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Cytokine Production by Intestinal Intraepithelial Lymphocyte Subsets in Celiac Disease

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One of the earliest signs of mucosal immune activation in celiac disease (CD) is an increase in the intraepithelial lymphocyte (IEL) count in the small intestinal epithelium. Though most of those IELs express T cell receptor (TcR)-$\alpha\beta$ chains, CD is characterized by an increase in TcR-$\gamma\delta^+$ IELs and by the loss of $CD3^-$ IELs. There is currently little evidence that these changes in IEL subset distribution are of relevance in the pathogenesis of CD. We aimed to determine the pattern of cytokine production by IEL subsets isolated from duodenal biopsy specimens from control subjects and CD patients at different stages of the disease. We quantified the capacity of IEL subsets to produce IFN-γ, TNF-α, IL-2, IL-4, and IL-10 by intracellular staining by flow cytometry. All IEL subsets studied displayed a type I cytokine profile in both CD and control subjects, with TcR-$\alpha\beta^+$ IELs being the main IFN-γ producers. Untreated CD exhibited a trend toward a superior accumulation of IFN-γ per cell but a reduced proportion of INF-γ^+ cells in vitro in association with a significantly increased apoptotic rate of IELs. IL-4 was almost undetectable in all cases and IL-10 showed a tendency to increase in treated and "silent" celiac patients. IEL subsets have a similar Th1 profile in controls and CD patients, and the superior in vitro apoptosis of IELs from CD patients may reflect their superior in vivo activation. The induction of IL-10-dependent regulatory Tr1 responses may be of potential clinical significance in this disease and merits further investigation.

KEY WORDS: celiac disease; cytokines; flow cytometry; IEL; IL-2; IL-10; IFN-γ; TNF-α.

Human small bowel mucosa contains a large number of intestinal intraepithelial lymphocytes (IELs) within its villous epithelium. There are three major IEL subsets in healthy mucosa: $CD7^+CD3^+$ IELs, which are T lymphocytes, either TcR$\alpha\beta^+$ or TcR$\gamma\delta^+$; and $CD7^+CD3^-$ IELs, which include NK cells. These IEL subsets experience notable changes in celiac disease (CD), such as an increase in TcR$\gamma\delta$ IELs (1–3) concomitant with a decrease in the NK subset (4, 5). $CD3^+$ and $CD3^-$ IEL subsets share activation markers such as CD45RO, CD69, CD38, and CD95 but differ in the expression of adhesion molecules such as CD44, LFA-1, and CD31, as well as in the selective expression of CD122 in the $CD3^-$ NK subset (6), suggesting potential differences in their function. Their strategic location and "in vivo" activated status suggest that IELs may play a role in the homeostasis of the intestinal epithelium and in the regulation of mucosal immune responses to foreign antigens. However, direct evidence for a role of IEL subsets in antigen-dependent immune activation or in intestinal pathology is still lacking.

CD is a small bowel enteropathy (7, 8) mediated by lamina propria $CD4^+$ T cells (9, 10) which elicit a T helper type I hyperreactive response against gluten in genetically predisposed individuals (11, 12). Hallmarks of CD include an early IEL increase followed by crypt hyperplasia and villous atrophy, all in response to oral gluten intake (13, 14). The increased IEL count (15, 16) is characterized by a permanent increase in the proportion of TcR$\gamma\delta$ IELs (1–3) and the loss of the $CD3^-$ NK IEL subset (17).

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In CD, TcR$\alpha\beta$ IEL numbers vary with disease activity, being elevated in untreated disease but normalized by a gluten-free diet (18).

The aim of our study was to investigate whether different IEL subsets have a different capacity for cytokine production, and if so, this may contribute to explain the pathogenesis of CD.

METHODS

Patients and IEL Isolation. Biopsy specimens from distal duodenum were obtained during upper gastrointestinal endoscopies performed for diagnostic purposes in subjects under study for suspected or known CD, at the Gastroenterology and Pediatric Departments of Ramón y Cajal Hospital (Madrid, Spain). The study was approved by the Ethics Committee of Ramón y Cajal Hospital and written informed consent was obtained from the subjects.

The biopsies were processed and analyzed by flow cytometry and the results were matched retrospectively with the corresponding final diagnosis for each subject. In total, 29 biopsies corresponded to patients with confirmed CD (as assessed by typical histopathologic findings (19), IEL profile (5, 17), presence of serum antitransglutaminase [ATG] antibodies (20), and subsequent response to gluten-free diet). Twenty biopsies corresponded to untreated disease ("active CD"; two of them without any symptoms, "silent CD"), seven were taken during a successful gluten-free diet ("GFD CD," with negativity for ATG and recovery of the villi), and two were "potential CD" patients (no histological lesion at the time of the study but positivity for IEL changes and the presence of serum ATG or antigliadin antibodies, both patients in a risk family history of CD; and subsequent development of classic CD with flat mucosa and ATG antibodies). Twenty-four of the biopsies originated from subjects who had no intestinal disease (normal histology, normal IEL profile, absence of ATG antibodies) and were included in the control group.

Isolation and Purification of IEL Subsets. Single cell suspensions were prepared from the epithelial layer of small intestinal biopsies using a previously described protocol (21) with minor modifications. Briefly, IELs and epithelial cells were liberated from mucosal specimens by incubation for 1 hr under stirring in RPMI 1640 medium (GibcoBRL Life Technologies, Vienna, Austria) containing 1 m*M* DDT and 1 m*M* EDTA and supplemented with 10% FCS (Gibco), 3 m*M* L-glutamine, and antibiotics (complete medium; CM). A fraction of the released suspension of cells was then washed and surface-labeled with the appropriate conjugated mAbs for four-color flow cytometric analysis (FCM). The remaining cells were kept in CM until cell cultures were established. Where indicated, IEL were expanded in vitro by culture with 10 ng/ml recombinant human IL-2 (rhIL-2) (R&D Systems, Minneapolis, MN, USA) for 2 weeks.

Intracellular Cytokine Detection by Flow Cytometry. This analysis was performed on IELs stimulated or not with phorbol 12-myristate 13-acetate (PMA; 5 ng/ml; Sigma, St. Louis, MO, USA) and calcium ionophore ionomycin (100 ng/ml; Sigma) for 12 hr in CM, in the presence of Monensin (GolgiStop; BD). After stimulation, cells were washed and labeled with conjugated mAbs (FITC, PerCP, PE, APC) against surface antigens: $\alpha\beta$-TcR (WT31), $\gamma\delta$-TcR (11F2), CD3ε (Leu4), CD69 (L78), CD45 (HI30), obtained from BD, and Annexin-V/FITC from MedSystems (Bender, Austria). The cells were then fixed and permeabilized (Cytofix/Cytoperm, BD) for 20 min, washed with WashPerm (BD), and labeled with cytokine-specific FITC- and PE-conjugated mAbs and isotype-matched Ig controls obtained from Beckton Dickinson: IL-2 (MQ1-17H), IL-4 (MP4-25D2), IL-10 (JES3-19F1), IFN-γ (4S.B3), and TNF-α (Mab11). Four-color analyses were performed using a FACSCalibur instruments (BD Biosciences, San Diego, CA, USA). A biparametric gate was drawn around IELs, according to their side scatter (SSC) and CD45$^+$ expression, and data were processed using CellQuest software (BD Biosciences). Apoptotic cells were excluded from the analysis on the basis of their surface expression of phosphatidylserine as evaluated by annexin-V staining. The mean fluorescence intensity (MFI) index was calculated as the ratio of the MFI of cytokine$^+$ cells to the MFI of cytokine$^-$ cells (nonspecific background).

Statistical Analysis. The results are expressed as the percentage of cytokine-positive cells, with mean and standard deviation. Significance of the differences between patient groups was performed using the Mann–Whitney test.

RESULTS

Minimal Spontaneous Cytokine Production by IELs. IELs extracted from biopsies and kept in culture in the absence of stimulation showed a negligible cytokine content (not shown) which was close to the limit of detection of FCM (1%). IFN-γ^+ IELs were observed at low frequencies (1–3% of CD3$^+$ IELs; negative in NK IELs) in 5 of 21 controls and in 4 of 6 celiac patients on GFD but not in active CD patients ($n = 7$). No detectable IL-2, TNF-α IL-4, IL-5, or IL-10 was spontaneously produced by duodenal IELs from controls ($n = 21$) or celiac patients ($n = 13$).

Cytokine Production by Stimulated IELs Shows a Th1 Pattern, TcR-$\alpha\beta^+$ IEL Being the Main Producers. Upon stimulation with PMA and Ionomycin, we observed superior in vitro apoptosis of stimulated TcR$\alpha\beta^+$ IELs from active CD patients, 40 ± 17%, vs. 13 ± 7% in controls and 18 ± 10% in CD patients on a GFD (not shown). Apoptotic IELs were excluded from all subsequent analysis, which showed how almost all viable stimulated IELs expressed the activation marker CD69 with a high intensity, unlike IELs kept in culture without stimulation, which were negative for this marker (not shown).

The mitogenic stimulation revealed a type I cytokine profile in both controls and CD mucosal samples (Figure 1) for the three subsets of IELs (Figure 2, Table I). Figure 2 illustrates that TcR$\alpha\beta$ IELs are the main source of IFN-γ, TNF-α, and IL-2, followed by NK IELs and TcR$\gamma\delta$ IELs. Neither TcR$\gamma\delta^+$ in celiac mucosa nor NK cells in controls seemed to produce substantial amounts of IL-2 (1.9 ± 2 and 1 ± 2%, respectively), IL-4, or IL-10

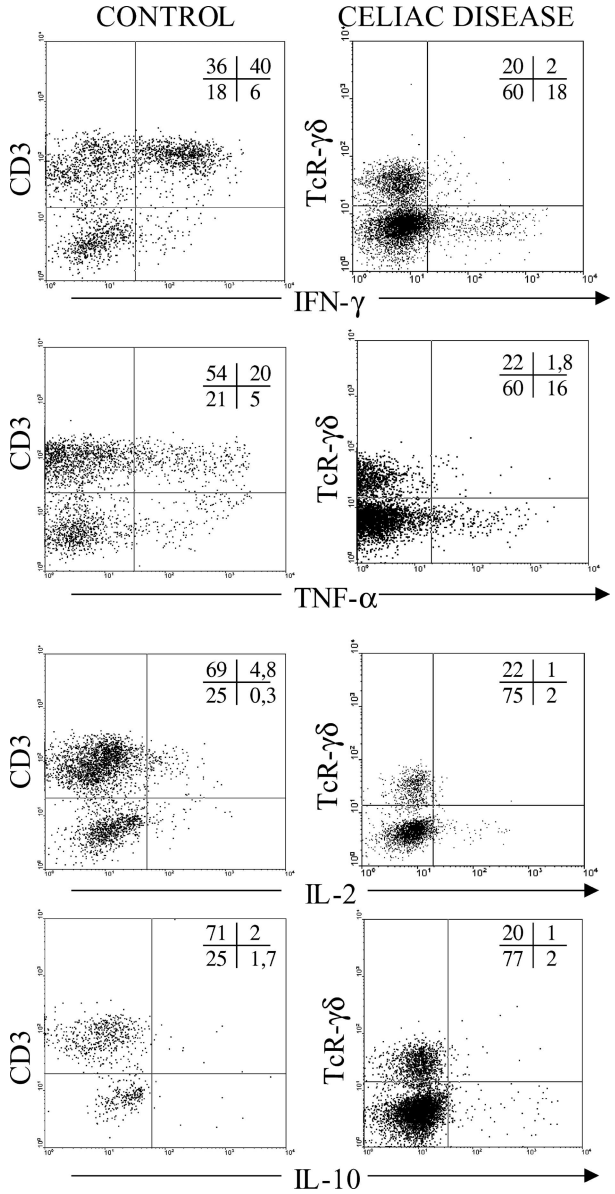

Fig 1. Intracellular cytokine content of in vitro stimulated IELs from controls and active celiac disease. Flow-cytometric dot plots are representative of the intracellular content of IFN-γ, TNF-α, IL-2, and IL-10, illustrating the predominant Th1 pattern. In the left column, $CD3^+$ cells (predominantly TcR-$\alpha\beta^+$; Table I and Figure 2) are the major cytokine producers in controls. In the right column, the same superior production by TcR-$\alpha\beta^+$ IELs was found in CD samples, which contain virtually no $CD3^-$ NK IELs (17). The numbers indicate the percentage of cells within each quadrant.

(Figures 1 and 2B). IL-4 (Figure 2B) and IL-5 (not shown) were barely produced by IELs from CD or control mucosa.

Comparing controls to celiacs, the proportions of cytokine-producing IELs were not significantly different (Figure 2), with one exception: the fraction of TcR$\alpha\beta^+$ IFN-γ^+-producing IELs was significantly reduced ($P = 0.0048$) in active CD with respect to controls (Figure 2A). However, TcR$\alpha\beta^+$ IELs from CD had a superior content of IFN-γ on a per-cell basis, displaying a higher mean fluorescence intensity (MFI) index, 28.6 ± 12, versus 12 ± 2.4 in controls.

Association Between the Absence of Symptoms and the Production of IL-10 by IELs in CD. IL-10-producing IELs were below the level of detection (<1%) in most specimens from controls or active CD patients (Figures 1 and 2B). In two of seven GFD CD samples, however, we detected a small but clear fraction of IL-10^+ IELs on both TcR$\alpha\beta^+$ (4% on each samplep and CD3-/NK (5 and 10%) IELs (Figures 2B and 3A). This was confirmed in a cell line derived from one of these patients (Figure 3A) but not in cell lines similarly derived from controls or active CD patients (not shown). Furthermore, both silent CD biopsies analyzed showed a tendency toward substantial IL-10 production after stimulation (Figures 2B and 3B). These findings preliminarily suggest that IL-10 may play a role in the suppression of pathology and symptomatology in CD.

DISCUSSION

CD, an extremely prevalent human enteropathy caused by immunologically mediated intolerance to gluten, is characterized by unique permanent changes in IEL subset distribution (1–3, 17, 18). We sought to investigate whether a correlation among cytokine production, IEL subset, and clinical status could be established in CD. In order to do so, we performed a flow cytometric analysis of intracytoplasmic cytokine content, which allows for characterization of the cellular source of the cytokine analyzed, unlike other sensitive techniques such as the detection of mRNA levels by RT-PCR or protein secretion by ELISPOT. In vitro mitogenic stimulation in the presence of Golgi blockers like monensin permits the sensitive detection of accumulated cytokines by cells already committed to their production (22–25).

The cytokine profile of CD has been studied predominantly in the lamina propria, where the pathogenic immune response is thought to occur. In this disease, the intake of gluten induces a T helper I response by lamina propria (LP) $CD4^+$ T cells (26, 27), with increased production of IFN-γ and less intense increases in TNF-α and IL-2 (28–30). Activated LP T cells play a crucial role in inducing villous atrophy and crypt hyperplasia (31, 32), and there is evidence for gluten-specific Th1 clones isolated from CD lesions (33, 34). Pro-inflammatory cytokines such as TNF-α have also been involved in the immunopathogenesis of CD, either

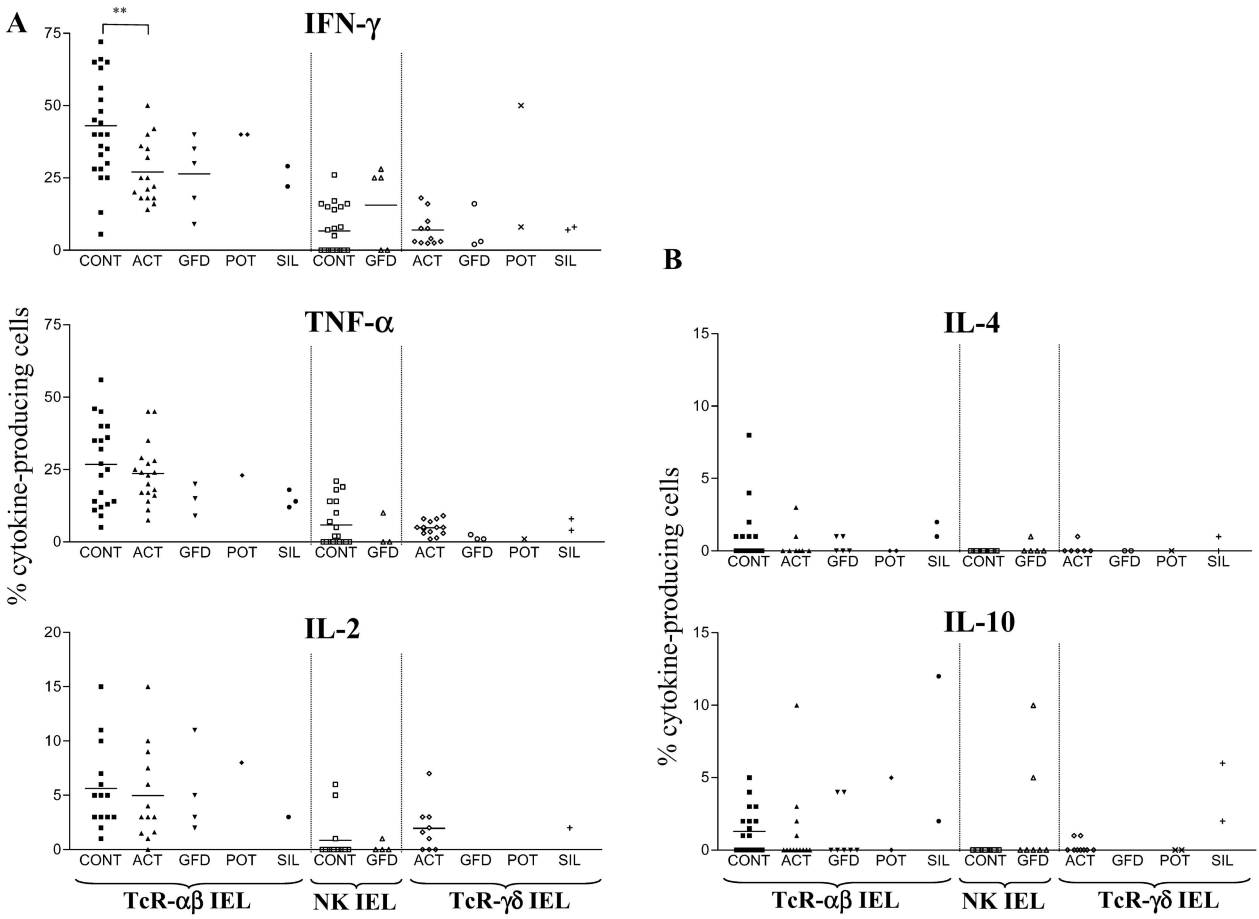

Fig 2. Cytokine production by IEL subsets in controls and in the different stages of celiac disease. (A) Column-scatter distribution of the percentages of IFN-γ^+, TNF-α^+, and IL-2$^+$ IELs (TcR$\alpha\beta$, CD3-/NK, and TcR$\gamma\delta$). (B) IL-4- and IL-10-producing IELs. CONT, controls; ACT, active CD; GFD, gluten-free diet; POT, potential CD; SIL, silent CD. The horizontal lines represent the mean. A significant difference ($P = 0.0048$) was observed between IFN-γ^+ TcR$\alpha\beta$ cells in controls vs. active CD. CD3−/NK IELs are not present in active CD epithelium, including potential and silent CD (17).

by a direct cytotoxic effect on epithelial cells (35) or indirectly due to their induction of perforin and FasL on Tc and the subsequent apoptosis of enterocytes (36, 37). Finally, some cytokines can also modulate epithelial proliferation (38).

In contrast, there is no evidence for gluten-specific IEL responses so far, and data on cytokine production by human IEL subsets are scarce (39–44) and sometimes controversial. The small spontaneous cytokine secretion by IEL isolated from normal human small intestine is reportedly

TABLE 1. CYTOKINE PRODUCTION BY IEL SUBSETS IN TREATED AND UNTREATED CELIAC DISEASE PATIENTS AND IN CONTROLS

IEL subset	*Groups*	*IL-4*	*IL-10*	*IL-2*	*TNF-α*	*IFN-γ*
TcR$\alpha\beta^+$	Controls	0.8 ± 1 ($n = 22$)	1.3 ± 1.5 ($n = 19$)	5.6 ± 4 ($n = 14$)	27 ± 14 ($n = 20$)	43 ± 18 ($n = 24$)
	Active CD	0.5 ± 1 ($n = 8$)	1.2 ± 2.8 ($n = 13$)	4.9 ± 4 ($n = 13$)	24 ± 10 ($n = 18$)	27 ± 10 ($n = 16$)
	GFD CD	0.5 ± 0.6 ($n = 5$)	1.1 ± 2 ($n = 7$)	5.2 ± 4 ($n = 4$)	14 ± 5 ($n = 3$)	26 ± 12 ($n = 5$)
CD3$^-$/NK	Controls	0.1 ± 0.1 ($n = 19$)	0.1 ± 0.1 ($n = 15$)	1 ± 2 ($n = 14$)	6 ± 7 ($n = 19$)	7 ± 8 ($n = 22$)
	GFD CD	0.1 ± 0.2 ($n = 5$)	2.2 ± 3 ($n = 7$)	0.2 ± 0.5 ($n = 4$)	3 ± 5 ($n = 3$)	15 ± 14 ($n = 5$)
TcR $\gamma\delta^+$	Active CD	0.2 ± 0.4 ($n = 6$)	0.2 ± 0.4 ($n = 9$)	1.9 ± 2 ($n = 9$)	5 ± 2 ($n = 14$)	7 ± 5 ($n = 11$)
	GFD CD	0 ($n = 2$)	ND	ND	1.5 ± 0.8 ($n = 3$)	7 ± 7 ($n = 3$)

Note. Results are expressed as the percentage of cytokine-producing cells for each IEL subset (mean ± SD; n). NK-IELs are not present in active CD epithelium (17). ND, not done.

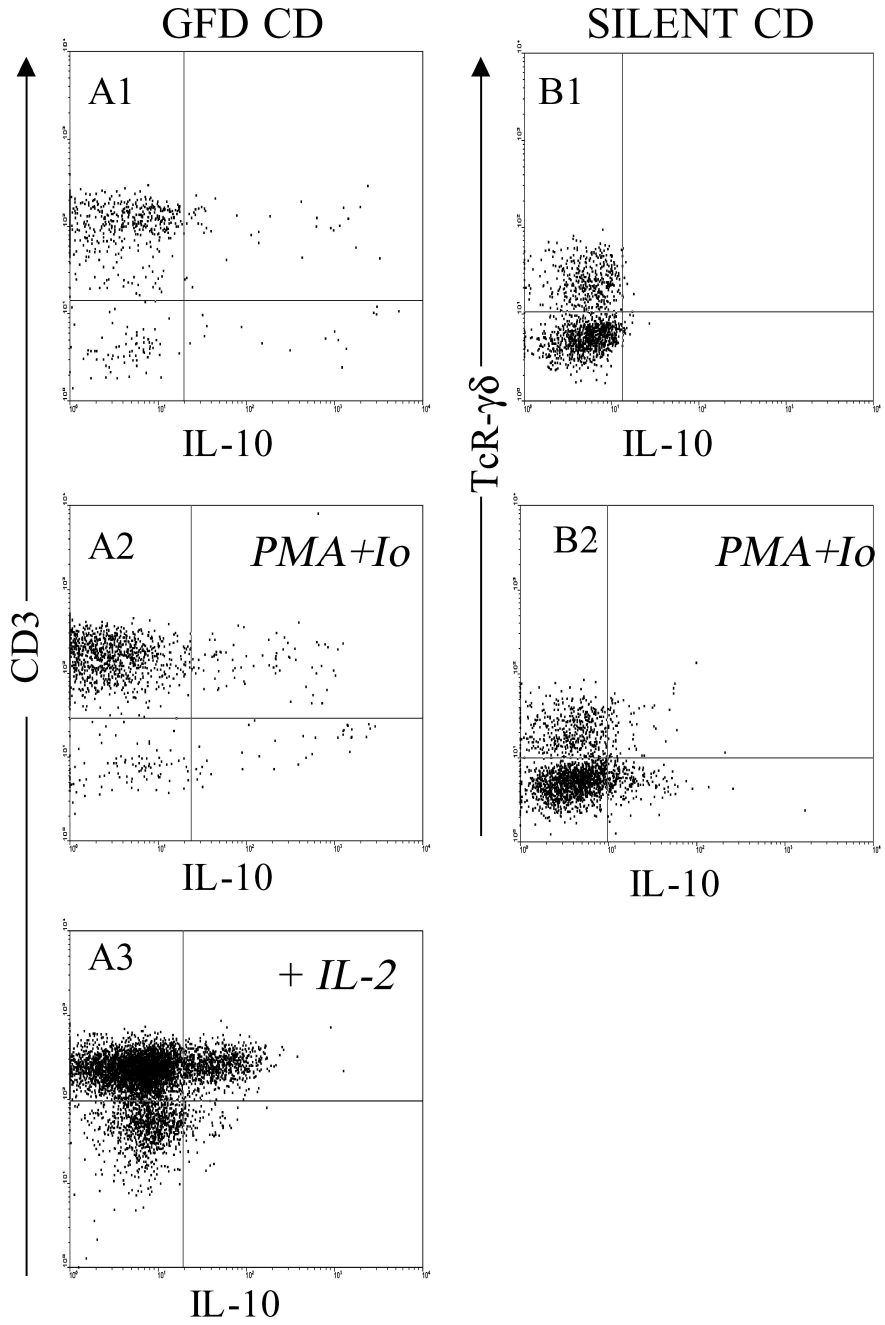

Fig 3. Association between the absence of symptoms and the production of IL-10 by IELs in CD. Representative examples of the detection of intracellular IL-10 in IELs obtained from (A) one patient with celiac disease on a gluten-free diet (GFD CD) and (B) one patient with silent celiac disease (silent CD). IELs were cultured in the absence (A1, B1) or presence (A2, B2) of PMA and ionomycin. (A3) Production of IL-10 by IELs obtained from the GFD CD patient and expanded in vitro with IL-2.

biased toward a Type I profile (40, 45), though the literature shows some discrepancies. IFN-γ mRNA transcripts have been deteceted by RT-PCR (39), but not by in situ hybridization (ISH) (30, 46). Basal IFN-γ protein has been detected in 3% of IELs by ELISPOT (40) and in 0.5% of IELs by IFN-γ secreting assay (42), but not by immunohistochemistry (IHQ) (12). Our study by FCM also showed very low numbers of IFN-γ^+ cells in nonstimulated IELs from controls, with no IL-4 or IL-10 detection. The latter is also in agreement with the

literature, which shows low (40, 41) or undetectable (12, 40, 43) IL-4 and IL-10 mRNA expression on IELs.

Considering the production of cytokines by stimulated IELs, our data also confirm that IFN-γ is one of the main cytokines produced in patients with CD, in agreement with reported data (29, 40, 42, 47). Our work identifies TcR$\alpha\beta^+$ IELs as the main producers of INF-γ, followed by CD3$^-$/NK and TcR$\gamma\delta$ IELs as minor contributors. We failed to detect IL-4-producing IELs in any sample, stimulated or not, while Carol *et al*. (40) estimated 1.3% of IL-4-secreting IELs by ELISPOT. Other studies also failed to detect IL-4 in the duodenal epithelium by either IHQ (12), ISH (48), or RT-PCR after mitogenic stimulation (39, 43). We could nor detect TNF-α or IL-2 basally, but their synthesis was induced by in vitro mitogenic activation, in agreement with most reports (40, 43, 46). We found normal levels of these two cytokines in CD IELs, as has been previously reported (43, 49). Finally, we observed a tendency toward IL-10 production in association with asymptomatic situations in CD. Data on this cytokine are contradictory, with one study reporting an increase in IL-10 production in the epithelium of active CD (43), although this was not confirmed (12, 39), and neither were differences observed in the LP (48). Even though our data are preliminary in this regard, we speculate that these IL-10-producing cells may be regulatory Tr1 cells (51, 52).

The superior in vitro apoptosis of stimulated TcR$\alpha\beta$ IELs in CD makes the interpretation of the differences between CD and controls difficult, and we cannot predict the net in vivo result of the superior per-cell cytokine content combined with their superior susceptibility to apoptosis. The reason behind the increased apoptosis may be intense in vivo stimulation, which induces apoptotic pathways (53) on highly activated and cytokine-secreting IELs (54, 55) or the lack of necessary growth factors in vitro (56, 57). Consistent with our finding is the reported decrease in the frequency of INF-γ-producing gut mucosal lymphocytes analyzed by intracellular flow cytometry in patients with spondyloarthropathy (41) and Crohn's disease (50).

In summary, our study shows that IEL subsets from CD mucosa and controls produce a Type I cytokine profile and that TcR$\alpha\beta$ IELs are the main contributors of INF-γ and TNF-α, followed by NK IELs in controls and TcR$\gamma\delta$ IELs in CD patients. The potential induction of IL-10-dependent regulatory responses in CD merits further investigation.

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