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Phosphodiesterase 4 in inflammatory diseases: Effects of apremilast in psoriatic blood and in dermal myofibroblasts through the PDE4/CD271 complex / Schafer, Peter H; Truzzi, Francesca; Parton, Anastasia; Wu, Lei; Kosek, Jolanta; Zhang, Ling Hua; Horan, Gerald; Saltari, Annalisa; Quadri, Marika; Lotti, Roberta; Marconi, Alessandra; Pincelli, Carlo. - In: CELLULAR SIGNALLING. - ISSN 0898-6568. - 28:(2016), pp. 753-763. [10.1016/j.cellsig.2016.01.007]

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23/04/2024 17:52

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

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ABSTRACT

Phosphodiesterases 4 (PDE4) act as proinflammatory enzymes via degradation of cAMP, while 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 to 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 to 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 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 β-amyloid, a CD271 ligand, in DM. Furthermore, apremilast significantly reduces NGF- and TGFβ_1-induced fibroblasts migration, and inhibits DF differentiation into DM mediated by NGF or TGFβ_1. Finally, in DM, apremilast significantly reduces cAMP degradation induced by treatment with β-amyloid. Taken together, these results indicate that PDE4 play an important role in psoriasis. In addition, the study reveals that the complex PDE4/CD271 could be important in modulating fibroblasts functions.

Keywords: PDE4, PBMC, apremilast, psoriasis, myofibroblasts, CD271

ABBREVIATIONS

AD = atopic dermatitis

ANOVA = analysis of variance

BSA = body surface area

cAMP = cyclic adenosine monophosphate

DF = dermal fibroblasts

DLE = discoid lupus erythematosus

DM = dermal myofibroblasts

DMSO = dimethyl sulfoxide

ELISA = enzyme-linked immunosorbent assay

IgM = immunoglobulin M

IHC = immunohistochemistry

IPF = idiopathic pulmonary fibrosis

LPS = lipopolysaccharide

MCP = monocyte chemotactic protein

MIP = macrophage inflammatory protein

NGF = nerve growth factor

NR = not reported

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

SEM = standard error of the mean

SLE = systemic lupus erythematosus

SMA = smooth muscle actin

sPGA = static Physician’s Global Assessment

TGF-β = transforming growth factor-beta

TLR4 = toll-like receptor 4

TNF-α = tumor necrosis factor alpha

VEGF = vascular endothelial growth factor

1. INTRODUCTION

cAMP is a pivotal second messenger that regulates inflammatory responses (Taskén K. Physiol Rev. 2004 Jan;84(1):137-67. Review. PubMed PMID: 14715913). cAMP is degraded through the activity of the large superfamily of phosphodiesterases (PDE) (Conti M, Annu Rev Biochem. 2007;76:481-511. Review. PubMed PMID: 17376027). 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 (Houslay MD. Drug Discov Today. 2005 Nov 15;10(22):1503-19. Review. PubMed PMID: 16257373). Encoded by the PDE4A, PDE4B, PDE4C, and PDE4D genes, there are also more than 20 splice variants due to differential mRNA splicing genes (Manganiello VC, Murata T, Taira M, Belfrage P & Degerman E. Perspectives in biochemistry and biophysics: Diversity in cyclic nucleotide phosphodiesterase isozyme families. Arch Biochem Biophys 1995; 322: 1–13 PubMed PMID: 7574662). PDE4 isoforms display a differential expression pattern in human testis, skeletal muscle, lung and brain (Obernolte R, Ratzliff J, Baecker PA, Daniels DV, Zuppan P, Jarnagin K, Shelton ER. Multiple splice variants of phosphodiesterase PDE4C cloned from human lung and testis. Biochim Biophys Acta. 1997 Sep 12;1353(3):287-97. PMID: 9349724; Enoksson S, Degerman E, Hagström-Toft E, Large V, Arner P. Various phosphodiesterase subtypes mediate the in vivo antilipolytic effect of insulin on adipose tissue and skeletal muscle in man. Diabetologia. 1998 May;41(5):560-8. PMID: 9628274; Pérez-Torres S, Miró X, Palacios JM, Cortés R, Puigdoménech P, Mengod G. Phosphodiesterase type 4 isozymes expression in human brain examined by in situ hybridization histochemistry and[3H]rolipram binding autoradiography. Comparison with

monkey and rat brain. J Chem Neuroanat. 2000 Dec;20(3-4):349-74. PMID: 11207431). 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 (Videla S,. J Pharmacol Exp Ther. 2006 Feb;316(2):940-5. Epub 2005 Oct 27. PubMed PMID: 16254133; Keshavarzian A, Expert Opin Investig Drugs. 2007 Sep;16(9):1489-506. Review.PubMed PMID: 17714033; Press NJ Prog Med Chem. 2009;47:37-74. Review. PubMed PMID:19328289). Apremilast is a novel, orally administered, small molecule phosphodiesterase 4 (PDE4) inhibitor approved for treatment of psoriatic arthritis (PsA) and moderate to severe plaque psoriasis (Abdulrahim H, Thistleton S, Adebajo AO, Shaw T, Edwards C, Wells A. Apremilast: a PDE4 inhibitor for the treatment of psoriatic arthritis. Expert Opin Pharmacother. 2015 May;16(7):1099-108. doi: 10.1517/14656566.2015.1034107. Epub 2015 Apr 11. Review. PMID: 25864487; Paul C, Cather J, Gooderham M, Poulin Y, Mrowietz U, Ferrandiz C, Crowley J, Hu C, Stevens RM, Shah K, Day RM, Girolomoni G, Gottlieb AB. Efficacy and safety of apremilast, an oral phosphodiesterase 4 inhibitor, in patients with moderate to severe plaque psoriasis over 52 weeks: a phase III, randomized, controlled trial (ESTEEM 2). Br J Dermatol. 2015 Sep 10. doi: 10.1111/bjd.14164. [Epub ahead of print] PMID: 26357944). Apremilast, by inhibiting PDE4, elevates intracellular cAMP levels and thus down-regulates the inflammatory response by modulating expression of a network of pro-inflammatory and anti-inflammatory mediators implicated

in psoriasis and PsA (Gooderham M, Papp K. Apremilast in the Treatment of Psoriasis and Psoriatic Arthritis. Skin Therapy Lett. 2015 Sep;20(5):1-6. PMID: 26382906). 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 (Selige J, J Cell Physiol. 2010 May;223(2):317-26. PubMed PMID: 20082309). Schafer et al showed that apremilast, a PDE4 inhibitor, does not inhibit proliferation in normal primary human epidermal keratinocytes. On the other hand, apremilast inhibited TNF-α production by keratinocytes upon UVB treatment (50 mJ/cm^2) (Schafer PH, Br J Pharmacol. 2010 Feb;159(4):842-55. PubMed PMID: 20050849). 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 (Sachs BD. J Cell Biol. 2007 Jun 18;177(6):1119-32. PubMed PMID: 17576803). CD271 is a neurotrophin (NT) receptor, also known as $p75^{NTR}$, which belongs to the TNF receptors 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 their survival signal. By contrast, CD271 alone can induce apoptosis upon specific binding, such as with β-amyloid, via its own transduction pathway and by interacting with a mounting number of downstream molecules (Dechant G. Nat Neurosci. 2002Nov;5(11):1131-6. Review. PubMed PMID: 12404007). NT and their receptors have autocrine and paracrine functions within the skin (Botchkarev VA.J Invest Dermatol. 2006 Aug;126(8):1719-27. Review. PubMed PMID: 16845411).

Moreover, our group has recently demonstrated that CD271 is expressed by basal keratinocytes, specially by transit amplifying (TA) cells (Truzzi et al, 2011, 18:948-958 Cell Death and Diff. PMID: 21151024). It should be noted that the crosstalk between the epidermal and dermal compartment 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 Trk receptors and that NTs exert a critical role in tissue remodelling and wound healing (Palazzo E et al 2012. PubMed PMID 21503896)

In the current set of experiments, we report gene expression patterns of PDE4 isoforms (A,B,C,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 psoriasis, atopic dermatitis and discoid lupus patients. We then examine PDE4 isoform binding to CD271 in fibroblasts cultured from healthy skin, and analyze the function of the CD271-PDE4 complex in fibroblasts.

2. MATERIALS AND METHODS

2.1. Gene Expression Analysis of PDE4 isoforms in PBMC

Viably frozen PBMC and autologous serum from healthy volunteers or patients with psoriasis, RA, IPF, sarcoidosis, scleroderma, Crohn's disease, or SLE (n=5 from

each disease) were purchased from Coversant Healthcare Systems (Huntsville, Alabama). 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. PBMC were thawed and cultured in RPMI-1640 supplemented with 5% of each patient's autologous serum for 24 hours to allow cells to recover from freezing and thawing. Cells ($1x10^6$ PBMC per patient) were collected for gene expression analysis by hybridization array. Cells were washed with cold phosphate-buffered saline, lysed with 350 µL RLT buffer (Qiagen, Gaithersburg, Maryland), and transferred to barcoded tubes. RNA was extracted and converted to cDNA using the Ovation Whole Blood Solution (NuGEN Technologies, San Carlos, California), and hybridized to HG-U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, California). Gene expression data were normalized and provided by Covance Genomics Laboratory (Seattle, Washington). Statistical comparison for PBMC from each disease versus normal control was by one-way ANOVA.

2.2. qRT-PCR

Quantitative RT-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 hours in 5% autologous serum. RNA was isolated, converted to cDNA and analyzed by qRT-PCR, and a comparison of PBMC from psoriasis patients versus normal controls was conducted by t-test.

2.3. Multiplexed Bead-Based Immunoassay

PBMCs from psoriasis patients (n=10) were obtained after informed consent by Conversant Healthcare Systems (Huntsville, Alabama). 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. PBMCs were thawed (minimum viability was 80%), plated at $2x10^5$ cells/well, and stimulated in duplicate via various immune receptors with or without apremilast (1 μM) in 0.1% dimethyl sulfoxide for 24 hours. Immune cell stimuli included 1 ng/mL LPS (Sigma), targeting TLR4 on monocytes, dendritic cells, and B cells; 10 ng/mL each of staphylococcal enterotoxin A and B (Toxin Tech), targeting T-cell receptors; 2 ng/mL each of TNF-α + IL-1β (Sigma), targeting TNF and IL-1 receptors on monocytes, dendritic cells, B cells, T cells, and natural killer cells; and 2 μg/mL anti-IgM (Jackson IR), 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 (IHC)

Immunohistochemistry was performed on dermis samples from healthy controls and from patients with psoriasis, atopic dermatitis (AD), and discoid lupus erythematosus (DLE) at University of Modena and Reggio Emilia, Modena, Italy. Paraffin embedded biopsies (4μm) were stained for protein analysis by IHC as follows: PDE4A, rabbit polyclonal antibody (1:100, Proteintech Group, Chicago, IL, USA); PDE4B, rabbit polyclonal antibody (1:40, Santa Cruz Biotechnology, INC, Santa

Cruz, CA); PDE4C, rabbit polyclonal (1:40, Santa Cruz Biotechnology); PDE4D, rabbit polyclonal antibody (1:40, Santa Cruz Biotechnology); CD271, mouse monoclonal antibody (1:100, Upstate, Lake Placid, NY, USA). After washes, slides were incubated with secondary antibody for 45 minutes at room temperature. Fast Red was used as cromogen, stainings were performed according to the UltraVision LP Detection System AP Polymer & Fast Red Chromogen assay (Thermo Fisher Scientific, Waltham, MA, USA). The expression intensity was quantitatively determined using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA). 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) 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 Room. Patient consent for experiments was not required because Italian laws 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- β_1 (TGF-β_1) (1 ng/mL, Sigma, St. Louis, Missouri) was added in fibroblast secondary culture for six days to promote differentiation into myofibroblasts (DM). The myofibroblast phenotype was checked by immunostaining in situ of α-smooth muscle actin (α-SMA).

For cell treatment, Apremilast was diluited in DMSO 10mM and used at the final concentration of 10μM in culture medium without serum. DF and MF were plated in

DMEM with FBS. 48h after seeding, Apremilast or DMSO were added to DMEM for 2 hours and, after that, diluent, 100 ng/ml human recombinant NGF (Sigma–Aldrich), 1ng/ml TGF-β_1 (Sigma-Aldrich), 40μM β-amyloid 25-35 (Bachem, Bubendorf, Switzerland) were added to medium. Cells were monitored and used for further experiments 48 hours later.

2.6. Immunofluorescence (IF) in situ

DF were plated on chamber slides and, 48 hours after seeding, were treated with TGF-β_1 (1 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) or diluent alone. Six days later, cells were fixed in situ in 4% paraformaldehyde for 20 min and air dried. After a rehydration in PBS, cells were permeabilized for 10 min with 0.5% Tryton X-100 in 0.1% sodium citrate, treated for 5 min with 50 mM NH4Cl and incubated with 1% bovine albumin serum for 20 min. Then slides were incubated for 1 hour at room temperature with the mouse monoclonal anti-α-SMA antibody (1:400, Sigma-Aldrich). Then cells were incubated with secondary antibody, Alexa Fluor 488 (1:100, Thermo Fisher Waltham, MA, USA). Finally, cell nuclei were stained with 1μg/ml Dapi (Sigma-Aldrich). Micrographs were taken on a Confocal Scanning Laser Microscopy (Leica TCS SP2) (Leica, Exton, PA, USA).

2.7. Co-Immunoprecipitation

DF and DM were cultured until pre-confluent condition. Cells were harvested with lysis buffer pH 7.5 (150 nM NaCl, 15 mM MgCl, 1 mM EGTA, 50 mM Hepes, 10% glycerol, 1% Triton) containing protease inhibitors. Monoclonal antibody CD271

(Upstate, Lake Placid, New York), was bound to sepharose beads for 1 h at 4 ºC under rotation. Thereafter, pre-cleared lysates were conjugated to sepharose beads-antibody complex or, as control, to sepharose beads alone or to antibody alone, overnight at 4 ºC under rotation. Immunocomplexes were washed with binding buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2 mM $MgCl_2$, 1 mM DTT, 0.5% Triton X-100, 0.5% bovine serum albumin) six times. Samples were eluted with 2x Laemnli sample buffer for 5 minutes at 90 ºC and Western blotting for PDE4A, B, C and D was performed as described below.

2.8. Western Blotting

Total proteins (20 µg) or immunocomplexes were analyzed under non-reducing conditions on 7.5% polyacrylamide gels. To obtaine total proteins, cells were washed with phosphate-buffered saline (PBS) and lysed on ice, in lysis buffer pH 7.5 (150 nM 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 hours 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), 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 α-SMA (1:3000, Sigma-Aldrich) or mouse monoclonal anti-β-actin (1:1000; Sigma) overnight at 4 ºC. Then membranes were washed in TBS/Tween 20, incubated with peroxidase-conjugated goat anti-mouse or

goat anti-rabbit or anti-mouse (1:3000, Biorad, 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, England). The band intensity was quantitatively determined using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA), and protein levels' intensity was normalized to β-actin expression.

2.9 MTT Assay

DF and DM were plated in a 96-well tissue culture plate (5000 cells/well). 48 hours after plating, cells were treated as described above with apremilast or DMSO for 2 hours, then treated with diluent (control), NGF (100 ng/mL), NT3 (100 ng/mL), or β-amyloid (40 μM). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed 48 hours later. Proliferative cells were detected by incubating with MTT (Sigma-Aldrich) solution at 37 ºC for 4 hours. The formazan dye produced after DMSO solubilization was evaluated by a multiwall scanning spectrophotometer at 540 nm. The results are expressed as viability percentage, as compared to control. Results are calculated as the mean ± SD of three different experiments. Student's t-test was performed for comparison of the means.

2.10. Propidium Iodide (PI) staining

At 48 hours after cell seeding, DF and DM were pretreated with DMSO or apremilast as described above, then diluent (control) or 40 μM β-amyloid were added. After 48 hours, cells were trypsinized and resuspended ($5x10^5$ cells) in 1 mL hypotonic fluorochrome

solution: 50 mg/mL PI containing 0.1% sodium citrate and 0.5% TrytonX-100 (Sigma). After 15 minutes, cells were analyzed using an Epics XL flow cytometer (Coulter Electornics Inc., Hialeah, FL). Apoptosis was detected by evaluating the fluorescence of hypodiploid cells counted in subG1 region.

2.11. TUNEL Assay

DF 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 are calculated as the mean ± SD of three different experiments.

2.12. Migration Assay

A total of $20x10^4$ DF were plated on 12-well tissue culture plates. 48 hours after seeding, cells were pretreated with DMSO or apremilast for 2 hours as described above, then cells were treated with 5 mg/mL mitomycin C for 1 hr 30 min. Subsequently, the cells were washed three times in serum-free medium and a line for each well was drawn along the cell monolayer with a sterile plastic tip. Plates were washed twice with serum-free medium to remove all detached cells and incubated in medium with BSA 0.1% with 1 ng/mL TGF-β_1, 100 ng/mL human recombinant NGF in presence of DMSO or apremilast. Cells were monitored at 24 and 48 hours 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 ± SD of three different experiments.

2.13. cAMP ELISA

At 48 hours 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 μM β-amyloid (Bachem) were added to the culture medium. 48 hours later, supernatants were collected, particulates were removed by centrifugation and stored at -20 ºC. 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 ± 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 systemic lupus erythematosus (SLE). PDE4B and PDE4D isoforms were increased at the mRNA level in PBMC from psoriasis patients relative to normal controls (**Figure B and D**, $p<0.05$).

PDE4D was also increased versus normal controls in PBMC from patients with SLE (**Figure 1D**, $p<0.05$). PDE4C mRNA was preferentially overexpressed in PBMC from patients with Crohn's disease versus normal controls (**Figure 1C**), while PDE4A mRNA expression levels were similar across PBMC from normal controls and patients with the various immune/inflammatory diseases (**Figure 1A**). 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 vs normal control), compared with 1.1-fold for PDE4A (p=NS) (**Figure 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 females and 5 males ranging in age from 26 to 42 years, with PASI scores ≤26 and ≤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-α/IL-1β, or anti-IgM are shown in **Figure 2A**.

Addition of 1 μM apremilast had the greatest effect on psoriatic PBMC cultures stimulated via TLR4 (ie, LPS stimulation), with 13 of the 25 analytes significantly affected versus LPS control (**Figure 2B**). Specifically, apremilast caused significant inhibition of LPS-induced TNF-α, IL-12/IL-23p40, IFN-γ, IP-10, MCP-1, MCP-3, MIP-1β, IL-1α, IL-1β, 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-α, IL-1α, and IFN-γ, and the chemokines MCP-1, MCP-3, MIP-1β, 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, that are markedly elevated in PBMC from psoriatic patients, in the dermal infiltrate from lesional psoriatic skin. Also dermis from healthy subjects, atopic dermatitis (AD), and discoid lupus erythematosus (DLE) was evaluated. In general, PDE4 staining was higher in diseased vs. normal skin when measured by ImageJ software (**Figure 3B**). 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 discoid lupus erythematous (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 as compared to controls. In this context, dermal fibroblasts appeared intensely positive. In dermis from DLE skin, PDE4B staining was markedly increased, as compared to healthy controls, dermal fibroblasts 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 (**Figure 3A**).

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 vs. 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 showed frequent 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 to normal skin, with PDE4A and PDE4D displaying strong staining in psoriatic endothelium, but only faint 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 vs. normal skin.

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-β_1, promote fibroblast differentiation into myofibroblasts, characterized by the expression of a-Smooth Muscle Actin (α-SMA). These cells are predominantly responsible for connective-tissue contracture and ECM reorganization in different physiopathologic processes in the skin, including wound healing and fibrosis (Verjee et al 2013/ PMID:23431165). We showed that all PDE4 isoforms are expressed in dermal fibroblasts (DF) and myofibroblasts (DM) cultured from healthy donors at the mRNA level (**Figure 4A, B, C**), while PDE4 protein levels are higher in DM than in DF (**Figure 4D**). PDE4A had previously been found to associate with the CD271 to enhance cAMP degradation in fibroblasts, and to regulate fibrotic responses [Sachs BD, Baillie GS, McCall JR, et al. J Cell Biol. 2007 Jun

18;177(6):1119-32. PubMed PMID: 17576803]. Because CD271 is also expressed in skin fibroblasts (Palazzo et al, 2012, J Cell Physiol), 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 (**Figure 4E**). However, in DF, CD271 formed only weak complexes with PDE4A and PDE4B and no complex 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 hours and then treated with NGF, NT3, β-amyloid, or diluent. Treatment with β-amyloid significantly decreased the percentage of proliferative cells for both DF and DM cultures compared with diluent control. In DM, the addition of apremilast significantly inhibited the effect of β-amyloid (p<0.05 vs DMSO), while apremilast had no effect versus DMSO in DF (**Figure 5A, B**). Similarly, the addition of apremilast significantly reduced the induction of apoptosis by β-amyloid treatment in DM (p<0.05 vs DMSO, **Figures 5D**, **F**) but not in DF (**Figures 5C E**).

It has been shown previously that neurotrophins mediate DF migration, and that neurotrophins as well as TGF-β_1 induce DF to differentiate into DM by expressing α-SMA.{Palazzo, 2012 3572 /id} We therefore analyzed whether the addition of apremilast could affect DF migration and α-SMA expression. Apremilast inhibited DF migration induced by both NGF and TGF-β_1 treatment (**Figure 6A, B**). Similarly, apremilast

inhibited the up-regulation of α-SMA expression induced by NGF and TGF-β_1, especially 6 days after treatment (**Figure 6C**).

cAMP ELISA was performed to evaluate if 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 (**Figure 7A**). On the other hand, in DM, Apremilast reduced cAMP degradation induced by treatment with β-amyloid (**Figure 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. PCR 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 receptor stimulation, with a similar pattern observed in B cell receptor-stimulated PBMC. Apremilast consistently decreases production of the cytokines TNF-α, IL-1α, and IFN-γ, and the chemokines MCP-1, MCP-3, MIP-1β, 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, a careful analysis of the skin was conducted. PDE4 protein expression is higher in the skin of patients psoriasis, atopic dermatitis, and discoid lupus, as compared to normal skin. In a detailed IHC analysis of PDE4 protein expression in psoriatic skin, the endothelium and fibroblasts, in particular, shows increased expression of PDE4A and PDE4D, as compared to normal skin. Little had previously been known about the expression of PDE4 isoforms in human dermal fibroblasts. Selige and co-workers showed that primary normal human lung fibroblasts (NHLF) express PDE4A, B, and D, while PDE4C is only barely detected (Selige J, J Cell Physiol.2011, 226:1970-80.). 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 level 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 (Micallef L, Vedrenne N, Billet F, Coulomb B, Darby IA, Desmoulière A. The myofibroblast, multiple origins for major roles in normal and pathological tissue repair. Fibrogenesis Tissue Repair. 2012, 5 Suppl 1:S5.). Indeed, DM play an important role in organogenesis, oncogenesis, inflammation, repair, and fibrosis in most organs and tissues (Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. Am J Physiol. 1999, 277:C1---9.). 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,{Palazzo, 2012 3572 /id}, 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 β-amyloid in DM, as well as β-amyloid-mediated induction of apoptosis and reduction of proliferative cells . Addition of apremilast also reduces DF migration and differentiation mediated by NGF and TGF-β_1. Taken together, these results indicate that PDE4 play an important role in psoriasis. In addition, the study reveals that the complex PDE4/CD271 could be important in modulating fibroblasts functions.

REFERENCES

[Please run RefMan]

Table 1. Detailed Summary of PDE4 IHC Staining Scores in Normal and Psoriatic Skin Skin sections were stained with antibodies specific for PDE4A, B, C, 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.

PDE4 Isozyme	Normal				Psoriatic			
	A	B	C	D	A	B	C	D
Squamous epithelium	4.0	NR	1.0	2.2	4.0	1.5	1.5	2.8
Arrector pili smooth muscle	3.3	2.0	2.0	2.5	NR	2.0	2.0	2.3
Sweat glands/ducts	3.3	2.0	2.0	2.3	4.0	2.0	2.5	2.5
Pilosebaceous units	2.8	2.0	2.0	2.0	4.0	2.0	2.5	NR
Vascular smooth muscle	2.5	2.0	2.0	2.0	4.0	2.0	2.0	NR
Endothelium	2.0	2.0	2.0	2.3	4.0	3.2	3.0	4.0
Inflammatory cells	2.5	3.0	2.0	2.5	4.0	2.5	2.5	2.3
Peripheral nerves	1.3	0.0	1.0	1.0	2.3	0.0	1.5	2.0
Adipocytes	0.5	0.5	0.3	1.3	0.0	0.0	0.3	0.0
Fibroblasts	0.0	1.0	1.0	2.0	3.5	1.0	1.9	3.0

FIGURE LEGENDS

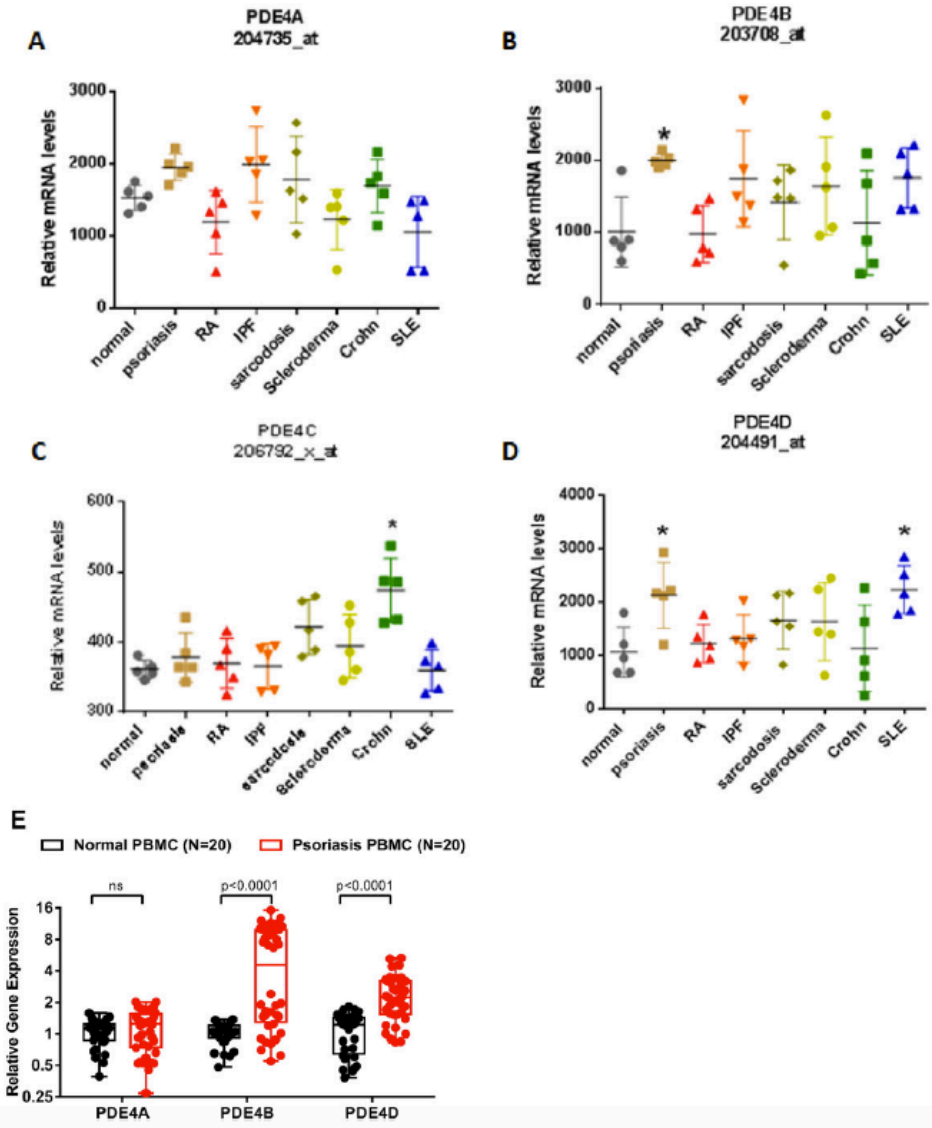


Figure 1. Gene expression analysis of PDE4 isoforms in PBMC from healthy volunteers or patients with psoriasis, RA, IPF, sarcoidosis, scleroderma, Crohn's disease, or SLE. (**A, B, C, D**) PBMC samples (n=5 from each immune/inflammatory condition) were cultured 24 hours in 5% autologous serum. RNA was 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 vs normal controls by one-way

ANOVA. (**E**) PBMC samples from healthy donors and psoriasis patients (N=20 each) were cultured for 24 hours in 5% autologous serum. RNA was isolated, converted to cDNA and analyzed by qRT-PCR. Dots represent individual data points. The box extends from the 25th to the 75th percentile, the whiskers represent the minimum and maximum, and the line in the middle of the box represents the median. (p<0.0001 vs normal controls, by t-test).

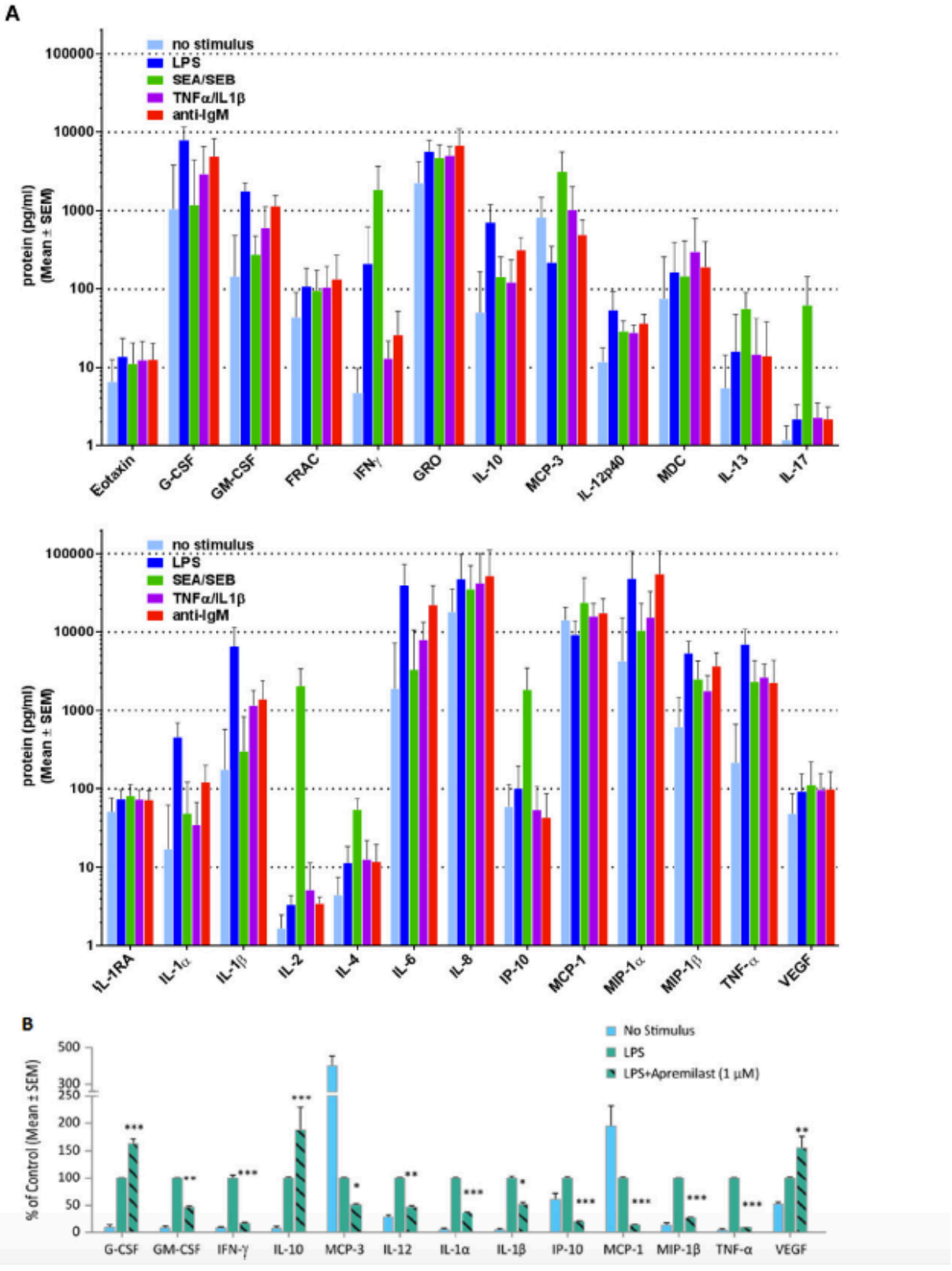
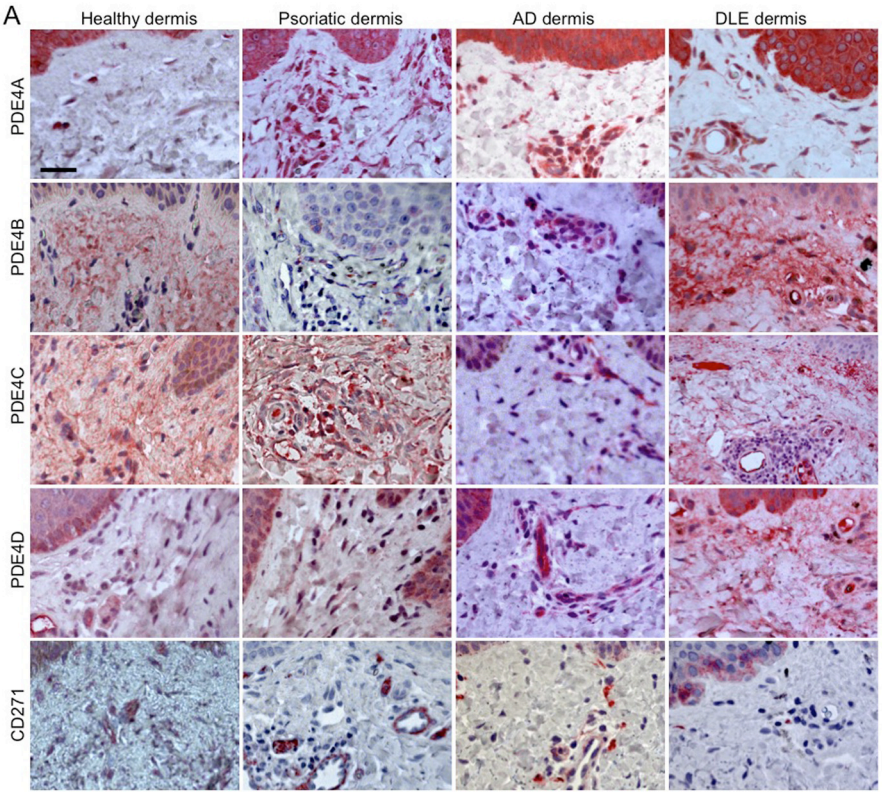

A
no stimulus
LPS
SEA/SEB
TNFα/IL1β
anti-IgM
protein (pg/ml) (Mean ± SEM)
100000
10000
1000
100
10
1
Eotaxin
G-CSF
GM-CSF
FRAC
IFNγ
GRO
IL-10
MCP-3
IL-12p40
MDC
IL-13
IL-17
no stimulus
LPS
SEA/SEB
TNFα/IL1β
anti-IgM
protein (pg/ml) (Mean ± SEM)
IL-1RA
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-8
IP-10
MCP-1
MIP-1α
MIP-1β
TNF-α
VEGF
B
No Stimulus
LPS
LPS+Apremilast (1 μM)
% of Control (Mean ± SEM)
500
300
250
200
150
100
50
0
G-CSF
GM-CSF
IFN-γ
IL-10
MCP-3
IL-12
IL-1α
IL-1β
IP-10
MCP-1
MIP-1β
TNF-α
VEGF

Figure 2. Modulation of production of inflammatory cytokines and chemokines in psoriatic PBMCs. **(A)** Production of 25 inflammatory cytokines and chemokines by psoriatic PBMCs. Data are the absolute mean ± SEM of 10 samples, each tested in duplicate, measured by cytometric bead array using Milliplex MAP Human Cytokine/Chemokine beads (EMD Millipore Corporation, Billerica, MA) on a MagPix instrument (Luminex, Austin, TX). **(B)** Effect of apremilast on LPS-stimulated PBMCs from patients with psoriasis. Data are the mean ± SEM percent relative to LPS control, from 10 psoriatic PBMC samples, each tested in duplicate. *p<0.05, **p<0.01, ***p<0.001 vs LPS control by 2-way ANOVA, followed by Bonferroni post-test to compare replicate means.

A



B

	Healthy Skin	Psoriasis	Atopic Dermatitis	Discoid Lupus
PDE4A	1.00	2.24	1.21	1.20
PDE4B	1.00	1.29	1.36	2.56
PDE4C	1.00	1.93	1.60	2.28
PDE4D	1.00	2.42	2.78	3.74
CD271	1.00	1.44	1.62	1.20

Figure 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, atopic dermatitis (AD), and discoid lupus erythematosus (DLE) were detected by immunohistochemistry. Fast red was used as cromogen. Bar = 200 μm (**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 healthy dermis for each individual antibody.

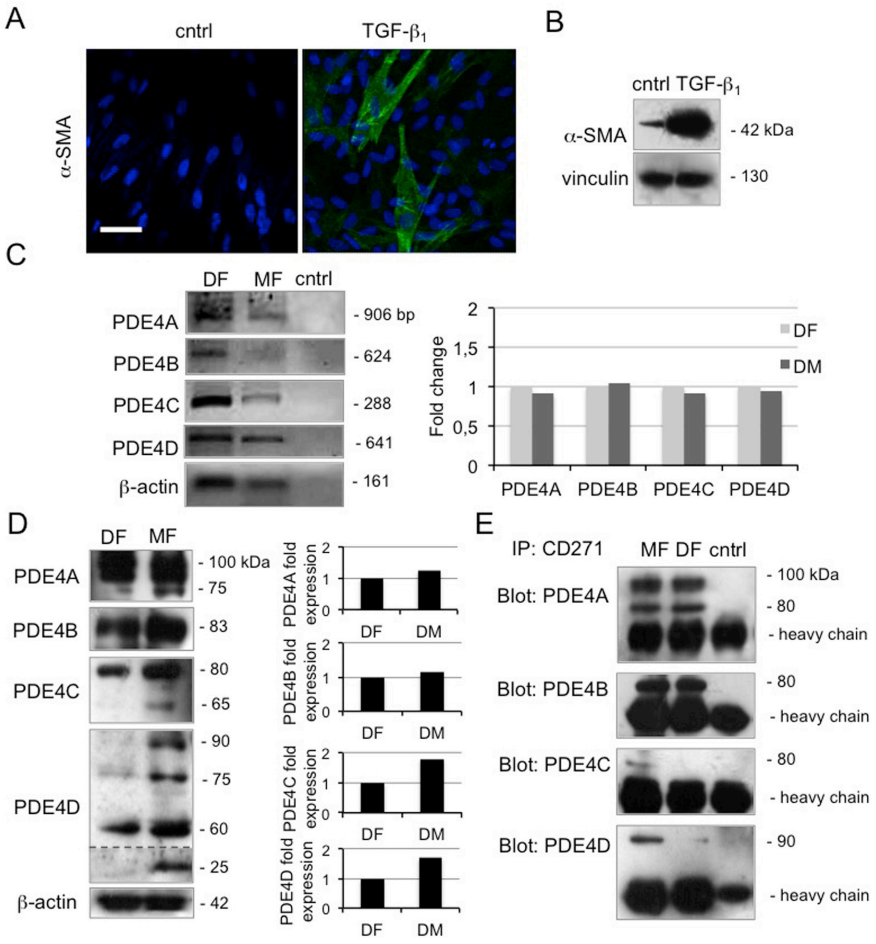


Figure 4. PDE4/CD271 expression in fibroblasts and myofibroblasts *in vitro* (**A**) α-SMA expression in dermal fibroblasts (DF) and myofibroblasts (DM) obtained after treatment with diluent (control) or TGF-β_1 (1 ng/mL) for 6 days by immunofluorescence in situ. Cell nuclei were counterstained with DAPI (blue); bar = 20 μm. (**B**) After diluent (control) or TGF-β_1 treatment, levels of α-SMA were determined by Western blotting 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 graphs show the average densitometry values normalized to β-actin. (**D**) PDE4 isoform expression in

DF and DM was determined by Western blot analysis. β-actin was used as a loading control. Bar graphs show the average densitometry values normalized to β-actin (fold-expression). (**E**) DF and DM protein extracts were immunoprecipitated with CCD271 antibody and immunoblotted with PDE4A, PDE4B, PDE4C, and PDE4D antibodies and with PAS-only as control.

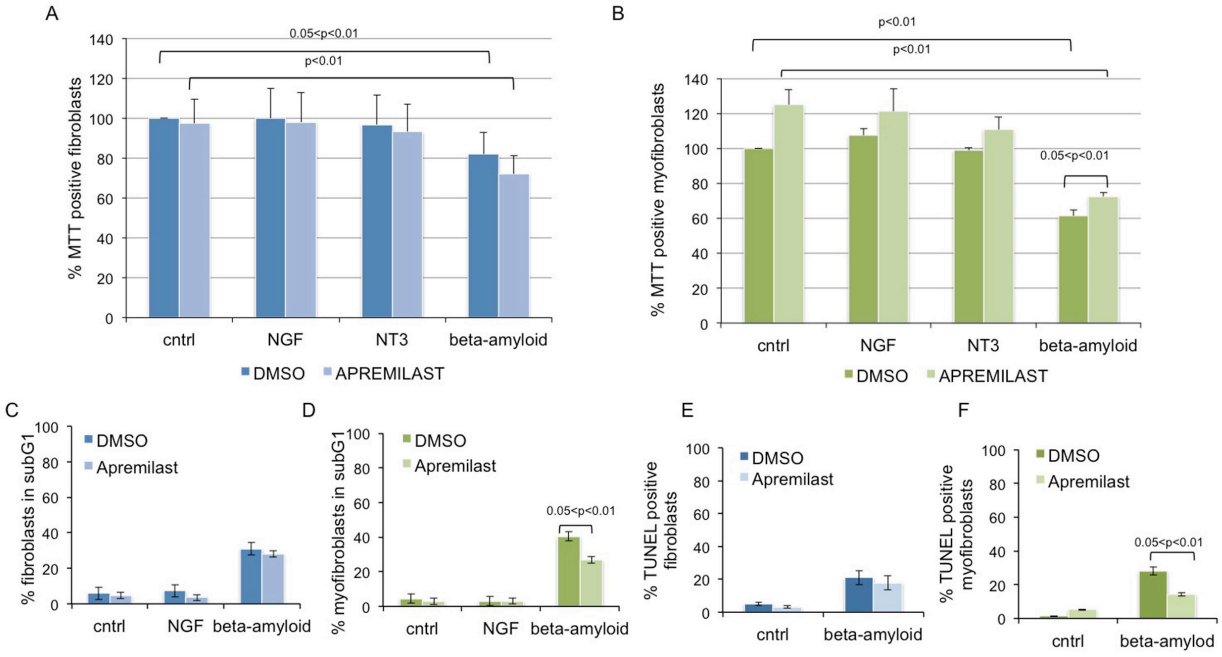


Figure 5. Apremilast modulation of apoptosis in DF or DM. Fibroblast (DF) or myofibroblast (DM) cultures were pretreated with apremilast (10 μM) or DMSO for 2 hours, then NGF (100 ng/mL), NT3 (100 ng/mL), and β-amyloid (40 μM) were added. MTT assay was performed 48 hours after stimulation of DF (**A**) or DM (**B**). Results are represented as percentage with respect to diluent. 48 hours after the addition of stimuli, propidium iodide (PI) staining was performed and subG1 peak of DF (**C**) or DM (**D**) was analyzed for FACS analysis. TUNEL assay was performed and apoptotic nuclei of DF (**E**) and DM (**F**) were counted. Data represent the mean ± SE of triplicate determinations.

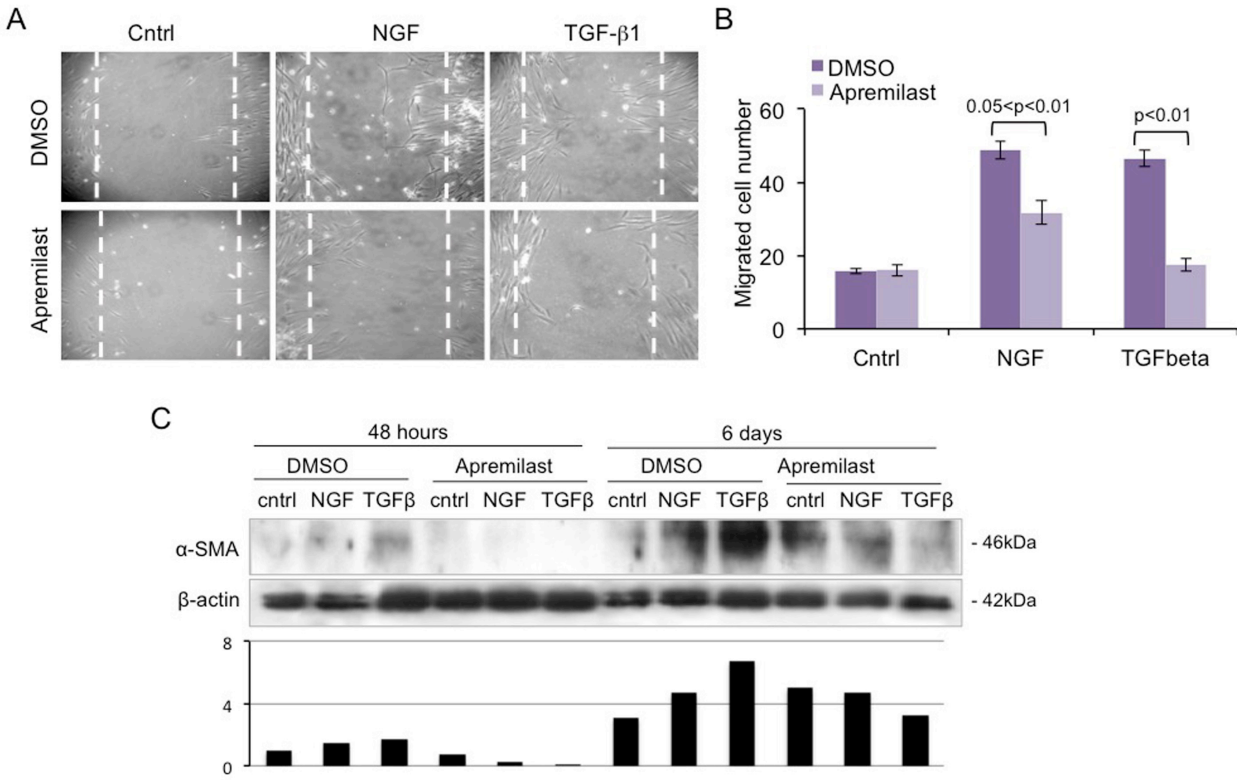


Figure 6. Effect of apremilast on DF migration and differentiation. DF were pretreated with apremilast (10 μM) or DMSO for 2 hours. After that, scratching assay was performed and diluent, NGF or TGF-β_1 were added to medium as described in Materials and Methods. Cell pictures were taken 48 hours later (**A**) and migrating cells were counted (**B**). (**C**) DF were pretreated with apremilast or DMSO for 2 hours, after which diluent, NGF or beta-amyloid were added to medium. Western blotting was performed for α-SMA and β-actin was used as internal control. Bars show the densitometric analysis of α-SMA normalized to β-actin. Data represent the mean ± SE of triplicate determinations.

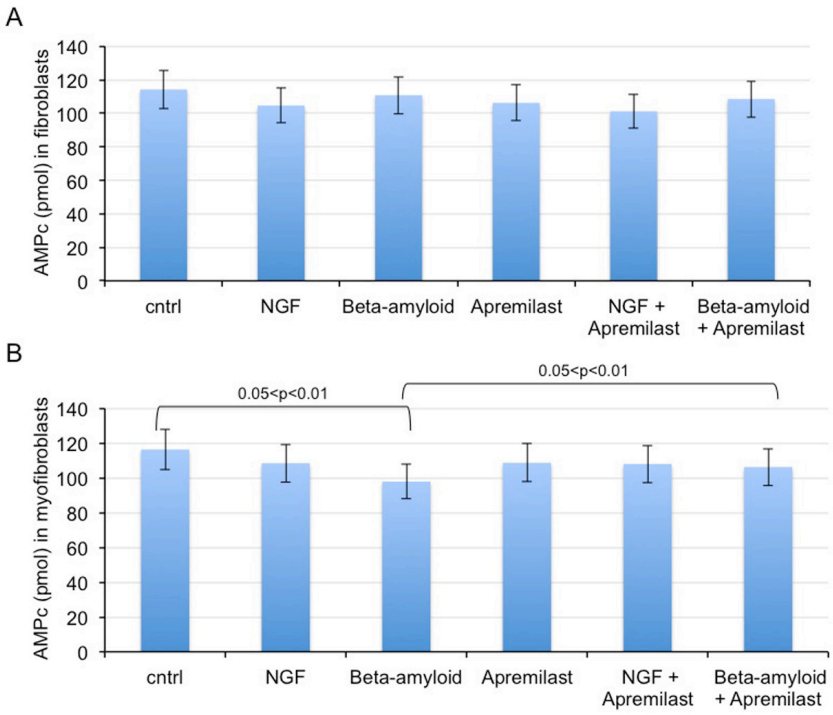


Figure 7. cAMP degradation by PDE4/CD271 complex activation in DF and DM. DF and DM were pretreated with apremilast (10 μM) or DMSO for 2 hours, then NGF or beta-amyloid were added to medium as described in Materials and Methods. Supernatants of DF (**A**) or DM (**B**) were collected 48 hours later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