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Reduction of Neutrophil Activation by Phosphodiesterase 4 Blockade in Behçet's Disease

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Objective. Behçet's disease (BD) is a systemic vasculitis with inflammatory lesions mediated by cytotoxic T cells and neutrophils. Apremilast, an orally available small-molecule drug that selectively inhibits phosphodiesterase 4 (PDE4), has been recently approved for the treatment of BD. We aimed to investigate the effect of PDE4 inhibition on neutrophil activation in BD.

Methods. We studied surface markers and reactive oxygen species (ROS) production by flow cytometry, and neutrophil extracellular traps (NETs) production and molecular signature of neutrophils by transcriptome analysis before and after PDE4 inhibition.

Results. Activation surface markers (CD64, CD66b, CD11b, and CD11c), ROS production, and NETosis were upregulated in BD patient neutrophils compared to healthy donor neutrophils. Transcriptome analysis revealed 1,021 significantly dysregulated neutrophil genes between BD patients and healthy donors. Among dysregulated genes, we found a substantial enrichment for pathways linked to innate immunity, intracellular signaling, and chemotaxis in BD. Skin lesions of BD patients showed increased infiltration of neutrophils that colocalized with PDE4. Inhibition of PDE4 by apremilast strongly inhibited neutrophil surface activation markers as well as ROS production, NETosis, and genes and pathways related to innate immunity, intracellular signaling, and chemotaxis.

Conclusion. We highlight key biologic effects of apremilast on neutrophils in BD.

INTRODUCTION

Behçet's disease (BD) is a chronic systemic vasculitis characterized by recurrent oral and genital ulcers, skin lesions, and articular, neurologic, vascular, and sight-threatening intraocular inflammation (1). BD is thought to share both autoimmune and autoinflammatory disease features. The natural course of the disease is characterized by spontaneous remissions and exacerbations, and it is usually more active during the initial years after onset. The pathophysiology of BD is poorly understood. Immune system imbalance is considered the main basis for BD development. Several pathogens can activate innate immunity, especially neutrophils and γ/δ T cells, and acquired immunity following antigen processing and presentation to naive T lymphocytes by antigen-presenting cells (2–5).

Neutrophils represent the main cells involved in the pathogenesis of the different BD clinical manifestations. Their abilities of chemotaxis, phagocytosis, and production of reactive oxygen species (ROS) have a key role in the BD pathogenesis (6,7). In fact, they are also extensively found in histologic specimens in BD. Moreover, neutrophils from BD patients were shown to be more prone to NETosis (8,9). During NETosis, neutrophils die by excreting cell-free DNA decorated with histones and granular components called neutrophil extracellular traps (NETs), which contain host nuclear material and neutrophilic granule proteins. These NETs may promote thrombin

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generation and endothelial dysfunction, and worsen the inflammation loop (8,9).

Phosphodiesterase 4 (PDE4) is an immune and inflammatory cell enzyme which, by degrading the key intracellular signaling messenger cAMP, promotes an increase in the production of pro-inflammatory mediators (10). Neutrophils represent an excellent target for PDE4 inhibitors as neutrophils almost exclusively express PDE4 (11). Research on the effects of PDE4 inhibition in neutrophils is scarce, and these effects have not been studied at all in BD. Apremilast is an orally administered small-molecule drug that acts as an inhibitor of PDE4 and has shown effectiveness in treating oral ulcers of BD in phase II and III studies (12,13) and has recently been approved by the Food and Drug Administration. However, mechanisms underlying its biologic effects in BD have not been established.

In this study, we aimed to study the effect of PDE4 inhibition in neutrophils of BD patients.

PATIENTS AND METHODS

Patients. The study population consisted of 20 BD patients (median age at diagnosis 49 years [interquartile range 38–55 years]) fulfilling the International Criteria for BD (14). Patients treated with disease-modifying antirheumatic drugs, biologics, or glucocorticoids >10 mg/day were excluded. Clinical characteristics of BD patients are presented in Table 1. Blood samples from 15 age- and sex-matched healthy donors, obtained from the Établissement Français du Sang (Hôpital Pitié-Salpêtrière), were used as controls. The study was approved by the Paris VI ethics review board and was conducted according to the Declaration of Helsinki. All patients gave informed consent.

Table 1. Characteristics of patients with Behçet's disease who provided samples for the study*

	Study population (n = 20)
Age, median (IQR) years	49 (38–55)
Male sex	16 (80)
HLA–B51 positive†	5 (50)
Geographic origin	
Europe	4 (20)
North Africa	16 (80)
Clinical features	
Oral ulcers	20 (100)
Genital ulcers	12 (60)
Skin involvement	15 (75)
Ocular involvement	8 (40)
Vascular involvement	8 (40)
Joint involvement	10 (50)
C-reactive protein >10 mg/liter	11 (55)
Medical therapy	
Steroids (<10 mg/day)	8 (40)
Colchicine	12 (60)

* Except where otherwise indicated, values are the no. (%) of patients. IQR = interquartile range.
† HLA–B51 data are for 10 patients.

Sample collection and neutrophil isolation. Blood samples from heathy donors or BD patients were collected by venipuncture into acid citrate dextrose Formula A blood collection tubes. Neutrophils were isolated by 2-step, Ficoll density-gradient centrifugation of whole blood <3 hours after venipuncture (15).

Visualization and quantification of NETs. Isolated neutrophils (10^5 cells) were seeded on glass coverslips and allowed to adhere for 30 minutes before stimulation with or without phorbol myristate acetate (25 n*M*) (positive control) for 3 hours at 37°C and 5% CO_2 and then fixed with paraformaldehyde for 30 minutes. For experiments with roflumilast and colchicine, neutrophils were cultured with roflumilast (1 μ*M*; Sigma-Aldrich) and colchicine (100 ng/ml; Thermo Fisher Scientific). Neutrophils were then stained for NETs markers (DNA and myeloperoxidase [MPO]). Briefly, after 1 hour blocking (phosphate buffered saline [PBS] with 3% goat serum, 1% bovine serum albumin [BSA]), NETs were detected using a mouse anti-MPO primary antibody (Abcam), diluted 1:100 in blocking buffer for 2 hours at 37°C. Slides were then incubated with 1:200 Alexa Fluor 488–conjugated goat anti-mouse antibody for 2 hours at 37°C. DNA was stained with Fluoroshield mounting medium with DAPI (Abcam). NETs were visualized by using a NanoZoomer imaging fluorescence microscope (Hamamatsu) and quantified by investigators who were blinded with regard to the sample conditions using ImageJ software version 8. Images were evaluated for MPO and DNA costaining; nuclear phenotypes and NETs were counted and expressed as percentage of the total number of cells in the fields.

Transcriptome analysis. Neutrophils were sequentially isolated from peripheral blood mononuclear cells of BD patients (n = 4, for untreated or treated conditions) and healthy donors (n = 5) as previously described. Sample purities were assessed and were all >95%. Total RNA from neutrophils was then extracted using the NucleoSpin RNA kit (Macherey-Nagel) and quantified by a NanoDrop 1000 spectrophotometer. The mean ± SEM RNA concentration in the processed samples was 94.54 ± 20.45 ng/μl. For quality control, RNA dilution was performed using an Agilent RNA 6000 Nano kit, and 1 μl of the sample was run on the nano chip using an Agilent 2100 electrophoresis bioanalyzer. The quality of total RNA was assessed using the profile of the electropherogram, and the RNA integrity number was calculated. The mean ± SEM RNA integrity number for the processed samples was 6.46 ± 0.43. Samples were then hybridized to an Affymetrix Clariom S Human microarray. Subsequent CEL files were processed and robust multiarray average–normalized using affy and oligo R software packages and concatenated into a single text file summarizing all samples and genes.

Flow cytometry analysis. Whole blood from healthy donors and BD patients were antibody stained following standard

protocol with anti-CD16, anti-CD15, anti-CD66b, anti-CD11b, anti-CD11c, and anti-CD69 (BioLegend).

ROS generation by flow cytometry. Neutrophils at 5×10^6/ml were preincubated in PBS with 3% fetal bovine serum–EDTA (2 m*M*) with or without roflumilast (1 μ*M*; Sigma-Aldrich) for 20 minutes. Then, neutrophils were incubated with a ROS probe (CM-H2DCFA) (Molecular Probes) and treated with or without roflumilast (1 μ*M*; Sigma-Aldrich) for 20 minutes. Reaction was stopped by adding PBS with 2% BSA and EDTA (2 m*M*). Fluorescence was then measured by flow cytometry using an Acurri C6 flow cytometer (BD Biosciences).

Immunofluorescence. Paraffin-embedded skin tissue sections from BD patient pseudo folliculitis or inflammatory skin lesions and from healthy donor controls were subjected to sequential indirect immunolabeling with a tyramide signal amplification technique using 3-plex immunofluorescence composed of DAPI, CD66b, and PDE4. Anti-CD8 (product no. MA5-13473; Thermo Fisher Scientific) and anti-PDE4 (product no. ab14628; Abcam) anti-human antibodies were used.

Before immunolabeling, slides were dewaxed at 72°C for 30 seconds before an antigen retrieval step at pH 9 and 95°C for 20 minutes was performed. After this, endogenous peroxidases and nonspecific binding sites were blocked with hydrogen peroxide for 10 minutes at room temperature and BSA solution for 10 minutes at room temperature, without rinse. Next, primary antibodies were sequentially applied for 45 minutes at 37°C, then secondary antibodies were incubated for 30 minutes at room temperature. After the completion of all immunolabeling, spectral DAPI (Akoya Biosciences) was added to the slides for 5 minutes as counterstaining, and coverslips were mounted with mounting medium. The immunolabeling detection was finally done using an Opal 4-Color Automation Immunohistochemistry kit (Akoya Biosciences). Slides were thereafter scanned with an Axio Scan Z1 slide scanner (Zeiss Microscopy), equipped with a Zeiss Colibri 7-LED illuminator and a Hamamatsu ORCA-Flash 4.0 CMOS camera using a Plan-Apochromat 20×, numerical aperture 0.8 objective lens. The method used to open, process, and analyze the subsequent CZI files is described below.

Once the slides were scanned, the obtained CZI files were opened using QuPath software to extract as many analyzable regions of interest as possible, which were directly sent to Fiji software version 1.53h. Using Fiji software, a semiautomated scripted analysis was performed to remove background noise and locally enhance contrast for each channel. Then, nuclei contours (edges) were determined using a difference of means method. Once the nuclei contours were identified, their shapes were dilated to take the cell cytoplasm area into account. After that, quantification of the remaining signals inside each determined cell shape was performed. Thereafter, fluorescence, spatial, and shape information for each identified cell in each region of interest and 3-plex immunofluorescence were exported from Fiji, imported to R software version 4.1, and analyzed with FlowJo software version 10.8. Neutrophils (CD66b+) and CD66b+PDE4+ cells were automatically counted and normalized to region of interest surface.

Statistical analysis. Continuous variables are presented with the median and range or the mean ± SEM. Categorical variables are presented with counts and proportions. Statistical comparisons were performed using Mann-Whitney test for unpaired quantitative data, and Wilcoxon's matched pairs test for paired quantitative data. All statistical tests were 2-tailed with a significance level of 0.05. Statistical significance was evaluated using GraphPad Prism version 5.00.

RESULTS

Molecular signature and functionality of neutrophils in BD patients. We aimed to study the activation status of BD patient neutrophils, so we assessed their surface activation markers, ROS production, NETs production, and transcriptomic profile.

We first analyzed activation markers of neutrophils from BD patients and healthy donors by flow cytometry. Surface markers such as CD11b, CD64, CD66b, and CD11c were increased in BD patients compared to healthy donors (mean ± SEM % CD11b+ neutrophils 4.1% ± 1.2% versus 0.4% ± 0.1% in healthy donors, $P < 0.001$; CD64+ 1.5% ± 0.2% versus 0.6% ± 0.2%, $P = 0.003$; $CD66b^{high}$ 6.9% ± 0.1% versus 2.3% ± 0.4%, $P < 0.001$; and CD11c+ 6.3% ± 0.9% versus 1.2% ± 0.3%, $P < 0.001$, respectively) (Figure 1A). Second, ROS production of rested neutrophils isolated from BD patients was increased compared to that in neutrophils isolated from healthy donors (mean fluorescence intensity [MFI] ± SEM 11.1 ± 5.7 versus 3.7 ± 1.1; $P = 0.003$) (Figure 1B). Then, we analyzed NETs production by isolated neutrophils and found an increased production of NETs in neutrophils from BD patients compared to that in neutrophils from healthy donors (mean ± SEM % NETs 30% ± 3% versus 7% ± 2.1%; $P < 0.001$) (Figure 1C). Last, we analyzed the transcriptomic profile of sorted neutrophils from healthy donors (n = 5) and BD patients (n = 4). Overall, there were 1,021 significantly dysregulated genes between healthy donors and BD patients (702 up-regulated and 319 down-regulated in BD patients). Among them, we observed that neutrophils from BD patients presented an increased expression of genes related to innate immunity (*TLR1*, *TLR5*, *TLR4*, *TLR8*, *LY96*, *TLR1*, *TLR4, IFIT5, IFITM1*), intracellular signaling (*IRAK4*, *JAK2*, *MAPK1*, *MAP3K1*, *STAT3*), and chemotaxis (*CCR1*, *CCR3*, *CXCR2*, *CXCR1, IL4R, IL10RB, IL18R1*) compared to their healthy donor counterparts (Figure 1D).

Together, these results show that neutrophils are activated in BD.

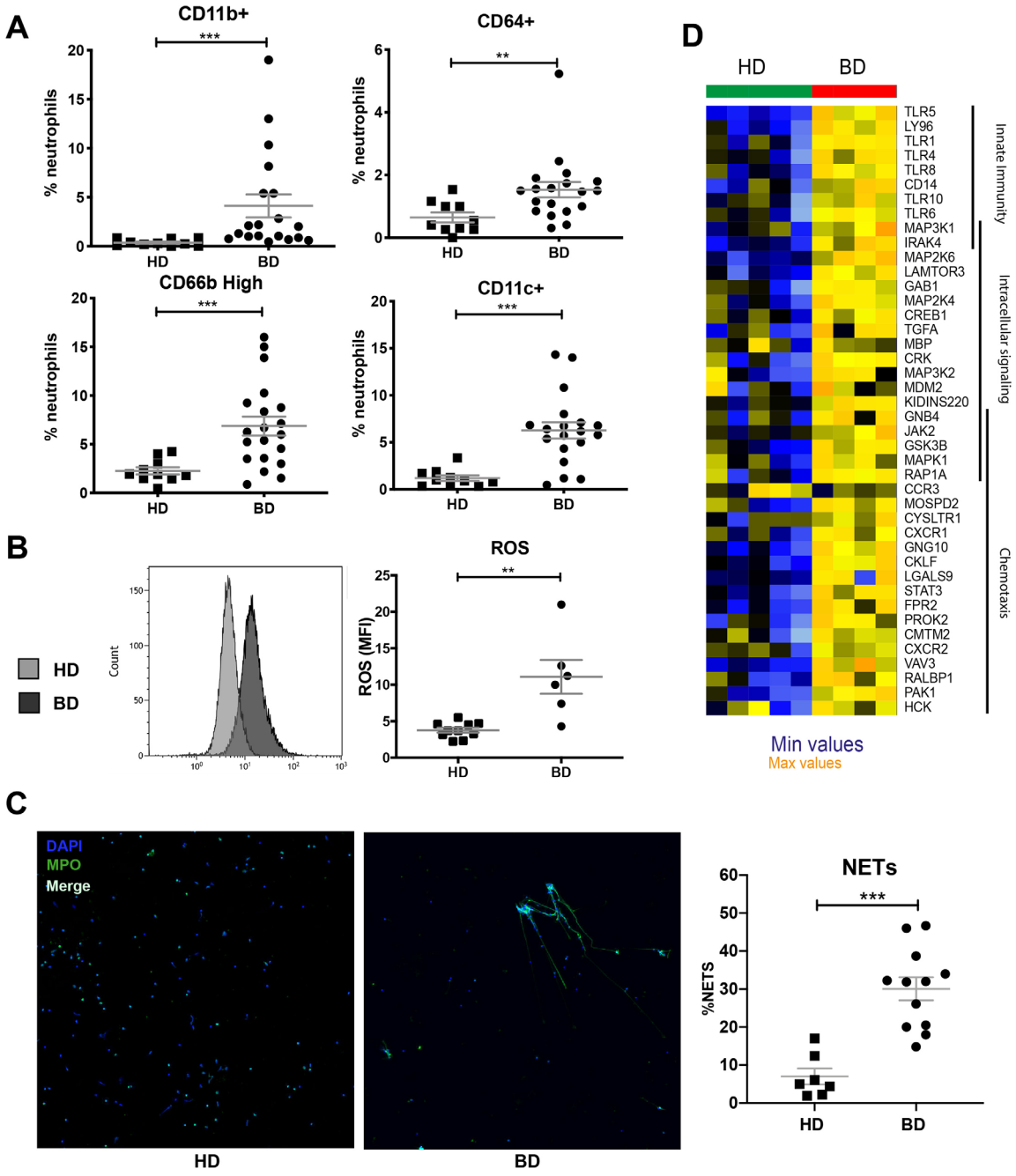

Figure 1. Molecular signature and functionality of neutrophils in Behçet's disease (BD). **A**, Whole blood from 19 BD patients and 10 healthy donors (HD) were antibody stained with anti-CD11b, anti-CD64, anti-CD66b, and anti-CD11c, and analyzed by flow cytometry to determine levels of the surface markers on neutrophils. **B**, Neutrophils isolated from BD patients (n = 6) and healthy donors (n = 10) were incubated with a reactive oxygen species (ROS) probe (CM-H2DCFA) and analyzed by flow cytometry for count and mean fluorescence intensity (MFI) values. **C**, Neutrophils isolated from BD patients (n = 12) and healthy donors (n = 7) were cultured for 3 hours and stained with anti-myeloperoxidase (anti-MPO) and DAPI. Extracellular MPO and DNA costaining were counted as neutrophil extracellular traps (NETs) and expressed as the percentage of the total number of cells in the field. **D**, Heatmap showing the most up-regulated gene functions in neutrophils of BD patients compared to those of healthy donors. In dot plots, symbols represent individual samples; lines with whiskers show the mean ± SEM. ** = $P < 0.01$ and *** = $P < 0.001$ by Mann-Whitney test.

Marked PDE4 expression by neutrophils in skin lesions of BD patients. To confirm neutrophil implication in BD, we first analyzed neutrophil expression by immunofluorescence of BD skin lesions and healthy skin slices (representative images shown in Figure 2A). Density of neutrophils (CD66+ cells) was increased in skin lesions of BD patients compared to healthy donor skin (mean ± SEM CD66+ cells 0.8 ± 0.9 /µm^2 versus 0.03 ± 0.002 /µm^2; $P < 0.0001$). Moreover, the number of

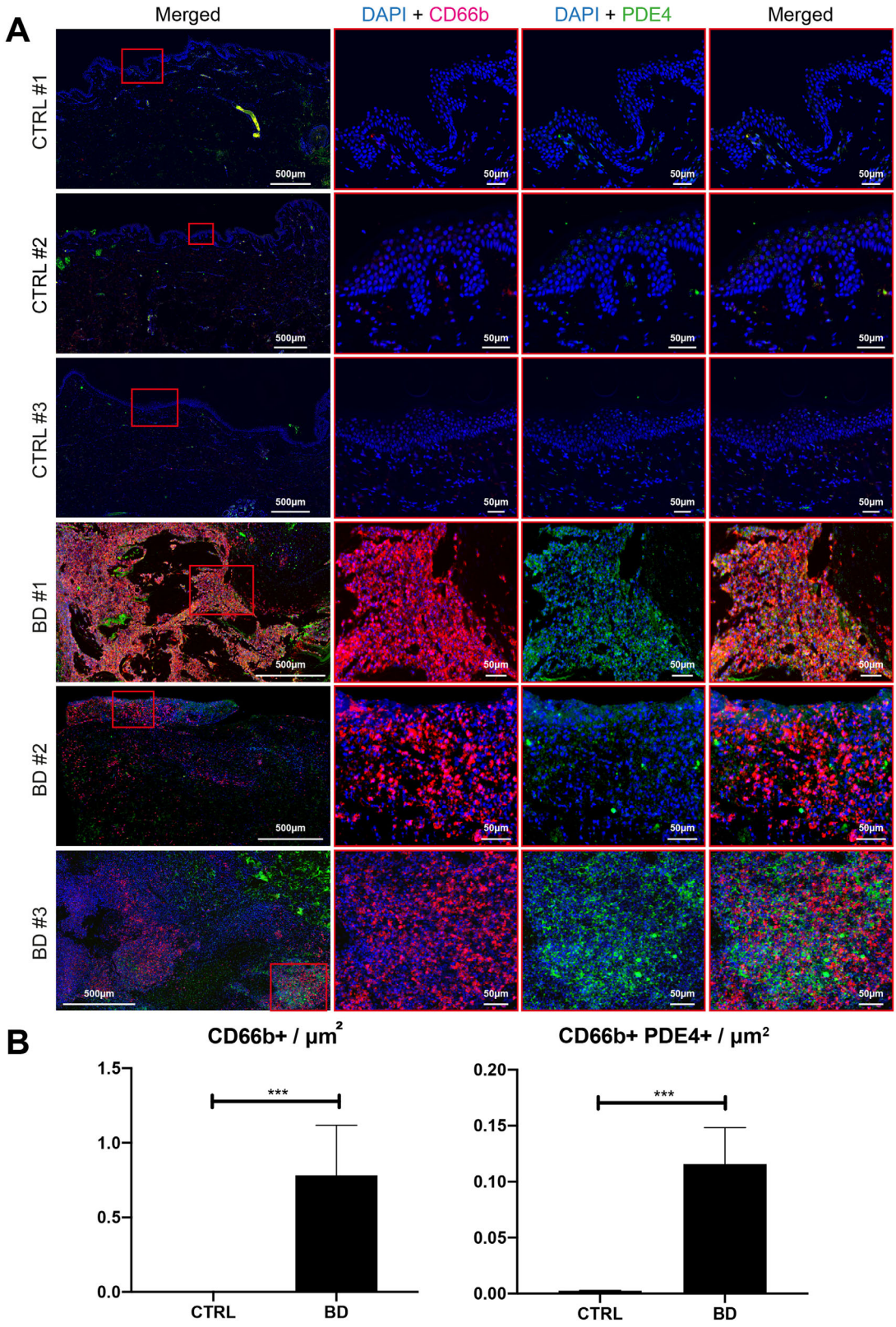

Figure 2. Neutrophils and phosphodiesterase 4 (PDE4) expression in skin lesions of Behçet's disease (BD) patients. **A**, Representative images of PDE4 immunostaining in BD skin lesions and healthy donor skin slices as control (CTRL). **B**, Number of neutrophils (CD66b+ cells) and PDE4+ neutrophils (CD66b+PDE4+ cells) in BD skin lesions versus healthy donor skin as control. In bar charts, bars show the mean ± SEM. *** = $P < 0.001$ by Mann-Whitney test. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42486/abstract.

PDE4+ neutrophils (CD66b+PDE4+ cells) was also increased in BD skin lesions compared to healthy donor skin (mean ± SEM CD66b+PDE4+ cells 0.1 ± 0.09 /μm^2 versus 0.002 ± 0.001 /μm^2; $P = 0.0001$) (Figure 2B).

Decreased in vitro activation of neutrophil ROS and NETs production by PDE4 inhibitors. We then aimed to test the in vitro effect of a PDE4 inhibitor, roflumilast, on production of neutrophil activation markers, ROS, and NETs. Colchicine was used as a control. Roflumilast and colchicine strongly reduced NETs production by BD neutrophils (mean ± SEM % NETs 15.2% ± 1.7% with roflumilast treatment versus 27% ± 2.9% without treatment, $P < 0.001$; and 18.9% ± 3.6% with colchicine treatment versus 27% ± 2.9% without treatment, $P = 0.03$). Roflumilast but not colchicine reduced NETs production by healthy donor neutrophils (mean ± SEM % NETs 6.8% ± 1.2% with roflumilast treatment versus 10.8% ± 1.4 without treatment, $P = 0.002$; and 9.6% ± 2.6% with colchicine treatment versus 10.8% ± 1.4% without treatment, $P = 0.75$) (Figure 3A).

Roflumilast abrogated ROS production by BD neutrophils (mean ± SEM MFI 11.1 ± 5.7 versus 5.5 ± 3.2 with roflumilast treatment; $P = 0.05$) but with no effect on healthy donor neutrophils (mean ± SEM MFI 3.8 ± 1.1 versus 3 ± 1.1 with roflumilast treatment; $P = 0.13$).

Inhibition of neutrophils activation, ROS and NETs production by apremilast in vivo. To further address the impact of PDE4 inhibitor on neutrophils in BD, we studied the in vivo effect of apremilast in BD patients. To do so, we treated BD patients with apremilast, an orally administered inhibitor of PDE4 (30 mg twice per day) and analyzed neutrophil activation at baseline (week 0) and 12 weeks after treatment. We also analyzed, as a control, patients treated with colchicine before and after 12 weeks of treatment. Compared to baseline (week 0), we observed a reduction of surface activation markers CD11b,

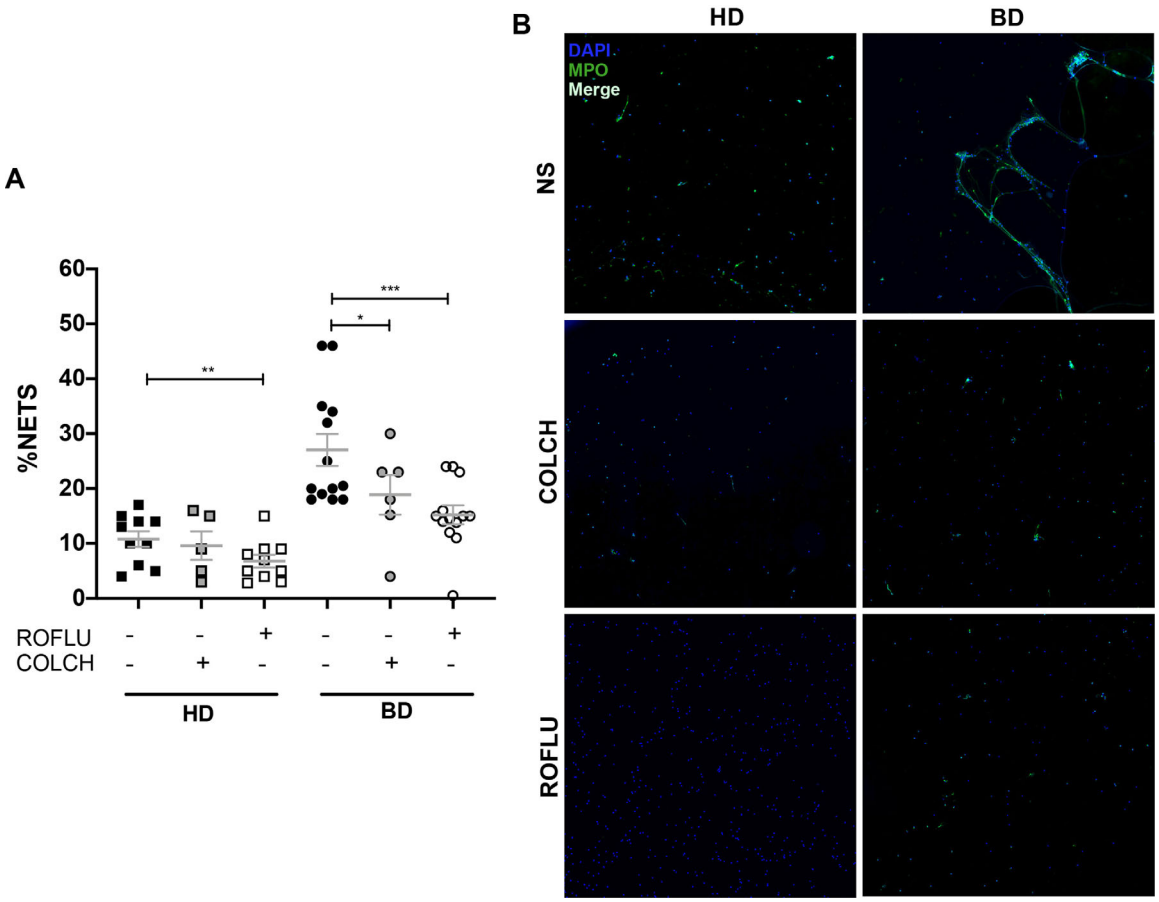

Figure 3. Phosphodiesterase 4 (PDE4) inhibitors decrease activation of neutrophils in vitro. **A–B**, Neutrophils isolated from Behçet's disease (BD) patients (n = 6) and healthy donors (HD) (n = 6) were cultured with or without roflumilast (ROFLU) (1 μM) or colchicine (COLCH) (100 ng/ml) as control and stained with anti-myeloperoxidase (anti-MPO) and DAPI. Extracellular MPO and DNA costaining were counted as neutrophil extracellular traps (NETs) and expressed as the percentage of the total number of cells in the field (**A**). Representative immunofluorescence images of neutrophils stained with anti-MPO and DAPI shown (**B**). In dot plot, symbols represent individual samples; lines with whiskers show the mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ by Mann-Whitney test. NS = non-stimulated. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42486/abstract.

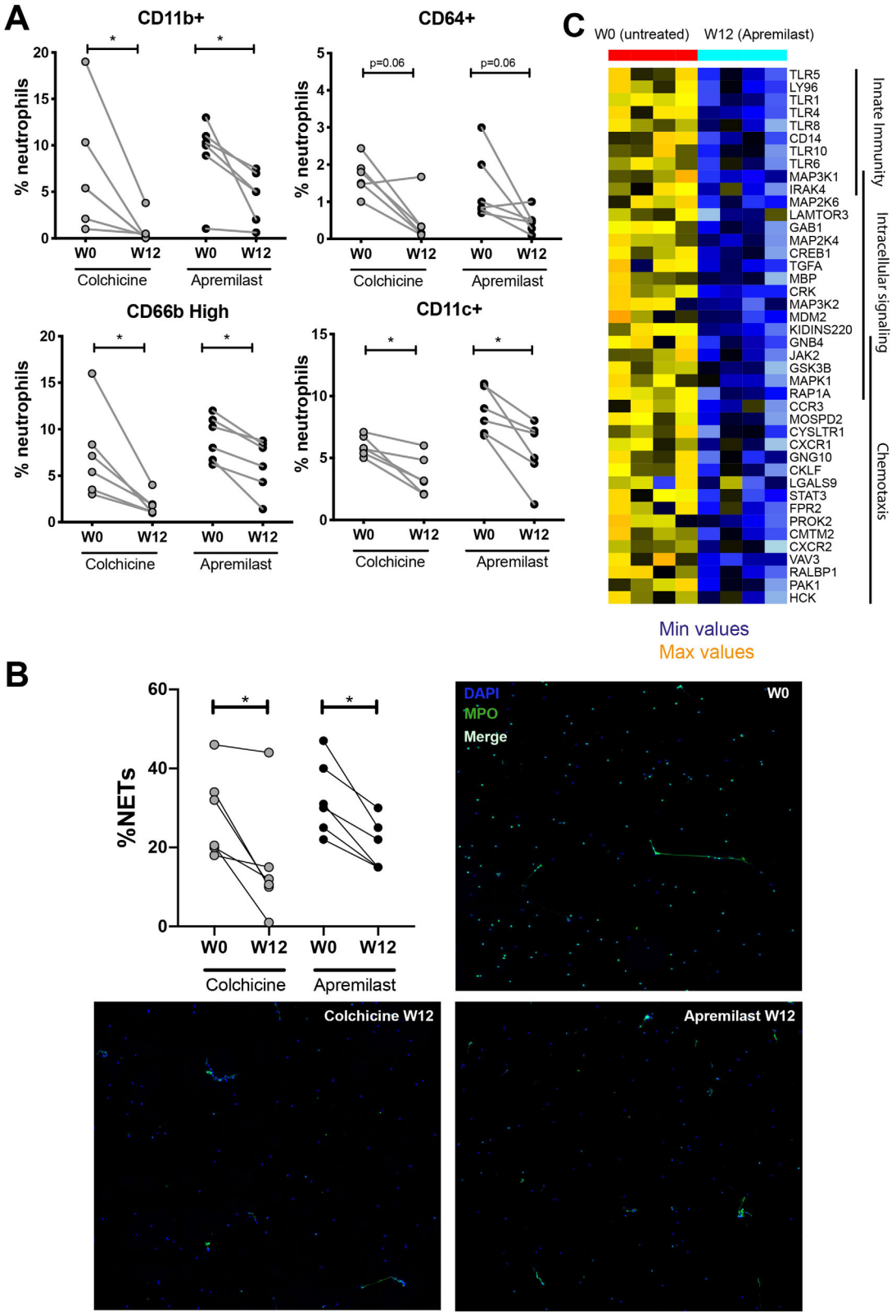

Figure 4. Apremilast inhibits neutrophil activation in vivo. **A–B**, Neutrophils from Behçet's disease (BD) patients treated with apremilast and colchicine were analyzed at week 0 (W0) and week 12 (W12) by flow cytometry for activation surface markers CD11b, CD64, CD66b, and CD11c (**A**), and by immunofluorescence after being cultured for 3 hours and stained with anti-myeloperoxidase (anti-MPO) and DAPI. Extracellular MPO and DNA costaining were counted as neutrophil extracellular traps (NETs) and expressed as the percentage of the total number of cells in the field (**B**). **C**, Heatmap showing the most down-regulated gene functions in neutrophils of BD patients before and after 12 weeks of treatment with apremilast. In paired dot plots, symbols represent individual patients. * = $P < 0.05$ by Wilcoxon's matched pairs test. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42486/abstract.

CD64, CD66b, and CD11c at week 12 of apremilast treatment (mean ± SEM % CD11b+ neutrophils 9% ± 4% versus 4.5% ± 2.7% at week 12, $P = 0.03$; CD64+ 1.4% ± 0.9% versus 0.5% ± 0.3%, $P = 0.06$; CD66b^{high} 9% ± 2.4% versus 6% ± 2.9%, $P = 0.03$; CD11c+ 8.8% ± 1.8% versus 5.5% ± 2.5%, $P = 0.03$, respectively) and at week 12 of colchicine treatment (mean ± SEM % CD11b+ neutrophils 7.2% ± 2.7% versus 0.8% ± 0.6% at week 12, $P = 0.03$; CD64+ 1.7% ± 0.2% versus 0.5% ± 0.3%, $P = 0.06$; CD66b^{high} 7.2% ± 2% versus 1.8% ± 0.46%, $P = 0.03$; CD11c+ 6% ± 0.3% versus 3.5% ± 0.6%, $P = 0.03$, respectively) (Figure 4A), as well as a reduction in NETs production with apremilast treatment at week 12 (mean ± SEM % NETs 32% ± 3.8% versus 20% ± 6.4% at week 12; $P = 0.03$) and colchicine treatment at week 12 (mean ± SEM % NETs 28.4% ± 4.4% versus 15.4% ± 6.0% at week 12; $P = 0.03$) (Figure 4B), and a reduction in ROS production with apremilast treatment at week 12 (mean ± SEM MFI 13 ± 5.9 versus 6.6 ± 2.5 at week 12; $P = 0.01$).

We also performed transcriptome analysis of sorted circulating neutrophils from untreated BD patients (n = 4) at week 0 compared to neutrophils obtained after apremilast treatment for 12 weeks. Overall, we found 2,407 dysregulated genes between untreated and treated BD samples, of which 1,254 genes were down-regulated and 1,153 were up-regulated in treated versus untreated BD samples. Of note, we assessed the expression of the genes previously showed in Figure 1D, and indeed observed a very marked decrease of all these gene expressions in samples at 12 weeks of treatment compared to baseline (week 0), clearly showing that apremilast dampens neutrophil activation in vivo (Figure 4C).

DISCUSSION

Neutrophils and their hyperfunction represent the main cells involved in the pathogenesis of BD. Matsumura et al showed that neutrophil chemotaxis was increased in BD and could be alleviated with colchicine (16). Many studies have highlighted the chemotaxis, phagocytosis, and production of ROS by neutrophils in BD (17–23). Beside these mechanisms, some studies have also pointed out the up-regulation of surface markers on BD neutrophils. Here, we provide new data on molecular signaling of neutrophils in BD. To our knowledge, isolated neutrophil transcriptome analysis has not been previously done in BD. Thus, our data confirm neutrophil activation in BD at the molecular level. Some studies have previously assessed the molecular signature of peripheral blood mononuclear cells in BD and showed an up-regulation of neutrophil chemotaxis genes (24).

We found that CD64, CD11b, CD11c, and CD66b are up-regulated on BD neutrophil surface. CD64 (Fcγ receptor I) and CD11b are expressed at very low levels on resting neutrophils but are up-regulated upon activation (25,26). CD11c is an integrin that is highly expressed on activated neutrophils, allowing them to adhere to fibrinogen (27). CD66b is a marker of granulocyte activation involved in adhesion to endothelial cells (28), degranulation, and ROS production. Beside chemophagocytosis and surface markers, increased ROS is a key mechanism of neutrophil function. In this study, we found that BD neutrophils produce significantly more ROS than their healthy donor counterparts. The consequences of ROS production in BD can be very wide and have not been extensively studied yet. Increased ROS production by BD neutrophils may be a direct cause of fibrinogen oxidation, which leads to slower fibrin polymerization and resistance to plasmin-induced lysis. This could therefore be an explanation of the prothrombotic state in BD (29). Last, our findings showed that NETosis was also increased in BD. The role of NETosis in BD has been studied by our group (8) and others (9,30–32). NETs can promote intravascular coagulation by promoting thrombin generation and also cause endothelial dysfunction via decreasing cell proliferation and increasing apoptosis (9). In the present study, we demonstrated in vivo the abundance of neutrophils in skin lesions of BD. Altogether, these data confirm the prominent role of neutrophil activation in BD physiopathology.

There is growing evidence of the implication of the NF-κB pathway in BD. The NF-κB pathway is tightly regulated through multiple posttranslational mechanisms. Dysfunction of key proteins of the NF-κB pathway leading to its up-regulation have recently been described in humans in a phenotype very close to BD (33). The NF-κB pathway could thus represent an interesting target in the pathophysiology of BD but has not yet been widely studied. PDE4 is an immune and inflammatory cell enzyme which, by degrading the key intracellular signaling messenger cAMP, promotes NF-κB pathway activation (10,34,35). Given the central role of PDE4 in the NF-κB pathway and the putative role of the NF-κB pathway in BD, we aimed to study this enzyme. PDE4 has not been studied in BD neutrophils, but apremilast, an orally administered small-molecule drug that acts as an inhibitor of PDE4, showed a therapeutic benefit in treating oral ulcers in phase II and III studies (12,13). We found in this study that neutrophils expressed PDE4 in skin lesions of BD patients.

To confirm the role of neutrophil PDE4 expression in BD, we demonstrated both in vitro and in vivo that PDE4 inhibition abrogated neutrophil activation (surface marker expression, ROS generation, and NETosis). PDE4 blockade has not been extensively studied on human neutrophils. It has previously been shown that PDE4 inhibition reduced neutrophil adhesion and migration, as well as cytokine, chemokine, and ROS release (36). In some animal models, genetic deficiency of PDE4 reduces neutrophilic inflammation (37) and reduces leukocyte recruitment at the site of inflammation (38,39). Recently, Totani et al showed for the first time in a human and in a mouse model of chronic obstructive pulmonary disease that PDE4 inhibition effectively reduced NETosis (40). We confirmed that colchicine (used as a control) inhibits neutrophil activation in BD (41) and decreases NETs formation (31).

Overall, our data confirm the instrumental role of neutrophils in the pathogenesis of BD and highlight for the first time the role of PDE4 and its inhibition on neutrophil function. We also highlight a possible mechanism behind the beneficial clinical effects of apremilast in BD.

In conclusion, our findings help to unravel the possible mechanism behind beneficial clinical effects of PDE4 inhibitors in BD and raise a strong rationale for apremilast use in BD.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content. All authors approved the final version to be published. Dr. Saadoun had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Le Joncour, Saadoun.

Acquisition of data. Le Joncour, Régnier, Maciejewski-Duval, Charles, Fouret, Rosenzwajg, Klatzmann, Saadoun.

Analysis and interpretation of data. Le Joncour, Régnier, Maciejewski-Duval, Charles, Barete, Fouret, Rosenzwajg, Klatzmann, Cacoub, Saadoun.

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