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Kv1.3 in Psoriatic Disease: PAP-1, a small molecule inhibitor of Kv1.3 is effective in the SCID mouse psoriasis - xenograft model

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Abstract

Kv1.3 channels regulate the activation/proliferation of effector memory T cells and thus play a critical role in the pathogenesis of autoimmune diseases. Using a combination of immunohistochemistry, confocal microscopy, flow cytometry and electrophysiology methods we observed a significant enrichment of activated $Kv1.3^{+}$ memory T cells in psoriasis plaques and synovial fluid from patients with psoriasis/psoriatic arthritis (PsA) compared to non-lesional psoriatic skin, normal skin or peripheral blood lympho-mononuclear cells. In *in vitro* studies performed with lesional mononuclear cells or T cells derived from skin and joints of psoriatic disease, the small molecule Kv1.3 blocker PAP-1 dose-dependently inhibited proliferation and suppressed IL-2 and IFN-γ production. To further substantiate the pathologic role of $Kv1.3^{high}$ T_{EM} cells in psoriatic disease we tested whether PAP-1 is able to improve psoriatic disease pathology in the SCID mouse-psoriasis skin xenograft model. Following four weeks of daily treatment with 2% PAP-1 ointment rete peg length was reduced by roughly 50% and the number of $CD3^{+}$ lymphocytes/mm^2 of dermis decreased by 85%. Vehicle treated and untreated plaques in contrast remained unchanged and showed no reduction in epidermis thickness and infiltrating $CD3^{+}$ T cells and $HLA\text{-}DR^{+}$ T cells. Based on these results we propose the development of Kv1.3 targeted topical immunotherapy for psoriasis and possibly for other inflammatory skin conditions, where effector memory T cells are involved in the pathogenesis.

Keywords - Kv1.3 channel, psoriatic diseases, pathogenesis, PAP-1, effector memory T cells, psoriasis skin xenograft model

1. Introduction

Psoriasis affects around 2% of the world population [1,2]. It is associated with a significant reduction in quality of life and is an enormous economic burden on the health care system for the "biologics" that are currently increasingly used for treatment [3,4]. Unfortunately, long term therapeutic efficacy (Psoriasis Area and Severity Index score reduction of 75%) for "biologics" like anti-TNF agents or the CD2-binding fusion protein Alefacept is only 60% and 20%, respectively and there currently still exists an urgent need for new psoriasis treatments [5-7]. There also is a need for effective treatments for patients with moderate psoriasis that do not qualify for use of biologics [8].

With the voltage-gated potassium channel Kv1.3 we have recently identified a new molecular target that allows for the pharmacological inhibition of $CCR7^-$ effector memory T (T_{EM}) cells and thus for the selective suppression of a T cell subset that makes up 80% of skin resident T cells [9] and plays a crucial role in the pathogenesis of psoriatic disease [10-12]. Expression of Kv1.3 is increased in activated $CD4^+$ and $CD8^+$ T_{EM} cells, which rely on this channel for their calcium signaling [13]. Kv1.3 blockers have accordingly been shown to preferentially suppress the proliferation and cytokine production by T_{EM} cell without impairing the function of naïve and central memory T (T_{CM}) cells [14-17]. Kv1.3 blocking peptides and small molecules further treat several other T_{EM} cell mediate autoimmune disease models such as chronic-relapsing experimental autoimmune encephalomyelitis (EAE), pristane-induced arthritis, spontaneous autoimmune diabetes and glomerulonephritis in rats [16-19], while $Kv1.3^{-/-}$ mice are resistant to EAE induction and are deficient in IFN-γ and IL-17 production [20]. One observation, that makes Kv1.3 particularly attractive as a target for psoriasis, is the finding that PAP-1, a small molecule Kv1.3 blocker (IC_{50} = 2 nM) developed in our laboratory, potently

suppresses allergic contact dermatitis (ACD) in rats when applied topically [21]. We here tested the therapeutic hypothesis that Kv1.3 constitutes a potential target of psoriasis by first demonstrating the presence of $Kv1.3^{+}$ effector memory T cells in psoriatic disease and then evaluating the therapeutic efficacy of PAP-1 in the SCID mouse-psoriasis xenograft model.

2. Material and methods

2.1. Subjects

This study was approved by the Institutional Review Board of the VAMC Sacramento and the University of California, Davis School of Medicine. Specimens were collected from patients with active psoriasis, psoriatic arthritis (PsA) and healthy controls. The enrolled psoriasis patients had plaque psoriasis, involving 5-10% of their body surface. PsA patients had either oligoarthritis or symmetric polyarthritis as described by Moll and Wright [22]. All PsA patients fulfilled the CASPAR classification criteria for PsA [23]. Patients had a complete physical examination, evaluation of severity of psoriasis and arthritis, appropriate blood tests and radiological studies. Synovial fluid (SF) was collected from PsA patients with active arthritis of knees. Active PsA was defined by the presence of at least 3 swollen and 3 tender joints. Patients were evaluated for swollen joint count, tender joint count, patient's assessment of pain, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). None of the patients were taking prednisone. Recruited psoriasis patients were not on biologic agents, PUVA (psoralen + UVA) and DMARDs (Disease-modifying antirheumatic drugs) in the last 6 months. Three active PsA patients were on methotrexate ($\leq$ 10 mg/week). As controls we used historic blood and SF data from osteoarthritis patients [16].

Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were obtained from psoriasis and PsA patients (n = 10). Paired samples of PBMC, skin biopsies and SFMC were obtained. All arthritis patients had active disease and required therapeutic joint aspiration and/or intra-articular injection of steroids. 4 mm size punch biopsies were collected from psoriasis plaques (n = 15) and non-lesional psoriatic skin (n = 15). We also used biopsies of normal skin as a control (n = 15) from our tissue bank. Demographics of the healthy controls and psoriasis patients are provided in the Supplementary Table S1.

2.2. Immunohistochemistry

A. Immunoperoxidase staining

To identify $Kv1.3^+$ lymphocytes in psoriatic lesions we performed immunoperoxidase staining with CD3 and Kv1.3 in serial sections. Psoriasis lesion (n = 15), non-lesional psoriasis skin (n = 15) and healthy skin tissue (n = 15) were studied. Snap frozen-cryosections were prepared and stained with a mouse monoclonal Kv1.3 antibody (1:50 dilution, clone: L23/27, NeuroMab) and a mouse monoclonal CD3 antibody (1:100 dilution, clone: UCHT-1, Sigma). Primary antibodies were detected with biotinylated secondary antibodies, followed by a horseradish peroxidase-conjugated avidin complex and peroxidase activity was visualized with 3,3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin. Cells positive for a specific phenotypic marker were quantified as number of positive cells per mm^2 of skin/synovial tissues as per our earlier reports [12,24].

B. Double labeling immunofluorescence studies to identify $CD3^+Kv1.3^+$ cells

Snap frozen-cryosections of psoriatic tissues (n = 5) were fixed with cold acetone for 7 minutes and then stained with a rat monoclonal anti-CD3 antibody (1:100 dilution, clone: CD3-12, Serotec) and a mouse monoclonal Kv1.3 antibody (1:100 dilution, clone: L23/27, NeuroMab) at

4°C overnight [25]. Bound primary antibodies were detected with anti-rat Alexa Fluor® 647-conjugated and anti-mouse 546-conjugated secondary antibodies (1:500, Molecular Probes). Sections were mounted in Fluoromount-G (SouthernBiotech) with DAPI and imaged with a Zeiss LSM-510 confocal microscope.

2.3. Flow Cytometry

Lymphocyte Isolation: Skin lymphocytes were isolated according to the protocol by Chang et al. [26]. Flow cytometry and isolation of lymphocytes from synovial fluid was performed based on our established protocols [12]. Briefly, lymphocytes from psoriasis lesions (n = 5), synovial fluid (n = 10) and peripheral blood (n = 10) were isolated by Ficoll spin. Cells were enriched for T cells ($CD3^+$) by removing unwanted cells (neutrophils, B cells, monocytes) with the EasySep® method (StemCell Technologies, Auburn, CA). Isolated $CD3^+$ T (1x 10^6 cells/ml) were activated with CD3 or CD3/CD28 (5 μg each/ml, UCHT1 clone for CD3, CD28.2 clone for CD28 antibody, BD Bioscience) coated wells in complete RPMI medium for 72 hours and Hi-D multiparameter FACS studies were performed to identify activated $Kv1.3^+$ peripheral memory T cells ($CD3^+CD11a^+CD45RO^+HLA^-DR^+$ $CCR7^-$ cells). All antibodies for flow cytometry were from BD Biosciences. The Kv1.3 antibody was purchased from Sigma-Aldrich and recognizes the extracellular amino acid residues 211-224 of human Kv1.3.

2.4. Electrophysiology

The average number of Kv1.3 channels per cell was determine by whole-cell patch-clamp as previously described for CSF samples and antigen-specific cells from MS and diabetes patients [16,27]. Briefly, mononuclear cells (MNCs) from psoriatic skin lesions or PsA synovial

fluid were activated with soluble anti-CD3/CD28 antibodies (5 μg each/ml) for 48 hours. In order to identify T cells, cells were stained with PE-conjugated anti-human CD3 (HIB19, BD Biosciences). $CD3^+$ cells were then visualized by fluorescence microscopy and patch-clamped in the whole-cell configuration of the patch-clamp technique with a holding potential of –80 mV using a HEKA EPC-10 amplifier. Kv1.3 currents were elicited by repeated 200-ms pulses from –80 mV to 40 mV applied every 45 or 1 sec.

2.5. Cytokine assays

Enriched $CD3^+$ T cells ($1x10^6$ cells/well) from psoriatic lesions (n = 4) or synovial fluid (PsA, n = 5) were cultured in triplicate in CD3/CD28 antibody (5 μg/ml each) coated 24-well plate with/without PAP-1 (1 μM) for 72 hours. Supernatants were collected and frozen until the ELISA was performed with IL-2 and IFNγ ELISA kits from Raybiotech Inc. (Norcross, GA). In other assays, PsA synovial fluid mononuclear cells were stimulated with PMA (10 nM) and ionomycin (175 nM) for 24 hours.

2.6. Proliferation assays

Mononuclear cells (5×10^5) from PsA synovial fluid or psoriasis skin were incubated with different concentrations of PAP-1 and stimulated with anti-CD3/CD28 antibodies (5 mg each/ml) for 48 hours. [^{3}H]-Thymidine incorporation was measured as previously described [14].

2.7. Psoriasis SCID xenograft model

All animal experiments were approved by the University of California, Davis, Institutional Animal Care and Use Committee and were in accordance with National Institute of Health guidelines. Female CB17 SCID mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Shave biopsies (2.5 X 2.5 cm) were obtained from active plaques located on the thigh or arm of 15 psoriatic patients. As per our earlier reports, each piece of biopsy was divided into four equal parts of approximately 1 sq.cm size so that 60 SCID mice could be transplanted using the shave biopsies obtained from 15 patients [24].

For preparation of the PAP-1 ointment, PAP-1 was first suspended in one part of embryo tested mineral oil and then worked mechanically at 2% into nine parts Eucerin® (Beiersdorf, Wilton, CT).

2.8. Statistical analysis

Analyses of FACS data such as calculation of absolute cell numbers and comparisons of means/medians of each surface phenotype under different experimental conditions were carried out using FlowJo software (TreeStar). Statistical analyses were performed with the JMP statistical software package (SAS Institute) or with Origin software. Histopathological data were analyzed by ANOVA. Flow cytometry data and ELISA data were evaluated by t-test. The differences between study and control groups were tested by Fisher's Exact test. A $P<0.05$ was considered statistically significant.

3. Results

3.1. Psoriatic plaques are enriched with Kv1.3$^+$ T cells

To determine whether Kv1.3$^+$ T cells are enriched in psoriatic plaques we stained psoriasis lesion (n = 15), non-lesional psoriasis skin (n = 15), and healthy skin tissue (n = 15) and counted the number of Kv1.3$^+$ positive cells/mm^2. Patients and healthy controls were age, sex and race ethnicity matched (Supplementary Table S1). We observed that psoriasis plaques were enriched with Kv1.3$^+$ lymphomonuclear cells (Fig. 1A) and that Kv1.3$^+$ cells were generally localized in the same areas as CD3$^+$ T cells in serial sections (Fig. 1C,D). Using double fluorescent immunostaining and confocal microscopy we confirmed co-localization of Kv1.3$^+$ and CD3$^+$ (Fig. 2) demonstrating that Kv1.3$^+$ cells are a subset of lesional T cell infiltrates.

The numbers of Kv1.3$^+$ T cells/mm^2 were significantly increased in psoriatic dermis (92 ±18) compared to the non-lesional psoriatic skin (6 ± 4) (P<0.01, ANOVA). We further stained biopsies harvested from SCID mouse-psoriasis xenografts, a widely used animal model for psoriasis [24, 28], and found that 8-week old grafts (n = 10) exhibited all salient features of psoriatic lesions and contained 72 ± 24 Kv1.3$^+$ cells/ mm^2 demonstrating that Kv1.3$^+$ cells are retained in this model and probably continue to contribute to its pathogenesis (Fig. 1E).

3.2. A high percentage of activated T cells at the disease site in psoriatic disease express Kv1.3 and are of a memory phenotype

To further characterize the phenotype of the Kv1.3$^+$ cells we studied CD3$^+$ cells extracted from synovial fluid (SF) from PsA patients (n = 10) or from psoriatic skin lesions (n = 5) by flow cytometry after activation with CD3/CD28 antibodies. Hi-D multiparameter FACS studies were

performed to identify Kv1.3$^+$ memory T cells (CD3$^+$CD11a$^+$CD45RO$^+$) in SF samples (Fig. 3). The majority of the Kv1.3$^+$ T cells were activated (HLA$^-$DR$^+$) and CCR7$^-$ (Fig. 3). We observed a significant enrichment of activated Kv1.3$^+$ memory T cells (6.3 ± 1.2%) within the SF as compared to the peripheral blood of the PsA patients (n = 10, Fig. 3). Similarly we found that the percentages of activated Kv1.3$^+$ memory T cells derived from psoriatic skin lesions (n = 5) were 7.5 ± 1%. In the PBMC of PsA (n = 10) or psoriasis (n = 10) patients and healthy controls (n = 10) activated Kv1.3$^+$ memory T cells were <1% of total T cells (data not shown). These results suggest that increased numbers of Kv1.3$^+$ T_{EM} cells are present at the site of disease pathology in psoriasis and PsA. The degree of enrichment did not vary with the severity of the psoriatic arthritis or extensiveness of psoriasis skin lesions. FACS experiments with synovial fluid samples from patients with OA showed that there were no Kv1.3+ memory cells present. While OA samples contained significant numbers of CD3+ cells, no cells were positive for CD45RO and HL-DR and therefore not of an activated memory phenotype (n = 10, data not shown).

3.3. Whole-cell patch-clamp confirms high Kv1.3 expression on T cells from psoriatic skin lesions and PsA biopsies

In the ion channel field patch-clamp is considered the "Gold-Standard" technique because instead of only providing information that a protein is present at low, medium or high levels, it allows to determine the number of functional channels on the cell membrane. We measured Kv1.3 currents using the whole-cell patch-clamp technique on T cells from 3 skin biopsies and 7 PsA synovial fluid samples, which contained sufficiently large numbers of cells for electrophysiological experiments. Mononuclear cells from these samples were stimulated for 48 h with soluble CD3/CD28 antibodies, stained with a PE-conjugated anti-CD3 Ab, and then

patch-clamped on a fluorescent microscope. Using a KF-based pipette solution, which eliminates contaminating calcium-activated K^+ currents and Cl^- currents, the cells exhibited large Kv currents that exhibited the characteristic biophysical and pharmacological properties of Kv1.3 (Fig. 4). The currents were "use-dependent", which means that they exhibited a decrease in current amplitude when currents were elicited by depolarizing steps every second (Fig. 4A). The currents were further sensitive to the Kv1.3 blockers PAP-1 (IC_{50} 2 nM) and ShK-L5 (IC_{50} 70 pM). Between 30 and 50% of the clearly activated cells with a membrane capacitance of more than 4 pF were found to contain more than 1000 Kv1.3 channels per cell (Fig. 4B). The average Kv1.3 expression in the activated T cells from psoriatic skin lesions was 1061 ± 70 channels (n = 45, mean ± SEM) and 1078 ± 195 (n = 150) in T cells from PsA SF samples. This is similar to what had been previously observed by us in myelin or diabetes antigen specific T cells from the blood of multiple sclerosis or type-1 diabetes patients and synovial T cells from RA patients [14,16] and significantly higher ($P < 0.001$ in all cases) than Kv1.3 expression in activated T cells of osteoarthritis synovial fluid (523 ± 35, n = 64), or peripheral blood T cells of healthy controls (465 ± 35, n = 104).

3.4. PAP-1 inhibits proliferation and IL-2 and IFN-γ production in T cells derived from psoriatic plaques and synovial fluid (SF) of psoriatic arthritis (PsA) patients

To determine whether activation of lesional T cells derived from skin and joints of psoriatic disease is regulated by Kv1.3 we investigated the effect of PAP-1 on proliferation and IL-2 and IFN-γ production. As shown in Figure 5A, PAP-1 dose-dependently inhibited CD3/CD28-antibody stimulated incorporation of [^{3}H]-thymidine by mononuclear cells from two psoriasis skin samples and one PsA synovial fluid sample. When CD3-enriched cells from skin

or synovial fluid were incubated in CD3/CD28-antibody coated 24-well-plates PAP-1 (1 μM) significantly inhibited both IL-2 and IFN-γ secretion (P<0.01, t-test, Fig. 5B). On mononuclear cells from a PsA synovial fluid incubated for 24h with the stronger but more calcium-dependent stimulus PMA plus ionomycin PAP-1 (1 μM) inhibited IL-2 and IFN- γ production more strongly (Fig. 4C).

3.5. Therapeutic efficacy of PAP-1 in the SCID-psoriasis xenograft model

Using the SCID mouse-psoriasis xenograft model we next tested whether Kv1.3 blockade with PAP-1 would be effective in treating psoriasis *in vivo*. For this purpose a total of 60 SCID mice were transplanted using shave biopsies obtained from 15 patients with chronic plaque-type psoriasis. Supplementary Table S1 shows the patient demographics. After successful acceptance (n = 40), grafts were selected for treatment on the 4th week of transplantation (Table 1). Eucerin® or Eucerin® containing 2% of PAP-1 was applied topically to the grafts twice daily for 4 weeks. Cyclosporine (4 mg/kg) was administered i.p. three times per week for 4 weeks as a positive control as per our earlier report [24]. We further compared the 2% PAP-1 preparation against the most potent topical corticosteroid preparation (Halobetasol propionate 0.05% ointment*)* (Table1).

In order to evaluate the therapeutic efficacy of PAP-1, punch biopsies (2mm) were obtained on day-0 (before treatment) and day-28 (after treatment) of the study period. Biopsies were snap-frozen and cryosections were prepared to determine rete peg length (epidermis thickness) by Hematoxylin and Eosin (H&E) staining and degree of activated T cell infiltration by staining for CD3, HLA-DR and Kv1.3.

Transplanted plaques treated with topical PAP-1 (n = 10) showed significant clinical and histological improvement compared to controls (n = 10). The histological improvement of the plaques treated with PAP-1 was evidenced by the reduction of hyperkeratosis, acanthosis and lymphomononuclear cellular infiltrates (Fig. 6). In plaques treated with 2% of PAP-1 rete peg lengths changed from 415.2 ± 59.6 μm to 231.4 ± 40.4 μm (P<0.01, paired t-test, Table 1 and supplemental data- Fig. S1), while vehicle treated plaques remained unchanged. As expected, transplanted psoriasis plaques (n = 10) treated with cyclosporine also showed significant improvement. We also observed that the inflammatory infiltrates (H&E staining), or $CD3^+$ and $HLA\text{-}DR^+$ dermal infiltrates were significantly reduced in the plaques treated with PAP-1 (Fig. 6). The number of $CD3^+$ and $HLA\text{-}DR^+$ lymphocytes/mm^2 of dermis was reduced from 166 ± 51 and 126 ± 56 respectively to 22 ± 8 and 15 ± 12 following 28 days of treatment (n = 10, P<0.01, Student's t test). Whereas in the vehicle control group, the numbers of CD3+ T cells in dermis before and after treatment were 158 ± 48/mm^2 and 140 ± 42/mm^2 respectively (n = 10, P = 0.1, Student's t test) and the $HLA\text{-}DR^+$ lymphocytes/mm^2 before and after treatment were respectively 130 ± 35 and 116 ± 18 (P = 0.1, Student's t test).

4. Discussion

Psoriasis is a multifactorial chronic inflammatory disease [2,29-31]. An active role of T cells in the pathogenesis of psoriasis is strongly substantiated by the following observations: (i) immunotherapy targeted specifically against $CD4^+$ T cells clears active plaques of psoriasis [32] (ii) in SCID mice, transplanted nonlesional psoriatic skin converts to a psoriatic plaque subsequent to intradermal administration of activated T cells [33] (iii) anti-CD28 antibody improves psoriasis in the SCID mouse-psoriasis xenograft model [24].

The voltage-gated K^+ channel Kv1.3 offers a novel approach of targeting T cells, which is particularly appealing because of the availability of potent and selective peptidic and small molecule inhibitors [15,34-37] that can interfere with T cell calcium signaling and activation. Engagement of the T cell receptor by antigen presentation triggers a Ca^{2+}-influx through the voltage-independent Ca^{2+}-release activated Ca^{2+} channel (CRAC) down-stream of IP_3-induced store depletion [13,38], which results in the sustained increase in cytosolic Ca^{2+} necessary for the translocation of NFAT to the nucleus and the initiation of new transcription ultimately resulting in cytokine secretion and T cell proliferation [13, 39]. However, this crucial Ca^{2+}-influx is only possible if the T cell can keep its membrane potential negative by a counterbalancing K^+ efflux through Kv1.3 and/or the other lymphocyte K^+ channel, the Ca^{2+}-activated KCa3.1 channel [13,40]. Interestingly, naïve and T_{CM} cells increase expression of KCa3.1 following activation, while T_{EM} cells up-regulate Kv1.3 and predominantly rely on this channel for their calcium signaling, migration, proliferation and cytokine production [13,14]. Kv1.3 blockade therefore seems to offer a powerful new way to selectively target T_{EM} cells in autoimmune disorders without affecting the function of naïve and T_{CM} T cells.

To define the role of Kv1.3 channels in the autoimmune process of psoriasis we carried out a series of *in vitro* studies. We demonstrated that psoriatic plaques are enriched with cells expressing high levels of Kv1.3 (Fig 1.), which were found to be T cells by double fluorescent immunostaining and by flow cytometry (Figs. 2 and 3). We were further able to identify with Hi-D FACS studies that the $Kv1.3^+$ T cells represent activated T cells (HLA^-DR^+) and that they are of a memory phenotype ($CD45RO^+/CCR7^-$) (Fig. 3). We confirmed high Kv1.3 expression at the functional level by subjecting T cells from psoriatic skin lesions and PsA synovial fluid to whole-cell patch-clamp experiments, which verified the molecular identity of the channel

through its characteristic biophysical and pharmacological properties (Fig. 4). It is believed that in psoriatic disease lesional specific memory T cells interact with a hypothetical antigen, which results in activation of these cells and initiation of signal transduction cascades for up-regulation of various inflammatory cytokines such as TNF-α, IFN-γ, IL-17, IL-22 [11, 12, 30]. To provide evidence for the functional significance of Kv1.3 we demonstrated that the Kv1.3 blocker PAP-1 inhibits the proliferation and IL-2 and IFN-γ production by mononuclear cells or T cells from PsA synovial fluid or psoriatic skin lesions (Fig. 5).

Several studies, including work from our research group, have successfully used the SCID mouse-psoriasis plaque xenograft model to evaluate the therapeutic efficacy of established and novel therapies [24,32,41,42]. Using the SCID mouse model we have demonstrated here that the Kv1.3 blocker PAP-1 exhibits significant therapeutic efficacy for psoriasis when administered topically. As seen in the Table 1, Fig. 6 and supplemental Fig. S1 four weeks of daily treatment with 2% PAP-1 ointment resulted in significant thinning of the epidermis along with reduced total numbers of infiltrating $CD3^+$ and activated HLA-DR^+ T cells. These results confirm and significantly expand the observations recently made by Gilhar et al. in a small study where SCID mice were grafted with normal human skin and then injected 4-weeks later with allogenic NK-like cells to induce psoriasis-form lesions [43]. Three of six grafts subsequently injected daily intradermally into the grafts with the Kv1.3 blocking *Stichodactyla helianthus* toxin (ShK) showed reduced immune cell infiltration and reduced epidermal thickening. However, since 50% of grafts did not respond to the ShK injections the results were not statistically significant [43]. Our study differs from this smaller study, in that we transplanted shave biopsies from patients with active psoriasis, which retained their histologic and immunologic features following acceptance of the grafts and did not have to induce psoriatic

changes by injecting IL-2 expanded NK-like cells. We further carried out extensive *in vivo/in vitro* studies to identify the role of Kv1.3 in psoriatic disease and instead of injecting a peptidic Kv1.3 blocker directly into the lesions applied a skin penetrant small molecule Kv1.3 blocker topically. This topical application is gentler and more practical in comparison to intradermal injections. Compared with systemic application of a Kv1.3 blocker, topical application also potentially has fewer systemic side effects. In addition to cells of the immune system Kv1.3 is also expressed in olfactory bulb mitral cells [44] and neurons of the auditory system [45], suggesting that Kv1.3 blockers could affect smell or high-frequency hearing. However, it should be pointed out here that the Kv1.3 blocker ShK-186 has gone through IND-enabling toxicity studies [19,37] and recently completed human phase 1A and phase 1B trials in healthy volunteers.

Both PAP-1 in our study and ShK in the Gilhar study [43] probably exerted their positive effects by preventing T_{EM} cell reactivation in the skin. As previously demonstrated by elegant *in vivo* two-photon imaging experiments with GFP-expressing ovalbumin-specific T_{EM} cells, Kv1.3 blockers "work" by inhibiting antigen-specific T_{EM} cell reactivation in tissues. In the presence of a Kv1.3 blocker T_{EM} cells still make occasional contact with antigen presenting cells, but the contact is not stable and the cells fail to enlarge and subsequently do not migrate through the inflamed tissue or proliferate and produce cytokines [17]. So by inhibiting Ca^{2+} influx during the initial APC contact Kv1.3 blockers prevent T_{EM} cells from becoming reactivated, immobilizes them and presumable induces "death by neglect" [13]. In our study we cannot completely exclude the possibility that in addition to T cells, PAP-1 might also have affected macrophages and dendritic cells which have been described to express Kv1.3 together with the related Kv1.5 channel [46-48]. However, we do not believe that PAP-1 affected any other cells in the skin

since we have not observed any positive Kv1.3 staining in normal human, mouse or rat skin that was not localized to lymphocytes (this study and [21]). Similarly, Gilhar et al. did not observe any Kv1.3 staining in skin grafts from healthy controls in a recent alopecia areata xenograft study, in which intradermal injection of our PAP-1 was found to reduce $CD3^+$ infiltrates around hair follicles [49].

Based on the results of our study we would like to propose the development of Kv1.3 targeted topical immunotherapy for psoriasis and possibly for other skin conditions such as contact dermatitis, alopecia areata and cutaneous T cell lymphoma, where effector memory T cells are involved in the pathogenesis. A topically applied small-molecule Kv1.3 would have the advantage that compared to the biologics it would be less expensive and could constitute an alternative to topical steroids.

Conflict of Interest

H.W. is an inventor on the University of California patent claiming PAP-1 for immunosuppression. This patent has been licensed by Airmid, Inc., a start-up company for which H.W. is a scientific founder. Airmid has licensed on the PAP-1 patent to Circassia Ltd. (Oxford, UK) for development as a topical for psoriasis.

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Figure Legends

Fig. 1. Identification of $Kv1.3^+$ lymphomononuclear infiltrates in psoriasis plaques. Arrows show $Kv1.3^+$ lymphocytes (A) $Kv1.3^+$ lymphocytes in the epidermis and dermis in a psoriatic plaque. (B) Negative control for panel A. (C, D) Diffuse $CD3^+$ lymphocyte infiltrates in a psoriatic plaque (C). A serial section from the same plaque (D) demonstrates $Kv1.3^+$ lymphocytes in a similar distribution. (E) A psoriasis plaque 8 weeks after transplantation on a SCID mouse demonstrating Kv1.3 T^+ cells are retained in the xenograft. (F) Lesion in panel E treated with PAP-1 demonstrates marked reduction of Kv1.3 T^+ cells. (G) **N**on-lesional psoriatic skin stained for $Kv1.3^+$ did not demonstrate any $Kv1.3^+$ T cells. Scale bar = 50 μm in panels A and B and 100 μm in panels C to G.

Fig. 2. Double fluorescent immunostaining demonstrating $CD3^+Kv1.3^+$ cell infiltrates in a psoriatic plaque. (A) Psoriatic skin stained with DAPI (left), Kv1.3 (middle) and CD3 (right). (B) Boxed area in A shown at higher magnification. (C) Close-up view of a $Kv1.3^+CD3^+$ cell showing co-localization of Kv1.3 and CD3.

Fig. 3. A representative FACS plot of Kv1.3+ T cells derived from psoriasis skin lesions (n=5) and psoriatic arthritis synovial fluid (n=10). (A) Gated live memory T cells ($CD3^+CD11a^+CD45RO^+$) derived from the synovial fluid of a psoriatic arthritis patient demonstrates that 7.37% cells are $Kv1.3^+$. These $Kv1.3^+$ memory T cells (C) were mostly $HLADR^+$ (activated) and $CCR7^-$ (D). Similarly we observed that lymphocytes derived from a psoriasis plaque had 7% $Kv1.3^+$ memory T cells (B).

Fig. 4. $CD3^+$ T cells from psoriasis skin biopsies and PsA synovial fluid (SF) express higher levels of Kv1.3 channels than controls. (A) Psoriasis skin T cells express a K^+ current that is use-dependent and sensitive to the Kv1.3 blockers PAP-1 and ShK-L5. (B) Average Kv1.3 channel number per cell in activated T cells from 3 psoriasis skin biopsies, 6 PsA SF samples. Osteoarthritis (OA) SF and mitogen stimulated PB T cells from healthy controls are shown for comparison (previously published by us in Beeton et al., 2006 and Wulff et al., 2003). Each data point represents the mean ± SEM from 15-30 cells per patient sample.

Fig. 5. Functional role of Kv1.3 channels in psoriatic disease: PAP-1 inhibits activation of lesional T cells derived from psoriatic plaques and synovial fluid. (A) PAP-1 inhibits [^{3}H]thymidine incorporation by mononuclear cells from a PsA synovial fluid sample and two psoriasis skin biopsies stimulated for 48 h with soluble anti-CD3/CD28 antibody. Shown are means ± SD of radiation counts per minute from triplicate wells. (B) Enriched $CD3^+$ T cells ($1x10^6$ cells/well) from psoriatic lesions (n = 3) or PSA synovial fluid (n = 5) were cultured in CD3/CD28-antibody coated 24-well plates for 72h. (C) PsA synovial fluid mononuclear cells from one patient were directly incubated for 24h with PMA/ionomycin in triplicate wells. In both cases PAP-1 (1 μM) significantly inhibited IL-2 and IFN-γ secretion ($P < 0.01$).

Fig. 6. Topical PAP-1 is therapeutically effective on SCID mouse-psoriasis plaque xenografts. Serial sections from a psoriasis plaque transplanted onto a SCID mouse demonstrate the typical histological features of psoriasis with CD3+ T Cell (**A**) and HLA-DR+ T cell (B) infiltrates. Topical PAP-1 treatment for 4 weeks reduced the thickness of the epidermis and the number of CD3+ T cells (C) and HLA-DR+ T cells (D). Sections from vehicle treated transplanted plaques

did not demonstrate thinning of epidermis or reduction of CD3+ T cells in the post treated plaque (E) compared to pre-treatment plaque (F). Scale bar = 100 μm in A to D and 80 μm in E and G.

Table 1.

Pre and post treatment rete peg length (thickness of epidermis) in different study groups: PAP-1 cream had a marked therapeutic effect on the transplanted psoriasis grafts.

Treatment agent and number of mice (n)	Schedule Duration	Route	Rete peg length [mean (μm) ± SD]		
			Pre-treat	Post-treat	*P* value
Eucerin® containing 2% of PAP-1 (n=10)	Twice a day for 4 weeks	Topical	415±59	231±40	*P*<0.01
Eucerin® (n=10)	Twice a day for 4 weeks	Topical	300±43	284±30	*P*=0.25
Untreated (n=10)	4 weeks		346±105	322±109	*P*=0.1
Cyclosporine 4 mg/kg (n=10)	3 times/week for 4 weeks	i.p.	361±104	171±55	*P*<0.01
Halobetasol propionate 0.05% (n=5, *Ultravate oint.)*	Twice a day for 4 weeks	Topical	405±45	175±40	*P*<0.001

Supplementary Table S1. Demographics of the healthy controls and psoriasis patients.

Study Population	Study Subjects (n)	Age Years ±SD	Sex Male	Female	Disease Duration Years ±SD
Psoriatic arthritis	10	48.5±14.5	8	2	8 ±4
Psoriasis Plaque	15	42±8.5	12	3	12 ±6
Non-lesional psoriasis skin	15	42±8.5	12	3	
Normal skin	15	44±10.5	12	3	

* All were Caucasians

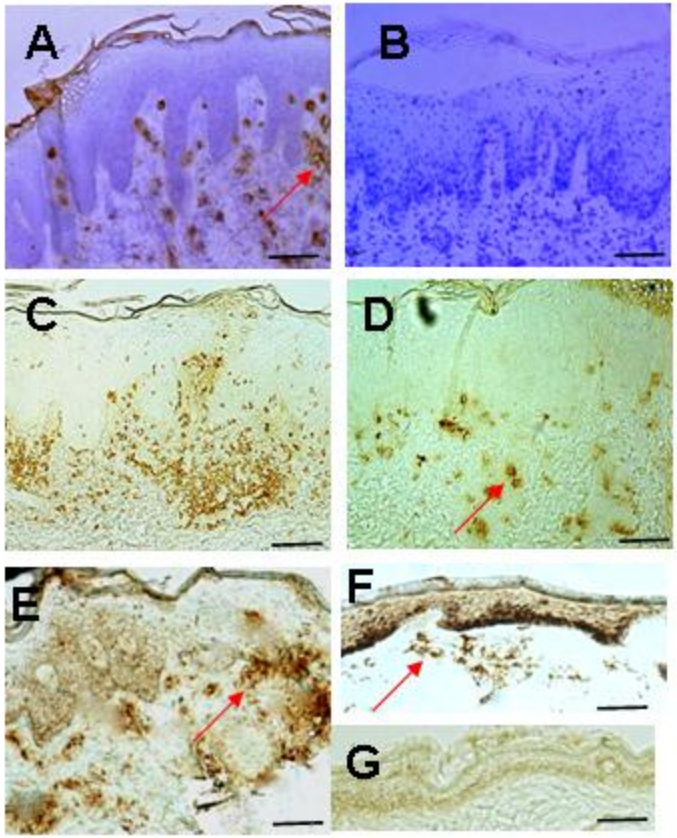

Figure 1

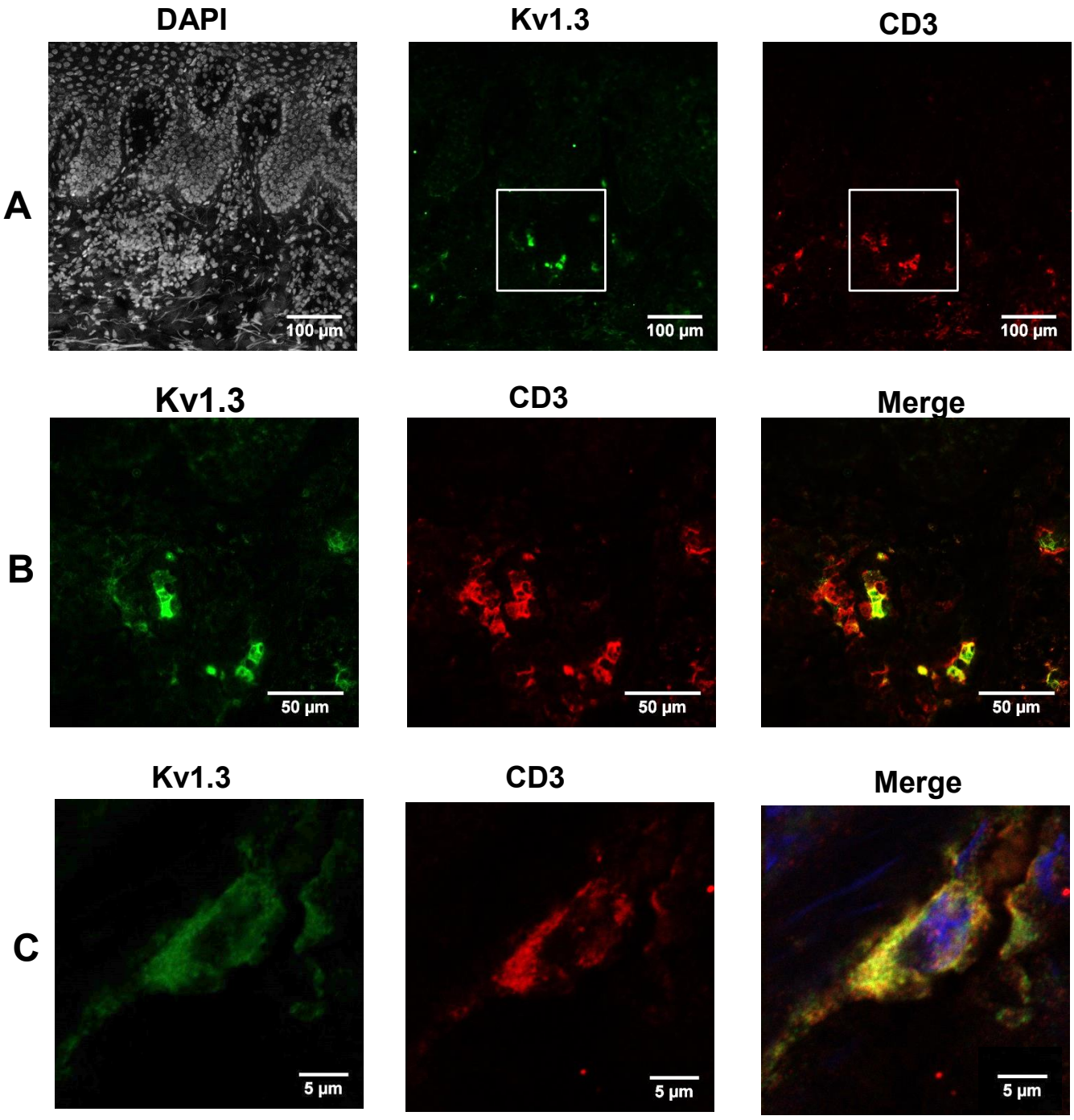
DAPI
Kv1.3
CD3
A
100 µm
100 µm
100 µm
Kv1.3
CD3
Merge
B
50 µm
50 µm
50 µm
Kv1.3
CD3
Merge
C
5 µm
5 µm
5 µm

Figure 2

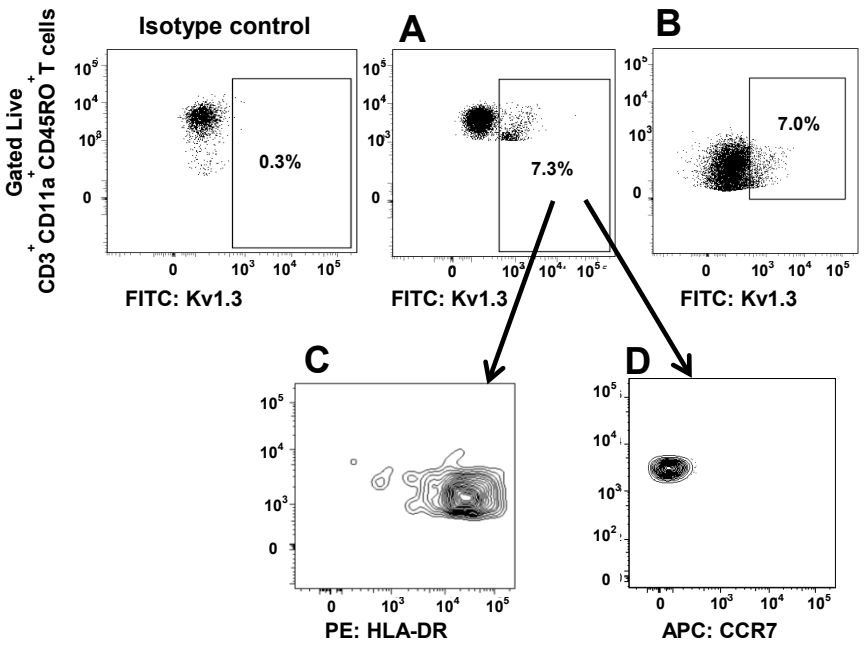
Gated Live CD3+ CD11a+ CD45RO+ T cells
Isotype control
A
B
0.3%
7.3%
7.0%
FITC: Kv1.3
FITC: Kv1.3
FITC: Kv1.3
C
D
PE: HLA-DR
APC: CCR7

Figure 3

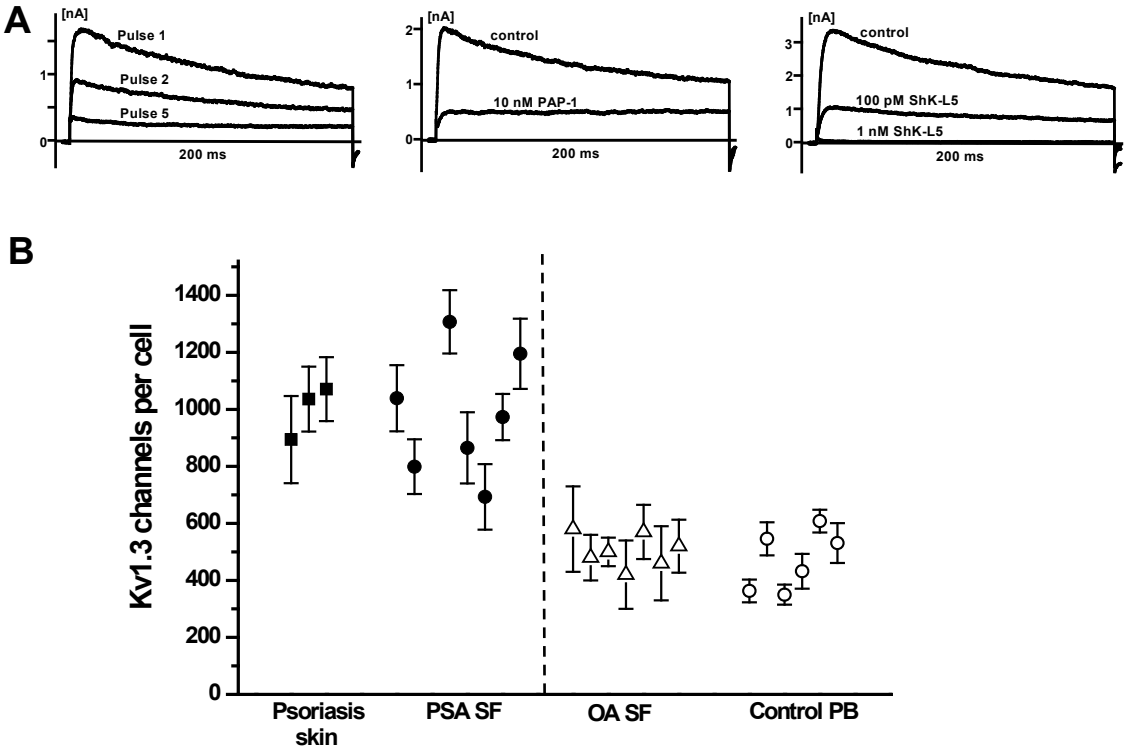
A
[nA]
Pulse 1
Pulse 2
Pulse 5
200 ms
control
10 nM PAP-1
100 pM ShK-L5
1 nM ShK-L5
B
Kv1.3 channels per cell
Psoriasis skin
PSA SF
OA SF
Control PB

Figure 4

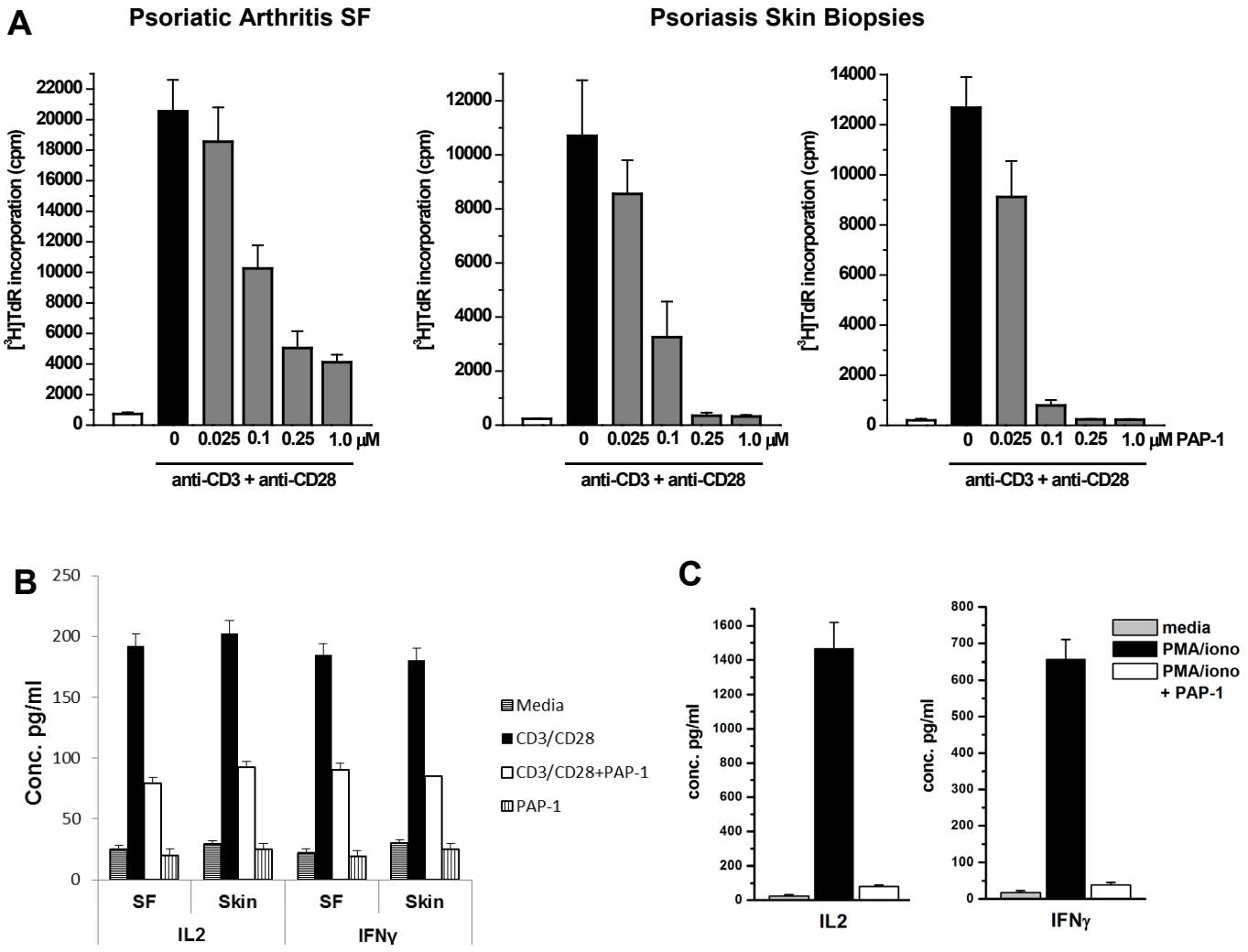
A
Psoriatic Arthritis SF
Psoriasis Skin Biopsies
[3H]TdR incorporation (cpm)
0 0.025 0.1 0.25 1.0 µM
anti-CD3 + anti-CD28
0 0.025 0.1 0.25 1.0 µM PAP-1
B
Conc. pg/ml
Media
CD3/CD28
CD3/CD28+PAP-1
PAP-1
SF
Skin
IL2
IFNγ
C
conc. pg/ml
media
PMA/iono
PMA/iono
+ PAP-1

Figure 5

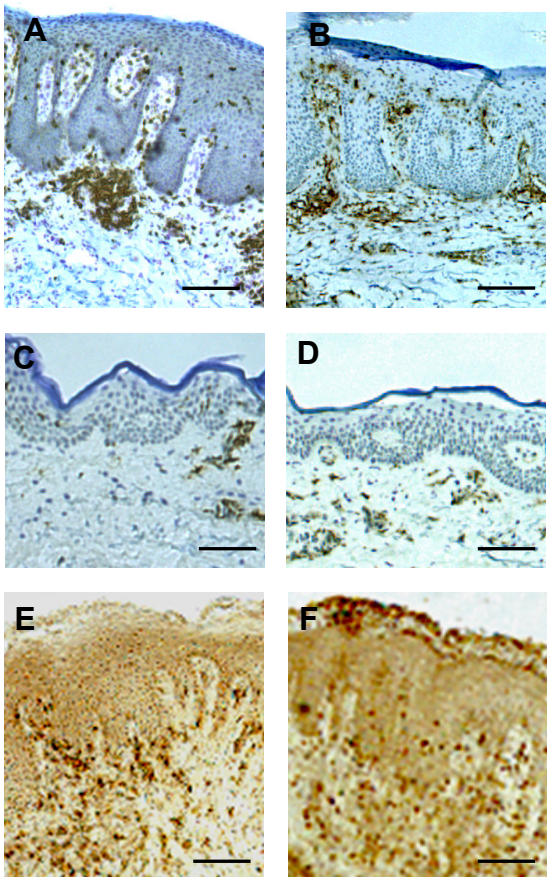

Figure 6

Figure S1.
Mean of the epidermal thickness (reti peg lengths) (µm) in each transplanted psoriasis plaque (n=10) in the pre or post treated tissues following treatment with the vehicle (A) or with the PAP-1 cream (B) are shown here.

A. Vehicle-treated psoriasis plaques did not show any significant changes in the reti peg lengths (µm).
B. PAP-1- treated psoriasis plaques showed significant reduction of reti peg lengths suggesting that blocking of Kv1.3 ion channels is therapeutically effective in psoriasis.