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Gliadin activates arginase pathway in RAW264.7 cells and in human monocytes

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ABSTRACT

Celiac disease (CD) is an autoimmune enteropathy triggered in susceptible individuals by the ingestion of gliadin-containing grains. Recent studies have demonstrated that macrophages play a key role in the pathogenesis of CD through the release of inflammatory mediators such as cytokines and nitric oxide (NO). Since arginine is the obliged substrate of iNOS (inducible nitric oxide synthase), the enzyme that produces large amount of NO, the aim of this work is to investigate arginine metabolic pathways in RAW264.7 murine macrophages after treatment with PT-gliadin (PTG) in the absence and in the presence of IFNγ. Our results demonstrate that, besides strengthening the IFNγ-dependent activation of iNOS, gliadin is also an inducer of arginase, the enzyme that transforms arginine into ornithine and urea. Gliadin treatment increases, indeed, the expression and the activity of arginase, leading to the production of polyamines through the subsequent induction of ornithine decarboxylase. This effect is strengthened by IFNγ. The activation of these pathways takes advantage of the increased availability of arginine due to a decreased system y^+_L-mediated efflux, likely ascribable to a reduced expression of Slc7a6 transporter. A significant induction of arginase expression is also observed in human monocytes from healthy subject upon treatment with gliadin, thus demonstrating that gluten components trigger changes in arginine metabolism in monocyte/macrophage cells.

1. Introduction

Celiac disease (CD, MIM: 212750) is a chronic, immune-mediated disorder caused by a permanent intolerance in genetically susceptible individuals to ingested gluten/gliadin, a protein of wheat and related cereals [1]. The disease ranges from asymptomatic to severely symptomatic, with clinical manifestations including typical gastrointestinal symptoms, such as diarrhea and abdominal pain or discomfort, but also extraintestinal manifestations, such as short stature, neurologic symptoms, or anemia [2]. The clinical heterogeneity of CD develops in the context of a sustained chronic inflammation of the small intestinal mucosa which is histologically characterized by villous atrophy, crypt hyperplasia, hypercellularity of the lamina propria and increased number of intraepithelial lymphocytes (IELs) [3,4]. All morphological changes are generally reversible during a gluten-free diet.

CD has been strongly associated with HLA-DQ2 or HLA-DQ8 isotypes, responsible of the inappropriate T cell-mediated immune response [5–7]. However, studies addressing the onset of CD in identical twins, as well as the fact that only 2–5% of individual expressing these HLAs develops the disease, clearly indicate that the expression of HLA-DQ2/8 is necessary but not sufficient to cause CD, while additional genetic or environmental factors may have a role in the pathogenesis of this autoimmune disease [8].

Clinical and experimental findings ascribe a primary role to the innate immune system in the activation of an early response against ingested gluten in CD, providing evidences that these events are critical for the priming of the subsequent adaptive immune responses [9]. Innate immune cells, mainly monocytes/macrophages and dendritic cells (DCs), are, indeed, supposed to interact with gliadin into the intestinal submucosa, leading to the activation of the Ag-specific adaptive immune response. Consistently, in vitro studies with intestinal organ cultures, primary APCs and epithelial and monocytic cell lines, support the notion that gluten affects the innate immune system to the same extent as T cells [7]. In particular, gliadin and its peptic fragments are reported to induce the expression of proinflammatory genes, cytokines secretion (TNFα, IL-12, IL-15), and zonulin release in murine macrophages [10] and to activate blood monocytes from patients with CD, in terms of TNF-α and IL-8 production [11].

Among the repertoire of inflammatory genes up-regulated by gliadin peptides, a significant up-regulation of inducible nitric oxide synthase (iNOS) has been described in the small intestinal mucosa of CD patients [12,13]. Consistently, increased levels of nitric oxide (NO) metabolites (nitrites and nitrates) are detected in serum and urine of children with both symptomatic CD or screening-detected, nonsymptomatic, "silent" CD, pointing to NO metabolites as a pathogenetic feature of CD [14–16].

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Nitric oxide is known to exert antibacterial and cytostatic effects; however, when produced in excessive amounts, it also acts as a free radical that can cause tissue damage, impairing the function and integrity of the gut barrier and, hence, increasing mucosal permeability [17].

Since the amino acid arginine is the obliged substrate for iNOS, both its intracellular availability and metabolism appear of peculiar relevance for the study of CD pathogenesis. Besides iNOS, that converts arginine to nitric oxide and citrulline, also arginase is involved in the catabolism of the amino acid converting it to urea and ornithine. The two enzymes are specifically induced during classical (type I or M1) or alternative (type II or M2) macrophage activation, respectively. In particular, classically activated macrophages produce copious amounts of nitric oxide, as well as of pro-inflammatory cytokines, as a defense against intracellular pathogens. In contrast, alternatively activated macrophages exert anti-inflammatory properties and mediate tissue homeostasis and repair through the arginase dependent production of polyamines and proline, which are involved in cell proliferation and collagen production, respectively.

Intracellular arginine levels are regulated by the uptake of the extracellular amino acid via specific transporters [18]. In particular, arginine transport in non-epithelial models is mediated by system y^+ and system y^+L. System y^+ activity, referable to the CAT family of monomeric transporters, mediates a sodium-independent, membrane potential-sensitive arginine transport. Conversely, system y^+L consists of a group of heterodimeric amino acid transporters formed by a heavy subunit (4F2hc/CD98, the product of SLC3A2 gene) and one of two alternative light chains, y^+LAT1 (encoded by SLC7A7) or y^+LAT2 (encoded by SLC7A6), and operates as an antiport that couples the flux of arginine with that of neutral amino acids and sodium [18]. While the activity of system y^+ is mainly responsible for the intracellular accumulation of arginine, system y^+L likely acts as an efflux route for the amino acid [19–21].

In the present paper we investigate the effects of the treatment with gliadin on arginine metabolism in RAW264.7 murine macrophages and in human monocytes.

2. Materials and methods

2.1. Cell culture and experimental treatments

RAW264.7 murine macrophages were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia-Romagna. Cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and kept at 37 °C, pH 7.4, in an atmosphere of 5% CO_2.

For the experiments, RAW264.7 cells were seeded in multi-well Falcon dishes at the density of 100,000/ml and incubated the day after in the presence of 10 ng/ml IFNγ, 1 mg/ml gliadin peptic–tryptic peptides (PTG) or both, as indicated.

Human peripheral blood mononuclear cells (PBMC) were isolated by Lympholite® (EuroClone, Milano, Italy) gradient separation of blood samples obtained from healthy donors (approved by Local Ethics Committee N° 37863 del 14/11/2013). Monocytes were purified from PBMC by positive selection using magnetic cell separation columns and CD14 MicroBeads (Miltenyi Biotec, Bologna, Italy). Highly enriched monocytes (10^6 cells/ml) were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C, pH 7.4, in an atmosphere of 5% CO_2 in the absence or in the presence of PTG.

PTG was obtained through enzymatic digestion of 50 g of gliadin (Sigma-Aldrich, Milano, Italy) by incubation for 2 h at 37 °C in the presence of 1 g pepsin (Sigma-Aldrich) in 500 ml HCl 0.2 N. The resulting product was further digested by addition of 1 g trypsin (Sigma-Aldrich) and the pH adjusted to 7.4 with NaOH 2 M. The solution was stirred vigorously at 37 °C for 4 h, boiled (100 °C) for 30 min, cooled off, stocked in aliquots and conserved at −20 °C. Endotoxin contamination, assessed with an E-TOXATE kit (Sigma-Aldrich), was below 4.8 EU/ml.

2.2. Intracellular arginine content and metabolism

The study of arginine conversion into its metabolites has been performed employing an original technique developed in our lab [22]. Cells were treated as required by the experimental plan in complete growth medium containing L-[^{14}C(U)]arginine (5 μCi/ml). At the end of the treatment, the intracellular amino acid pool was extracted in a 5% solution of acetic acid in ethanol, lyophilized, and re-suspended in 150 μl of Lithium Loading Buffer pH 2.2 (Biochrom, Cambridge, UK). 50 μl were employed immediately for measuring whole radioactivity with a Wallac MicroBeta TriLux^2 liquid scintillation spectrometer (PerkinElmer, Monza, Italy), while the remaining 100 μl were injected on Biochrom 20 Amino Acid Analyzer and separated by employing a High Resolution Column Bio 20 Peek Lithium and the Physiological Fluid Chemical Kit (Biochrom). The resulting eluting fractions were collected into 96-well trays (192 samples; 40 s/fraction), dried by heating at 45–50 °C and resuspended in 50 μl of ethanol. Radioactivity in each well was determined and CPM in each fraction were plotted as a function of their Retention Time (T_r). Standard samples containing known radiolabeled amino acids were run in parallel and employed to identify the metabolite in each fraction. Finally, extracted cells were dissolved with 0.5% sodium deoxycholate in 1 M NaOH and protein content was determined directly in the well using a modified Lowry procedure [23].

2.3. NO production

The production of NO was assessed through the quantification of nitrites, stable derivatives of NO, in the culture media, as already described [24]. A fluorimetric approach was employed, based on the production of the fluorescent molecule 1-(H)-naphthotriazole from DAN in the presence of nitrite in an acid environment. Fluorescence was determined with EnSpire® Multimode Plate Reader (PerkinElmer). Nitrite production was expressed in nmoles per ml of extracellular medium (μM).

2.4. Arginase activity

Arginase activity was determined with QuantiChrom™ Arginase Assay Kit by BioAssay Systems (Valter Occhiena, Torino, Italy). Lysates obtained from 10^6 cells were incubated for 2 h at 37 °C in the presence of 5× Substrate Buffer. The reaction was then stopped by the addition of Urea Reagent and, after 1 h, optical absorbance at 430 nm was read with EnSpire® Multimode Plate Reader (PerkinElmer). Protein content was determined for each sample using a modified Lowry procedure [23] and arginase activity was expressed as U/L/μg of protein.

2.5. qRT-polymerase chain reaction

1 μg of total RNA, extracted with GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) was reverse transcribed and 40 ng of cDNA were amplified as described previously [25]. The sequence of the forward and reverse primers employed is listed in Table 1. The expression of the gene of interest under each experimental condition was normalized to that of the housekeeping gene *Gapdh* (for RAW264.7 cells) or RPL-15 (for human monocytes) and shown relatively to its expression level in control, untreated cells (=1).

2.6. Western Blot analysis

Cells were washed with ice-cold PBS and suspended in Laemmli buffer (62.5 M Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.2 M DTT). Western Blot analysis was performed as previously described [26], using anti-iNOS (1:1000) and anti-Arginase II (1:500) purified rabbit

Table 1
Primers employed for qRT-PCR analysis.

	Forward primer	Reverse primer
ArgII	CTGGCTGAAGTGGTTAGTAGA	GGGCGTGACCGATAAT
Nos2	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Oat	GGAGTCCACACCTCAGTCG	CCACATCCCACATATAAATGCCT
Odc	GACGAGTTTGACTGCCACATC	CGCAACATAGAACGCATCCTT
Slc7a1	GGAGTGCGACTTTTGACGAG	ACCAGGACATTGATACAGGTGA
Slc7a2	TCCCAATGCCTCGTGTAA	CCCGACGACAAAGTAGCA
Slc7a6	CCTACTCGGGCTGGGACAC	AGGTAGATGATGGTGACGATTGGC
Slc7a7	TTATTAGGAATGTGCTGGCTACTG	GGTTTAGGGAACTGGTCTTTG
Gapdh	TGTTCCTACCCCCAATGTGT	GGTCCTCAGTGTAGCCCAAG
ARGII	AAGCTGGCTTGATGAAAAGGC	GCGTGGATTCACTATCAGGTTGT
RPL-15	GCAGCCATCAGGTAAGCCAAG	AGCGGACCCTCAGAAGAAAGC

polyclonal antibodies by Santa Cruz Biotechnology (DBA, Segrate MI, Italy) in blocking solution. GAPDH or actin, detected with a polyclonal antibody (1:1000, Sigma-Aldrich), were employed as internal standard. Immunoreactivity was visualized with enhanced chemiluminescence (Millipore, Milano, Italy).

2.7. Arginine influx

For transport studies, experiments were carried out on cell cultures resulting from 3×10^4 cells seeded into 96-well trays. Cells, washed once with a modified bicarbonate-free Earle's Balanced Salt Solution (EBSS) buffered at pH 7.4 with 20 mM Tris/HCl, were incubated for 1 min in the same solution containing L-[^{3}H]arginine (5 μCi/ml, 100 μM) in the absence or in the presence of 2 mM leucine or 2 mM leucine + 2 mM lysine. The experiment was terminated by two rapid washes (<10 s) in ice-cold urea and cell monolayers were extracted in ethanol. The radioactivity in cell extracts was determined with Wallac Microbeta Trilux^2 liquid scintillation spectrometer (PerkinElmer). Protein content was determined directly in the well using a modified Lowry procedure [23]. Arginine influx is expressed as nmol/mg of protein/min. The contribution of system y^+L, the leucine-inhibitable component, was calculated from the difference between total influx and the influx measured in the presence of leucine, while the activity of system y^+ corresponds to the difference between the influx measured in the presence of leucine and the influx measured in the presence of leucine + lysine (see [27] for details).

2.8. L-arginine efflux

Cells, loaded with [^{3}H]-arginine (50 μM, 10 μCi/ml) in EBSS for 10 min, were washed twice with arginine-free EBSS (efflux medium) and incubated in 0.15 ml of the same solution in the absence or in the presence of 1 mM leucine [28]. At the indicated times efflux medium was removed, replaced with fresh medium and counted for radioactivity. Cell monolayers were extracted in 50 μl of ethanol and radioactivity in the extracts was counted so as to measure arginine remained within the cells. The percent of arginine efflux at each time was calculated with the formula: [efflux medium counts/(intracellular counts + total efflux media counts)], %. System y^+L-mediated efflux was calculated as the difference between efflux measured in the presence and in the absence of leucine at any time.

2.9. Statistical analysis

Student *t* test for unpaired data was used for statistical analysis of differences between control and each single treatment. Differences were considered significant when the associated probability value (p) was <0.05.

2.10. Materials

RPMI-1640 and endotoxin-free FBS were purchased from EuroClone (Milano, Italy). [L-2,3,4-^{3}H]arginine (45–70 Ci/mmol) and L-[^{14}C(U)]arginine (274 mCi/mmol) was obtained from PerkinElmer (Monza, Italy). IFNγ was purchased from VWR International PBI s.r.l. (Milano, Italy). Microtech s.r.l. (Napoli, Italy) provided Falcon plastic disposable. Sigma-Aldrich (Milano, Italy) was the source of gliadin, and, unless otherwise specified, of all the other chemicals.

3. Results

3.1. Effects of PTG and IFNγ on intracellular arginine accumulation and metabolism in RAW264.7 cells

Since arginine is the obliged substrate for the synthesis of nitric oxide (NO), we have first evaluated whether the treatment of murine macrophages with gliadin peptic–tryptic peptides (PTG) modifies the intracellular bioavailability of the amino acid. To this aim RAW264.7 cells were incubated in medium containing radiolabeled [^{14}C]arginine and treated for 24 h with PTG, in the absence and in the presence of IFNγ, the cytokine secreted by activated CD4$^+$ T-cells. At the end of the treatment intracellular radioactivity was measured under all experimental conditions. Results, shown in Fig. 1A, clearly indicate that the incubation with IFNγ, but not PTG, causes a significant accumulation of radiolabeled [^{14}C]arginine within the cells. The simultaneous addition of the two compounds further enhances the amount of intracellular radioactivity, which is doubled with respect to control, untreated cells, and significantly higher than in IFNγ-treated cells.

To evaluate which amount of the radioactivity measured within the cells is actually arginine or is rather due to a transformation of the amino acid into its metabolites, we have performed a chromatographic separation of the extracted pool and measured radioactivity in all elution fractions. The resulting plot (Fig. 1B) indicates that, beside the peak of arginine (T_r = 115 min), only another one, corresponding to polyamines (T_r = 130–140 min), is readily distinguishable in untreated, control cells. This result points to a basal activity of arginase in this cell model. As expected, the addition of IFNγ causes the appearance of the peak of citrulline (T_r = 47 min) that demonstrates the activation of NO pathway. No significant change of polyamines production is detectable under this condition. On the contrary, the treatment with PTG causes an increase of polyamines fractions that suggests the stimulation of arginase activity, and no change of citrulline peak. When IFNγ and PTG are simultaneously present, the effects of the two compounds appear reciprocally strengthened: peaks corresponding to citrulline and polyamines are, indeed, higher than those observed upon incubation with either compounds alone.

3.2. Effects of PTG and IFNγ on iNOS and arginase expression and activity in RAW264.7 cells

The metabolic profiles obtained for arginine under the different experimental conditions have been, then, validated at functional level.

Consistently with previously reported studies performed in mouse peritoneal macrophages and in RAW264.7 cells [29–32], the treatment of the cells with PTG, which causes per se an only modest increase of nitric oxide (NO) production, significantly strengthens the stimulatory effect of IFNγ (Fig. 2A), through the induction of iNOS enzyme at both gene and protein levels (Fig. 2B–C). As revealed by the time course analysis of *Nos2* mRNA, the synergic effect of PTG on IFNγ-dependent stimulation of gene expression is particularly evident for short term treatments (4 and 8 h), while lowering at 24 h.

The increased production of polyamines observed upon incubation with PTG prompt us to explore also the pathway of arginase (ARG). As shown in Fig. 3A, a significant stimulation of the enzymatic activity occurs upon treatment with PTG alone, but not with IFNγ. When the

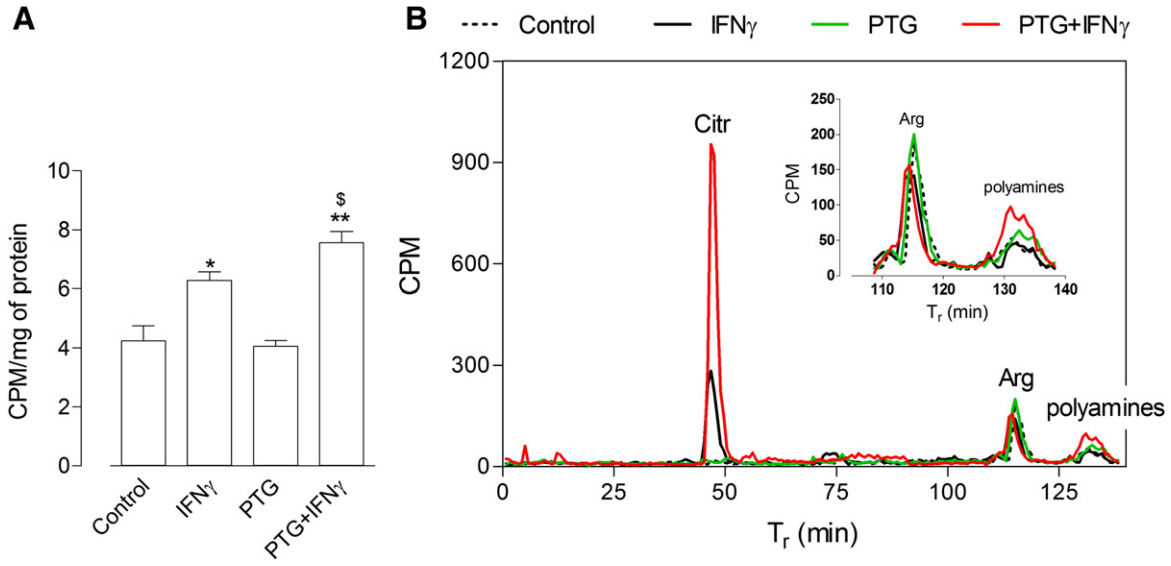

Fig. 1. *Effects of IFNγ and PTG on arginine accumulation and metabolism.* RAW264.7 cells were either untreated (control) or treated with 10 ng/ml IFNγ, 1 mg/ml PTG or both in complete growth medium added with L-[^{14}C(U)]arginine (5 μCi/ml). After 24 h, the intracellular amino acid pool was extracted. Radioactivity under each condition was counted directly (Panel A) or separated in 192 fractions through HPLC, measured in each fraction and plotted as function of retention time (T_r), as described in Methods (Panel B). A magnification of peaks corresponding to arginine (Arg) and polyamines is given in the Insert. Data of Panel A are means ± SEM of 4 experiments, one of which is shown in Panel B. *$p < 0.05$, **$p < 0.01$ vs. control, untreated cells; $^{\$}p < 0.05$ vs. IFNγ-treated cells.

cytokine is added to PTG-treated cells, a further stimulation of ARG activity can be observed. The activation of arginase is associated to an increased expression of the enzyme at both mRNA and protein level (Fig. 3B–C). In particular, a significant increase of arginase transcript is appreciable starting from 8 h of treatment with PTG. The simultaneous presence of IFNγ and PTG causes a late hyper-expression of the mRNA whose levels, after 24 h, becomes at least five folds higher than in PTG-treated cells (Fig. 3B). The Western Blot analysis of the protein confirms these results: poorly detectable in control and in IFNγ-treated cells, ARG expression becomes evident upon treatment with PTG and, even more, in the simultaneous presence of IFNγ (Fig. 3C).

In order to pursue the metabolic pathway of arginase products, we investigated the expression of ornithine decarboxylase (*Odc*), the enzyme that converts ornithine to polyamines (putrescine, spermine and spermidine) and ornithine aminotransferase (*Oat*) that transforms ornithine to glutamate and proline. Results, presented in Fig. 4, show that *Odc* expression is significantly stimulated by PTG, confirming the activation of the pathway leading to polyamines production; IFNγ does not modify PTG effect. On the contrary, the expression of *Oat* is slightly decreased by IFNγ, both in the absence and in presence of PTG.

3.3. Effects of IFNγ and PTG on arginine transport activity in RAW264.7 cells

A preliminary characterization of arginine transport in RAW264.7 has been performed, according to the strategy already used in other cell models [24,25,27]. As shown in Fig. 5A, the addition of leucine, a substrate of system y^+L in the presence of Na^+, inhibits arginine influx by more than 70% indicating that most of arginine uptake occurs through this route. The further addition of lysine produces a modest further decrease of arginine transport, thus indicating a marginal contribution of system y^+ in these cells.

The effects of PTG and IFNγ on the discriminated arginine transport has been then evaluated. As shown in Fig. 5B, the activity of system y^+ is not modified by any of the experimental treatments, whereas the activity of system y^+L decreases upon incubation with IFNγ, both in the absence or in presence of PTG.

Under the same experimental conditions, the efflux of arginine through system y^+L has also been evaluated. While control cells exhibit a marked system y^+L-mediated arginine efflux, in IFNγ–treated cells the efflux of the amino acid appears significantly reduced. After 1 min, indeed, control cultures release about 60% of pre-accumulated arginine, while IFNγ–treated cells only 20%. The simultaneous addition of PTG, that per se only slightly reduces arginine efflux, further strengthens the effect of IFNγ (Fig. 6).

The effects of the compounds have been assessed also on the expression of genes coding for arginine transporters. As shown in Fig. 7, the treatment with PTG does not change the expression of any of the arginine carriers, while IFNγ modifies both system y^+- and system y^+L-related transporters. In particular, the expression of Slc7a2 and Slc7a7 are significantly induced by the cytokine, while Slc7a1 and Slc7a6 appear clearly decreased.

3.4. Effects of PTG on arginase expression in human monocytes

In additional experiments, the effect of PTG has been preliminary investigated on healthy human monocytes. As shown in Fig. 8A, the treatment with PTG causes the induction of arginase mRNA in monocytes isolated from six different donors, with expression levels 2 to 12 fold higher than in control, untreated cells. Consistently, an increased expression of the enzyme at protein level can be observed in PTG-treated monocytes, as confirmed by Western Blot analysis (Fig. 8B). Comparable results have been obtained employing a synthetic peptide whose sequence corresponds to a part of the 33-mer gliadin peptide known to be immunogenic for humans [33] (data not shown).

4. Discussion

In the present study we provide evidence, for the first time, about the ability of gliadin to stimulate arginine metabolism through the activation of arginase pathway in RAW264.7 mouse macrophages and in human monocytes.

Previous studies have demonstrated that gluten/gliadin and their proteolitic fragments strengthens the stimulatory effect of IFNγ on

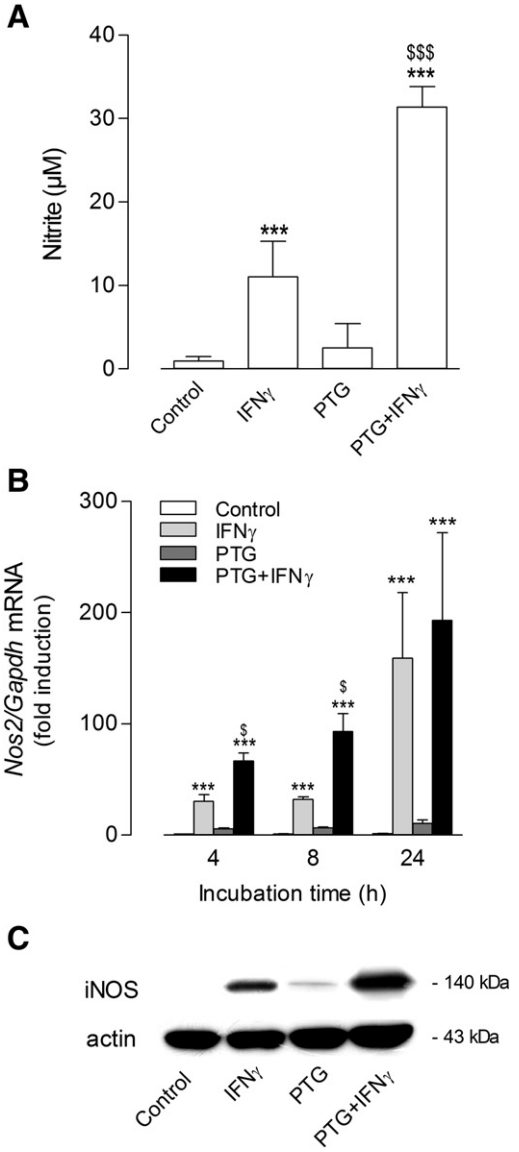

Fig. 2. *Effects of IFNγ and PTG on nitric oxide production and iNOS expression.* Cells were either untreated (control) or treated with 10 ng/ml IFNγ, 1 mg/ml PTG or both in complete growth medium. After 24 h the production of nitric oxide (NO) was assessed through the quantization of nitrites in the incubation medium (Panel A). At the times indicated, the expression of *Nos2* was determined through qRT-PCR analysis and shown, after normalization to *Gapdh*, as fold induction relatively to control, untreated cells (=1) (Panel B). Data are means ± SEM of 3 experiments, each performed in triplicate. After 24 h-treatment, Western Blot analysis was performed using anti-iNOS and anti-actin antibodies (see Methods). A representative Western Blot is shown (Panel C). The experiment was repeated three times with comparable results. $^{***}p < 0.001$ vs. control, untreated cells; $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. IFNγ-treated cells at the corresponding time-point.

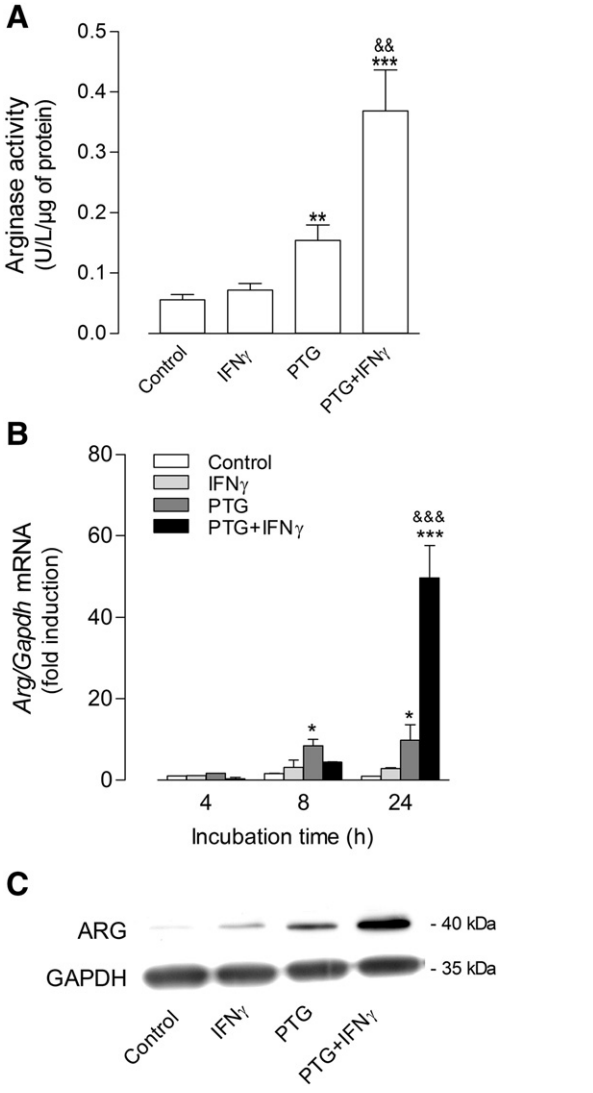

Fig. 3. *Effects of IFNγ and PTG on arginase pathway.* RAW264.7 were left untreated (control) or incubated in the presence of 10 ng/ml IFNγ, 1 mg/ml PTG or both in complete growth medium. After 24 h arginase activity was measured as described in Methods (Panel A). Data are means ± SEM of 3 experiments, each performed in triplicate. The expression of *Arg* was determined through RT-qPCR at the times indicated (Panel B). Data, shown after normalization to *Gapdh* as fold induction relatively to control, untreated cells (=1), are means ± SEM of 3 experiments, each performed in triplicate. After 24 h-treatment, Western Blot analysis was performed using anti-ARG and anti-GAPDH antibodies (see Methods). A representative Western Blot is shown (Panel C). The experiment was repeated three times with comparable results. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control, untreated cells; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. PTG-treated cells at the corresponding time-point.

iNOS expression and, thus, on arginine conversion to nitric oxide (NO) in mouse peritoneal macrophages [29,31]. Accordingly, high levels of NO derivatives are detectable in vivo, in the serum and urine of children with celiac disease, and correlate with an increased expression of iNOS in the small intestine [15,16,34,35]. Although the precise mechanisms through which NO may directly or indirectly induce tissue damage in CD are not completely understood [14], those findings point to a pathogenetic role of NO in the onset of celiac disease.

By addressing the whole metabolic profile of arginine in RAW264.7 cells, we show here that, besides the expected conversion of the amino acid to citrulline and NO upon incubation with IFNγ, another metabolic pathway is activated in the presence of PTG that leads to an increased production of polyamines (see Fig. 1). As demonstrated at both molecular and functional levels, this effect is attributable to the activation of arginase, the enzyme that converts arginine to ornithine and urea, and to the subsequent induction of *Odc*, which synthesizes polyamines from ornithine (see Figs. 3 and 4).

Interestingly, the effects of IFNγ and PTG on arginine metabolism, which diverge when the two compounds are employed alone, appear mutually strengthened during a combined treatment (see Figs. 2–4). Thus, when RAW264.7 cells are exposed to PTG and IFNγ, they present features of both classically M1 and alternatively M2 activated macrophages. The pro-inflammatory (M1) phenotype, likely responsible for tissue damage, is mainly ascribable to IFNγ, while the anti-inflammatory (M2) phenotype is induced by PTG and leads to the synthesis of polyamines, well known modulators of cell proliferation and differentiation.

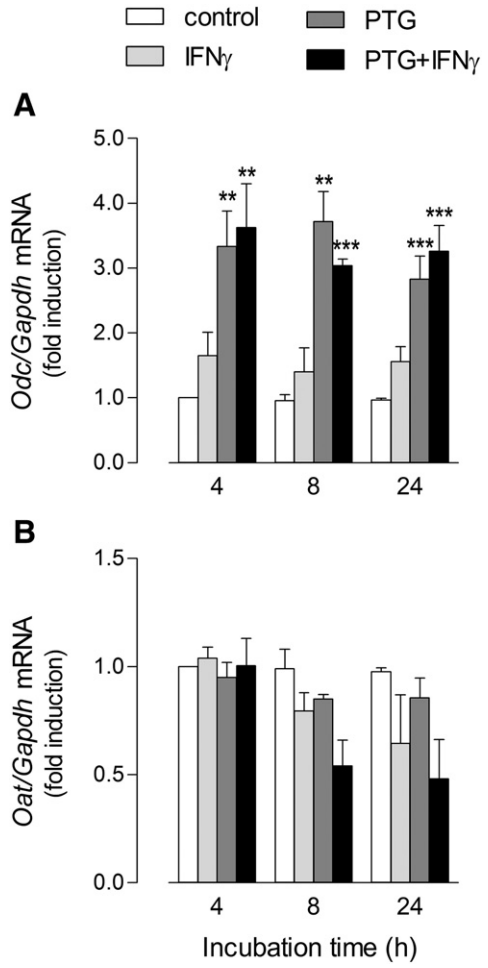

Fig. 4. *Effects of IFNγ and PTG on Odc and Oat expression.* RAW264.7 were left untreated (control) or incubated in the presence of 10 ng/ml IFNγ, 1 mg/ml PTG or both in complete growth medium. At the indicated times, the expression of *Odc* (Panel A) and *Oat* (Panel B) were determined through RT-qPCR as described in Methods. Data, shown after normalization to *Gapdh* as fold induction relatively to control, untreated cells (=1), are means ± SEM of 3 experiments, each performed in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control, untreated cells at the corresponding time-point.

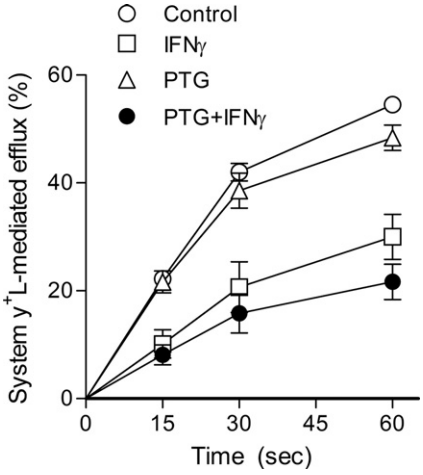

Fig. 6. *Effects of IFNγ and PTG on arginine efflux through system y^+L.* RAW264.7 were left untreated (control) or treated with 10 ng/ml IFNγ, 1 mg/ml PTG or both in complete growth medium. After 24 h cells were loaded with L-[^{3}H]arginine (50 μM; 10 μCi/ml) for 10 min. After washing, cells were incubated in 100 μl EBSS in the absence or in the presence of 1 mM leucine and radioactivity in the efflux medium was measured at the indicated times. At each time, arginine efflux corresponds to the fraction in % of the pre-accumulated [^{3}H]arginine escaped from the cells. System y^+L-mediated arginine efflux is calculated as described in Methods. Data are means ± SEM of 3 independent experiments, each performed in triplicate.

The simultaneous induction of iNOS and arginase is quite uncommon in macrophages, as the induction of one enzyme usually leads to the inhibition of the other due to the competition for the common substrate arginine. However, it is also known that the upregulation of arginase expression in macrophages is employed by several pathogens as a strategy to limit iNOS-dependent host defense [36]. For example, studies concerning gastritis induced by *Helicobacter pylori* have shown that the bacterial infection induces the expression of both arginase II (*Arg2*) and *Odc* in macrophages in vitro and in vivo, and causes an increase in polyamine levels [37]. The same study also demonstrated that the production of NO derived from iNOS in RAW 264.7 cells stimulated by *H. pylori* depends on the arginine concentration in the culture medium. Under our experimental conditions, i.e. in the presence of non-limiting concentrations of arginine, the activation of the two metabolic pathways takes advantage from the increased availability of the amino acid inside the cells (see Fig. 1). This condition can be achieved through either an increased influx or a decreased efflux through the plasma membrane. Previous studies attributed a central role to system y^+ and, specifically, to Slc7a2/CAT2 in providing arginine for the synthesis of NO in activated macrophages [37,38]. Accordingly, our results indicate that the incubation with IFNγ produces a significant induction of

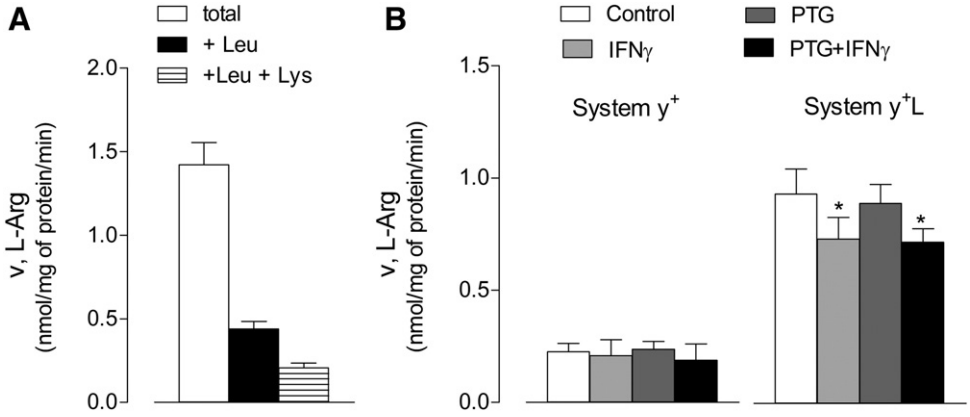

Fig. 5. *Arginine transport in RAW264.7: effects of IFNγ and PTG.* In Panel A a preliminary characterization of arginine influx has been performed as described in Methods. Arginine uptake was assayed in untreated RAW264.7 with 1-min incubations in EBSS supplemented with L-[^{3}H]arginine (50 μM; 5 μCi/ml) in the absence (total) or in the presence of 2 mM leucine or 2 mM leucine + 2 mM lysine, as indicated. Data are means ± SEM of 3 experiments, each performed in quadruplicate. In Panel B RAW264.7 were left untreated (control) or incubated in the presence of 10 ng/ml IFNγ, 1 mg/ml PTG or both for 24 h in complete growth medium. At this time arginine uptake was measured as described in Panel A and the activity of system y^+ and y^+L was calculated as described in Methods. Data are means ± SEM of 3 experiments, each performed in quadruplicate $^*p < 0.05$ vs. control, untreated cells.

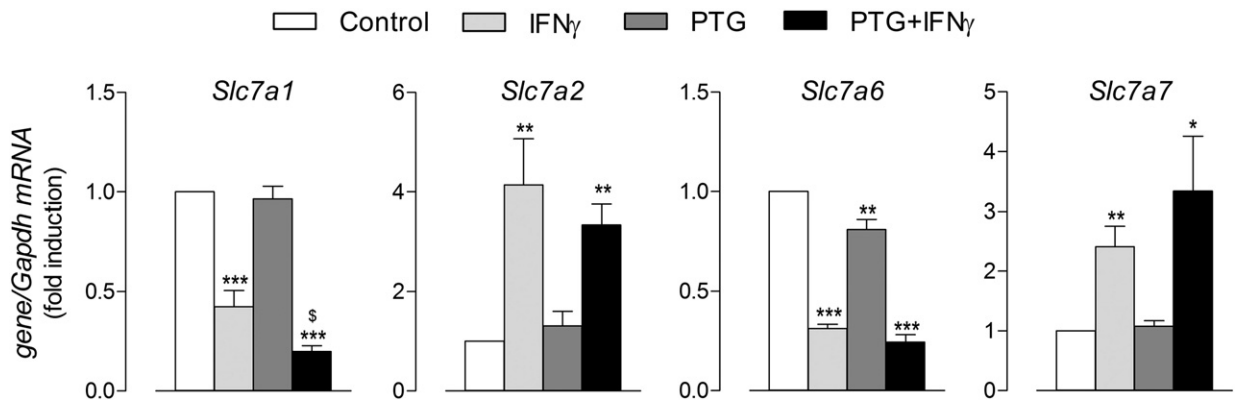

Fig. 7. *Effects of IFNγ and PTG on arginine transporters.* Cells were left untreated (control) or treated with 10 ng/ml IFNγ, 1 mg/ml PTG or both in complete growth medium. After 24 h the expression of the indicated genes was evaluated with qRT-PCR and shown, after normalization to *Gapdh*, as fold induction relatively to control, untreated cells (=1). Data are means ± SEM of 3 experiments, each performed in triplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control, untreated cells; $^{\$}p < 0.05$ vs. IFNγ-treated cells.

Slc7a2/CAT2 expression (see Fig. 7); however, in our hands, this stimulation results ineffective at functional level, given that the low basal activity of system y^+ is not modified by the addition of the cytokine, both in the absence and in the presence of gliadin (see Fig. 5). Since a concomitant decrease of Slc7a1/CAT1 expression is detectable in IFNγ-treated cells (see Fig. 7), we can speculate that the opposite effects of the cytokine on the expression of the two genes cancel each other, leaving the activity of system y^+ unaltered under any experimental condition.

Conversely, our findings clearly indicate that the treatment with PTG and IFNγ markedly affects arginine transport through system y^+L (see Figs. 5 and 6). Since system y^+L is known to physiologically mediate arginine efflux, rather than influx, exchanging intracellular arginine with extracellular neutral amino acid plus sodium [19–21], the decrease of its activity upon incubation with IFNγ and, even more, with IFNγ + PTG, justifies the increased amount of intracellular arginine observed. At molecular level, this effect is paralleled by an impressive decrease of Slc7a6/y^+LAT2 and a concomitant induction of Slc7a7/y^+LAT1 by IFNγ (see Fig. 7). A comparable stimulation of Slc7a7/y^+LAT1 has been previously reported by our group in freshly isolated human monocytes [39] in which, however, a parallel increase of system y^+L activity was observed. In the present study, IFNγ and even more IFNγ + PTG, causing a marked decrease of Slc7a6 expression, determines a decrease in system y^+_L-mediated arginine transport, demonstrating that, unlike human monocytes, in mouse macrophages the activity of system y^+L is attributable to Slc7a6 more than Slc7a7 expression. Our results point then to a central role of Slc7a6 in the regulation of arginine availability in RAW264.7.

Interestingly, preliminary experiments performed on healthy human monocytes confirmed the results obtained in mouse macrophages concerning the effects of PTG. The treatment causes, indeed, an evident induction of arginase expression at both gene and protein levels (Fig. 8), even if a great variability of response can be observed among the different donors enrolled. On the contrary, unlike mouse macrophages, the simultaneous addition of IFNγ in these cells does not strengthen the effect of PTG (results not shown).

In conclusion our results demonstrate that also arginase, besides iNOS, is a target of PTG in monocyte/macrophage cells. Recent literature evidences ascribe a role to polyamines in the control of epithelial permeability [40]. In light of this study and of our results we can thus speculate that arginase activation and polyamine production, triggered in innate immune cells by ingested gluten, might contribute to the impairment of intestinal epithelial permeability in vivo. Whether these findings may have an implication in the comprehension of CD pathogenesis appears intriguing, although it deserves further investigations.

Conflicts of interest

The authors declare that they have no competing interests.

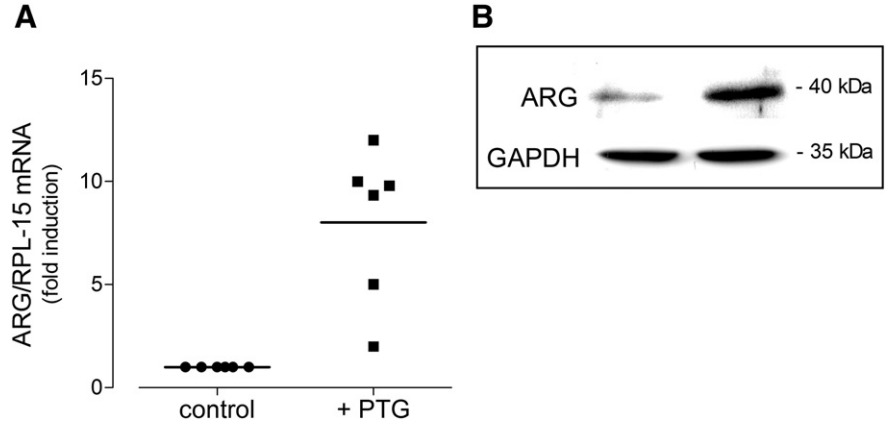

Fig. 8. *Effects of PTG on arginase expression in human monocytes.* Freshly isolated human monocytes were incubated in complete growth medium in the absence or in the presence of 1 mg/ml PTG (Panel A, n = 6 healthy subjects). After 24 h, arginase (ARG) expression was determined through RT-qPCR. Data are shown after normalization to RPL-15 as fold induction relatively to control, untreated cells (=1). A representative Western Blot analysis, performed using anti-ARG and anti-GAPDH antibodies (see Methods), is shown in Panel B.

References

[1] J.S. Trier, Diagnosis of celiac sprue, Gastroenterology 115 (1998) 211–216.
[2] P.H. Green, C. Cellier, Celiac disease, N. Engl. J. Med. 357 (2007) 1731–1743.
[3] B. Diosdado, E. van Oort, C. Wijmenga, "Coelionomics": towards understanding the molecular pathology of coeliac disease, Clin. Chem. Lab. Med. 43 (2005) 685–695.
[4] F. Koning, D. Schuppan, N. Cerf-Bensussan, L.M. Sollid, Pathomechanisms in celiac disease, Best Pract. Res. Clin. Gastroenterol. 19 (2005) 373–387.
[5] L.M. Sollid, G. Markussen, J. Ek, H. Gjerde, F. Vartdal, E. Thorsby, Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer, J. Exp. Med. 169 (1989) 345–350.
[6] A. Spurkland, K.S. Ronningen, T. Leivestad, F. Vartdal, E. Thorsby, HLA-DR-DQ haplotype frequencies in a Norwegian population, Transplant. Proc. 24 (1992) 298–299.
[7] B. Jabri, L.M. Sollid, Tissue-mediated control of immunopathology in coeliac disease, Nat. Rev. Immunol. 9 (2009) 858–870.
[8] L. Greco, R. Romino, I. Coto, N. Di Cosmo, S. Percopo, M. Maglio, F. Paparo, V. Gasperi, M.G. Limongelli, R. Cotichini, C. D'Agate, N. Tinto, L. Sacchetti, R. Tosi, M.A. Stazi, The first large population based twin study of coeliac disease, Gut 50 (2002) 624–628.
[9] A. Di Sabatino, G.R. Corazza, Coeliac disease, Lancet 373 (2009) 1480–1493.
[10] K.E. Thomas, A. Sapone, A. Fasano, S.N. Vogel, Gliadin stimulation of murine macrophage inflammatory gene expression and intestinal permeability are MyD88-dependent: role of the innate immune response in Celiac disease, J. Immunol. 176 (2006) 2512–2521.
[11] J. Cinova, L. Palova-Jelinkova, L.E. Smythies, M. Cerna, B. Pecharova, M. Dvorak, P. Fruhauf, H. Tlaskalova-Hogenova, P.D. Smith, L. Tuckova, Gliadin peptides activate blood monocytes from patients with celiac disease, J. Clin. Immunol. 27 (2007) 201–209.
[12] I. Daniels, D. Cavill, I.A. Murray, R.G. Long, Elevated expression of iNOS mRNA and protein in coeliac disease, Clin. Chim. Acta 356 (2005) 134–142.
[13] K. Holmgren Peterson, K. Falth-Magnusson, K.E. Magnusson, L. Stenhammar, T. Sundqvist, Children with celiac disease express inducible nitric oxide synthase in the small intestine during gluten challenge, Scand. J. Gastroenterol. 33 (1998) 939–943.
[14] G. Ferretti, T. Bacchetti, S. Masciangelo, L. Saturni, Celiac disease, inflammation and oxidative damage: a nutrigenetic approach, Nutrients 4 (2012) 243–257.
[15] T. Sundqvist, P. Laurin, K. Falth-Magnusson, K.E. Magnusson, L. Stenhammar, Significantly increased levels of nitric oxide products in urine of children with celiac disease, J. Pediatr. Gastroenterol. Nutr. 27 (1998) 196–198.
[16] E.A. van Straaten, L. Koster-Kamphuis, I.M. Bovee-Oudenhoven, R. van der Meer, P.P. Forget, Increased urinary nitric oxide oxidation products in children with active coeliac disease, Acta Paediatr. 88 (1999) 528–531.
[17] M.B. Grisham, K.P. Pavlick, F.S. Laroux, J. Hoffman, S. Bharwani, R.E. Wolf, Nitric oxide and chronic gut inflammation: controversies in inflammatory bowel disease, J. Investig. Med. 50 (2002) 272–283.
[18] F. Verrey, E.I. Closs, C.A. Wagner, M. Palacin, H. Endou, Y. Kanai, CATs and HATs: the SLC7 family of amino acid transporters, Pflugers Arch. 447 (2004) 532–542.
[19] A. Broer, C.A. Wagner, F. Lang, S. Broer, The heterodimeric amino acid transporter 4F2hc/y^+LAT2 mediates arginine efflux in exchange with glutamine, Biochem. J. 349 (Pt 3) (2000) 787–795.
[20] B.M. Rotoli, O. Bussolati, R. Sala, G.C. Gazzola, V. Dall'Asta, The transport of cationic amino acids in human airway cells: expression of system y^+L activity and transepithelial delivery of NOS inhibitors, FASEB J. 19 (2005) 810–812.
[21] B.M. Rotoli, E.I. Closs, A. Barilli, R. Visigalli, A. Simon, A. Habermeier, N. Bianchi, R. Gambari, G.C. Gazzola, O. Bussolati, V. Dall'Asta, Arginine transport in human erythroid cells: discrimination of CAT1 and 4F2hc/y^+LAT2 roles, Pflugers Arch. 458 (2009) 1163–1173.
[22] A. Barilli, R. Visigalli, B.M. Rotoli, O. Bussolati, G.C. Gazzola, A. Parolari, V. Dall'Asta, Radiochemical high-performance liquid chromatography detection of arginine metabolism in human endothelial cells, Anal. Biochem. 424 (2012) 156–161.
[23] G.C. Gazzola, V. Dall'Asta, R. Franchi-Gazzola, M.F. White, The cluster-tray method for rapid measurement of solute fluxes in adherent cultured cells, Anal. Biochem. 115 (1981) 368–374.
[24] R. Sala, B.M. Rotoli, E. Colla, R. Visigalli, A. Parolari, O. Bussolati, G.C. Gazzola, V. Dall'Asta, Two-way arginine transport in human endothelial cells: TNF-alpha stimulation is restricted to system y(+), Am. J. Physiol. Cell Physiol. 282 (2002) C134–C143.
[25] A. Barilli, B.M. Rotoli, R. Visigalli, O. Bussolati, G.C. Gazzola, V. Dall'Asta, Arginine transport in human monocytic leukemia THP-1 cells during macrophage differentiation, J. Leukoc. Biol. 90 (2011) 293–303.
[26] R. Visigalli, A. Barilli, A. Parolari, R. Sala, B.M. Rotoli, O. Bussolati, G.C. Gazzola, V. Dall'Asta, Regulation of arginine transport and metabolism by protein kinase Calpha in endothelial cells: stimulation of CAT2 transporters and arginase activity, J. Mol. Cell. Cardiol. 49 (2010) 260–270.
[27] B.M. Rotoli, V. Dall'asta, A. Barilli, R. D'Ippolito, A. Tipa, D. Olivieri, G.C. Gazzola, O. Bussolati, Alveolar macrophages from normal subjects lack the NOS-related system y+ for arginine transport, Am. J. Respir. Cell Mol. Biol. 37 (2007) 105–112.
[28] A. Barilli, B.M. Rotoli, R. Visigalli, O. Bussolati, G.C. Gazzola, R. Gatti, C. Dionisi-Vici, D. Martinelli, B.M. Goffredo, M. Font-Llitjos, F. Mariani, M. Luisetti, V. Dall'asta, Impaired phagocytosis in macrophages from patients affected by lysinuric protein intolerance, Mol. Genet. Metab. 105 (2012) 585–589.
[29] D. De Stefano, M.C. Maiuri, B. Iovine, A. Ialenti, M.A. Bevilacqua, R. Carnuccio, The role of NF-kappaB, IRF-1, and STAT-1alpha transcription factors in the iNOS gene induction by gliadin and IFN-gamma in RAW 264.7 macrophages, J. Mol. Med. 84 (2006) 65–74.
[30] M.C. Maiuri, D. De Stefano, G. Mele, B. Iovine, M.A. Bevilacqua, L. Greco, S. Auricchio, R. Carnuccio, Gliadin increases iNOS gene expression in interferon-gamma-stimulated RAW 264.7 cells through a mechanism involving NF-kappa B, Naunyn Schmiedeberg's Arch. Pharmacol. 368 (2003) 63–71.
[31] L. Tuckova, Z. Flegelova, H. Tlaskalova-Hogenova, Z. Zidek, Activation of macrophages by food antigens: enhancing effect of gluten on nitric oxide and cytokine production, J. Leukoc. Biol. 67 (2000) 312–318.
[32] L. Tuckova, J. Novotna, P. Novak, Z. Flegelova, T. Kveton, L. Jelinkova, Z. Zidek, P. Man, H. Tlaskalova-Hogenova, Activation of macrophages by gliadin fragments: isolation and characterization of active peptide, J. Leukoc. Biol. 71 (2002) 625–631.
[33] L. Shan, O. Molberg, I. Parrot, F. Hausch, F. Filiz, G.M. Gray, L.M. Sollid, C. Khosla, Structural basis for gluten intolerance in celiac sprue, Science 297 (2002) 2275–2279.
[34] L. Hogberg, C. Webb, K. Falth-Magnusson, T. Forslund, K.E. Magnusson, L. Danielsson, A. Ivarsson, O. Sandstrom, T. Sundqvist, Children with screening-detected coeliac disease show increased levels of nitric oxide products in urine, Acta Paediatr. 100 (2011) 1023–1027.
[35] I.A. Murray, I. Daniels, K. Coupland, J.A. Smith, R.G. Long, Increased activity and expression of iNOS in human duodenal enterocytes from patients with celiac disease, Am. J. Physiol. Gastrointest. Liver Physiol. 283 (2002) G319–G326.
[36] R. Chaturvedi, T. de Sablet, L.A. Coburn, A.P. Gobert, K.T. Wilson, Arginine and polyamines in *Helicobacter pylori*-induced immune dysregulation and gastric carcinogenesis, Amino Acids 42 (2012) 627–640.
[37] R. Chaturvedi, M. Asim, S. Hoge, N.D. Lewis, K. Singh, D.P. Barry, T. de Sablet, M.B. Piazuelo, A.R. Sarvaria, Y. Cheng, E.I. Closs, R.A. Casero Jr., A.P. Gobert, K.T. Wilson, Polyamines impair immunity to *Helicobacter pylori* by inhibiting L-arginine uptake required for nitric oxide production, Gastroenterology 139 (2010) 1686–1698 (1698 e1681-1686).
[38] D.K. Kakuda, M.J. Sweet, C.L. Mac Leod, D.A. Hume, D. Markovich, CAT2-mediated L-arginine transport and nitric oxide production in activated macrophages, Biochem. J. 340 (Pt 2) (1999) 549–553.
[39] B.M. Rotoli, O. Bussolati, R. Sala, A. Barilli, E. Talarico, G.C. Gazzola, V. Dall'Asta, INFgamma stimulates arginine transport through system y^+L in human monocytes, FEBS Lett. 571 (2004) 177–181.
[40] A. Orlando, M. Linsalata, M. Notarnicola, V. Tutino, F. Russo, *Lactobacillus* GG restoration of the gliadin induced epithelial barrier disruption: the role of cellular polyamines, BMC Microbiol. 14 (2014) 19.