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Circulating CD4⁺ CD25^{bright}FOXP3⁺ regulatory T-cells are significantly reduced in bullous pemphigoid patients

P. Quaglino, E. Antiga, A. Comessatti, M. Caproni, T. Nardo`, R. Ponti, M. Novelli, S. Osella-Abate, P. Fabbri, M. G. Bernengo

Abstract

Bullous pemphigoid (BP) is the most frequent autoimmune bullous skin disease, characterised by auto-antibodies against the hemidesmosome complex. Recently, regulatory T cells (Tregs) have been implicated in the development of several autoimmune diseases; few data are available in BP, failing to demonstrate a role of this subset in disease pathogenesis. The aim of this study was to investigate the expression and phenotypes of different Tregs (CD4⁺ CD25^{bright}FOXP3⁺ and CD8⁺ CD28⁻ cells) in BP to clarify whether the depletion of this subset constitutes one mechanism of tolerance loss. The CD4⁺ CD25^{bright}-FOXP3 and CD8⁺ CD28⁻ circulating subsets were determined by flow-cytometry in 26 untreated BP patients and compared with a group of age- and sex-matched healthy controls (HC, n = 30). Absolute and percentage values of the CD4⁺ CD25^{bright}FOXP3⁺ cells were significantly reduced in BP compared with HC (median CD25^{bright}- FOXP3⁺ expression within CD4⁺ cells: 1.8 vs. 3.5%, p = 0.002); conversely, BP patients were characterised by a significant expansion of the CD25^{bright}FOXP3⁻ “activated” T-cell subset. CCR4 and CD62L were expressed on the majority of CD4⁺ CD25^{bright}FOXP3⁺ cells (75.2 and 82.3%, respectively). No differences in the CD8⁺ CD28⁻ subset were found between BP and HC. This is the first report showing a significant reduction of circulating CD4⁺ CD25^{bright}FOXP3⁺ Treg frequency in BP patients.

Keywords: Bullous pemphigoid - Regulatory T cells - FoxP3 - CCR4

Introduction

Bullous pemphigoid (BP), the most common autoimmune blistering disease, is characterised by presence of auto-antibodies directed against components of the hemidesmosome adhesion complex, the BP230 and collagen XVII/BP180 proteins. The pathogenesis of disease involves also development of BP180-specific auto-reactive T cells, accumulation of other inflammatory cells, complement activation, mast-cell degranulation and protease release [6, 8, 11, 38].

Numerous immune-mediated diseases have been shown to present a reduction and/or function impairment of regulatory T cells (Tregs) [21, 28], a subset of effector cells which play an important role in tolerance control by silencing self-reactive T cells. In previous studies, we found a significant Treg down-modulation in immune-mediated skin diseases (psoriasis, scleroderma, dermatomyositis, lupus and Graft-versus-Host-Disease (GvHD))[1–3, 25, 26].

Distinct Treg subsets have been described [10, 19]. The bright CD25 expression identifies both naturally occurring Tregs (nTregs), characterized by the intracellular expression of transcription factor forkhead-box-P3 (FOXP3) and “antigen induced” Tregs, whose suppressive functions are mediated by IL-10 and TGF- β release. CD8⁺ Suppressor lymphocytes are CD28^{negative}, do not determine apoptosis and induce immune suppression through an antigen- or non-antigen-specific mechanism [5, 9, 13, 14].

To date few studies focused on Tregs in autoimmune bullous skin diseases. A significant depletion of Tregs was demonstrated in pemphigus vulgaris (PV) [31, 35]. Experimental studies confirmed that FoxP3 inhibition abrogated the suppressor activity on desmoglein3-specific cell clones [36, 37] and that anti-desmoglein-3 auto-antibodies were suppressed by the adoptive transfer of Tregs[39]. As for BP, only two papers were published, reporting conflicting results [27, 4]. One study [27] failed to demonstrate significant differences in Treg number in both skin and blood of 14 BP patients with respect to healthy donors, Tregs from BP patients maintaining also their ability to suppress T-cell proliferation [27]. A recent paper [4] showed, however, a significant lower expression of FOXP3⁺ Treg cells in the lesional skin of BP compared with PV patients, coupled with an accumulation of Th17 cells.

The aim of this study was to investigate the expression and phenotypes of two distinct Treg circulating subsets (CD4⁺ CD25^{bright}FOXP3⁺ and CD8⁺ CD28⁻) in BP patients to clarify whether the depletion of this subset constitutes one potential mechanism of tolerance loss induction.

Materials and methods

Patients

Twenty-six BP patients (17 males, 9 females; median age 74 years, range: 59–88) were enrolled, after getting their written informed consent, in two Italian dermatological centres (Florence and Turin). The study was approved by the Ethics Committee in each institution. All the patients were included at the time of first BP diagnosis before receiving steroids or other immuno-suppressive therapies. BP diagnosis was based on the following criteria: typical clinical features, with skin blistering in elderly patients; subepidermal blistering and eosinophil/neutrophil inflammatory infiltrate; linear IgG/C3 deposits along the dermal-epidermal junction at direct immunofluorescence examination, linear IgG deposits at the blister roof of sodium chloride split human skin at indirect immunofluorescence; circulating anti-BPAg2 (collagen XVII/BP180) antibodies at enzyme-linked immunosorbent assay (ELISA). Disease extent and severity was graded according to the ABSIS score (autoimmune bullous skin disorder intensity score) [24] (scoring range 0–206, 150 points maximum for skin involvement, 11 for oral involvement and 45 for subjective discomfort).

Flow cytometric analysis

Peripheral blood sampling was performed during the first visit before systemic treatment was started. A group of age- and sex-matched healthy subjects (n = 30) were used as healthy controls (HC). Peripheral blood lymphocyte samples were evaluated according to their immunofluorescence reactivity using FACSCalibur/FACSCanto TM II cytometers and analyzed with CellQuest/FACSDiva (Becton–Dickinson, San Jose, CA, USA). Surface markers were performed by four- or six-colour immunofluorescence analyses, using simultaneously antibodies conjugated to FITC, PE, PerCP or PerCP Cy5.5, Pe-Cy7, APC and APC-H7. At least 10,000 lymphocytes were collected for each antibody combination. Lymphocyte purity was verified by standard forward and sideways scattering parameters, using a CD45 gating analysis. The following MoAbs were analysed: CD3 FITC, PerCP Cy5.5 or APC-H7 (SK7, mouse IgG1), CD4 PerCP or Pe-Cy7 (SK3, mouse IgG1) CD8 APC-H7 (SK1, mouse IgG1), CD16 FITC (NKP15, mouse IgG1), CD19 APC (SJ25C1, mouse IgG1), CD28 PE (CD28.2, IgG1), CD56 PE (MY31, IgG1), anti-CCR4 PE (IG1, IgG1), anti CD25 PE or APC (M-A251, mouse IgG1), anti CD62L PE or APC (Dreg 56, mouse IgG1), all purchased from BD Biosciences (San Diego, CA, USA).

The CD4⁺ CD25⁺ population can be divided into two different levels of expression, i.e. low CD25 level (defined CD4⁺ CD25^{low}) and high CD25 level (defined CD4⁺ CD25^{bright}) [9–13] that appear to have a tail to the right of the major population containing both CD4⁺ CD25^{low} and CD4⁺ CD25⁻ cells. FOXP3 expression was analysed using mononuclear cells purified from peripheral blood using Lymphoprep (1.077 g mL; Axis-Shield, Oslo, Norway) density gradient centrifugation. The cells were incubated with surface antibodies, anti-CD4 PerCP or PE-Cy7 and anti-CD25 APC for 10 min at room temperature, then stained with anti-FOXP3 PE (clone PCH101, rat IgG2a; eBiosciences, San Diego, CA, USA), according to the manufacturer's protocol, using fixation and permeabilisation buffers from the same provider. nTreg values were determined as the percentage of CD25^{bright}FOXP3⁺ within the CD3⁺ CD4⁺ compartment. Statistical analysis The results are presented as medians, 25th- and 75th-percentile. The Mann–Whitney U test and the Kruskal–Wallis with Dunn posthoc test were used to compare data, giving similar results. p<0.05 was considered statistically significant.

Results

Circulating T-cell subsets

No significant differences in the median percentage values of circulating T CD3⁺ CD4⁺ and CD3⁺ CD8⁺, B and natural killer (NK) cells were found between BP patients and HC (Table 1). By contrast, CD25^{bright}FOXP3⁺ expression within the CD4⁺ subset was significantly lower in BP patients (median: 1.8%, 25th–75th percentile: 1–3.1%) than in HC (median: 3.5%, 25th–75th percentile: 3–4%) (p = 0.002) (Fig. 1a). The same differences were demonstrated when considering absolute values of the circulating CD4

+CD25^{bright}FOXP3⁺ subset (median 20 cells/ll, 25th–75th percentile: 13–35, in BP versus 28 cells/ll, 21–31, in HC) ($p = 0.05$) (Fig. 1b). No relationship was found between levels of CD4⁺ CD25^{bright}FOXP3⁺ and the disease extent as determined by the ABSIS score. No significant differences in the percentage expression of CD28⁻ cells within the CD8⁺ T-cell population were found between BP patients and HC.

CD25 expression intensity and CCR4, CD62L and FOXP3 phenotype characterization

On the basis of CD25 expression, three separate sub-populations have been singled out, CD4⁺ CD25^{bright}, CD4⁺ CD25^{low} and CD4⁺ CD25⁻ cells. CD4⁺ CD25⁻ absolute circulating cells were significantly higher in HC (median: 502 cells/ll, 25th–75th percentile: 428–545) than in BP (median 281 cells/ll, 25th–75th percentile: 237–367) ($p = 0.001$). No differences between BP and HC could be found in absolute CD4⁺ CD25^{low} cells (median BP: 370 cells/ll, 25th–75th percentile, vs. 412 cells/ll, 313–542 in HC). On the other hand, CD4⁺ CD25^{bright} cells constituted a small subgroup, with a significantly higher percentage (median = 4.7 and 2.8%, respectively) and absolute expression (median 244 cells/ll, 25th–75th percentile: 106–575 and 37 cells/ll, 21–98) in BP than in HC ($p = 0.001$).

CCR4⁺, CD62L⁺ and FOXP3⁺ expression were analysed within these three different T-cell subsets in both BP patients and HC (Table 2). CCR4 was expressed on CD4⁺ CD25^{bright} cells in both BP and HC (median 71.2 and 74.8%, respectively) and significantly decreased in CD4⁺ CD25^{low} (47.9 and 34.6%, respectively) and, particularly, CD4⁺ CD25⁻ cells (16 and 18%, respectively) ($p = 0.0001$ in BP and $p = 0.0001$ in HC; Kruskal–Wallis test), without differences between BP patients and HC.

CD62L was expressed on both CD4⁺ CD25^{bright} and CD4⁺ CD25^{low} cells without significant differences between BP and HC. A significant CD62L reduction was found in the CD4⁺ CD25⁻ subset only in BP patients (64.9%) with respect to HC (85.2%; $p = 0.003$). FOXP3 expression, as expected, was mainly confined to the CD4⁺ CD25^{bright} subset, with less than 5% of FOXP3⁺ cells being observed in both CD4⁺ CD25^{low} and CD4⁺ CD25⁻ cells ($p < 0.001$). FOXP3 expression within the CD4⁺ CD25^{bright} subset was significantly reduced in BP with respect to HC (median: 35.7 vs. 57.9%; $p = 0.008$) (Fig. 2). This implies that BP patients are characterised by a significant expansion of the CD25^{bright}FOXP3⁻ “activated” T-cell subset with respect to HC. CCR4 and CD62L were expressed on the majority of CD4⁺ CD25^{bright} FOXP3⁺ cells (median: 75.2 and 82.3%, respectively).

Discussion

Our study shows for the first time that BP patients are characterised by a significant reduction in the percentage and absolute values of circulating CD4⁺ CD25^{bright}FOXP3⁺ Tregs, associated with an opposite up-regulation of the CD4⁺ CD25^{bright} “inflammatory” cell subset. On the other hand, we failed to demonstrate a significant modification in CD8⁺ CD28⁻ Treg cells [22, 34]. However, this latter subset has been found reduced in immune-driven diseases such as type-1 juvenile diabetes mellitus and systemic lupus erythematosus affecting predominantly young people [22, 34]; the age of the patients can play a role in this phenomenon as it is known that the CD8⁺ CD28⁻ subset increases with age [12, 30] and BP affects predominantly elderly people (74 years median age in this study). The CD25^{bright} expression does not exclusively identify this T-cell population as it can be found also on activated T cells and APC [10, 19], whilst FOXP3 is the most exclusive Treg marker as the inhibitory properties lie in the FOXP3⁺ subset [10, 18, 20, 32, 33]. Indeed, BP patients were characterised by a significant expansion of the CD4⁺ CD25^{bright} and reduction of the CD4⁺ CD25⁻ subset with respect to HC, which was coupled by an opposite marked down-regulation of the FOXP3⁺ compartment (64.3% of the CD4⁺ CD25^{bright} subset were FOXP3 negative). From a biological point of view, these data suggest that in BP patients, the Treg reduction is associated with an expansion of the “activated” CD25^{bright}FOXP3⁻ inflammatory compartment. These findings can at least partially explain the discrepancies between our study and that of Rensing-Ehl et al. [27]. In that paper, circulating Tregs were analysed on the basis of the CD25^{bright} expression; even if FOXP3 expression was reported to be higher among the CD4⁺ CD25^{bright} subset, no specific comparison data as to FOXP3 expression in BP and HC are shown. The paper by Rensing-Ehl et al. [27]

reports two other main findings which are not available in our study, the presence of significant amounts of FOXP3⁺ cells in the lesional BP skin and the absence of functional Treg impairment. Indeed, patients with rheumatoid arthritis demonstrated a significant Treg increase in synovial fluid suggesting that recruitment to inflammatory sites may be responsible for circulating Treg reduction [23]. This phenomenon has not been demonstrated in BP, as the recent data by Arakawa et al. [4] showed a significant reduction in FOXP3⁺

cells in BP lesional skin compared with pemphigus vulgaris. As to the effective functional properties of these cells, the phenotypic characterisation performed in our study can give some more insights. Our data showed that the large majority (70–80%) of CD4⁺ CD25⁺ bright cells expressed CCR4 and CD62L. CCR4 is a chemokine receptor involved in the recruitment of skin-specific lymphocytes [7]. Hirahara et al. [16] showed that virtually all circulating CD4⁺ CD25^{bright}FOXP3⁺ Treg expressed high CCR4 levels, and that this subset showed marked suppressive properties. CCR4 expression may drive Treg cells to sites of antigen presentation (such as the skin in BP patients) supporting the down-regulation of T-cell activation [17]. As to CD62L expression, CD4⁺ CD25^{bright} nTregs positive for L-selectin display a significantly stronger suppressor activity than the negative ones [15, 29]. Taken together, the CD4⁺ CD25^{bright}- FOXP3⁺ cells shown to be reduced in BP patients exhibited the Tregs characteristic phenotypic features. The Treg phenotypic modulation shown by our study in BP patients is similar to that reported in PV patients where a reduction in circulating Tregs and a preferential CCR4 and CD62L expression on the CD4⁺ CD25^{bright} cells were reported [31].

In conclusion, this study shows for the first time a significant down-regulation of circulating Tregs in BP patients. Future studies are mandatory to identify the effective role of this modification in the disease pathogenesis as well as its relationship with the clinical response.

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Conflict of interest

none.

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Fig. 1 Expression percentage levels of the CD25^{bright}FOXP⁺ subpopulation within the CD4⁺ cell subset (Fig. 1a) and absolute values of the CD4⁺CD25^{bright}FOXP⁺ cells (Fig. 1b) in BP patients and healthy controls. Each dot represents a patient; horizontal bars indicate median values. p = 0.002 (Fig. 1a) and p=0.05 (Fig. 1b)

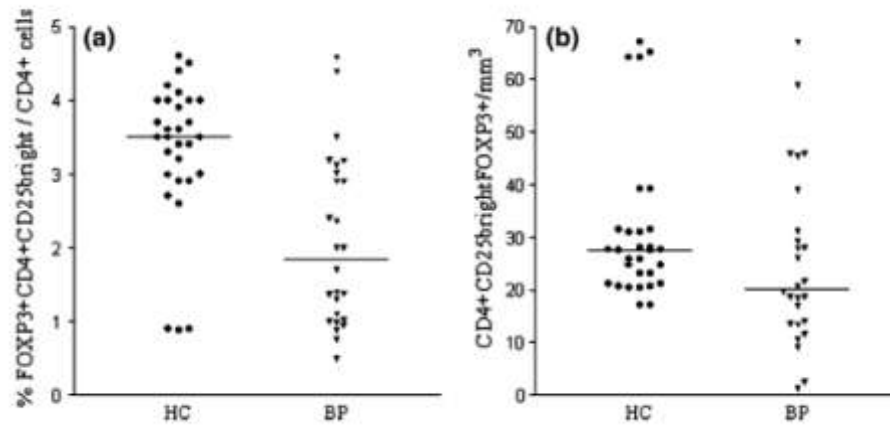


Fig. 2 Representative dot plots from a BP patient (Fig. 2a) and a normal control (HC, Fig. 2b) showing the reduction of the FOXP3+ cells within the CD25 bright subset

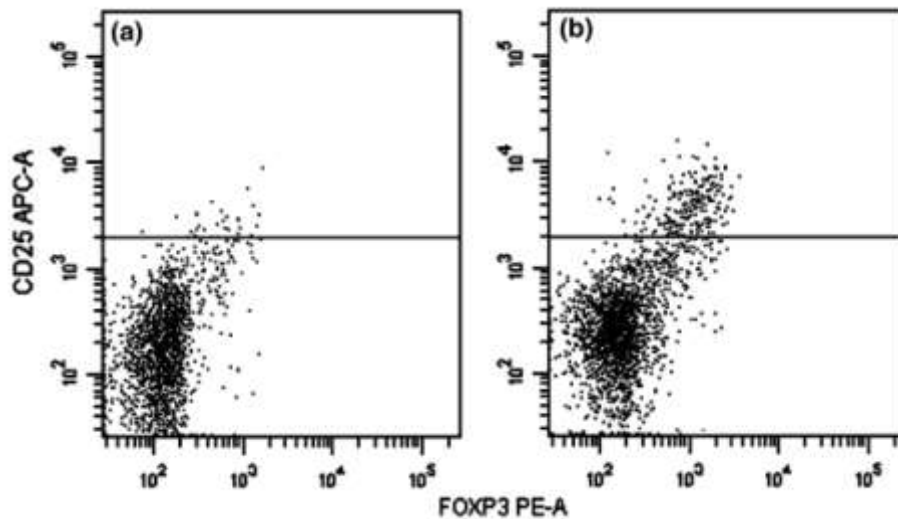


Table 1 Circulating lymphocyte subsets in BP and HC

Parameters	PB group (%)		Healthy control group (%)	
	Median	25th–75th percentile	Median	25th–75th percentile
CD3+	75	72–80	73	69–76
CD3+CD4+	49	43–57	51	43–53
CD3+CD8+	26	17–33	20.5	18–20.5
NK (CD3-CD16+/CD56+)	12	6–14	14	11–16
B cells (CD19+)	9	4–12.5	10.5	8.7–13
CD4+CD25brightFOXP3+a	1.8*	0.9–3.2	3.5*	3–4
CD8+CD28-b	74	62.5–90	65	48–76.5

* p=0.002

a expressed as a percentage of CD25brightFOXP3+ cells within the CD4+ circulating compartment

b expressed as a percentage of CD28negative cells within the CD8+ circulating compartment

Table 2 Phenotype characterisation of circulating subsets on the basis of CD25 expression intensity

	CD4+CD25bright			CD4+CD25low			CD4+CD25-		
	CCR4	CD62L	FOXP3	CCR4	CD62L	FOXP3	CCR4	CD62L	FOXP3
Healthy controls	74.8 (61.8–83)	88.6 (84.9–94.4)	57.9 (52.4–63.4)	47.9 (37.2–52.7)	75.4 (71.5–79.3)	4.7 (3.4–5.6)	18.0 (12.8–22.6)	85.2 (80.2–89.2)	2.5 (1.3–3.7)
BP patients	71.2 (51.6–84)	81.7 (70.6–89)	35.7 (20.0–61.9)	34.6 (19.5–39.3)	77.6 (59.7–83.9)	1.6 (0.7–5.0)	13.8 (6.7–17.1)	64.9 (43.0–75.7)	1.4 (0.7–2.4)

Values are expressed as percentage median (25th–75th percentile in brackets)