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*J Immunol* 2006; 177:4178-4186; doi: 10.4049/jimmunol.177.6.4178
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Gliadin-Specific Type 1 Regulatory T Cells from the Intestinal Mucosa of Treated Celiac Patients Inhibit Pathogenic T Cells

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Celiac disease (CD) is a common intestinal disorder caused by altered immune responses to dietary gliadin present in wheat gluten and to proteins from the related cereals barley and rye (1). The hallmark of CD is enteropathy of the small intestine, characterized by flattening of the villous architecture and massive infiltration of T cells which release proinflammatory cytokines, such as IL-2 and IFN-γ, in response to gliadin (1, 2). Currently, the only available treatment for CD is withdrawal of gliadin from the diet, and in patients following a strict gluten-free diet the intestinal architecture is completely restored. Nevertheless, even after many years of a gluten-free diet, CD patients never acquire tolerance to gliadin, and re-exposure to this Ag results in recurrence of acute disease. These findings suggest that pathogenic T cells remain in the intestine throughout the life of treated CD patients.

The mechanisms responsible for normal intestinal homeostasis toward harmless intestinal Ags such as gliadin are not well understood (3). Recently, several studies have suggested that the immunosuppressive cytokines such as IL-10 and TGF-β have an important role in maintaining intestinal tolerance (4–11). For example, mice which are genetically deficient for IL-10 develop a severe form of enterocolitis, similar to human inflammatory bowel disease (IBD) (12), and have increased susceptibility to autoimmune diseases such as rheumatoid arthritis (13) and experimental autoimmune encephalomyelitis (14). In contrast, colitis, which develops in SCID mice after transfer of CD4+CD45RBhigh T cells, can be prevented by IL-10 (15).

The immunomodulatory properties of IL-10 are at least partially due to its role in the differentiation of a subset of CD4+ T regulatory cells, known as type 1 regulatory T cells (Tr1) (16, 17). Tr1 cells possess a unique profile of cytokine production characterized by high levels of IL-10 and TGF-β, normal levels of IFN-γ, very low amounts of IL-2, and little or no IL-4 (18). Importantly, Tr1 cells down-regulate naive and memory T cell responses upon local secretion of IL-10 and TGF-β (18). Thus, via the induction of Tr1 cells, IL-10 has a central role in maintaining intestinal tolerance. It is possible that a dysfunction or deficiency in Tr1 cells is involved in the breakdown of tolerance in intestinal disorders such as CD and IBD (19–21).

Interestingly, we and others have recently shown that, in addition to proinflammatory cytokines, the inflamed CD mucosa also contains high levels of T cell-derived IL-10 when compared with treated CD or normal donors (22, 23). It is possible that in acute CD, although IL-10 is produced, the levels are insufficient to down-regulate the massive Th1/Th0 immune responses induced by gliadin. Indeed, addition of exogenous IL-10 to mucosal cultures from treated CD patients can suppress gliadin-induced T cell activation and cytokine production ex vivo. (22) Therefore, similar to
what has been described with alloantigens, (24), IL-10 can induce hyporesponsiveness to food Ags, and under noninflammatory conditions may also induce the differentiation of Tr1 cells in the gut.

Although it is widely assumed that IL-10 and Tr1 cells have a central role in regulating responses to intestinal Ags, including those from commensal bacteria and dietary proteins, (6, 7, 20), to date the derivation of dietary Ag-specific Tr1 cells from human intestinal mucosa has not been described. We therefore investigated whether gliadin-specific Tr1 cells are present in the mucosa of CD patients and whether they can mediate suppression of T cell responses to dietary gliadin.

Materials and Methods

Organ culture and generation of gliadin-specific T cell lines (TCL)

Jejunal explants were obtained from 12 CD patients (7 treated, mean age, 31.1 years; range, 18–49 years; 5 untreated, mean age, 23.6 years; range, 10–34 years) and 5 healthy subjects (mean age, 25.8 years; range, 10–50 years). Both treated CD and control subjects were serum negative for anti-endomysium Abs and had a noninflamed mucosa. All subjects gave informed consent to the proposed study. Mucosal explants were either immediately digested or cultured for 24 h in vitro with a peptic-trypsin digest of purified tissue (cub biopsy) or presence of recombinant human IL-10 (IL-10 biopsy) (50 ng/ml; R&D Systems). Biopsies were digested with 1 mg/ml collagenase A for 1 h at 37°C. Cells obtained from fresh biopsies and ctb biopsies were stimulated with 1 × 10^6 irradiated (3500 rad) autologous PBMC and 50 µg/ml translglutenaminase-treated peptid-tryptic-gliadin (indicated as gliadin) for an additional 7 days. Cells from the IL-10 biopsy were stimulated with gliadin and PBMC in the presence of IL-10 (200 ng/ml; IL-10-TCL). To generate short-term TCLs, cells were restimulated with irradiated autologous PBMC and gliadin (50 µg/ml). Every 3–4 days, medium (X-Vivo15 medium supplemented with 5% AB+ pooled human serum and antibiotics all provided from BioWhittaker) was replenished and IL-15 (10 ng/ml) was added. After 18–21 days of culture, TCLs were assayed for gliadin specificity and either cryopreserved or kept in culture as long-term TCLs by restimulating with gliadin followed by cyclic (14 days) restimulation with a feeder-cell mixture containing PHA (1 µg/ml) and IL-2 (100 IU/ml) as previously described (25).

IFN-γ ELISPOT assay

TCLs were tested for gliadin recognition by an IFN-γ ELISPOT assay as previously described (22). Briefly, 0.3–0.5 × 10^5 T cells were plated in the presence of 1 × 10^6 autologous PBMC that had been pulsed overnight with medium or gliadin (50 µg/ml) in 96-well plates (Millipore) coated with anti-IFN-γ mAbs. In the experiments with neutralizing mAbs, T cells were preincubated with anti-IL-10R (10 µg/ml, clone 1D11; R&D Systems) or anti-TGF-β (10 µg/ml, clone 1D11; R&D Systems) mAbs before addition of gliadin-pulsed APC. All experiments were performed in duplicate. After 36–40 h of incubation, spot-forming cells (SFC) were counted by an immunospot analyzer (A.E.L. VIS).

Cloning of intestinal TCLs

Ctl-TCLs and IL-10-TCLs from two treated CD patients (CD041001, male, 28 years old, DR3/5, DQ2/DQ90401 female, 27 years old, DR3/14, DQ2) and the normal donor ND090102 (male, 31 years old, DR5/D87, DQ2) were plated at 1 cell/well in the presence of 5 × 10^4 allogeneic irradiated (6000 rad) PBMC, 5 × 10^4 irradiated (10000 rad) JY (an EBV-lymphoblastoid cell line (LCL)), and 0.05 µg/ml PHA (Roche). At days 3, 7, and 10, cells were fed with fresh medium containing IL-2 (40 IU/ml), T cell clones (TCCs) were screened after 14 days for IL-10 and IL-4 production following activation of one-half of each well with immobilized anti-CD3 (10 µg/ml) and soluble anti-CD28 (1 µg/ml) mAbs in a final volume of 200 µl of complete medium. IL-10-producing TCCs were evaluated by cyclic restimulation with feeder cells as described above.

Proliferation and cytokine production

Ten to 14 days after restimulation, TCLs and TCCs were analyzed for proliferation and/or cytokine production in response to gliadin and/or polyclonal activation. Cells (0.3–0.5 × 10^5) were plated in 96-well round-bottom plates in the presence of 0.3–0.5 × 10^5 irradiated autologous EBV-LCLs, which had been pulsed overnight with gliadin (50 µg/ml) or medium alone, or stimulated with immobilized anti-CD3 (10 µg/ml) and soluble anti-CD28 (1 µg/ml) mAbs in 200 µl of complete medium. After 48 h of incubation, T cells were pulsed for 16 h with 1 µCi/well [3H]thymidine (Amersham Pharmacia).

In parallel, supernatants from TCCs were collected after 24 h (for IL-2) or 48 h (for IL-10, IL-4, IFN-γ, and TGF-β). For detection of IL-2, IL-10, IL-4, or IFN-γ, specific capture and detection mAbs were used (BD Pharmingen) and ELISAs were performed as described previously (25). Amounts of TGF-β were determined by a commercial ELISA kit (R&D Systems). Sensitivity of assays was as follows: IL-10, 9 pg/ml; IL-4, 9 pg/ml; IL-2, 15 pg/ml; IFN-γ, 62 pg/ml; and TGF-β, 62 pg/ml.

TCLs and TCCs were considered responsive to gliadin when proliferation and/or IFN-γ (ELISPOT/ELISA) production were 2-fold greater than those of cells cultured in medium alone.

For suppression experiments, CD3-depleted PBMC from DQ2+ healthy donors were used as APC. Increasing numbers of effector Th0 or Tr1 cell clones (up to 1 × 10^5) were added to 0.5 × 10^5 effecter Th0 cell clones and cocultured in the presence of 0.5 × 10^5 APC, which had been pulsed overnight with gliadin. Proliferation was evaluated after 48 h as described above.

Statistical analysis

Data are expressed as mean ± SD, mean (range) or median (upper and lower quartile) as indicated. Statistical analysis was performed by a non-parametric Wilcoxon test for paired data or Mann-Whitney U test for independent samples, as appropriate (26). Two-sided p values <0.05 were considered statistically significant. Statistical analysis was performed by SPSS 11.0 (SPSS).

Results

IL-10-induced unresponsiveness in TCLs from treated CD patients results in low proliferation and cytokine production following gliadin stimulation

We previously reported that incubation of treated CD mucosa with exogenous IL-10 for 24 h inhibits gliadin-induced T cell activation and IFN-γ production (22). Herein, we further characterized the gliadin-specific T cells generated in the presence of IL-10. Short-term TCLs were generated from mucosal explants of three treated CD patients (CD041001, CD090401, CD140102) and three normal donors (ND090102, ND140102, ND200203) upon organ culture with gliadin in the absence (ctl-TCLs) or presence of IL-10 (IL-10-TCLs) (see Materials and Methods). Upon restimulation with gliadin (in the absence of IL-10), the ctl-TCLs from only two CD patients (CD041001, CD090401) proliferated (mean stimulation index (SI) ± SD: 3.6 ± 1.8), whereas the corresponding IL-10-TCLs showed low Ag-dependent proliferation (mean SI ± SD: 1.5 ± 0.7) (Fig. 1A). As expected, TCLs obtained from normal mucosa failed to proliferate in response to gliadin in any condition (mean SI ± SD: 1.1 ± 0.4 and 1.3 ± 0.3 in ctl-TCLs and IL-10-TCLs, respectively) (Fig. 1A).

To confirm that IL-10 treatment did not simply result in deletion of T cells, we analyzed the percentages of CD3+ CD4+, and CD3− CD8+ cells in short-term, ctl- and IL-10-TCLs from CD patients (CD041001, CD090401, CD140102) and normal donors (ND140102, ND200203). In CD patients, an average (range) of 79.3% (71–91%) vs 85.3% (68–95%) CD3+ cells, and 37.3% (31–47%) vs 26% (7–49%) CD3− CD4+ cells were found in ctl-TCLs and IL-10-TCLs, respectively (Fig. 1B). Similar findings were observed in TCLs from normal mucosa, although the percentages of either CD3+ and CD3− CD4+ cells were found to be slightly reduced compared with those of CD mucosa: CD3+: 55.6% (44–67%) in ctl-TCLs vs 65.5% (60–71%) in IL-10-TCLs; CD3− CD4+: 31% (18–44%) in ctl-TCLs vs 19.2% (18.5–20%) in IL-10-TCLs. In contrast, the percentage of CD3− CD8+ cells was found to be enhanced in IL-10-TCLs compared with ctl-TCLs in both CD patients and normal donors: 38.3% (13–69%) in ctl-TCLs vs 55.7% (30–75%) in IL-10 TCLs; 13.5% (7–20%) in ctl-TCLs vs 34% (21–47%) in IL-10 TCLs in CD patients and normal donors, respectively (Fig. 1B).
**FIGURE 1.** Gliadin-dependent T cell responses are present in the intestinal mucosa of treated CD patients and are susceptible to IL-10-induced hyporesponsiveness. Short-term TCLs were obtained from small intestinal mucosa of three celiac patients (CD) and three normal donors (ND). Mucosal explants were cultured for 24 h with gliadin alone (ctl-TCLs) or in presence of IL-10 (IL-10-TCLs). TCLs obtained from IL-10-cultured biopsies underwent an additional 7 days of in vitro treatment with IL-10. A, Proliferative responses of TCLs from patient CD041001 and normal donor ND090102 are illustrated. T cells (3 × 10⁵) were plated in the presence of 5 × 10⁵ autologous EBV-LCLs, which were pulsed with medium or gliadin (50 μg/ml). Proliferative responses, evaluated after 48 h of incubation, were considered positive when the SI was >2. Numbers above the columns indicate the corresponding SI values. One representative experiments of three performed for each TCL is shown. B, Cellular phenotypes of short-term ctl-TCLs and IL-10-TCLs from one representative CD patient (CD041001) of three analyzed are shown. Numbers indicate percentage of double-positive cells present in the R1 (alive cells) region.

**IL-10-induced unresponsiveness to gliadin in intestinal T cells is mediated by IL-10 and TGF-β**

Ag-driven activation of T cells in the presence of IL-10 results in a long-lasting Ag-specific unresponsiveness mediated by Tr1 cells which secrete both IL-10 and TGF-β (18, 24). We therefore investigated whether the unresponsiveness of the IL-10-TCLs was mediated by endogenous IL-10 and/or TGF-β production. Similar to Fig. 1, gliadin-dependent IFN-γ-secreting T cells were present at high frequencies in short-term ctl-TCLs from the three CD patients analyzed (CD090401, CD041001, CD140102), as determined by ELISPOT: median (range) of IFN-γ-secreting cells (IFN-γ-SFC)/10⁶ cells: 200 (170–1380) (Fig. 2A). By contrast, a reduction of the IFN-γ response to gliadin stimulation was observed in IL-10-TCLs: median IFN-γ-SFC: 40 (40–220) (p < 0.05, Wilcoxon test) (Fig. 2A). Addition of neutralizing anti-IL-10 receptor or anti-TGF-β mAbs in the IL-10-TCLs reversed the anergic state of the gliadin-specific cells and led to a positive response to gliadin stimulation (>2-fold response to medium alone). More specifically, the addition of anti-IL-10R rescued IFN-γ responses in two of three CD patients (CD090401, CD041001); the addition of anti-TGF-β rescued IFN-γ responses in all three CD patients tested (CD090401, CD041001, CD140102): median IFN-γ-SFC/10⁶ cells: 120 (50–1990) and 150 (140–2520) in the presence of anti-IL-10R and anti-TGF-β mAbs, respectively, in comparison to 50 (40–410) in the presence of medium alone (Fig. 2A). Interestingly, we also observed a consistent trend to increased production of IFN-γ from the ctl-TCLs upon addition of either anti-IL-10R or anti-TGF-β mAbs: median IFN-γ-SFC: 510 (300–2210) and 400 (200–2050) in the presence of anti-IL-10R and anti-TGF-β mAbs, respectively. Isotype Abs were used as control in stable intestinal TCL and no effect was observed (data not shown).

Similar experiments with short-term TCLs obtained from three normal donors revealed that gliadin-responsive IFN-γ-secreting T cells continued to be undetectable even in the presence of neutralizing anti-IL-10R or anti-TGF-β mAbs (data not shown).

To further investigate whether IL-10- and/or TGF-β-producing T cells may be present in the TCLs derived from ctl-biopsies (i.e., without addition of exogenous IL-10), we generated long-term TCLs from a larger panel of both treated (n = 5, CD230204, CD041001, CD310504, CD041005, CD041005.2) and untreated (n = 5, CD171204, CD151004, CD061204, CD011204, CD200503) patients by restimulating short-term TCLs with gliadin and thereafter with PHA (see Materials and Methods). Thereafter, long-term TCLs were tested for gliadin recognition in the absence or presence of either anti-IL-10R or anti-TGF-β or both mAbs. As shown in Fig. 2, B–E, both proliferation (B and D) and production of IFN-γ, assessed by ELISA (C and E), were significantly enhanced in the presence of both neutralizing mAbs in treated (B and C) as well as in untreated (D and E) TCLs. Statistical significance was evaluated with the Wilcoxon test comparing proliferative responses (SI) and IFN-γ production (level of IFN-γ detected in cell supernatants) in response to gliadin in the presence of anti-IL-10R and anti-TGF-β mAbs with responses in the presence of gliadin alone in both groups of patients. In treated CD, SI (median (and lower and upper quartile)) was: 17.6 (1.7–95.3) in cultures with anti-IL-10R and anti-TGF-β mAbs vs 8.9 (1.1–62.8) in cultures with gliadin alone (n = 5, p < 0.05); IFN-γ (net IFN-γ/10⁶ cells, median (and upper and lower quartile)) was: 7.5 ng/ml (6.8–8.7) vs 2.8 ng/ml (2.1–3.6), (n = 5, p < 0.05). Similarly, in untreated CD, SI was:
1.6 (1.3–3.5) vs 1.2 (1.1–2.5), (n/H11005, p/H11021 < 0.05) and IFN-\(\gamma\) was: 7.7 ng/ml (0.6 –13.5) vs 3.4 ng/ml (0.09 –7.7), (n/H11005, p/H11021 < 0.05). Although a large variability in the magnitude of proliferative responses and IFN-\(\gamma\) production to gliadin was observed among CD patients, a trend toward higher proliferative responses in treated compared with untreated CD was detected. However, the differences between the two cohorts of patients were not statistically significant in any experimental condition (p/H11022 > 0.5, Mann-Whitney U test).

These data suggest that although gliadin-specific T effector cells may predominate in the intestinal mucosa of CD patients, Tr1 cells are also present in vivo both in treated (noninflamed) and untreated (inflamed) CD mucosa.

**Gliadin-specific T cells are restricted by the CD-associated HLA-DQ2 molecule**

Several studies have found that T cells isolated from CD mucosa recognize gliadin peptides in the context of HLA-DQ2 or DQ8 (27–32). To assess the MHC restriction of the TCLs isolated in this study, DQ2+ APC from three normal donors were used individually (ND.1 DR4/17, DQ2/3; ND.2 DR3/11, DQ2; ND.3 DR1/DR7, DQ1/2), or as a pool (ND.1 + ND.2), to present gliadin to ctl-TCLs. When gliadin was presented by APC from any of the three DQ2+ normal donors, the CD ctl-TCLs proliferated vigorously at levels equivalent to those obtained when gliadin was presented by autologous EBV-LCLs (Fig. 3A). Similar results were obtained upon analysis of cytokine production (Fig. 3B). Importantly, no responses were observed when DQ2neg APC from two different normal donors (ND.4 DR7/8, DQ3; ND.5 DR11, DQ7) were used. Because DQ2 is the only MHC II allele shared by these three normal donors, these data indicate that the response in these CD patient-derived TCLs is restricted to this allele.

**Isolation of gliadin-specific Tr1 cell clones from intestinal mucosa**

Based on our findings that anti-IL-10R and/or anti-TGF-\(\beta\) mAbs could enhance gliadin-induced proliferation and cytokine production in both ctl- and IL-10-TCLs, derived from CD patients, we...
next investigated whether gliadin-specific Tr1 cells are present in
ctl-TCLs and/or IL-10-TCLs. TCCs were isolated by limiting dilution from ctl-TCLs and IL-10-TCLs from two different CD patients (CD041001, DR3/5) and a DQ2+ normal donor (ND090102). To evaluate the frequency of Tr1 cells, TCCs were screened after 14 days for their capacity to produce IL-10 following stimulation with anti-CD3/28 mAbs (data not shown).

FIGURE 3. T cells from CD patients recognize gliadin in the context of the HLA-DQ2 heterodimer. To assess the MHC restriction of the gliadin-specific immune response, ctl-TCLs from patient CD041001 (DR3/5) were stimulated with APC (PBMC depleted of CD3+ cells) from three different DQ2+ normal donors (ND.1 DR4/17, DQ2/3; ND.2 DR3/11, DQ2; ND.3 DR1/DQ7, DQ1/2). PBMC were used either individually or as a pool of two donors (ND.1 and ND.2). CD3-depleted PBMC from two different DQ2+ normal donors (ND.4 DR7/8, DQ3; ND.5 DR11, DQ7) were used as negative control. APC were pulsed overnight with medium or gliadin (50 μg/ml). As a positive control, TCL was stimulated with autologous EBV-LCLs that had been pulsed with medium or gliadin (50 μg/ml). After 48 h, proliferation (A) or production of IFN-γ (B) was determined. Numbers above the columns in A indicate the SI. Representative results of one (mean ± SD of duplicates) of three experiments performed are illustrated.

![Image of Figure 3](http://www.jimmunol.org/)

The cytokine production profile of gliadin-specific TCCs upon stimulation with gliadin-pulsed EBV or anti-CD3/28 mAbs is shown in Table II. Importantly, all TCCs displayed very similar cytokine production profiles following Ag-specific or polyclonal stimulations. Three TCCs, TCC.7, TCC.9 (from patient CD041001) and TCC.15 (from patient CD090401) which proliferated in response to EBV/gliadin (Fig. 4 and data not shown), displayed a typical Tr1 cytokine production profile and produced significant levels of IL-10, and IFN-γ, low levels of IL-2, and no IL-4. The Tr1 clones TCC.7 and TCC.9 were also assayed for TGF-β production in response to gliadin in a separate set of experiments. Both Tr1 clones produced significant, although low, levels of TGF-β in responses to gliadin: 170 and 220 net pg/ml for TCC.7 and TCC.9, respectively. The majority of the other TCCs produced all cytokines tested (IL-2, IL-4, IFN-γ, and IL-10) and were classified as Th0 cells, with the exception of TCC.19 from CD090401, which was classified as a Th1 cell clone. All TCCs produced high levels of IFN-γ likely due to the fact that they were expanded in the presence of IL-15 (33). All gliadin-specific Th0/1 and Tr1 cell clones were CD3+CD4+TCRαβ+ and expressed the memory (CD45RO) phenotype (data not shown). Furthermore, Th0 and Tr1 cell clones expressed comparable levels of the IL-2α chain receptor (CD25) and IL-2/IL-15Rβ and γ common chains, which were up-regulated following activation with anti-CD3/28 mAbs (data not shown).

We also investigated whether, similar to the TCCs (Fig. 3), the gliadin-specific TCCs were restricted by HLA-DQ2. TCCs were stimulated with gliadin presented by a pool of DQ2+ APC from two normal donors and tested for their ability to proliferate and produce IFN-γ. Results from representative Th0 and Tr1 cell clones are shown in Fig. 4, B and C. Th0 cell clones displayed significant proliferation and production of IFN-γ under these activation conditions. Typical of Tr1 cells, (18) gliadin-induced proliferation was minimal whereas significant amounts of IFN-γ were produced. These data indicate that both Th0 and Tr1 cell clones from CD patients recognize gliadin in the context of HLA-DQ2. Interestingly, the proliferative response of Tr1 cell clone TCC.9 was significantly higher when gliadin was presented by autologous EBV-LCLs, in contrast to freshly isolated APC (cf Fig. 4, A and B). These data suggest that unknown factors expressed by EBV-transformed B cells can overcome the anergic state of Tr1 cells.
although they have no evident effects on their cytokine production pathway (Table II).

**Intestinal mucosa Tr1 cell clones suppress gliadin-reactive Th0 cell clones**

We next investigated whether the gliadin-specific Tr1 cell clones could suppress Ag-specific proliferative responses of pathogenic T cells. Suppression experiments with two representative Tr1 cell clones (TCC.9 and TCC.7) are shown in Fig. 5. In the case of Tr1 clone TCC.9, 57% suppression of the proliferative response of the Th0 cell clone TCC.3 was observed at a 1:1 ratio (Fig. 5A). A similar magnitude of suppression was observed with Tr1 clone TCC.7 on the gliadin-induced proliferative response of Th0 cell clone TCC.6. Importantly, the suppressive effects of the Tr1 cells were found to be dose dependent (Fig. 5B). The Tr1 cell clones were capable of suppressing the gliadin-induced proliferation of T

![FIGURE 4. Both gliadin-specific Tr1 and Th0 T cells are present in intestinal mucosa of treated CD patients. A, IL-10+ TCCs derived from intestinal TCLs generated in the presence of gliadin alone (ctl-TCLs) or gliadin plus IL-10 (IL-10-TCLs) were tested for their capacity to proliferate in response to gliadin presented by autologous EBV-LCLs after 48 h of culture. TCCs 1–13 from ctl-TCLs and TCCs 10/1, 10/2, 10/3 obtained from IL-10 TCLs derived from patient CD041001 are illustrated. B and C, The MHC restriction of representative Th0 (TCC.6) and a representative Tr1 (TCC.9) TCC was tested. TCCs were stimulated with a pool of APC from two different DQ2+ normal donors that had been pulsed with medium or gliadin (50 μg/ml). After 48 h, cultures were tested for proliferation (B) or production (C) of IFN-γ. Numbers above the columns in B indicate the SI. Representative results of one (mean ± SD of duplicates) of three experiments performed for each TCC are reported.

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* Cytokine levels are shown as net value (amount of cytokine in the presence of gliadin minus amount of cytokine in the presence of medium).

Sensitivity of ELISA assays: IL-10; 9 pg/ml; IL-4; 9 pg/ml; IL-2; 15 pg/ml; IFN-γ; 62 pg/ml.
Interestingly, no evidence of gliadin-specific T cells, either in terms of proliferative or cytokine responses, was detected in normal donors. Upon cloning of cells from the normal intestinal mucosa, we observed a small increase in the frequency of IL-10^- TCC derived from the IL-10-TCLs in comparison to ctl-TCLs. However, we were unable to expand and characterize any IL-10-producing TCC from normal mucosa. Therefore, although we cannot formally exclude that Tr1 cells are present in the mucosa of normal donors, we can conclude that gliadin-specific T cells are not detectable, which is consistent with previous observations (Refs. 28–30 and C. Gianfrani, unpublished observations). This may be due to the fact that gliadin-specific T cells are only generated in CD patients and that the normal immune system simply ignores this food Ag. Alternatively, the frequency of gliadin-specific T cells in the normal mucosa may be extremely low and/or they may have an intrinsically low proliferative capacity which hampers the possibility to clone them in vitro (34, 35). By contrast, Tr1 cells specific for desmoglein 3, the autoantigen of pemphigus vulgaris, are found at high frequency in the peripheral blood of healthy donors and only in a minority of patients (36, 37). Our results are consistent with the hypothesis that gliadin-specific Tr1 cells are recruited to/differentiated in the inflamed intestinal mucosa during the acute disease, possibly as consequence of local production of high levels of IL-10 (22). However, in the acute stage of disease, Tr1 cells are clearly unable to efficiently down-regulate the massive inflammatory response driven by gliadin. Nevertheless, they must remain in the treated mucosa as long-lived memory T cells. In accordance with this hypothesis, we observed that TCLs generated from the intestinal mucosa of treated as well as untreated CD patients contained both gliadin-specific T effector and Tr1 cells. Indeed, proliferation and production of IL-10- and IFN-γ in response to gliadin increased in the presence of anti-IL-10R and/or anti-TGF-β-neutralizing mAbs, and Tr1 cell clones were isolated from CD patients. Interestingly, challenge of treated mucosa with gliadin in the presence of exogenous IL-10 resulted in further down-regulation of gliadin-specific responses, (22), indicating that, at least in treated patients, the pathogenic T cells are still susceptible to some form of immune modulation. Although exogenous IL-10 was able to modulate gliadin-specific responses in vitro, an increase in the percentage of Tr1 cell clones was observed in cultures from only one of the two treated CD patients tested. We have previously shown that although IL-10 is necessary for the differentiation of Tr1 cells, it is not sufficient (17). Therefore, these in vitro cultures may have lacked an essential cofactor(s) which led to inefficient differentiation of Tr1 cells. To clarify the effect of IL-10 on differentiation/expansion of intestinal mucosa Tr1 cells, cloning from a larger cohort of CD patients is required.

The role of Ag-specific Tr1 cells in controlling immune responses in murine intestinal mucosa is well established. For example, IL-10-secreting cells from Peyer’s patches are responsible for active suppression in low-dose oral tolerance to OVA Ag, (10), andecal bacterial Ag-specific Tr1 inhibit the immune response to enteric flora (38). Moreover, mucosal administration of IL-10 along with repetitive feeding of low doses of myelin basic protein or insulin results in prevention of experimental autoimmune encephalomyelitis or diabetes in NOD mice, respectively, and in both cases is associated with enhanced production of IL-10 by T cells (39). Reports on the role of IL-10 and Tr1 cells in the human intestinal mucosa are more limited. Khoo et al. (35) showed that lymphocytes from human intestinal mucosa, but not from mesenteric lymph nodes, inhibited peripheral responses to Escherichia coli via an IL-10- and TGF-β-dependent mechanism. Our data are consistent with these findings and demonstrate that Tr1 cells arise...
in response to dietary Ags in human intestine. Moreover, these Ag-specific Tr1 cells suppressed the responses of Ag-specific effector T cells, a phenomenon that has not been previously reported.

In vivo administration of IL-10 has already been used in an attempt to decrease inflammation in a variety of intestinal diseases. Clinical trials in which IL-10 was delivered s.c. in CD patients who were refractory to gluten-free diet regimen proved ineffective (40). Furthermore, i.v. or s.c. administration of IL-10 to patients with active CD or ulcerative colitis gave unclear results on the therapeutic efficacy of IL-10 treatment (41–46). Several reasons can account for the overall failure of IL-10 therapy to treat inflammatory mucosal diseases, including an inappropriate route of administration, the short serum half-life of IL-10, and its pleiotropic activities (47). The current study has important implications for an IL-10-based therapy for CD. A future therapeutic approach to silence gliadin-induced intestinal inflammation could include administration of IL-10 through the intestinal lumen, to recruit and/or differentiate gliadin-specific Tr1 cells in treated (i.e., noninfamned) CD patients. In addition, in vitro expanded gliadin-specific Tr1 cells that could be reintroduced into CD patients. In this context, it is noteworthy to mention a recent study from Van Montfrans et al. (48) who described the generation of IL-10-producing CD4+ Tr cells with a specific gut-homing capacity as a novel tool to deliver IL-10 to the intestinal mucosa of IBD patients.

In addition to Tr1 cells, many regulatory T cell subsets have been described to control inflammation and maintain immune tolerance to harmless Ags in intestinal tissues. Among these, the natural occurring CD4+CD25+ cells have been the most extensively characterized (6–11). Although the role of this CD4+CD25+ cell population in controlling auto- and alloimmunity has been largely demonstrated, only recent studies reported their involvement in orally induced tolerance (49–52). The proliferation of CD4+CD25+Ag+ cells to enterointerrogants was markedly suppressed by CD4+CD25+ Tr cells in mesenteric lymph nodes (50). Interestingly, Brandzaerg and coworkers (52) found an increased percentage of circulating CD4+CD25+ cells in children who outgrew milk allergy (tolerant), compared with children who retained a clinically active milk allergy. The gliadin-specific Tr1 cell clones isolated in this study did not express high levels of CD25, a marker that we have previously found to be a hallmark for naturally occurring CD4+CD25+ Tr cells (25). However, we cannot exclude that CD4+CD25+ Tr cells may also have a role in controlling the immune response to gliadin. Further studies are underway to address this question.

In conclusion, our data demonstrate that gliadin-reactive Tr1 cells are present in the intestinal mucosa of CD patients and suggest that methods to enhance their numbers and/or function can be a novel tool to suppress immune responses toward gliadin or other Ags that are introduced daily into the gastrointestinal system.

Acknowledgments
We thank Dr. Beatrice De Giuliio for assistance in gliadin preparation and Drs. Caterina Anania and Giuseppe Falanga at the Malzoni Clinic in Avelino for their help in cell irradiation. We gratefully thank Drs. Massimo Conese and Katherina Fleischhauer for helpful discussions.

Disclosures
The authors have no financial conflict of interest.

References


