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ORIGINAL PAPER

Regulatory T cells in type 1 diabetic patients with autoimmune chronic atrophic gastritis

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Abstract Type A chronic atrophic gastritis (CAG) is increased in type 1 diabetic patients (DM1). To address this issue, we determined and analyzed the number of peripheral blood regulatory T cells (Tregs) in 15 DM1-CAG patients, 15 DM1 patients without associated autoantibodies (DM1) and 15 healthy controls by flow cytometry and compared gastric Tregs expression (CD4+Foxp3+/CD4+) in DM1-CAG patients with that observed in 10 control *Helicobacter pylori* CAG-infected biopsies. The percentage of peripheral Tregs was higher in DM1-CAG patients compared to DM1 and controls (CD4+Foxp3+: 7.67 ± 1.91% vs. 5.38 ± 1.57% and 5.65 ± 1.76%, $P < 0.001$, respectively), with no differences between DM1 and controls. Gastric mucosal Tregs were higher in *H. pylori* CAG than in DM1-CAG patients (31.31 ± 5.52% vs. 7.68 ± 3.70%; $P < 0.001$). Data suggest that Tregs are stimulated in patients with more than one autoimmune disease (DM1 + CAG) in an ineffectual attempt to control autoimmune response and that the number of Tregs in gastric mucosa implicated in the chronification of gastritis differs according to the etiology.

Keywords Regulatory T cells · Gastritis · Type 1 diabetes mellitus · Pepsinogen I · Gastrin · Parietal cell antibodies

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Introduction

Type 1 diabetes mellitus (DM1) is an autoimmune disease due to a complex interaction of immune, genetic, and environmental factors [1] in which there is dysregulation of the basic processes designed to maintain self-tolerance [2, 3]. Tolerance is maintained in the periphery via a variety of mechanisms, including the action of regulatory T cells (Tregs), which suppress autoreactive T cell function. Tregs are divided into two major subtypes, thymus-derived natural Tregs (nTregs) and peripherally inducible Tregs (iTregs), and can be identified by their phenotypic CD4 expression, high CD25 expression (interleukin 2 receptor alpha chain) [4], CD27 [5], and transcription factor forkhead box P3 (Foxp3) [6]. In functional human Tregs, the low/− concentrations of CD127 (interleukin-7 receptor

alpha chain) on their cell surface has recently been found to correlate well with Foxp3 expression [7].

In recent years, in a series of mouse and human genetic studies, many investigators have firmly established that Foxp3 mutations gene are linked to the autoimmune manifestations observed in scurfy mice and humans with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) disease [8]. Subsequent studies in mice showed that Foxp3-deficient animals lacked Tregs, whereas overexpression of the Foxp3 protein led to profound immune suppression. Development of early-onset DM1 in Foxp3-deficient IPEX patients suggests an important role for Tregs in the development of DM1.

Most studies investigating the role of Tregs in human DM1 found no differences in peripheral blood frequency in these cells between DM1 patients and control subjects [9–11], when the CD4 and CD25 coexpression was used as the criterion for Tregs identification. These results were recently confirmed using the lineage marker Foxp3 [12].

DM1 patients show an increased prevalence of other associated organ-specific autoimmune diseases [13, 14]. Type A chronic atrophic gastritis (CAG) is an autoimmune disease that involves the fundus and body of the stomach and spares the antrum. It is characterized by the presence of circulating parietal cell antibodies (PCA) and a chronic mononuclear cell infiltrate in gastric mucosa, with CD4+ T cells being the pathogenic cells [15]. Histologically, type A CAG shows a progressive disappearance of oxyntic glands and is associated with iron deficiency and pernicious anemia [16]. In adult DM1 patients PCA prevalence is high, 15–25% [17, 18]. Experimental autoimmune gastritis has been established as a highly defined model of organ-specific autoimmunity. In fact, it has played a pivotal role in defining the characteristics of CD4+CD25+ Tregs [19–21]. To our knowledge, Tregs in human autoimmune gastritis have not been previously analyzed.

In the present study, we aimed to ascertain whether the number of peripheral blood Tregs is altered in DM1 patients with a second autoimmune endocrinopathy, more specifically type A CAG, compared with DM1 patients without other associated autoimmune disease and healthy controls. Moreover, we evaluated Tregs expression at the inflammation site in the target tissue, i.e., gastric mucosa of DM1 patients with CAG (DM1-CAG).

Results

Characterization of peripheral Tregs in DM1-CAG patients

We addressed the question of whether Tregs frequency in peripheral blood was altered in DM1-CAG patients compared to DM1 patients and controls. Peripheral blood T

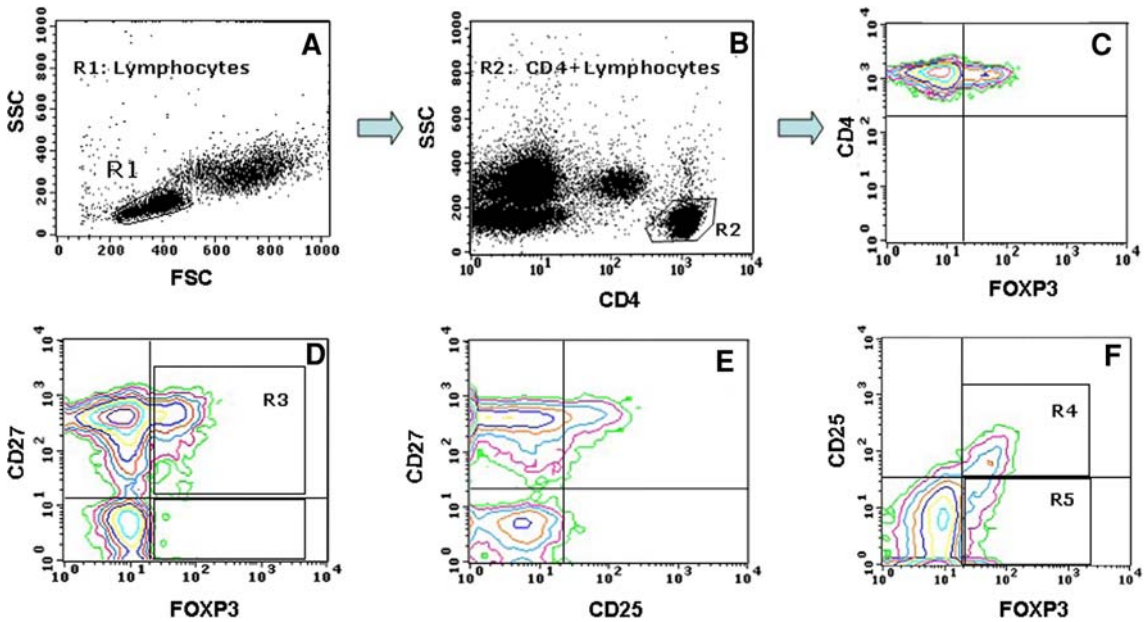

Fig. 1 Flow cytometric analysis of fresh peripheral blood Tregs. Cells were stained with anti-CD4-APC, FOXP3-FITC, CD27-PE, and CD25-PE Cy5 as specified in "Material and Methods" section. For the cytometric analysis we first selected the lymphocyte subpopulation from peripheral blood cells on the basis of Forward Scatter (FSC) and Side Scatter (SSC) (see R1, Fig. 1a). CD4+ lymphocytes were gated by positive staining of CD4+ cells and SSC (R2, Fig. 1b). From CD4+ cells, we selected those that were Foxp3+ (Upper right quadrant, Fig. 1c). As can be observed in Fig. 1d, most Foxp3+ cells were CD27+. Most CD25+ cells were also CD27+ (Upper right quadrant, Fig. 1e). Figure 1f shows in a dot-plot the expression of CD25 and Foxp3 in relation to the total population of CD4+ cells: Foxp3+CD25+ (R3) and Foxp3+CD25− (R4). The graphics are from a representative DM1-CAG patient

cells were stained with CD4, CD25, CD27, and Foxp3 in all DM1-CAG patients and DM1 patients and healthy controls as indicated in Fig. 1. Foxp3+ cells were CD27+ (upper right quadrant, Fig. 1d). Most CD25+ cells were also CD27+ (upper right quadrant, Fig. 1e). DM1-CAG patients had a significantly higher percentage of CD4+ Foxp3+ T cells (7.67 ± 1.91%) compared with DM1 patients (5.38 ± 1.57%; $P < 0.001$) and controls (5.65 ± 1.76%; $P < 0.001$). In contrast, no differences were found in the percentage of CD4+Foxp3+ T cells between DM1 patients and controls (Fig. 2a). The percentage of CD4+ lymphocytes that were Foxp3+CD25+ (R3, Fig. 1f) was also higher in DM1-CAG patients (3.62 ± 1.1%) versus DM1 patients (2.65 ± 0.95%, $P = 0.024$) and did not differ from controls (2.86 ± 0.91%, $P = 0.09$) (Fig. 2b). Surprisingly, an increased expression of CD4+Foxp3+ was also observed in the CD25− subpopulation (CD4+ Foxp3+CD25−) (R4, Fig. 1f), in DM1-CAG patients (2.84% [1.8–4.56]) compared with DM1 patients (1.75% [1.37–2.76], $P = 0.019$) and controls (1.68% [1.12–2.46], $P = 0.002$) (Fig. 2c).

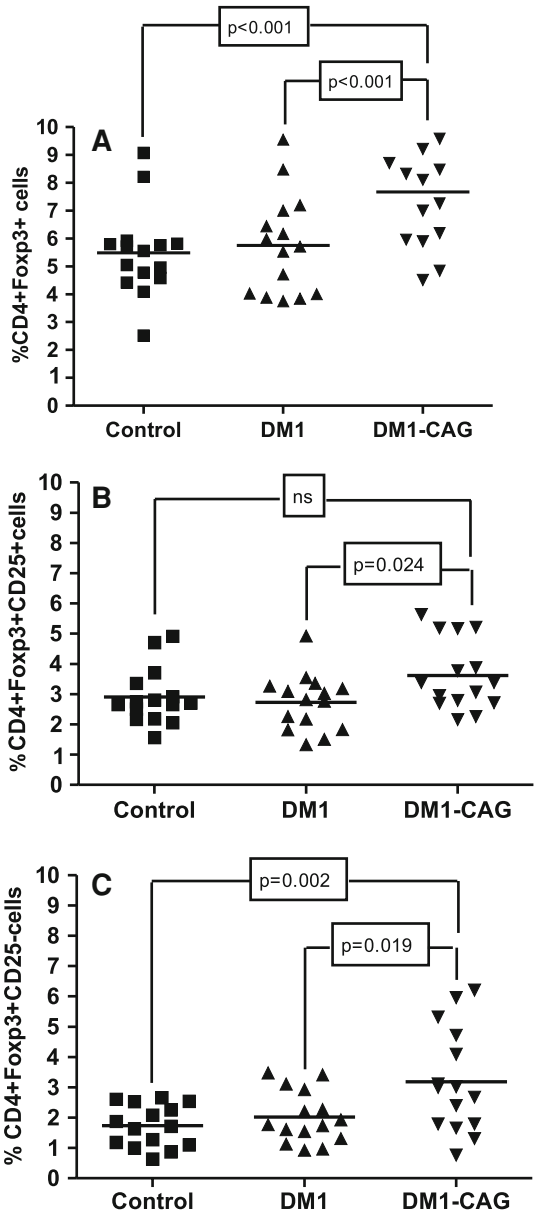

Fig. 2 Percentage of CD4+Foxp3+ (**a**), CD4+Foxp3+CD25+ (see R3 in Fig. 1) (**b**) and CD4+Foxp3+CD25− lymphocytes (see R4 in Fig. 1) (**c**) in peripheral blood from DM1-CAG patients, DM1 patients and healthy controls

To better characterize Tregs, expression of other Tregs-related cellular markers was investigated in a subgroup of DM1-CAG patients ($n = 6$), DM1 ($n = 6$), and controls ($n = 6$). Therefore, we analyzed the cellular distribution of CD127 (the α-chain of the IL-7 receptor), the down-regulation of which has been shown to be a useful marker for identifying human Tregs in addition to Foxp3 [6, 7]. CD4+Foxp3+CD25+ cells of the three groups expressed low/negative CD127 levels. In fact, the descriptive analysis of Tregs using the phenotypical marker CD127 (CD4+ CD25+, Foxp3+CD127$^{low/neg}$ cells) in relation to the total population of CD4+ lymphocytes, confirmed the increased percentage of Tregs in DM1-CAG patients versus DM1 (2.14 ± 1.29% vs. 0.80 ± 0.62%, $P = 0.035$) and versus controls (0.52 ± 0.32%, $P = 0.007$). The percentage of the CD127high subpopulation, which may represent activated T cells, was also analyzed and, of the total population of CD4+ cells, CD4+CD25+Foxp3+CD127^{high} cells were found to be almost undetectable. The percentage of CD4+CD25+Foxp3+CD127^{high} of the total CD4+ Foxp3+ lymphocytes was therefore analyzed and found to be slightly increased in DM1-CAG patients (1.06 ± 0.77%) compared to DM1 (0.40 ± 0.39%) and controls (0.41 ± 0.35%), although not statistically significant.

The expression of other less specific Tregs markers such as GITR, CD49d, CD122, CCR5, CD62L, CCR4, CD45RO, CD95, and CD152 (CTLA-4) was also analyzed in CD4+Foxp3+CD25+ lymphocytes. In agreement with the literature, we found that a high proportion of these cells expressed these markers, albeit across a continuum of levels, thereby confirming that these cells were Tregs. No differences in the expression of these markers were found among groups.

The ratio of memory/activated T cells (CD4+ CD45RO+CD25+) to regulatory T cells (CD4+Foxp3+ CD25+ cells) was analyzed in DM1-CAG patients ($n = 6$), DM1 ($n = 6$) and controls ($n = 6$) with no significant differences being observed among groups, although a higher ratio was observed in the control group (3.06 ± 1.04) and in DM1 (2.86 ± 0.86) compared with DM1-CAG patients (1.98 ± 0.38). Differences in the percentages of Tregs were not due to differences in male/female ratios among groups, given that all the groups had the same distribution among sexes (see Table 1).

Table 1 Clinical and biochemical parameters of DM1-CAG and DM1 patients and controls

	DM1-CAG	DM1	Control	*P*-value
N (male/female)	15 (6/9)	15 (6/9)	15 (6/9)	N.S.
Age (years)	36.87 ± 13.5	37.4 ± 13	35.5 ± 13	N.S.
Diabetes duration (years)	17.9 ± 6.3	18.7 ± 6.8		
HbA1c (%)	7.7 ± 0.7	7.7 ± 1.1	–	N.S.
Pepsinogen I (μg/l)	18.5 ± 15[a]	113.7 ± 33	125.5 ± 32.6	<0.001
Pepsinogen I-II ratio	1.4 ± 1.0[b]	11 ± 5.3	10.2 ± 4.5	<0.001
Gastrin (ng/l)	469 (225–708)[c]	26 (22–30)	21 (17.2–30)	<0.001

DM1-CAG Type 1 diabetes mellitus with chronic autoimmune gastritis, *DM1* Type 1 diabetes mellitus

[a,b,c] Significantly different from DM1 ($P < 0.001$) and control group ($P < 0.001$)

Foxp3 expression in gastric mucosa of DM1-CAG patients

To ascertain whether Tregs were present in gastric mucosa of DM1-CAG patients, a representative section of a biopsy specimen obtained by gastroscopy was studied in each patient. Positive cells in 10 high power fields from lamina propria were analyzed for quantitative analysis. Tregs were detected immunohistochemically by double immunoenzymatic labeling as lamina propria lymphocytes expressing both CD4 and Foxp3. The analysis confirmed the presence of a moderate percentage of CD4+Foxp3+ cells in inflammatory infiltrates of DM1-CAG patient gastric mucosa (7.68 ± 3.70%) of the total CD4+ cells (Fig. 3b). These results were clearly different from those obtained in biopsy specimens from controls with normal gastric mucosa, in whom we found few CD4+ lymphocytes with no Foxp3 expression (Fig. 3a). Biochemical, pathologic and immunohistochemical features of DM1-CAG patients are shown in Table 2. To obtain clear evidence for specific and accurate detection of mucosal Tregs, Foxp3 expression in gastric mucosal T lymphocytes was confirmed with two different anti-Foxp3 monoclonal antibodies by either immunohistochemistry or immunofluorescence, with similar results. We next tried to compare DM1-CAG cellular infiltrate with that of another CAG inflammatory condition due to H. *pylori* infection. In the latter, a higher frequency of CD4+Foxp3+ T cells (31.31 ± 5.52%) (Fig. 3c) was observed compared to gastric mucosa DM1-CAG patients (7.68 ± 3.70%; $P < 0.001$) (Fig. 4).

Discussion

Autoimmune diseases are characterized by failure in the mechanisms of tolerance to self-antigens. There is

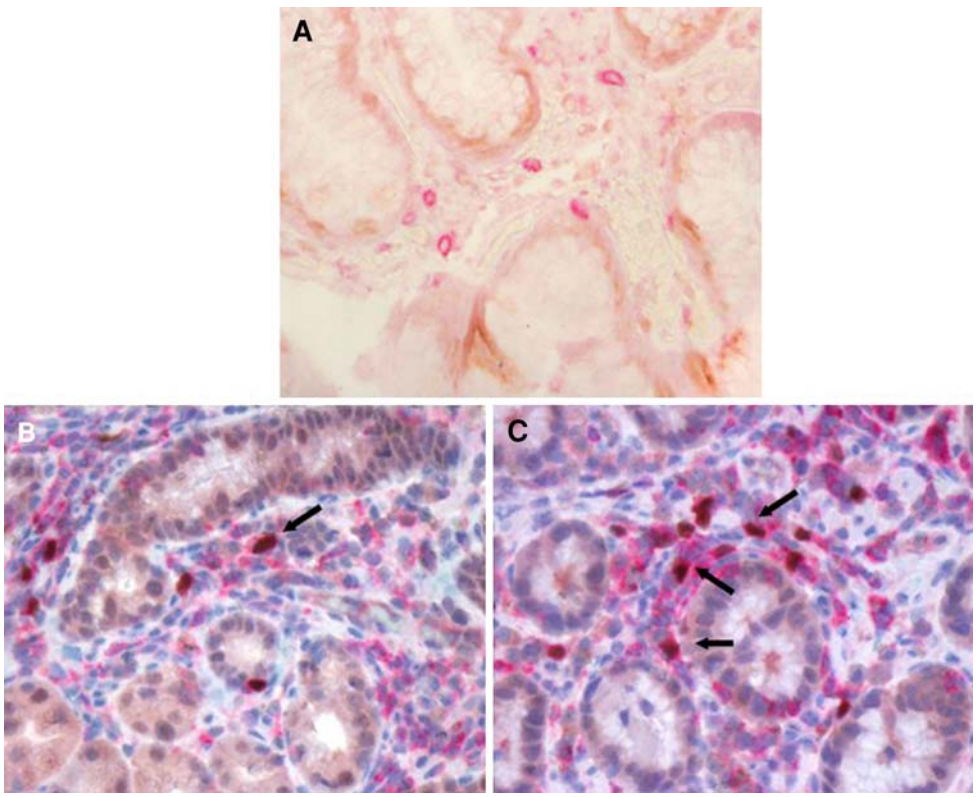

Fig. 3 Expression of Tregs in gastric tissue. Normal mucosa (**a**), DM1-CAG (**b**) and chronic *H. pylori* gastritis (**c**). The number of CD4+ cells (red staining) coexpressing Foxp3 PHC101 MAb (brown nuclear staining) is higher in *H. pylori* gastritis cases than in DM1-CAG cases (×400)

Table 2 Biochemical, pathologic, and immunohistochemical features of type 1 diabetes mellitus patients with type A CAG

Patient no.	Sex/age (yr.)	Corpus atrophy	Serum PI (μg/l)	PI/II ratio	Gastrin (ng/l)	PCA titer	IFAb	Serum CD4+ Foxp3	CD4+ Foxp3+ biopsy (%)
1	M/28	Moderate	3.80	0.50	222	1/160	–	8.33	6.6
2	M/33	Mild	29.50	4.04	32	1/640	+	9.22	10
3	M/51	Mild	18.70	1.57	225	1/1280	–	7	4.5
4	F/31	Severe	6.90	1.17	314	1/640	–	9.59	15
5	F/37	Mild	8.40	1.06	708	1/640	–	8.71	8
6	F/72	Moderate	58.60	2.90	490	1/1280	–	10.43	10
7	F/28	Moderate	23	1.15	514	1/160	–	8.48	7
8	F/29	Mild	9	0.78	469	1/1280	–	4.85	0
9	F/34	Severe	11.8	0.62	1649	1/640	–	8.11	5
10	F/23	Moderate	37.7	2.22	199	1/1280	–	6.20	5.5
11	M/20	Moderate	30	1.99	446	1/1280	–	4.51	10
12	F/48	Severe	10.60	0.78	436	1/640	–	10.46	10
13	M/30	Mild	5.40	0.51	683	1/640	–	7.26	6.6
14	F/51	Severe	11.40	0.56	717	1/320	–	5.90	4.5
15	M/38	Severe	12.50	1.51	1085	1/1280	–	5.96	12.5

Serum pepsinogen I (PI): normal values: 30–117 ug/l

PI/PII ratio: normal values > 3

Serum gastrin:normal values:undetectable-100 ng/l

PCA Parietal cell antibodies. Normal values < 1/40

IFAb : intrinsic factor antibodies. Normal values < 1

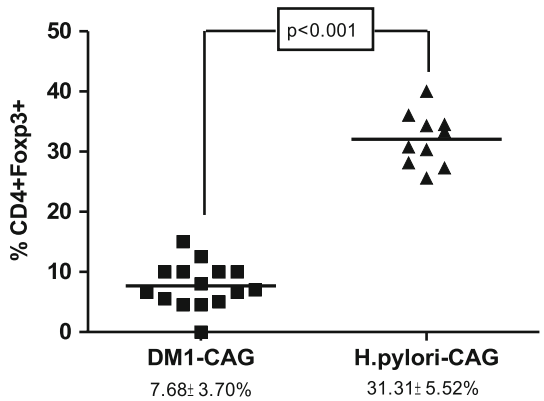

Fig. 4 Percentage of CD4+Foxp3+ cells of the total CD4+ cells in inflammatory infiltrates of gastric mucosa in DM1-CAG patients (7.68 ± 3.70%) and *H. pylori*-gastritis (31.31 ± 5.52%)

accumulating evidence that Tregs actively suppress the activation and expansion of self-reactive T cells, thereby preventing autoimmune disease [22]. The present study examined Tregs in DM1 patients with a second autoimmune disease (type A CAG) both in peripheral blood and in gastric mucosa.

The number of Tregs in peripheral blood in most autoimmune diseases investigated to date has either been normal [9–11, 23, 24] or decreased [25–27]. However, comparison of results among the different studies is complicated by the fact that some investigators studied the total CD4+CD25+ T cell population while others only evaluated the CD4+CD25^{high} T cells and few examined the more specific Tregs marker Foxp3 [28]. Data on Tregs in peripheral blood of patients with autoimmune gastritis are lacking.

Type 1 diabetes mellitus is the result of an autoimmune reaction that develops against pancreatic β-cell antigens [29]. Most studies using antibodies against CD4 and CD25 and, more recently, Foxp3, to evaluate Tregs in DM1 patients found no differences in the number of these cells in peripheral blood compared to healthy controls [9–12]. Our results concur with these findings in that the percentage of peripheral blood Tregs in DM1 patients was similar to controls with the phenotypic marker Foxp3. On the other hand, the CD4+CD25+Foxp3+ subpopulation in DM1-CAG patients was significantly higher compared with DM1 patients and controls. These cells expressed low/– levels of CD127, an excellent marker of Tregs in human peripheral blood [7], and high levels of other markers such as CD45RO, CD95, CCR4, CD62L, or CD152, which supports the regulatory nature of these cells. On the other hand, the increase in CD4+Foxp3+ cells found in DM1-CAG patients is probably not explained by an increase in activated T cells since no differences were observed among groups on analysis of the CD127^{high}CD25+ cells of the total CD4+Foxp3+ cells.

On analyzing the percentage of peripheral Tregs (CD4+Foxp3+CD25+CD127$^{low/-}$) in 16 long-standing DM1 patients, Liu et al. [7] found no differences between these patients and control subjects. However, in that study several of the DM1 patients had high Tregs numbers compared with the bulk of the control and DM1 patients. The presence of a second autoimmune disease, such as autoimmune gastritis, might explain the high Tregs levels observed in these DM1 patients although this point was not addressed by the authors. To our knowledge, only one study examined Tregs in patients with at least two autoimmune endocrine diseases [24]. That study analyzed peripheral CD4+CD25+ T cells in eight patients with polyglandular syndrome type II (APS-II) and found no differences compared to healthy subjects. In contrast to the present study, Foxp3 expression in peripheral blood cells was not analyzed. In addition, the study did not take into account whether patients had associated type A CAG. To determine whether the increase in Tregs was linked to CAG or to polyendrocrinopathy (DM1 + CAG), it would have been necessary to include a CAG group without DM1. However, the advanced age at which autoimmune CAG is diagnosed in patients with no other autoimmune diseases, but with age-related comorbidities, renders comparison with our young (mean age: 36.8 years) DM1-CAG patients difficult.

Factors required for survival of Tregs in the periphery remain controversial. Several studies have suggested that the generation and/or survival of Tregs require the presence of autoantigens in peripheral tissues [30]. This could explain why DM1-CAG patients compared with DM1 patients and controls showed an increase in peripheral Tregs, possibly due to the persistence of gastric mucosa antigens in autoimmune gastritis. This increase might also be, as postulated by others, a reflection of an attempt to stem the increasingly aggressive effector cells against gastric antigens, similar to what has been suggested for other inflammatory conditions [31]. The absence of differences among groups in the ratio of memory/activated T cells to regulatory T cells would not support this hypothesis. Nonetheless, the presence of an imbalance in regulatory processes cannot be discarded because of the low number of individuals analyzed. This issue should be addressed in further studies.

Most studies on Tregs in human autoimmune diseases have focused primarily on peripheral blood samples and few have evaluated Tregs expression in inflammatory tissue. However, several reports on patients with autoimmune diseases described the presence and characterization of Tregs both in peripheral blood and in target tissue. In active rheumatoid arthritis (RA) [32] and in active chronic inflammatory bowel diseases [26] reduced levels of CD4+CD25+ Tregs were observed in peripheral blood of these patients compared to target tissue. However, in these cases it has been postulated that the recruitment or migration of Tregs from blood to the inflammatory site may have been responsible for the decreased number of Tregs in peripheral blood. Indeed, studies on patients with RA suggest that Tregs migrate into joints and are inactivated at the site by inflammatory mediators [32]. The study by Marazuela et al. [33] analyzed Tregs in peripheral blood mononuclear cells and in thyroid cell infiltrates from 20 patients with autoimmune thyroid disease (AITD). They showed an increased percentage of CD4+Foxp3+ in AITD patients compared with controls and moderate Foxp3 expression in thyroid inflammatory cell infiltrates. In contrast, Nakano et al. [34] found no differences in the proportion of CD4+CD25+Foxp3+ T cells between AITD patients and controls, although the analysis was performed in a small number of patients ($n = 5$).

The present study is the first to determine Foxp3 expression in gastric mucosa of DM1-CAG patients. Analysis of gastric biopsies of DM1-CAG patients showed a higher proportion of Tregs in situ than that observed in normal gastric mucosa. Following the observation that Tregs number was higher in gastric mucosa of DM1-CAG patients, we then studied Tregs expression in gastric mucosa of another CAG inflammatory condition due to *H. pylori* infection. Interestingly, the percentage of gastric Tregs in DM1-CAG patients in this study was lower than that observed in patients with chronic gastritis by *H. pylori* infection. Current understanding of Tregs suggests that these cells may play a crucial role in suppressing the overall T-cell response to infection and cancer. In this regard, higher frequencies of CD4+CD25^{high} T cells were recently found in gastric biopsy specimens in *H. pylori*-infected individuals compared to uninfected controls, suggesting that these cells may suppress mucosal immune response and, therefore, contribute to the persistence of this infection [35]. Taking into account the results obtained in the present study, it could be argued that the number of Tregs implicated in the chronification of gastritis differs according to the etiology, i.e., higher if it is infectious (*H. pylori*) and lower if it is autoimmune (type A CAG).

One of the limitations of our study was that functional assays were not performed to test differences in Tregs function in patients with DM1-CAG compared with DM1 and controls. Most, albeit not all [10], studies carried out in DM1 patients showed an impaired ability of Tregs to suppress autologous effector T cells. However, recent in vitro studies in peripheral blood from DM1 patients suggested that, more than a defective suppressor function of peripheral Tregs, there is a resistance of effector T cells to Tregs [36]. Furthermore, studies on rheumatoid arthritis [32] and experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis [37], have stressed the

relevance of the inflammatory cytokine milieu in the target tissue which may render effector T cells resistant to the suppressive capacity of Tregs. Thus, analysis of Tregs function should ideally be performed in gastric Tregs. Nevertheless, despite the controversies surrounding functional regulatory T-cell studies, mix-and-matched functional experiments with allogenic and autologous CD4+ T cells and regulatory T cells might resolve some of these issues for further experiments.

A second limitation is that esophagogastroduodenoscopy was not performed in DM1 patients without CAG to rule out gastric corpus atrophy. However, a low serum pepsinogen I concentration and a low pepsinogen I to II ratio are widely accepted as good biochemical markers of gastric corpus atrophy. In the present study, all DM1 patients without CAG had normal pepsinogen I, pepsinogen I to II ratio and gastrin concentrations, which permitted gastric corpus atrophy to be ruled out and obviated fibrogastroscopy on ethical grounds.

In summary, we showed that DM1-CAG patients had a higher frequency of Tregs in peripheral blood compared to DM1 patients with no evidence of type A CAG or other associated autoantibodies, and healthy controls. Moreover, these cells were also present in the gastric mucosa of DM1-CAG patients, suggesting that the immune system recruits Tregs in an active attempt to control the inflammatory response which, however, fails possibly due to the inflammatory milieu of the gastric mucosa that would make effector T cells resistant to regulation. Interestingly, the number of Tregs in gastric mucosa of DM1-CAG patients was lower than in those with *H. pylori*-induced CAG. This fact underlines the influence of the percentage of Tregs in situ necessary for controlling the chronification of an autoimmune or infectious gastritis. Further studies are required to analyze the factors involved in Tregs homing to tissues and the different inflammatory milieux in autoimmune diseases as well as their potential therapeutic manipulation.

Materials and methods

Patients

Peripheral blood samples were obtained from 15 DM1-CAG patients whose characteristics are described in Table 1. Patients positive for nuclear antibodies (ANA), thyroperoxidase antibodies (TPOa), tyroglobulin antibodies (TGa), and transglutaminase antibodies or with autoimmune disease-related clinical symptoms were excluded. These patients were recruited from the Department of Endocrinology–Diabetology outpatient clinic of the University Hospital Germans Trias i Pujol (Badalona, Spain) [38, 39]. The diagnosis of CAG was based on the presence of low-serum pepsinogen I concentration, hypergastrinemia, pepsinogen I/II ratio under 3, positive PCA, and histologic confirmation as previously described [39]. Peripheral blood samples were also obtained from 15 DM1 patients (DM1) and 15 age- and sex-matched controls in whom the previously mentioned organ-specific autoantibodies as well as PCA were also negative. Endoscopic gastric biopsy specimens demonstrating different degrees of gastric mucosa atrophy according to the Updated Sydney System were obtained from the 15 DM1-CAG patients. In addition to the DM1-CAG group, gastric biopsies of 10 patients with confirmed *Helicobacter pylori*-induced CAG infection and 10 normal gastric mucosa biopsy specimens retrieved from the files of the Department of Pathology, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain and histologically identified by an experienced pathologist were also investigated for Foxp3 mucosal gastric expression. The *H. pylori* CAG-infected gastric biopsies were used as positive controls to detect Tregs in gastric mucosa. Briefly, during the procedure, two pairs of biopsy specimens were obtained from the antrum and corpus for histopathologic assessment of gastritis and for the presence of *H. pylori*. Sections of these biopsied specimens were used for immunohistochemical and immunofluorescence examination. Biopsy specimens were fixed in 10% formalin and embedded in paraffin. The sections (4 μm thick) were deparaffinized, rehydrated, and stained with hematoxylin-eosin. Giemsa staining was used to detect *H. pylori*.

Organ-specific autoantibodies, pepsinogen I, pepsinogen I/II ratio, and gastrin were determined as previously described [38, 39].

This study was approved by the local ethics committee and written informed consent was obtained from patients and controls.

Flow cytometry analysis

Lymphocyte staining was performed on 100 μl samples of whole peripheral blood following standard protocols. Briefly, cells were stained with surface molecules with the corresponding monoclonal antibodies: CD4-allophycocyanin (APC), CD27-phycoerythrin (PE), CD25-PE-Cychrom (PECy5), (all from BD Biosciences, San Jose, CA, USA) for 20 min at room temperature. After lysis of erythrocytes (FACS lysing solution, Becton Dickinson (BD), San Jose, CA, USA), cells were washed and fixed/permeabilized (fixation/permeabilization buffer, eBiosciences Inc., San Diego, CA, USA) for 45 min in the dark and washed twice with permeabilization buffer (eBiosciences). After centrifugation, a blocking step was performed with 2% normal mouse serum in 1× permeabilization buffer at 4°C for

15 min. Afterwards, 20 µl of fluorescein isothiocyanate (FITC)-conjugated anti-human Foxp3 antibody (PCH101 eBioscience) or isotype control were added and incubated for 45 min at 4°C in the dark. Cells were then washed twice in permeabilization buffer, resuspended in flow cytometry staining buffer and analyzed on a FACScalibur flow cytometer (BD). Data were collected and analyzed with CellQuest software (BD). The lymphocyte population was gated on the basis of forward and side scatter and CD4 expression.

In a subgroup of patients and controls, surface expression of CD127-PE, GITR-PE, CD49d-PE, CD122-PE, CCR5-PE, CD62L-PE, CCR4-PE, CD45RO-PE or CD95-PE and cytoplasmic staining of CD152 (CTLA-4)-PE were also analyzed. All studies were performed and analyzed in a blinded manner.

Immunofluorescence on tissue sections

For immunofluorescence staining, 4-µm-thick, formalin-fixed and paraffin-embedded blocks of gastric mucosa and tonsil (positive control) were cut, deparaffinized, and subjected to a heat-induced epitope retrieval step (30 min, 100°C in microwave in sodium citrate buffer solutions at pH 6.0). Prior to incubation with antibodies, sections were incubated with 10% normal goat serum for 30 min to reduce non-specific staining. Primary antibody (mouse anti-human Foxp3 (236A/E7 (IgG1), AbCAM) was added at 1:20 dilution in PBS supplemented with 1% bovine serum albumin and 10% normal goat serum and incubated overnight (ON) at 4°C. After three washing steps, sections were incubated with goat anti-mouse IgG1 Alexa fluor-488 antibody (Southern Biotechnology, Birmingham, AL) 1:400 in PBS/1% BSA for 30 min at room temperature. Sections were washed (× 3) and further incubated with mouse anti-human-CD3 (IgG2a, Novocastra Laboratories, Newcastle, UK) at 1:20 dilution for 1 h. Sections were washed and further incubated with goat-anti-mouse IgG2a-TRIC (Dako, Roskilde, Denmark) at 1:400 for 30 min at room temperature. Slides were examined with a Zeiss Axioplan® microscope equipped with UV and connected to a Leica high-sensitivity photographic camera. Negative controls were performed by adding an irrelevant isotype control antibody or omitting the primary antibodies.

Immunohistochemistry

For immunohistochemical staining, 4-µm sections of gastric mucosa were cut and deparaffinized as previously described. The primary antibodies included anti-Foxp3 (clone PHC101 IgG2a, dilution 1:20, eBiosciences) and anti-CD4 (clone 1F6, dilution 1:25, Novocastra). Sections were incubated with anti-Foxp3 antibody for 1 h. EnVision system (DAKO, Roskilde, Denmark) was used as visualization system and diaminobenzidine (DAB) as chromogen for Foxp3 staining. Subsequently, sections were incubated with anti-CD4 antibody for 1 h and then with a rabbit anti-mouse secondary antibody. Alkaline phosphatase anti-alkaline phosphatase (DAKO) was used as visualization system and permanent red (DAKO) as chromogen for CD4 staining. The number of CD4+/Foxp3 positive cells within the mucosal lamina propria was evaluated by counting the positive cells in 10 high-power fields in every section. The frequency of Foxp3+ T cells per mucosal CD4+ T cells was determined by dividing the number of CD4+Foxp3+ by the number of mucosal CD4+ cells. Quantification was performed by two independent investigators unaware of the diagnosis.

Statistical analysis

Descriptive results are expressed as mean ± SD or medians (interquartile range). Departure from normality was assessed by the Kolmogorov-Smirnov distribution test. Differences between groups were tested by the non-parametric Mann–Whitney *U* test. Differences among groups were examined by the Kruskal–Wallis test and when the test was significant at 0.05 level, pairwise comparisons were based on the Mann–Whitney *U* test followed by Bonferroni correction. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS/Windows version 12.0; SPSS Inc., Chicago, Il, USA).

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