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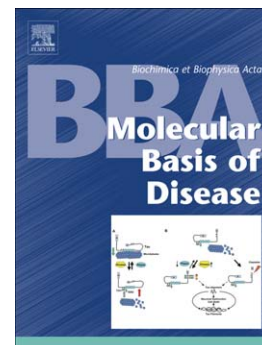
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Celiac anti-tissue transglutaminase antibodies interfere with the uptake of alpha gliadin peptide 31-43 but not of peptide 57-68 by epithelial cells

Running title: tTG and uptake of gliadin peptides

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Abstract

Celiac disease is characterized by the secretion of IgA-class autoantibodies that target tissue transglutaminase (tTG). It is now recognized that anti-tTG antibodies are functional and not mere bystanders in the pathogenesis of celiac disease. Here we report that interaction between anti-tTG antibodies and extracellular membrane-bound tTG inhibits peptide 31-43 (but not peptide 57-68) uptake by cells, thereby impairing the ability of p31-43 to drive Caco-2 cells into S-phase. This effect did not involve tTG catalytic activity. Because anti-tTG antibodies interfered with epidermal growth factor endocytosis, we assume that they exert their effect by reducing peptide 31-43 endocytosis. Our results suggest that cell-surface tTG plays a hitherto unknown role in the regulation of gliadin peptide uptake and endocytosis.

Key words

transglutaminase, anti-transglutaminase antibodies, gliadin peptides, celiac disease, endocytosis

Abbreviations

tTG, tissue transglutaminase; CD, celiac disease; p31-43, peptide 31-43; p57-68, peptide 57-68; PBS, phosphate-buffered saline; PFA, paraformaldehyde; BrdU, bromodeoxyuridine; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; M- β -CD, methyl- β -cyclodextrin; MDC, monodansylcadaverine.

1. Introduction

Celiac disease (CD) is an enteropathy triggered by the ingestion of gliadin and of other related prolamines in genetically predisposed individuals [1, 2]. Gliadin peptides exert damaging effects through various mechanisms: they are resistant to gastrointestinal enzymes [3], they have amino acid sequences that are specific for human leukocyte antigen (HLA)-DQ2, which is a class II major histocompatibility complex, they also have preferred glutamine residues for tissue transglutaminase (tTG)-mediated deamidation [4], and, lastly, they affect intestinal permeability [5]. Some gliadin peptides are toxic and others are immunogenic for CD patients [5]. Toxic gliadin peptides exert direct effects on cells. In particular, they can agglutinate undifferentiated K562, can maintain tissue damage in the intestinal mucosa of CD patients, and can activate innate immune mechanisms in patients on a gliadin-free diet [6-8]. On the other hand, immunogenic gliadin peptides, after passing the intestinal epithelial border, trigger a dysregulated adaptive immune response at gut level dominated by T cells of the T helper 1 type. However, the innate and adaptive immune systems may respond synergistically to gliadin peptides [5-7]. The prototype of peptides effective on innate response is peptide 31-43/49, which has been shown to be toxic for CD patients both in vitro and in vivo [8, 9]. Peptide 31-43 (p31-43) stimulates the synthesis and release of the proinflammatory cytokine IL-15 that can promote an adaptive immune response [6] involving CD4⁺ T cells that recognize various deamidated gliadin peptides [4]. Unlike p31-43 which is not immunogenic for T cells, peptide 57-68 (p57-68), which binds to HLA-DQ2/8 molecules, is one of the dominant epitopes recognised by T cells isolated from the intestine of CD patients. [4].

Celiac disease is considered an autoimmune disease because of the presence of tTG-targeting autoantibodies in the serum and the intestinal mucosa of patients. tTG is a ubiquitously expressed multifunctional protein [10] that prevalently catalyzes the formation of isopeptide linkages between the γ -carboxamide group of protein-bound glutamine residue and

the ϵ -amino group of protein-bound lysine residue. Glutamine residues can be deamidated to glutamic acid as a side-reaction in the absence of suitable amines or at low pH. Furthermore, tTG is capable of both binding and hydrolyzing GTP, so that the enzyme can function as a cell signal transducer in association with the $\alpha_{1\beta}$ -adrenoreceptor [11]. Also disulphide isomerase activity [12] and kinase activity [13], have been attributed to tTG.

In CD, anti-tTG autoantibodies are produced in the small-intestinal mucosa and are deposited in the small bowel mucosa below the basement membrane and around capillaries in untreated patients [14, 15]. Interestingly, even seronegative patients have these mucosal anti-tTG deposits when on a gluten-containing diet [16]. During gluten consumption, anti-tTG antibodies can be detected also in the serum of patients and slowly disappear from the circulation consequent to a gluten-free diet [17]. After a gluten-free diet, serum autoantibodies disappear from the circulation more rapidly than do the small-intestinal mucosal autoantibody deposits [14, 15].

It is now recognized that anti-tTG antibodies are functional and not mere bystanders in the pathogenesis of CD. In fact, they inhibit intestinal epithelial cell differentiation [18], increase epithelial permeability and activate monocytes [19], and impair angiogenesis [20]. Moreover, we previously demonstrated that anti-tTG antibodies induce intestinal epithelial cell proliferation [21]. Interestingly, the effect of epithelial cell proliferation evoked by anti-tTG antibodies is similar to the effect evoked by some gliadin peptides [22]. In particular, p31-43, but not p57-68, causes actin alterations and cell proliferation, both of which depend on activation of the epidermal growth factor (EGF)-receptor, in several cell types, and in organ culture of celiac mucosa [22]. These effects of gliadin peptides are regarded as non-T-cell mediated, and most likely play an important role in the very early phases of CD. However, little is known about the mechanism underlying p31-43 and p57-68 uptake into cells [23].

The aim of this study was to investigate the relationship between anti-tTG antibodies and gliadin peptides, both of which are present in the small-intestinal mucosa of patients in the early phase of CD.

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2. Materials and Methods

Antibodies and peptides – The commercial monoclonal tTG-antibody clone CUB 7402 (200 µg/ml) was from NeoMarkers (Freemont, CA, USA). The recombinant anti-tTG miniantibody (clone 2.8) was derived from a library of CD intestinal lymphocytes [24]. The cloning and purification procedure is reported elsewhere [25]. Control antibodies were non-specific mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Unlabelled and lissamine-labelled synthetic peptides were from Inbios (Naples, Italy). The sequences of p31-43 and p57-68 from α -gliadin were LGQQQPFPPQQPY and QLQPFPPQQLPY, respectively.

Cell culture – Caco-2 cells were cultured in 100x10-mm Petri dishes containing Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids, 0.2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (Invitrogen SRL, Milan, Italy). Cells were maintained at 37°C in a 5% CO₂, 95% air-humidified atmosphere and passaged twice a week.

Cell staining – The localization of tTG in non-permeabilized living cells seeded on glass coverslips was determined by incubating cells with 20 µg/ml of clone 2.8 in 1% bovine albumin in phosphate-buffered saline (PBS) and with a mouse secondary TRITC-conjugated antibody (1:50). After fixing with 3% paraformaldehyde (PFA) for 10 min, coverslips were washed several times with PBS and mounted with Moviol (Sigma-Aldrich, Milan, Italy). Stained cells were observed with an Axioplan 2 fluorescent microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany). Images were acquired and processed with the KS300 software (Carl Zeiss MicroImaging Inc.).

S-phase entry assay – The bromodeoxyuridine (BrdU) (Roche Diagnostics SpA, Monza, Italy) incorporation study was carried out as reported elsewhere [21] with some modifications. Briefly, Caco-2 cells were seeded on coverslips and, 24-48 h later, starved by culturing cells in 0.1% FBS medium for 72 h. Cells were then challenged with antibodies to tTG, or control antibodies, or gliadin peptides, for 24 h. 10% FBS was used as positive

control. Three h before PFA fixing and triton X-100 permeabilizing, BrdU was added to the medium (final concentration, 100 μ M). BrdU incorporation was monitored by treating cells with an anti-BrdU antibody 1:100 (Invitrogen) and a mouse secondary TRITC-conjugated antibody (Invitrogen) 1:100. Finally, cells were stained for 5 min with Hoechst (Sigma-Aldrich) and mounted with Moviol. The number of cells that entered into S-phase was expressed as the ratio between the number of cells incorporating BrdU and the total number of cells. Microscope observations were carried out as reported above. Over 300 cells in several fields were evaluated in each sample.

In vitro tTG assay - Enzyme activity was assayed by a radiometric method based on Ca^{2+} -dependent incorporation of [^3H]spermidine (Nen Life Science Products, Boston, MA, USA) into *N,N*-dimethylated casein, as reported elsewhere [26]. Cellular extract from Caco-2 cells (15 μ g) was used as enzymatic source and incubated without (basal activity) and with antibodies and amines at 37 °C for 30 min.

Immunoprecipitation of surface tTG from Caco 2 cell membranes - We used the immunoprecipitation strategy (slightly modified) reported by Akimov et al. [27]. Briefly, Caco-2 cells (about 20×10^6) were extensively washed with PBS without calcium and magnesium, and detached with 0.5% EDTA to preserve surface tTG. We biotinylated the cell surface using PBS containing 4 mM Sulfo-NHS-Biotin (Pierce, Milan, Italy) for 30 min at room temperature, and the reaction was quenched with 100 mM glycine in PBS. After washing with PBS, cells were disrupted by osmotic shock in deionized water and the cell membrane fraction was enriched by centrifugation (14,000 g for 90 min). Pellets were resuspended in 0.25 ml of RIPA buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgCl_2 , 0.1% sodium dodecyl sulphate, 1% triton X 100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM ortovanadate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin [Sigma-Aldrich]), or in the same buffer without sodium dodecyl sulphate and triton X 100 but containing 0.1% Nonidet P-40 (Sigma-Aldrich), for 30 min at 4°C. An aliquot of the extracted

proteins was tested in an ELISA assay to verify the presence of a biotinylated protein that could be recognized by antibody clone 2.8 (not shown). The remaining surface proteins were immunoprecipitated with CUB 7402 (3.0 µg) or clone 2.8 (3.0 µg). Mouse IgG (3.0 µg) was used in control immunoprecipitation.

Western blot – For the detection of the phosphorylated form of extracellular signal-regulated kinase (P-ERK), cell lysates were prepared as described previously [28], as well as electrophoresis and immunoblotting procedures [28]. P-ERK was detected using anti-P-ERK mouse monoclonal antibody (clone E4, Santa Cruz). Total ERK was detected using ERK rabbit polyclonal antibody anti-ERK K23 (clone K23, Santa Cruz). The samples resulting from immunoprecipitation experiments were separated by 10% sodium dodecyl sulphate gel electrophoresis and transferred to a membrane. Biotinylated proteins were visualized on the blot with horseradish peroxidase-conjugated streptavidin 1:2000 (Pierce) according to the procedure described elsewhere [29]. The blot was then reprobed with CUB 7402, as described previously [30], to visualize tTG. Immunocomplexes were revealed using the chemiluminescence detection kit (Pierce) according to manufacturer's instructions.

Visualization of peptide uptake by Caco-2 cells – Caco-2 cells were seeded on glass coverslips ($5 \times 10^3/\text{cm}^2$) and three days later were treated for 15 min at 37°C with 100 µg/ml gliadin lissamine-peptides (p31-43 or p57-68). When requested, inhibitors of endocytosis, such as methyl-β-cyclodextrin (M-β-CD) and filipin (Sigma-Aldrich), were added to the medium 15 min before treatment with peptides. To investigate the effect of anti-tTG antibodies on gliadin peptide uptake by cells, 2 µg/ml of clone CUB 7402, or clone 2.8, or non specific IgG, were added to the medium 15 min before treatment with peptides. Coverslips were then washed with PBS, cells were fixed in 3% PFA for 5 min, and, after washing, coverslips were mounted with Moviol. Confocal images were acquired with a LSM 510 Zeiss microscope. Fluorescence intensity was measured on an area of $5000 \mu\text{m}^2$, randomly selected for every sample.

Endocytosis – Caco-2 cells were seeded on glass coverslips ($5 \times 10^3/\text{cm}^2$) and 3 days later used for endocytosis assay. Endocytosis was carried out with 20 ng/ml EGF-Alexa Fluor 488 (Invitrogen). After washing cells with cold PBS, EGF-receptors were loaded with the ligand at 4°C for 1 hour. Then cells were washed with cold PBS and incubated for 15 min at 37°C with warm medium containing unlabelled EGF. To investigate the effect of anti-tTG antibodies, 2 $\mu\text{g}/\text{ml}$ clone 2.8 (or CUB 7402) were added during the incubation at 4°C . Antibodies were added again during the incubation at 37°C . Finally, coverslips were washed with PBS and cells were fixed with 3% PFA for 10 min. Mounted coverslips were observed with a LSM 510 Zeiss microscope and confocal images were acquired. Fluorescence intensity was measured on an area of $5000 \mu\text{m}^2$, randomly selected for every sample.

Statistics – Statistical analysis was performed where appropriate by using the Student's t test. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1 ANTI-tTG ANTIBODIES, AS WELL AS GLIADIN PEPTIDES, INDUCE S-PHASE IN CACO-2 CELLS

We recently demonstrated that anti-tTG antibodies, as well as gliadin-peptide 31-43, induce $G_0 \rightarrow S$ transition in G_0 -synchronized NIH 3T3 fibroblasts [21, 22]. In the present study, we investigated whether Caco-2 cells, which are human epithelial cells that share several functions with small intestinal enterocytes [31, 32], responded in the same way to tTG antibodies and to gliadin peptides.

First, we verified that the antibodies we used in this study (the commercial CUB 7402 and the recombinant mini-antibody from a CD patient, named “clone 2.8”) specifically recognize cell surface tTG on Caco-2 cells. We biotinylated cell surface proteins and specifically immunoprecipitated tTG from a membrane-enriched fraction. With clone 2.8, we used a more bland buffer containing Nonidet P-40 to extract proteins, because clone 2.8 is a conformational antibody and only recognizes a native tTG [33]. Figure 1A shows the profile, in the range of 75-100 kDa, of surface biotinylated proteins immunoprecipitated with clone 2.8 and CUB 7402. The control lane shows the profile of surface biotinylated proteins immunoprecipitated with non specific mouse IgG. The asterisks indicate a band that has the same molecular weight as tTG. We verified the identity of this band with protein tTG by reprobing the blot with CUB 7402 (lower part of the blot). Visualization of the interaction between the recombinant miniantibody and surface tTG in not-permeabilized living Caco-2 cells is shown in Fig. 1B. Membrane-bound tTG had a punctuated distribution and was prevalently localized at the periphery of cell clusters, as previously reported for staining with CUB 7402 [21].

Then, we evaluated the S-phase entry of Caco-2 cells, measured in the presence of increasing concentrations of the commercial clone CUB 7402 and the recombinant mini-antibody clone 2.8. Fig. 2A shows that both CUB 7402 and clone 2.8 induced BrdU

incorporation. CUB 7402 was most effective at 2.0 $\mu\text{g/ml}$ ($54.2 \pm 4.8\%$ BrdU incorporation) whereas clone 2.8 was most effective at 4.0 $\mu\text{g/ml}$ ($50.5 \pm 2.1\%$ of BrdU incorporation). The induction of S-phase entry was specific because non specific IgG did not induce any effect ($40.0 \pm 2.6\%$ of BrdU incorporation).

To verify that the interaction of tTG antibodies with the extracellular membrane-bound tTG triggers an intracellular response, we treated Caco-2 cells with 100 $\mu\text{g/ml}$ of CUB 7402 or clone 2.8 for 5 min and then performed a western blot analysis on Caco-2 cell extracts to visualize ERK-phosphorylation. Non specific mouse IgG and 20% FBS were used as negative control and positive control of the experiment, respectively. As shown in Fig. 2B, as expected, ERK phosphorylation increased rapidly after brief treatment with clone CUB 7402 or clone 2.8, and with FBS, but was not influenced by mouse IgG. This finding indicates that anti-tTG antibodies mediate out-in cell signaling by interacting with cell surface tTG.

We next carried out experiments to evaluate whether the toxic peptide p31-43 and the T-cell immunogenic peptide p57-68 were able to drive Caco-2 cells into S phase. As shown in Fig. 2C, p31-43 induced BrdU incorporation in Caco-2 cells in a dose-dependent manner. In fact, BrdU incorporation increased up to about 50% in cells treated with 100 $\mu\text{g/ml}$ p31-43. Peptide 57-68 did not induce S-phase entry ($40.3 \pm 2.1\%$ of BrdU incorporation) when used at a concentration of 100 $\mu\text{g/ml}$.

3.2 ANTI-tTG ANTIBODIES IMPAIR THE CAPABILITY OF P31-43 TO INDUCE CACO-2 CELLS INTO S-PHASE

Because we found that anti-tTG antibodies and p31-43, but not p57-68, drive Caco-2 cells into S-phase, we stimulated intestinal cells with clone 2.8 and p31-43 separately and in combination. As reported in Fig. 3, concentrations of clone 2.8 (2 $\mu\text{g/ml}$) and p-31-43 (100 $\mu\text{g/ml}$) induced $47.0 \pm 1.0\%$ and $48.7 \pm 1.5\%$ BrdU incorporation respectively when used

separately and $48.3 \pm 2.9\%$ BrdU incorporation when used together (light grey bars). This result suggests that anti-tTG antibodies and p31-43 interacted at some level. Therefore we evaluated the effect of clone 2.8 at a suboptimal concentration ($0.2 \mu\text{g/ml}$) combined with p31-43 at a concentration that induced the highest BrdU incorporation ($100 \mu\text{g/ml}$). Under these conditions, BrdU incorporation was reduced to $40.5 \pm 0.7\%$, which suggests that anti-tTG antibodies, at a suboptimal concentration, affected p31-43 stimulation (Fig. 3, white bars). We also tested the effect of clone 2.8 at a concentration that induced the highest BrdU incorporation ($2.0 \mu\text{g/ml}$) combined with a suboptimal concentration of p31-43 ($20 \mu\text{g/ml}$). In this case, BrdU incorporation was not affected ($46.0 \pm 1.0\%$ versus $47.0 \pm 0.8\%$ produced by clone 2.8 alone) (Fig. 3, dark grey bars) which indicates that p31-43 did not influence the effects induced by anti-tTG antibodies. We obtained similar results when we used the commercial clone CUB 7402 (not shown) instead of clone 2.8.

3.3 RELATIONSHIP BETWEEN tTG'S EXTRACELLULAR TRANSAMIDATING ACTIVITY AND THE EFFECTS EVOKED BY GLIADIN PEPTIDES

We next investigated if the transamidating activity associated with the extracellular membrane surface of tTG could be involved in the modulation of cell cycle progression. To this aim, we carried out an S-phase entry assay with cystamine, a TG irreversible inhibitor, and monodansylcadaverine (MDC), a TG competitive substrate. As shown in Fig. 4A, cystamine did not affect BrdU incorporation. Similarly, MDC did not induce S-phase entry at any of the concentrations tested (Fig. 4B); indeed, BrdU incorporation was slightly reduced at $50 \mu\text{M}$. Notably, both amines, like CUB 7402 and clone 2.8, exerted a strong inhibitory effect in an *in vitro* enzymatic assay (Fig. 4C).

Finally, we investigated whether cystamine and MDC affected p31-43-induced S-phase entry. Neither $50 \mu\text{M}$ cystamine together with $100 \mu\text{g/ml}$ p31-43 (light grey bars) nor

10 μ M MDC with 100 μ g/ml p31-43 (dark grey bars) significantly inhibited BrdU incorporation versus p31-43 alone (Fig. 4D).

3.4 ANTI-tTG ANTIBODIES DERANGE P31-43 (BUT NOT P57-68)

INTERNALIZATION BY CACO-2 CELLS

We next evaluated whether anti-tTG antibodies impaired p31-43's capability to drive Caco-2 cells into S phase by interfering with gliadin-peptide uptake by cells. To this aim, we examined the mechanisms of transport in Caco-2 cells using lissamine-labelled p31-43 (p31-43-liss) and p57-68 (p57-68-liss) in the absence and presence of anti-tTG antibodies.

When we pre-treated Caco-2 cells with M- β -CD, a general inhibitor of endocytosis, for 15 min and then added p31-43-liss to the medium and incubated the mix at 37°C for a further 15 min, p31-43-liss entry into Caco-2 cells was dramatically reduced (Fig. 5A). On the contrary, when we pre-treated the cells with filipin, an inhibitor of lipid raft/caveolae-mediated endocytosis, p31-43-liss entry into Caco-2 cells was not affected (Fig. 5A). We concluded that p31-43 enters Caco-2 cells by endocytosis and excluded the involvement of lipid raft/caveolae-mediated endocytosis. As shown in Fig. 5B, when we pre-treated cells with M- β -CD, p57-68-liss uptake was slightly reduced, whereas filipin-pretreatment reduced p57-68-liss uptake in a more consistent way (Fig. 5B). Thus, under our conditions, p57-68 was endocytosized by Caco-2 cells, probably through a lipid raft/caveolae-mediated mechanism.

To evaluate whether or not anti-tTG antibodies affect gliadin-peptide uptake into Caco-2 cells, we carried out peptide uptake experiments in the presence of CUB 7402 and clone 2.8. As reported in Fig. 6A, pre-treatment with clone 2.8 (2 μ g/ml) resulted in weak p31-43-liss staining, which indicates that the antibody affected the entry of p31-43-liss into the cells. A similar effect was obtained with CUB 7402. Conversely, non specific IgG did not affect p31-43-liss uptake (Fig. 6A). Quantification of the percent of fluorescence intensity

(mean of 3 experiments) relative to p31-43-liss uptake, in the presence of clone CUB 7402 or clone 2.8 (2 $\mu\text{g/ml}$), showed that both antibodies reduced fluorescence by about 50%, whereas non specific IgG did not affect fluorescence intensity (Fig. 6C). These effects were less pronounced, but still significant, at lower concentration (0.2 $\mu\text{g/ml}$ and 1.0 $\mu\text{g/ml}$) of antibodies (Fig. 6C). Pre-treatment with clone 2.8 or CUB 7402 did not affect p57-68-liss entry into Caco-2 cells (Fig. 6B, C). In addition, As expected, cystamine and MDC did not affect either p31-43-liss or p57-68-liss entry into cells (not shown). Interestingly, when we treated cells with antibodies to $\beta 1$ -integrin (10 $\mu\text{g/ml}$), which is non-covalently complexed with tTG on the cell surface [27], p31-43 uptake but not p57-68 uptake was reduced (Fig. 6D).

3.5 ANTI-tTG ANTIBODIES AND ENDOCYTOSIS

To determine how the tTG antibodies interfered with endocytosis, we studied their effect on EGF internalization by cells evaluated with the labelled-ligand EGF-Alexa Fluor 488, which is prevalently internalized by cells through clathrin-mediated endocytosis [34]. After incubation at 0°C for 1 hour with and without clone 2.8 (2 $\mu\text{g/ml}$), cells were treated with EGF-Alexa Fluor 488 at 37°C for 15 min, then fixed. In the presence of clone 2.8, the number of endocytic vesicles containing EGF-Alexa Fluor 488 after 15 min of incubation at 37°C was lower than in cells not exposed to antibodies (Fig. 7A). To quantify fluorescence, we measured its intensity relative to a random area of 5000 μm^2 in each coverslip. As shown in Fig. 7B, there was no significant difference between fluorescence intensity at time zero and at 15 min in the presence of clone 2.8. Similar results were obtained with CUB 7402 (Fig. 7 B).

4. Discussion

We investigated the relationship between anti-tTG antibodies and gliadin peptides, both of which are present in the small-intestinal mucosa of patients in the early phase of CD, probably even at luminal level. Our results indicate a hitherto unknown role of celiac anti-tTG antibodies, i.e., they impair the uptake of p31-43, but not of p57-68, by epithelial cells. We previously reported that celiac anti-tTG antibodies and α -gliadin p31-43 induce the G₀-S transition in murine fibroblasts and enterocytes from celiac patients [21, 22]. We hypothesized that autoantibodies to tTG and gliadin peptides together could produce an additive or synergic proliferative effect in cells of epithelial origin. To test our hypothesis, we first investigated whether the response of Caco-2 cells to tTG-antibodies is similar to that of NIH 3T3 cells and of other cells we previously used [21]. Here we report that both commercial clone CUB 7402 and a recombinant mini-antibody from a CD patient induce proliferation in Caco-2 cells. Moreover, we found that p31-43 but not p57-68, induce Caco-2 cell proliferation.

Surprisingly, when we stimulated intestinal cells with effective concentrations of p31-43 combined with anti-tTG antibodies, there was no additive effect. On the contrary, suboptimal concentration of antibodies together with effective concentration of p31-43 inhibited BrdU incorporation. In conclusion, our results indicate that anti-tTG antibodies interfered with p31-43 stimulation whereas p31-43 did not affect the response induced by anti-tTG antibodies.

In an attempt to explain these effects, we investigated the relationship between tTG-antibodies and gliadin peptide uptake. A critical step in the pathogenesis of CD is the epithelial handling of gliadin peptides. It is known that gliadin peptides enter the cells, interact with the vesicular compartment (M.V. Barone, unpublished) and are transcytosed to the basal part of the cells [23, 35]. However, the mechanism of uptake is not completely understood. Our results on the comparative uptake of two different gliadin fragments (p31-43

and p57-68), in the presence of inhibitors of endocytosis, show that p31-43 and p57-68 act differently in relation to entry into epithelial cells. M- β -CD is considered a non-specific inhibitor of endocytosis and can be used to block all endocytic pathways, whereas the antibiotic filipin specifically blocks lipid raft/caveolae-mediated endocytosis [36-41]. Our confocal images of lissamine-labeled peptides show that both p31-43 and p57-68 enter Caco-2 cells, but while p57-68 uptake was reduced by filipin, which indicates the occurrence of a lipid raft/caveolae-mediated mechanism, p31-43 uptake was not (Fig. 5).

We also show that anti-tTG-antibodies affect only p31-43 uptake. This effect is specific because a non-specific antibody did not alter the transport pathway (Fig. 6). Interestingly, anti- β 1 integrin antibodies had a similar effect on p31-43 uptake, which suggests that the tTG-integrin complex on the cell surface is implicated in the cellular transport of p31-43 but not of p57-68.

Taken together, our results show that cells handle p31-43 and p57-68 differently, which is in line with other differences we identified between the two peptides. In fact, p31-43 and p57-68 are located in different sites during maturation of endocytic vesicles both in Caco-2 cells and in intestinal biopsies from CD patients (M.V. Barone, unpublished), [42]. Staining with markers of the endocytic pathway showed that p31-43 remains in early endocytic vesicles whereas p57-68-carrying vesicles mature into late endosomes (M.V. Barone, unpublished). Furthermore, studies on the interaction of “toxic” and “immunogenic” gliadin peptides with a membrane-like environment in vitro showed that only p31-43 was able to bind micelles, whereas p56-68 did not appear to interact with them [43].

Since gliadin peptides enter the cells by endocytosis and anti-tTG antibodies interfere with p31-43 entry, we evaluated whether anti-tTG antibodies could interfere with endocytosis. Using EGF-Alexa Fluor 488, which is prevalently internalized by cells through a clathrin-mediated endocytosis [34], we demonstrate that anti-tTG-antibodies interferes with endocytosis. These results provide strong evidence that cell surface tTG is involved in the

mechanism of endocytosis. In 1980, Davies et al., reported that tTG was involved in the mechanism of endocytosis [44]. However, at that time, the existence of an extracellular membrane bound form of tTG was unknown.

We previously suggested [21] that anti-tTG antibodies, by interacting with the extracellular membrane-bound tTG, cause $G_0 \rightarrow S$ transition in synchronized NIH 3T3 fibroblasts through a phosphorylation pathway. We also hypothesized that tTG catalytic activity was not involved in this process. Although tTG is prevalently an intracellular enzyme mainly localized in the cytosol, relevant amounts of the protein are present also on the cell surface. Externalized tTG is in part removed from the cell surface and in part constitutes the pool of the membrane-bound protein [45]. Surface tTG functions often are independent of catalytic activity. However, the issue is highly debated. In fact, owing to the availability of high calcium levels and low GTP concentrations in the extracellular space, tTG should be basically active, although the effects of anchorage to fibronectin and the actions of metalloproteinases have not been fully explored. It has been demonstrated that, being an outside membrane-bound protein, tTG could mediate the interaction of $\beta 1$ and $\beta 2$ integrins with fibronectin in a catalytic independent manner [27]. Through this interaction, tTG could modulate cell-matrix adhesion, spreading, integrin-mediated signaling, and cell migration, proliferation or differentiation [27, 46, 47]. Remarkably, the cross-linking activity of tTG is not required for inhibition of p31-43 uptake or for its proliferating effect. In fact, neither cystamine nor MDC, which are inhibitors of tTG catalytic activity, affected peptide entry into Caco-2 cells or cell cycle progression. In addition, we provide a direct demonstration that the interaction of tTG antibodies with the extracellular membrane-bound tTG triggers an intracellular response that leads to a rapid increase of ERK phosphorylation (Fig. 2B). Therefore, the effects we observed probably result from the interaction of antibodies with tTG on the cell surface without the involvement of catalytic activity.

4.1 CONCLUSIONS

The results we report herein show that anti-tTG antibodies interfere with p31-43 endocytosis, which suggests that cell-surface tTG could play a hitherto unknown role in the regulation of gliadin peptide uptake and, more generally, in endocytosis.

5. Acknowledgements

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7. Figure legends

Fig. 1 – (A) Surface biotinylated proteins immunoprecipitated with clone 2.8, CUB 7402 and non specific mouse IgG and visualized with horseradish peroxidase-conjugated streptavidin (upper part of the blot). The asterisks (*) indicate a band that has the same molecular weight as tTG. Surface tTG was detected after reprobing the blot with CUB 7402 (lower part of the blot). **(B)** Surface tTG staining of unpermeabilized living Caco-2 cells obtained with clone 2.8. 40X magnification. Non-specific mouse IgG served as control.

Fig. 2- (A) CUB 7402 (black bars) and clone 2.8 (light grey bars) dose-dependently induced the S-phase entry of Caco-2 cells. Non-specific mouse IgG served as control. Data are reported as means \pm s.d. of at least three independent experiments. **(B)** Western blot of Caco-2 total proteins, stained by ERK or P-ERK antibodies showed a rapid increase of ERK phosphorylation after CUB 7402 or Clone 2.8 treatment. Non specific mouse IgG and 20% FBS served as negative and positive control, respectively. The blot shows the result of two parallel experiments for each stimulus. **(C)** Induction of S-phase entry of Caco-2 cells by different concentrations of p31-43. The effect of 100 μ g/ml p57-68 is also reported. Data are reported as means + s.d. of 3 separate experiments.

Fig. 3- Effect of the simultaneous presence of clone 2.8 and p31-43 on BrdU incorporation by Caco-2 cells. Effective concentrations of clone 2.8 and p31-43 (2 μ g/ml and 100 μ g/ml, respectively) did not produce an additive or a synergic effect (light grey bars). A suboptimal concentration of clone 2.8 (0.2 μ g/ml) inhibited the p31-43 proliferating effect (white bars), whereas a suboptimal concentration of p31-43 (20 μ g/ml) did not affect the clone 2.8 proliferating effect (dark grey bars). Data are reported as means \pm s.d. of at least three independent experiments.

Fig. 4- Effect of cystamine **(A)** and MDC **(B)** on BrdU incorporation by Caco-2 cells. **(C)** Effect of CUB 7402 (20 μ g/ml), clone 2.8 (20 μ g/ml), MDC (50 μ M) and cystamine (50 μ M) on tTG activity. **(D)** Effect of the simultaneous presence of cystamine, or MDC, and p31-43

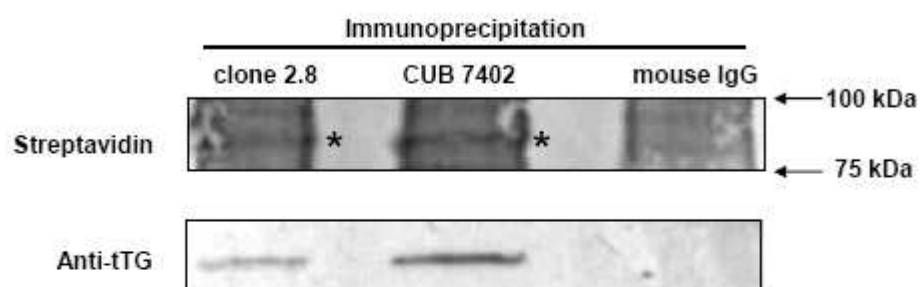
on BrdU incorporation by Caco-2 cells. All data are reported as means \pm s.d. of at least 3 independent experiments.

Fig. 5- Effect of 10 mM M- β -CD and 5 μ g/ml filipin on p31-43 uptake (**A**) and p57-68 uptake (**B**) by Caco-2 cells. Representative confocal images of several similar experiments (more than 3). 63X magnification.

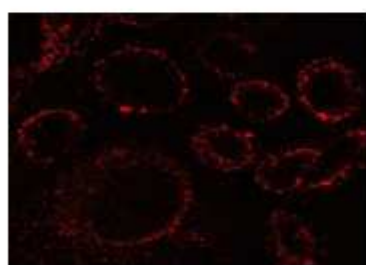
Fig. 6 – Representative confocal images of similar experiments (more than 3). 63X magnification. Effect of clone 2.8 or CUB 7402 or non specific IgG (2 μ g/ml) on p31-43 uptake (**A**) and p57-68 uptake (**B**) by Caco-2 cells. (**C**) Quantification of the inhibitory effect of anti-tTG antibodies on p31-43 and p57-68 uptake by Caco-2 cells. The bars represent the percent of fluorescence intensity calculated with respect to liss-peptide uptake in the absence of antibodies (100% of fluorescence intensity). Data are reported as means \pm s.d. of 3 independent experiments. Asterisk (*) indicates significantly different values ($p < 0.05$). (**D**) Effect of anti- β 1-integrin antibodies (10 μ g/ml) on p31-43 and p57-68 uptake by Caco-2 cells.

Fig. 7- (A) Endocytic vesicles containing EGF-Alexa Fluor 488 were less abundant in Caco-2 cells after incubation at 37°C for 15 min in the presence of clone 2.8 (2 μ g/ml). Representative confocal images of similar experiments (more than 6). 63X magnification. (**B**) Quantification of the inhibitory effect of anti-tTG antibodies on EGF-Alexa Fluor 488 uptake by Caco-2 cells. The bars represent the fluorescence intensities relative to 6 independent experiments.

A



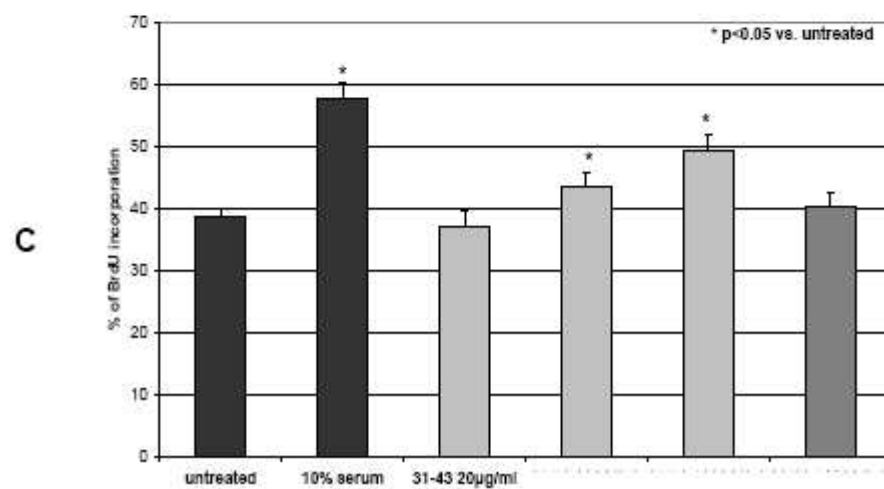
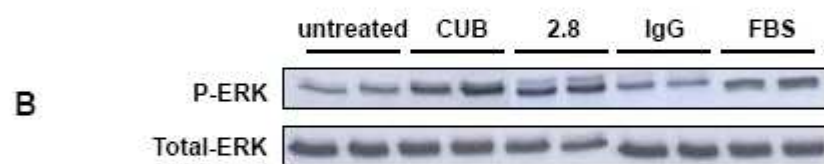
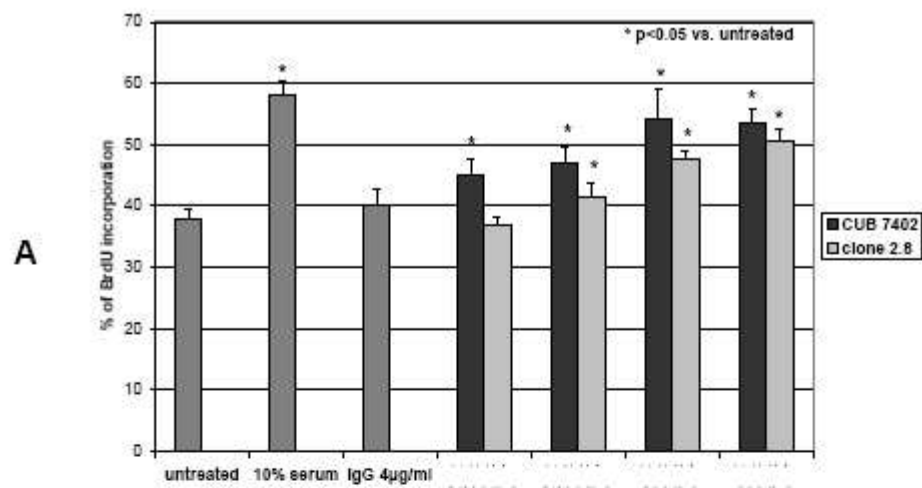
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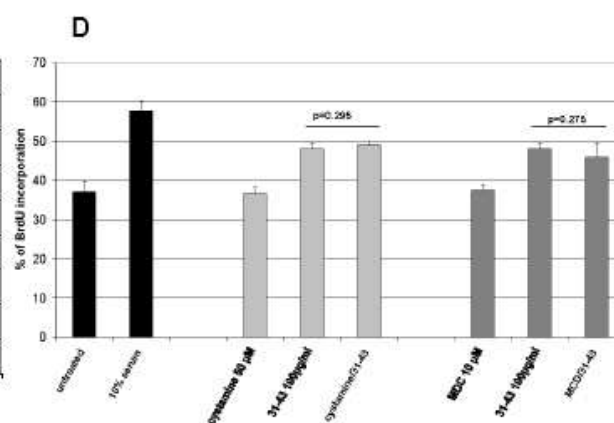
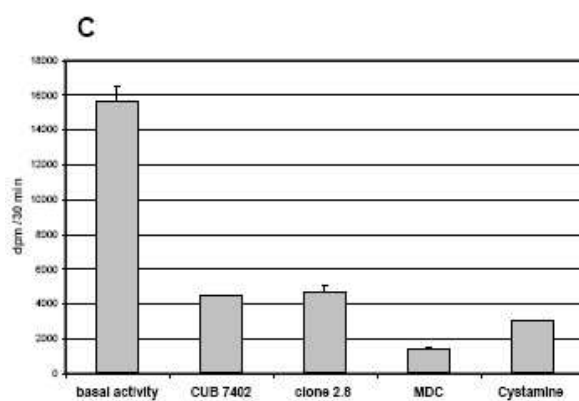
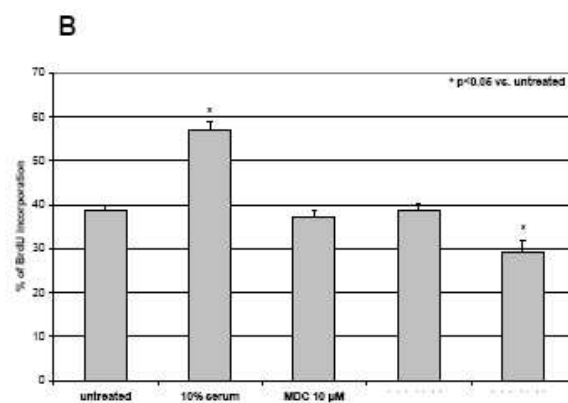
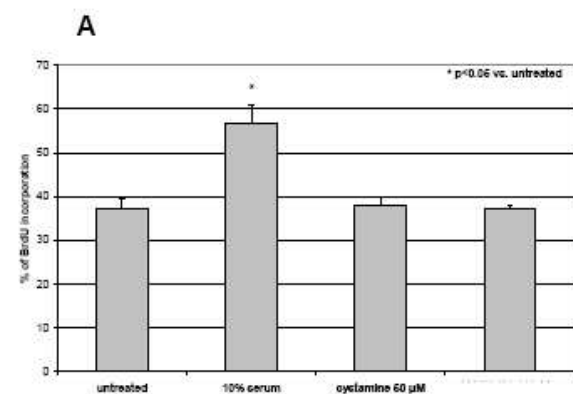
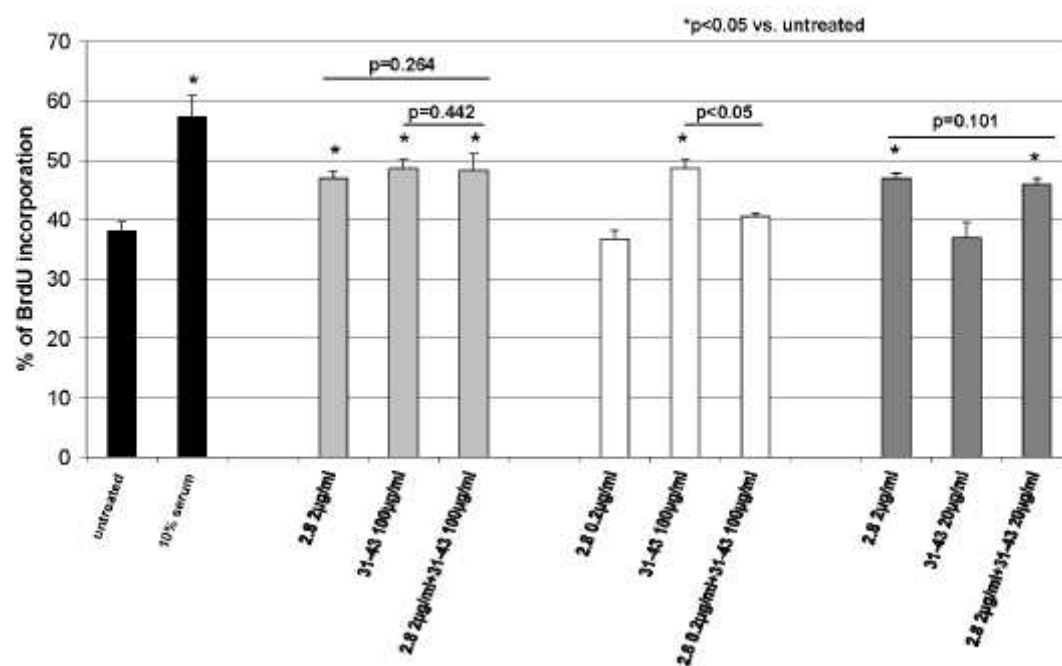


Staining with clone 2.8

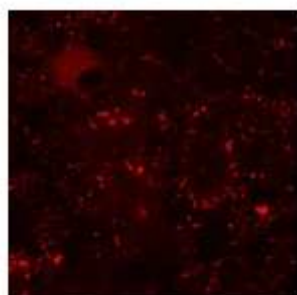


Staining with mouse IgG

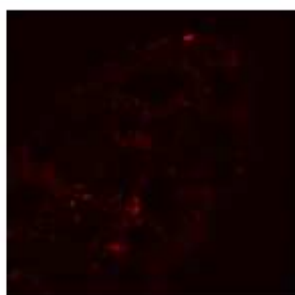




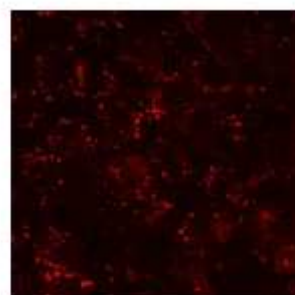
A



P31-43

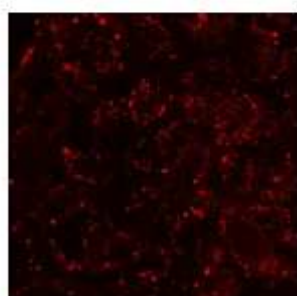


P31-43 + M-β-CD

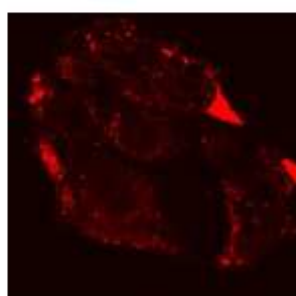


P31-43 + Filipin

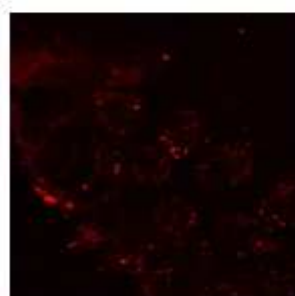
B



P57-68



P57-68 + M-β-CD



P57-68 + Filipin

Figure 6

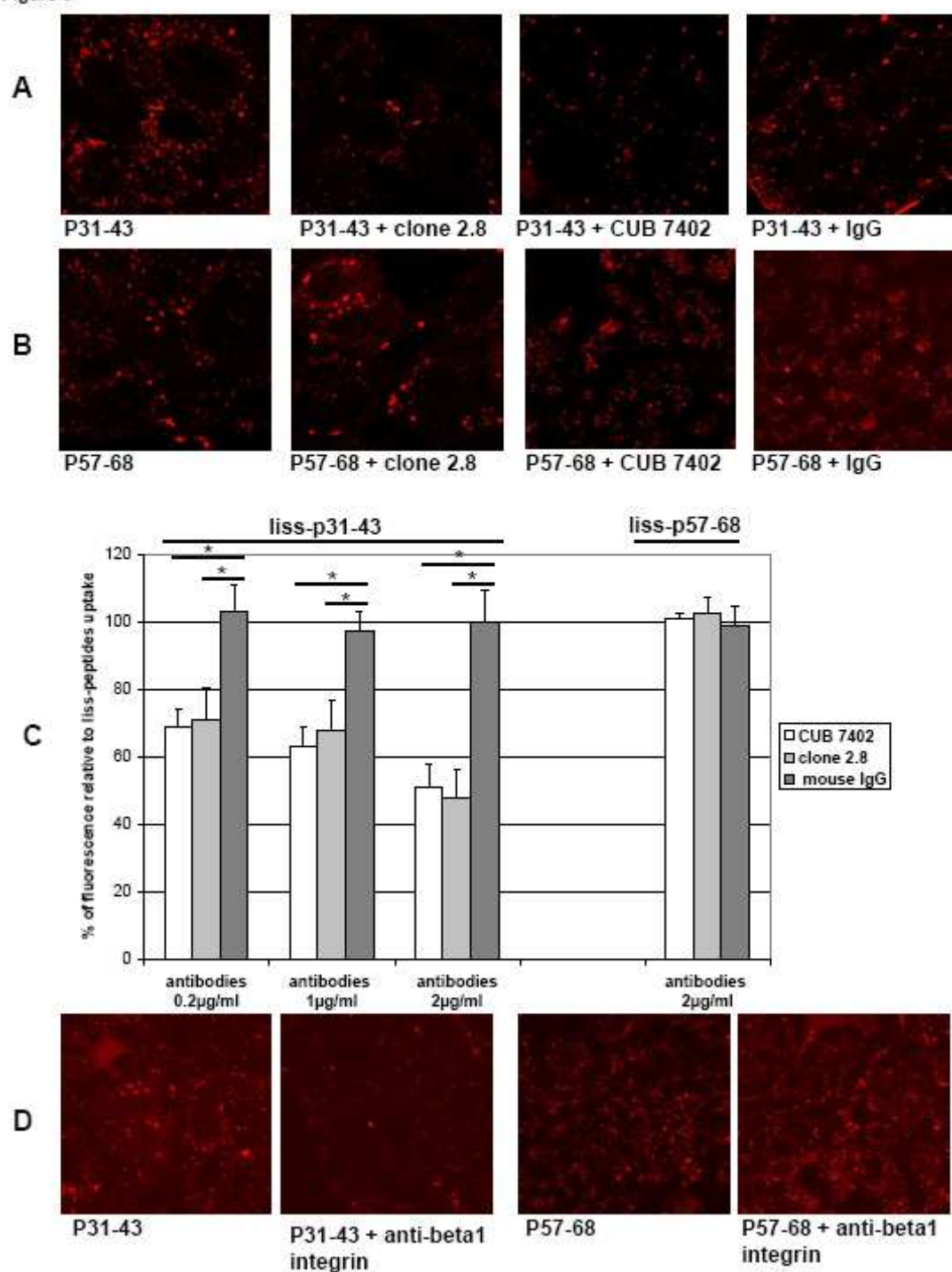


Figure 7

