

## Phosphodiesterase 4 in inflammatory diseases: Effects of apremilast in psoriatic blood and in dermal myofibroblasts through the PDE4/CD271 complex



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### ABSTRACT

Phosphodiesterases 4 (PDE4) act as proinflammatory enzymes via degradation of cAMP, whereas PDE4 inhibitors play an anti-inflammatory role in vitro and in vivo. In particular, apremilast has been recently approved for the treatment of psoriasis and psoriatic arthritis. However, little is known on the expression pattern of PDE4 in psoriasis. We report that PDE4B and PDE4D mRNA are overexpressed in peripheral blood mononuclear cells (PBMC) from psoriasis, as compared with normal controls, while apremilast reduces PBMC production of a number of pro-inflammatory cytokines and increases the levels of anti-inflammatory mediators. PDE4 expression is up-regulated in psoriatic dermis as compared with normal skin, with particular regard to fibroblasts. This is confirmed in vitro, where both dermal fibroblasts (DF) and, to a greater extent, myofibroblasts (DM) express all PDE4 isoforms at the mRNA and protein level. Because PDE4 interacts with the nerve growth factor (NGF) receptor CD271 in lung fibroblasts, we evaluated the relationship and function of PDE4 and CD271 in normal human skin fibroblasts. All PDE4 isoforms co-immunoprecipitate with CD271 in DM, while apremilast inhibits apoptosis induced by  $\beta$ -amyloid, a CD271 ligand, in DM. Furthermore, apremilast significantly reduces NGF- and transforming growth factor- $\beta_1$  (TGF- $\beta_1$ )-induced fibroblast migration, and inhibits DF differentiation into DM mediated by NGF or TGF- $\beta_1$ . Finally, in DM, apremilast significantly reduces cAMP degradation induced by treatment with  $\beta$ -amyloid. Taken together, these results indicate that PDE4 play an important role in psoriasis. In addition, the study reveals that the PDE4/CD271 complex could be important in modulating fibroblast functions.

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**Abbreviations:** AD, atopic dermatitis; ANOVA, analysis of variance; BSA, body surface area; cAMP, cyclic adenosine monophosphate; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DF, dermal fibroblasts; DLE, discoid lupus erythematosus; DM, dermal myofibroblasts; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO, growth-regulated oncogene; IFN- $\gamma$ , interferon gamma; IgM, immunoglobulin M; IHC, immunohistochemistry; IL1, interleukin 1; IP-10, IFN- $\gamma$ -inducible protein 10 (C-X-C motif chemokine 10); IPF, idiopathic pulmonary fibrosis; LPS, lipopolysaccharide; MDC, macrophage-derived chemokine; MDC, macrophage-derived chemokine; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor; NR, not reported; NT, neurotrophin; NT3, neurotrophin-3; PAS, preautophagosomal structure; PASI, Psoriasis Area and Severity Index; PBMC, peripheral blood mononuclear cells; PDE, phosphodiesterase; PI, propidium iodide; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase-polymerase chain reaction; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SEM, standard error of the mean; SLE, systemic lupus erythematosus; SMA, smooth muscle actin; sPGA, static Physician Global Assessment; TA, transit amplifying; TBS, tris buffered saline; TGF- $\beta$ , transforming growth factor-beta; TLR4, toll-like receptor 4; TNF- $\alpha$ , tumor necrosis factor-alpha; TRK, tyrosine kinase; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine, 5'-triphosphate (dUTP) nick end labeling; VEGF, vascular endothelial growth factor.

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## 1. Introduction

cAMP is a pivotal second messenger that regulates inflammatory responses [1]. cAMP is degraded through the activity of the large superfamily of phosphodiesterases (PDE) [2]. Within this group, enzymes of the four-gene PDE4 family play a key role in degrading cAMP in inflammatory cells, as well as endothelial cells, smooth muscle cells, and keratinocytes [3]. There are also more than 20 splice variants encoded by the PDE4A, PDE4B, PDE4C, and PDE4D genes, due to differential mRNA splicing genes [4]. PDE4 isoforms display a differential expression pattern in human testis, skeletal muscle, lung, and brain [5–7]. However, the expression patterns of PDE4 isoforms have not been studied in blood or skin of patients with inflammatory disease such as psoriasis.

PDE4 inhibitors are a well-characterized class of pharmaceutical agents, with a broad range of anti-inflammatory activities *in vitro*, and in *in vivo* preclinical models of asthma, lung neutrophilia, arthritis, inflammatory bowel disease, multiple sclerosis, osteoporosis, and other conditions [8–10]. Apremilast is a novel, orally administered, small molecule PDE4 inhibitor approved for treatment of psoriatic arthritis (PsA) and moderate to severe plaque psoriasis [11,12]. By inhibiting PDE4, apremilast elevates intracellular cAMP levels and thus down-regulates the inflammatory response by modulating expression of a network of pro- and anti-inflammatory mediators implicated in psoriasis and PsA [13–15]. Selige et al. found different PDE4 subtypes with distinct functions in human lung fibroblasts and highlighted the predominant role of PDE4B in controlling pathophysiological processes of human lung fibroblasts. This provides a rationale for targeting of PDE4B to treat respiratory diseases with fibrotic lesions in the lung [16]. Schafer et al. showed that apremilast does not inhibit proliferation in normal primary human epidermal keratinocytes; however, apremilast inhibited tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production by keratinocytes upon UVB exposure (50 mJ/cm<sup>2</sup>) [17]. Sachs et al. found a new mechanism in which PDE4A4/5 interacts with CD271 to enhance cAMP degradation. The CD271-dependent down-regulation of cAMP results in a decrease in extracellular proteolytic activity [18]. CD271 is a neurotrophin (NT) receptor, also known as p75<sup>NTR</sup>, that belongs to the TNF receptor superfamily and shares with the other members of the family the death domain. The role of CD271 is complex: it may increase high-affinity binding of Trk receptors to NTs, and enhances the survival signal. By contrast, CD271 alone can induce apoptosis upon specific binding, such as with  $\beta$ -amyloid, via its own transduction pathway and by interacting with a mounting number of downstream molecules [19]. Neurotrophins and their receptors have autocrine and paracrine functions within the skin [20]. Moreover, our group has recently demonstrated that CD271 is expressed by basal keratinocytes, specifically by transit-amplifying (TA) cells [21]. It should be noted that the crosstalk between the epidermal and dermal compartments is necessary to sustain epidermal homeostasis, and that some factors that are produced in the dermal compartment act on stem cells that reside in the epidermis. Moreover, Palazzo et al. showed that both dermal human fibroblasts and myofibroblasts express NT, CD271, and tyrosine kinase (Trk) receptors, and that NTs exert a critical role in tissue remodeling and wound healing [22].

In the current set of experiments, we report gene expression patterns of PDE4 isoforms (A, B, C, and D) in peripheral blood mononuclear cells (PBMC) from healthy donors and from patients with a variety of immune-mediated or inflammatory diseases including psoriasis, as well as the effect of apremilast on cytokine and chemokine production from PBMC of patients with psoriasis. We further report immunohistochemistry studies showing the isoform distribution of PDE4 in skin samples from healthy donors and patients with psoriasis, atopic dermatitis (AD), and discoid lupus erythematosus (DLE). We then examine PDE4 isoform binding to CD271 in fibroblasts cultured from healthy skin, and analyze the function of the PDE4/CD271 complex in fibroblasts.

## 2. Materials and methods

### 2.1. Gene Expression Analysis of PDE4 Isoforms in PBMC

Vially frozen PBMC and autologous serum from healthy volunteers or patients with psoriasis, rheumatoid arthritis (RA), idiopathic pulmonary fibrosis (IPF), sarcoidosis, scleroderma, Crohn's disease, or systemic lupus erythematosus (SLE; n = 5 from each disease) were purchased from Conversant Healthcare Systems (Huntsville, AL). The diagnosis of disease was made on a case report form by the responsible physician in a network of medical clinics, research clinics, hospitals, and outpatient medical facilities. Peripheral blood mononuclear cells were thawed and cultured in RPMI-1640 supplemented with 5% of each patient's autologous serum for 24 h to allow cells to recover from freezing and thawing. Cells ( $1 \times 10^6$  PBMC per patient) were collected for gene expression analysis by hybridization array. Cells were washed with cold phosphate-buffered saline, lysed with 350  $\mu$ L RLT buffer (Qiagen, Gaithersburg, MD), and transferred to barcoded tubes. RNA was extracted and converted to cDNA using the Ovation Whole Blood Solution (NuGEN Technologies, San Carlos, CA), and hybridized to HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). Gene expression data were normalized and provided by Covance Genomics Laboratory (Seattle, WA). Statistical comparison for PBMC from each disease versus normal control was by one-way analysis of variance. Affymetrix probes were annotated using the GeneAnnot microarray gene annotation database at the Weizmann Institute of Science (<http://genecards.weizmann.ac.il/geneannot/index.shtml>) and identified in specific gene splice variants using BLAST at National Center for Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### 2.2. Quantitative RT-PCR

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to confirm overexpression of PDE4B and PDE4D isoforms in PBMC from patients with psoriasis. PBMC samples (n = 20 each from psoriasis patients and healthy donors) were cultured for 24 h in 5% autologous serum. RNA was isolated, converted to cDNA, and analyzed by qRT-PCR, and a comparison of PBMC from patients with psoriasis versus normal controls was conducted by *t* test.

### 2.3. Multiplexed bead-based immunoassay

Peripheral blood mononuclear cells from patients with psoriasis (n = 10) were obtained after informed consent by Conversant Healthcare Systems. The diagnosis of psoriasis was made on a case report form by the responsible physician in a network of medical clinics, research clinics, hospitals, and outpatient medical facilities. Peripheral blood mononuclear cells were thawed (minimum viability was 80%), plated at  $2 \times 10^5$  cells/well, and stimulated in duplicate via various immune receptors with or without apremilast (1  $\mu$ M) in 0.1% dimethyl sulfoxide (DMSO) for 24 h. Immune cell stimuli included 1 ng/mL lipopolysaccharide (LPS; Sigma, St. Louis, MO), targeting toll-like receptor 4 (TLR4) on monocytes, dendritic cells, and B cells; 10 ng/mL each of staphylococcal enterotoxin A and B (Toxin Tech, Sarasota, FL), targeting T-cell receptors; 2 ng/mL each of TNF- $\alpha$  + interleukin (IL)-1 $\beta$  (Sigma), targeting TNF and IL-1 receptors on monocytes, dendritic cells, B cells, T cells, and natural killer cells; and 2  $\mu$ g/mL anti-immunoglobulin M (IgM; Jackson ImmunoResearch, West Grove, PA), targeting B-cell receptors.

The production of 25 protein analytes was measured by cytometric bead array using Milliplex MAP Human Cytokine/Chemokine beads (EMD Millipore Corporation, Billerica, MA) on a MagPix instrument (Luminex, Austin, TX).

#### 2.4. Skin immunohistochemistry

Immunohistochemistry (IHC) was performed on dermis samples from healthy controls and from patients with psoriasis, AD, and DLE at University of Modena and Reggio Emilia, Modena, Italy. Paraffin embedded biopsies (4  $\mu$ m) were stained for protein analysis by IHC as follows: PDE4A, rabbit polyclonal antibody (1:100; Proteintech Group, Chicago, IL); PDE4B, rabbit polyclonal antibody (1:40; Santa Cruz Biotechnology); PDE4C, rabbit polyclonal (1:40; Santa Cruz Biotechnology); PDE4D, rabbit polyclonal antibody (1:40; Santa Cruz Biotechnology); and CD271, mouse monoclonal antibody (1:100; Upstate, Lake Placid, NY). After washes, slides were incubated with secondary antibody for 45 min at room temperature. Fast Red was used as chromogen; stainings were performed according to the UltraVision LP Detection System AP Polymer & Fast Red Chromogen assay (Thermo Fisher Scientific, Waltham, MA). The expression intensity was quantitatively determined using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD). Detailed histopathology and immunohistochemistry of PDE4A, PDE4B, and PDE4D protein expression in psoriatic skin was performed by LifeSpan Biosciences, Inc. (Seattle, WA) using the primary antibodies as described above. The principal detection system consisted of a Vector anti-rabbit secondary (BA-1000, Vector Laboratories, Peterborough, UK) and a Vector ABC-AP kit (AK-5000) with a Vector Red substrate kit (SK-5100).

#### 2.5. Isolation of primary fibroblasts and cell treatment

Normal human dermal fibroblasts (DF) were isolated from healthy skin biopsies obtained from waste materials from operating rooms. Patient consent for experiments was not required because Italian law considers human tissue left over from surgery as discarded material. Briefly, DF were obtained by explant culture and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (FBS). Transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ; 1 ng/mL; Sigma) was added in fibroblast secondary culture for 6 days to promote differentiation into dermal myofibroblasts (DM). The myofibroblast phenotype was checked by immunostaining in situ of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA).

For cell treatment, apremilast was diluted in DMSO 10 mM and used at the final concentration of 10  $\mu$ M in culture medium without serum. Dermal fibroblasts and MF were plated in DMEM with FBS. At 48 h after seeding, apremilast or DMSO was added to DMEM for 2 h and, after that, diluent, 100 ng/mL human recombinant nerve growth factor (NGF; Sigma-Aldrich, St. Louis, MO), 1 ng/mL TGF- $\beta_1$  (Sigma-Aldrich), and 40  $\mu$ M  $\beta$ -amyloid 25–35 (Bachem, Bubendorf, Switzerland) were added to medium. Cells were monitored and used for further experiments 48 h later.

#### 2.6. Immunofluorescence in situ

Dermal fibroblasts were plated on chamber slides and, 48 h after seeding, were treated with TGF- $\beta_1$  (1 ng/mL, Sigma-Aldrich) or diluent alone. Six days later, cells were fixed in situ in 4% paraformaldehyde for 20 min and air dried. After a rehydration in phosphate-buffered saline (PBS), cells were permeabilized for 10 min with 0.5% Triton X-100 in 0.1% sodium citrate, treated for 5 min with 50 mM NH<sub>4</sub>Cl, and incubated with 1% bovine albumin serum for 20 min. Then slides were incubated for 1 h at room temperature with the mouse monoclonal anti- $\alpha$ -SMA antibody (1:400; Sigma-Aldrich). Then cells were incubated with secondary antibody, Alexa Fluor 488 (1:100; Thermo Fisher Scientific). Finally, cell nuclei were stained with 1  $\mu$ g/ml DAPI (Sigma-Aldrich). Micrographs were taken on a Confocal Scanning Laser Microscopy (Leica TCS SP2; Leica, Exton, PA).

#### 2.7. Co-immunoprecipitation

Dermal fibroblasts and DM were cultured until pre-confluent condition. Cells were harvested with lysis buffer pH 7.5 (150 mM NaCl, 15 mM MgCl, 1 mM ethylene glycol tetraacetic acid [EGTA], 50 mM Hepes, 10% glycerol, 1% Triton) containing protease inhibitors. Monoclonal antibody CD271 (Upstate, Lake Placid, NY) was bound to Sepharose beads for 1 h at 4 °C under rotation. Thereafter, precleared lysates were conjugated to Sepharose beads-antibody complex or, as control, to Sepharose beads alone or to antibody alone, overnight at 4 °C under rotation. Immunocomplexes were washed with binding buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2 mM MgCl<sub>2</sub>, 1 mM DTT, 0.5% Triton X-100, 0.5% bovine serum albumin) 6 times. Samples were eluted with 2 $\times$  Laemmli sample buffer for 5 min at 90 °C and Western blotting for PDE4A, B, C, and D was performed as described below.

#### 2.8. Western blotting

Total proteins (20  $\mu$ g) or immunocomplexes were analyzed under non-reducing conditions on 7.5% polyacrylamide gels. To obtain total proteins, cells were washed with PBS and lysed on ice, in lysis buffer pH 7.5 (150 mM NaCl, 15 mM MgCl, 1 mM EGTA, 50 mM Hepes, 10% glycerol, 1% Triton) containing protease inhibitors. Briefly, after the electrophoretic separation, total proteins or immunocomplexes were blotted onto nitrocellulose membranes. The blots were blocked for 2 h in blocking buffer (PBS buffer, pH 7.4 with 0.2% Tween 20 and 5% nonfat milk) and incubated with rabbit polyclonal anti-human PDE4A antibody (1:1000; Proteintech Group, Chicago, IL), rabbit polyclonal anti-human PDE4B antibody (1:300; Santa Cruz Biotechnology), rabbit polyclonal anti-human PDE4C antibody (1:300; Santa Cruz Biotechnology), rabbit polyclonal anti-human PDE4D antibody (1:300; Santa Cruz Biotechnology) or mouse monoclonal  $\alpha$ -SMA (1:3000; Sigma-Aldrich) or mouse monoclonal anti- $\beta$ -actin (1:1000; Sigma) overnight at 4 °C. Then membranes were washed in TBS/Tween 20, incubated with peroxidase-conjugated goat anti-mouse or goat anti-rabbit or anti-mouse (1:3000; Bio-Rad, Hercules, CA) antibodies for 45 min at room temperature. Finally, membranes were washed and developed using the ECL chemiluminescent detection system (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, UK). The band intensity was quantitatively determined using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD), and protein level intensity was normalized to  $\beta$ -actin expression.

#### 2.9. MTT Assay

Dermal fibroblasts and DM were plated in a 96-well tissue culture plate (5000 cells/well). At 48 h after plating, cells were treated as described above with apremilast or DMSO for 2 h, then treated with diluent (control), NGF (100 ng/mL), neurotrophin-3 (NT3; 100 ng/mL), or  $\beta$ -amyloid (40  $\mu$ M). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed 48 h later. Proliferative cells were detected by incubating with MTT (Sigma-Aldrich) solution at 37 °C for 4 h. The formazan dye produced after DMSO solubilization was evaluated by a multiwell scanning spectrophotometer at 540 nm. The results are expressed as viability percentage, as compared with control. Results are calculated as the mean  $\pm$  SD of three different experiments. Student's *t* test was performed for comparison of the means.

#### 2.10. Propidium iodide Staining

At 48 h after cell seeding, DF and DM were pretreated with DMSO or apremilast as described above, then diluent (control) or 40  $\mu$ M  $\beta$ -amyloid was added. After 48 h, cells were trypsinized and resuspended ( $5 \times 10^5$  cells) in 1 mL hypotonic fluorochrome solution: 50  $\mu$ g/mL propidium iodide (PI) containing 0.1% sodium citrate and 0.5% TritonX-100 (Sigma). After 15 min, cells were analyzed using an Epics

XL flow cytometer (Coulter Electronics Inc., Hialeah, FL). Apoptosis was detected by evaluating the fluorescence of hypodiploid cells counted in subG1 region.

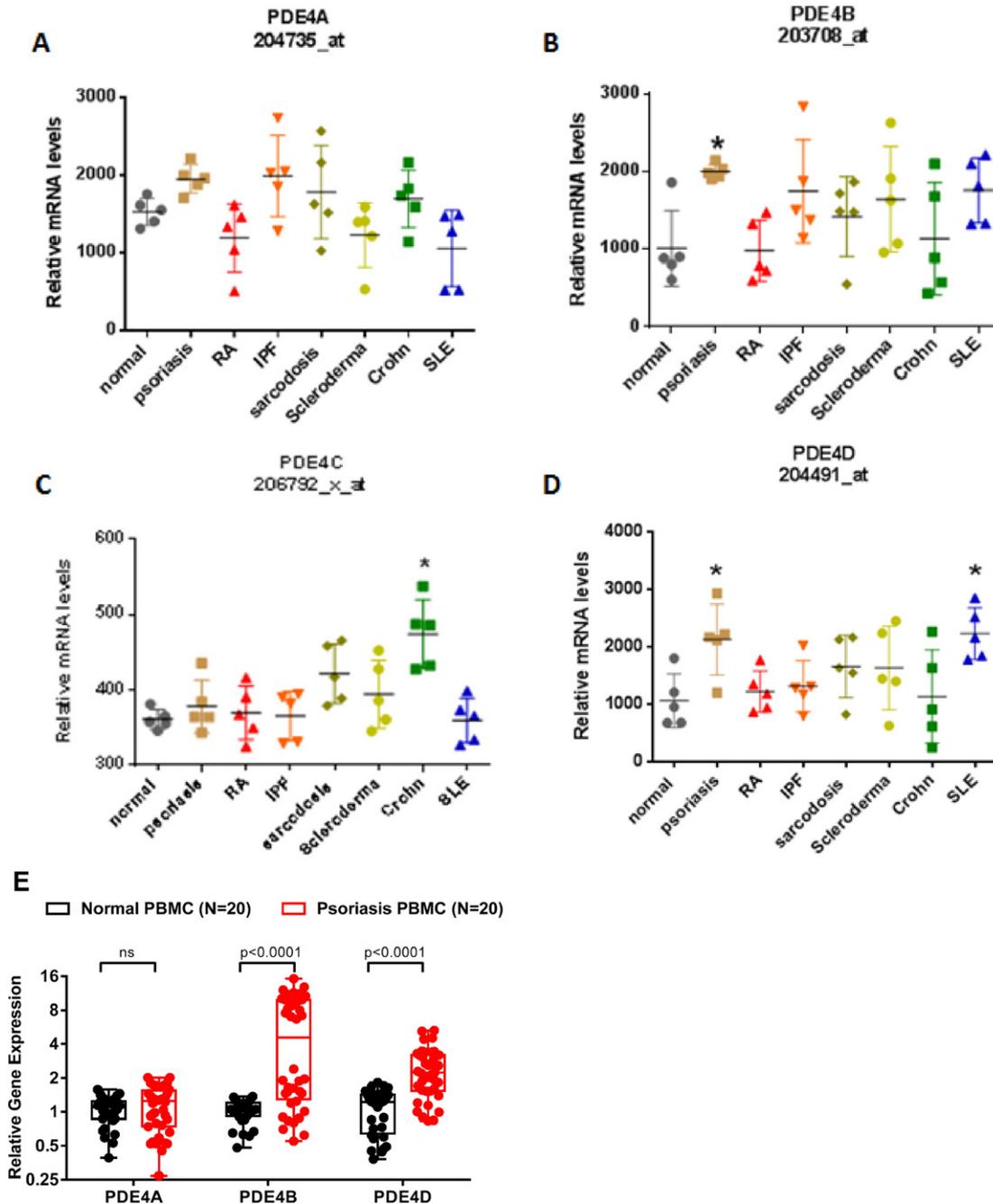
### 2.11. TUNEL assay

Dermal fibroblasts and DM after treatment were fixed in situ in paraformaldehyde (4% in PBS) and air-dried. The slides were stained with the In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) as recommended by the manufacturer. Fluorescent specimens were analyzed by confocal scanning laser microscopy (Leica TCS

SP2, Leica Microsystems, Wetzlar, Germany) in conjunction with a conventional optical microscope (Leica DM IRBE, Leica). Results were calculated as the mean  $\pm$  SD of three different experiments.

### 2.12. Migration assay

A total of  $20 \times 10^4$  DF were plated on 12-well tissue culture plates. At 48 h after seeding, cells were pretreated with DMSO or apremilast for 2 h as described above, then cells were treated with 5  $\mu$ g/mL mitomycin C for 1.5 h. Subsequently, the cells were washed three times in serum-free medium and a line for each well was drawn along the cell



**Fig. 1.** Gene expression analysis of PDE4 isoforms in PBMC from healthy volunteers or patients with psoriasis, RA, IPF, sarcoidosis, scleroderma, Crohn's disease, or SLE. (A, B, C, D) PBMC samples ( $n = 5$  for each immune/inflammatory condition) were cultured 24 h in 5% autologous serum. RNA was isolated, converted to cDNA, and hybridized to Affymetrix HG-U133 Plus 2.0 arrays. Representative results from one hybridization probe per gene are shown. \* $p < 0.05$  versus normal controls by one-way ANOVA. (E) PBMC samples from healthy donors and psoriasis patients ( $N = 20$  each) were cultured for 24 h in 5% autologous serum. RNA was isolated, converted to cDNA, and analyzed by qRT-PCR. Dots represent individual data points. The boxes extend from the 25th to the 75th percentile, the whiskers represent the minimum and maximum, and the lines in the middle of each box represent the median ( $p < 0.0001$  versus normal controls, by  $t$  test).

monolayer with a sterile plastic tip. Plates were washed twice with serum-free medium to remove all detached cells and incubated in medium with 0.1% bovine serum albumin with 1 ng/mL TGF- $\beta$ 1, 100 ng/mL human recombinant NGF in presence of DMSO or apremilast. Cells were monitored at 24 and 48 h from stimulation. The result of each experiment was expressed as the mean of migrated cells from three different areas. The final results are expressed as the mean  $\pm$  SD of three different experiments.

### 2.13. cAMP ELISA

At 48 h after cell seeding, DF and DM were pretreated with DMSO or apremilast as described above, then diluent, 100 ng/mL human recombinant NGF (Sigma-Aldrich), or 40  $\mu$ M  $\beta$ -amyloid (Bachem) were added to the culture medium.

48 h later, supernatants were collected, particulates were removed by centrifugation and stored at  $-20^{\circ}\text{C}$ . cAMP quantitation was performed by a competitive enzyme immunoassay (R&D Systems cAMP immunoassay, R&D Systems, Minneapolis, MN) according to manufacturer instructions. The sample concentration was determined by absorbance at 540 nm against a standard curve. cAMP levels are given in pmol/mL of cell lysate and results are expressed as mean  $\pm$  SEM of triplicate from three different experiments.

## 3. Results

### 3.1. Gene expression analysis of PDE4 Isoforms in PBMC

We first wanted to analyze gene expression of PDE4 isoforms (A, B, C, and D) in PBMC from healthy donors and from patients with 7 different immune-mediated or inflammatory diseases: psoriasis, rheumatoid arthritis (RA), idiopathic pulmonary fibrosis (IPF), sarcoidosis, scleroderma, Crohn's disease, and SLE. PDE4B and PDE4D isoforms were increased at the mRNA level in PBMC from psoriasis patients relative to normal controls (Fig. 1B and D,  $p < 0.05$ ). The PDE4B probe (203708\_at) corresponds with the National Center for Biotechnology Information (NCBI) reference sequence NM\_002600, found in PDE4B variants A and D. The PDE4D probe (204491\_at) corresponds with NCBI reference sequence ID R40917, found in PDE4D variants 1 through 9. PDE4D was also increased versus normal controls in PBMC from patients with SLE (Fig. 1D,  $p < 0.05$ ). PDE4C mRNA was preferentially overexpressed in PBMC from patients with Crohn's disease versus normal controls (Fig. 1C), while PDE4A mRNA expression levels were similar across PBMC from normal controls and patients with the various immune/inflammatory diseases (Fig. 1A). The PDE4A probe (204,735-at) corresponds with NCBI reference sequence ID NM\_006202, found in PDE4A variants 1 through 5, X1 and X3. The PDE4C probe (206792\_x\_at) corresponds with NCBI reference sequence ID NM\_000923, found in PDE4C variants 1 through 4. To confirm the overexpression of PDE4B and PDE4D mRNA in PBMC from patients with psoriasis relative to normal controls, quantitative RT-PCR was performed using 20 samples each from psoriasis patients and healthy donors. Mean relative gene expression in PBMC from psoriasis patients was 5.4-fold higher for PDE4B, and 2.2-fold higher for PDE4D ( $p < 0.0001$  for both versus normal control), compared with 1.1-fold for PDE4A ( $p = \text{NS}$ ) (Fig. 1E). There were no significant correlations between PDE4A, PDE4B, or PDE4D gene expression in the psoriatic PBMC with PASI, BSA, or sPGA scores (data not shown).

### 3.2. Effect of apremilast on the production of inflammatory mediators by PBMC from patients with psoriasis

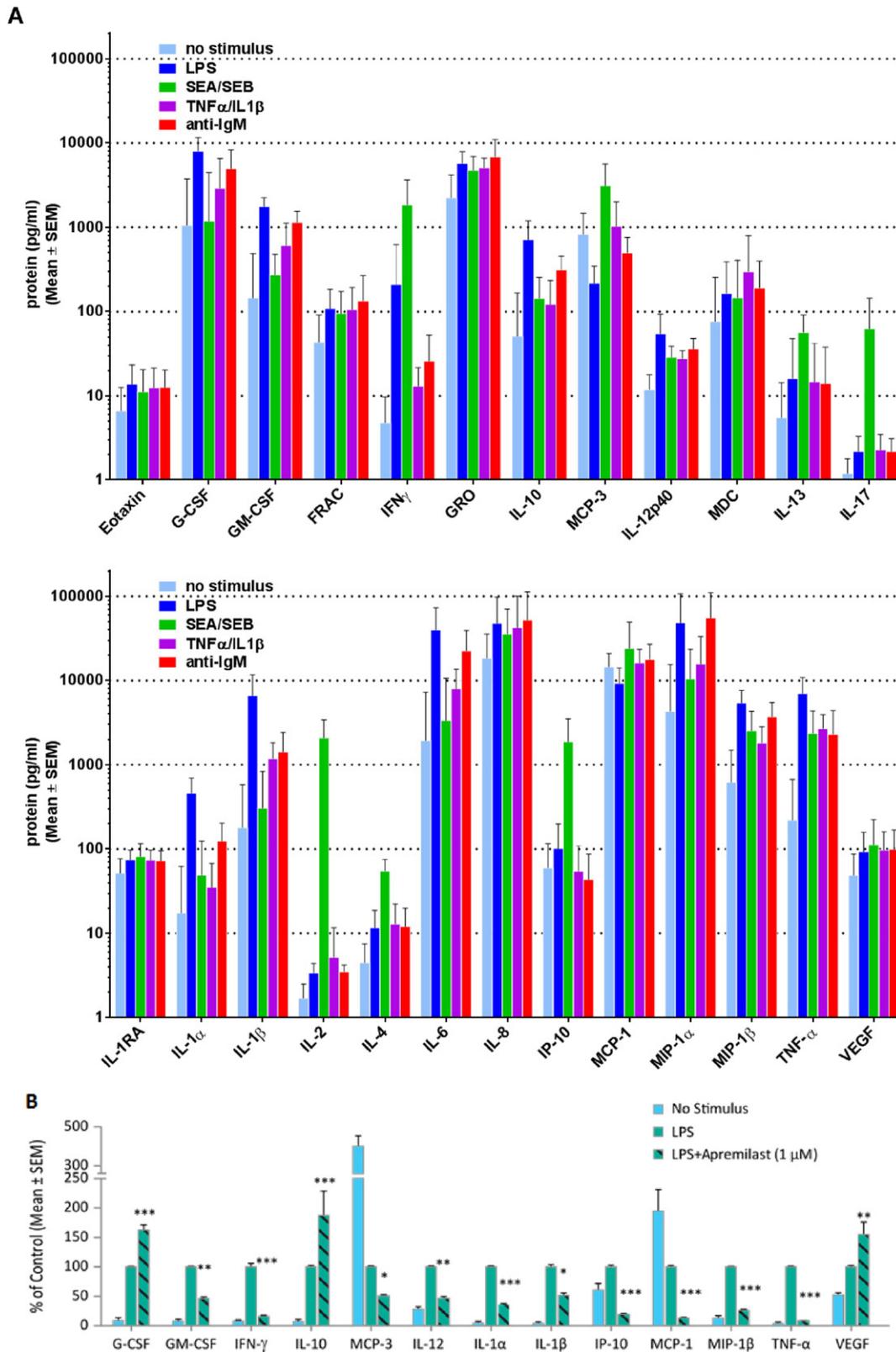
Given the high PDE4 expression in psoriasis, we investigated the effect of apremilast on cytokines produced by PBMC from psoriatic patients. Psoriasis patient PBMC donors ( $n = 10$ ) included 5 women and 5 men ranging in age from 26 to 42 years, with PASI scores  $\leq 26$  and

$\leq 33\%$  body surface area affected. Two patients also had a diagnosis of psoriatic arthritis. Changes in production of various cytokines and chemokines upon stimulation of PBMCs from these psoriasis patients with LPS, staphylococcal enterotoxin A and B, TNF- $\alpha$ /IL-1 $\beta$ , or anti-IgM are shown in Fig. 2A. Addition of 1  $\mu$ M apremilast had the greatest effect on psoriatic PBMC cultures stimulated via TLR4 (i.e., LPS stimulation), with 13 of the 25 analytes significantly affected versus LPS control (Fig. 2B). Specifically, apremilast caused significant inhibition of LPS-induced TNF- $\alpha$ , IL-12/IL-23p40, interferon gamma (IFN- $\gamma$ ), IP-10, MCP-1, MCP-3, MIP-1 $\beta$ , IL-1 $\alpha$ , IL-1 $\beta$ , and GM-CSF production, and increased IL-10, G-CSF, and VEGF production. In psoriatic PBMC stimulated with anti-IgM to ligate the B-cell receptor, apremilast decreased production of the cytokines TNF- $\alpha$ , IL-1 $\alpha$ , and IFN- $\gamma$ , and the chemokines MCP-1, MCP-3, MIP-1 $\beta$ , and IP-10, and increased IL-10, G-CSF, VEGF, and IL-6. In psoriatic PBMC cultures stimulated via the T-cell receptor or TNF and IL-1 receptor, apremilast had far fewer effects (data not shown).

### 3.3. PDE4 expression in inflammatory skin infiltrates

Psoriasis is an immune-mediated disease characterized by a dense dermal infiltrate extravasating from the blood stream. Immuno-inflammatory infiltrate in turn is responsible for the epidermal alterations. For this reason, we focused on the expression of PDE4 proteins, which are markedly elevated in PBMC from psoriatic patients, in the dermal infiltrate from lesional psoriatic skin. Also, dermis from healthy subjects, AD, and DLE was evaluated. In general, PDE4 staining was higher in diseased versus normal skin when measured by ImageJ software (Fig. 3A). PDE4A was expressed at the cytoplasmic level in a few dermal cells, most likely fibroblasts, in healthy skin, while it was markedly increased in fibroblasts from psoriasis, AD, and DLE. PDE4B was slightly expressed by dermal cells both in healthy and psoriatic skin. In AD, PDE4B staining was somewhat more expressed in the dermis of AD skin compared with controls. In this context, dermal fibroblasts appeared intensely positive. In dermis from DLE skin, PDE4B staining was markedly increased compared with healthy controls, DF being strongly positive. PDE4C was considerably more expressed in the dermis from psoriatic or AD sections than in skin from healthy controls. In DLE, PDE4C staining intensity was higher than in AD or psoriasis, fibroblasts being intensely stained. PDE4D expression was markedly higher in dermal infiltrate (fibroblasts) from psoriasis, AD, or DLE than in healthy skin. Finally, CD271 appeared more expressed in fibroblasts from psoriasis and AD than in healthy skin or DLE (Fig. 3B).

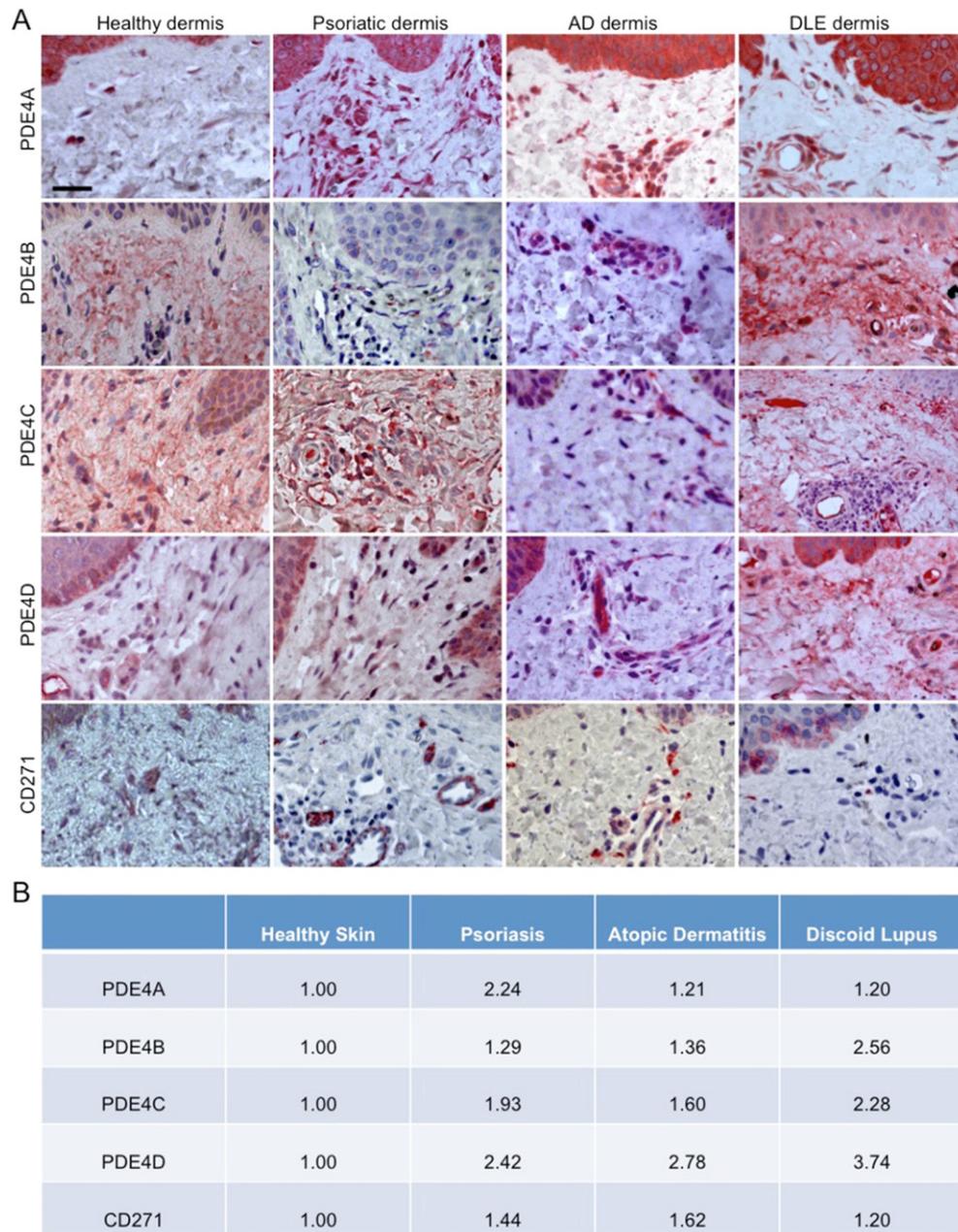
In order to better characterize PDE4 protein expression in psoriatic skin, a more detailed analysis was performed in the dermis. PDE4A, B, and D staining was evaluated and interpreted by a pathologist, and each antibody was evaluated for the presence of specific signal and level of background. Staining intensity was recorded on a 0–4 scale (0 = negative, 1 = blush, 2 = faint, 3 = moderate, 4 = strong) (Table 1). Overall, lymphocytes present in the psoriatic skin were moderately to strongly positive for PDE4A and PDE4B, and faintly positive for PDE4D. Pilosebaceous units, sweat glands, sweat ducts, hair follicles, and arrector pili smooth muscle were positive. Fibroblasts were positive, and endothelium stained strongly for PDE4A, PDE4B, and PDE4D. Specifically, PDE4A expression showed increased intensity and prevalence of staining in nearly all cell types in psoriatic versus normal samples, although neutrophils within microabscesses were exceptionally negative. PDE4B staining in psoriatic samples was very prominent in vessels in the superficial dermis, and positively stained inflammatory cells were more abundant than in normal samples. PDE4D staining in psoriatic samples was present in fibroblasts and endothelium. Lymphocytes frequently showed staining in both normal and psoriatic skin samples, but were far more prevalent in the psoriasis samples. The endothelium of psoriatic skin showed a relatively large increase in PDE4 expression compared with normal skin, with PDE4A and PDE4D displaying strong staining in psoriatic endothelium, but only faint



**Fig. 2.** Modulation of production of inflammatory cytokines and chemokines in psoriatic PBMC. (A) Production of 25 inflammatory cytokines and chemokines by psoriatic PBMC. Data are the absolute means  $\pm$  SEM of 10 samples, each tested in duplicate, measured by cytometric bead array using Milliplex MAP Human Cytokine/Chemokine beads on a MagPix instrument. (B) Effect of apremilast on LPS-stimulated PBMC from patients with psoriasis. Data are the means  $\pm$  SEM percent relative to LPS control, from 10 psoriatic PBMC samples, each tested in duplicate. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus LPS control by two-way ANOVA, followed by Bonferroni post-test to compare replicate means.

staining in normal endothelium. In fibroblasts, PDE4A expression was moderate to strong in psoriatic skin, but absent in normal skin. PDE4D expression was moderate in psoriatic fibroblasts, but faint in normal

fibroblasts. In overall terms of staining intensity, the endothelium and fibroblasts showed the largest consistent increase in PDE4 expression in psoriatic versus normal skin.



**Fig. 3.** PDE4/CD271 expression in healthy and pathologic dermis. (A) PDE4A, PDE4B, PDE4C, PDE4D isoforms and CD271 in dermis from healthy controls and patients with psoriasis, AD, and DLE were detected by IHC. Fast red was used as chromogen. Bar (shown in top left image = 200  $\mu$ m) (B) The expression intensity of PDE4A, PDE4B, PDE4C, PDE4D isoforms and CD271 in (A) was quantitatively measured using ImageJ software as described in the Materials and Methods. Relative staining intensities are shown, normalized to intensities in healthy dermis for each individual antibody.

#### 3.4. PDE4 Isoforms Co-immunoprecipitate with CD271 in fibroblasts

Due to the large increase in PDE4 staining observed in psoriatic fibroblasts, this cell type was selected for functional studies to determine the role of PDE4 in cellular responses. Different proinflammatory cytokines, including TGF- $\beta_1$ , promote fibroblast differentiation into myfibroblasts, characterized by the expression of  $\alpha$ -SMA. These cells are predominantly responsible for connective-tissue contracture and extracellular matrix reorganization in different physiopathologic processes in the skin, including wound healing and fibrosis [23]. We showed that all PDE4 isoforms are expressed in dermal fibroblasts (DF) and myfibroblasts (DM) cultured from healthy donors at the mRNA level (Fig. 4A, B, C), while PDE4 protein levels are higher in DM than in DF (Fig. 4D). PDE4A had previously been found to associate with CD271 to enhance cAMP degradation in fibroblasts, and to regulate

fibrotic responses [18]. Because CD271 is also expressed in skin fibroblasts [22], we wanted to determine if PDE4 isoforms (PDE4A, B, C, and D) co-localize with CD271 in dermal fibroblasts. We showed that PDE4 isoforms co-immunoprecipitate with CD271 in DM (Fig. 4E). However, in DF, CD271 formed only weak complexes with PDE4A and PDE4B and no complexes with PDE4C or PDE4D.

#### 3.5. PDE4/CD271 complex function in fibroblasts in Vitro

To evaluate the effect of the PDE4/CD271 complex on proliferation and apoptosis in fibroblasts, DF and DM were pretreated with apremilast or DMSO for 2 h and then treated with NGF, NT3,  $\beta$ -amyloid, or diluent. Treatment with  $\beta$ -amyloid significantly decreased the percentage of proliferative cells for both DF and DM cultures compared with diluent control. In DM, the addition of apremilast

**Table 1**

Detailed summary of PDE4 IHC staining scores in normal and psoriatic skin. Sections were stained with antibodies specific for PDE4A, B, or D and interpreted by a pathologist. Each antibody was evaluated for the presence of specific signal and level of background. Staining intensity was recorded on a 0–4 scale (0 = negative, 1 = blush, 2 = faint, 3 = moderate, 4 = strong). Data shown are the mean values determined by staining of skin sections from 3 normal subjects and 3 psoriatic patients. NR = not reported.

PDE4 isozyme	Normal			Psoriatic		
	A	B	D	A	B	D
Squamous epithelium	4.0	NR	2.2	4.0	1.5	2.8
Arrector pili smooth muscle	3.3	2.0	2.5	NR	2.0	2.3
Sweat glands/ducts	3.3	2.0	2.3	4.0	2.0	2.5
Pilosebaceous units	2.8	2.0	2.0	4.0	2.0	NR
Vascular smooth muscle	2.5	2.0	2.0	4.0	2.0	NR
Endothelium	2.0	2.0	2.3	4.0	3.2	4.0
Inflammatory cells	2.5	3.0	2.5	4.0	2.5	2.3
Peripheral nerves	1.3	0.0	1.0	2.3	0.0	2.0
Adipocytes	0.5	0.5	1.3	0.0	0.0	0.0
Fibroblasts	0.0	1.0	2.0	3.5	1.0	3.0

significantly inhibited the effect of  $\beta$ -amyloid ( $p < 0.05$  versus DMSO), while apremilast had no effect versus DMSO in DF (Fig. 5A, B). Similarly, the addition of apremilast significantly reduced the induction of apoptosis by  $\beta$ -amyloid treatment in DM ( $p < 0.05$  versus DMSO, Fig. 5D, F) but not in DF (Fig. 5C, E).

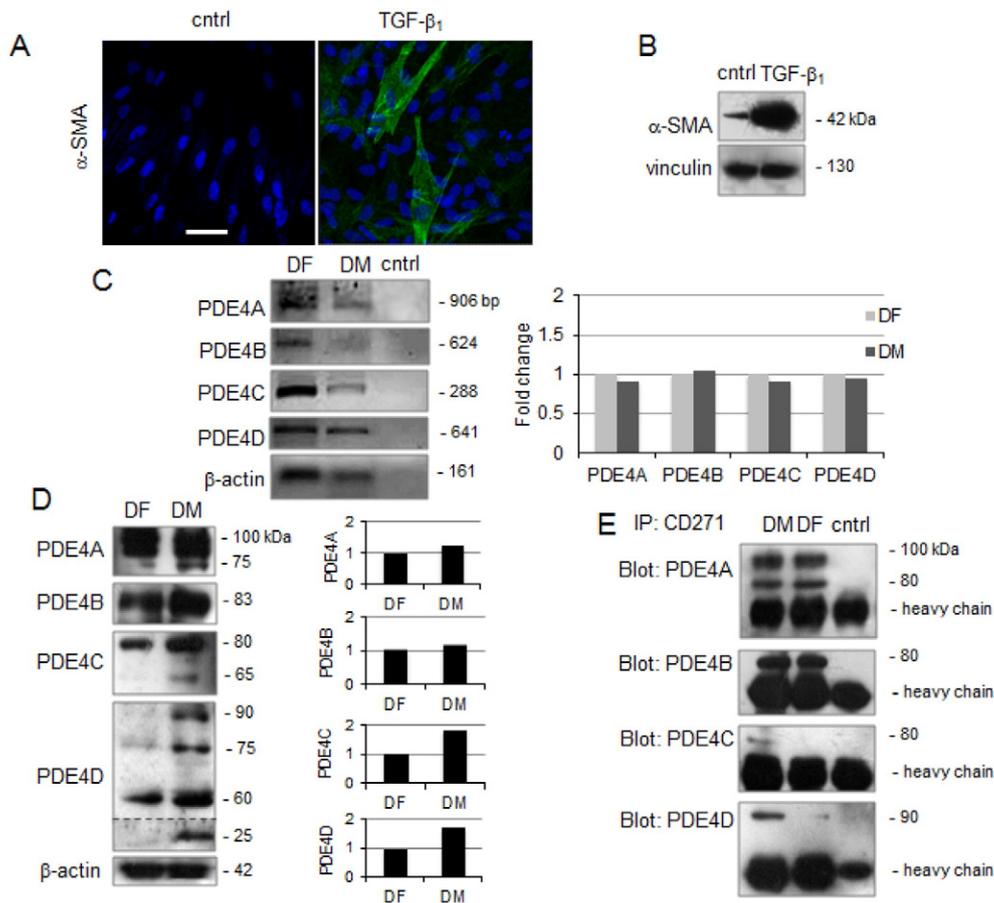
It has been shown previously that neurotrophins mediate DF migration, and that neurotrophins as well as TGF- $\beta_1$  induce DF to differentiate

into DM by expressing  $\alpha$ -SMA [22]. We therefore analyzed whether the addition of apremilast could affect DF migration and  $\alpha$ -SMA expression. Apremilast inhibited DF migration induced by both NGF and TGF- $\beta_1$  treatment (Fig. 6A, B). Similarly, apremilast inhibited the up-regulation of  $\alpha$ -SMA expression induced by NGF and TGF- $\beta_1$ , especially 6 days after treatment (Fig. 6C).

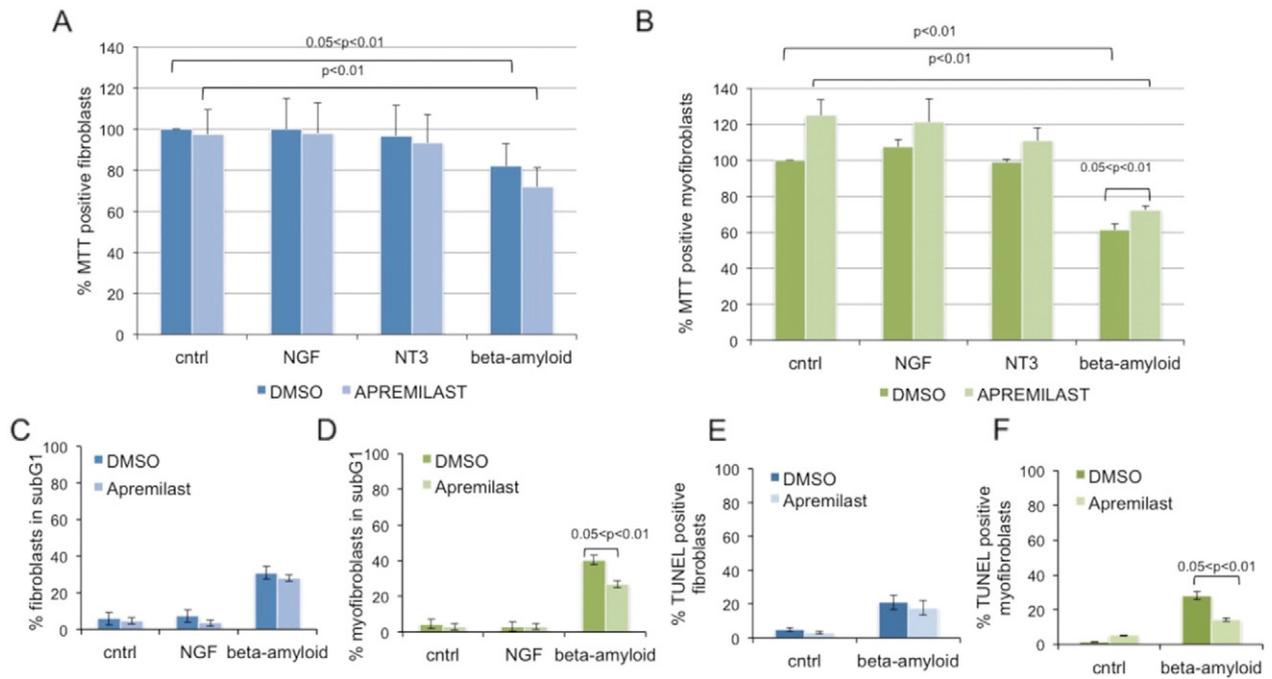
cAMP ELISA was performed to evaluate whether activation of the CD271/PDE4 complex leads to cAMP degradation. In this analysis, neither apremilast alone nor apremilast in combination with NGF exerted any significant effect on release of cAMP by either DF or DM (Fig. 7A). On the other hand, in DM, Apremilast reduced cAMP degradation induced by treatment with  $\beta$ -amyloid (Fig. 7B).

#### 4. Discussion

In this series of experiments characterizing the role of PDE4 isoforms in psoriasis, gene expression studies indicate that PDE4B and PDE4D mRNA are overexpressed in PBMC from donor patients with psoriasis relative to normal controls (healthy volunteers) or patients with other inflammatory and immune-mediated diseases. Polymerase chain reaction experiments confirmed that, on average, the overexpression of the PDE4B gene is 5.4-fold, and the overexpression of the PDE4D gene is 2.2-fold, compared with normal PBMC. Regarding the effect of the PDE4 inhibitor apremilast on cytokine/chemokine production of stimulated PBMCs from patients with psoriasis, apremilast has the greatest effect on responses to TLR4 stimulation, with a similar pattern observed in B-cell receptor-stimulated PBMC. Apremilast consistently decreases



**Fig. 4.** PDE4/CD271 expression in fibroblasts and myofibroblasts in vitro. (A)  $\alpha$ -SMA expression in DF and DM obtained after treatment with diluent (control) or TGF- $\beta_1$  (1 ng/mL) for 6 days by immunofluorescence in situ. Cell nuclei were counterstained with DAPI (blue); bar = 20  $\mu$ m. (B) After diluent (control) or TGF- $\beta_1$  treatment, levels of  $\alpha$ -SMA were determined by Western blot analysis. Vinculin was used as a loading control. (C) Levels of PDE4A, PDE4B, PDE4C, and PDE4D mRNA in DF and DM were determined by RT-PCR analysis. Bar graph shows the average densitometry values normalized to  $\beta$ -actin. (D) PDE4 isoform expression in DF and DM was determined by Western blot analysis.  $\beta$ -Actin was used as a loading control. Bar graphs show the average densitometry values normalized to  $\beta$ -actin (fold-expression). (E) DF and DM protein extracts were immunoprecipitated with CD271 antibody and immunoblotted with PDE4A, PDE4B, PDE4C, and PDE4D antibodies and with PAS-only as control.

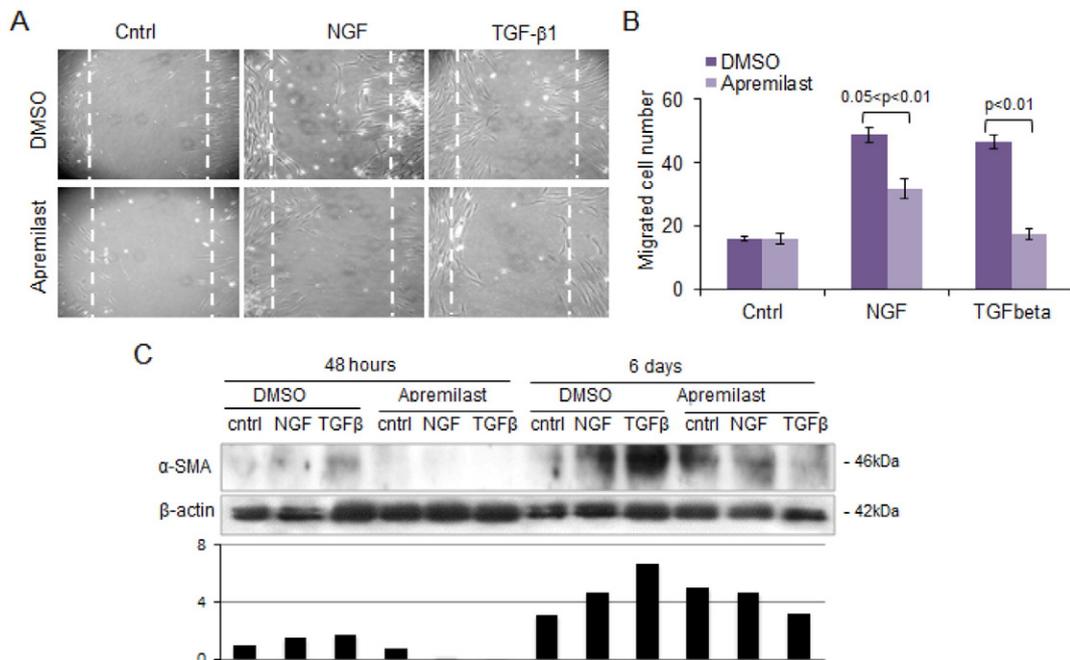


**Fig. 5.** Apremilast modulation of apoptosis in DF or DM. DF or DM cultures were pretreated with apremilast (10  $\mu$ M) or DMSO for 2 h, then NGF (100 ng/mL), NT3 (100 ng/mL),  $\beta$ -amyloid (40  $\mu$ M), or diluent (control) were added. MTT assay was performed 48 h after stimulation of DF (A) or DM (B). Results are represented as percentages with respect to diluent. At 48 h after the addition of stimuli, PI staining was performed and subG1 peaks of DF (C) or DM (D) were analyzed by FACS analysis. TUNEL assay was performed and apoptotic nuclei of DF (E) and DM (F) were counted. Data represent the means  $\pm$  SE of triplicate determinations.

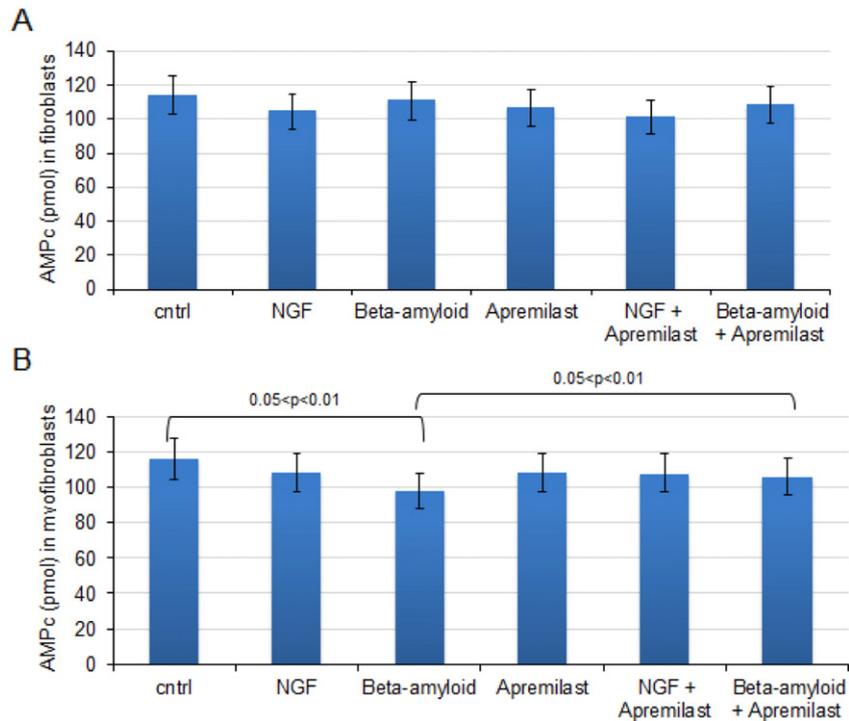
production of the cytokines TNF- $\alpha$ , IL-1 $\alpha$ , and IFN- $\gamma$ , and the chemokines MCP-1, MCP-3, MIP-1 $\beta$ , and IP-10. In psoriatic PBMC cultures stimulated via the T-cell receptor or TNF and IL-1 receptors, apremilast has far fewer effects. On the other hand, apremilast induces an up-regulation of IL-10 and G-CSF production under all conditions.

Because of the higher PDE4 gene expression observed in psoriasis patient blood, an analysis of the skin was conducted. PDE4 protein

expression is higher in the skin of patients with psoriasis, AD, and DLE as compared with normal skin. In a detailed IHC analysis of PDE4 protein expression in psoriatic skin, the endothelium and fibroblasts, in particular, show increased expression of PDE4A and PDE4D as compared with normal skin. Little had previously been known about the expression of PDE4 isoforms in human dermal fibroblasts. Selige and colleagues showed that primary normal human lung fibroblasts (NHLF) express



**Fig. 6.** Effect of apremilast on DF migration and differentiation. DF cultures were pretreated with apremilast (10  $\mu$ M) or DMSO for 2 h. After that, scratching assay was performed and diluent, NGF, or TGF- $\beta$ <sub>1</sub> were added to medium as described in Materials and Methods. Cell pictures were taken 48 h later (A) and migrating cells were counted (B) Data represent the means  $\pm$  SE of triplicate determinations. (C) DF cultures were pretreated with apremilast or DMSO for 2 h, after which diluent, NGF, or beta-amyloid were added to medium. Western blot analysis was performed for  $\alpha$ -SMA and  $\beta$ -actin was used as internal control. Bars show the densitometric analysis of  $\alpha$ -SMA normalized to  $\beta$ -actin. The experiment was repeated three times with similar results.



**Fig. 7.** cAMP degradation by PDE4/CD271 complex activation in DF and DM. DF and DM were pretreated with apremilast (10  $\mu$ M) or DMSO for 2 h, then NGF or  $\beta$ -amyloid were added to medium as described in Materials and Methods. Supernatants of DF (A) or DM (B) were collected 48 h later and cAMP levels were evaluated by ELISA analysis. Data represent the means  $\pm$  SE of triplicate determinations.

PDE4A, B, and D, while PDE4C is only barely detected [16]. In the present study, we found that all PDE4 isoforms are expressed in both DF and DM, even if at different levels. Regarding dermal compartment, all PDE4 isoforms are expressed at the mRNA level both in DF and DM. DM express higher levels of PDE4 isoform proteins than DF. DM invade and repair injured tissues by secreting and organizing the extracellular matrix and by developing contractile forces. At the end of the normal repair process, DM disappear by apoptosis. In pathologic conditions, DM likely remain, leading to excessive scarring [24]. Indeed, DM play an important role in organogenesis, oncogenesis, inflammation, repair, and fibrosis in most organs and tissues [25]. We found that all four PDE4 proteins are up-regulated in cells of the dermal compartment in biopsies from inflammatory conditions. We postulate that these cells could be DM, and suggest that PDE4 proteins play a role in the above-mentioned DM functions, although more functional studies are definitely needed.

Because PDE4 isoforms are expressed in dermal fibroblasts and evidence has indicated an interaction between PDE4 and the CD271, which may be important in tissue remodeling and wound healing [22], we assessed the effect of PDE4 inhibition on the function of the PDE4/CD271 complex in DF and DM. We first showed all PDE4 isoforms co-immunoprecipitate with CD271 in DM. Inhibition of PDE4 with apremilast reduces cAMP degradation mediated by  $\beta$ -amyloid in DM, as well as  $\beta$ -amyloid-mediated induction of apoptosis and reduction of proliferative cells. Addition of apremilast also reduces DF migration and differentiation mediated by NGF and TGF- $\beta$ <sub>1</sub>. Taken together, these results indicate that PDE4 play an important role in psoriasis. In addition, the study reveals that the PDE4/CD271 complex could be important in modulating fibroblast functions.

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