



8-Methoxypsoralen Plus Ultraviolet A Reduces the Psoriatic Response to Imiquimod in a Murine Model

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The effects of 8-Methoxypsoralen plus ultraviolet A (PUVA) or ultraviolet B (UVB) alone on imiquimod-induced psoriasis were examined in a mouse model. Mouse skin was treated with repetitive sub-phototoxic doses of PUVA or UVB before or during the induction of toll-like receptor 7/8 activation and psoriasis through the application of imiquimod. PUVA, to a greater degree than UVB, suppressed the established imiquimod-induced psoriatic phenotype, but pretreatment with PUVA prior to administration of imiquimod also reduced the susceptibility of murine skin to respond to imiquimod to a greater degree than did pretreatment with UVB. PUVA downregulated baseline levels of miR-NA27a and 29a, as well as interferon- γ , interleukin-17 and -9, cytokines, which drive psoriatic inflammation. Microarray analysis showed enrichment of senescence pathway genes linked to upregulation of p16/p21 proteins after PUVA pretreatment. However, the anti-psoriatic effect of PUVA was lost when there was an interval of 7 days between final exposure to PUVA and the start of administration of imiquimod. This indicated that (UVB and) PUVA diminished imiquimod-induced established psoriatic inflammation, but also primed the skin in favour of a reduced responsiveness to toll-like receptor activation.

Key words: psoriasis; PUVA; UVB; imiquimod; interleukin-9, senescence.

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8-Methoxypsoralen plus ultraviolet A (PUVA) or ultraviolet B (UVB) alone are well-established and effective phototherapeutic treatments for mild to severe psoriasis (1–6). However, the exact therapeutic mechanism(s) of phototherapy in psoriasis is unknown (7, 8). Phototherapy has both proapoptotic and immunomodulatory actions, which may, singly or together, be responsible for its therapeutic efficacy (9, 10). Exposure to UV radiation (with or without psoralen photosensitization) is immunosuppressive at the local and even the systemic level, depending on the dose administered (11–14). Similar to biologics, several studies have indicated

that UVB and PUVA photochemotherapy downregulate the interleukin (IL)-23/T-helper (Th)17 axis, which is thought to be the essential driver in the pathophysiology of psoriasis (15–19). However, a mathematical model has suggested that keratinocyte apoptosis and elimination alone may be responsible for the therapeutic effects of UVB treatment (20).

The aim of the current study was to investigate the mechanism of phototherapy in an imiquimod (IMQ) psoriasis model (21). Topical application of IMQ on the skin of humans and mice induces inflammation and pathology, which closely resembles plaque-type psoriasis (22). The immunomodulatory effects of IMQ are predominantly attributed to stimulation of toll-like receptor (TLR) 7/8 on plasmacytoid dendritic cells (pDCs). This IMQ-induced inflammation leads to production of large amounts of type I interferons (IFNs) from pDCs, along with interleukin (IL)-23 and IL-17RA signalling and IL-22. Thus, the IMQ mouse model enables research into the development and persistence of psoriasis, which would be difficult in patients (23). To date, the IMQ model has helped to increase our knowledge of the early pathophysiology of psoriasis (21). The action of tumour necrosis factor (TNF) antagonists (including adalimumab, etanercept and infliximab), and other important anti-cytokine antibodies, such as anti-IL-17 mAb, as well as IL-4, rapamycin, and narrow-band ultraviolet B (NB-UVB) have been examined with this model (19, 24–28).

MATERIALS AND METHODS

Animals and psoriatic model

BALB/c mice were purchased from Charles-River, Sulzfeld, Germany and housed in the animal facility of the Centre for Medical Research, Medical University of Graz, Austria. All procedures to which the mice were subjected were approved by the Austrian Government, Federal Ministry for Science and Research (protocol number BMWF-66-010/0032-11/3b/2013). Female mice aged 7–8 weeks had their back skin shaved 48 h before the start of the study. Aldara® (IMQ) 5% cream (MEDA Pharmaceuticals, Vienna, Austria) was used to induce psoriatic skin changes on the shaved back of the mice, as described previously (28). A total amount of 62.5 mg IMQ per treatment and mouse was applied in the morning whereas PUVA or UVB treatment, when given on the same days, was administered in the afternoon at an interval of 6 h after IMQ administration. All animals were maintained with alternating 12-h light-and-dark cycles and at controlled temperature and humidity

in facilities approved by the Austrian Government. Water and food were provided *ad libitum*. Mice were euthanized with an overdose of isoflurane and all efforts were made to minimize suffering.

PUVA and UVB treatment and determination of MPD

8-Methoxypsoralen (8-MOP) plus ultraviolet A (PUVA) or ultraviolet B (UVB) alone were examined in the mouse model. Groups of mice were painted on their backs with 200 μ l 8-methoxypsoralen (8-MOP) (Sigma-Aldrich, St Louis, MO, USA) in ethanol (0.1 mg/ml) or vehicle (95% ethanol) or were left untreated. The mice were then kept for 15 min in individual compartments of standard cages to allow penetration of 8-MOP prior to irradiation with UVA.

UVA irradiation was performed using a Waldmann UV236A irradiation system, equipped with 2 fluorescent PL 36W/09-PUVA light tubes (emission range 315–400 nm; peak 365 nm; Waldmann Medizintechnik, Villingen-Schwenningen, Germany) at a mean irradiance of 8.55 mW/cm² at a distance of 15 cm from the dorsal skin of the mouse to the glass cover of the irradiation system (which was positioned upside down on top of the cage). Irradiance was monitored with a Waldmann PUVA photometer, calibrated for the irradiation system.

UVB radiation was performed using a Waldmann 236 light source, equipped with 2 Waldmann UV6 fluorescent tubes (emission range 280–360 nm; peak 320 nm) (29). This light source emitted a spectrum of approximately 40% UVB and 60% UVA radiation; 99% of the erythema-producing radiation of this light source was derived from the UVB region (280–320 nm), as determined by spectral measurement (MSS 2040 UV Spectral Radiometer, Fröndenberg, Germany) and calculation of the CIE-weighted erythema spectrum (29). The mean UVB irradiance of the lamp was 1.7 mW/cm², as measured by a Waldmann UV photometer with UV6 detector head, appropriate for the radiation device.

To determine the minimal phototoxic dose (MPD) of PUVA and UVB, kinetic and dose-response studies were performed in BALB/c mice, as described previously (15). The MPD on the back of the mice was 0.5 J/cm² for UVA after topical psoralen photosensitization and 0.4 J/cm² for UVB without photosensitization (Fig. 1). For repeated treatment of the mice, PUVA and UVB was given at a sub-phototoxic dose level of 0.25 J/cm² (mean exposure time 29 s) and 0.20 J/cm² (mean exposure time 120 s), respectively, twice a week for 2 weeks.

In order to elucidate the mechanism of phototherapy in psoriatic inflammation, PUVA and UVB pretreatment were used to investigate whether the therapeutic effect was direct or indirect. As outlined in the study plan (Fig. S1¹), PUVA pretreatment with a single exposure (Group VII) as well as PUVA or UVB pretreatment with 4 repetitive exposures over 2 weeks was given until 3 days (or more) before the start of an IMQ cycle over a period of 3 days (Groups II and IX). In order to study the duration of the effect of phototherapy, in certain study groups the start of IMQ treatment was delayed by 7 days after the final phototherapy exposure (Groups III and X). Detailed information about all other experimental procedures, reagents, and statistical analyses are given in Appendix S1¹.

RESULTS

Both PUVA and UVB treatment reduced psoriatic inflammation in the IMQ model when given simultaneously. Double skinfold thickness (DSFT) revealed that IMQ-induced psoriatic inflammation was suppressed when

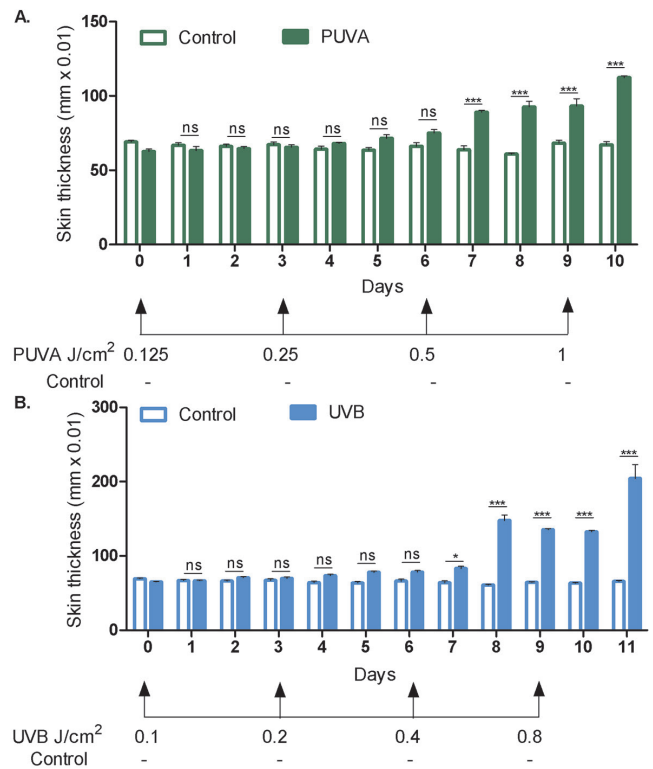


Fig. 1. Determination of the minimal phototoxic dose (MPD) of 8-methoxypsoralen plus ultraviolet A (PUVA) and ultraviolet B (UVB) treatment in BALB/c mouse skin. (A) Mice were treated topically with increasing doses, starting from 0.125 J/cm² to a maximum of 1 J/cm² for PUVA, and (B) 0.1 J/cm² to a maximum of 0.8 J/cm² for UVB. Data shown are from one representative experiment, with 5 mice per treatment group. Statistical differences were determined by 2-way analysis of variance (ANOVA) using Bonferroni post-test (* $p \leq 0.05$; *** $p \leq 0.001$).

2 cycles of IMQ and repetitive sub-phototoxic PUVA or UVB exposures were given simultaneously over the 16-day period (Fig. S2¹). During the first cycle of IMQ application psoriatic inflammation was maximally suppressed on day 5, by 90.5% and 54.8%, at 0.25 J/cm² and 0.5 J/cm² PUVA, respectively, and by 57.5% at 0.2 J/cm² UVB (Fig. S2C¹). After the second cycle of IMQ treatment the suppression (on day 16) was 67.1% and 80.8% at 0.25 J/cm² and 0.5 J/cm² PUVA, respectively, and 19.4% at 0.2 J/cm² UVB. Since the sub-phototoxic dose of 0.25 J/cm² PUVA showed overall greater anti-psoriatic efficacy than the phototoxic dose of 0.5 J/cm² PUVA, sub-phototoxic doses were used (at the level of 50% of the MPD, i.e. 0.25 J/cm² PUVA and 0.2 J/cm² UVB) in all subsequent studies.

In the pretreatment experiments, both PUVA and UVB significantly reduced the susceptibility of the skin to mount an inflammation due to IMQ (Fig. 2). Similar to the simultaneous protocol (Fig. S2¹), pretreatment with PUVA was superior to pretreatment with UVB in reducing this susceptibility (Fig. 2A, E and Table S1¹). The overall mean suppression of IMQ-induced psoriatic skin swelling after an interval of 3 days between the final pretreatment and the start of IMQ was 62.9% for PUVA and 22.6% for UVB (Table S1¹). However, with an interval

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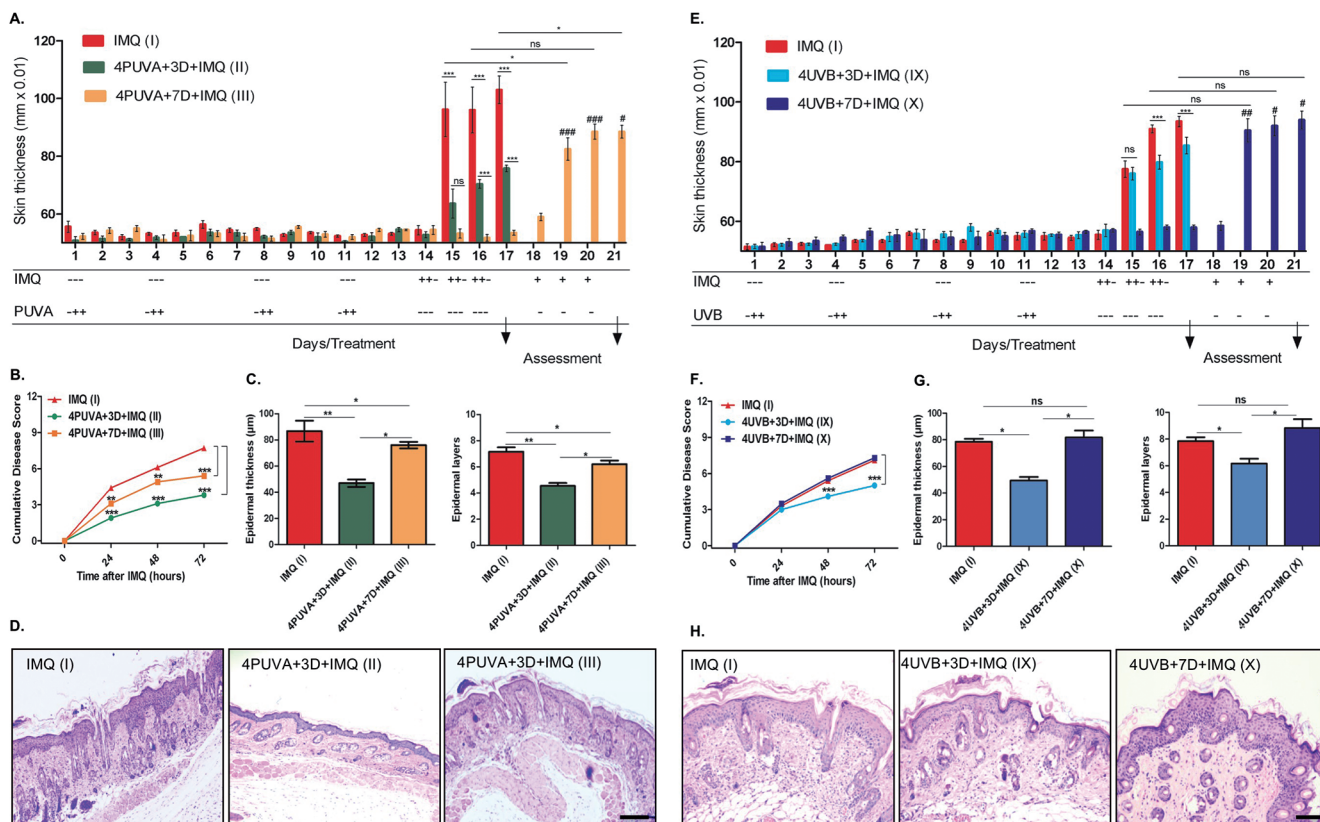


Fig. 2. Effect of phototherapy pretreatment on imiquimod (IMQ)-induced inflammation and hyperplasia. Double skinfold thickness (DSFT) measurement to quantify the change in IMQ-induced thickening of the skin after pretreatment with: (A) 8-methoxypsoralen plus ultraviolet A (PUVA) and (E) UVB. (B&F) Cumulative disease severity score based on erythema and scaling during the course of the experiment for PUVA and UVB. (C) Evaluation of histological features, such as epidermal thickness (μm) and epidermal layers from PUVA haematoxylin and eosin (H&E)-stained section and (G) UVB. Representative image of H&E-stained section from dorsal skin of a mouse pretreated with (D) PUVA and (H) UVB (scale bar: 200 μm). Data shown are from 1 representative experiment with 5 mice. Statistical differences between the groups for both studies were determined by 2-way analysis of variance (ANOVA) using Bonferroni post-test. (For PUVA, $*p < 0.05$; $**p \leq 0.01$; $***p \leq 0.001$ for Group I vs. Group II or Group III and $\#p < 0.05$; $##p \leq 0.001$ for Group III vs. Group II. While for UVB, $*p < 0.05$; $***p \leq 0.001$ and $\#p < 0.05$ for Group I vs. Group IX or Group X; $##p \leq 0.01$ for Group X vs. Group IX).

of 7 days between final UVB exposure and start of IMQ this therapeutic effect of PUVA was partially sustained, compared with the effect of UVB, which was entirely lost. After a 7-day gap, the overall mean suppression of psoriatic skin swelling was 22.6% for PUVA, whereas there was worsening of macroscopic skin swelling for UVB (with an overall negative value of 21.6% suppression in skin swelling) (Table SI¹). The effect of PUVA and UVB on overall skin thickness was paralleled by the effect on cumulative disease score (similar to human PASI), as described previously (28) for IMQ-induced psoriatic inflammation (Fig. 2B, F).

These data were consistent with the results of histological examination of the skin samples taken 24 h after the final administration of IMQ in the phototherapy pretreatment model (Fig. 2D, H and Table SI¹). Application of IMQ led to epidermal hyperplasia with strong epidermal thickening, hyperkeratosis along with elongated rete ridges and parakeratosis and cellular infiltration of the dermis. The mean epidermal thickness was 18.4 μm in control mice (data not shown) vs. up to 78.6 μm and 86.7 μm in IMQ-treated mice, respectively (Fig. 2C, G). The mean number of epidermal layers was

1.3 in control mice (data not shown) vs. up to 7.2 and 7.8 layers in IMQ-treated mice in the experiment shown in Fig. 2C, G, respectively. The overall mean suppression of IMQ-induced epidermal hyperplasia was 58.1% and 48.5% for PUVA and UVB (reduction in skin thickness to 46.9 and 49.4 μm , respectively), as measured by epidermal thickness, and 45.3% and 25.3%, as measured by epidermal layers (Table SI¹). Similar to the effect of PUVA on overall macroscopic skin thickness, the effect of PUVA in reducing epidermal hyperplasia was partially sustained, whereas the effect of UVB was entirely lost after a gap of 7 days between final UVB exposure and start of IMQ. With the 7-day gap, for PUVA the overall mean suppression of epidermal hyperplasia was 15.6% for epidermal thickness and 16.2% for the increase in epidermal layers, whereas for UVB there was worsening of epidermal hyperplasia (with overall negative values of 5.1% and 15.9%) (Table SI¹). Pretreatment with a single dose of PUVA (Group VII) also resulted in less IMQ-induced inflammation, but its effect was smaller than that after repetitive PUVA exposure (data not shown). Thus, overall, PUVA suppressed the IMQ-induced psoriatic phenotype to a greater degree than did UVB.

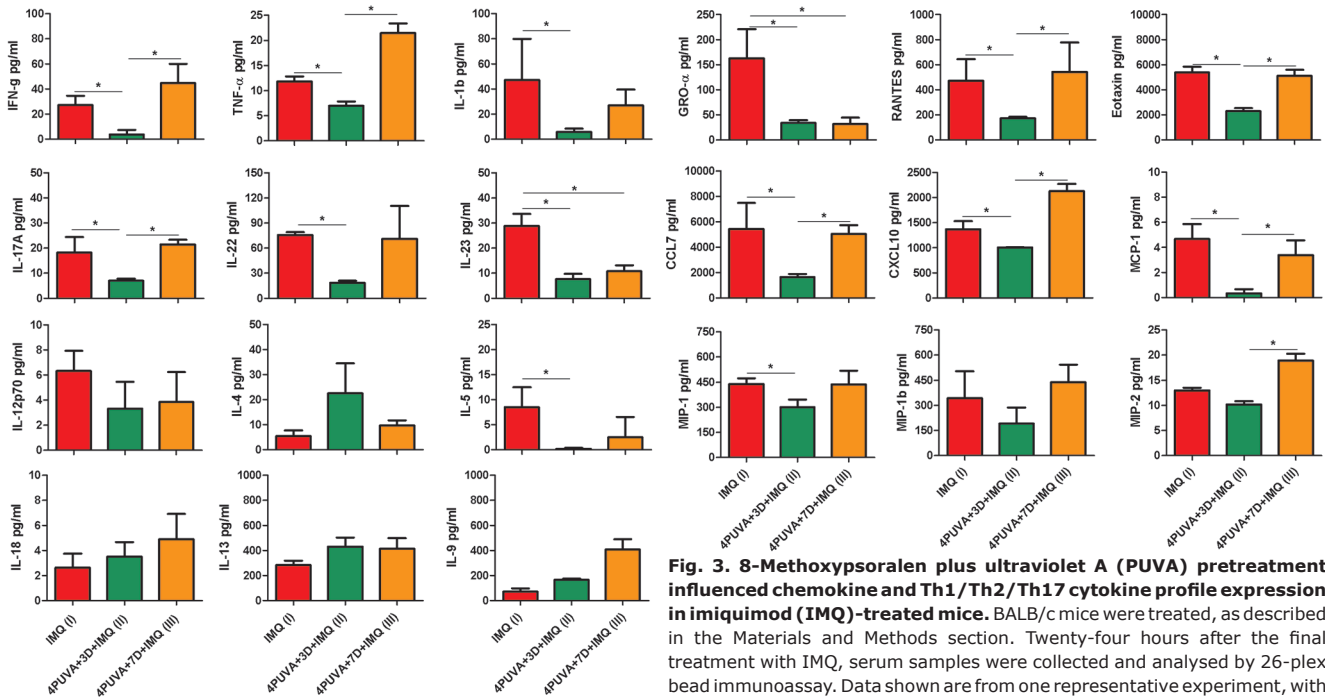


Fig. 3. 8-Methoxypsoralen plus ultraviolet A (PUVA) pretreatment influenced chemokine and Th1/Th2/Th17 cytokine profile expression in imiquimod (IMQ)-treated mice. BALB/c mice were treated, as described in the Materials and Methods section. Twenty-four hours after the final treatment with IMQ, serum samples were collected and analysed by 26-plex bead immunoassay. Data shown are from one representative experiment, with 5 mice. Statistical analysis was performed using unpaired *t*-test ($*p < 0.05$).

Furthermore, the effect of PUVA pretreatment on the systemic Th1/Th2/Th17 cytokine and chemokine expression profile in IMQ-treated mice was evaluated. A downregulatory effect of PUVA pretreatment on the serum levels of cytokines of the Th1 and IL-23/Th17 axis was identified, including IFN- γ , IL-1 beta, TNF-alpha, IL-17A, IL-22, IL-23 as well as chemokines such as GRO alpha, IP-10, MCP-1, MCP-3, MIP-1 alpha, MIP-1 beta, MIP-2, RANTES and eotaxin (Fig. 3). In contrast, there was an ambivalent response of Th2 cytokines with a trend of upregulation of IL-4 ($p = 0.057$) and downregulation of IL-5 ($p = 0.05$) after PUVA treatment. For most cytokines (except IL-23 and GRO-alpha) the effect of PUVA was lost when they were measured at the end of the experiment with an interval of 7 days between final PUVA and first IMQ exposure. IL-2, IL-6, IL-10, IL-27 and GM-CSF levels were below the detection limit of the immunobead assay in all samples. This systemic effect of PUVA on cytokine levels was paired with its effect on spleen size by reducing splenomegaly (data not shown). We also studied the cytokine and chemokine levels after PUVA treatment (before the start of IMQ treatment) and observed a significant downregulation of IFN- γ , IL-17A, IL-9, and upregulation of GRO-alpha compared with untreated controls (Table I).

To assess the gene signature development due to PUVA pretreatment, we performed microarray analysis of the RNA from dorsal skin for the samples, as outlined in the Materials and Methods section. As shown in Fig. 4, pretreatment with PUVA significantly altered the expression of 30 genes that were identified based on the fold change of >1.5 and a p -value of <0.05 in the array.

Those genes included *Col1A1*, *Col1A2*, *Col3A1*, *Col5A1*, *Col5A2*, *Col6A3*, *Col6A2*, *TIMP1* and *MMP13*, which were overexpressed compared with untreated controls (Group VIII) and Group VI (Fig. 4). It is notable that the

Table I. Change in serum levels of cytokines and chemokines due to pretreatment with 8-methoxypsoralen plus ultraviolet A (PUVA) before exposure to imiquimod in a murine model

Cytokine/chemokine	Serum levels, pg/ml		
	Control (VIII) Mean \pm SEM	4PUVA+3D (V) Mean \pm SEM	<i>p</i> -value
IFN- γ	8.9 \pm 1.1	1.2 \pm 4.2	0.03
IL-17A	26.2 \pm 14	0 \pm 1	0.05
IL-9	63.3 \pm 18	0 \pm 0	0.05
GRO-alpha	12.3 \pm 10.7	34 \pm 9.6	0.05
Eotaxin	3,620 \pm 1,219	6,067 \pm 1,373	0.11
IL-23	0.17 \pm 0.29	0.83 \pm 0.76	0.13
IL-13	13.4 \pm 20.6	32 \pm 16.8	0.20
IL-27	219 \pm 74.4	95 \pm 139	0.20
IP-10	978 \pm 324	618 \pm 113	0.23
MCP-3	2,886 \pm 1,805	1,248 \pm 755	0.23
IL-18	7.9 \pm 5.9	4.2 \pm 3.1	0.24
MCP-1	13.3 \pm 10.7	4.7 \pm 2.1	0.27
IL-22	10.1 \pm 5.6	7.2 \pm 6.3	0.31
MIP-2	29.1 \pm 8	36.8 \pm 5.5	0.40
IL-4	8.3 \pm 6	5.3 \pm 1.5	0.43
IL-5	11.3 \pm 8.2	43.8 \pm 41.6	0.50
IL-1 beta	1.8 \pm 1.3	2.8 \pm 2.1	0.59
MIP-1 beta	25 \pm 8.2	29 \pm 3.3	0.70
RANTES	285.2 \pm 177	378 \pm 55.8	0.70
IL-12p70	3.8 \pm 1.5	3 \pm 1.8	0.82
TNF-alpha	11.7 \pm 7.5	8 \pm 4.5	0.86
MIP-1 alpha	214.6 \pm 58.3	226.8 \pm 37.3	0.86

Control (VIII) vs. 4PUVA+3D (V) samples were analysed using multiplex bead immunoassay. Entries represent values of detected cytokines and chemokines. IL-2, IL-6, IL-10 and GM-CSF were not detected by the assay. *p*-values were determined using Mann-Whitney *U* test. SEM: standard error of the mean; IFN: interferon; IL: interleukin; GRO-alpha: growth-related oncogene-alpha; IP-10 inducible protein 10; MCP: monocyte-chemotactic protein; MIP: macrophage inflammatory protein.

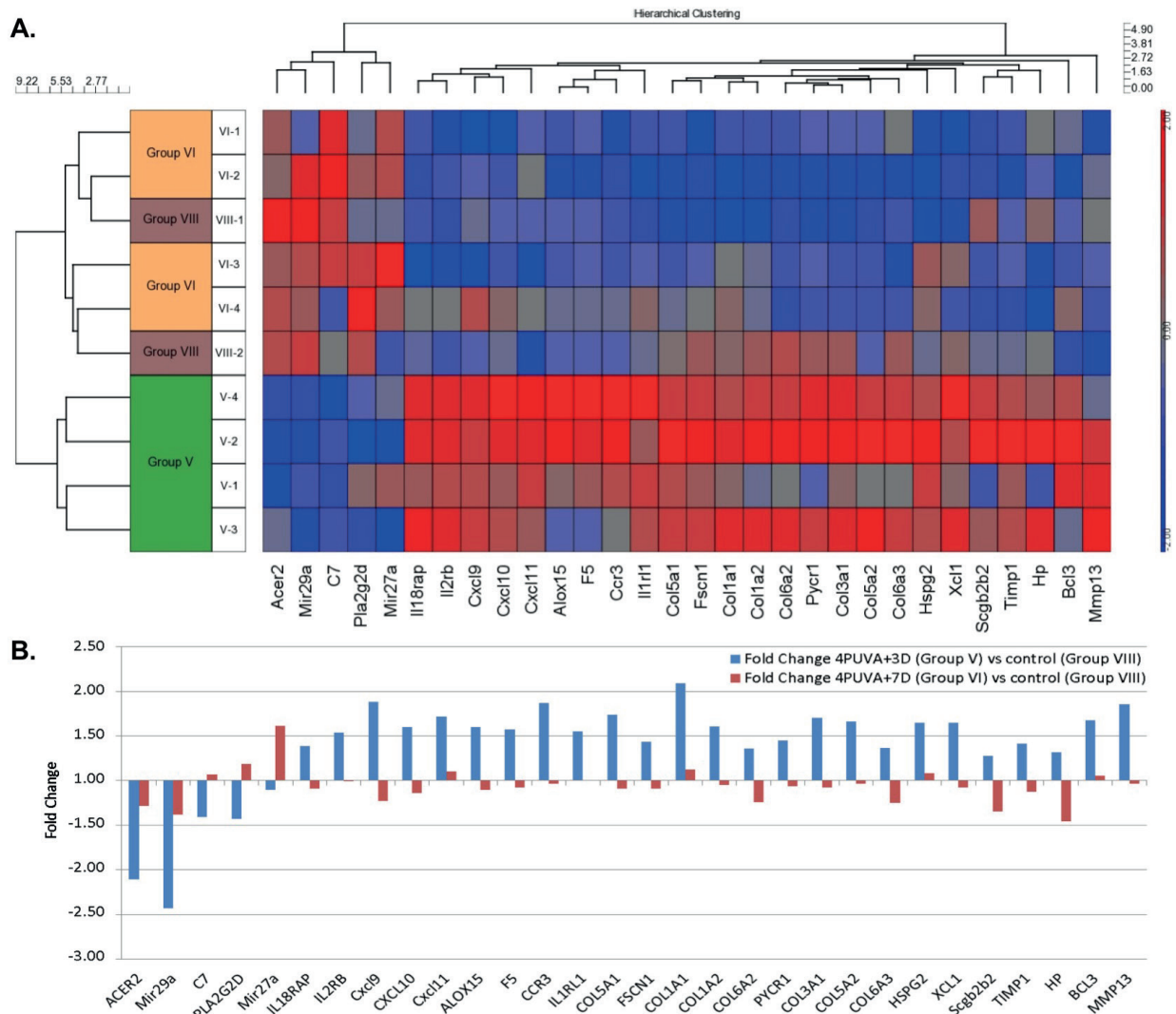


Fig. 4. Heat map of gene array expression with changes in molecular signature due to pretreatment with 8-methoxypsoralen plus ultraviolet A (PUVA). (A) Genes identified on the basis of the fold change of >1.5 and a p -value <0.05 are plotted. Heat map of expression values for 30 genes is shown by 2-way clustering of expression levels for Group V (4PUVA+3D) compared with Group VI (4PUVA+7D) and Group VIII (Control) (2 outlier samples from Group VIII had to be excluded due to hair cycling) ($n=4$ per group). Red denotes increased relative gene expression levels for the indicated genes per mouse and group, while blue denotes decreased expression levels. (B) Bar diagram indicates the fold changes in gene expression after PUVA pretreatment in comparison with controls (Group VIII). Statistical analysis for this data set was performed using Partek Software v.6.6, as outlined in the Materials and Methods section.

majority of these genes belong to collagen (Col), matrix metalloproteinase (MMPs) and tissue inhibitor of metalloproteinase (TIMP 1) families, which are associated with senescence and apoptosis. In contrast, alkaline ceramidase 2 (*Acer2*), which mediates cell proliferation, differentiation, apoptosis, adhesion, and migration, belonging to the sphingosine and sphingosine-1-phosphate (S1P) metabolism pathway (30), and miRNA-29a, which is a negative regulator of *TIMP 1*, were down-regulated (31). The fold-change bar diagram in the lower part of Fig. 4 highlights the differences in the expression of the 30 genes (normalized to control) 3 days after the final PUVA treatment of Group V, compared with Group VI, which had a 7-day gap between final PUVA exposure and collection of skin samples.

To confirm the activation of the senescence pathway, immunohistochemistry was used to quantify the expression of the key upstream markers p21 and p16 in the dorsal skin of the mice. The presence of p21 in the basal layer of the epidermis was observed in all treatment groups (Fig. 5). PUVA treatment increased the expression of p21 in the entire epidermis, and in the dermis mainly in epithelial cells of hair follicles. Similarly, PUVA treatment also increased the expression of p16 in the epidermis and dermis. In contrast, UVB pretreatment did not significantly affect the expression of p21 and p16 protein in the skin (data not shown). As outlined in the graphic panel of Fig. 5, the increase in senescence after PUVA was lost in mice that had a 7-day gap between final PUVA exposure and collection of tissue samples.

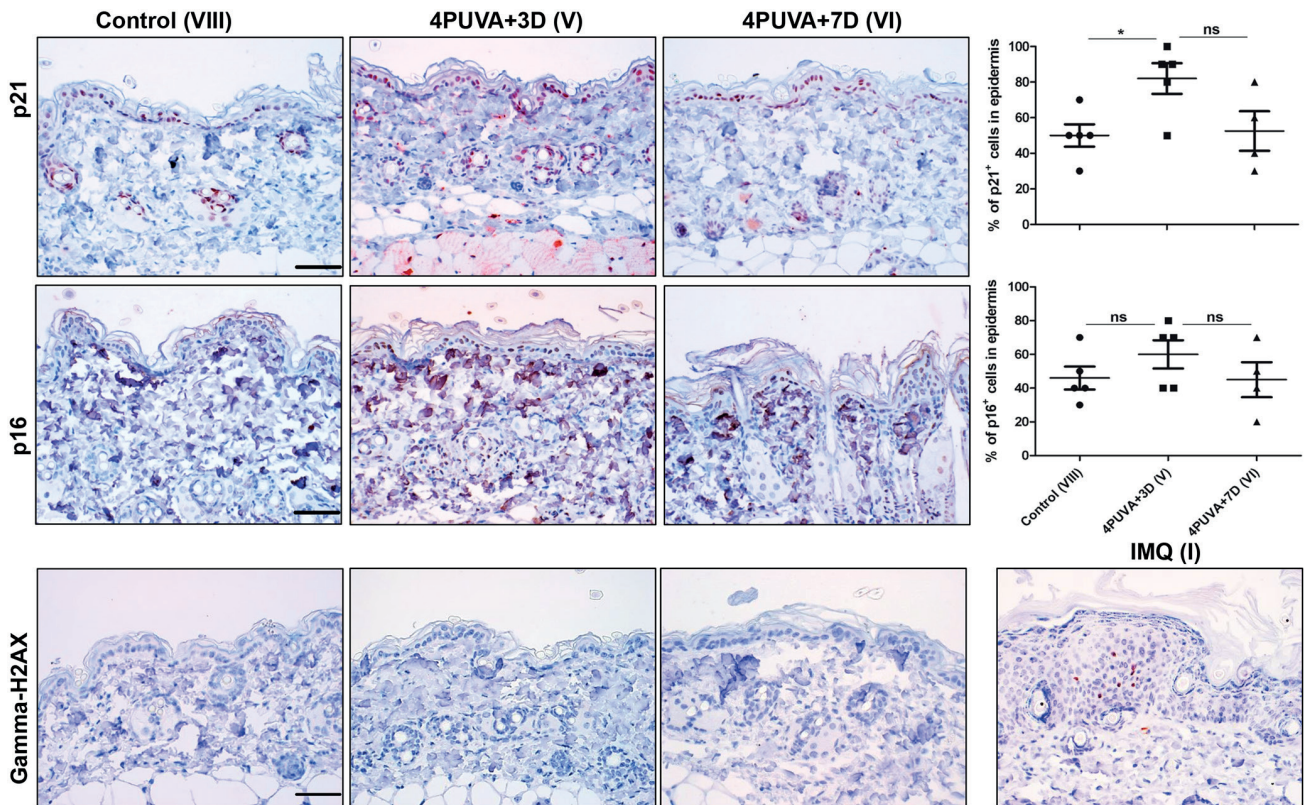


Fig. 5. Expression of p21/p16 and γ H2AX associated with cellular senescence in 8-methoxypsoralen plus ultraviolet A (PUVA)-pretreated skin. Representative skin sections for p21, p16 and γ H2AX staining in 4PUVA+3D (Group V) and 4PUVA+7D (Group VI) is shown in comparison with untreated control (Group VIII) with 4–5 mice per group. Magnification 20 \times , scale bar 100 μ m for p21 and p16 and magnification 40 \times , scale bar 50 μ m for γ H2AX. Semi-quantitative scoring for p21 and p16 expression was performed as outlined in the Materials and Methods section. Statistical analysis was performed using unpaired *t*-test ($*p < 0.05$).

It is notable that staining for the phosphorylated form of H2AX, called γ -H2AX, as p53/p21 upstream regulator (32), was completely absent (Fig. 5) in PUVA-pretreated skin (before the start of IMQ treatment), while it was present in clusters in IMQ-treated skin (as positive control) (33).

In addition, microarray data (Fig. 4) indicated that PUVA pretreatment at a sub-phototoxic level upregulated *Fscn1*, a gene related to dendritic cell maturation. Thus, we performed multiplex immunohistochemical staining for dendritic cell maturation using MHC-II expression as the marker in the skin samples taken after PUVA pretreatment, but did not detect any statistically significant differences among the different treatment groups (data not shown).

DISCUSSION

Elucidation of the cellular networks and molecular signals that promote the development of psoriasis depends on the use of appropriate animal models. Regardless of the fundamental differences in the immune response, cellular interaction and function of the skin between humans and mice, there are indisputable benefits of *in vivo* models of psoriasis (34). The repetitive application of IMQ re-

veals a complex biological outcome involving multiple cell types, cytokines, and inflammatory pathways, and represents an acute model of skin inflammation that captures the initial stages of development of psoriasis. During the initial phase, application of IMQ instigates erythema and infiltration of the skin, which reaches a maximum after the final day of repetitive application of IMQ (35). The skin of different strains of mice may exhibit different levels of susceptibility to environmental stimuli including UV radiation (36, 37); thus, we initially identified the MPD in BALB/c mice in this series of experiments (Fig. 1) and subsequently mainly used sub-inflammatory doses in the study. The PUVA MPD (0.5 J/cm²) was identical to that determined in a previous study for wild-type controls with ICR (CD-1R) background of the K5.hTGF- β 1 psoriasis model, in which we also investigated the anti-psoriatic effect of PUVA (15). As in the previous study with K5.hTGF- β 1 mice (15), the lower PUVA dose of 0.25 J/cm² was more effective than the higher dose of 0.5 J/cm² in clearing psoriatic lesions. Overall, PUVA was able to suppress established IMQ-induced psoriatic inflammation in the simultaneous treatment protocol to a greater degree than UVB radiation (Fig. S2¹), but also pretreatment with PUVA (before administration of IMQ) was, to a greater degree than UVB

radiation, able to suppress the susceptibility of murine skin to mount psoriatic inflammation (Fig. 2). Since the antipsoriatic effect of PUVA in this study was, in general, greater than that of UVB (consistent with the results of clinical studies (2, 3, 38–41)) we focused on the former in investigating mechanistic aspects.

The effect of PUVA on IMQ-induced macroscopic psoriatic skin changes (Fig. 2A and B) was paired with a reduction in histological epidermal hyperplasia and inflammation (Fig. 2C and D) and downregulation of psoriatic cytokines of the Th1 and Th17 axis, including IFN- γ , TNF- α , IL-17, IL-23 and IL-22 (Fig. 3), consistent with the results of our previous study in K5.hTGF- β 1 transgenic mice (15). Moreover, repetitive pretreatment with PUVA downregulated the pro-psoriatic cytokines IFN- γ , IL-9 and IL-17 at baseline before the start of IMQ treatment (Table I). The effect of PUVA on IL-23 and GRO- α was sustained, as measured in Group III 10 days after final PUVA exposure and 24 h after final exposure to IMQ (Fig. 3). Since IL-23 is crucial for driving the IL-23/Th17 axis, and GRO- α (IL-8) is essential for growth promotion of keratinocytes (42), the inhibition of these cytokines was consistent with the partially sustained effect of PUVA in reducing macroscopic and microscopic inflammation of the skin and epidermal hyperplasia (Fig. 2C, D).

The observation that PUVA before the start of IMQ treatment induced the downregulation of IL-9 (Table I) is intriguing, since this cytokine is known to be produced transiently and prior to the activation-induced upregulation of other downstream inflammatory pro-psoriatic cytokines (43). IL-9 functions indirectly by regulating the induction of Th1/Th2/Th17 cytokines, such as IFN- γ , IL-5, IL-13 and IL-17 (43). In agreement with this, dermal injection of IL-9 enhanced Th17-related psoriasiform inflammation in K5.hTGF- β 1 transgenic mice and its blockade by anti-IL-9 antibody suppressed disease and expression of IL-17A (44). In the K5.hTGF- β 1 model, PUVA was effective in diminishing psoriatic inflammation, in parallel with strong downregulation of IL-9 (15, 44). It is notable that IL-9 and its receptor are expressed in the infiltrate of lesional skin in patients with psoriasis (43, 44), indicating its potential role in the pathophysiology of psoriatic skin. Moreover, the IL-9 axis has been shown to drive γ delta T-cell activation in patients with psoriatic arthritis (45).

High throughput genome microarray analysis showed enrichment of senescence pathway genes after pretreatment with PUVA. This was validated by staining of 2 CDKIs i.e. p21 and p16, which play a role in cell cycle arrest leading to senescence (46). It is notable that the staining for the phosphorylated form of H2AX (known as γ -H2AX), which is a variant of histone H2A in mammalian cells (phospho-Histone H2A.X (Ser139)) and a p53/p21 upstream regulator protein of DNA damaging responses (32), was completely absent in PUVA pretreated

skin, while it was present in clusters in the IMQ-treated epidermis (Fig. 5), consistent with previous studies (33). This was not entirely unexpected, since we collected the skin samples 3 days after the final exposure to PUVA, a time-point at which any PUVA-induced upregulation of γ -H2AX was mostly likely exhausted due to its short-life kinetics and rapid dephosphorylation, along with p53/p21 induction (32, 47, 48).

Cellular senescence requires functional p53 and pRB family proteins, both of which regulate growth signalling. The CDKI p21 belongs to the CIP/KIP family of CDKIs and is best known as an inhibitor of cell proliferation. p21 plays a complex role in apoptosis, differentiation and cancer biology (49) and treatment. *In vitro* studies have shown that PUVA treatment causes cellular senescence, leading to growth arrest of fibroblasts, with enlarged cytoplasm and increased synthesis of MMPs (50). These responses are mainly regulated by the 2 potent CDKIs p21 and p16, where the former is a direct target of p53 transactivation, while the latter is known to be induced by stress without DNA damage (33, 47, 48). The current study found that p21 was stronger and more intensely expressed in the dermis and epidermis after PUVA treatment (Fig. 5), paralleling its reducing effect on psoriatic hyperplasia and inflammation. Senescence is known to stimulate cytokines and chemokines, a result termed senescence-associated secretory phenotype (SASP). This phenotype can affect surrounding cells by activating cell-surface receptors and corresponding signal transduction pathways that lead to irreversible senescence induced by soluble signalling interleukins and chemokines (51), including IL-1, IL-5, IL-6, IL-13, GRO- α , GM-CSF, MIP-1 (α & β) and MIP-2 (52). While the levels of GRO- α were seen to be significantly stimulated after PUVA pretreatment, the cytokines (except INF- γ , IL-17A, and IL-9) were affected only insignificantly or remained undetectable (IL-6 and GM-CSF) in our studies (Table I).

MMPs and TIMPs are other products of genes associated with senescence and apoptosis (53). Elevated concentrations of MMPs have been found in the scales of psoriatic lesions, but their levels decreased in patients with more severe disease, consistent with inverse PASI correlation (54). Similarly, serum levels of TIMP-1 and TIMP-2 decreased with severity of psoriasis. In our study, PUVA-induced MMP gene expression (Fig. 4) was paralleled by improvement in the psoriatic skin phenotype. In addition, our microarray data indicated that PUVA downregulated miR-27a and miR-29a (Fig. 4), uncovering its correlation in the regulatory networks of PUVA. miRs are known as essential controllers of cell fate decisions in immune responses. They act by coordination and repression of multiple target genes. miR-27a has been identified as an inhibitor of Th2 cell differentiation and function, thereby limiting IL-4 production in T cells and the network that regulates IL-4

production (55). Knocking down miR-27a significantly downregulates expression levels of TNF- α and IL-6 via reducing the phosphorylation level of NF- κ B p65. Moreover, miR-27a neutralization with anti-miR-27a hairpin downregulates TNF- α expression, which is consistent with our cytokine results (Fig. 3) (56). In addition, the role of miR-29a is well documented in association with extracellular matrix (ECM) induction (31). miR-29a overexpression in murine hepatic stellate cells resulted in downregulation of collagen expression, thereby directly targeting the mRNA expression of ECM genes (57). Collagen-associated genes were significantly expressed, possibly due to downregulation of miR-29a. PUVA also downregulated the *Acer2* gene that regulates sphingolipid metabolism and the S1P signalling pathway (58). S1P has attracted attention for its effects in promoting cell proliferation and survival in psoriasis (30, 59, 60).

In conclusion, this study identifies the potential dual action of phototherapy. Simultaneous treatment, in particular with PUVA, was able to suppress established psoriatic inflammation. In addition, pretreatment with phototherapy (before administration of IMQ) was able to suppress the susceptibility of murine skin to mount a psoriatic inflammation. This action of phototherapy may, in addition to the direct elimination of keratinocytes (20) and lymphocytes (61), be relevant in humans, because it suppresses the continuous response to endogenous or exogenous psoriatic triggers that act through the TLR pathway, which is responsible for the initiation and persistence of the disease (62). The current mechanistic study indicates that PUVA-induced senescence, along with downregulation of IL-9, IL-17 and IFN- γ , may make cells less susceptible to stimulation by IMQ, resulting in less psoriatic inflammation and hyperplasia.

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