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CONCISE COMMUNICATION

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Evaluation of neutrophil extracellular trap deregulated formation in pyoderma gangrenosum

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Abstract

Pyoderma gangrenosum (PG) is a neutrophilic dermatose (ND) characterized by a dense neutrophilic infiltrate in the affected tissue. Neutrophil extracellular traps (NETs) are web-like structures released by neutrophils and composed of cytosolic and granule proteins assembled on a scaffold of decondensed chromatin. Very little is known about the role of NETosis in PG. Here, we assessed the possible implication of NETosis in the pathogenesis of PG by investigating the NETosis in the ulcers of 26 PG patients. We demonstrated that neutrophils in the PG skin lesions undergo an aberrant level of NETosis in 100% of the analysed cases (N = 26). All control and abscess biopsies were instead negative for the NETosis. In addition, neutrophils from peripheral blood of PG patients showed a significantly higher rate of spontaneous, but not induced, NETosis. Overall, this study suggests that the NETosis may contribute to systemic inflammation and tissue destruction in PG, thus representing a possible novel therapeutic target.

KEYWORDS
inflammation, inflammatory skin diseases, neutrophil extracellular trap, neutrophilic dermatoses, pathogenesis

1 | BACKGROUND

Neutrophilic dermatoses are a group of skin inflammatory disorders, characterized by a neutrophilic infiltrate in the affected tissue with no evidence of infection or vasculitis. Skin lesions in ND can be localized or diffuse and include pustules, plaques, nodules or ulcerations.[1] ND can be divided in different subgroups on the basis of clinical and histopathological differences.

In PG, the initial lesion can be a papule, a pustule or a nodule that evolves into an ulcerative lesion with necrotic borders. According to the appearance of lesions, five subtypes of PG can be diagnosed: ulcerative, bullous, pustular, vegetative and peristomal.[2,3] In half of the cases, PG is associated with an underlying systemic disorder, such as inflammatory bowel disease, systemic lupus erythematosus, ANCA-associated vasculitis or rheumatoid arthritis, or with haematologic malignancies.[4] In the remaining 50% of the cases, the disorder can be considered idiopathic.

The pathogenesis of PG is complex and probably multifactorial, but the neutrophil-rich inflammatory infiltrate in the absence of infection suggests a central role of neutrophils.[5,6] However, the final mechanism involved in neutrophil activation and damage to tissue has not been fully characterized. NETosis is a recently discovered function of neutrophils, critical for the protection against bacterial, fungal and parasitic infections but also potentially harmful to surrounding cells and tissues.[7]

Netting neutrophils undergo disruption of intracellular membranes, with mixing of nuclear and cytoplasmic content and permeabilization of plasma membrane. These events precede the release of decondensed chromatin fibres containing histones as well as antimicrobial granular and cytoplasmic proteins that form a net around the cell.[8] NET components such as histones, antimicrobial peptides and reactive oxygen species kill pathogens but can also damage host cells and tissues.

Netting neutrophils are been already linked to different immune-mediated disorders such as systemic lupus erythematosus, ANCA-associated vasculitis and rheumatoid arthritis.[9]

Moreover, low-density granulocytes that are known to be more prone to NETosis are increased in the circulation of patients affected by inflammatory bowel disease, often associated with PG.[10]

Here, we studied NET formation in lesional skin and circulating neutrophils of PG patients.

2 | QUESTIONS ADDRESSED

We aimed to understand whether the NETosis represents a mechanism of damage in PG, therefore contributing to the disease pathogenesis.

3 | EXPERIMENTAL DESIGN

3.1 | Patients

We enrolled 26 PG patients. In 2 of those patients, the disease was associated with rheumatoid arthritis and in one with systemic lupus.

As control, we used three patients with skin abscess (one pilonidal cyst and two inflammatory abscesses) and two biopsies from non-lesional skin.

3.2 | NET analysis on skin tissues

All skin sections were H&E-stained and analysed to confirm that the histology was in line with the clinical diagnosis. Sections were further analysed for NETs by performing a triple immunofluorescence for MPO (myeloperoxidase), H2B and DAPI (4′,6-diamidino-2-phenylindole).

3.3 | NET analysis on circulating neutrophils

Neutrophils were isolated from peripheral blood, and spontaneous and induced NETosis was assessed by a double immunofluorescence for MPO and DAPI.

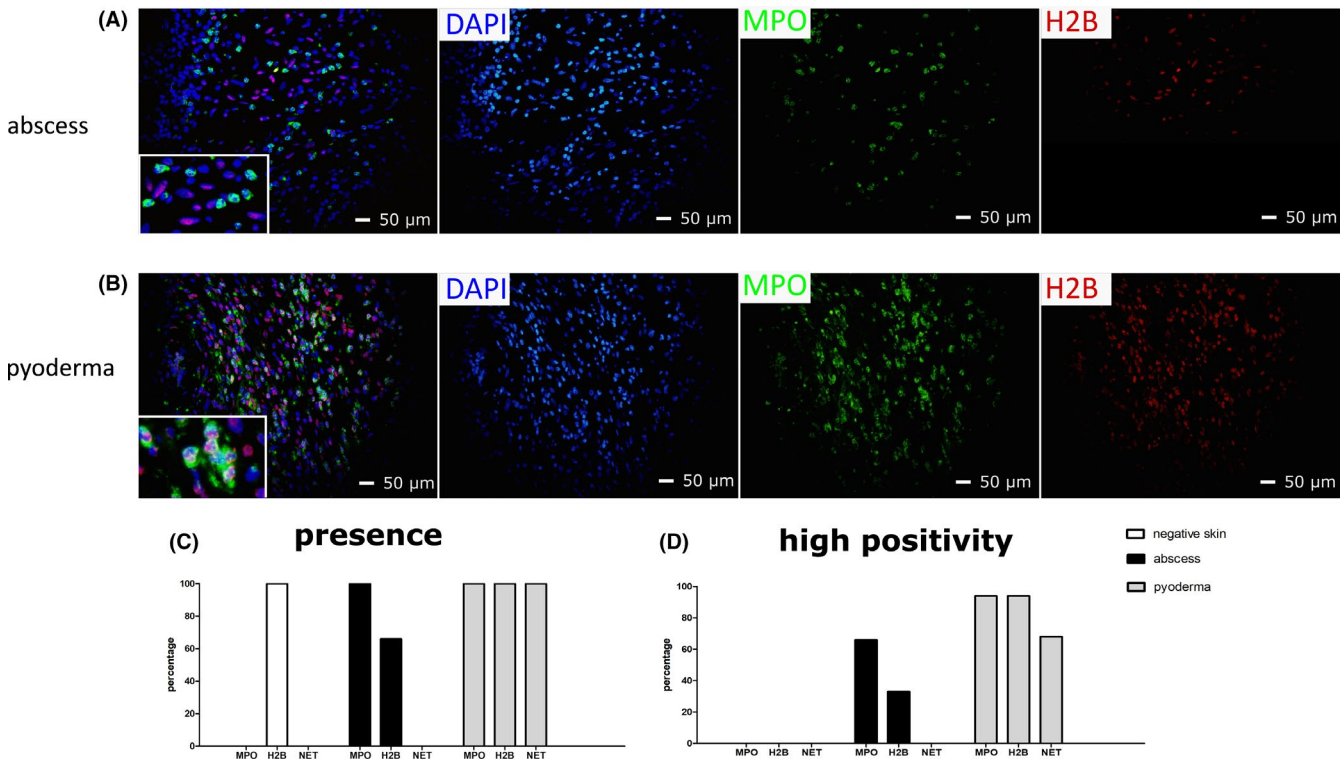

FIGURE 1 Pyoderma gangrenosum skin biopsies show a higher rate of NETosis. MPO and H2B IF double staining shows some single positive cells in an abscess skin sample; the lack of colocalization (inset) indicates the absence of NETosis (A). In B, a representative image of a PG skin sample revealing numerous Netting cells. The colocalization of the two antigens is better illustrated in the inset of B. All images are 20×. Table C represents the different percentage of NETosis in normal, abscess, PG and SS biopsies. Table D indicates the percentage of samples extremely rich in NETs

Details about patients, protocols and statistics are provided in Appendix S1.

4 | RESULTS

4.1 | Aberrant level of NETosis in PG skin biopsies

Skin biopsies were obtained from 26 PG. The clinical diagnosis was supported by histological analysis of the tissue (data not shown).

As recently described by Brinkmann et al,[11] the correct identification of NETs in tissue sections requires the colocalization of two different immunofluorescent signals, one from a nuclear constituent and the other from granular proteins contained in NET. Thus, to detect NET is tissue biopsies we set up a combination of two antibodies, one against the nuclear histone H2B, a nuclear component of NET, and the other against MPO, a granular component of NET.

We demonstrated that 100% of the skin biopsies from PG show signs of NETosis (Figure 1B,C). Moreover, 65% of the PG skin biopsies can be classified as "extremely rich in NET," since extended and compact areas of MPO+H2B+ cells were observed (Figure 1D and Figure S1). We did not detect NET in abscesses or in normal skin, although a variable level of MPO and H2B expression was found (Figure 2A, C).

4.2 | Increased number of NETotic neutrophils in PG blood

We analysed the rate of spontaneous and stimuli-induced NETosis in circulating neutrophils from 3 PG and 5 healthy controls by performing MPO IF on fixed cells. We found that the rate of spontaneous NETosis in PG neutrophils was 5 times higher than in control neutrophils ($p = 0.04$) (Figure 2A, B, C). Once stimulated, the neutrophils of PG patients and controls did not show any significant difference in the rate of NETosis (Figure 2A, D, E).

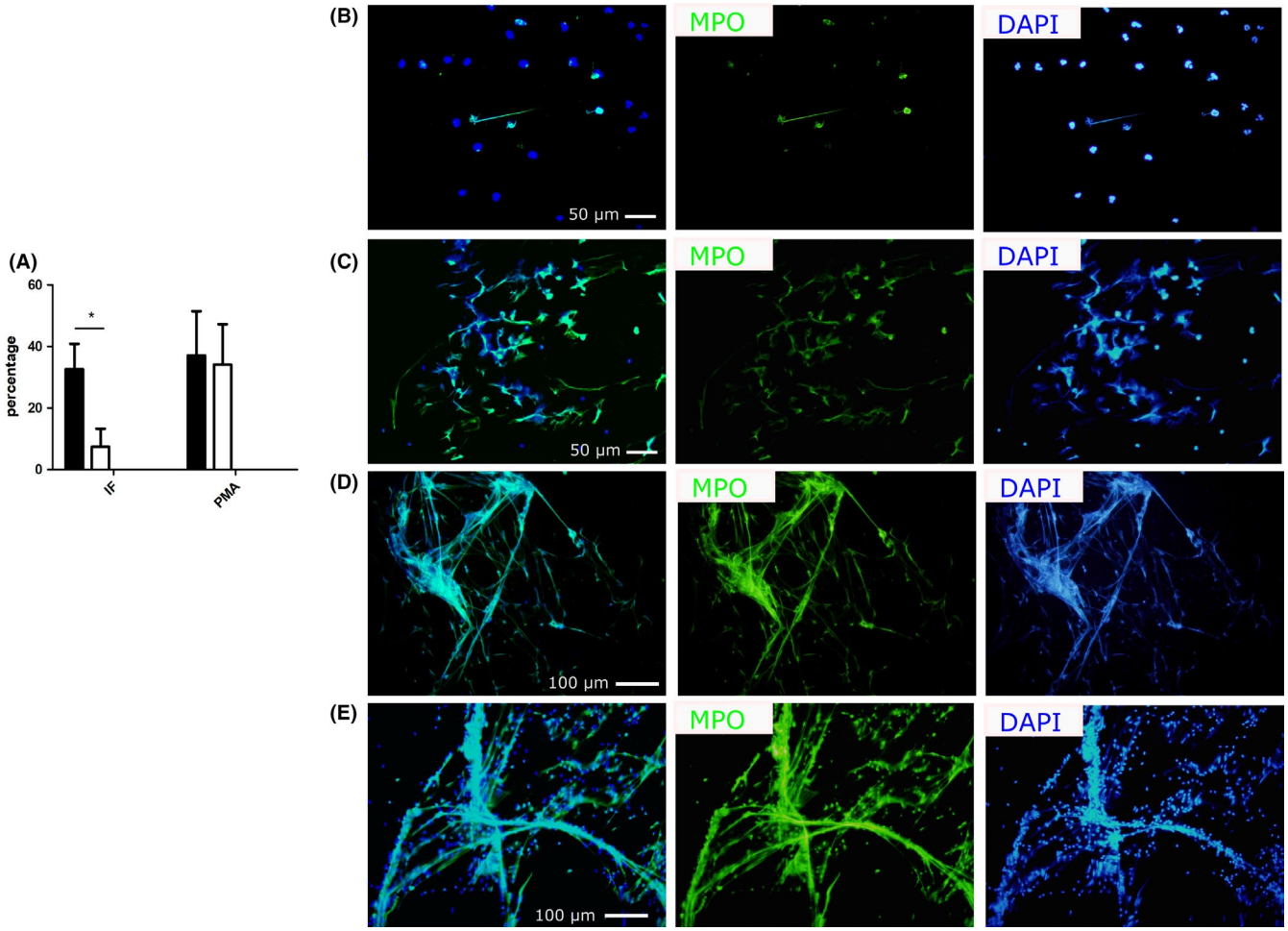

FIGURE 2 Neutrophils from PG patients undergo a higher rate of spontaneous but not PMA-induced NETosis. Graph in A shows the different percentage of spontaneous and induced NETosis in neutrophils from PG versus normal patients. Very few NETting neutrophils were observed in the immediately fixed neutrophils from healthy subjects (B), while a high number of circulating neutrophils undergoing NETosis were observed in PG patients (C). No significant difference was observed in the NETosis from healthy or PG neutrophils if cells were pretreated with PMA (D, E). All images are 20×. *$p < .05$

5 | CONCLUSION & PERSPECTIVE

The data obtained in this paper indicate that neutrophils undergo an aberrant level of NETosis in the lesions of PG patients, in the vast majority idiopathic PG, suggesting that NET may play an important role in inducing skin damage in this disorder. We analysed 26 PG tissue sections using as control abscess tissue, heavily infiltrated with neutrophils. The inclusion of abscess as control strengthens our conclusions. In fact, although in the abscess the neutrophil infiltrate is very dense, we did not find any sign of NETosis, thus excluding that neutrophils migrated into tissues undergo NETosis with a higher rate. Moreover, the absence of NET in the abscess excludes that artifacts due to tissue fixation and staining can be a possible cause of the immunofluorescence positivity.

Spontaneous NETosis was also evaluated in peripheral blood of a small number of PG patients and was found to be increased in 3/3 patients. Thus, neutrophils from PG patients seem to be more prone to NETosis, as indicated by the higher number of netting neutrophils in peripheral blood as well as in lesions. Previous data, obtained in a small number of PG patients, are consistent with our findings.[12] Although the limit of this analysis is clearly the small amount of blood samples analysed, the results suggest that the stimuli leading to NETosis act systemically in PG.

This high prevalence of NETosis detected in PG lesions is shared by other autoinflammatory disorders with skin involvement and also by psoriasis.

In Schnitzler's syndrome and in PAPA syndrome, an increased number of netting neutrophils are detected in skin lesions and peripheral blood; serum from patients with PAPA syndrome displays a reduced ability to degrade NET.[12,13]

A higher NETosis rate has been described also in peripheral blood neutrophils from patients with psoriasis, correlated with disease activity.[14] Patient serum is able to induce NETosis of normal neutrophils but has a normal ability to degrade NET.[14] NET remnants are increased in serum and netting neutrophils are detected in skin lesions, in close proximity to keratinocytes.[15]

Neutrophils are induced to release NET by different stimuli that engage a variety of receptors and act through different pathways. The stimuli involved in NET formation in the different skin diseases are not yet fully characterized, while more data are available on the mechanisms of tissue damage induced by NET.

Proinflammatory cytokines like IL-1β, TNF-α, IL-17A, IL-18 and IFNγ play a critical role in NET induction.[14] On the other hand, neutrophils are an important source of IL-17 and in psoriasis IL-17 is abundantly released during NETosis.[16] NETs activate caspase-1 in macrophages leading to the release of active IL-1β and IL-18.[17]

In psoriasis, RNA-LL-37 complexes contained in NET and abundant in psoriatic skin trigger TLR8-mediated cytokine release and induce further NET formation, thus fuelling a process of self-sustained inflammation.[18]

Besides hyperproduction of pro-inflammatory cytokines, NET components like LL-37 induce pDC maturation, with possible enhancing of autoimmune responses. This could explain the production of ANCA in patients with ND, associated with ulcerative colitis or RA.[19,20]

Thus, NET may represent an important mechanism of damage in an ample spectrum of neutrophil mediated diseases, that includes autoinflammatory diseases, neutrophilic dermatoses and also psoriasis.

Overall, our data offer new insight into the role of NETosis in pyoderma gangrenosum suggesting new therapeutic perspectives such as drugs interfering with neutrophil activation.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

CC performed all experiments and assisted in the data analysis and manuscript writing. MR, EM and VD recruited the patients and provided the clinical data and have been involved in drafting and critically revised the manuscript. BL provided the patient biopsies and has been involved in drafting and critically revised the manuscript. PM designed the research and wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

ETHICAL APPROVAL

The study received the local ethics committee approval (protocol 9041) and was conducted according to the Declaration of Helsinki. Patients gave their informed consent to participate in the study.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1. A representative image of a sample extremely rich in NET. A dense infiltrate (more than 100 cells) of extremely bright double positive MPO/H2B cells in a single 20× field of a PG skin sample.

Appendix S1. Reports the clinical characterization of patients, describes the staining of tissue section and details thje detection of NET in tissue neutrophils and in isolated peripheral blood neutrophils.

Supplementary Material.

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