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Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

The Treg/Th17 cell ratio is reduced in the skin lesions of patients with pyoderma gangrenosum / Caproni M; Antiga E; Volpi W; Verdelli A; Venegoni L; Quaglino P; Fabbri P; Marzano AV.. - In: BRITISH JOURNAL OF DERMATOLOGY. - ISSN 0007-0963. - STAMPA. - 173:(2015), pp. 275-278.

*Availability:*

This version is available at: 2158/969192 since: 2015-11-09T11:05:10Z

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Accepted Date : 27-Dec-2014

Article type : Research Letter

### **The Treg/Th17 cell ratio is reduced in the skin lesions of patients with pyoderma gangrenosum**

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**Funding:** The study was funded by a grant Ministry of Instruction, University and Research of the Italian Government within the funding program PRIN 2008 (project code: 2008EW3FHK).

**Conflict of interests:** None

**Keywords:** pyoderma gangrenosum, Sweet's syndrome, regulatory T cells, T helper 17 cells

Pyoderma gangrenosum (PG) is a rare, inflammatory skin disease that, together with other conditions such as Sweet's syndrome (SS), is included within the group of neutrophilic dermatoses.<sup>1</sup> Although its pathogenesis remains poorly understood,<sup>2</sup> the treatments with the best clinical evidence are tumor necrosis factor (TNF)-inhibitors, high-dose systemic corticosteroids and cyclosporine, suggesting the pivotal role of inflammatory pathways in the development of PG.

Interestingly, recent studies highlighted the role of T helper 17 (Th17) cells in neutrophilic dermatoses,<sup>2</sup> and an increase of IL-17<sup>3</sup> and IL-23<sup>4</sup> expression was found in PG lesions.

Together with Th17 cells, regulatory T cells (Tregs) play a major role in human disease.<sup>5</sup> Accordingly, recent reports suggest that controlling the balance between Treg and Th17 cells may be a promising therapeutic strategy for inflammatory diseases.<sup>6</sup> However, no data are present in the literature about Tregs in PG.

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In this study, we investigated the proportions of Tregs and Th17 cells in the skin of 15 PG patients (7 males, 8 females; age range 27-69 years), not on immunosuppressive treatment nor topical steroids for at least 4 weeks prior to entering the study.

As control, skin samples from 5 SS patients (2 males, 3 females; age range 31-64 years) and 6 normal subjects (NS) (3 males, 3 females; age range 28-59 years) were collected.

The trial was approved by ethical committee and conducted according to the Declaration of Helsinki. All the patients and controls provided written informed consent.

Treg and Th17 markers were analysed by immunohistochemistry using monoclonal antibodies (mAb) anti-CD4 (1:20; Dako, Copenhagen, Denmark), anti-CD25 (1:25; Histo-Line Laboratories, Milan, Italy), anti-CD161 (1:80; AbD Serotec, Oxford, UK), anti-FoxP3 (1:80; Abcam, Cambridge, UK), anti-IL10 (1:300; Dako), anti-IL-17 (1:1000; Abcam), anti-ROR $\gamma$ t (1:2000; R&D Systems, Minneapolis, MN, USA), anti-TGF $\beta$ 1 (1:2000; Abcam), as described previously.<sup>7</sup>

The stained cells were counted in three consecutive microscopic fields (400 $\times$ ). Furthermore, FOXP3<sup>+</sup>/CD4<sup>+</sup>, TGF- $\beta$ <sup>+</sup>/CD4<sup>+</sup>, IL-10<sup>+</sup>/CD4<sup>+</sup> and ROR $\gamma$ t<sup>+</sup>/CD4<sup>+</sup> cell ratios were calculated. The results were analysed with Mann-Whitney *U* test and considered significant with a *p* value <0.05.

In PG and SS, CD4<sup>+</sup> and CD25<sup>+</sup> cells were located in the whole dermis with some cells scattered in the epidermis (Fig. 1a,b,d,e). The number of CD4<sup>+</sup> cells in PG was significantly higher than in SS (*p*=0.0003), while no differences were found for CD25<sup>+</sup> cells. By contrast, CD4<sup>+</sup> and CD25<sup>+</sup> cells were significantly less represented in NS than in the other two groups (*p*<0.0001) (Fig. 2a).

Some FOXP3<sup>+</sup> cells were detected within the superficial dermis of patients with PG (Fig. 1g); their number was higher than in NS (*p*=0.004), but lower than in SS (*p*=0.0001) (Fig. 2a). Interestingly, the FOXP3<sup>+</sup>/CD4<sup>+</sup> cell ratio was significantly lower in PG compared to SS (*p*<0.0001) and NS (*p*<0.0001) (Fig. 2a).

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Some IL-10<sup>+</sup> cells were found in PG superficial dermis (Fig. 1j); their number was significantly lower than in SS ( $p < 0.0001$ ) and higher than in NS ( $p < 0.0001$ ). Moreover, the IL-10/CD4<sup>+</sup> cell ratio was reduced in PG than in SS and NS ( $p < 0.0001$  and  $p = 0.03$ , respectively) (Fig. 2a).

TGF- $\beta$  staining was diffusely distributed within the superficial and medium dermis in PG and SS. Moreover, some TGF- $\beta$ <sup>+</sup> cells could be detected in the same areas (Fig. 1m,n). Their number was similar in both PG and SS. By contrast, the TGF- $\beta$ <sup>+</sup>/CD4<sup>+</sup> cell ratio was significantly reduced in PG ( $p = 0.01$ ). Moreover, although NS showed a lower number of TGF- $\beta$ <sup>+</sup> cells than PG ( $p < 0.0001$ ) and SS ( $p < 0.0001$ ), their TGF- $\beta$ <sup>+</sup>/CD4<sup>+</sup> cell ratio was significantly higher (NS vs PG:  $p = 0.001$ ; NS vs SS:  $p = 0.0001$ ) (Fig. 2a).

Regarding Th17 markers, ROR $\gamma$ t<sup>+</sup> cells were distributed in the upper dermis in PG (Fig. 1p); their number was similar to that found in SS. By contrast, the ROR $\gamma$ t<sup>+</sup>/CD4<sup>+</sup> ratio was significantly lower in PG than in SS ( $P = 0.008$ ) (Fig. 2a). As expected, no ROR $\gamma$ t expression was found in NS. The numbers of both CD161<sup>+</sup> and IL-17<sup>+</sup> cells, that were predominantly distributed in the superficial and medium dermis (Fig. 1s,t), were similar in PG and SS, while no CD161 nor IL-17 expression was detected in NS (Fig. 2a).

Finally, in order to quantify the balance between Tregs and Th17 cells, we calculated the ratio between FOXP3 and ROR $\gamma$ t, that was significantly lower in PG than in SS ( $p < 0.0001$ ) (Fig. 2b).

Our study demonstrated a reduced proportion of Tregs in PG skin, that may be responsible for an impairment of the suppressive activity, leading to the development of the lesions. Interestingly, Treg reduction was not found in SS, where the skin inflammation is less strong and the tissue damage milder.

Accordingly, we found significantly reduced IL-10<sup>+</sup>/CD4<sup>+</sup> and TGF- $\beta$ <sup>+</sup>/CD4<sup>+</sup> cell ratios in PG. IL-10 and TGF- $\beta$  are involved in the biology of Tregs, and their defective signaling is associated with

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inflammatory conditions.<sup>8-10</sup> In agreement with our results, PBMCs from patients with PAPA syndrome (pyogenic sterile arthritis, PG, and acne) showed a diminished production of IL-10 after stimulation, suggesting an impairment of IL-10 pathway.<sup>11</sup>

As a result, in PG, the impaired T cell regulation due to the reduction of Tregs and of the regulatory cytokines may allow the uncontrolled activation of effector T cells, such as Th17 cells.

Interestingly, in PG skin, we found augmented numbers of Th17 cells, that may play a role in the disease *via* the recruitment of neutrophils and monocytes,<sup>12</sup> and the induction of MMPs.<sup>13</sup>

Accordingly, some Authors reported the effectiveness of therapies targeting the Th17 pathway in the treatment of PG.<sup>4,14</sup> In this view, our findings may pave the way to the use of novel Th17-oriented therapies such as IL-17 antagonists for PG therapy.

Finally, we found an impairment of the balance between Tregs and Th17 cells in PG but not in SS, that could explain the most severe clinical course of the former. Interestingly, a reduced ratio of Tregs to Th17 cells was recently found even in patients with inflammatory bowel diseases,<sup>15</sup> that are often associated to PG, suggesting that common mechanisms could be implicated in immune system dysregulation in both conditions.

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**Legend to figures:**

**Figure 1.** Immunohistochemical staining for CD4, CD25 and FOXP3 as markers of Tregs (a-i), for the regulatory cytokines TGF- $\beta$  and IL-10 (j-o), as well as for the Th17 cell markers ROR $\gamma$ t, CD161 and IL-17 (p-x) in skin biopsy specimens from patients with pyoderma gangrenosum (PG) and Sweet's syndrome (SS). Scale bar = 100  $\mu$ m

**Figure 2.** (a) Numbers of CD4<sup>+</sup>, CD25<sup>+</sup>, FOXP3<sup>+</sup>, TGF- $\beta$ <sup>+</sup>, IL-10<sup>+</sup>, ROR $\gamma$ t<sup>+</sup>, CD161<sup>+</sup> and IL-17<sup>+</sup> cells expressed as medians, as well as FOXP3/CD4, TGF- $\beta$ /CD4, IL-10/CD4 and ROR $\gamma$ t/CD4 cell ratios expressed as % of CD4<sup>+</sup> cells in the inflammatory infiltrate of skin biopsy specimens from patients with pyoderma gangrenosum (PG), Sweet's syndrome (SS) and normal subjects (NS).

(b) FOXP3/ROR $\gamma$ t cell ratios in the inflammatory infiltrate of skin biopsy specimens from patients with PG and SS. PG showed a significantly reduced ratio than SS. \*: p<0.05.



**Supplementary table 1.** Clinical findings in 15 patients with pyoderma gangrenosum.

N	Sex	Age (years)	Disease duration (months)	Disease severity (BSA%)	Associated diseases	Family history
1	M	65	6	12	Klinefelter	Negative
2	M	61	3	8	-	Negative
3	F	28	12	18	Cystic fibrosis	Negative
4	M	30	18	6	IBD	Negative
5	F	35	10	5	-	Negative
6	F	30	4	8	IBD	Negative
7	M	67	3	20	-	Negative
8	M	62	24	8	-	Negative
9	M	57	18	3	-	Negative
10	F	44	48	2	-	Negative
11	F	69	40	3	-	Negative
12	M	42	6	6	-	Negative
13	F	48	16	1	-	Negative
14	F	53	36	10	-	Negative
15	F	38	36	6	-	Negative

IBD: inflammatory bowel disease

**Supplementary table 2.** Quantitative analysis on the numbers of positive cells for field (400X) in skin lesions of patients with pyoderma gangrenosum and Sweet's syndrome, as well as in normal subjects as assessed by immunohistochemistry

	<b>PG</b>	<b>SS</b>	<b>NS</b>
<i>CD4</i>	103 [90.7-143.5]	69 [54-77]	4.5 [2-7]
<i>CD25</i>	18.5 [14-23.2]	22 [17.5-27]	1.5 [0.25-2]
<i>FOXP3</i>	11 [2.2-16]	19 [18-23.5]	1 [0-2]
<i>% FOXP3/CD4</i>	8.5 [3-12.7]	28 [26-29]	25 [6.2-33.3]
<i>TGF-<math>\beta</math></i>	28 [23.7-35.2]	28 [24.5-31]	3 [1.25-4]
<i>% TGF-<math>\beta</math>/CD4</i>	23.9 [18.9-27.3]	36.8 [32.64-43]	63.6 [50-73.2]
<i>IL-10</i>	16 [11.2-18]	24 [18-27]	1 [0-1.75]
<i>% IL-10/CD4</i>	14.3 [12.1-16.6]	30.4 [22.4-39.5]	20 [11.7-38]
<i>ROR<math>\gamma</math>t</i>	15 [13-18]	16 [11.2-32.2]	0 [0-0]
<i>% ROR<math>\gamma</math>t/CD4</i>	12.1 [8.3-22.1]	23.3 [17.4-40.8]	0 [0-0]
<i>CD161</i>	32 [23.5-65.5]	27 [17.5-31.5]	0 [0-0]
<i>IL-17</i>	20.5 [17-25.2]	17.5 [13.2-23.5]	0 [0-0]
<i>% FOXP3/ROR<math>\gamma</math>t</i>	32.4 [13.1-76.4]	122.1 [67.3-156.2]	0 [0-0]

Positive-cell counts are expressed as medians [25th–75th percentile].

PG, pyoderma gangrenosum; SS, Sweet's syndrome; NS, normal subjects.



