduces the degradation of EGFR and other receptor tyrosine kinases (RTK) and prolongs their activation, which in turn results in actin modification, increased cell proliferation and other biological effects.[11,14] The alteration of the recycling pathway is able to direct more IL15 to the cell surface. Gliadin peptide P31-43 also increases the levels of IL15 mRNA.16 By increasing the synthesis of IL15 and the amount of the cytokine that is presented to neighboring cells, P31-43 affects both enterocyte proliferation, which is EGFR- and

IL15-dependent, and the activation of innate immunity.[16] The reason the effects of these peptides on the endocytic compartment are extremely disruptive to the CD mucosa is not clear. Our hypothesis is that, in CD mucosa, an alteration exists that may represent a predisposing condition to the damaging effects of gliadin. According to this hypothesis, in the present work we have attempted to determine whether constitutive morphological and functional alterations occur in the endocytic compartment of CD enterocytes and skin-derived fibroblasts of CD patients on gluten-free diet (GFD). The alterations found are independent of the presence of gluten in the diet and of the inflammation site.

METHODS

Cell culture

Fibroblast cell lines were cultured from skin biopsies from patients, all of whom gave informed consent to use of biopsy tissue in the study. We obtained fibroblasts from five celiac patients on gluten-free diet and from four HLA DQ2/8 negative healthy controls. Detailed description of fibroblasts and CaCo-2 cells culture is in the supplementary material.

Pulse-chase experiments on fibroblasts

Pulse-chase experiments were performed as described previously14 and are reported in detail in the supplementary material.

EEA1 and phosphotyrosine staining

EEA1 and phosphotyrosine staining in fibroblasts and in biopsies from CD patients and controls is decribed in the supplementary material. Briefly acetone-fixed (10 min, Sigma-Aldrich, Milan, Italy) 5-µm frozen sections from CD biopsies and controls and, fibroblasts seeded on glass coverslips fixed with 3% paraformaldehyde, were examined by immunofluorescence, after staining with antibody against EEA1 (C-15) (Santa Cruz, DBA, Milan, Italy), with anti-pTyr antibody (Santa Cruz Biotechnology, CA, USA) and realative fluorescinated secondary antibodies. Fluorescence intensity (Fi) analysis of the samples respect to the background was carried out using AIS Zeiss software.

Immunohistochemistry of pY-ERK in biopsies

For the immunohistochemical study, 4-µm biopsy sections from CD patients and controls were fixed in 3% paraformaldehyde (Sigma-Aldrich, Milan, Italy) and stained as reported in the supplementary material.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed as described previously, [11,16] and detailed in the supplementary material.

Organ culture studies

For organ culture studies, biopsy fragments from duodenum were obtained from 8 CD patients with villus atrophy, 8 controls (affected by gastroesophageal reflux), 11 CD patients on GFD and 11 potential CD patients. Informed written permission was obtained from all patients. The biopsy fragments were cultivated as reported elsewhere [11,17] and detailed in the supplementary material.

Pulse-chase experiments on biopsies

To examine endocytosis of EGF, all biopsies were treated as described previously11 and detailed in the supplementary material.

Ethical approval

The protocol of the study was approved by the Ethical Committee of the University "Federico II", Naples, Italy (ethical approval certification C.E. n. 230/05).

RESULTS

The morphology of the early endocytic compartment is altered in CD mucosa

It has previously been shown that gliadin peptide P31-43 can interfere with endocytic trafficking in CaCo2 cells.[11,14] We therefore analyzed the endocytic compartment in enterocytes of CD mucosa to determine whether an alteration in this compartment represents a predisposing condition to the damaging effects of gliadin peptides in CD patients. EEA1 and LAMP 2 were used as markers of the early and late endocytic compartments, respectively, in duodenal biopsies from controls and from CD patients in the active phase of the disease with villus atrophy, on a GFD and also from potential CD patients. Potential CD patients are those on a gluten-containing diet with predisposing HLA DQ2 or DQ8 who are positive for anti-TTG antibodies in the serum but who do not show intestinal alterations. As shown in figure 1A, EEA1- positive vesicles are increased in crypts and villi of CD enterocytes compared to controls. Interestingly, the enterocytes of potential CD patients also display increased EEA1-positive vesicles, indicating that alteration of the endocytic compartment is a marker of the disease in the absence of intestinal atrophy. In CD patients on GFD, the amount of EEA1-positive vesicles is also increased, suggesting that an alteration of the endocytic compartment could be present in CD intestinal mucosa independent of the gluten content of the diet. As shown in figure 1B, the fluorescence intensity of EEA1 is increased in all CD patients with respect to controls both in villi (CD with villus atrophy 1123 ± 507.4, GFD 698.3 ± 377.7, potential 750.7 ± 339.5, controls 511.1 ± 255.6) and in crypt enterocytes (CD with villus atrophy 1207 ± 166.3, GFD 1426 ± 144.2, potential 1594 ± 365.5, controls 884.4 ± 185.3). Increased EEA1 staining is also present in the lamina propria cells of CD patients. Nevertheless, we focused our study on the enterocytes because this cell type represents a homogeneous cell population and is the first cellular compartment to meet gliadin and the compartment in which cellular stress has been described.

Because EEA1 is a structural marker of early vesicles, [18] we measured the levels of EEA1 protein in intestinal mucosa from CD patients and controls. Western blotting revealed that the EEA1 protein is increased by 5- to 8-fold in intestinal biopsies from CD patients compared to controls (figure 1C). Staining of Lamp 2, a marker of late vesicles, shows a slight increase only in the surface epithelium of CD with villus atrophy (supplementary figure 1).

Endocytic trafficking is altered in CD mucosa

EEA1 staining and Western blotting highlighted a morphological alteration in the endocytic compartment in CD enterocytes that occurs even in the absence of gluten from the diet. We next investigated endocytic trafficking in CD mucosa using EGF as a marker of endocytosis. Intestinal biopsies from controls and CD patients were treated with EGF labeled with the fluorescent marker Alexa 488. Biopsy cultures were pulsed for 3 h with EGF-Alexa 488 and chased for 24 h in media alone. As shown in figure 2, EGF-Alexa 488 can be seen in the crypt enterocytes from CD patients, whether on gluten-containing or gluten-free diets, after 24 h of chase, whereas in the control biopsies EGF-Alexa 488 is no longer visible after 24 h. This indicates that the trafficking of EGF in endocytic vesicles is delayed in CD.

In CD fibroblasts both the morphology and the trafficking of the early endocytic compartment are altered

The previously described alterations in endocytic trafficking in CD enterocytes could result, in the GFD patients, from residual inflammation and not from a constitutional defect of the celiac cells. We therefore investigated the endocytic compartment in skin-derived fibroblasts of CD patients. These cells represent a cellular compartment that is located far from the inflammation site. We measured the fluorescence intensity of EEA1-positive early endocytic vesicles (figure 3A), the levels of EEA1 protein (figure 3B) and EGF-Alexa 488 trafficking in these cells (figure 3C). The fluorescence intensity of EEA1-positive vesicles is increased in fibroblasts from CD patients (2104 ± 905.4) in comparison to controls (1272 ± 673.6) (figure 3Ac). As expected from the increase of EEA1-positive vesicles in fibroblasts from CD patients, we found that the level of EEA1 protein also increased in CD fibroblasts. This is shown in the Western blot in figure 3B. To measure endocytic trafficking in these cells, we loaded early vesicles with EGF-Alexa 488 in pulse-chase experiments. Fibroblasts were pulsed for 30 min with EGF-Alexa 488 and, after intensive washing, were chased for 3 hours in medium alone. As shown in figure 3C, after 3 h chase EGF-Alexa 488 fluorescence is strongly reduced in fibroblasts from controls but is still present in those from CD patients. Statistically significant differences were found in the fluorescence intensity/cell of EGF-Alexa 488; after 3 h chase, the fluorescence intensity was 225 ± 189 in controls and 2350 ± 208 in GFD CD fibroblasts.

The results with CD mucosa and skin-derived fibroblasts suggest that there is a morphological and functional alteration of the endocytic compartment in CD patients. This is constitutive and independent of the inflammation site, because it can be found in a cellular compartment far from the intestine. Interestingly, we have

obtained similar results with dendritic cells derived from blood mononuclear cells (supplementary figure 2).

Signaling is altered in CD cells

The alterations of the endocytic pathway we have described in CD cells might be expected to delay the decay of signaling molecules, allowing them to continue to signal downstream from the endocytic compartment. We found that total phosphorylated proteins are increased in crypt and villus enterocytes of CD patients, both those on a gluten- containing diet (CD patients with villus atrophy and potential CD with normal mucosa) as well as patients on a GFD (figure 4, A and B). This suggests that there is a constitutive activation of signaling molecules in CD patients that is independent of diet. Villi present more positive staining for phosphotyrosine when patients are on a gluten-containing diet (CD with villus atrophy and potential) (figure 4B, villi).

Some of the increased phosphorylation that we observed is due to an increase in the phosphorylation of the downstream effector of EGFR signaling, ERK1/2 (figure 4, C/D). When ERK1/2 is activated, it migrates to the nucleus. Using an antibody against the phosphorylated form of ERK (pY-ERK), we stained biopsies from CD in the active phase of the disease (both CD with villus atrophy and potential CD) and from patients on a GFD, in the remission state of the disease. The percentage of nuclei positive for the activated form of ERK 1/2 are increased in crypt enterocytes from all CD mucosas (CD with villus atrophy 59.58%+/-18.86%, GFD CD 77.28%+/-9.97%, potential 69%+/-16.45%) compared to controls (38.75%+/-17.66%). In villi enterocytes, a similar trend is present but reaches statistical significance only in cells derived from patients with CD with villus atrophy. Biochemical analysis of pY-ERK in biopsies from CD patients and controls confirms the immunohistochemical analysis. As shown in figure 4E, blotting of proteins from lysates of biopsies from CD patients and controls indicates that there is a significant increase of pY-ERK not only in CD with villus atrophy and potential CD but also in CD patients on GFD. Taken together, these results indicate that signaling molecules are activated in CD mucosas of patients whether they are on GFD or gluten containing diet (GCD).

To analyze whether similar alterations are present in cells outside the intestine, we investigated the level of phosphorylation of total proteins and of ERK and EGFR in skin fibroblasts from GFD CD patients and

controls (figure 5 A, B). Figure 5A shows staining for total phosphorylated proteins in fibroblasts. The fluorescence intensity/cell indicates that there are more phosphorylated proteins in fibroblasts from GFD CD patients (655.2 ± 229.1) than in controls (510.0 ± 164.1). To confirm the increment in phosphorylated proteins in CD fibroblasts, we immunoprecipitated total phosphoproteins from the cell lysate using an anti-phosphotyrosine antibody. The results, which are shown in supplementary figure 3, demonstrate that there is an increase in the total amount of phosphorylated proteins in CD fibroblasts compared to control fibroblasts. Using specific antibodies, two of the proteins that showed increased phosphorylation were identified as ERK and EGFR.

To further confirm the increased phosphorylation of the active signaling molecules ERK and EGFR in these cells, we used Western blotting to specifically analyze their phosphorylated state. As shown in figure 5B, pY-ERK was identified in total cell lysates of CD and control fibroblasts using specific antibodies that recognize the phosphorylated form of ERK. Densitometric analysis (figure 5Bb) shows a significant increase in the phosphorylated form of ERK in CD fibroblasts. Phosphorylated EGFR was identified by immunoprecipitating EGFR with a specific antibody and then staining the immunoprecipitated proteins with an anti-phosphotyrosine antibody (figure 5Bc). Densitometric analysis (figure 5Bd) shows an increase of almost 6-fold in the phosphorylated form of EGFR in CD fibroblasts.

Taken together, the data presented here imply that, in CD cells, alterations occur in the phosphorylation of signaling proteins such as EGFR and the downstream effector ERK. These alterations are present even in patients on GFD, and they are independent of the site of inflammation.

Proliferation of crypt enterocytes is increased in CD

We have shown that EGF-Alexa remains in enterocytes of CD patients longer than in controls, indicating that the decay of this potent mitogen is delayed in CD cells. Furthermore, an increase in EGF mRNA is found not only in enterocytes isolated by laser microdissection from biopsies of patients with CD with villus atrophy but also in enterocytes from patients in remission on GFD. This finding indicates that a positive autocrine loop19 occurs between EGFR activation and EGF production, independently of gluten intake and crypt hyperplasia (supplementary figure 4). We therefore considered the possibility that proli-

feration of crypt enterocytes might be a possible consequence of the alterations shown above.

We studied the proliferation of crypt enterocytes by measuring BrdU incorporation in cultured biopsies from CD patients and controls. We found that proliferation of crypt enterocytes is increased in CD patients compared to controls (figure 6 A-B). This proliferation is increased in enterocytes from patients with CD with villus atrophy (17.0%±3.5%), potential patients (10.8%±2.7%) and in CD patients in remission on a gluten-free diet (15.9%±9.1%) with respect to controls (7.7%±2.5%). This finding indicates that the increased proliferation of crypt enterocytes seen in CD is partially independent of the crypt hyperplasia (that does not occurs in potential CD) and of the presence of gluten in the diet. As expected, the increased proliferation of crypt enterocytes in cells from patients with CD with villus atrophy is dependent both on EGFR and IL15 signaling; this is shown by the fact that it can be prevented by anti-EGFR and anti-IL15 antibodies (data not shown).

DISCUSSION

In this paper, we describe a constitutive alteration of the endocytic pathway in enterocytes and fibroblasts of patients with celiac disease. This alteration consists of an increase in EEA1-positive vesicles (early endosomes) and a delay of EGF endocytic trafficking at this level. In biopsies from these patients, we also found an increase in total phosphorylated proteins including EGFR and the downstream signaling molecule ERK, an increase in EGF mRNA and increased proliferation of enterocytes. These alterations are present in patients on GFD as well as those on regular diets and, as shown by their presence in skin fibroblasts, are independent of the inflammation site. We have studied the endocytic pathway both morphologically and functionally. The fluorescence intensity of early endocytic vesicles stained with EEA1 was increased in intestinal crypt enterocytes and in skin fibroblasts from CD patients. An increase in EEA1 protein levels was also demonstrated, suggesting that there is a net increase in the early endocytic compartment in CD cells. The increase in EEA1-positive vesicles in intestinal biopsies from CD patients was found mainly in crypt enterocytes and was present not only in tissue from active CD patients but also in tissue from CD patients in the remission state of the disease and in potential CD patients (those with normal intestinal mucosa, positive serology for CD and on a GCD). This alteration therefore occurs in the absence of gluten in the diet and in the normal mucosa of potential CD patients. Other endocytic compartments, such as the compartment that includes Lamp2-containing vesicles (late vesicles), appeared altered mainly in mucosa from CD patients with villus atrophy.

To test the function of endocytic trafficking in CD cells, we performed pulse-chase experiments in which we loaded early vesicles with EGF-Alexa 488.[11,14] The retention of EGF-Alexa 488 in the endocytic compartment of fibroblasts and enterocytes from patients in the acute or the remission phase of the disease was found to be delayed compared to the retention of the compound in cells from control patients. This shows that the endocytic pathway is not only morphologically altered, as demonstrated by EEA1 accumulation, but is also functionally impaired in CD cells.

Morphological and functional alteration of the endocytic pathway can have several biological consequences, including delay in the decay of signaling molecules that can continue to signal downstream from the endocytic compartment.[20] In particular, interference with endocytic vesicle maturation results in delay of the decay of tyrosine kinase receptors such as EGFR and a consequent increase in the level of phosphorylated proteins.[21,22,23] In the present work, we found that total phosphorylated proteins were increased in the enterocytes of CD patients both on gluten-containing diets

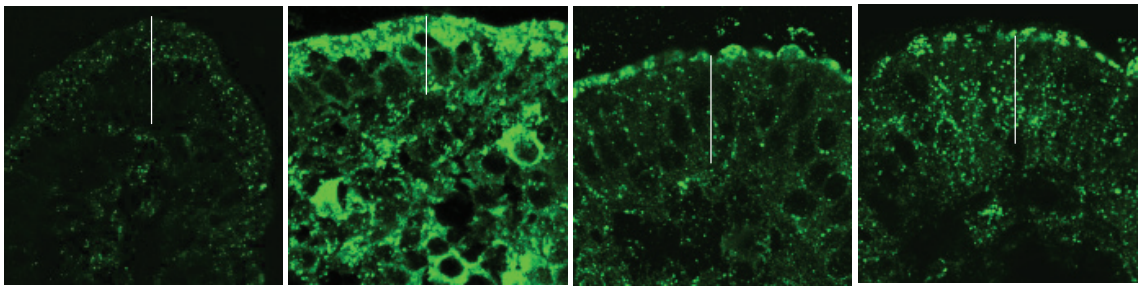
(including patients with atrophic mucosa and potential CD patients with normal mucosa) as well as in the enterocytes of patients on GFD. We found that ERK phosphorylation was increased in biopsies from CD patients and showed by immunohistochemistry that the increase in pY-ERK mainly occurs in crypt enterocytes, strongly suggesting that signal activity is increased in enterocytes from CD patients. An increase was also found in total phosphorylated proteins in fibroblasts from CD patients, and some of the phosphorylated proteins whose phosphorylation is increased were identified. In particular, phosphorylated EGFR and ERK are increased in fibroblasts from CD patients. Proliferation of enterocytes is a hallmark of CD.[11,16] In this paper, we have shown that EGF-Alexa remains in enterocytes of CD patients longer than in controls, indicating that, in these cells, the decay of a potent mitogen such as EGF is delayed independently from gliadin treatment. EGFR can still signal downstream after internalization.[11] Consistent with this idea, we found increased activation of ERK, an EGFR downstream effector,[23,24] in fibroblasts and intestinal mucosa of CD patients. Moreover, in CD fibroblasts, there was increased activation of EGFR. As a readout of EGFR-ERK pathway activation, we measured crypt enterocyte proliferation by measuring BrdU incorporation in organ culture experiments. As expected, crypt enterocyte proliferation was found to be increased in active CD patients with crypt hyperplasia. In potential CD patients, crypt enterocyte proliferation was also increased, although the small intestine apparently had a normal architecture in these patients. Interestingly, increased proliferation was also found in the absence of gluten from the diet. Moreover, increased levels of EGF mRNA occurred not only in enterocytes of CD patients with villus atrophy but also in patients in remission on GFD, indicating that there is a positive autocrine loop [19] between EGFR activation and EGF production in enterocytes that is independent of gluten intake and crypt hyperplasia. Taken together, these results show that increased proliferation, together with increased phosphorylation of several proteins including EGFR and ERK, is an intrinsic characteristic of CD cells. The MAPK-ERK 1-2, like all mitogen-activated-kinases (MAPKs), is one of the essential signaling molecules that converts environmental inputs into influences on a plethora of cellular programs. [25] Moreover, most of the MAPK, including ERK, are stress sensors that can be activated by different inputs. [26] . Taken as a whole, our data suggest that in CD there is a constitutive derangement of the endocytic pathway that can also be found outside the intestine and is independent of gluten intake. We also found an increase in protein phosphorylation, with EGFR and ERK activation, and an increase in enterocyte proliferation. Emerging evidence connects endocytosis to complex cellular programs that control proliferation, apoptosis, cell motility, cell fate determination, and immunologic response to infections and other agents.[15] Alteration of vesicular trafficking has been demonstrated to play a role in the pathogenesis of another intestinal in-

flammatory disease, Crohn's disease, in which defective vesicular transport results in impaired macrophage function, reduced cytokine secretion and decreased autophagy.[27] Thus, vesicular trafficking seems to represent an important aspect of cell regulation, and the disruption of this pathway is predicted to play an important role in human disease. It has been proposed that to develop villus atrophy, patients must have an intestinal stress/innate immune response to certain gliadin peptides in association with adaptive anti-gluten immunity. [2,3] In CD enterocytes, signs of distress such as an increase in heat shock proteins and in the non-classical MHC class 1 molecules HLA E and MIC-A, as well as signs of innate immunity activation such as an increase in IL15 levels, have been reported.[3]. Increasing evidence from our laboratory and those of others [11-14,16] suggests an effect of certain gliadin peptides (e.g., P31-43) on the function of early endocytic vesicles and implicates EGFR signaling as an important pathway in celiac intestine. The so-called gliadin toxic peptide P31-43 enters early endocytic vesicles of CaCo-2 cells and intestinal enterocytes [11,14], delays endocytic vesicle maturation and consequently reduces epidermal growth factor receptor (EGFR) degradation and prolongs EGFR activation, which in turn results in actin modification and increased cellular proliferation in celiac crypt enterocytes. [11,14,16] In normal subjects, gliadin peptide P31-43 does not induce a significant increase of proliferation in crypt enterocytes, [11,16] although it is able to cause short-term effects on the endocytic compartment such as accumulation of EEA1 vesicles and protein, delay of EGF-Alexa 488 trafficking and ERK activation (manuscript in preparation). This shows that gliadin is an activator of various stress signals at the cellular level independent of the celiac background but that only in the celiac background it is able to produce long-term damage including overproliferation and stress/innate immune response activation.The reason the stress/innate immune and proliferative responses to certain gliadin peptides are so disruptive in celiac and not in control intestine is unknown. It has been shown that a stress/innate immune response to gliadin exists in family members of CD patients in the absence of anti-gluten T-cell-mediated immunity. [3] We looked for the presence in CD mucosa of a constitutive alteration that may represent a predisposing condition to the damaging effects of gliadin. In accord with this hypothesis, we show in this paper that the endocytic compartment is altered in CD cells and that this alteration implies an increase in signaling, with increases in the level of stress molecules such as pY-ERK. We know from previous work that the endocytic compartment is also the target of the P31-43 peptide. Thus, the same pathway that gliadin peptides (e.g., P31-43) can interfere with is constitutively altered in CD cells, potentially explaining the specificity of the damaging effects of certain gliadin peptides on CD mucosa.

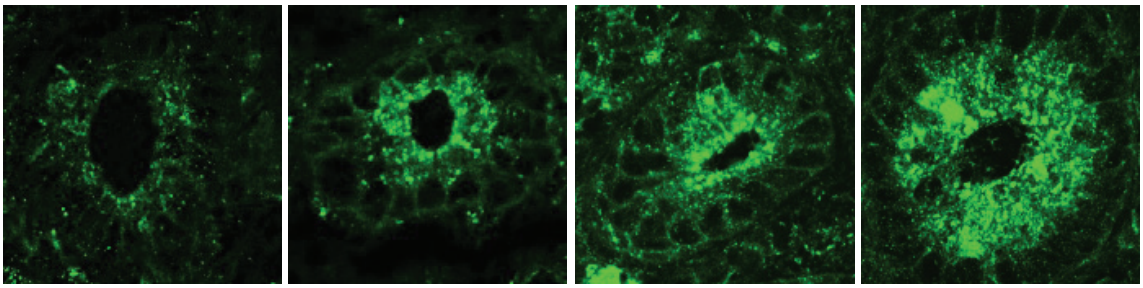
These data are currently submitted .

Figure 1A

Villi



Crypts



Control **Villus atrophy CD** **GFD CD** **Potential CD**

Figure 1B

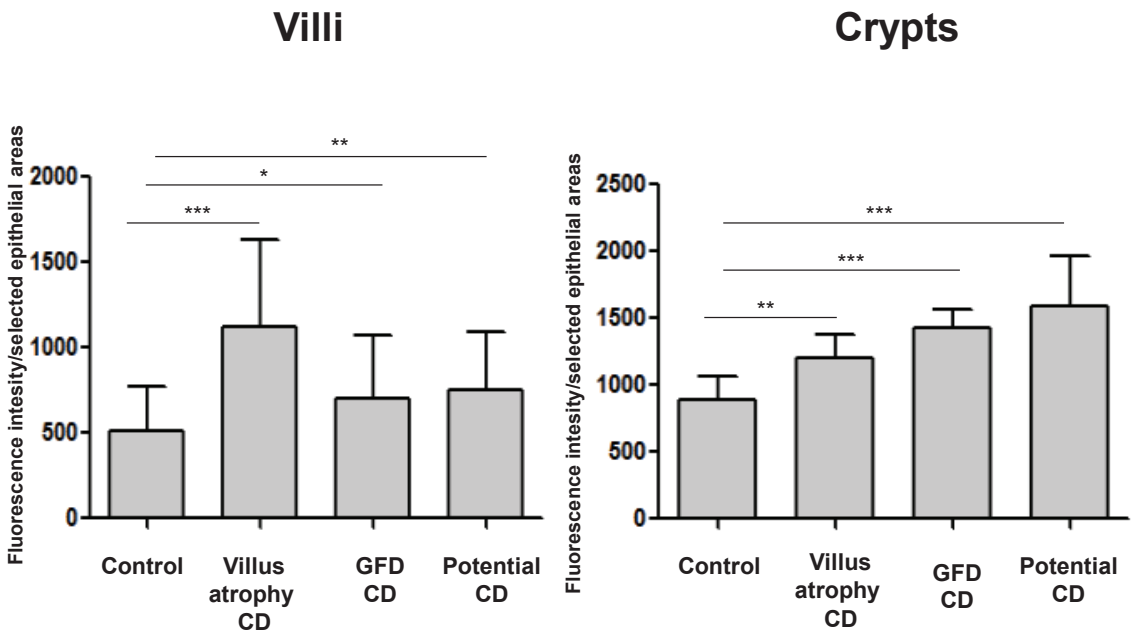


Figure 1C

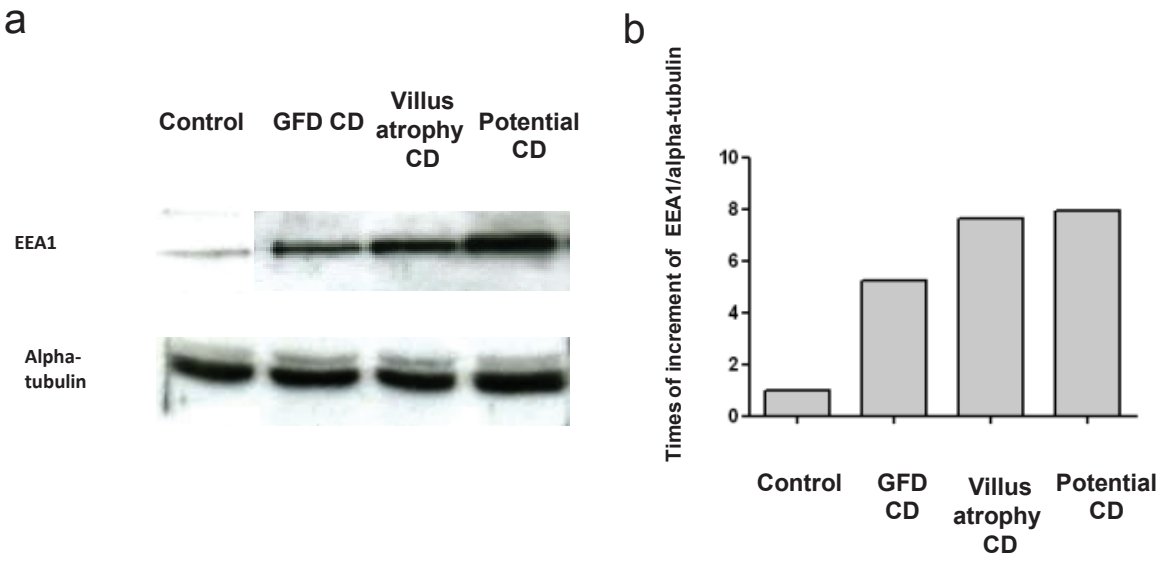


Figure 1. EEA1-positive vesicles are increased in CD enterocytes.

A. EEA1 staining of duodenal biopsies from controls, from CD patients with villus atrophy, from potential CD and from CD patients on GFD. 63x objective (2x digital zoom) images from villi and crypts are shown. The white lines indicate the height of the epithelium. Of 5 independent experiments, one representative experiment is shown.

B. Statistical analysis of fluorescence intensity in selected epithelial areas. For each group of patients and controls, 5 subjects were examined. For each subject, 3 independent experiments were performed to measure the fluorescence intensity of the selected epithelial areas. Columns represent mean values, and bars represent the standard deviation. * = $P<0.05$, ** $p< 0.001$, *** = $P<0.0001$

C (a) Western blot analysis of EEA1 levels in biopsies from CD patients and controls. Alpha-tubulin was used as a loading control. (b) Densitometric analysis of Western blots shown in (a). The fold increase in EEA1 (iEEA1) signal intensity in CD patients respect to the control for each sample was calculated as follows: iEEA1 = [CD patients (EEA1)/control (EEA1)]/[CD patients (alpha-tubulin)/control (alpha-tubulin)]. For each group, similar results were obtained in 5 subjects.

Figure 2

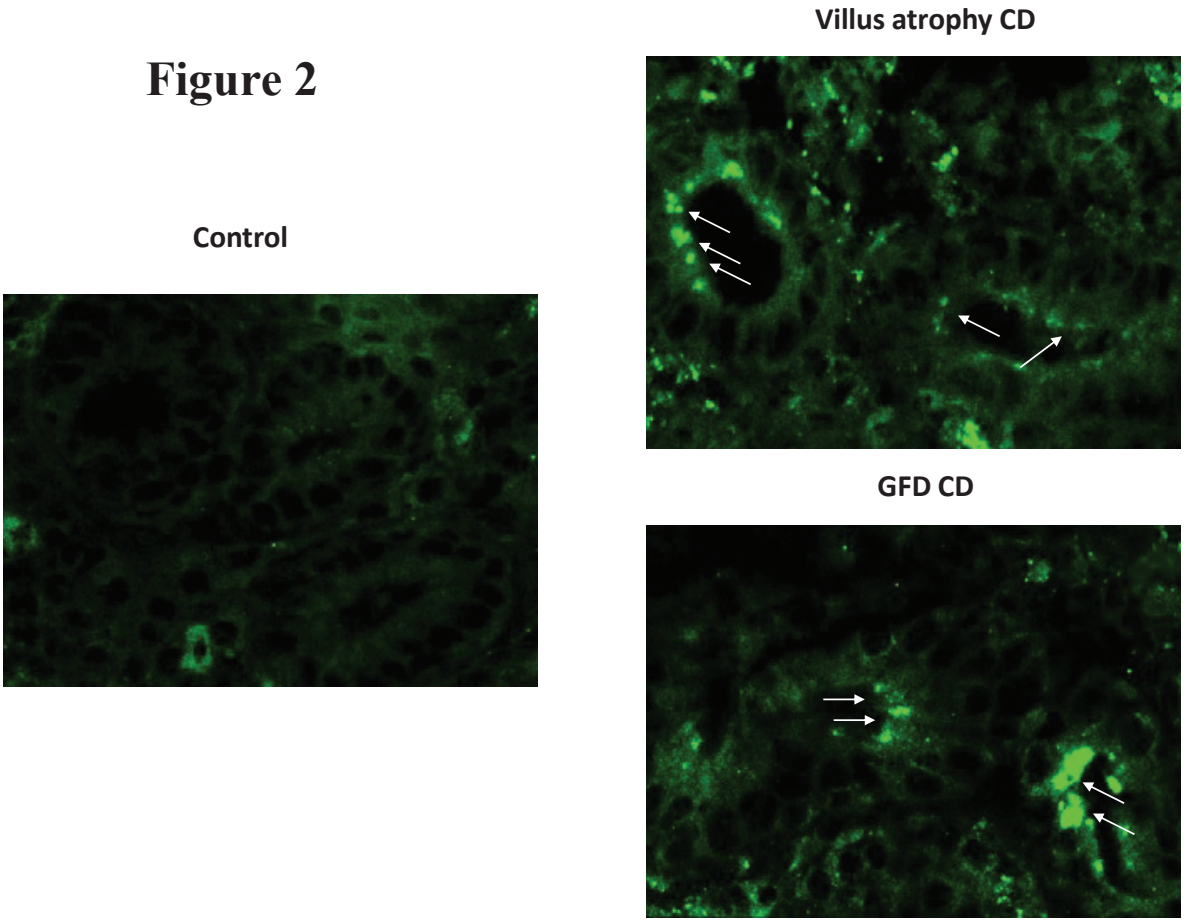


Figure 2. EGF-Alexa-488 is delayed in endocytic compartments of CD enterocytes in both the acute and remission phases of the disease.

Biopsies from CD patients with villus atrophy who were on a gluten-containing diet and from GFD CD patients were cultured for 24 h after a 3 h pulse with EGF-Alexa-488. Images of crypts obtained with a 63x objective (2x digital zoom). One representative experiment out of 5 independent ones are shown. White arrows indicate EGF-Alexa-488 accumulation in vesicles of the apical portions of the crypt enterocytes.

Figure 3A

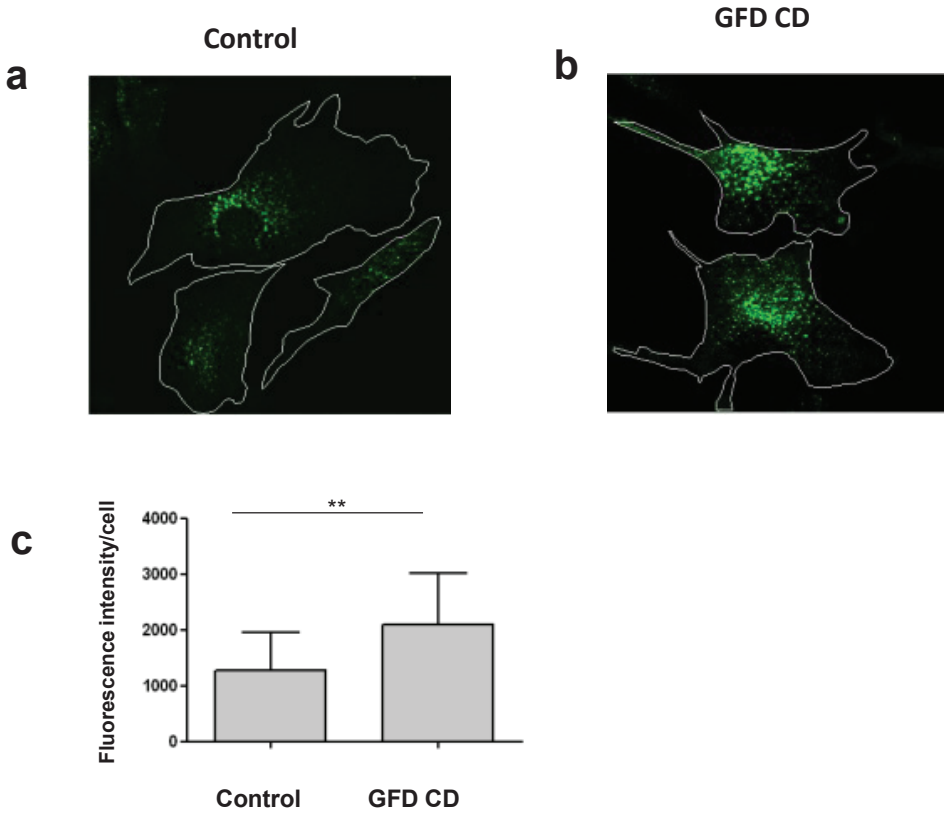

a
Control
b
GFD CD
c
Fluorescence intensity/cell
4000
3000
2000
1000
0
**
Control
GFD CD

Figura 3B

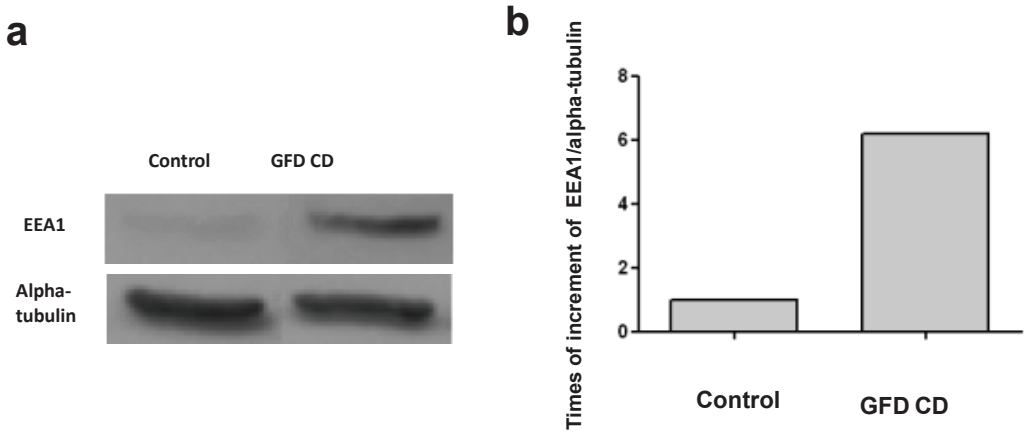


Figure 3C

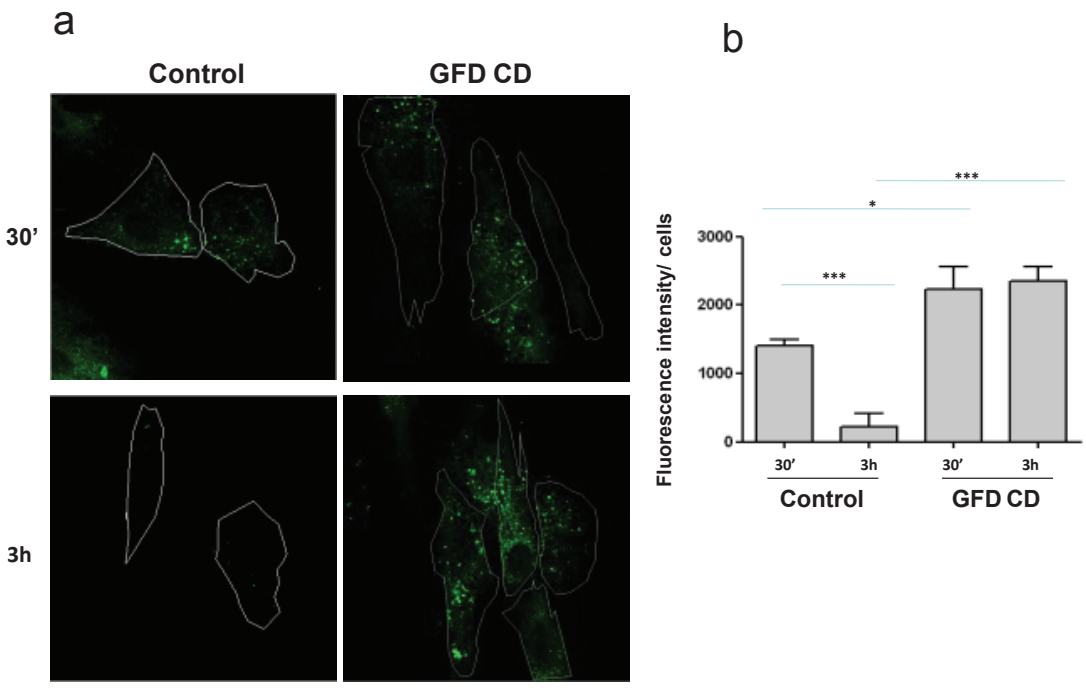


Figure 3. Morphological and functional alterations of the early endocytic compartment in skin fibroblasts from CD patients.

A. EEA1 staining of early endocytic vesicles is increased in skin-derived fibroblasts from CD patients on GFD in comparison to controls. (a, b) Immunofluorescence images of EEA1 staining (63x objective, 2x digital zoom). White lines indicate single cells in a representative field. (c) Statistical analysis of fluorescence intensity/cell. Three independent experiments were carried out for each of 5 patients and 4 controls; in each experiment, the fluorescence intensity of 10 cells in random fields was measured. Columns represent means; bars represent standard deviation. $**p< 0.001$ (Student t-test).

B. EEA1 protein is increased in fibroblasts of CD patients in comparison to controls. (a) Western blot analysis of EEA1 levels in fibroblasts from CD patients and controls. Alpha-tubulin was used as a loading control. (b) Densitometric analysis of Western blots shown in (a). The fold increase in EEA1 (iEEA1) in CD fibroblasts with respect to control in each sample was calculated as follows: iEEA1 = [Fibroblasts GFD CD (EEA1)/control (EEA1)]/ [Fibroblasts GFD CD (alpha-tubulin)/ control (alpha-tubulin)]. Similar results were obtained in 5 patients and 4 controls.

C. EGF-Alexa-488 is delayed in the endocytic compartment of CD skin fibroblasts.

(a) Skin fibroblasts from CD patients on GFD and controls were pulsed for 30 min with EGF-Alexa-488 and chased for 3 h with medium alone. The area showing the cells is highlighted by a white line. Representative fields obtained using a 63x objective, 2x digital zoom are shown. (b) Statistical analysis of the fluorescence intensity/cell at indicated time points. For each of 5 patients and 4 controls, 3 independent experiments were done; in each experiment, 10 cells in random fields were counted. Columns represent means and bars standard deviations. $* = P<0.05$, $*** = P<0.0001$

Figure 4A

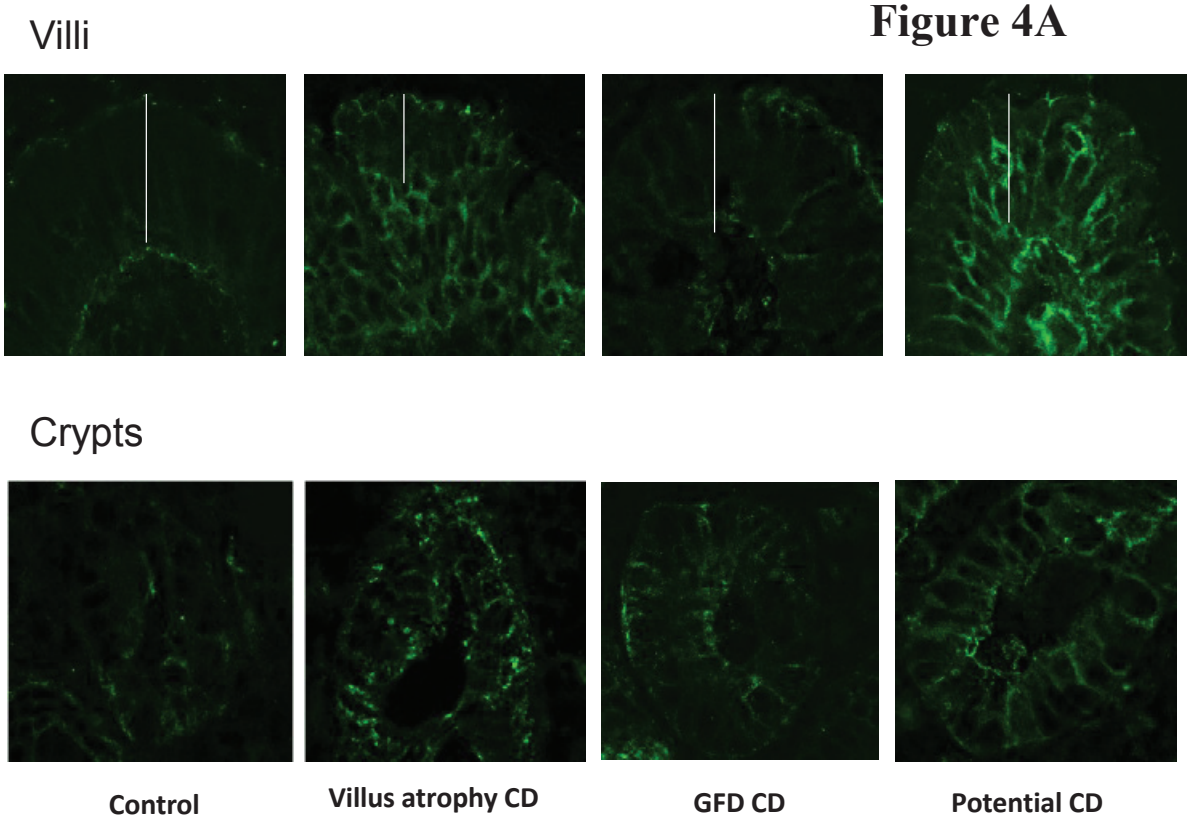


Figure 4B

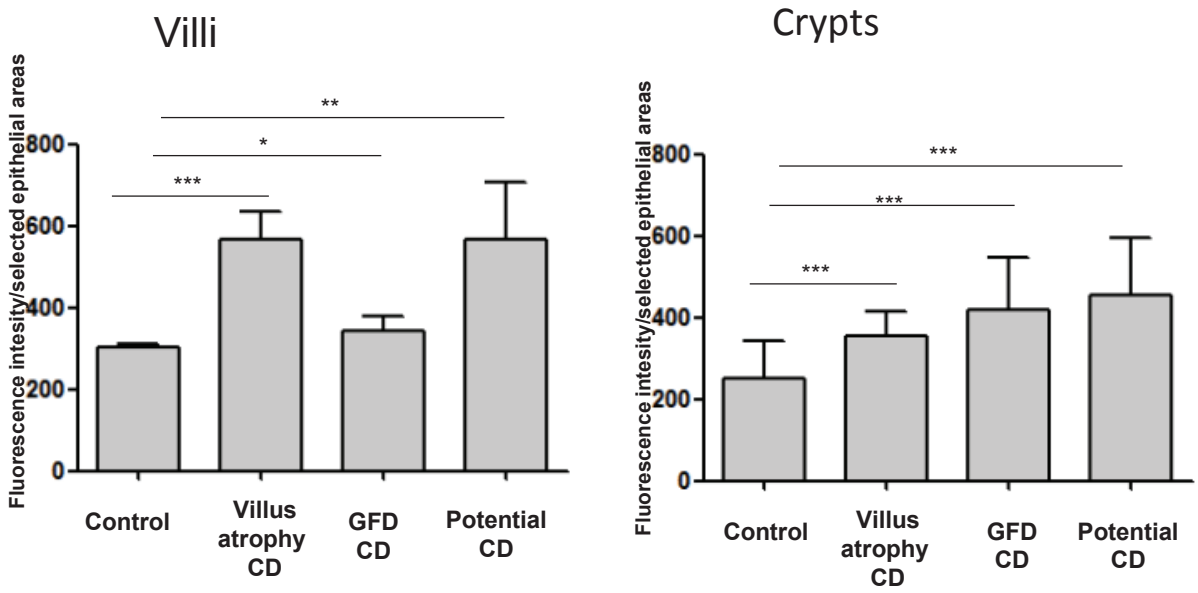


Figure 4C

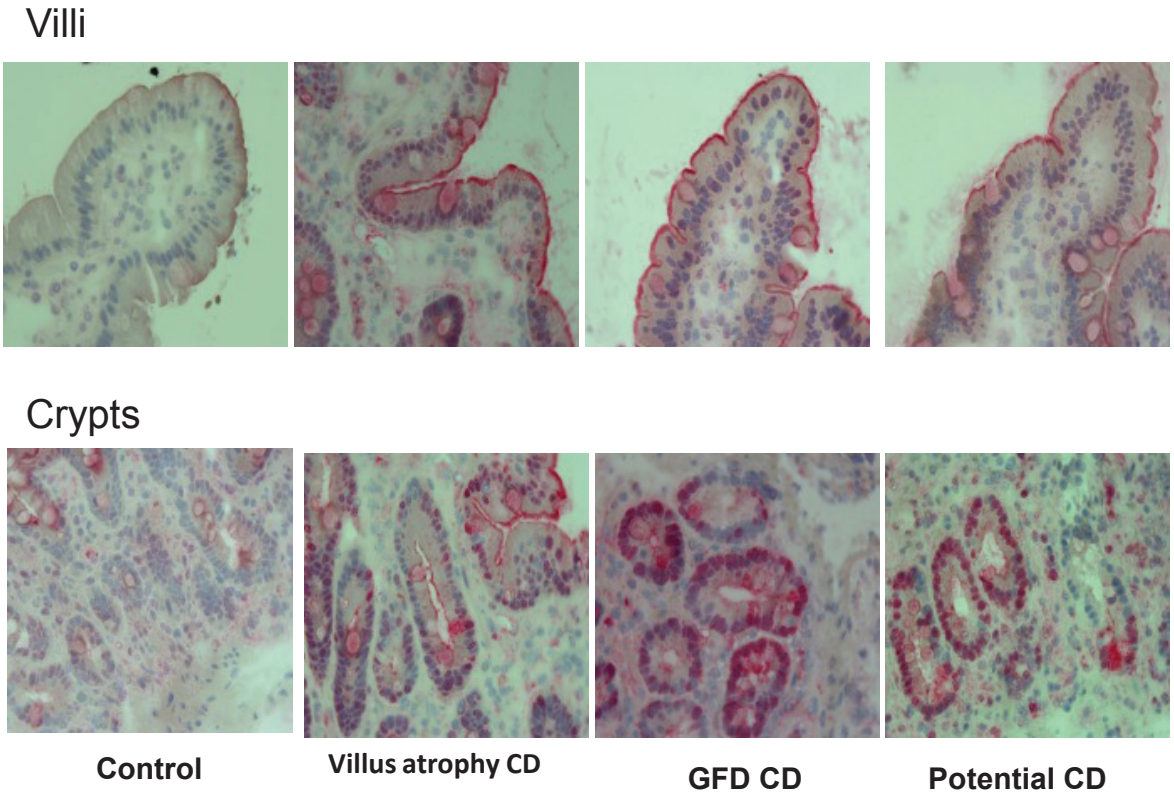


Figure 4D

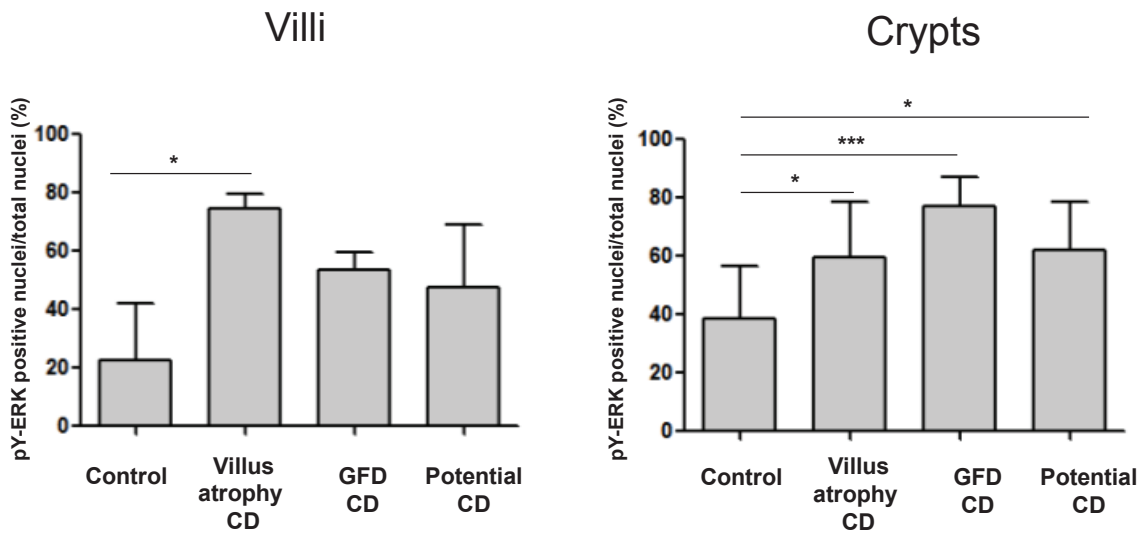


Figure 4E

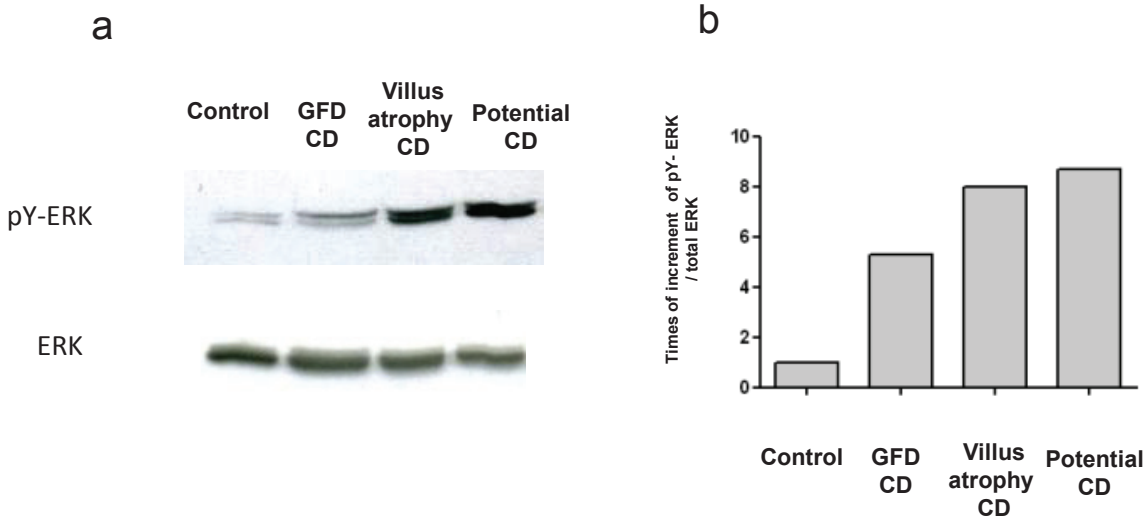


Figure 4. Phosphorylation of proteins is increased in enterocytes of CD patients.

A. Duodenal biopsies from CD patients with villus atrophy and potential CD patients, both on a gluten-containing diet and from GFD CD or from controls were stained with anti-phosphotyrosine antibody. 63x objective (2x digital zoom) images from villi and crypts obtained in one representative experiment out of 5 independent ones are shown. White lines indicate the height of the epithelium.

B. Statistical analysis of fluorescence intensity/selected epithelial area. For each group of patients and controls, 5 subjects were examined. For each subject, 5 independent experiments in which the fluorescence intensity of the selected epithelial areas was measured were performed. Columns represent means and bars standard deviation. * = $P<0.05$, ** = $P<0.001$, *** = $P<0.0001$

C. Immunohistochemical images of crypts and villi of intestinal biopsies from CD patients and controls stained with an antibody that recognizes the phosphorylated form of ERK 1/2 (pY-ERK) and with hematoxylin/eosin. One representative experiment out of 5 independent experiments is shown.

D. Statistical analysis of pY-ERK positive nuclei with respect to total nuclei in the enterocytes of the crypts and villi of 5 CD patients for each group and 5 controls. More than 300 pY-ERK- positive nuclei were counted in several fields in each sample on several slides. Columns represent means and bars standard deviation. * = $P<0.05$; *** = $P<0.0001$ (Student's t-test).

E. (a) Western blot analysis of biopsies from CD patients and controls stained with anti-pY-ERK and anti-ERK antibodies. (b) Densitometric analysis of Western blots shown in (a). The fold increase in pY-ERK (i pY-ERK) in CD patients with respect to controls was calculated as follows: ipY-ERK = [CD patients (pY-ERK)/control (pY-ERK)]/ [CD patients (total ERK)/control (total ERK)]. Similar results were obtained in 5 subjects in each group.

Figure 5A

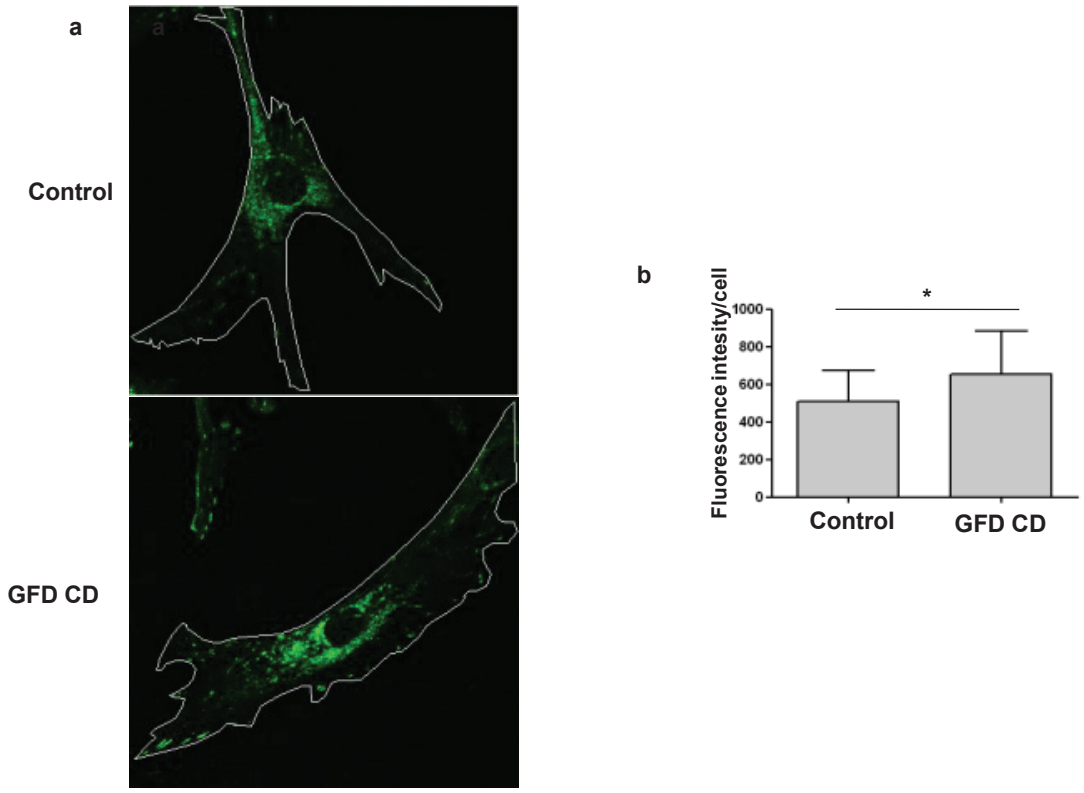


Figure 5 B

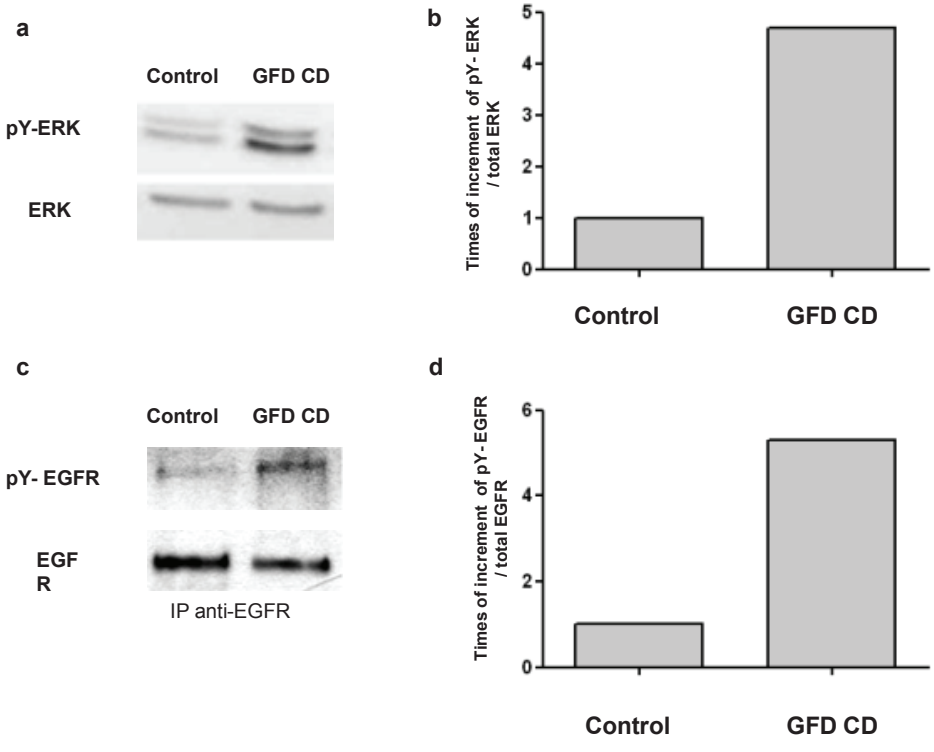


Figure 5. Phosphorylation of proteins is increased in skin fibroblasts of CD patients.

A. Staining of total phosphorylated proteins in CD fibroblasts. a. Immunofluorescence of total phosphorylated proteins in single cells. Images obtained using a 63x objective (2x digital zoom) are shown; white lines indicate single cells in a representative field. b. Statistical analysis of fluorescence intensity/cell. For 5 patients and 4 controls, 3 independent experiments were done; in each experiment, the fluorescence intensity of 10 cells in random fields was measured. Columns represent means and bars standard deviation. * = $P<0.05$ (Student's t-test).

B. Western blot analysis of phosphorylated ERK and EGFR in skin fibroblasts from CD patients on a GFD and from controls.

(a) Western blot analysis of skin fibroblasts from CD patients and controls stained with anti-pY-ERK and anti-ERK antibodies.

(b) Densitometric analysis of Western blots shown in (a). The fold increase in pY-ERK (ipY-ERK) with respect to total ERK in GFD CD fibroblasts was calculated as follows: ipY-ERK = [Fibroblasts GFD CD (pY-ERK)/control (pY-ERK)]/ [Fibroblasts GFD CD (total ERK)/control (total ERK)]. Similar results were obtained in 5 CD patients and 4 controls.

(c) Western blot analysis of EGFR immunoprecipitated from skin fibroblasts and stained with anti-pY antibody. (d) Densitometric analysis of Western blots shown in (c). The fold increase of pY-EGFR (i pY-EGFR) with respect to total EGFR in GFD CD fibroblasts was calculated as follows: ipY-EGFR = [Fibroblasts GFD CD (pY-EGFR)/control (pY-EGFR)]/[Fibroblasts GFD CD (EGFR)/control (EGFR)]. Similar results were obtained in 5 CD patients and 4 controls.

Figure 6A

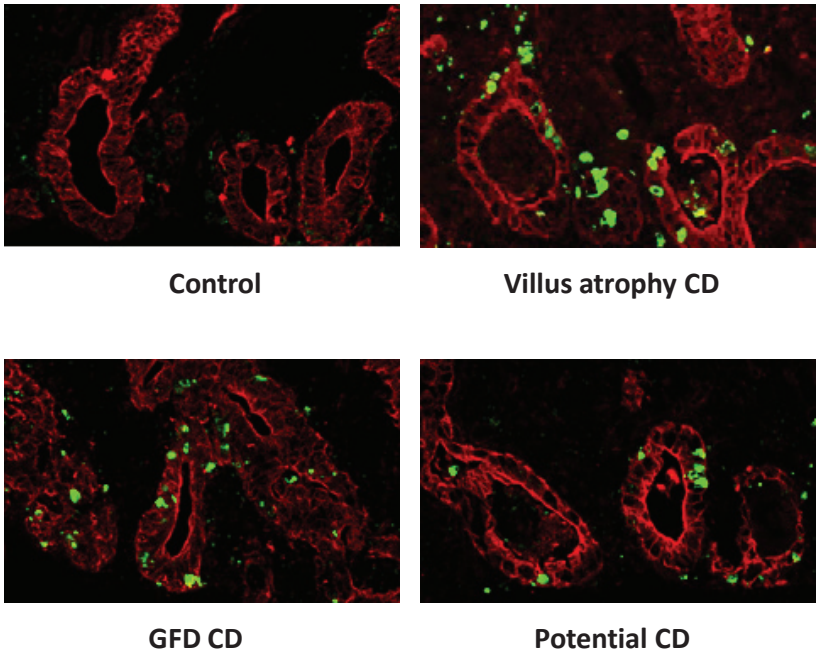


Figure 6B

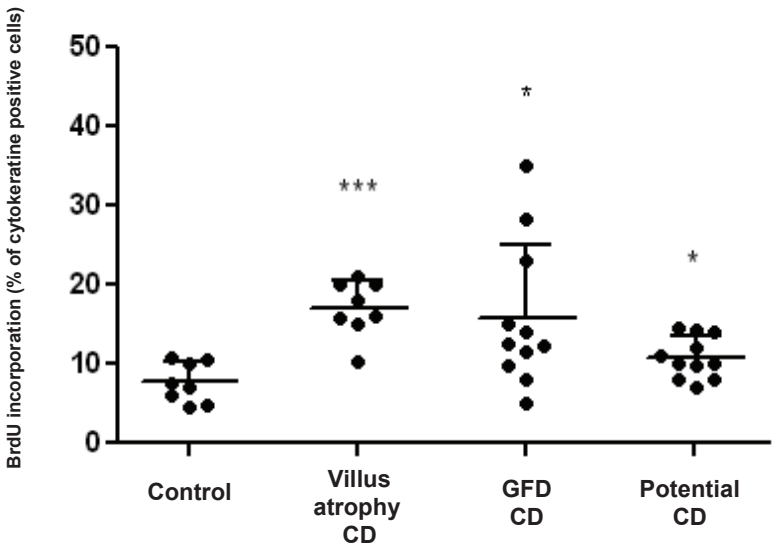
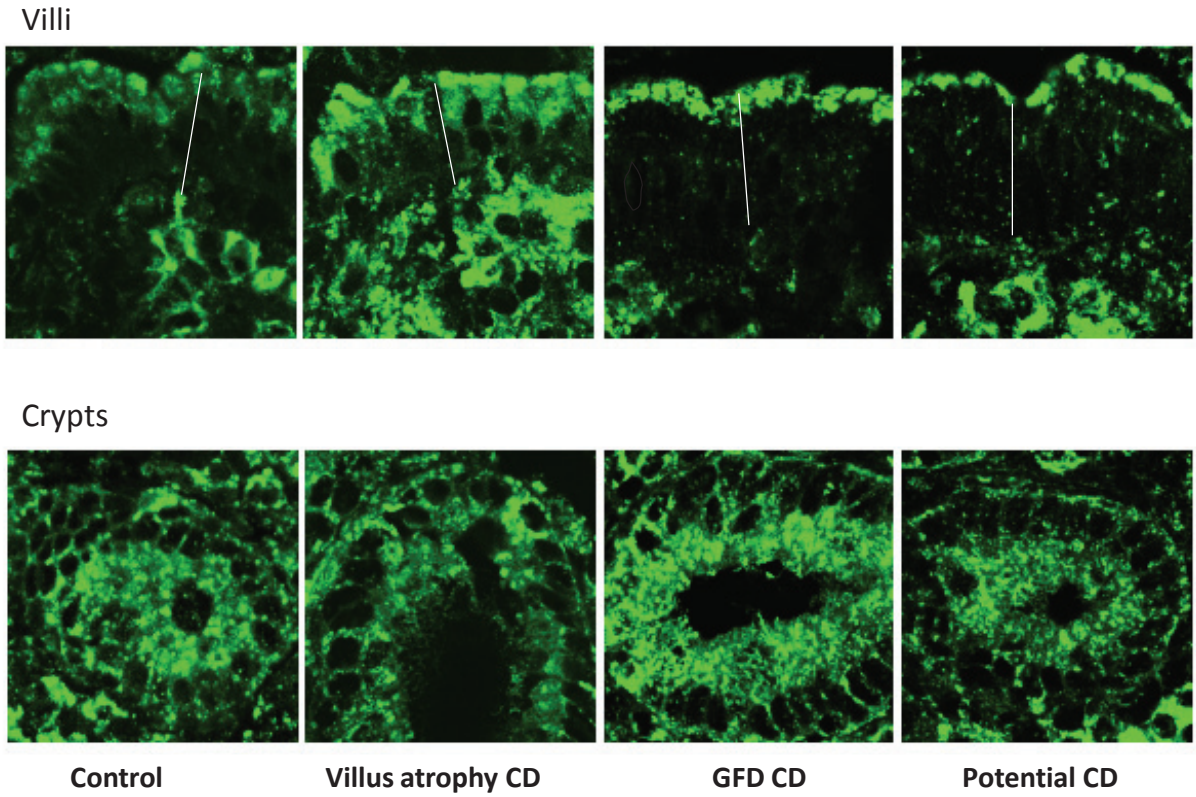


Figure 6.: Proliferation of crypt enterocytes is increased in CD.

A. Immunofluorescence images of duodenal biopsies from a control, from a CD patient with villus atrophy, from a potential CD patient who were on a gluten-containing diet and from a GFD CD patient. Biopsies were cultured for 24 h with BrdU and then stained for cytokeratin to identify epithelial cells (red) and for BrdU (green) to identify proliferating cells. One representative experiment is shown. B. Quantitation of BrdU incorporation by intestinal biopsies. More than 300 cytokeratin-positive cells were counted in several fields in each sample; the number of BrdU- positive cells was expressed as a proportion of the total cytokeratin-positive cells. Bars represent mean and standard deviation; each dot represents a single CD patient or control. * = $P<0.05$; * * * = $P<0.0001$ (Student's t-test).

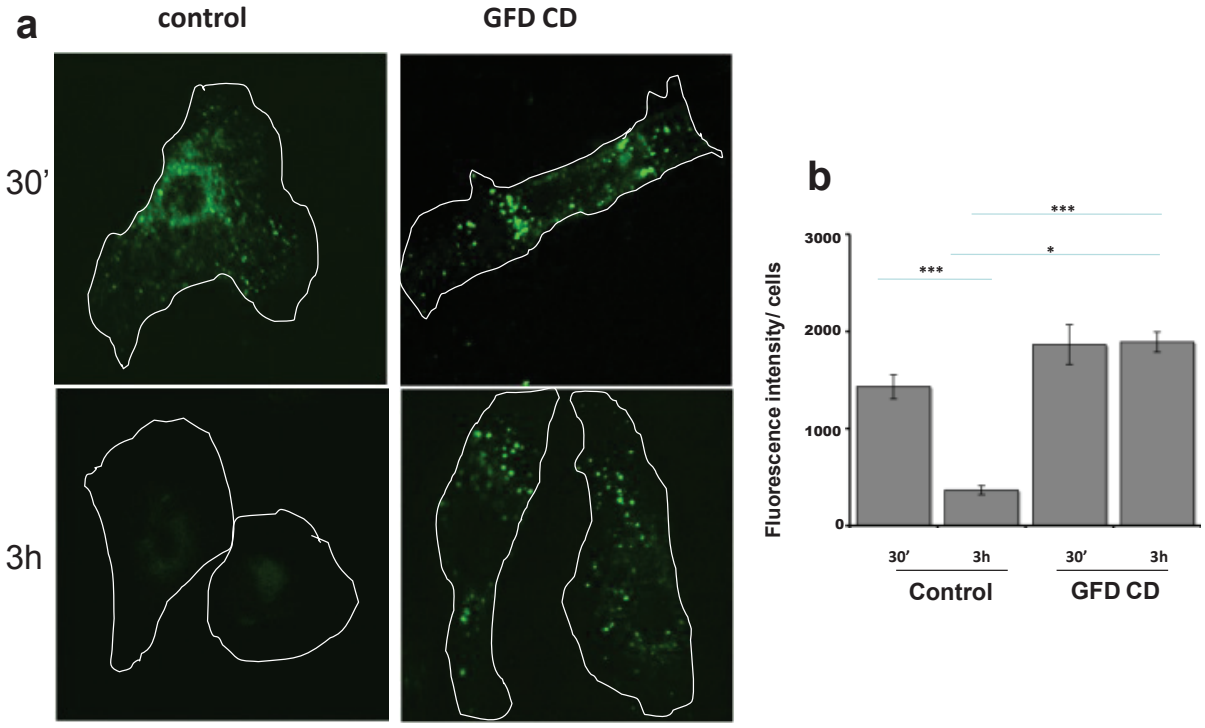
Supplemetary Figure 1



Supplementary Figure 1. LAMP2-positive vesicles are increased in surface enterocytes of mucosa from CD patients with villus atrophy.

Lamp2 staining of duodenal biopsies from controls, from CD patients in the active phase of the disease on a gluten-containing diet with villus atrophy and crypt hyperplasia (CD with villus atrophy), from patients with normal mucosa (potential CD) and from CD patients on a gluten-free diet with normal mucosa (GFD CD). 63x objective (2x digital zoom) images from villi and crypts are shown. White lines indicate the height of the epithelium. One representative experiment out of 5 independent ones is shown.

Supplementary Figure 2



Supplementary Figure 2. EGF-Alexa 488 is delayed in the endocytic compartment of CD dendritic cells. a) Dendritic cells from CD patients on GFD and from controls were seeded on fibronectin and pulsed for 30 min with EGF-Alexa 488, then chased for 3 h with medium alone. After fixation with 3% paraformaldehyde, they were mounted with Mowiol and observed by confocal microscopy (LSM Zeiss 510). The area of the cells is highlighted by a white line. Representative fields obtained using a 63x objective (2x digital zoom) are shown. Seven patients and seven controls were tested with similar results.

(b) Statistical analysis of fluorescence intensity/cell at indicated time points. For all patients and controls, 3 independent experiments were done; in each experiment, 10 cells in random fields were counted. Columns represent means and bars standard deviation. * = $P<0.05$, * * *= $P<0.0001$.

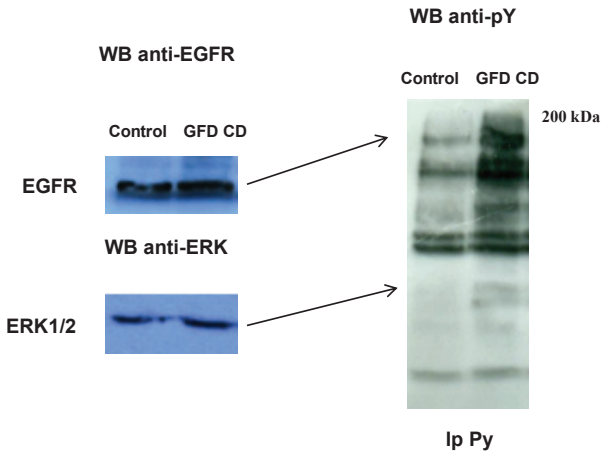
Method for dendritic cells differentiation:

Peripheral blood mononuclear cells were isolated from heparinized peripheral blood by density gradient centrifugation on lymphocyte separation medium (MP Biomedicals, LLC, Ohio). After 1 hour of incubation at 37˚C, the nonadherent cells were removed with a gentle rinse and discarded.

The adherent monocytes were used to generate dendritic cells (DCs) cells. Briefly, 5x105 monocytes/

ml were cultured in 24-well plates with RPMI 1640/10% FCS (Cambrex, Charles City, IA, USA) for 7 days; recombinant human IL-4 and recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (Invitrogen, San Giuliano Milanese, Italy) were added to final concentrations of 1000 U/ml and 800 U/ml, respectively, on days 0 and 4.

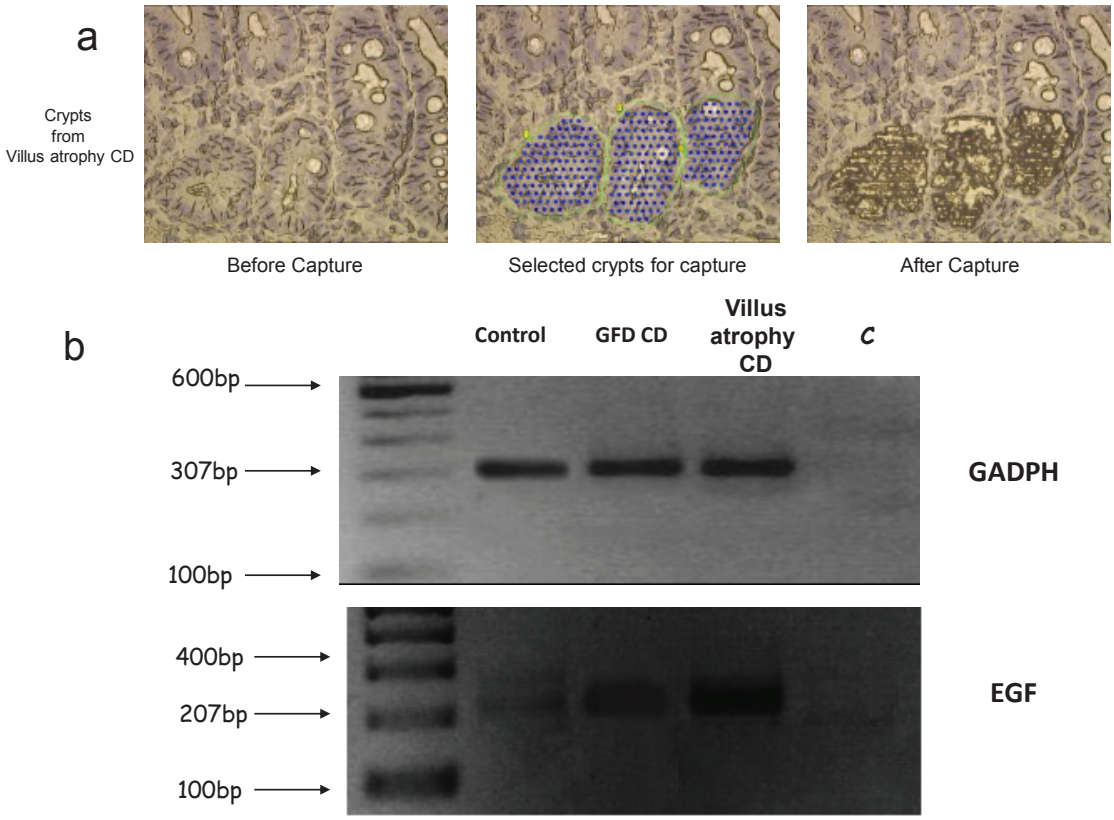
Supplementary Figure 3



Supplementary Figure 3. Western blot analysis of total phosphorylated proteins.

Skin fibroblasts from CD patients on GFD and from controls were lysed, and phosphoproteins in the lysates were immunoprecipitated, blotted and stained with anti-phosphotyrosine antibodies. The blots were stained again with anti-EGFR and anti-ERK antibodies to identify the corresponding phosphorylated proteins. One representative experiment of 3 independent ones is shown for each subject (4 controls and 5 patients).

Supplementary Figure 4



Supplementary Figure 4. EGF mRNA levels are increased in intestinal mucosa of CD patients with villus atrophy who are on gluten-containing diet and in GFD CD patients.

a) Example of selected crypt enterocytes from 5-micron sections of intestinal biopsies frozen and air dried before capture. For each sample, 300 crypt epithelial cells were captured.

b) Semiquantitative PCR analysis of a biopsy from a CD patient and a biopsy from a control. A representative experiment is shown. Similar results were obtained in 3 CD patients on gluten- containing diet (GCD), 3 CD patients on gluten-free diet (GFD) and 3 controls with gastro-esophageal reflux.

Methods:

The laser capture microdissection (LCM) method allows the selection of individual or clustered cells from intact tissues. Total RNA was extracted from 300 captured crypt epithelial cells from biopsies from 3 CD patients on gluten-containing diet (GCD), 3 CD patients on gluten-free diets (GFD) and 3 controls with gastroesophageal reflux. For each sample, cDNAs were transcribed using AmpliTaq Gold (Applied Biosystems, Foster City, CA). Semiquantitative PCR was carried out using oligonucleotide primers that reco-

gnize the EGF sequence: EGF, 5′-GCCAACAAACACACTGGAAA-3′ (forward) and 5′-CATGCACA-AGTGTGACTGGA-3′ (reverse). The GAPDH gene was used as an example of a housekeeping gene, with the following primers: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′(forward) and 5′-AGCCTTCTC-CATGGTGGTGAAGAC -3′ (reverse). The PCR conditions were as follows: 1 cycle of 95 °C for 10 minutes, 40 cycles of 95 °C for 1 min, 60 °C for 1 minute, and 72 °C for 1 minute followed by 1 cycle of 72 °C for 4 minutes.

SUPPLENTARY MATERIAL

Cell culture

The skin explants were immediately placed in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, San Giuliano Milanese, Italy), 20% fetal bovine serum (FBS) (GIBCO, San Giuliano Milanese, Italy), 100 units/ml penicillin-streptomycin (GIBCO, San Giuliano Milanese, Italy), and 1 mM glutamine (GIBCO, San Giuliano Milanese, Italy) and incubated for 24 hours. Subsequently, each skin explant was divided into about 50 small fragments; these fragments were plated on Petri dishes and incubated in the presence of 95% oxygen and 5% CO2 at a temperature of 37° C to allow adhesion and subsequent release of fibroblasts. Seven-ten days later, fibroblasts began to emerge from the fragments. When fibroblasts had reached confluence, they were harvested with trypsin and frozen. In all experiments, the fibroblasts were used between the 2nd and the 4th passage.

CaCo-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, San Giuliano Milanese, Italy), with the same additions as described above except that 10% instead of 20% fetal calf serum was used.

Pulse-chase experiments on fibroblasts

Fibroblasts were seeded on glass coverslips and pulsed for 30 minutes at 37° C with 20 nanograms/ml of EGF-Alexa-488 (Molecular Probes, San Giuliano Milanese, Italy), then washed several times and incubated for 3 h with unlabeled EGF at 37°C (chase). The coverslips were then mounted on glass slides and observed using a confocal microscope (LSM 510 Zeiss).

Pulse-chase experiments on biopsies

Briefly, 5 intestinal biopsies from CD patients with villus atrohy, from patients on a GFD and from control subjects affected by gastroesophageal reflux were cultured for 3 hours with Alexa-488 fluorochrome-labeled EGF (pulse). After careful washing to eliminate the EGF-Alexa-488, all samples were chased for 24 h and prepared for cryo-sectioning; air-dried 5-µm sections were analyzed using a confocal microscope (LSM 510 Zeiss).

EEA1 and phosphotyrosine staining

Acetone-fixed (10 min, Sigma-Aldrich, Milan, Italy) 5-µm frozen sections from CD biopsies and controls were examined by immunofluorescence. After a 15-min pre-incubation with normal goat serum (1:100; Dako Milan, Italy), the sections were incubated with a polyclonal IgG goat antibody against early endocytic antigen 1 (EEA1) (1:100; Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature and with mouse monoclonal antibody against anti-pTyr (1:300; Santa Cruz Biotechnology, CA, USA) overnight in a humidified chamber. The sections were then washed with PBS (phosphate buffer solution) containing BSA (bovine serum albumin, Sigma Aldrich, Milan, Italy) for 10 min and incubated with a secondary antibody, donkey anti-mouse Alexa 488 (1:100), for 30 min in a dark humid chamber. Finally, the sections were washed in PBS and mounted with glycerol/PBS (1:10). The preparations were analyzed by confocal microscopy (LSM510; Zeiss).

Fibroblasts seeded on glass coverslips were fixed with 3% paraformaldehyde (Sigma Chemical Co., Milan, Italy) for 5 min at room temperature, permeabilized with 0.2% Triton (Biorad, Milan, Italy) for 3 min at room temperature and stained14 for 1 h at room temperature with goat polyclonal antibody against EEA1 (C-15) (Santa Cruz, DBA, Milan, Italy) or with mouse monoclonal antibody against LAMP2 (H4B4) (Santa Cruz, DBA, Milan, Italy) or with anti-pTyr antibody (Santa Cruz Biotechnology, CA, USA) all of them at 2 µg /ml. Alexa-488-conjugated secondary antibodies (Invitrogen, San Giuliano Milanese, Italy) at a dilution of 1:100 were added to the coverslips for 1 h at room temperature. The coverslips were then mounted on glass slides and observed by confocal microscopy (LSM 510 Zeiss). A total of 40 to 50 cells were observed in each sample, and all images were generated with the same confocal microscope. Fluorescence intensity (Fi) analysis of the samples respect to the background was carried out using AIS Zeiss software. Magnification of the micrographs is the same for all figures shown (63x objective) unless stated differently in the legends.

Immunohistochemistry of pY-ERK in biopsies

For the immunohistochemical study, 4-µm biopsy sections were fixed in 3% paraformaldehyde (Sigma-Aldrich, Milan, Italy) for 10 min. After incubation with normal rabbit serum (1:200, Dako, Copenha-

gen, Denmark) for 20 min, sections were covered with pY-ERK polyclonal rabbit antibodies (1:80, Cell Signaling, Euroclone Milan, Italy) overnight. All incubations were carried out at room temperature in a humid chamber. As a negative control, some sections were not treated with the primary antibody but with buffer solution instead. After washing with TBS (Tris- buffered solution, 0.15 M, pH 7.36, Sigma-Aldrich, Milan, Italy) + saponin (0.1%, Carlo Erba, Milan, Italy), the sections were incubated for 30 min with biotinylated goat anti-rabbit antibody (1:300; Dako, Milan, Italy) and then with streptavidin AP (1:400; Dako, Milan, Italy) for 30 min. New fuchsin was used as the peroxidase substrate. Finally, sections were counterstained with Mayer's hematoxylin (Sigma Diagnostic, St Louis, USA) and mounted with Aquamount (BDH, Poole, England). The preparations were analyzed using transmitted light microscopy (Nikon Eclipse 80, Nikon instruments, USA)

Immunoprecipitation

Cells lysates were prepared as described previously, [11,16] and protein concentration was measured using a Bio-Rad protein assay kit (Hercules, CA, USA). Equal amounts of cell lysates (2 mg protein/ml) were used for immunoprecipitation. EGFR was immunoprecipitated using anti-EGFR (Cell Signalling, Euroclone Milan, Italy). Proteins were immunoblotted with specific antibodies as described below.

Western blotting

Briefly, fibroblast cells cultured in DMEM containing 20% FBS at 37° C were washed twice with PBS and resuspended in lysis buffer. Cell lysates were analyzed by SDS-PAGE and transferred to nitrocellulose membranes (Whatman Gmbh, Dassel, Germany). The membranes were blocked with 5% nonfat dry milk and probed with anti p-Tyr(P99), anti pY-ERK, anti ERK, anti EEA1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti tubulin (Sigma-Aldrich, Milan, Italy), and anti-EGFR (Cell Signaling EuroClone Celbio, Milan, Italy). Bands were visualized using the ECL system (GE Healthcare, Amersham, Buckinghamshire, UK). Band intensity was evaluated by integrating all the pixels of the immunostained band without the background, which was calculated as the average of the pixels surrounding the band. [11,16]

Biopsy fragments (5 mg wet weight each) from duodenum obtained from 5 CD with villus atrophy, 5 controls (affected by gastroesophageal reflux), 5 patients in remission and 5 potential CD patients were homogenized in 100 µL homogenization buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgCl2, 1% TritonX100, and protease inhibitors) using a 2-mL conical Wheaton glass tube with a Teflon pestle.

Organ culture studies

For organ culture studies, biopsy fragments from duodenum were obtained from 8 CD patients with villus atrophy, 8 controls (affected by gastroesophageal reflux), 11 CD patients on GFD and 11 potential CD patients. Informed written permission was obtained from all patients. The biopsy fragments were cultivated as reported elsewhere. [11,17] The intestinal samples were cultured for 24 h with medium alone. All cultures were enriched with 10 µM BrdU (Sigma-Aldrich, Milan, Italy). Specimens were harvested, snap-frozen in liquid nitrogen, embedded in OCT and stored at –80 °C until required.

We used double immunofluorescence to evaluate crypt proliferation in 5-µm cryostat sections from cultured biopsies.[11,17] After a short (3 min) treatment with 1.5 N HCl, the sections were incubated with mouse monoclonal anti-BrdU (1:150, GE Healthcare Amersham, Buckinghamshire, UK) for 1 h followed by 30 min incubation with Alexa488-conjugated anti-mouse IgG (1:150, Invitrogen, San Giuliano Milanese, Italy) to identify BrdU-positive cells. After several washes in phosphate buffer solution, specimens were fixed with 3% paraformaldehyde (Sigma-Aldrich, Milan, Italy) for 5 min and incubated for 1 h with polyclonal rabbit anti-cow cytokeratin (1:50, Dako, Milan, Italy) to stain epithelial cells. Slides were then covered for 30 min with Alexa-633-labeled goat anti-rabbit immunoglobulin (1:200, Invitrogen, San Giuliano Milanese, Italy), contrasted with Hoechst (Sigma-Aldrich, Milan, Italy) and mounted in Mowiol 4-88. All incubations were carried out at room temperature in a dark humid chamber. The number of BrdU-positive cells divided by the total number of cytokeratin-positive cells gave the percentage of BrdU-positive cells.

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SUMMARY

Celiac Disease is an interesting model of a disease induced by food. It consists in an immunogenic reaction to wheat gluten and glutenins that has been found to arise in a specific genetic background; however, this reaction is still only partially understood. Damage to the intestinal mucosa in celiac disease (CD) is mediated both by inflammation due to the adaptive and innate immune responses, with IL-15 as a major mediator of the innate immune response, and by proliferation of crypt enterocytes as an early alteration of CD mucosa causing crypts hyperplasia. Activation of innate immunity by gliadin peptides is an important component of the early events of the disease. In particular the so-called "toxic" A-gliadin peptide P31-43 induces several pleiotropic effects including Epidermal Growth Factor Receptor (EGFR)-dependent actin remodelling and proliferation in cultured cell lines and in enterocytes from CD patients. These effects are mediated by delayed EGFR degradation and prolonged EGFR activation in endocytic vesicles.

Many biological activities have been associated with gliadin peptides in several cell types including reorganisation of actin and increased permeability in the intestinal epithelium. It has yet to be established to what extent these properties relate to the ability of these A-gliadin peptides to activate innate immunity mechanisms. Virtually nothing is known about the mechanisms underlying the biological properties of P31-43 or about the metabolic pathways involved in the activation of innate immunity in CD. Similarly, it is not known why celiac patients are particularly sensitive to these biological activities.

During the three years of my PhD program I have contribuited in same measure to clarify the "Relationship between proliferative effects and activation of innate immunity induced by gliadin", through the combination of cellular, functional and molecular approaches.

In particular, in the first chapter of my thesis it has been shown that both P31-43 and P57-68 enter CaCo 2 cells and interact with endocytic compartment, but only P31-43 interferes with the endocytic pathway by delaying maturation of early endosomes to late endosomes. We also show that the P31-43 sequence is similar to hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a key protein necessary for endocytic maturation. P31-43 is localised at the vesicles membranes and interferes with the correct localisation of Hrs to endocytic vesicles thus delaying the maturation of early endosomes to late endosomes.

Consequently the activation of EGFR and other receptors is expanded with multiple effects on various metabolic pathways and cellular functions.

Moreover, during my PhD I have tried to better characterize the role of P31-43 in the induction of cellular proliferation and innate immune activation on celiac enterocytes and cells. In particular, in the second chapter of my thesis has been shown that P31-43 induces at least two main effects by interfering with the trafficking of cell vesicular compartments. This leads to overexpression of the trans-presented IL-15/ IL5R alpha complex, an activator of innate immunity, and, due to cooperation of IL-15 and EGFR, the proliferation of crypt enterocytes with consequent remodelling of the CD mucosa.

These observations are relevant to our understanding of the early events occurring in the celiac mucosa exposed to gliadin because the increase of IL-15 and IL-15R alpha is a major event in the initial phases of CD. Our observation that in the celiac intestine IL-15 plays a major role in the gliadin-induced proliferation of epithelial cells, one of the hallmarks of CD, increasing our understanding of the pathogenesis of CD. Why the celiac mucosa seems to be particularly sensitive to the effects of some gliadin peptides, such as peptide P31-43, remains to be elucidated. Preliminary data suggest that in CD cells, the endocytic compartment is morphologically and functionally altered. We hypothesize that in CD mucosa, an alteration of the vesicular compartment renders the tissue more sensitive to the effects of gliadin.

Endocytosis has many effects on signalling: in fact, signalling pathways and endocytic pathways are regulated in a reciprocal manner. It is now widely accepted that the "Endocytic Matrix" is a master organiser of signalling, governing the resolution of signals in space and time. Consequently endocytosis affects several cell functions that range from proliferation to cell motility (Scita, Di Fiore 2010). Growing evidences point to an effect of certain gliadin peptides (i.e. P31-43) on the endocytic compartment.

In conclusion, we can say that by interfering with Hrs localisation to the endocytic membranes, P31-43 induces two important effects:

a) it delays endocytic maturation, as observed by the data produced in the first year of my PhD

b) it alters the recycling pathway, as observed by the data produced in the second year of my PhD

By delaying the maturation of endocytic vesicles P31-43 reduces EGFR and other RTK degradation and prolongs their activation. The biological consequeces of the delay of the vesicles maturation are increased proliferation, actin modification and other biological effects. In addition, the alteration of the recycling

pathway is able to direct more transferrin receptor and likely other recycling receptors such as IL15 to the membranes, this leads to an increase of proliferation of epithelial cells and activation of the innate immunity.

During my PhD I also paid a particular attention to investigate the presence and the suppressive function of Treg cells in the celiac small intestinal mucosa, their correlation with the state of the disease and the inducibility by gliadin stimulation in an organ culture system; moreover, we tried to define whether interleukin 15, that is overexpressed in CD, could influence the regulatory activity of such cells.

In Celiac Disease, beside the Th1 response, it has been highlighted the fundamental role of other pro-inflammatory cytokines, such as IL15. More recently also other cytokines, such as IL21, bridging innate and adaptive immunity, have been found to play an important role. In these studies an important contribution to the comprehension of the mechanisms leading to the disease has come from in vitro studies based on ex vivo organ cultures of intestinal biopsies taken from CD patients on a gluten-free diet (GFD).

In particular in this study we observed a higher density of CD4+CD25+Foxp3+ T cells (Treg) in duodenal biopsies from active CD patients in comparison to treated CD and controls. In co-culture CD4+CD25+ T cells were functionally suppressive, but their activity was impaired by IL-15. Furthermore, we demonstrated an expansion of Foxp3 in treated CD mucosa following in vitro challenge with gliadin.

These data suggest that CD4+CD25+Foxp3+ T cells are induced in situ by gliadin. However, their suppressor capacity might be impaired in vivo by IL-15, this phenomenon contributing to maintain and expand the local inflammatory response in CD.

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APPENDIX

List of publication

I. Maria Vittoria Barone, Merlin Nanayakkara, Giovanni Paolella, Maria Antonia Maglio, Virginia Vitale, Raffaele Troiano, Maria Teresa Silvia Ribecco, Giuliana Lania, Delia Zanzi, Sara Santagata, Renata Auricchio, Riccardo Troncone, Salvatore Auricchio :"Gliadin peptide P31-43 localizes to endocytic vesicles and interferes with their maturation". (PLoS ONE, August 2010)

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