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Phenotypical characterization of circulating cell subsets in pyoderma gangrenosum patients: the experience of the Italian immuno-pathology group

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Abstract

Background No data are available as to the phenotype of circulating lymphocyte subsets in pyoderma gangrenosum (PG).

Aim To analyse the expression of different chemokine receptors associated to T-helper (Th)1 (CCR5), Th2 (CCR4) and Th17 (CCR6), as well as the regulatory T-cell subset (Treg) and dendritic cell polarization in the blood of newly diagnosed untreated PG patients.

Materials and methods Multi-parameter flow cytometry was performed on blood samples from 10 PG patients collected at first diagnosis among centres belonging to the Italian Immuno-pathology Group. Blood samples from 10 age and sex-matched healthy controls (HC) were used as controls.

Results PG patients are characterized by an over-expression in the blood of the CD4+CCR5+ and CD4+CCR6+ and a down-regulation of CD4+CCR4+ counts with respect to healthy subjects. Moreover, they show increased levels of myeloid derived dendritic cells type1 and reduced levels of the Treg CD4+CD25^{high}FOXP3+ subset.

Conclusions The pattern of chemokine expression argues in favour of a Th1 (CCR5+) and Th17 (CCR6+) polarization with a down-regulation of Th2 (CCR4+).

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Conflicts of interest None declared.

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Introduction

Pyoderma Gangrenosum (PG) is a rare skin disease which affects middle aged mainly female patients, clinically characterized by recurrent deep erythematous to violaceous painful necrotic ulcers with well-defined borders frequently localized to the lower limbs^{1–3} and histopathologically by the presence of a diffuse dermal infiltrate mainly constituted by neutrophils.

Besides neutrophils, however, the skin infiltrate of PG is also composed by lymphocytes predominant in the wound edge of the ulcerative lesions.^{4,5} Moreover, expanded cell clones circulating between the blood and the skin were found in PG patients by means of complementarity spectratyping, suggesting a trafficking of these cells between the two compartments.⁶ The cytokine lymphocyte pattern is not well known. In a previous study, we demonstrated by means of a sandwich-based protein antibody array method, an over-expression of cytokines/chemokines such as IL-1, IL-8, IL-17, RANTES, CXCL1/2/3 and CXCL16 in the PG cutaneous infiltrate, as well as of molecules amplifying the inflammatory network such as TNF- α , metalloproteinases, CD40/CD40 ligand and Fas/Fas ligand.⁷ No data are available as to the pattern of lymphocyte activation in the peripheral blood.

This study was designed to analyse the expression of different chemokine receptors associated to T-helper (Th)1 (CCR5), Th2 (CCR4) and Th17 (CCR6), as well as the regulatory T-cell subset (Treg) and dendritic cell polarization in the blood of PG patients.

Materials and methods

Patients

Ten PG patients (four males and six females; median age: 53.5 years) (Table 1) were included after written informed consent and study approval by the Ethical Committees of each institution.

Blood samples were collected from the centres of Milan, Florence, Terni and Turin belonging to the Italian Immuno-pathology Group, in all cases at first diagnosis before both systemic and topical specific treatment. Flow cytometry analyses were performed in the Dermatologic Clinic of Turin. Blood samples from 10 age- and sex-matched healthy controls (HC) were used as controls.

The diagnosis of PG was established on the basis of clinicopathologic criteria.^{1–4} Patients were categorized according to the clinico-therapeutic algorithm proposed by Marzano et al.⁸ and all presented with the classic ulcerative variant.

Flow Cytometry

Peripheral blood lymphocytes were evaluated according to their immuno-fluorescence reactivity using FACSCalibur/FACSCanto TM II cytometres and analysed with CellQuest/FACSDiva (Becton-Dickinson, San Jose, CA, USA). Surface markers were performed using whole blood. Surface markers were performed by four or six-colour immuno-fluorescence 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, IgG1), CD4 PerCP or Pe-Cy7 (SK3, IgG1) CD8 APC-H7 (SK1, IgG1), anti-CCR4 PE (IG1, IgG1), anti-CD25 PE or APC (M-A251, IgG1), anti-CCR5 PE (2D7/CCR5), CCR6 PE-Cy7 (11A9), CD11c APC (B-ly6, IgG1), CD123 PE (9F5, IgG1), HLA-DR PE-Cy7(L243) all purchased from BD Biosciences (San Diego, CA, USA). Flow cytometry determinations were performed using whole blood for all the markers except FOXP3.

The peripheral blood dendritic cell (DC) subsets were identified as myeloid derived DCs (DC1) characterized by a CD11c+CD123-HLA-DR+ phenotype, and lymphoid (or “plasmacytoid”) derived DCs (DC2) expressing a CD11c-CD123+HLA-DR+ phenotype.⁹

The CD4+CD25+ population can be divided into two different levels of CD25 expression (low and high)¹⁰ 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. Treg 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 post hoc test were used to compare data, giving similar results. $P < 0.05$ was considered statistically significant.

Results

The median values of the CD3+CD4+ and CD3+CD8+ subsets were respectively 48% (25th–75th percentile: 39–49%) and 26% (26–36%). The CD3+CD4+/CD3+CD8+ ratio ranged from 1.2 to 4.5 (median 1.45). No difference in the distribution of these values was found between PG and HC. On the other hand, significant differences were found between PG patients and HC in the expression of other parameters studied (Fig. 1). The percentage values of the myeloid (type 1) DCs were significantly higher in PG (median 3.2%; 25th–75th percentile: 2.4–4.3) than in HC (Mann–Whitney U-test: $P < 0.001$), whilst no differences were found in the percentage levels of the lymphoid/plasmacytoid (type 2) DCs between PG patients and HC. In PG patients, the values of myeloid (type 1) were significantly higher than those of lymphoid (type 2) DCs (median values: 0.35%; 25th–75th percentile: 0.2–0.9) (Mann–Whitney U-test: $P < 0.001$). As a consequence, the ratio of myeloid to lymphoid/plasmacytoid DCs was significantly higher in PG patients than in HC (9.1 vs. 1.6; $P < 0.001$). A statistically significant different distribution of CD4+CCR4+, CD4+CCR5+, CD4+CCR6+ and CD4+CD25+highFOXP3+ subsets was found (Kruskal–Wallis, Dunn post hoc test: $P < 0.0001$) (Fig. 1). The CD4+CCR5+ and CD4+CCR6+ subsets resulted significantly over-expressed in PG patients (median CD4+CCR5+: 16.5%, 25th–75th percentile: 13–25.5; median CD4+CCR6+ 23.7%, 25th–75th percentile: 15.7–29.6) when compared to HC ($P = 0.0015$ and $P = 0.001$ respectively); on the other hand, the CD4+CCR4+ subset (median: 8.5%, 25th–75th percentile: 6–12.6) was significantly reduced in PG with respect to the CD4+CCR5+ and CD4+CCR6+ populations (Mann–Whitney U-test: $P = 0.0011$ and $P < 0.0001$, respectively), showing values similar to those found in HC. The CD4+CD25+highFOXP3+ subset showed statistically significant lower percentage values when compared to HC (median: 1.6%; 25th–75th percentile: 1.2–2%) ($P = 0.0011$).

Discussion

This study shows for the first time that patients with a prototypic neutrophilic dermatosis such as PG show distinct abnormalities in the peripheral blood expression of chemokines and DC subsets, supporting a definite lymphocyte polarization towards a Th1/Th17 phenotype with a Th2 and Tregs down-regulation. Indeed, data retrieved from this multicentric study document that newly diagnosed untreated PG patients are characterized by an over-expression in the blood of the CD4+CCR5+ and CD4+CCR6+ and a down-regulation of CD4+CCR4+ counts with respect to healthy subjects. Moreover, they show increased levels of myeloid derived DC1 and reduced levels of the Treg CD4+CD25highFOXP3+ subset. It has been shown that chemokine receptors are differentially expressed by the Thelper cell subsets. Namely, CXCR3 and CCR5 are selectively expressed by Th1 lymphocytes, whilst CCR3, CCR4, CCR8 are mostly present on Th2 lymphocytes.¹¹ As to Th17 cells, it has been supported that CCR6 is the signature chemokine receptor for this subset.¹² The pattern of chemokine expression argues therefore in favour of a Th1 (CCR5+) and Th17 (CCR6+) polarization with a down-regulation of Th2 (CCR4+). It is of interest to highlight that Th1/Th17 cells have been implied as mediators in the formation of other neutrophilic dermatoses such as pustular psoriasis.¹³ Only a few studies analysed Th17 expression in PG, all in skin lesions. Marzano et al.⁵ found an increase in IL-17 expression in PG lesions, whilst Guenova et al.¹⁴ reported an augmented expression of IL-23 at protein level by confocal laser scanning microscopy and at mRNA level by polymerase chain reaction in a patient with recalcitrant PG. The over-expression of the DC1 cells found in PG patients is in agreement with these results: actually, the DC1 subset has been shown to predominantly prime a Th1 cell response, whilst the DC2 cells induce the generation of Th2 cells.¹⁶ The down-regulation of Tregs is consistent with a process of immune activation and particularly with the up-regulation of the Th17 cell subset. Numerous immune-mediated diseases have been shown to present a reduction and/or function impairment of Tregs,¹⁷ 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 downmodulation in psoriasis¹⁸ as well as other immune-mediated skin diseases.¹⁹ As an altered balance between Th17 and Treg cells has been shown to contribute to the development of auto-immunity and inflammation,²⁰ the skewed phenotype of our PG patients towards a Th17 rather than Treg activation clearly is in keeping with this process. The blood findings reported in this study are completely superimposable to those obtained in the skin by our group in a recent paper¹⁵: in a series of 15 PG patients, we found by immunohistochemistry a significant reduction

in both FOXP3+ and IL-10/TGF-beta+ CD4+ infiltrating cells, thus meaning an impairment of regulatory T-cell functions, coupled with a marked over-expression of Th17 subsets in the skin as shown by the increase in RORct staining. It is noticeable that psoriasis patients before anti-TNF blocker treatment were characterized by a similar blood amplification of RORct, specific transcription factor for Th17 T-cell lineage differentiation.²¹ Even if recent reports suggest that Th17 cells and peripherally induced Tregs represent competing fates of naïve T-cell differentiation,²⁰ however, it has also been shown that Treg cells can in turn easily differentiate into IL-17 producing cells on ex-vivo stimulation in psoriasis patients²² meaning that the plasticity of T-cell subsets limits undoubtedly the relevance of phenotypic data. Other limits of our study are represented by the relatively small number of patients enrolled, by the difficulties to correctly identify T-cell subsets based on the expression of single chemokines and by the absence of functional evidences beyond the phenotypic patterns. Future studies should also confirm these preliminary results by investigating serum expression of cytokines related to T-cell lineage differentiation as well as the modifications induced by the treatment and associated with the clearance of cutaneous lesions. On the basis of the present as well as literature data,^{5–7,14,15} we hypothesize that PG could be driven by an up-regulation of highly pro-inflammatory cell subsets such as Th1/Th17, potentially induced by a concurrent DC1 preferential activation, trafficking between the skin and blood. This T-cell activation and the imbalance between Th17 and Tregs could modulate the cytokine network in skin lesions and pave the way for neutrophil recruitment and activation.

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Table 1 Clinical findings in 10 patients with pyoderma gangrenosum†

| N | Gender/Age | PG subset‡ | Lesion Number | Sites | Associated disorders | Treatment | Disease evolution |
|----|------------|---------------|---------------|-------------|----------------------|---------------|----------------------|
| 1 | F/55 | Localized | 3 | Legs | – | Pred/Aza | Chronic-relapsing/PR |
| 2 | M/43 | Disseminated | 12 | Trunk, Legs | IBD | Cyc | Single episode/CR |
| 3 | F/61 | Localized | 1 | Trunk | – | Pred/Cyc | Single episode/CR |
| 4 | M/56 | Multilesional | 4 | Legs | IBD | Pred/ivIG | Chronic-relapsing/CR |
| 5 | F/52 | Multilesional | 6 | Legs | – | Pred/Cyc | Chronic-relapsing/CR |
| 6 | F/70 | Localized | 3 | Legs | – | Pred | Single episode/CR |
| 7 | F/28 | Localized | 3 | Legs | Cystic fibrosis | Pred | Chronic-relapsing/CR |
| 8 | M/30 | Localized | 3 | Legs | IBD | Pred/anti-TNF | Single episode/CR |
| 9 | F/78 | Localized | 3 | Scalp | – | Pred/Cyc | Chronic-relapsing/CR |
| 10 | M/18 | Disseminated | 12 | Trunk, Legs | – | Pred | Chronic-relapsing/CR |

IBD, inflammatory bowel diseases; Pred, prednisone; Cyc, oral cyclosporin; Aza, azathioprine; ivIG, high-dose Immunoglobulins; TNF, tumour-necrosisfactor; CR, complete remission; PR, partial remission.

†Patients numbered from 6 to 10 were included in the previous study 7.

‡According to Marzano et al.8

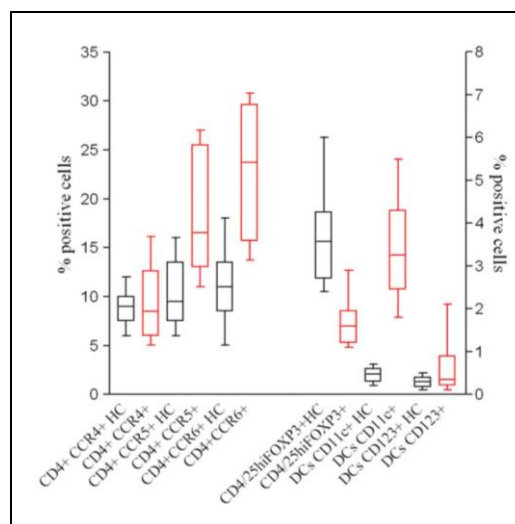


Figure 1 Percentages of the circulating T- and DC- cell subsets in patients with pyoderma gangrenosum compared with healthy controls (HC). *P = 0.001; **P < 0.001. Value distribution is represented as box and whiskers (PG patients in red colour, HC in black): the horizontal bar inside the box represents the median, the bottom and the top the 25th and 75th percentile, the ends of the whiskers the minimum and maximum of all data.