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Cell Injury, Repair, Aging and Apoptosis

Platelet-Activating Factor Is Crucial in Psoralen and Ultraviolet A-Induced Immune Suppression, Inflammation, and Apoptosis

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Psoralen plus UVA (PUVA) is used as a very effective treatment modality for various diseases, including psoriasis and cutaneous T-cell lymphoma. PUVA-induced immune suppression and/or apoptosis are thought to be responsible for the therapeutic action. However, the molecular mechanisms by which PUVA acts are not well understood. We have previously identified platelet-activating factor (PAF), a potent phospholipid mediator, as a crucial substance triggering ultraviolet B radiation-induced immune suppression. In this study, we used PAF receptor knockout mice, a selective PAF receptor antagonist, a COX-2 inhibitor (presumably blocking downstream effects of PAF), and PAF-like molecules to test the role of PAF receptor binding in PUVA treatment. We found that activation of the PAF pathway is crucial for PUVA-induced immune suppression (as measured by suppression of delayed type hypersensitivity to *Candida albicans*) and that it plays a role in skin inflammation and apoptosis. Downstream of PAF, interleukin-10 was involved in PUVA-induced immune suppression but not inflammation. Better understanding of PUVA's mechanisms may offer the opportunity to dissect the therapeutic from the detrimental (ie, carcinogenic) effects and/or to develop new drugs (eg, using the PAF pathway) that act like PUVA but have fewer side effects. *(Am J Pathol 2006, 169:795–805; DOI: 10.2353/ajpath.2006.060079)*

Psoralen and UVA (PUVA) photochemotherapy consists of the topical or oral application of a photosensitizing psoralen (ie, a furocoumarin compound), such as 8-methoxypsoralen, followed by exposure to photoactivating UVA light.[1] PUVA has been used now for more than 2 decades as a very effective therapeutic modality for various diseases such as psoriasis, chronic eczema, cutaneous T-cell lymphoma and other primary and secondary lymphoproliferative disorders, and cutaneous graft-versus-host disease after allogeneic stem cell transplantation. PUVA has strong proapoptotic[2] yet profound immunosuppressive effects,[3] but the mechanisms by which PUVA leads to clearance of skin lesions are not well understood. For many years, the interest in the molecular effects of PUVA has mainly been focused on the photobinding properties of psoralens to DNA, in which pyrimidine bases are the main targets for the photochemical reaction, leading to mono-adducts and/or interstrand crosslinks.[4] However, simple DNA photobinding may not be sufficient to explain all of the different activities of PUVA, and thus other targets have been hypothesized.[4,5] For instance, unsaturated lipids undergo photobinding by psoralens as well. Cyclobutane-like adducts are formed (similar to those between psoralens and thymine) between one of the reactive double bonds of psoralen and one or more of the double bonds of fatty acid residues.[4,5] Moreover, PUVA-induced alterations may involve oxidative processes such as the production of activated species of oxygen including singlet oxygen. These highly oxidizing agents can affect various biological substrates, including nucleic acids, proteins, and

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lipids. Recent work from our laboratory has shown that platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a potent phospholipid lipid mediator, is involved in UVB-induced immune suppression.[6] *De novo* synthesis of PAF occurs via a two-step pathway. Phospholipase-A2 enzymatically cleaves arachidonic acid from the sn-2 position of cell membrane phosphatidylcholine (PC), and an acetyl residue is subsequently transferred to the free hydroxyl from acetyl-CoA to form biologically active PAF.[6] PAF binds to a specific receptor, a seven transmembrane-spanning G-coupled protein, found on a variety of PAF-responding cells, including platelets, monocytes, mast cells, and polymorphonuclear lymphocytes[6] as well as keratinocytes.[7] In addition to PAF, this receptor also recognizes structural analogs of PAF, so called PAF-like molecules, generated by oxidation of PC. Both PAF and PAF-like molecules have been shown to activate the PAF receptor, inducing a variety of downstream effects, such as activation of the mitogen-activated protein kinase pathway and phospholipases, activating the transcription of cytokine genes and the production of several soluble factors, such as prostaglandin E_2 and interleukin (IL)-10 that play important roles in cellular communication.[6] UVB exposure has also been shown to induce phospholipase A2 activity[8] as well as reactive oxygen species, up-regulating PAF production.[9]

Based on the knowledge that many of the effects occurring after PUVA exposure (ie, inflammation, immune suppression, and apoptosis) are also induced by UVB radiation, and share similar characteristics, we hypothesized that the activation of the PAF pathway may be an important event in PUVA photochemotherapy. We used PAF receptor knockout mice, a PAF receptor antagonist, and a selective COX-2 inhibitor (presumably blocking downstream effects of PAF), and prospective PAF-like molecules to test the role of the PAF pathway in PUVA effects. We found that PAF receptor binding is crucial for PUVA-induced immune suppression and plays a role in inflammation and apoptosis.

Materials and Methods

Animals

Specific pathogen-free female C3H/HeNCr (MTV^-) mice and male and female C57BL/6 mice were purchased from the National Cancer Institute Frederick Cancer Research Facility Animal Production Area (Frederick, MD). PAF receptor knockout mice were received from J. Travers, Department of Dermatology, Indiana University, Bloomington, IN, and bred in our own animal facility. All animals were maintained with alternating 12-hour light and dark cycles and controlled temperature and humidity in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with current regulations of the United States Department of Health and Human Services. Water and food were provided *ad libitum*. All animal procedures were approved by the University of Texas M.D. Anderson Cancer Center Institutional Animal Care and Use Committee. Mice were 8 to 20 weeks old at the start of and were age- and sex-matched within each experiment.

Reagents

The PAF receptor antagonist PCA-4248 was purchased from Biomol Research Labs, Inc. (Plymouth Meeting, PA). Dr. Peter Isakson (G.D. Searle and Co., St. Louis, MO) provided the selective COX-2 inhibitor SC-236.[10] Stock solutions were prepared by dissolving each in a 50% dimethyl sulfoxide/phosphate-buffered saline (PBS) buffer and diluted further in PBS before injection into mice. The doses of SC-236 (0.2 μg per mouse) and PCA-4248 (500 nmol per mouse) injected intraperitoneally immediately before PUVA exposure were based on previous studies in which these concentrations totally blocked UV-induced immune suppression.[6] Anti-mouse IL-10 antibody (JES5-2A5.11, rat IgG) (Bioscience, San Diego, CA) was used for *in vivo* injection to neutralize IL-10 activity in mice. As a control, an isotype-matched antibody (rat IgG antibody; Sigma-Aldrich, St. Louis, MO) was used. Hen egg yolk PC (L-α lecithin) was purchased from Sigma-Aldrich (catalogue no. P 7718) for *ex vivo-in vivo* PC PUVA experiments.

PUVA Treatment

The backs of the mice were shaved with electric clippers 1 day before PUVA treatment. Groups of mice were painted on their backs with either 100 μl of vehicle (100% ethanol) or 8-MOP (Sigma-Aldrich) in ethanol (at a concentration of 1 mg/ml) or were left untreated. The mice were then kept for 30 minutes in individual compartments of standard cages, separated with Plexiglas dividers to allow penetration of 8-MOP. UVA irradiation was provided by a bank of six F15T8/BLB lamps (emission range, 345 to 400 nm; peak, 365 nm; Sylvania, Danvers, MA). During UVA irradiation, the mice were housed five per cage, individually separated, on a shelf 20 cm below the fluorescent light bulbs under a wire cage top. The mice were UVA-irradiated at a mean UVA irradiance of 4.5 mW/cm^2, as measured by an IL 700 spectroradiometer with a SEE 033 UVA detector (International Light Inc., Newburyport, MA). Solar-simulated UV radiation was provided by an Oriel 1000 W xenon solar simulator (Oriel, Stanford, CT) with a set-up and dose monitoring, as previously described.[11] For certain delayed type hypersensitivity (DTH) experiments a thin layer of PC (5 mmol/L in PBS) supplemented or not with 8-MOP (at a final concentration of 1 mg/ml) was spread into a polystyrene dish and exposed to different doses of UVA radiation of the F15T8/BLB lamps, under conditions as used for PUVA treatment of mice *in vivo*, as outlined above. PUVA- or UVA-treated solutions of PC are referred as to PUVA-PC or UVA-PC. PUVA-PC and UVA-PC were used for intraperitoneal injection into mice 5 days before sensitization with *Candida albicans* in DTH experiments.

Quantification of Macroscopic Skin Inflammation

Inflammation was assessed by measuring the double skin-fold thickness[12] of dorsal skin of the mice with a spring-loaded engineer's micrometer (Swiss Precision Instruments, Garden Grove, CA) before and at different time points after PUVA, UVA, or solar-simulated UV treatment. To determine the minimal inflammatory PUVA dose, groups of mice were PUVA-treated with double-dose increments. Skin swelling was determined for individual mice by subtracting the double skin-fold thickness before PUVA treatment from that after PUVA treatment. The skin swelling values of individual mice were averaged for the different treatment groups. The minimum inflammatory PUVA dose was defined as the minimum dose required to elicit statistically significant skin swelling in PUVA-treated mice.

DTH

Mice were treated with PUVA on day 0. Five days later they were immunized by the subcutaneous injection of 2×10^7 formalin-fixed *C. albicans* into each flank. Nine days later, each hind footpad was measured with the engineer's micrometer, the thickness was recorded, and the animals were challenged by injecting 50 μl of Candida antigen (Antigen Supply House, Northridge, CA) into each hind footpad. Thickness of each hind footpad was measured again 18 to 24 hours later and the mean footpad swelling for each mouse (left foot + right foot/2) was recorded. The mean footpad swelling ± SD was calculated for each experimental group. The specific footpad swelling was calculated by subtracting the mean footpad swelling found in control mice that were not immunized, but were challenged, from the swelling observed in groups of mice that were both immunized and challenged. The percent suppression of DTH was determined by the following formula: $[1 - (A/B)] \times 100$, where A represents the specific footpad swelling in sensitized and PUVA (or solar-simulated UV)-treated mice (ie, experimental group), and B represents that in sensitized, unirradiated mice (ie, positive control group).

Histological Examination

Two to three mice per treatment group were sacrificed before and at various time points (24, 48, 72, 120, and 360 hours) after PUVA exposure. Approximately 1 cm^2 of dorsal skin was excised per mouse, fixed immediately in 4% buffered formaldehyde, processed routinely, and sectioned at 5 μm for hematoxylin and eosin (H&E) staining. At least two nonsequential sections of each specimen were examined for histological alterations. Sunburn cells were counted in the interfollicular epidermis in a total of at least 10 random high-power fields per section (at ×100 microscopic magnification). Counts were expressed as the number of sunburn cells per centimeter length of epidermis, as determined with a calibrated eyepiece micrometer (Nikon Inc., Garden City, NY). Epidermal hyperplasia was assessed by counting epidermal cell layers and measuring the thickness of the epidermis from the basal layer to the stratum corneum with the eyepiece micrometer. A total of at least 10 measurements per section were made at randomly selected sites. To quantitate inflammatory cellular infiltration in the dermis of the specimens, an eyepiece counting grid (Olympus, Vienna, Austria) was used at ×200 microscopic magnification. The grid provided an area of 0.04 mm^2. The grid was placed at 10 randomly selected dermis locations per specimen. All infiltrating cells (including leukocytes, macrophages, and fibroblasts) within the grid were counted. The counts were averaged per specimen and then multiplied by 25 to give the total number of cells per mm^2.

Terminal dUTP Nick-End Labeling (TUNEL) Assay

Five-μm sections of paraffin-embedded skin tissue were deparaffinized, and TUNEL assay was performed using a commercial kit according to the manufacturer's protocol (Promega Corp., Madison, WI).

Immunohistochemical p53 Staining

Deparaffinized 5-μm tissue sections were used for immunohistochemical p53 staining, as described previously.[13]

Immunohistochemical IL-10 Protein Staining

Deparaffinized 5-μm tissue sections were placed in a humid chamber, and endogenous peroxidase was blocked with 3% hydrogen peroxide. Slides were incubated with protein blocking solution (5% horse serum in 1% normal milk made from 1 g of milk powder in 100 ml of PBS) for 30 minutes at room temperature, followed by overnight treatment at 4°C with diluted (5 μg/ml) goat anti-mouse IL-10 monoclonal antibody (R&D Systems, Minneapolis, MN). The slides were washed and incubated with biotin-labeled diluted (2 μg/ml) swine anti-goat antibody (Boehringer Mannheim, Indianapolis, IN) for 40 minutes, with peroxidase-labeled streptavidin for 30 minutes, and with diaminobenzidine (Research Genetics, Huntsville, AL) for 5 to 10 minutes. Sections were counterstained with hematoxylin.

IL-10 Enzyme-Linked Immunosorbent Assay (ELISA)

Two to three mice per treatment group were bled at various time points (24, 48, 72, and 120 hours) after PUVA exposure. Serum levels of IL-10 were measured by Opteia mouse IL-10 ELISA kit (Pharmingen, San Diego, CA), according to the manufacturer's instructions. The minimum detectable dose of IL-10 was ~125 pg/ml.

Image Analysis

Photographs of p53-stained sections were subjected to computerized, digitized image analysis. Optimate image

analysis software version 6.2 (Media Cybernetics, Silver Spring, MD) was used to quantify the staining. At least three photographs were used per section and sample. The amount of specific antibody binding was visualized and quantified by image analysis and expressed in terms of integrated mean optical density of a cell nucleus. The nuclear staining in at least 100 representative nuclei was measured per specimen.

Statistical Analysis

The readings of the DTH experiments as well as histological and immunohistochemical stainings were conducted in a blinded manner. All data presented are expressed as means ± SD. Statistical differences among control and experimental groups were determined by use of analysis of variance (StatView 5.01; SAS Institute Inc., Cary, NC). Statistical significance was set at a P level <0.05.

Results

Determination of the Minimal Phototoxic PUVA Dose

PUVA is used for the treatment of skin diseases such as psoriasis at sub- and/or near erythematogenic dosages. For instance, the original European PUVA study protocol calls for the minimal phototoxic dose (MPD) as the starting dose for PUVA treatment. The MPD is determined in a test, in which the skin of patients is exposed to a test ladder of increasing UVA dosages after topical and/or oral psoralen administration.[1,14] The MPD is defined as the smallest UVA dose required to produce a clearly demarcated and perceptible erythema, as determined 48 to 120 hours after psoralen administration. To assure that we used clinically relevant PUVA dosages in the experiments, we performed kinetic and dose-response studies to determine the MPD in mice. Because erythema is difficult to read in the pigmented skin of C3H and C57BL/6 mice, skin swelling was used as a surrogate end point for PUVA-induced inflammation and erythema. Those studies revealed that maximum skin swelling was present in mice 48 hours after PUVA exposure and the MPD in the C3H mice as well as C57BL/6 mice was 50 kJ/m^2 (data not shown). This dosage falls in the range of MPD values found in patients in the clinical set-up of minimal phototoxicity testing.

PUVA-Induced PAF Receptor Activation Is Crucial for PUVA-Induced Immune Suppression

We used the mouse model of induction of DTH to investigate the effect of different PUVA doses on immune function in the mice. Whereas significant inflammation (ie, skin swelling) after PUVA was observed in mice exposed to 50 kJ/m^2 (Figure 5a), significant immune suppression was observed at 25 kJ/m^2 (Figure 1a), indicating that higher PUVA doses are necessary to induce inflammation than immune suppression. Because in the clinic the dose

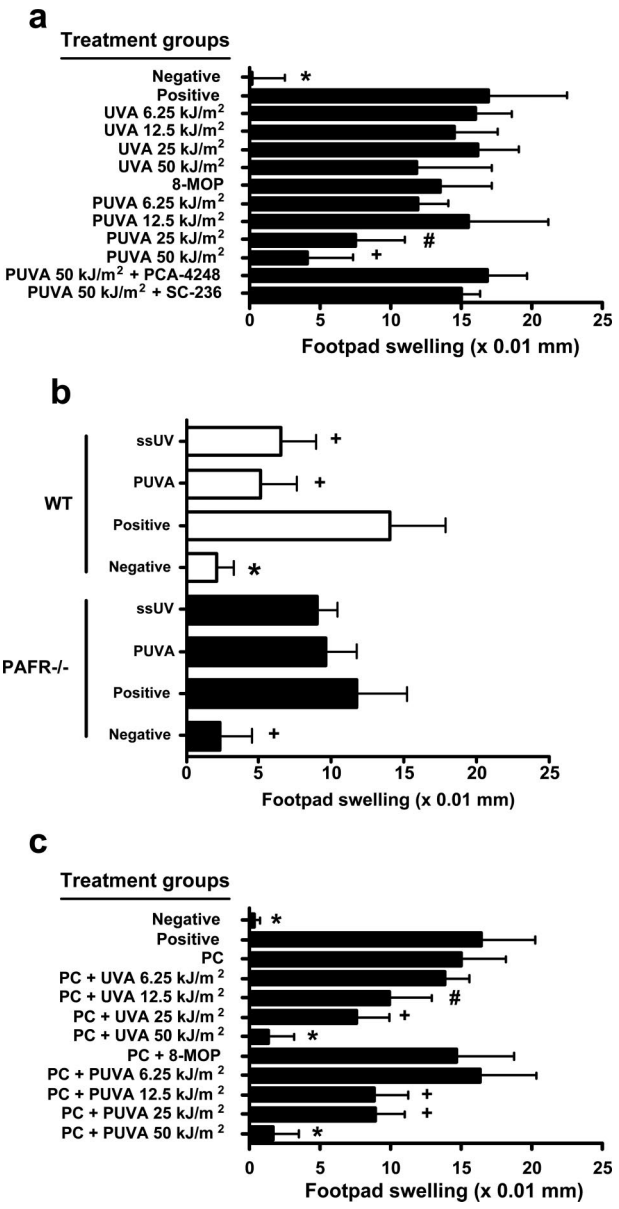

Figure 1. PUVA-induced PAF receptor activation is crucial for PUVA-induced immune suppression. PUVA-induced systemic immune suppression was studied in the model of DTH to *C. albicans*. Groups of C3H, C57BL/6, or PAF receptor knockout mice ($n = 5$ to 6 per group) were left untreated or treated with topical 8-MOP and different doses of UVA, ranging from 6.25 to 50 kJ/m^2, solar-simulated UV radiation (15 kJ/m^2), or were injected with PUVA-treated, UVA-treated, or untreated PC 5 days before immunization with *C. albicans*. Nine days later the mice were challenged with Candida antigen. DTH was measured 24 hours after challenge. The background response (negative control) was measured in mice that were not immunized but challenged. Mice that were immunized (but not PUVA-treated) and challenged served as positive control group. PUVA exposure was highly immunosuppressive and suppressed DTH by up to more than 90% at the PUVA dose of 50 kJ/m^2. **a:** The intraperitoneal injection of C3H mice immediately before PUVA exposure with either the PAF receptor antagonist PCA-4248 or the COX-2 inhibitor SC-236 totally blocked PUVA-induced immune suppression. $^*P < 0.0001$; $^+P = 0.0005$; $^\#P < 0.01$ versus positive control group. **b:** C57BL/6 (WT) but not PAF receptor knockout (PAFR$^{-/-}$) mice were susceptible to immune suppression induced by either PUVA at a dose of 50 kJ/m^2 or solar-simulated UV at 15 kJ/m^2. $^*P < 0.005$; $^+P < 0.05$ versus respective positive control group. **c:** The injection of C3H mice with PUVA-treated PC 5 days before sensitization with *C. albicans* resulted in significant immune suppression, in a dose-dependent manner similar to that in the *in vivo* PUVA experiments. The injection of UVA-treated PC (without psoralen) also led to immune suppression in a very similar dose-dependent manner than PUVA-treated PC. $^*P < 0.0001$; $^+P < 0.005$; $^\#P < 0.01$ versus positive control group.

of PUVA used is based on the minimal inflammatory dose, in subsequent studies we chose to use 50 kJ/m^2, which induces both immune suppression and inflammation.

To test the hypothesis that, similar to UVB exposure, PAF receptor activation may be involved in PUVA-induced immune suppression, we injected C3H mice in DTH experiments with the selective PAF receptor antagonist PCA-4248 and the selective COX-inhibitor SC-236, presumably blocking effects downstream of PAF activation (ie, PAF induction of COX-2).[15] As evident from Figure 1a, the intraperitoneal injection of mice immediately before PUVA exposure with either the PAF receptor antagonist or the COX-2 inhibitor nearly completely blocked PUVA-induced immune suppression. The injection of the dimethyl sulfoxide/PBS vehicle did not significantly affect PUVA-induced immune suppression in any experiment (data not shown).

However, because certain PAF antagonists have been shown to have dual activities in blockading PAF receptor-mediated activation and also inhibiting other enzymes, including COX, lipoxygenase, phospholipase A2, acetylcholinesterase, and intracellular PAF acetylhydrolase,[16] we have used PAF receptor knockout mice to substantiate the significance of the PAF pathway in PUVA-induced immune suppression. PAF receptor knockout mice, which have been created by targeted gene disruption of the PAF receptor gene in E14-1 embryonic stem cells derived from 129/Ola mouse on C57BL/6 genetic background, do not show gross morphological abnormalities in any organ system, including the skin.[16] Figure 1b shows that exposure to PUVA at a dose of 50 kJ/m^2 or solar-simulated UV radiation at a dose of 15 kJ/m^2 did not lead to significant immune suppression in PAF receptor knockout mice but only in their C57BL/6 wild-type littermates, substantiating the importance of an intact PAF pathway for immune suppression.

To test the hypothesis that not only PUVA-induced PAF itself but also PUVA-induced PAF-like molecules can lead to immune suppression, we performed an *ex vivo-in vivo* PUVA experiment with PC. PC was treated *in vitro* in the presence or absence of 8-MOP with different UVA doses before intraperitoneal injection into C3H mice. Similarly on UVB exposure[6] this presumably may lead to the formation of PAF-like molecules. These studies revealed that PUVA-treated PC on intraperitoneal injection 5 days before sensitization with *C. albicans* led to suppression of DTH in a dose-dependent manner, similar to that after *in vivo* PUVA treatment of the mice (Figure 1c), suggesting that PUVA-induced PAF-like molecules may indeed be involved in PUVA-induced immune suppression. However, intraperitoneal injection of UVA-treated PC alone (without psoralen) surprisingly led to immune suppression in a similar dose-dependent manner compared to PUVA treatment. This contrasted the finding that the very same UVA doses that were used in the PC experiment did not led to immune suppression in the pure *in vivo* PUVA experiment (Figure 1a). The reasons for this discrepancy remain unclear at present; however, possible explanations are outlined in the Discussion. Importantly, the intraperitoneal injection of UVA-PC in the presence or absence of psoralen did not led to inflammation of the skin, as measured by skin swelling (data not shown). Taken together, these findings unambiguously indicate that PUVA-induced immune suppression and inflammation follow overlapping but not identical pathways.

IL-10 Is Involved Downstream of PAF Receptor Activation in PUVA-Induced Immune Suppression but Not Inflammation

Next, we investigated which events may be involved in PUVA-induced immune suppression downstream of PAF. We hypothesized that similar to UVB[17] IL-10 may be involved in PUVA-induced immune suppression. First, we demonstrated by immunohistochemical staining that PUVA exposure led to up-regulation of IL-10 in the skin of C3H mice *in situ*. IL-10 protein was increased in the skin starting at 24 hours (Figure 2a) and remained elevated to a similar degree up to 120 hours (data not shown) after PUVA exposure. ELISA of serum taken from the mice at different time points after PUVA exposure revealed that PUVA also led to an increase of serum IL-10 at 120 hours after exposure (Figure 2b). Importantly, the injection of either the PAF antagonist PCA-4248 or the COX-2 inhibitor SC-236 nearly completely blocked PUVA-induced up-regulation of IL-10 in skin and serum (Figure 2, a and b). Next we determined if there was a functional role for IL-10 in PUVA-induced immune suppression in the DTH model. Injection of C3H mice with anti-IL-10 antibody abrogated PUVA-induced immune suppression whereas injection of an isotype-matched antibody had no significant effect (Figure 2c). In contrast, the administration of anti-IL-10 antibody did not significantly affect the PUVA-induced skin-swelling response (Figure 5b), indicating once more that immune suppression and inflammation involve overlapping but different mechanisms.

PAF Receptor Activation Is Involved in PUVA-Induced Apoptosis Possibly by Interfering with p53

PUVA-induced apoptosis of specific target cells such as keratinocytes, Langerhans cells, and/or lymphocytes within the skin is considered as one of the possible mechanisms involved in the therapeutic action of PUVA in skin diseases.[2] We therefore asked the question whether blockade of the PAF pathway may also influence PUVA-induced apoptosis. TUNEL staining of skin of C3H mice showed that PUVA-induced apoptosis of keratinocytes could be diminished by intraperitoneal injection of mice with either the PAF receptor antagonist PCA-4248 or the COX-2 inhibitor SC-236 (Figure 3). The crucial role of the PAF pathway in PUVA-induced apoptosis also became evident by the effect of both the PAF receptor antagonist and the COX-2 inhibitor on PUVA-induced sunburn cell formation. Sunburn cells are considered as the hallmark of apoptosis.[12] As evident from the results of examination of H&E-stained skin sections of mice both the PAF an-

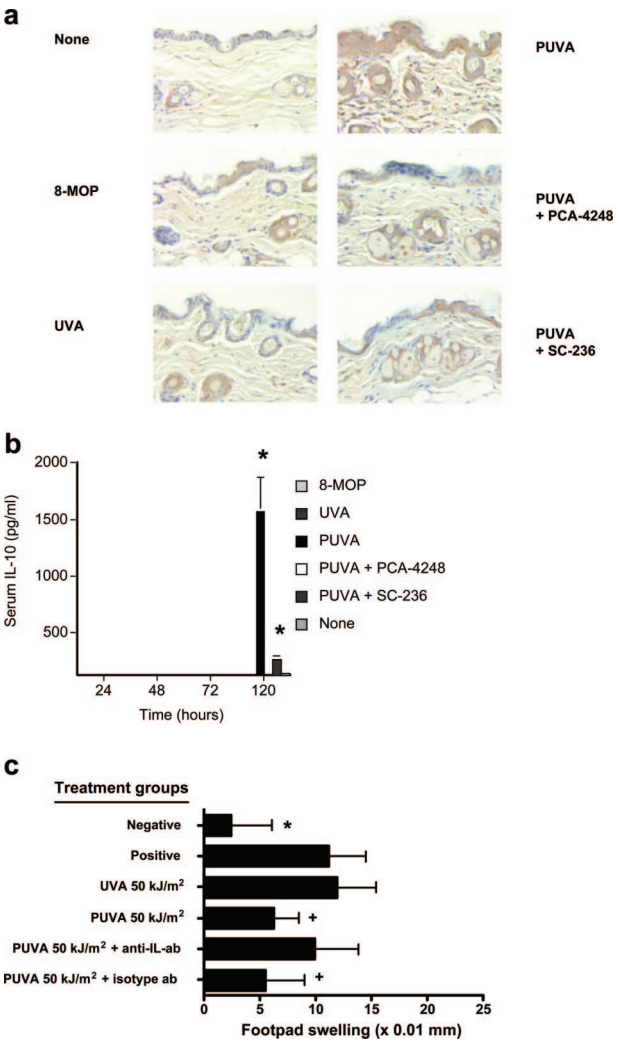

Figure 2. IL-10 is essential downstream of PAF receptor activation for PUVA-induced immune suppression. **a:** PUVA-induced IL-10 protein expression in the skin of C3H mice was studied by immunohistochemical anti-IL-10 staining. Note that there is diffuse brownish staining (indicating the presence of IL-10 protein) of keratinocytes and dermal cells in PUVA-treated skin. The intraperitoneal injection of mice immediately before PUVA exposure either with the PAF receptor antagonist PCA-4248 or the COX-2 inhibitor SC-236 inhibited PUVA-induced IL-10 up-regulation in the skin. Only patchy epidermal staining and occasional IL-10-positive dermal cells were present in the skin of mice injected with either the PAF receptor antagonist or the COX-2 inhibitor, similar to the staining pattern observed in untreated control mice (none) or UVA- or 8-MOP-treated mice. Photographs shown are from skin samples taken 24 hours after PUVA exposure to 50 kJ/m^2. **b:** There was significant p53 up-regulation of IL-10 protein (as measured by ELISA) in the serum at 120 hours after PUVA exposure to 50 kJ/m^2. This IL-10 up-regulation was not found in serum of mice injected immediately before PUVA exposure with either the PAF receptor antagonist or the COX-2 inhibitor. n = 2 to 3 mice per group. **c:** Immune function was studied in the model of DTH to *C. albicans* (for description of positive and negative control group see legend of Figure 1). The intraperitoneal injection of mice immediately before PUVA exposure with an anti-IL-10 antibody abrogated PUVA-induced immune suppression, whereas an isotype control antibody had no significant effect. n = 5 mice per group. $^*P < 0.0001$; $^+P = 0.05$ versus positive control group.

tagonist PCA-4248 and the COX-2 inhibitor SC-236 nearly completely blocked PUVA-induced sunburn cell formation (Table 1). The p53 gene and protein takes a crucial position in apoptosis in general by stopping the cell cycle to allow DNA repair after damage, so therefore we asked how blockade of PAF-receptor activation might

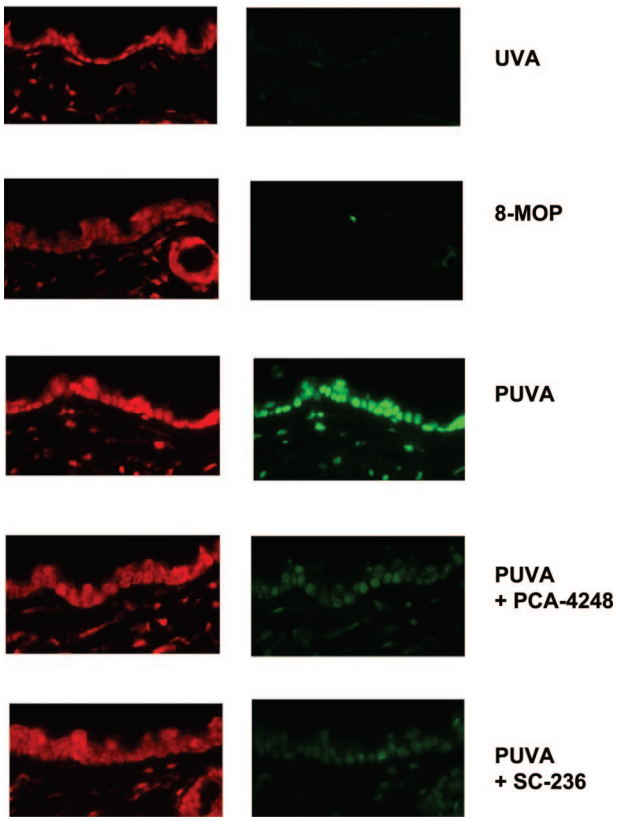

Figure 3. PAF receptor activation is involved in PUVA-induced apoptosis. Apoptosis was determined by TUNEL staining. Results shown are from skin samples taken 24 hours (time point of maximum effect) after PUVA exposure to 50 kJ/m^2. PUVA-induced apoptosis (green nuclear staining; right) was diminished in the skin of mice injected intraperitoneally immediately before PUVA exposure with either the PAF receptor antagonist PCA-4248 or the COX-2 inhibitor SC-236. The left panel (red nuclear staining) shows the propidium iodide controls. Treatment with UVA or 8-MOP alone did not lead to significant levels of apoptosis.

influence the p53 status. Immunohistochemical p53 protein staining of murine skin revealed that PUVA-induced up-regulation of p53 protein could be diminished by the administration of either the PAF receptor antagonist PCA-4248 or the COX-2 inhibitor SC-236 (Figure 4).

PAF Pathway Blockade Can Reduce PUVA-Induced Inflammation

Finally, we were interested in determining how blocking PAF receptor binding affects PUVA-induced inflammation. This is an important clinical question because accidental PUVA overdosage can result, 2 to 3 days later, in severe, life-threatening phototoxic burning of the skin, and presently, no effective treatment is available. In C3H mice blocking the activation of the PAF pathway by using either a PAF receptor antagonist PCA-4248 or targeting a downstream effect (by the COX-2 inhibitor SC-236) not only reduced PUVA-induced macroscopic skin swelling (as the clinical sign of PUVA phototoxicity) (Figure 5a), but also clearly diminished inflammatory skin infiltration (by up to ~90%) and hyperplasia (by up to 95%) on the microscopic level (Figure 6 and Table 1). However, whereas both the PAF receptor antagonist and the COX-2

Table 1. Effect of PAF Receptor Antagonist PCA-4248 and COX-2 Inhibitor SC-236 on PUVA-Induced Histological Skin Alterations

Treatment	Histological parameter			
	SBC per cm epidermis length	Number of epidermal skin layers	Epidermal thickness (μm)	Total dermal cell density per mm^2
None	0.0 ± 0	1.8 ± 0	26.9 ± 4.4	513 ± 18
8-MOP	0.0 ± 0	1.8 ± 0	26.6 ± 1.1	596 ± 101
UVA	1.3 ± 1.8	1.8 ± 0	32.8 ± 8.2	534 ± 48
PUVA	45 ± 14.4*	4 ± 0.4*	121.7 ± 5.0*	1075 ± 60*
PUVA + PCA-4248	2.5 ± 1.8	2.5 ± 0.8	40.4 ± 10.5	741 ± 164
PUVA + SC-236	0.0 ± 0	2 ± 0.1	31.7 ± 1.8	625 ± 131

Groups of C3H mice were treated with PUVA, 8-MOP, or UVA alone. The UVA dose used was 50 kJ/m^2. Immediately before PUVA exposure, certain groups of mice were injected intraperitoneally with either the PAF receptor antagonist PCA-4248 or the COX-2 inhibitor SC-236. Two to three mice per treatment group were killed at various time points after PUVA exposure (24, 48, 72, 120, and 360 hours). Data shown are from days with the maximum PUVA effect for a specific histological parameter; ie, sunburn cells (SBCs) at 24 hours; number of epidermal skin layers, skin thickness, and total dermal cell density at 48 hours after PUVA exposure.

*$P < 0.05$ versus untreated control group (none).

inhibitor more consistently protected against PUVA-induced immune suppression, they variably protected against inflammation (Figure 5b). We also looked for PUVA-induced and solar-simulated UV radiation-induced inflammation in PAF receptor knockout mice. Both PUVA and solar-simulated UV led to significant skin inflammation (as measured by skin swelling) surprisingly with no difference between PAF receptor knockout mice and their wild-type controls for both treatments (Figure 5c). In a control experiment, we used phorbol myristate acetate (PMA) (75 μl of a 200 μg/ml acetone solution per mouse applied topically to dorsal skin) to induce inflammation but did not find a difference either in the skin-swelling response between PAF receptor knockout mice and their wild-type controls (data not shown).

Discussion

This study revealed that PAF receptor binding is a crucial event in PUVA-induced immune suppression (Figure 1) and involved in other PUVA-induced effects, including IL-10 production (Figure 2), apoptosis (Figure 3), p53 up-regulation (Figure 4), and inflammation (Figures 5 and 6; and Table 1). Downstream of PAF activation, IL-10 seems to be involved in immune suppression but not inflammation. This finding is consistent with the role of this cytokine in UVB radiation-induced immune suppression,[17] in which PAF has been shown to activate the transcription of COX-2 and IL-10, two important immunological mediators.[6] Previous studies from our laboratory suggest that an early step in the UV-induced cytokine cascade is PGE_2 production, which then causes downstream effects, including the secretion of IL-4 and IL-10.[18] We also know from previous work that application of other dermal immunotoxic agents, such as jet fuel induces immune suppression via a PAF-, COX-2-, and IL-10-dependent mechanism.[19,20] Many PUVA-induced effects (including immune suppression) may be attributable to the generation of PAF and/or PAF-like molecules by degradation of PC by reactive oxygen species.[9] For instance, phospholipase-A2, a key enzyme in PAF production, can be induced by UVB exposure[8] and possibly also by PUVA.[21,22] The possibility that reactive oxygen species-associated effects are responsible for PUVA-induced effects such as immune suppression is supported by the observation that the antioxidative polypodium leucotomos extract has been shown to inhibit PAF production[23] and several types of UV and/or PUVA-induced immunological alterations, such as depletion of antigen-presenting Langerhans cells in human skin.[24] Moreover, the isoflavone genistein, a specific inhibitor of tyrosine kinase, has been shown to inhibit chemical carcinogen-induced reactive oxygen species and PUVA-induced photodamage, including inflammatory skin changes such as dermal neutrophilic and lymphocytic infiltration.[25]

We observed that *ex vivo* PUVA-treated and UVA-treated PC but not unirradiated PC induced immune suppression in a dose-dependent manner (Figure 1c). Surprisingly however, there was no difference in the UVA dose response dependency or the UVA threshold dose for immune suppression, irrespective whether or not psoralen was added to the UVA-irradiated PC solution before UVA irradiation. In contrast, the same PUVA, but not UVA, doses did induce immune suppression in the *in vivo* experiment (Figure 1a). This observation suggests that PUVA and UVA *ex vivo* treatment of PC produce PAF-like molecules (presumably via oxidative damage) that induce immune suppression. It also suggests that additional events (for instance DNA damage)[6] may be necessary and/or prerequisite for immune suppression, which only occurs after PUVA (but not UVA only) treatment *in vivo*. For example, UVB-induced DNA damage activates mitogen-activated protein kinase p38[26] which may initiate a cascade of events, including cell-cycle arrest at the G^2/M checkpoint (to allow for DNA repair) and the activation of phospholipase-A2,[27] the first enzymatic step of PAF synthesis.[6] A similar scenario might take place on PUVA exposure. Alternatively, we suggest that PUVA treatment may inhibit detoxifying mechanisms involved in PAF degradation (for instance by PAF-acetylhydrolase)[28] and therefore may lead to immune suppression that may not occur after *in vivo* UVA treatment.

The observation that IL-10 is involved downstream of PAF in PUVA-induced immune suppression (Figure 2) is intriguing because recombinant IL-10 has been shown to be clinically effective in the treatment of psoriasis,[29] one

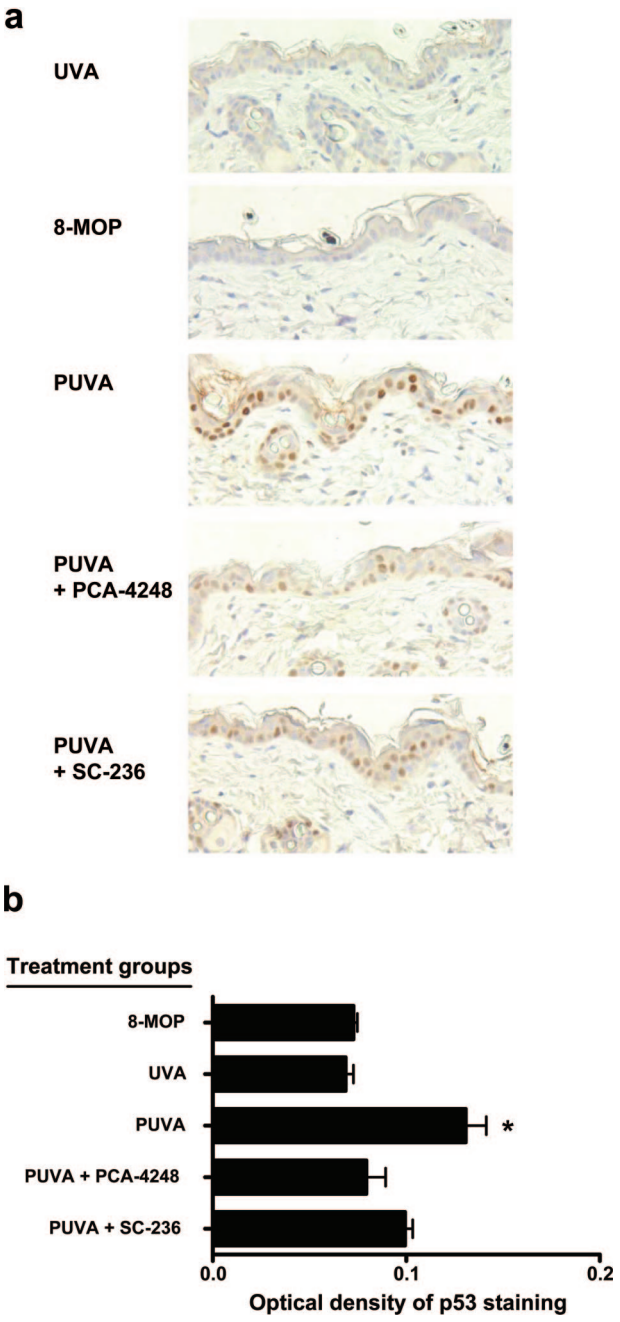

Figure 4. PAF receptor activation is also involved in PUVA-induced p53 up-regulation. PUVA-induced p53 protein expression in the skin of mice was studied by immunohistochemical staining. Results shown are from skin samples taken 24 hours (time point of maximum effect) after PUVA exposure to 50 kJ/m^2. **a:** PUVA-induced p53 up-regulation (brownish nuclear staining) was reduced in the skin of mice injected intraperitoneally immediately before PUVA exposure with either the PAF receptor antagonist PCA-4248 or the COX-2 inhibitor SC-236. Treatment with UVA or 8-MOP alone did not lead to significant apoptosis. **b:** Computerized image analysis quantifying the optical density of nuclear staining of epidermal cells revealed that the effect of the PAF receptor antagonist and the COX-2 inhibitor on PUVA-induced p53 expression was stronger than evident from the differences in the numbers of p53-positive epidermal cells in **a**. $n = 2$ to 3 mice per group. $^*P < 0.05$ versus 8-MOP- or UVA-treated mice.

of the diseases most often treated by PUVA.[1,14] The clinical response to IL-10 in patients with psoriasis was associated with a significant decrease of cutaneous T-cell infiltration and the lesional expression of type 1 cytokines such as interferon-γ and tumor necrosis factor-α. IL-10 inhibited the epidermal IL-8 pathway by down-regulating the expression of this potent chemoattractant and its receptor CXCR2.[29] On the other hand, activation of the epidermal PAF receptor, for instance by UVB radiation, can also lead to the production of IL-8.[15,30] However, different dose levels of an immunotoxic agent such as UVB and/or PUVA may be responsible for an ambivalent outcome in cytokine regulation. For instance, suprerythemal inflammatory doses of UVB and/or PUVA exposure may lead to a predominance of inflammatory cytokines such as IL-6 and IL-8, whereas suberythemal or near-erythemal doses of exposure (as used in the model of this study) may rather lead to the production of immunomodulatory cytokines such as IL-4[31] and IL-10. This goes well in line with the clinical knowledge that suberythemal PUVA exposure is beneficial for psoriasis but exposure to PUVA above the phototoxicity dose can lead to aggravation of the disease by a process called koebnerization.[32]

The finding that PAF pathway blockade (Figure 3) does reduce PUVA-induced apoptosis[33,34] is intriguing as well, because the proapoptotic effect of PUVA may contribute to the treatment's clinical efficacy at least in certain PUVA-responsive diseases.[2] For instance, PUVA can selectively induce cell-cycle arrest and subsequent apoptosis in human T lymphocytes,[2,35] a process that might be particularly important for therapeutic efficacy in cutaneous T-cell lymphoma.[36,37] Previous work from our laboratory using knockout mice has shown that p53 and Fas/Fas ligand interactions are required for PUVA-induced apoptosis in epidermal cells.[2] Interestingly, however, in a recent study induction of psoriasis was inhibited in a mouse xenotransplantation model by injection of blocking anti-Fas or anti-Fas ligand antibody.[38] Importantly, in the present study the reduction of apoptosis was paralleled by inhibition of p53 protein expression in the epidermis (Figure 4). A role for the PAF pathway in apoptosis on PUVA exposure is consistent with a role for PAF in apoptosis induction by UVB. Barber and colleagues[7] have reported that the expression of PAF receptor resulted in enhanced UVB radiation-induced apoptosis in a human epidermal cell line, consistent with very recent *in vivo* findings in PAF receptor knockout mice by the same group of investigators (Travers JB, Zhang Q, Konger RL: Involvement of the platelet-activating factor system in ultraviolet B-radiation-mediated cytokine production, apoptosis, and immunosuppression. Abstract presented at the 67th Annual Meeting of the Society for Investigative Dermatology, May 3 to 6, 2006 Philadelphia, PA). Interestingly, however, Brewer and colleagues[39] have shown that PAF can have anti- and proapoptotic effects in cells, initiated by its G-protein-coupled receptor or occurring independently of this receptor. Furthermore, Southall and colleagues[40] have shown that the activation of the epidermal PAF receptor protected from apoptosis induced by either TNF-α or TNF-related apoptosis-inducing ligand and that this protective effect was inhibited by pretreatment with PAF receptor antagonists mediated by a nuclear factor-κB-dependent pathway.

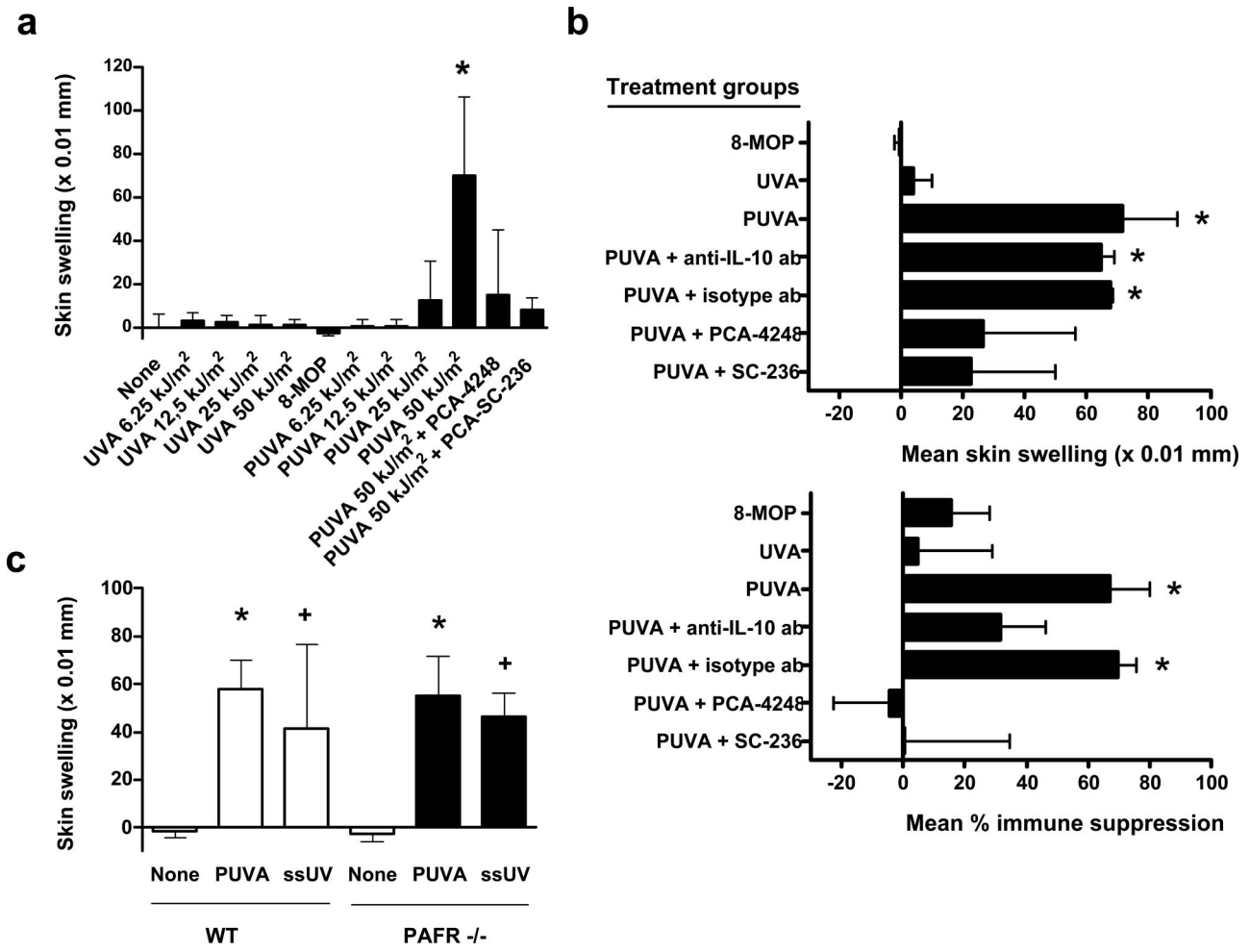

Figure 5. PAF pathway blockade does reduce skin inflammation on the macroscopic level. **a:** Groups of C3H mice ($n = 5$ per group) were treated with topical 8-MOP and/or different doses of UVA, ranging from 6.25 to 50 kJ/m^2. Skin swelling was determined by measuring double skin-fold thickness of dorsal skin of the mice before and at 48 hours after PUVA exposure. The skin swelling data are from the DTH experiment presented in Figure 1a. The skin swelling response was greatly diminished in the skin of mice injected intraperitoneally immediately before PUVA exposure with either he PAF receptor antagonist PCA-4248 or the COX-2 inhibitor SC-236. Note that treatment with UVA or 8-MOP alone did not lead to significant skin swelling. $^*P < 0.05$ versus none (no treatment). **b:** Both the PAF antagonist PCA-4248 and the COX-2 inhibitor SC-236 more consistently protected in repeat experiments against PUVA-induced immune suppression, but they gave only variable protection against inflammation (at the dose of 50 kJ/m^2). This is evident from the plotted graphs showing mean skin swelling (top graph) versus mean percent immune suppression (bottom graph). Results are means from several independent experiments ($n = 2$ to 7 per treatment with at least five mice per experimental group per experiment). $^*P < 0.01$ versus 8-MOP- or UVA-treated mice (top graph). $^*P < 0.0005$ versus 8-MOP- or UVA-treated mice (bottom graph). **c:** Swelling of dorsal skin was also determined in PAF receptor knockout mice (PAFR$^{-/-}$) and their C57BL/6 littermates (WT) at 48 hours after exposure to PUVA at a dose of 50 kJ/m^2 or solar-simulated UV (ssUV) radiation at a dose of 15 kJ/m^2. The presented skin swelling data are means from three independent experiments with at least five mice per experimental group per experiment. $^*P < 0.01$; $^+P < 0.05$ versus respective untreated control group (none).

Our study also showed that blockade of the PAF pathway reduced inflammation and hyperplasia of the skin in C3H mice (Figure 5, a and b; Figure 6; and Table 1). These findings are consistent with the results of studies in which PAF receptor antagonists have been shown to modulate neutrophil responses with thermal injury in rat skin *in vivo*[41] and inhibit UVB-induced inflammation in mouse ears.[42] PUVA can lead to severe phototoxic side effects, and although these effects are rarely lethal, are painful and are observed after accidental PUVA overdose.[43] We suggest that the administration of selective COX-2 inhibitors and/or PAF receptor antagonists may be clinically useful to suppress PUVA-induced acute and chronic phototoxicity. Importantly, in a long-term study in hairless mice, the PAF antagonist PCA-4248 and COX-2 inhibitor SC-236 not only reduced PUVA-induced chronic inflammation and hyperplasia but also suppressed carcinogenesis (P. Wolf, D.X. Nghiem, J.P. Walterscheid, S. Byrne, H.N. Ananthaswarmy, and S.E. Ullrich, unpublished preliminary data). In view of the detected anti-apoptotic effect of PAF inhibition, this anti-carcinogenic effect is intriguing but may be attributable to simultaneous reduction of inflammation and/or immune suppression after PAF antagonist application. Interestingly however, PUVA and solar-simulated UV exposure led to significant inflammation (as measured by skin swelling) with no difference between PAF receptor knockout mice and their wild-type littermates for both treatments (Figure 5c). This seems to be a general phenomenon (not only related to PUVA and UV) because a similar result was observed when we used PMA to induce inflammation in a control experiment. However, taken together with the results of the studies with the PAF receptor antagonist PCA-4248

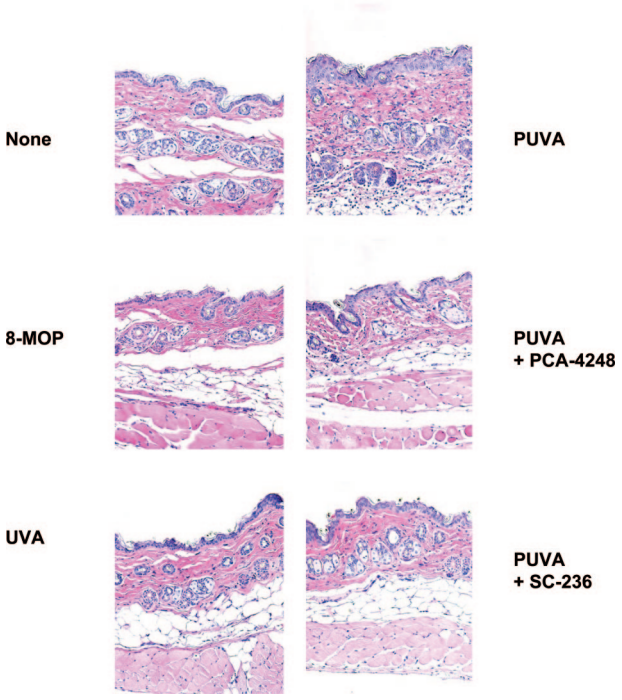

Figure 6. PAF pathway blockade does reduce PUVA-induced skin inflammation and other alterations on the microscopic level. Like macroscopic skin inflammation (ie, skin swelling), the inflammatory skin infiltration was also maximum at 48 hours after PUVA exposure (data shown), as examined on the microscopic level in H&E stainings. PUVA-induced skin infiltration and hyperplasia were greatly diminished in C3H mice injected intraperitoneally immediately before PUVA exposure with the PAF receptor antagonist PCA-4248 or the COX-2 inhibitor SC-236. The drug effects are quantified in Table 1. Treatment of mice with UVA or 8-MOP alone did not lead to significant levels of inflammation or hyperplasia, neither on the macroscopic nor on the microscopic level.

and the COX-2 inhibitor SC-236 in C3H mice (Figure 5b), this indicated that inflammation is less susceptible than immune suppression to blockade of the PAF pathway.

A better understanding of PUVA's mechanisms is of great clinical importance because it is a highly effective clinical treatment for a large number of diseases and remains the best therapeutic modality for early stages of cutaneous T-cell lymphoma[44] and severe forms of psoriasis, despite the introduction of new treatments such as the biologics.[32,45,46] For instance, we have recently shown that PUVA treatment resulted in the reduction of psoriasis activity severity index of greater than 75% (now considered as gold standard of therapeutic response) in more than 90% of patients with psoriasis,[14] a result, which is hardly obtained with any other type of antipsoriatic treatment. Therefore it seems highly desirable to investigate how the agents described here affect PUVA's therapeutic efficacy using newly available animal models of psoriasis[47–51] and eczema.[52] A better understanding of PUVA's mechanisms may not only offer the opportunity to dissect the beneficial (ie, therapeutic) effects from the detrimental (ie, carcinogenic) side effects[53,54] but also lead to the development of new drugs (eg, using the PAF pathway) acting like PUVA but having less side effects.

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