8-Methoxypsoralen Plus Ultraviolet A Therapy Acts via Inhibition of the IL-23/Th17 Axis and Induction of Foxp3+ Regulatory T Cells Involving CTLA4 Signaling in a Psoriasis-Like Skin Disorder

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8-Methoxypsoralen Plus Ultraviolet A Therapy Acts via Inhibition of the IL-23/Th17 Axis and Induction of Foxp3+ Regulatory T Cells Involving CTLA4 Signaling in a Psoriasis-Like Skin Disorder

Tej Pratap Singh,* Michael P. Schön,†,‡ Katrin Wallbrecht,‡ Kai Michaelis,‡ Beate Rinner,§ Gerlinde Mayer,* Ulrike Schmidbauer,* Heimo Strohmaier,§ Xiao-Jing Wang,†,§,#,** and Peter Wolf*

To elucidate the molecular action of 8-methoxypsoralen plus UVA (PUVA), a standard dermatological therapy, we used K5.hTGF-β1 transgenic mice exhibiting a skin phenotype and cytokine abnormalities with strong similarities to human psoriasis. We observed that impaired function of CD4+CD25+ regulatory T cells (Tregs) and increased cytokine levels of the IL-23/Th17 pathway were responsible for the psoriatic phenotype in this mouse model. Treatment of K5.hTGF-β1 transgenic mice with PUVA suppressed the IL-23/Th17 pathway, Th1 milieu, as well as transcription factors STAT3 and orphan nuclear receptor RORγt. PUVA induced the Th2 pathway and IL-10–producing CD4+CD25+Foxp3+ Tregs with disease-suppressive activity that was abolished by anti-CTLA4 mAb treatment. These findings were paralleled by macroscopic and microscopic clearance of the diseased murine skin. Anti–IL-17 mAb treatment also diminished the psoriatic phenotype of the mice. This indicated that both induced Tregs involving CTLA4 signaling and inhibition of the IL-23/Th17 axis are central for the therapeutic action of PUVA. The Journal of Immunology, 2010, 184: 7257–7267.

Despite the introduction of new therapeutic strategies such as TNF-α inhibiting biologics (1, 2) or anti–IL-12/23 Ab (3) treatment, 8-methoxy psoralen plus UV A (PUVA) photochemotherapy and other forms of phototherapies have kept their prominent places in the dermatological treatment armamentarium of psoriasis and many other skin diseases (4). However, the mechanisms by which PUVA leads to clearance of skin lesions are still not well understood. For many years, the interest in the molecular effects of PUVA has mainly focused on the binding of psoralen to DNA, in which pyrimidine bases are the main targets for a photochemical reaction (5). Thus, similar to DNA-alkylating agents, PUVA was thought to be effective mainly by a direct antiproliferative effect on skin cells through apoptosis (e.g., induced by p53 and Fas ligand interaction [6]). However, similar to UV radiation (7), PUVA has also been known for a long time to have profound immunosuppressive properties, potentially initiated through cytokine release after both DNA and/or cell membrane alterations (8). For instance, Vallat et al. (9) have shown that PUVA can exert distinct suppressive effects on infiltrating immune cells in psoriatic skin, with virtually total elimination of IL-2R+ T cells in some patients. In comparison with the immunosuppressive agent cyclosporine (another treatment option for psoriasis), PUVA has been found to have more complete reversal of pathological epidermal and lymphocytic activation, changes proposed to be the cellular basis of the sustained remission of psoriatic disease after PUVA treatment.

To elucidate the therapeutic and immunologic mechanisms of PUVA, we used K5.hTGF-β1 transgenic mice (10), which develop scaly erythema and inflammatory skin lesions, similar to the appearance of psoriasis in human patients. K5.hTGF-β1 overexpression leads to multiple molecular changes and cytokine abnormalities that typically occur in human Th1-type inflammatory skin disorders such as psoriasis (11). The skin lesions in K5.hTGF-β1 transgenic mice are characterized by epidermal hyperproliferation, massive infiltration of neutrophils, CD4+ lymphocytes, and macrophages to the epidermis and superficial dermis, subcorneal microabscesses, basement membrane degradation, and increased angiogenesis. K5.hTGF-β1 transgenic mice exhibit a more severe psoriasis-like phenotype than other transgenic models, in which individual growth factors or cytokines are targeted in the skin (12), rendering this model very attractive to study antipsoriatic therapies. Indeed, therapeutic approaches effective for human psoriasis, such as TNF-α antagonist etanercept or peroxisome proliferator-activated receptor γ agonist rosiglitazone, have been shown to be effective in alleviating the skin symptoms of K5.hTGF-β1 transgenic mice (13).
The IL-23/Th17 axis has been linked to autoimmune conditions such as psoriasis (14) and exerts many proinflammatory effects in a wide variety of cells, including keratinocytes, macrophages, and endothelial cells. Downstream effects of IL-17 include production of IL-1, IL-6, IL-8, TNF-α, G-CSF, and GM-CSF, as well as anti-microbial peptides (15). However, TGF-β is critical for generation of CD4+CD25-Foxp3+-induced regulatory T cells (iTregs) and controls Th17 immunity in the presence of IL-6 (16–18). Phenotypically Tregs express IL-2Rα (CD25), cell survival factor glucocorticoid-induced tumor necrosis factor receptor, and the T cell activation marker CTLA4, which is crucial for the maintenance and/or suppressive function of these cells (19). We therefore investigated whether PUVA treatment of K5.hTGF-β1 transgenic mice clears psoriasis-like phenotype, and if so, the underlying mechanisms engage inhibition of the inflammatory IL-23/Th17 axis and iTregs involving CTLA4 signaling.

Materials and Methods

Animals

K5.hTGF-β1 transgenic mice expressing wild-type (lentig) TGF-β1 were generated as described previously (10). Mice were crossed to the Icr:ICR(CD-1R) background. All animals were maintained with alternating 12-h light-and-dark cycles and controlled temperature and humidity in facilities approved by the Austrian government. Food and water were provided ad libitum. All animal procedures were approved by the Austrian government, Federal Ministry for Science and Research, through protocol numbers BMBWK-66.010/0034-BrGT/2006 and 66.010/79-C/GT/2007. Mice were 12–16 wk old at the start of the experiment and were age- and sex-matched within each experiment.

PUVA treatment

The backs of the mice were shaved 1 d before PUVA treatment with an electric clipper. The remaining hair was then removed by the application of a commercial depilation cream for 3–5 min (Depilan sensitive) and subsequent cleaning with water. Groups of mice were painted on their backs with 200 μl 8-methoxypsoralen (8-MOP) (Sigma-Aldrich, St. Louis, MO) in ethanol (at a concentration of 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. PUVA irradiation was performed using a Waldmann UV236A irradiation system carrying two 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 of the irradiation system positioned upside down on the top of the cage. Irradiance was monitored by a Waldmann PUVA photometer, calibrated for the irradiation system. During UVA irradiation, the mice were housed individually per cage. To determine the minimal phototoxicity of PUVA treatment, we performed kinetic and dose-response studies in K5.hTGF-β1 transgenic mice and wild-type (WT) controls as previously described (15). As control, an isotype-matched Ab (rat IgG Ab; R&D Systems) was used in other strains of mice (8). Those studies revealed that maximum transgenic mice and wild-type (WT) controls as previously described.

Histologic examination

Epidermal hyperplasia was assessed by counting epidermal cell layers and measuring the thickness of the epidermis from the basal corneum with the calibrated eyepiece micrometer of a microscope. Epidermal cell layers were counted at 10–15 randomly selected consecutive microscopic fields (at final magnification, ×200). For quantification of epidermal thickness, 10 randomly selected measurements per H&E-stained cross-section of dorsal or ear skin from each mouse were performed. Semi-quantitative scoring of the inflammatory infiltrate in the upper or lower dermis was performed as follows: 0, within normal limits; 1, mild; 2, moderate; and 3, severe at 10–15 randomly selected areas per section (at final magnification, ×100). All measurements were performed in a blinded manner. Results were first averaged per mouse and then averaged per treatment group for statistical analysis. Images were acquired by using a DP71 digital camera (Olympus, Melville, NY) attached to an Olympus BX51 microscope.

Immunohistochemistry and immunofluorescence

Stainings were performed on paraffin-embedded sections of mouse dorsal skin with monoclonal anti-human TGF-β (clone 1D11; R&D Systems) (1/50), monoclonal anti-mouse Foxp3 (clone 2A11G9; Abcam, Cambridge, U.K.) (1/100), monoclonal rat anti-mouse IL-10 (clone NYR-mL10; Abcam) (1/50), polyclonal rabbit anti-mouse STAT3 (Abcam) (1/50), or polyclonal rabbit anti-mouse IL-23p19 (Abcam) (1/500) Ab. Briefly, primary Ab was applied after pretreatment of the sections with EDTA at pH 8 (for anti–IL-10, anti–TGF-β, and anti–IL-23p19 staining) or citric acid pH 6 (for anti–IFN-γ staining). Dako Multilink System (biotinylated polyclonal swine anti-goat, mouse, rabbit IgGs, code E 0553) at a concentration of 1/50 and Dako REAL Detection System, Peroxidase/AEC, Rabbit/Mouse (code K5003) (DakoCytomation, Carpinteria, CA) were used for Ab detection (for anti–TGF-β, anti–IL-23p19, and anti–Foxp3 staining), according to the manufacturer’s instructions. For immunofluorescent IL-10 and STAT3 staining, rabbit anti-rat IgM FITC (clone MAR54-R; Abcam) (1/100) and goat anti-rabbit IgG FITC (Abcam) (1/100), respectively, were used as secondary Ab. Coverslips were mounted onto the slides using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were acquired by using a DP71 digital camera (Olympus) attached to an Olympus BX51 microscope.

Bead immunoassay

Mouse Cytokine/Chemokine Luminex bead immunoassay kit, LINCOplex, 22 Plex from Millipore (Bedford, MA) was used to measure the levels of cytokines and chemokines in serum and tissues. The concentrations of the following soluble mediators were measured: G-CSF, GM-CSF, IL-1α, IL-1β, IL-4, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17, IFN inducible protein-10 (IP-10), keratinocyte-derived chemokine (KC), MCP-1, RANTES, TNF-α, IFN-γ, IL-2, IL-5, IL-7, IL-15, and MIP-1α. Each sample was measured in duplicate according to the manufacturer’s specifications. Standard curves for each analyte were generated using the reference analytic concentration supplied by the manufacturer. In addition to the serum measurement, was performed using the Bio-Plex system in combination with Bio-Plex Manager software, version 4.1, using five-parametric curve fitting (Bio-Rad, Hercules, CA).

ELISA

IL-23 levels in serum were measured by mouse IL-23 (p19/p40) ELISA kit (E Bioscience, San Diego, CA); IL-10 levels in serum and culture supernatants were measured by mouse IL-10 ELISA kit (BD OptEIA; BD
Biosciences, San Jose, CA), according to the manufacturer’s instructions. A TGF-β1–specific ELISA kit (eBioscience) was used to quantify levels of human and mouse TGF-β1. Serum samples were acidified with 1 N HCl and neutralized with 1 N NaOH to assay for the amount of total (i.e., the sum of latent and active) TGF-β1 protein and analyzed according to the manufacturer’s instructions.

**RNA isolation and quantitative RT-PCR**

RNA was isolated from 20 mg mouse dorsal skin biopsies using Qiagen fibrous mini kit (Qiagen, Valencia, CA). RNA was reversely transcribed using a First-Strand cDNA synthesis kit (Roche, Basel, Switzerland), and quantitative RT-PCR for cytokine transcripts and transcription factors were performed using pretested primers to IL-17A, IL-17F, IL-12p35, IL-12p40, IL-23p19, IL-4, IFN-γ, RORγt, STAT3, keratin 5, keratin 14, filaggrin, and loricrin (Super Array Biosciences, Frederick, MD). The reactions were run on an Applied Biosystems 7900HT system by using RT² SYBR Green/ROX qPCR Master Mix (Super Array Biosciences). The ΔΔCt method was used to normalize transcript to GAPDH and to calculate fold change compared with WT control.

**Cell preparation and isolation**

Single-cell suspension of spleen and lymph node were prepared by using Cell Dissociation Sieve (Sigma-Aldrich), followed by RBC lysis buffer (eBioscience) for spleen. Single-cell suspensions were prepared by mechanical disruption in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) FCS, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 1-glutamate (2 μm; all from PAA Laboratories, Pasching, Austria). Prepared cells were used either for intracellular staining or for Treg isolation by a mouse Treg isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with autoMACS, according to the manufacturer’s instructions.

**Abs, intracellular staining, and FACS analysis**

The following Abs used for FACS were from eBioscience: Alexa Fluor-conjugated anti–IL-17A (clone eBio1B7), PE-conjugated anti–IL-17F (clone eBio18F10), PE-conjugated anti–IFN-γ (clone XMG1.2), FITC-conjugated anti–CD4 (clone RM4-5), PE-conjugated anti–CD25 (clone PC 61.5), PE-conjugated anti—I–L-selectin (CD62L) (clone MEL-14), allophycocyanin-conjugated anti–MHC class II (MHC-II) (clone MS/114.15.2), PE-conjugated anti–CCR7 (clone 4B12), and allophycocyanin-conjugated anti–Foxp3 (clone, FJK-16s). PE-conjugated anti–IL-10 Ab (clone JES5-16E3) was from BD Pharmingen (San Diego, CA).

Intracellular cytokine staining was performed on a spleen single-cell suspension. Cells (1 x 10⁷) were stimulated first with plate-bound 5 μg/ml anti–CD3ε (clone 145-2C11) and 2.5 μg/ml soluble anti–CD28 (clone 37.51; both from BD Pharmingen) in RPMI 1640 medium supplemented with 10% FCS (v/v), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 1-glutamate (2 μm) in triplicates for 4 d, then leukocyte-activating mixture (BD Pharmingen) was added for 5 h in a round-bottom 96-well plate (Nunc, Roskilde, Denmark). Cells were first stained for CD4 surface Ag and then treated with Fix/Per buffer (eBioscience),
according to the manufacturer’s instructions. Intracellular cytokine staining was performed using Abs to IFN-γ, IL-17A, and IL-17E. Lymph node or spleen cells were stained for Treg phenotype (CD4+CD25+Foxp3+) by using mouse Treg staining kit (eBioscience) according to the manufacturer’s instructions. For IL-10 and Foxp3 staining, isolated CD4+CD25+ T cells (1×10⁶) were cultured in triplicates, as described above. Cells were first stained for CD4 surface Ag and then fixed in eBioscience Fix/Per buffer after the in vitro stimulation. This was followed by permeabilization and staining for IL-10. Finally, the cells were again permeabilized in eBioscience permeabilization buffer and stained for Foxp3 according to the manufacturer’s instructions. FACS data were acquired on a FACS Calibur flow cytometer, and data were analyzed with CellQuest Pro software (BD Biosciences). All plots were gated on CD4+ or CD4+CD25+.

Treg suppression assay

The purity of isolated Tregs was checked before every proliferation assay. It was found to be in all cases >95%, as determined by FACS. To analyze proliferation and suppressive capacity of CD4+CD25+ Tregs in response to polyclonal activation, CD4+CD25+ or CD4+CD25- T cells (1×10⁵) were cultured in RPMI 1640 medium supplemented, as described above, either each alone or together at 1:1 ratio in a round-bottom 96-well plate at a final volume of 200 µl. Cells were stimulated with plate-bound 5 µg/ml anti-CD3ε and 2.5 µg/ml soluble CD28, with 1 µCi per [3H]thymidine (Amersham Biosciences, Piscataway, NJ) per well added for the final 16 h of the 72-h incubation.

Statistical analysis

All macroscopic readings and scorings of microscopic slides were conducted in a blinded fashion. Each experiment was repeated at least once with similar results. Data presented are expressed as means ± SEM. Statistical differences among experimental groups were determined by use of ANOVA or paired or unpaired two-tailed t test after testing for normality, whatever appropriate. Survival analysis was performed using log-rank test. Statistical significance was set at a level of p < 0.05.

Results

PUVA clears psoriasis-like skin lesions in K5.hTGF-β1 transgenic mice

PUVA is used for the treatment of skin diseases such as psoriasis at sub- or near-erythematogenic doses. For instance, the original European PUVA study protocol recommends the MPD as the starting dose for PUVA treatment (20). The MPD is determined prior to beginning the therapeutic treatment using a test, in which the skin of patients is exposed to a test ladder of increasing UVA doses after topical or oral psoralen administration. The MPD is defined as the smallest UVA dose required to produce a clearly demarcated and perceptible erythema, as determined 48–72 h after...
psoralen administration. To apply a clinically relevant treatment dose, we have determined the MPD in K5.hTGF-β1 transgenic mice with a protocol similar to that used in humans and have then administered for the treatment of the mice a subphototoxic dose of 0.25 J/cm² or a near-phototoxic dose of 0.50 J/cm² twice per week for 4 wk. These doses are in the range of MPD values used for starting PUVA therapy in patients. Fig. 1A shows that both PUVA doses initially caused skin swelling in the transgenic mice in a dose-dependent manner, similar to WT mice (Supplemental Fig. 1B–D). However, during the 4-wk treatment regimen, this swelling was resolved, and PUVA then prevented a further increase of the thickened skin (Supplemental Fig. 1A) that occurred in transgenic mice compared with WT mice. Likewise, both PUVA doses significantly improved the severity of skin disease in the mice (Fig. 1B). The macroscopic improvement of the skin status (Fig. 1C) of the mice was paralleled by a decrease of histological alterations (Fig. 1D, 1E, Supplemental Fig. 4). Whereas the skin of vehicle-treated K5.hTGF-β1 transgenic mice exhibited a hyperplastic epidermis (i.e., acanthosis) with hyperkeratosis and/or parakeratosis, a superficial mixed inflammatory infiltrate consisting of mononuclear cells and scattered neutrophils, and increased blood vessels in the dermis, such alterations were nearly absent in the skin of PUVA-treated transgenic mice. UVA given alone had no effect on the presence of psoriasis-like skin alterations (Fig. 1A, 1B, 1D, 1E). Notably, PUVA treatment significantly prolonged the survival of mice (Fig. 1F), a result that is consistent with observations in humans, in whom PUVA can be life saving for severe cases of erythrodermic or pustular psoriasis.

**Effect of PUVA on the IL-23/Th17 pathway and Th1 and Th2 cytokines, chemokines, and growth factors**

We were able to identify an effect of PUVA on the IL-23/Th17 axis that was disturbed in the K5.hTGF-β1 transgenic mice compared with WT mice. At the mRNA level, PUVA depressed the abnormally elevated mRNA levels of IL-17A, IL-17F, IL-12p35, IL-12p40, and IL-23p19 and orphan nuclear receptor RORγt and STAT3 transcription factors in the skin of transgenic mice (Fig. 2A). These results were paralleled by the depression of serum protein levels of IL-17, IL-12p70 (i.e., the bioactive heterodimer composed of the p40 and p35 IL-12 subunits), IL-23, IL-6, IL-1α, and IL-1β (Fig. 2B). UVA given alone had no significant effect on these factors. Immunohistochemical staining with anti–IL-23p19 Ab revealed that the keratinocytes of the lower layers of the epidermis as well as certain infiltrating cells of the dermis stained positive in the vehicle-treated transgenic mice. PUVA depressed this IL-23 positivity (Fig. 2C, 2D) as well as STAT3 in the dorsal skin (Supplemental Fig. 6). To determine the influence of PUVA...
treatment on the CD4+IL-17+ T cell population, we performed intracellular cytokine staining on pooled splenocytes obtained at the end of the 4-wk PUVA treatment regimen. PUVA decreased IL-17A- and IL-17F-producing CD4+ T cells in transgenic mice in a dose-dependent fashion by up to ~2- and 6-fold, respectively (Fig. 2E). We also found that PUVA downregulated abnormally elevated serum levels of G-CSF, GM-CSF, MCP-1, RANTES, KC, IP-10, MIP-1α, IL-9, TNF-α, and IFN-γ (Fig. 3A). In contrast, PUVA increased protein levels of IL-4, IL-10, and IL-13 in the serum. IL-2, IL-5, IL-7, and IL-15 levels were below the detection limit in all samples analyzed by bead immunoassay. Both PUVA doses upregulated IL-4 and IL-10 but downregulated IFN-γ in the serum, consistent with the decrease of those cytokines in the skin (Fig. 3B). In addition, PUVA decreased the IFN-γ content of CD4+ T cells in splenocytes in a dose-dependent fashion by up to >10-fold in transgenic mice (Supplemental Fig. 2).

An important question was whether PUVA affected TGF-β1 because it is the crucial driving factor in this disease model. Indeed, ELISA revealed that PUVA reduced total TGF-β1 (i.e., active and latent, human and mice) serum levels (Fig. 3A), but the levels after PUVA exposure were still significantly higher in transgenic mice compared with WT mice (~6-fold at the PUVA dose of 0.25 J/cm² and 3-fold at the PUVA dose of 0.50 J/cm²). Immunohistochemical staining of the skin revealed that there was no marked difference in the intensity of TGF-β staining between PUVA-treated and nontreated transgenic mice in the epidermis (Supplemental Fig. 3), indicating that PUVA did not per se inhibit transgenic keratinocyte production of hTGF-β1. However, PUVA decreased TGF-β protein in the dermis. The effect of PUVA on TGF-β expression in the skin appeared to be specific because other genes such as keratin 5, keratin 14, filaggrin, and loricrin were not significantly affected by the treatment (Supplemental Fig. 5). Collectively, these results indicated that PUVA treatment downregulates the IL-23/Th17 pathway and Th1 cytokines but upregulates Th2 cytokines in the K5.hTGF-β1 mouse model, at least partially independent of a direct effect on hTGF-β1 itself.

To study the functional relevance of the IL-23/Th17 pathway (Fig. 4), K5.hTGF-β1 transgenic mice were injected twice weekly over a 4-wk period with an anti–IL-17 mAb. The anti–IL-17 treatment stopped the disease progression in the K5. hTGF-β1 mice, compared with injection with an isotype control Ab (Fig. 4A–C). However, anti–IL-17 Ab injection did not affect IL-10 and Foxp3 mRNA expression in the dorsal skin of the mice (Fig. 4D).

PUVA-induced IL-10–producing Foxp3+ Tregs and IL-10 production

CD4+CD25+Foxp3+ Tregs, which have been shown to play a central role in autoimmunity, can be induced by TGF-β under certain circumstances (i.e., in the absence of IL-6) (21–23). To study the role of Tregs in the K5.hTGF-β1 mouse model, we first compared CD4+CD25+Foxp3+ Treg populations from lymph node cells or splenocytes between WT and K5.hTGF-β1 transgenic mice and did not find any significant differences (Fig. 5A). However, PUVA did increase the percentage of CD4+CD25+Foxp3+ Tregs by a similar rate at both PUVA doses in the spleens, whereas in the lymph nodes, the effect of PUVA appeared to be greater for the dose of 0.50 J/cm² compared with 0.25 J/cm² (Fig. 5B), most likely without biologic significance because there was no difference in the function of Tregs isolated from spleen between the two doses (Fig. 5C). Tregs induced by UV radiation have been shown to express the lymph node homing receptor CD62L but, like other T cells, not E- and P-selectin (24). Moreover, their homing is also controlled by expression of CCR7 and MHC-II on their surface. In this context, we found a higher expression of CD62L, MHC-II, and CCR7 on the CD4+...
T cell population of lymph nodes from PUVA-treated mice, with up to a 4-fold increased expression of CD62L in the case of mice treated with 0.50 J/cm² PUVA compared with vehicle-treated K5.hTGF-β1 (Fig. 6A, 6B). Quantitative RT-PCR analysis of RNA extracted from dorsal skin revealed a 3.5-fold increase of Foxp3 transcript in PUVA-treated compared with vehicle-treated K5.hTGF-β1 transgenic mice (Fig. 5D). In addition, PUVA treatment increased the percentage of CD4⁺CD25⁺Foxp3⁺IL-10⁺ Tregs among splenocytes in transgenic mice, compared with vehicle treatment (Fig. 5B). This was consistent with higher in vitro production of IL-10 by CD4⁺CD25⁺ Tregs from transgenic mice upon PUVA treatment, as measured by ELISA in cell culture supernatants (Fig. 5E). Furthermore, PUVA did increase IL-10 positivity of keratinocytes in the epidermis and cells in the inflammatory infiltrate of the dermis (Fig. 5C), consistent with the result of a previous study (8). The exact type and origin of PUVA-induced IL-10-positive cells in the epidermis remains unclear at present; however, they may represent macrophages, dendritic cells, and/or even certain T cells (25, 26). However, there was no significant difference neither in IL-10 expression (Fig. 3B) nor in IL-10 protein levels in the skin (Fig. 3C) and serum (Fig. 3A) between vehicle-treated WT and transgenic mice.

It has been shown previously that Tregs from patients with psoriasis were dysfunctional in suppressing the proliferation of T responder cells (27). We therefore aimed to study the function of Tregs in the K5.hTGF-β1 mouse model. We first examined the proliferative capacity of Tregs or T responder cells alone upon polyclonal stimulation. Neither Tregs from WT nor PUVA-treated or untreated K5.hTGF-β1 transgenic mice proliferated when stimulated with plate-bound anti-CD3 Ab alone or combined with plate-bound anti-CD3 and soluble anti-CD28 Ab. However, T responder cells had full capacity of proliferation upon stimulation with plate-bound anti-CD3 and soluble anti-CD28 Ab. Tregs from K5.hTGF-β1 transgenic mice failed to suppress T responder cell proliferation during later stage of the disease development but not at the onset of or submaximal disease development. To investigate whether PUVA is able to induce Tregs with suppressive activity, we performed coculture experiments with Tregs and T responder cells at a 1:1 ratio (Fig. 5C). Tregs from vehicle-treated K5.hTGF-β1 transgenic mice failed to suppress the proliferation of T responder cells. In contrast Tregs from K5.hTGF-β1 transgenic mice treated with PUVA were able to suppress the proliferation of T responder cells to levels that are comparable to WT cells (Fig. 5C). This is consistent with the findings by Schwarz et al. (24), who showed that Tregs induced in mice through exposure to UV radiation did not only have the capacity to inhibit the induction phase but also the effector phase of contact hypersensitivity to an allergen.

**CTLA4 signaling is crucial for PUVA-induced Treg function**

CTLA4 is an important molecule that is expressed on the surface of activated T cells and essential for the regulatory function of
Tregs. Like CD28, CTLA4 binds to B7-1 and B7-2 but at rates reported to be 500- to 2500-fold higher than those of CD28, thus giving CTLA4 a competitive advantage over CD28 (28, 29). Data from earlier studies indicated that treatment of mice with CTLA4Ig, a soluble form of CTLA4 that effectively blocks CD80/CD86 engagement with CD28, suppressed antitumor immunity, transplant rejection, and autoimmune responses (30). Moreover, blocking CTLA4 signaling has been shown to inhibit the suppressive function of UV-induced CD4+CD25+ T cells (31). We therefore used anti-CTLA4 mAb treatment to test the functional relevance of PUVA-induced Tregs. K5.hTGF-β1 transgenic mice were treated with PUVA (twice a week for 4 wk; 0.25 J/cm²) (Fig. 7A). When the mice were injected with anti-CTLA4 mAb, PUVA lost its disease-suppressive activity, whereas injection with an isotype control Ab did not affect the beneficial effect of PUVA. This effect was confirmed on the microscopic level (Fig. 7C) and inflammatory infiltrate in the dermis between anti-CTLA4 Ab-injected PUVA-treated or untreated transgenic mice.

We then investigated whether blocking of CTLA4 signaling directly affected the inhibitory function of PUVA-induced Tregs. CD4+CD25+ Tregs were prepared from PUVA-exposed transgenic mice and cultured alone or cocultured with CD4+CD25− T cells, each in the absence or presence of anti-CTLA4 mAb (Fig. 7E). Notably, we found that anti-CTLA4 treatment abrogated the suppressive activity of PUVA-induced Tregs upon stimulation with plate-bound anti-CD3 and soluble anti-CD28. Importantly, FACS analysis revealed that the injection of anti-CTLA4 mAb did not alter the number of CD4+CD25+Foxp3+ Tregs in the spleens of PUVA-treated transgenic mice, compared with isotype control Ab injection (Fig. 7D). The importance of IL-10 for the functional activity of Tregs was confirmed by analysis of cell culture supernatant of the cultured splenocytes upon TCR stimulation (Fig. 7F). Anti-CTLA4 (but not isotype control) Ab injection of the mice abrogated the PUVA-induced increase of IL-10 in the supernatant.

**Discussion**

This study addresses the molecular mechanisms of the therapeutic action of PUVA in K5.hTGF-β1 transgenic mice (Fig. 1), a highly ranked murine model with a skin disease of psoriasis-like character (10, 12, 13, 32). Besides many abnormalities that also occur in human psoriasis patients, such as preponderance of the IL-23/Th17 axis (14) (Fig. 2) and a Th1 milieu including abnormally elevated cytokines such as IFN-γ and TNF-α (Fig. 3) (likewise to certain other psoriasis mouse models) (33), we detected normal levels of natural Tregs (Fig. 5) but a dysfunctional activity of those cells in K5.hTGF-β1 transgenic mice at maximal disease manifestation. PUVA had a reciprocal effect by inhibiting the IL-23/Th17 axis and the Th1 milieu (e.g., by downregulating IFN-γ expression 10-fold) (Figs. 2, 3) and by simultaneously inducing an IL-10–producing CD4+CD25+Foxp3+ Treg population with disease-suppressive activity (Fig. 5B), which was abrogated by targeting Treg function with anti-CTLA4 (19, 31) mAb treatment (Fig. 7), providing the proof that PUVA-induced Tregs were indeed primarily responsible for the clearance of the psoriatic skin lesions in the K5.hTGF-β1 mice. This is intriguing because the role of the immune system in the formation of the psoriatic phenotype in K5.hTGF-β1 mice has been controversial. Han et al. (13) found that depletion of T cells by crossing K5.hTGFβ1tm mice with Ragl−/− mice delayed skin inflammation and associated epidermal hyperplasia. In contrast, T cells had a limited role in the formation of psoriasiform skin lesions in K5.hTGF-β1 mice (M.P. Schön and K. Michaelis, unpublished data). They found that eradication of T lymphocytes with CD4-depleting Abs did not alleviate the psoriatic skin phenotype. However, the results of our study unambiguously indicate that the induction of a specific immune response (i.e., iTregs) by PUVA ameliorates the psoriatic skin phenotype in diseased K5.hTGF-β1 mice, although the immune system may in the beginning not necessarily cause this phenotype.

That the findings on Tregs and the influence of PUVA in the model can be translated to human’s level appears likely because of many pathomechanical similarities between K5.hTGF-β1 mice and human psoriasis patients. Similar to the mouse model, 1) CD25high Tregs make up similar proportions of CD4+ T cells, 2) psoriatic CD4+CD25high Tregs differ from normal and untreated psoriatic patients express or produce similar amounts of IL-10 (27). Another interesting aspect of the K5.hTGF-β1 mouse model concerns the homing of CD4+ T cells, highlighted by the increased expression of CD62L, MHC-II, and CCR7 from lymph nodes of PUVA-exposed animals (Fig. 6). This finding suggests migration of CD4+ T cells from skin to peripheral lymph node and/or vice versa. In addition, PUVA exposure was linked to an upregulation of the transcription factor Foxp3 expression in the skin (Fig. 5D) and its level in cells from spleens or lymph nodes (Fig. 5A). Foxp3 programs the development and function of CD4+CD25+ Tregs in controlling autoimmunity. Its expression is a key feature of natural Treg cells (34), which arise during thymic development through high-affinity recognition of self-Ags (35). In contrast, iTregs or so-called adaptive Tregs are Foxp3+ cells that develop extrathympically and share many phenotypic and functional characteristics of natural Tregs but exert their regulatory activity mainly by IL-10 and TGF-β secretion (23). TGF-β is critical for the
generation of induced Foxp3+ T cells (22, 36) whereas the presence of IL-6 inhibits the conversion of T cells into Foxp3+ Tregs and favors Th17 immunity (16, 18). In this context, it needs to be noted that PUVA downregulated IL-6 expression in the skin of transgenic mice, an activity that may have culminated in the downregulation of IL-17 and induction of adaptive Tregs with antipsoriatic activity.

The therapeutic significance of PUVA-induced Tregs goes well in line with a recent study (37), in which epidermal RANKL induced upon UV exposure was shown to control Treg numbers coupled to local and systemic immune suppression. Notably, RANKL expression is a strong inducer of vitamin D₃, and vitamin D₃ derivatives are well-known immunomodulators (13, 38). Moreover, UV phototherapy may augment the functional activity of Tregs in polymorphic light eruption in pre-seasonal photohardening. This activity is possibly associated with normalization of skin sensitivity in this presumably autoimmune-mediated condition (39). It is also worth mentioning that the infusion of expanded Tregs (e.g., by photopheresis) (25) has been suggested as a therapeutic concept to control transplantation.

We also observed in the skin of K.5hTGF-β1 transgenic mice an increased level of STAT3 (Fig. 2A, Suppelmental Fig. 6), a transcription factor that is thought to link activated keratinocytes and immunocytes required for development of psoriasis in another transgenic mouse model (40), as well as orphan nuclear receptor RORγt, which is known for orchestrating the differentiation program of proinflammatory IL-17+ Th cells (41). Both transcription factor levels decreased upon PUVA exposure what was accompanied by a downregulation of the IL-17 pathway, as evident by lowered levels of IL-17A and IL-17F transcripts in the skin and IL-17 protein in the serum (Fig. 2A, 2B). The importance of the IL-17 pathway in the K5.hTGF-β1 model is supported by the observation that blocking by anti-IL-17 mAb injection stopped the progression of the skin disease but did not clear existing lesions in the skin of the transgenic mice (Fig. 4). However, the anti-IL-17 Ab treatment did not affect Foxp3 levels and IL-10 mRNA levels in the skin (Fig. 4D). Importantly, TGF-β in the context of an inflammatory cytokine milieu has been shown to support de novo differentiation of IL-17-producing T cells (42). IL-17 itself has many proinflammatory effects, including downstream production of IL-1, IL-6, IL-8, TNF-α, G-CSF, and GM-CSF as well as antimicrobial peptides (43). The significance of the IL-17 pathway in human psoriasis patients has been recognized recently (44). For instance, psoriasis lesions in human patients contain discrete populations of Th1 and Th17 cells (15), which are activated on increased production of IL-17A, IL-17F, and IL-22 mRNA (15, 44). Anti–IL-17-Ab treatment is in the clinical test phase for its antipsoriatic activity.

IL-23, a regulatory key cytokine in the differentiation and survival of Th17 cells (18), has been found upregulated in psoriatic skin lesions (45), consistent with our observations in the K5.hTGF-β1 mouse model (Fig. 2C, 2D). Both mRNA and
immunohistochemical analysis showed that IL-23p19 and IL-12p40 subunits (the latter shared by IL-12 and IL-23) were increased in human psoriatic plaques compared with noninvolved skin; however, no difference was observed in human subjects for the IL-12p35 subunit (45), suggesting that IL-23 is playing a more dominant role than IL-12 in psoriasis. This is absolutely in line with the results presented in this paper, showing that IL-12p40 and IL-23p19 expression and/or protein was increased (and responded to PUVA) (Fig. 2A–D) but IL-12p35 expression was not increased in the diseased skin of the transgenic mice (Fig. 2A). At first glance, these results seem to be contradictory to the recent study by Fitch et al. (46), reporting that the inflammatory skin disease in their K5.hTGF-β1 mice did not depend on the IL-23/Th17 pathway. However, they admitted major limitations to their study because of potential changes in transgene expression after years of breeding. Indeed, the animals they used exhibited eosinophilic skin infiltration and highly elevated esional IL-4 and high-serum IgE levels, contrasting to the characteristics of the K5.hTGF-β1 mice originally described by Li et al. (10) and confirmed in our study. The effect of PUVA on IL-23 levels in the skin is consistent with that of other antiinflammatory treatments such as UVB 311-nm therapy (47), cyclosporin A, and biological agents modulating this cytokine (14). Th22 cells represent another distinct human T cell subset uniquely able to regulate epidermal responses in inflammatory skin disease (48, 49); however, the effect of antiinflammatory treatments such as PUVA on this subset remains to be studied.

IL-10 is an immunoregulatory cytokine that mediates both UV- and PUVA-induced immune suppression (8). Indeed, one of its main biological functions seems to be the limitation and termination of inflammatory responses. Remarkably, a relative deficiency in IL-10 expression has been found in psoriasis (50), and IL-10 (and likewise IL-4) treatment has been shown to exhibit clinical efficacy in psoriasis (51, 52). It was therefore consistent that upregulation of IL-10 and IL-4 took a central place in the efficacy of PUVA in the K5.hTGF-β1 mouse model (Fig. 3), being crucial for the formation of iTregs with antipsoriatic activity (Figs. 5, 6). However, PUVA downregulated several other cytokines and chemokines such as IL-1a, IL-6, IL-9, IP-10, MCP1, KC, RANTES, G-CSF, MCP, and GM-CSF, identifying them as potential therapeutic targets in autoimmune diseases such as psoriasis and other conditions, which are responsive to PUVA (53). Interestingly, the higher PUVA dose of 0.50 J/cm² was more effective in increasing or reducing the levels of most cytokines (Figs. 2, 3), whereas the lower PUVA dose of 0.25 J/cm² was more effective in clearing the psoriatic lesions in the mice (Fig. 1B). However, this seemingly paradox observation is consistent with the clinical knowledge that suberythemal PUVA exposure can be more beneficial in psoriasis than exposure to higher doses near to or above phototoxicity, because the latter can trigger or aggravate the disease by the so-called Koebner phenomenon.

Taken together, it is fascinating that a therapy such as PUVA that has its roots thousands of years back in natural medicine works via the very same pathways that the most modern treatments are based on, such as biologics interfering with cytokines, including TNF-α (1), IL-12/23 (3), or IL-17. PUVA and other forms of phototherapy may act as a type of natural anti/procytokine therapy, and therefore, the combination with synthetic biologics may actually represent a novel synergistic multifunctional treatment strategy. Indeed, the therapeutic effects of PUVA may involve multiple mechanisms including suppression of Th1 and Th17 and stimulation of Th2 and Treg activities. The fact that IL-10 and Foxp3 was unchanged in anti–IL-17 Ab-treated mice indicates that those pathways may be parallel and independent from each other in the pathogenesis of K5.hTGF-β1 mouse model. The observation that anti–IL-17 Ab treatment prevented disease progression whereas PUVA improved the skin status of the transgenic mice, an effect that was abrogated by anti-CTLA4 mAb treatment, suggested that the primary therapeutic effect of PUVA is mediated via IL-10–producing Tregs. It remains to be determined how the PUVA-induced regulation of the disturbance of the IL-17/23 axis and the iTregs relate to carcinogenesis upon long-term treatment (54, 55). Interestingly, upon chronic UV exposure, Tregs were found to be involved in skin cancer formation in mice (31) and are targeted in human cancer immunotherapy (28). In contrast, IL-23 and IL-17 are increased in human tumors and have been implicated in causing cancer (56).

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Disclosures

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